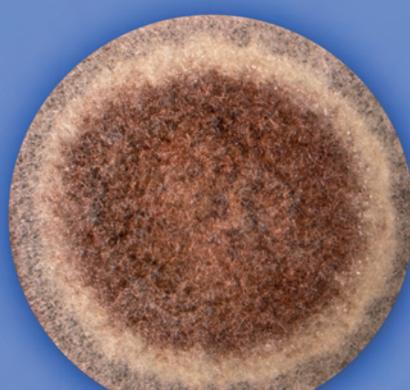
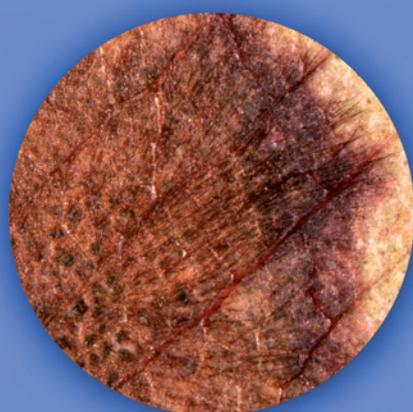


# Taxonomy and phylogeny of the genus *Mycosphaerella* and its anamorphs

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# Taxonomy and phylogeny of the genus *Mycosphaerella* and its anamorphs

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**Promoter:** Prof. M.J. Wingfield

## Declaration

I, the undersigned, hereby testify that the publications submitted for this doctoral degree have not previously been submitted to this or any other tertiary institution for such a doctoral degree; are my own work, and with regard to such publications of which I am co-author, my personal contribution to those works is clearly stated; takes place with due recognition given to the author's copyright in accordance with the case.

P.W. Crous

March 2009

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# Taxonomy and phylogeny of the genus *Mycosphaerella* and its anamorphs

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## Summary

The genus *Mycosphaerella* has been linked to more than 30 anamorphic form genera, which together represent several thousand species, the majority of which are plant pathogens. Historically species have been regarded as novel based on their hosts, with fungal morphology accepted as important among taxa occurring in specific plant families. Host specificity and anamorph-teleomorph connections have proven difficult to study, largely due to the relatively few fungal cultures available. During the course of the past 20 years a concerted effort has been made to collect these fungi, and devise methods to cultivate them to enable these questions to be addressed. Major findings from this work are that *Mycosphaerella* is polyphyletic, incorporating several anamorphic genera with formerly unknown affiliations. Teleomorph morphology was shown to be too narrowly defined in some cases, and again too widely in others. Species of *Cladosporium*, which have a characteristic conidial hilum and scar structure (coronate-type), were excluded from *Mycosphaerella*, and placed in a new genus, *Davidiella*, which is distinguished from *Mycosphaerella* by having irregular, somewhat angular lumens inside the ascospore cells, versus the normal guttules found in *Mycosphaerella*. Species of *Sphaerulina* that have 3-septate ascospores, but form *Pseudocercospora* anamorphs, were found to belong to *Mycosphaerella*, suggesting ascospore septation to be more variable. Several taxa that occur in extreme environments (especially on hosts with hard, leathery leaves), were shown to belong to *Teratosphaeria*, distinguished from *Mycosphaerella* by having hamathecial remnants, a multi-layered ascad endotunica, and ascospores with sheaths, that frequently turn brown while still in their asci. Anamorphs were shown to also differ between *Mycosphaerella* and *Teratosphaeria*. Among *Mycosphaerella* anamorphs, *Passalora* was shown to include species formerly placed in *Mycovellosiella* and *Phaeoramularia*, while *Pseudocercospora* was again shown to include species placed in *Cercostigmina*, *Stigmina*, and *Phaeoisariopsis*. Anamorph genera newly linked to *Mycosphaerella* include *Trochophora*, *Verrucisporota*, *Ramichloridium*, *Periconiella* and *Phaeophleospora*. *Teratosphaeria*, on the other hand, had anamorph genera such as *Batcheloromyces*, *Catenulostroma*, *Cibiessia*, *Colletogloeopsis*, *Davisoniella*, *Kirramyces*, *Nothostrasseria*, *Phaeothecoidea* and *Readeriella*. *Mycosphaerella*-like species with *Dissoconium* anamorphs appeared to represent a separate lineage. Finally, several species were revealed to not be host specific, while others were again strictly host specific, suggesting that no general rule can be applied. By designing degenerate mating type primers, proof could also be obtained that several apparently asexual species such as *Cercospora beticola*, *C. zaeae-maydis*, *C. zeina* and *Septoria passerini* are apparently undergoing cryptic sex, while others such as *Dothistroma septospora*, *D. pini* and *Passalora fulva* are heterothallic, with both mating types only occurring in some continents. Even though being polyphyletic, *Mycosphaerella* remains the largest genus of ascomycetous fungi known, with current species numbers of around 10 000 taxa shown to be conservative. Because most species are plant pathogens, detailed knowledge of their host specificity, sexual cycle and distribution will remain of paramount importance to plant pathologists and quarantine officers who must control the diseases associated with *Mycosphaerella* on the one hand, and enhance free trade in agricultural and forestry produce on the other.

## What is *Mycosphaerella*?

Species of *Mycosphaerella* have adapted in various ways to different ecosystems, and vary from being saprobic, plant pathogenic to hyperparasitic (de Hoog et al. 1991, Goodwin et al. 2001, Jackson et al. 2004, Arzanlou et al. 2007b). *Mycosphaerella* spp. are among the most common and destructive plant pathogens known, causing considerable economic losses on a wide variety of host plants worldwide, including economically important crops such as banana, cereals, sugar beet, strawberry, soybean, citrus, eucalypts, acacia, pines and many others (Farr et al. 1995, Crous & Braun 2003). Plant pathogenic *Mycosphaerella* species are mainly foliicolous, although some are associated with stem cankers (Cortinas et al. 2006), fruit lesions (Pretorius et al. 2003) or blemishes, spots and specks (Batzer et al. 2008). Damage is usually due to defoliation, which reduces the photosynthetic capacity of the crop, leading to growth loss. Some species, such as *M. citri*, affect both leaves and fruits. Others such as *M. fijiensis*, infect banana leaves, thereby reducing the photosynthetic capacity of the crop, and also induces physiological changes resulting in premature ripening of fruit (Carrier et al. 2000, Marin et al. 2003).

The first generic description for *Mycosphaerella* (1884) was that of *Sphaerella* (1882). Saccardo placed all species of *Sphaeria* with presumably 1-septate, hyaline ascospores in *Sphaerella*. The genus *Sphaerella* was, however, already in use for green algae, and thus all these taxa had to be placed in *Mycosphaerella* (Aptroot 2006), which is based on *M. punctiformis* (Verkley et al. 2004). Despite the hyaline, 1-septate ascospores reported in the type by Persoon (1794), most authors at the beginning of the 19th century worked without microscopes, and thus what they described as a *Sphaeria* or *Sphaerella* species, literally meant a 'spherical' fruiting body (Aptroot 2006). Soon it became standard to also describe collections from different hosts as new species, which later led to many taxa being reduced to synonymy (Von Arx 1949, Barr 1972, Tomilin 1979, Corlett 1991, Aptroot 2006). In the recent revision of *Mycosphaerella* names, Aptroot (2006) treated close to 10 000 taxa, recognising around 3 000 species.

In her treatment of North American taxa, Barr (1972) recognised two subgenera, *Eu-Mycosphaerella* and *Didymellina* (including the section *Cymadothea*), and 10 sections. The sub-genera were separated on the basis of the shape of their asci and anamorphs, and the sections based on ascospore shape, and/or parasitic or saprobic habit. Von Arx (1983) found the subdivision unsatisfactory, because the characters were inordinately divergent.

The sections of Barr were refined by Crous et al. (2000) as follows:

**Section *Mycosphaerella*:** cylindrical asci and mostly uniseriate, thin-walled, often small ascospores that are constricted at the septum and inequilateral, with rounded upper ends. Anamorphs: typically *Ramularia* with *Asteromella* spermatial states. Representative species: the common polyphagous *M. punctiformis*.

**Section *Tassiana*:** pyriform asci and irregularly arranged, thick-walled ascospores that are often large and constricted at the septum and nearly equilateral, relatively broad with rounded ends, containing irregular lumina. Anamorph: *Cladosporium* s. str. Representative species: the common polyphagous species *Davidiella tassiana*. Further research supported the decision of David (1997) to place *Heteroconium* anamorphs in *Cladosporium*, while section *Tassiana* was elevated to generic level as *Davidiella* (*Cladosporium* anamorphs) (Braun et al. 2003, Crous et al. 2007a, Schubert et al. 2007a, b, Zalar et al. 2007), for which the family

*Davidiellaceae* was established (Schoch et al. 2006).

**Section *Caterva*:** cylindrical asci and irregularly arranged, thin-walled, often medium-sized ascospores that are rarely constricted at the septum and inequilateral, with more or less pointed ends. *Asteromella* spermatial forms are typical. Representative species: the common polyphagous *M. subradians*.

**Section *Longispora*:** cylindrical asci with aggregated, thin-walled, long and slender ascospores that are rarely constricted at the septum and mostly equilateral, long but slender ascospores, characteristically with rounded upper and pointed lower ends. Anamorphs: *Phloeospora* or *Septoria* s. lat. Representative species: *M. eryngii* (with short spores), *M. latebrosa* and *M. populi* (with longer spores). The phylogenetic position of *Sphaerulina*, which differs by having additional ascospore septa, still needs to be resolved.

**Section *Fusispora*:** pyriform asci and irregularly arranged, thin-walled ascospores that are rarely constricted at the septum and mostly equilateral, fusiform, pointed ascospores. Anamorphs have not been proven. Representative species: the common *M. lineolata* on *Poaceae*.

**Section *Plaga*:** (incl. Section *Macula*) incorporates endophytic species sporulating on leaf spots, many of which are described as plant pathogens. This section is characterised by obovoid to ellipsoidal or cylindrical asci, small to medium sized ascospores, fusiform to obovoid with rounded ends. Many species have been described in this section, the majority of which originate from warm-temperate and tropical areas. Anamorphs include *Colletogloeopsis*, *Kirramyces*, *Passalora*, *Phaeophleospora*, *Pseudocercospora*, *Pseudocercospora*, *Sonderhenia*, *Stenella*, etc. Several representative species are listed by Crous (1998) on *Eucalyptus*.

**Section *Cymadothea*:** This section is now accepted as the genus *Cymadothea* (*Polytrincium* anamorphs) (Simon et al. 2009). *Cymadothea* has superficial ascomata situated on a stroma of pseudoparenchymatal cells, and ascospores that can become pale brown with age. Representative species: the genus is monotypic, with *C. trifolii* occurring on *Trifolium*.

Von Arx (1949) proposed separating species with separate ascomata immersed within the host tissue, and those with ascomata occurring in pseudoparenchymatous stromata. This idea certainly has merit, but too few taxa in the latter category have been subjected to DNA analyses to fully test this proposal. The original hypothesis of separating species with pigmented ascospores into *Phaeosphaerella*, while retaining those with hyaline ascospores in *Mycosphaerella*, should also be reinvestigated. This separation was also followed by Tomilin (1979). However, Müller and von Arx (1962) found that the type species of *Phaeosphaerella*, *P. maculosa* was identical to *Venturia macularis*. A new generic name would thus have to be introduced for species with pigmented ascospores. Species with hyaline and slightly pigmented ascospores are currently retained in *Mycosphaerella*, though some are now placed in *Teratosphaeria* (see below). Although ascospore germination patterns have thus far only been used at species level (Crous 1998, Crous et al. 2004a), many species with ascospores that turn dark and verruculose during germination (Crous et al. 1993a, b, Crous & Wingfield 1996, Crous et al. 2008a, b) have in fact been shown to belong to *Teratosphaeria*, not *Mycosphaerella*, suggesting that this character may have value at the generic level as well.

Klebahn (1918) and Laibach (1922) proposed that species be classified in different genera according to their anamorphs, and proposed *Septorisphaerella* (*Septoria* anamorphs), *Ramularisphaerella* (*Ramularia* anamorphs), *Cercosphaerella* (*Cercospora* anamorphs) and *Ovosphaerella* (*Ovularia* anamorphs),

= *Ramularia* fide Braun 1998). This approach was not accepted by subsequent workers. Von Arx (1983) stated that the presence or absence of anamorphs should not be used to separate genera, subgenera or sections in *Mycosphaerella*. Crous (1998) suggested that anamorph morphology, rather than features such as ascus and ascospore shape be used to separate genera within *Mycosphaerella*, though initial DNA phylogenies based on ITS sequence data refuted this (Crous et al. 2000, Goodwin et al. 2001, but see below). Although Sutton and Hennebert (1994) suggested that different anamorph conidiogenous events and conidiomatal types could prove useful in grouping species at some subgeneric level, the presence of synanamorphs (Crous et al. 2007a, c), and general plasticity observed in conidiogenesis and conidiomatal structure when studied in culture, suggests that these characters should be used with caution.

Barr (1996) placed two species occurring on pine needles in a new genus, *Eruptio* (*Lecanosticta* and *Dothistroma* anamorphs) based on their elongate, erumpent ascostromata that open via schizogenously formed ostioles. The taxa presently accommodated in *Eruptio* are, however, not congeneric, and thus genus will have to be evaluated further in future studies.

Although *Sphaerulina* was established to accommodate taxa with primarily 3-septate ascospores, Crous et al. (2003b) showed that some elements of '*Sphaerulina*' clearly belong in *Mycosphaerella*, and that ascospore septation is not as definitive as previously thought. The most significant finding, however, was that *Mycosphaerella* is an assemblage of numerous genera that are morphologically similar. These genera are more easily separated based on their anamorphs (Crous 1998, Crous et al. 2007a), though the same anamorph morphology could also evolve in more than one lineage (genus), making morphological identifications cumbersome in certain groups.

The separation of *Davidiella* teleomorphs (Braun et al. 2003) with their angular lumens, remnants of hamathecial tissue, and *Cladosporium* s.str. anamorphs was a significant step in redefining *Mycosphaerella*. The next was the separation of *Teratosphaeria* from *Mycosphaerella* (Crous et al. 2007a). Species of *Teratosphaeria* are distinguished from *Mycosphaerella* by frequently having hamathecial remnants, a multi-layered ascus endotunica, and ascospores with sheaths that frequently turn brown while still in their asci. Anamorph genera were shown to also not overlap between *Mycosphaerella* and *Teratosphaeria*. In a recent paper by Schoch et al. (2006), further support was provided for the separation of the *Teratosphaeriaceae* from the *Mycosphaerellaceae* and *Davidiellaceae*, while all families were shown to be members of the *Capnodiales* (*Dothideomycetes*).

The genus *Mycosphaerella* s.str. *Ascomata* are submerged, separate, somewhat erumpent, small, simple, globose, black, with walls of 2–4 layers of textura angularis. *Ostioles* are central, frequently lined with periphyses. *Asci* are bitunicate, fasciculate, lacking hamathecial filaments. *Ascospores* are 1-septate, hyaline, lacking appendages and sheaths. *Anamorphs* are accommodated in *Ramularia*.

Contemporary taxonomy of *Mycosphaerella* is based on a range of characters, including symptoms on host plants, morphological characters of both anamorphs and teleomorphs, cultural characteristics, and DNA phylogeny (Crous 1998, Stewart et al. 1999, Crous et al. 2000).

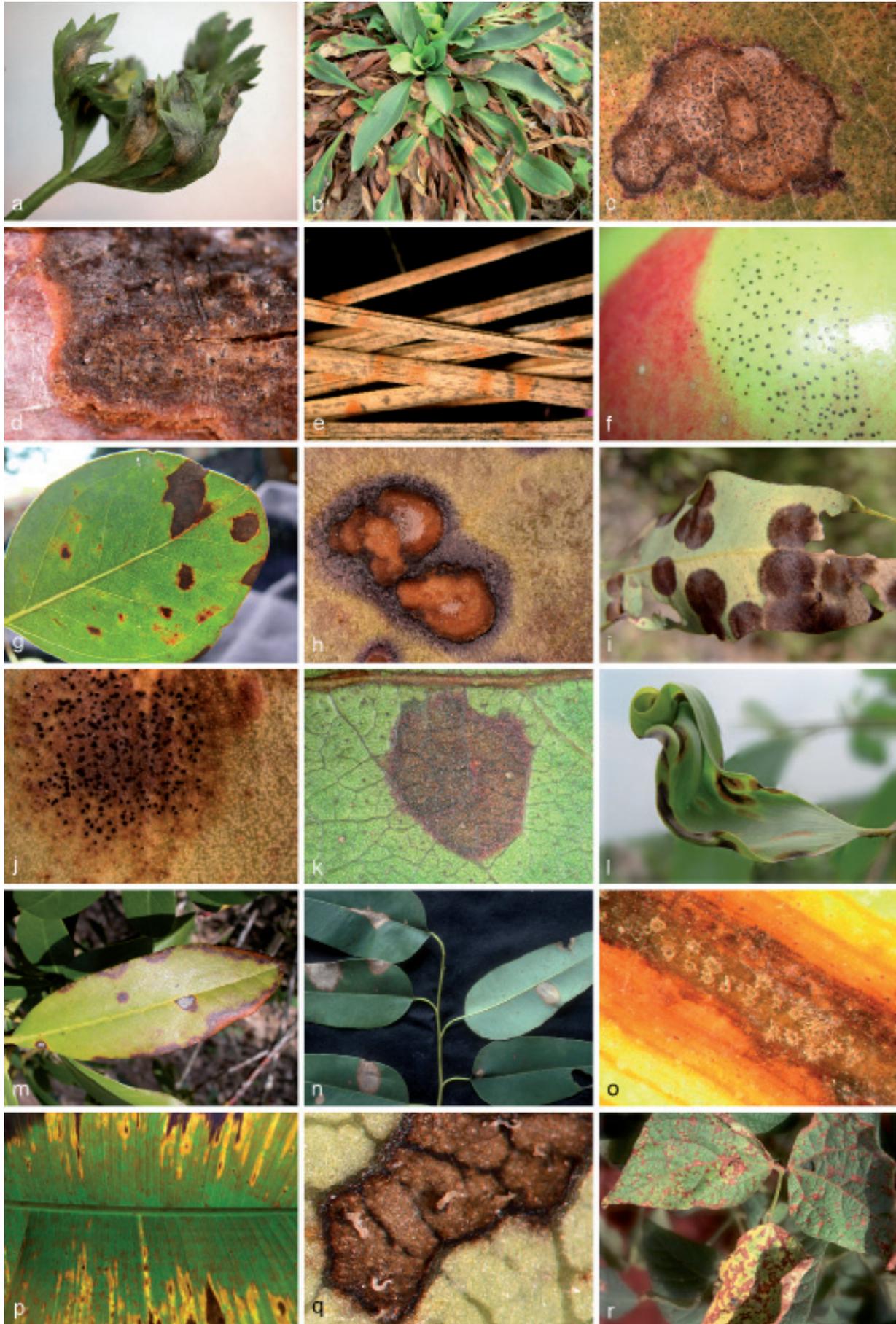
**Symptomatology:** Lesions vary in shape from being angular to circular or irregular, and in size from specks to spots or larger coalescing blotches, causing a distortion of the leaf lamina. Lesions also vary in colour at different stages of development,

and can be smooth and amphigenous, or corky and not extending through the lamina (Fig. 1). Borders of lesions can be raised, and frequently darker in colour, and margins can be absent, or vary from a chlorotic yellow to red or red-purple. Many *Mycosphaerella* spp. seem to occur only on foliage of defined age, namely on juvenile, intermediate or mature foliage. Lesions can also occur on fruit (spots or rot), or on twigs or stems, associated with dieback or cankers. Many species occur as symptomless endophytes, and are only observed to sporulate on plant debris.

**Teleomorph characters:** *Ascomata* of different species frequently vary in size between the larger- and smaller-spored species. *Ascomatal* distribution (upper or lower leaf surface), and aggregation (dense, sparse) and association with stromatic tissue, are very characteristic features among different taxa. Dimensions of the *ascomatal* wall cells tend to vary little among small- or large-spored species. However, some taxa have characteristically thick walls, consisting of more layers than the general 3–4 cell layers observed in common species. *Periphyses* are commonly present, lining ostiolar canals, and their level of development varies among taxa. *Asci* are paraphysate, bitunicate, sessile, and formed in a fascicle, vary in shape from obovoid to narrowly or broadly ellipsoidal, or narrowly ellipsoidal to cylindrical (Fig. 2). *Ascospores* are mostly hyaline (*Mycosphaerella* s.str.), or slightly olivaceous in some taxa. They are usually bi- to triseriate in *asci* of large-spored species, or multiseriate in those with small-spored taxa. *Ascospores* can either be straight, curved, or frequently both curved and straight. They vary from being strongly guttulate to non-guttulate, thin- to thick-walled, and prominently, slightly or not constricted at the septum. *Ascospores* are mostly medianly 1-septate, but in some species the basal cell is slightly longer than the apical cell. The widest point in the *ascospore* can either be at the median septum, in the middle of the apical cell, or closer to the apex. The apical cell can also be asymmetrical. *Ascospores* vary in shape from narrowly ellipsoidal, fusoid-ellipsoidal, or obovoid. They taper from the middle toward both ends, or more prominently from the tip or middle of the upper cell toward the base.

**Ascospore germination:** *Ascospore* germination patterns represent a valuable feature to help distinguish morphologically similar species (Crous et al. 1993a, b, Carnegie & Keane 1994, Crous & Alfenas 1995, Crous & Wingfield 1996, Crous 1998). Crous et al. (1991) studied *ascospore* germination by letting spores shoot from leaf lesions onto 2 % malt extract plates. *Ascospores* were usually ejected within 24 h, enabling germination patterns to be determined the following day. If left too long, *ascospores* from some faster growing species become totally distorted, clouding their germination patterns. For some species, germination is most characteristic at the very onset, whereas others tend to form lateral branches 24–48 h after they have been shot onto the agar surface (Crous & Wingfield 1996). These germination patterns have been found to be stable and reproducible, even when spores produced *in vitro* are germinated on agar. However, the patterns change when spores germinate in water or on different media, or are left for inordinately long after *ascospore* discharge. Standardisation to ensure reproducibility is, therefore, essential, and the time from discharge to observation must be carefully monitored. In studying the species occurring on *Eucalyptus*, Crous (1998) observed 14 different germination patterns (Fig. 3). *Ascospores* tend to become slightly swollen, or completely distorted, with one to several germ tubes emerging, growing at various angles to the long axis of the spore, remaining hyaline, or turning brown upon germination.

**Colony growth in culture:** Colony characteristics and *ascospore* morphology are generally consistent *in vitro*. *Mycosphaerella*



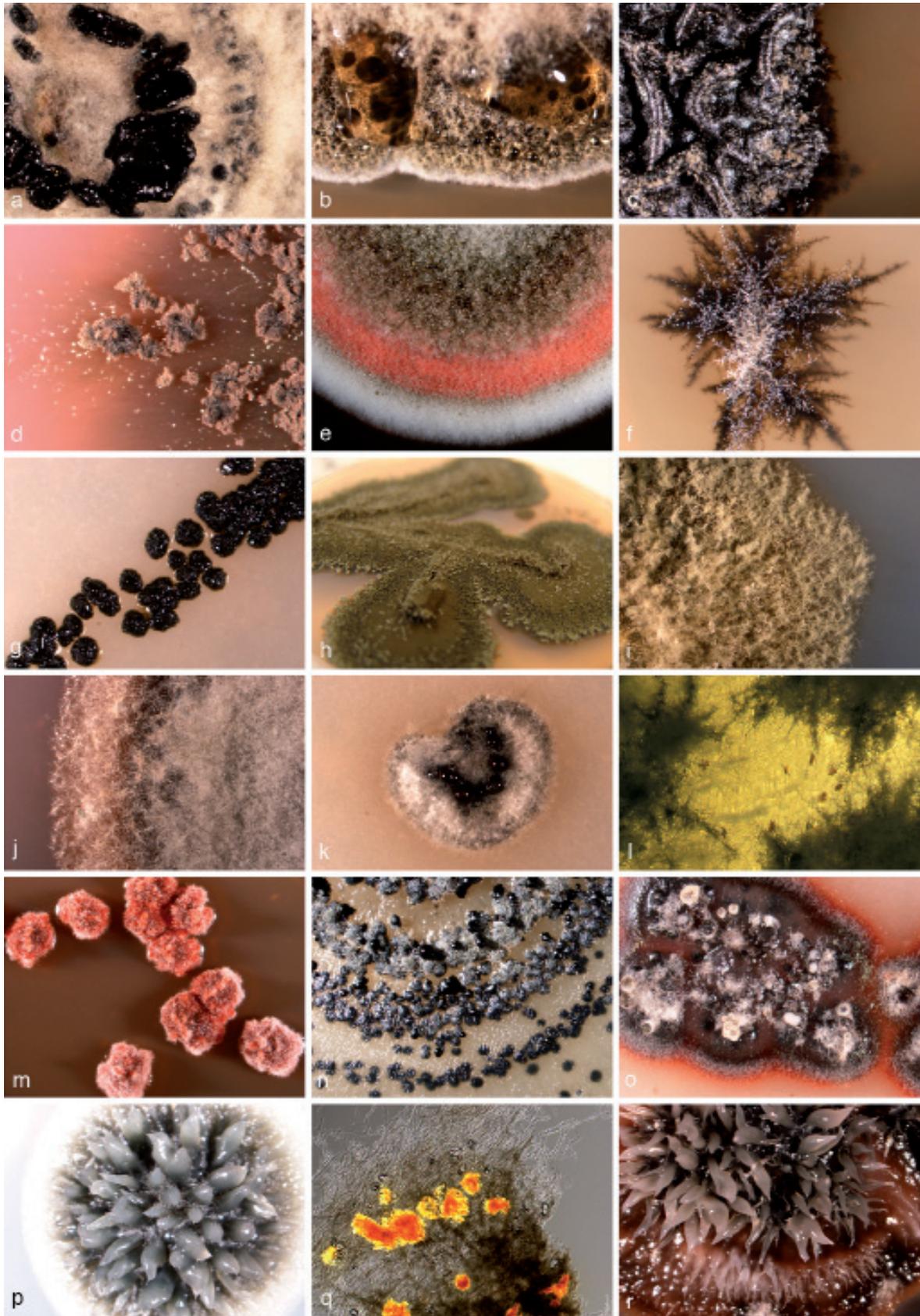
**Fig. 1** Disease symptoms associated with species of the *Mycosphaerella* complex. a. *Cercospora apii* leaf spots on *Apium graveolens* (M. Groenewald); b. *Cercospora beticola* spots on *Goniolimon tataricum* (S.G. Bobev); c. *M. sumatrensis* spot on *Eucalyptus*; d. *Teratosphaeria gauchensis* canker on *Eucalyptus* (M-N. Cortinas); e. red band needle blight of *Pinus* caused by *Dothistroma pini*; f. *Schizothyrium pomi* causing flyspeck of apple (J. Batzer); *T. verrucosa* spots on *Eucalyptus* (M.J. Wingfield); h. *Pseudocercospora lilacis* spot on *Ligustrum*; i. *T. fimbriata* spots on *Eucalyptus*; j. *Batcheloromyces protea* spot on *Protea*; k. *M. sphaerulinae* spot on *Eucalyptus*; l. *Passalora perplexa* blight of *Acacia* (M.J. Wingfield); m. *Mycosphaerella handelii* spot on *Rhododendron*; n. *Teratosphaeria parkii* spots on *Eucalyptus*; o. *Mycosphaerella fijensis* spot on *Musa*; p. *Mycosphaerella* spots on *Musa*; q. leaf spot with conidial cirri of *Septoria provencialis* on *Eucalyptus*; *Pseudocercospora griseola* causing angular leaf spot of *Phaseolus* (M.M. Liebenberg).



**Fig. 2** Teleomorphs. a. Ascomata of *M. gracilis*; b. squashed ascoma of a *Teratosphaeria* sp. on *Eucalyptus*; c. asci as arranged inside ascoma of *M. acaciigena*; d. asci of *Mycosphaerella* sp. occurring with *Cercospora acaciae-mangii*; e. asci of *M. gracilis*; f. asci of *T. toledana*; g. asci of *Teratosphaeria* sp. on *Eucalyptus*; h. asci of *T. pseudocryptica*; i. Ascus of *M. cussonia*; j. asci of *Davidiella tassiana*; k. ascus of *T. jonkershoekensis*; l. ascospores of *M. longibasalis*. — Scale bars: a = 80; b, d–h = 40; c, i–l = 10  $\mu$ m.



**Fig. 3** Ascospore germination patterns sensu Crous (1998). a. *Teratosphaeria cryptica* (Type A); b. *Mycosphaerella mozambica* (Type A); c. *M. gracilis* (Type B); d. *M. cussonia* (Type B); e. *Davidiella tassiana* (Type C); f. *T. alstairii* (Type C); g. *T. jonkershoekensis* (Type G); h. *M. elaeocarpi* (Type H); i. *M. graminicola* (Type D); j. *T. suberosa* (Type E); k. *M. parkii* (Type F); l. *T. nubilosa* (Type F); m. *T. africana* (Type G); n. *M. colombiensis* (Type J); o. *T. parva* (Type N). — Scale bars = 10  $\mu$ m.



**Fig. 4** Species of the *Mycosphaerella* complex in culture. a. *Teratosphaeria molleriana* on oatmeal agar (OA); b. *Stenella eucalypti* on malt extract agar (MEA); c. *Readeriella brunneotengens* on MEA; d. *Cibiessia minutispora* on OA; e. *Cercospora* sp. on MEA; f. *Cercospora ipomoeae* on OA; g. *Phaeothecoidea proteae* on OA; h. *Cibiessia dimorphospora* on PDA; i. *Teratosphaeria majorizuluensis* on MEA; j. *Teratosphaeria dendritica* on OA; k. *Phaeophleospora stonei* on OA; l. *Mycosphaerella heimii* on water agar (note crystals); m. *Ramularia* sp. on MEA; n. *Readeriella eucalypti* on OA; o. *Septoria* sp. on OA; p. *Teratosphaeria* sp. on OA; q. *Teratosphaeria alistairii* on potato-dextrose agar (note cysts); r. *Septoria proteae* on OA.

spp. commonly have colonies that are various shades of grey or olivaceous-grey on MEA, though taxa with cream to brown or red colonies also exist, and some form diffuse pigments in the agar (Fig. 4). Distinct differences exist between species in growth rate, temperature requirements for growth (Groenewald et al. 2005), presence of aerial mycelium and colony morphology (margins, colour, mycelium spreading or erumpent, chlamydo-spores, surface smooth or sectored, crystal formation; Crous 1998), formation of spermatogonia and (syn)anamorphs (Crous et al. 2007a, c), and smooth or verrucose nature of creeping hyphae. Aerial hyphae can vary completely from those occurring on the agar surface in texture, pigmentation, width, constriction at septa, etc.

## Anamorphic *Mycosphaerella* in the post-Chupp era

*Asteromella* is now commonly accepted as the spermatial state that occurs with species of *Mycosphaerella* when studied in culture, or on host material (Crous & Wingfield 1996), and it is possible that spermatial states have also been described as anamorphs in genera such as *Ascochyta*, *Asteroma* and *Phoma*. Most attention to date has been directed towards the hyphomycetous anamorphs of *Mycosphaerella*. Crous et al. (2000, 2001) listed 30 anamorph genera which had been linked to *Mycosphaerella* (Fig. 5, 6). Since then, many have been reduced to synonymy, namely *Uwebraunia* and *Dissoconium* (Crous et al. 2004a), *Paracercospora* and *Pseudocercospora* (Stewart et al. 1999), *Cercostigmina*, *Stigmina*, *Phaeoisariopsis* and *Pseudocercospora* (Crous et al. 2004b, 2006b, Braun & Crous 2006), *Ovularia*, *Ophiocladium* and *Ramularia* (Sutton & Waller 1988, Braun 1998, Crous et al. 2000, 2001). Several anamorph genera have been newly introduced to accommodate *Mycosphaerella* anamorphs, newly linked to *Mycosphaerella*, or newly introduced to accommodate anamorphs formerly linked to *Mycosphaerella* (Table 1). These include *Lecanosticta*, *Lecanostictopsis* (Sutton & Crous 1997, Verkley & Priest 2000), *Xenostigmina* (Crous 1998, Crous & Corlett 1998), *Metulocladosporiella* (Crous et al. 2006c), *Cladoriella* (Crous et al. 2006d), *Helgardia* (Crous et al. 2003a), *Batcheloromyces* (Taylor et al. 2003), *Cibiessiae*, *Phaeothecoidea*, (Crous et al. 2007c), *Pseudotaeniolina*, *Devriesia*, *Capnobotryella*, *Hortaea*, *Readeriella*, *Staninwardia*, *Penidiella* (Summerell et al. 2006, Crous et al. 2007a, c), *Rachicladosporium*, *Toxicocladosporium*, *Verrucocladosporium*, *Ochrocladosporium*, *Rhizocladosporium*, *Graphiopsis* (= *Dichocladosporium*) (Schubert et al. 2007a, Braun et al. 2008), *Zasmidium*, *Ramichloridium*, *Periconiella* (Arzanlou et al. 2007b), *Dothistroma* (Barnes et al. 2004, Groenewald et al. 2007), *Parapericoniella*, *Digitopodium* (Heuchert et al. 2005), *Trochophora*, *Verrucisporota* (Beilharz & Pascoe 2002, Crous et al. 2009a), *Baudoinia* (Scott et al. 2007), *Ramulispora* (Crous et al. 2003a), and *Colletogloeum* (Crous et al., unpubl. data).

Considerably fewer genera of coelomycetes have been linked to *Mycosphaerella*. Although the *Septoria* – *Phloeospora* – *Stagonospora* complex has not yet been resolved (Verkley & Priest 2000), other coelomycetous genera associated with the *Mycosphaerella* complex include *Lecanosticta* (Sutton & Crous 1997, Verkley & Priest 2000), *Phaeophleospora* (Crous et al. 1997, Crous 1998), *Colletogloeopsis* (Crous & Wingfield 1997), *Kirramyces* (Walker et al. 1992, Andjic et al. 2007), *Clypeispora* (Ramaley 1991), *Sonderhenia* (Park & Keane 1984, Swart & Walker 1988), *Readeriella*, *Staninwardia* and *Nothostrasseria* (Summerell et al. 2006, Crous et al. 2007a).

Most hyphomycetous genera linked to *Mycosphaerella* have

traditionally been dealt with as part of the cercosporoid complex (Table 1) (Braun, 1995, 1998, Crous & Braun 2003). These anamorph genera have been separated into more 'natural' or recognisable units based on features such as the presence or absence of superficial mycelium, and its texture. Conidiophore characteristics include arrangement, branching, pigmentation, conidiogenous cell placement, proliferation, scar type and conidial formation, shape, septation, wall texture and pigmentation. In most cases cercosporoid fungi have been treated as asexual fungi, and teleomorphs have been confirmed for only a few species. As is the case with their *Mycosphaerella* teleomorphs, cercosporoid fungi are associated with leaf spots, but can also cause necrotic lesions on flowers, fruits, bracts, seeds and pedicels of numerous hosts in most climatic regions. Furthermore, other than important pathogens of major agricultural crops, cercosporoid fungi are also known to be hyperparasitic to other plant pathogenic fungi (Shin & Kim 2001), and are also employed as biocontrol agents of alien weeds (Morris & Crous 1994, Den Breeÿen et al. 2006).

Chupp (1954) proposed a broad concept for the genus *Cercospora*, simply recording if hila were thickened or not, and if conidia were pigmented, single or in chains. As very little was known about the sexual states and relationships of cercosporoid fungi, Chupp chose a more practical approach by retaining all these taxa in *Cercospora*. Subsequent workers such as Deighton (1973, 1976, 1979, 1987, 1990) and Braun (1995, 1998) divided the *Cercospora*-complex into smaller, more morphologically similar units based on a combination of characters including conidiomatal structure (sporodochia, synnemata, etc.), mycelium (presence or absence of superficial mycelium and texture thereof), conidiophores (arrangement, branching, pigmentation and ornamentation), conidiogenous cells (placement, proliferation and scar type) and conidia (formation, shape, septation, ornamentation, pigmentation and catenulation).

The abandonment of the 'Chupp concept' has resulted in close to 50 genera being recognised in this complex (Braun 1995, Crous & Braun 2003). One of the reasons for this was the strict interpretation of the numerous conidiogenous events as defined by Sutton and Hennebert (1994), as well as the additional characters discussed above. Several anamorph genera have been found to have species with conidiomata varying from mononematous, scattered conidiophores to sporodochia with a basal stroma, or from pycnidia to sporodochia and synnemata. Based on similar observations Sutton (1980) and Nag Raj (1993) saw the need to abandon the distinction between hyphomycetes and coelomycetes, as acervuli were frequently found to form a continuum with more stromatic, sporodochial forms. If this plasticity is taken into consideration when examining the 23 anamorph genera accepted by Crous et al. (2000), many appear superfluous. However, recent phylogenetic studies have shown that many of the current generic concepts are represented as paraphyletic clades within some families in the *Capnodiales* (e.g. *Mycosphaerellaceae* or *Teratosphaeriaceae*) (Arzanlou et al. 2007b, Crous et al. 2007a), suggesting that some of these anamorph concepts still represent more than one genus. In other families in the order, such as *Schizothyriaceae* (Batzer et al. 2008) and *Davidiellaceae* (Crous et al. 2007b, Schubert et al. 2007a, b), this appears not to be the case, and the teleomorph is thus far linked to a single anamorph.

Characters such as the presence or absence of superficial mycelium, the formation of stromata, conidiomatal structure (conidiophores solitary, fasciculate to synnematos, sporodochia to pycnidia and acervuli), conidial shape, size and septation (even eusepta vs. distosepta), as well as solitary vs. catenate conidia,

saprobic, hyperparasitic and phytopathogenic habit, were rejected as single characters at the generic level by Crous & Braun (2003). These recent findings suggest, however, that all these characters again need to be re-evaluated in light of novel DNA data.

From these studies it was shown that most of these cercosporoid genera (with the possible exception of *Cercospora* and *Ramularia*), evolved more than once in the *Mycosphaerellaceae*. The majority of the 'anamorph genera' linked to *Mycosphaerella* in the broad sense, therefore, represent several phylogenetic units, e.g. *Pseudocercospora*, *Passalora*, *Septoria* and *Stenella*. To reduce the number of novel anamorph genera being introduced, Crous et al. (2007a) accepted the concept of paraphyletic anamorph genera within a specific family. This approach, however, has not been widely accepted, which means that many more genera will be introduced as *Mycosphaerella* is further separated into natural units. Teleomorph, as well as anamorph characters will have to be re-evaluated. The characters used by Crous & Braun (2003) to delineate anamorph genera still apply, namely the structure of conidiogenous loci (scars) and hila, and the presence or absence of pigmentation in conidiophores and conidia. In cases where genera are paraphyletic, however, these characters require further refinement.

## DNA phylogeny of *Mycosphaerella* species complexes on different hosts

Although the *Mycosphaerella* complex accommodated several thousand species, very few are known from culture. Largely due to the lack of cultures, the first DNA phylogeny paper on *Mycosphaerella* was that published by Stewart et al. (1999). Based on ITS phylogenetic data, subsequent workers (Crous et al. 1999, Goodwin et al. 2001) concluded that *Mycosphaerella* was monophyletic. This research was continued by Crous et al. (2000, 2001), wherein the anamorph concepts were re-evaluated, and based on the limited number of species available, most genera were shown to represent well-defined clades within *Mycosphaerella*. Once multi-gene data were employed (Hunter et al. 2006, Schoch et al. 2006, Arzanlou et al. 2007b, Crous et al. 2007a, b, Batzer et al. 2008), *Mycosphaerella* was shown to be polyphyletic, and the well-defined anamorph genera were shown to have evolved in several clades, within and outside the order, suggesting that in many cases the generic circumscriptions would have to be revised.

DNA phylogenetic techniques further revealed, that for all hosts investigated, there were a surprisingly high number of novel species. This was true for example on *Citrus* (Pretorius et al. 2003), *Acacia* (Crous et al. 2004b), *Chromolaena* (Den Breeÿen et al. 2006), *Eucalyptus* (Crous et al., 2004a, 2006e, 2007c, Cheewangkoon et al. 2008), *Zea mays* (Crous et al., 2006a), *Encephalartos* (Crous et al. 2008b), *Proteaceae* (Crous et al. 2008a), and *Musa* (Arzanlou et al. 2008), to name but a few. From these various studies, the same pattern emerged, namely that many morphologically similar species occur on the same host, and that based on morphology alone, it is typically very difficult or impossible to distinguish them. What this in turn implies for species numbers, is that in coming years there will be a significant expansion in the number of novel taxa described, and that the *Mycosphaerella* complex accommodates far more species than the 10 000 taxa described to date.

## Host specificity in *Mycosphaerella*

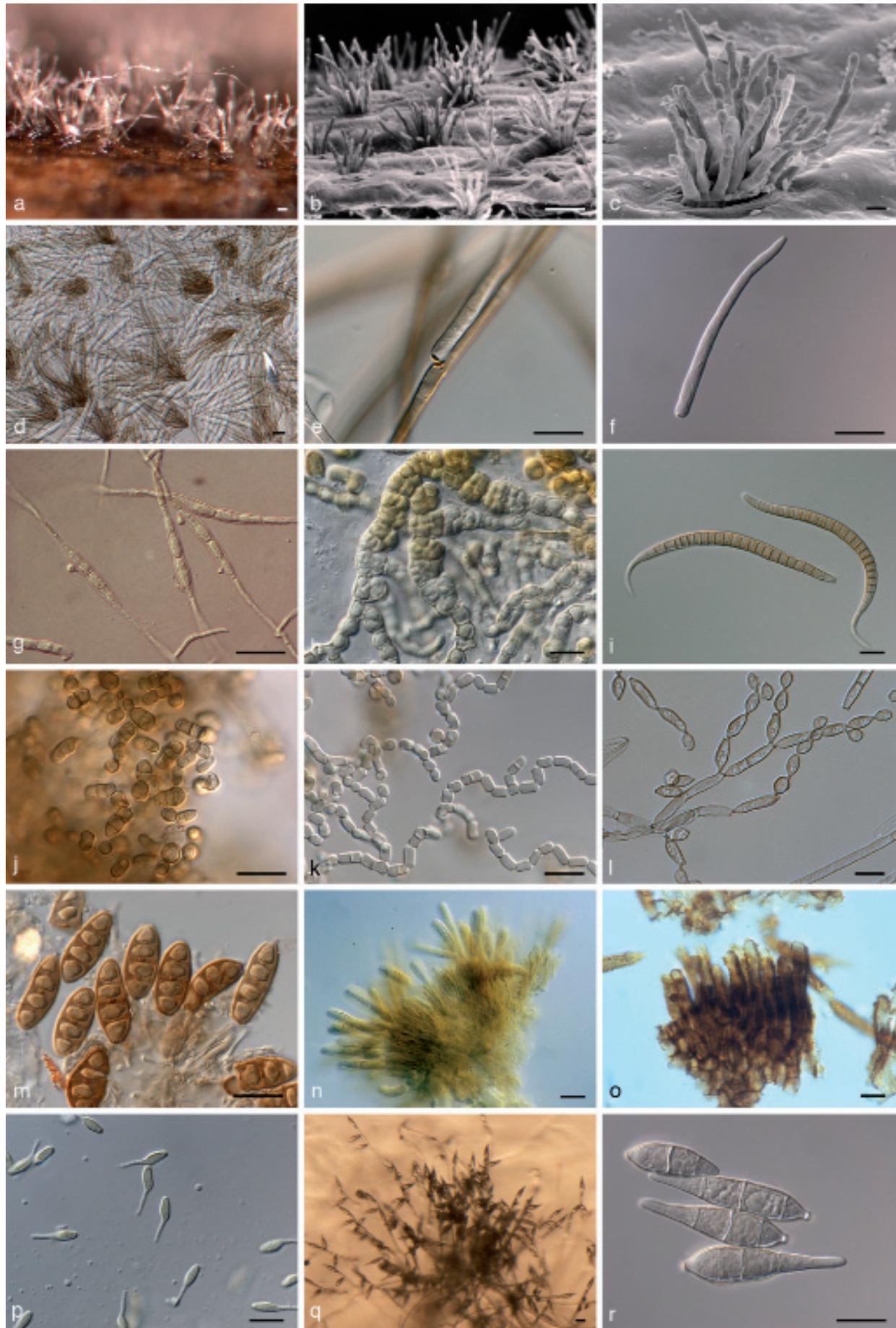
A significant problem pertaining to the taxonomy of *Mycosphaerella* is the degree of host specificity of the various species. Most species are still defined based on host, and they are assumed to be host-specific or restricted at least to a family of phanerogamic plants (Chupp 1954, Corlett 1991, Braun 1995). However, the tenability of many species may be called into question because some taxa, including *M. punctiformis*, the type species of *Mycosphaerella*, have been shown to be non-host specific (Verkley et al. 2004).

Although many may be host specific, some *Mycosphaerella* species are able to colonise different and even unrelated hosts. In some cases this appears to be due to the endophytic nature of these fungi (Crous 1998, Verkley et al. 2004), while in others species appear to actively undergo host shifts in the process of locating their ideal hosts (Crous et al., 2004b; Crous & Groenewald, 2005). Crous et al. (2008a) reported that many host-specific necrotrophic pathogenic species of *Mycosphaerella* and *Teratosphaeria* appeared to also exhibit a facultative saprobic behaviour. It was concluded, therefore, that the definitions of 'necrotroph' or 'saprobe' do not clearly define all species of *Mycosphaerella* and *Teratosphaeria*, as some have obviously retained the ability to also grow on dead tissue when they lose the connection to their real host.

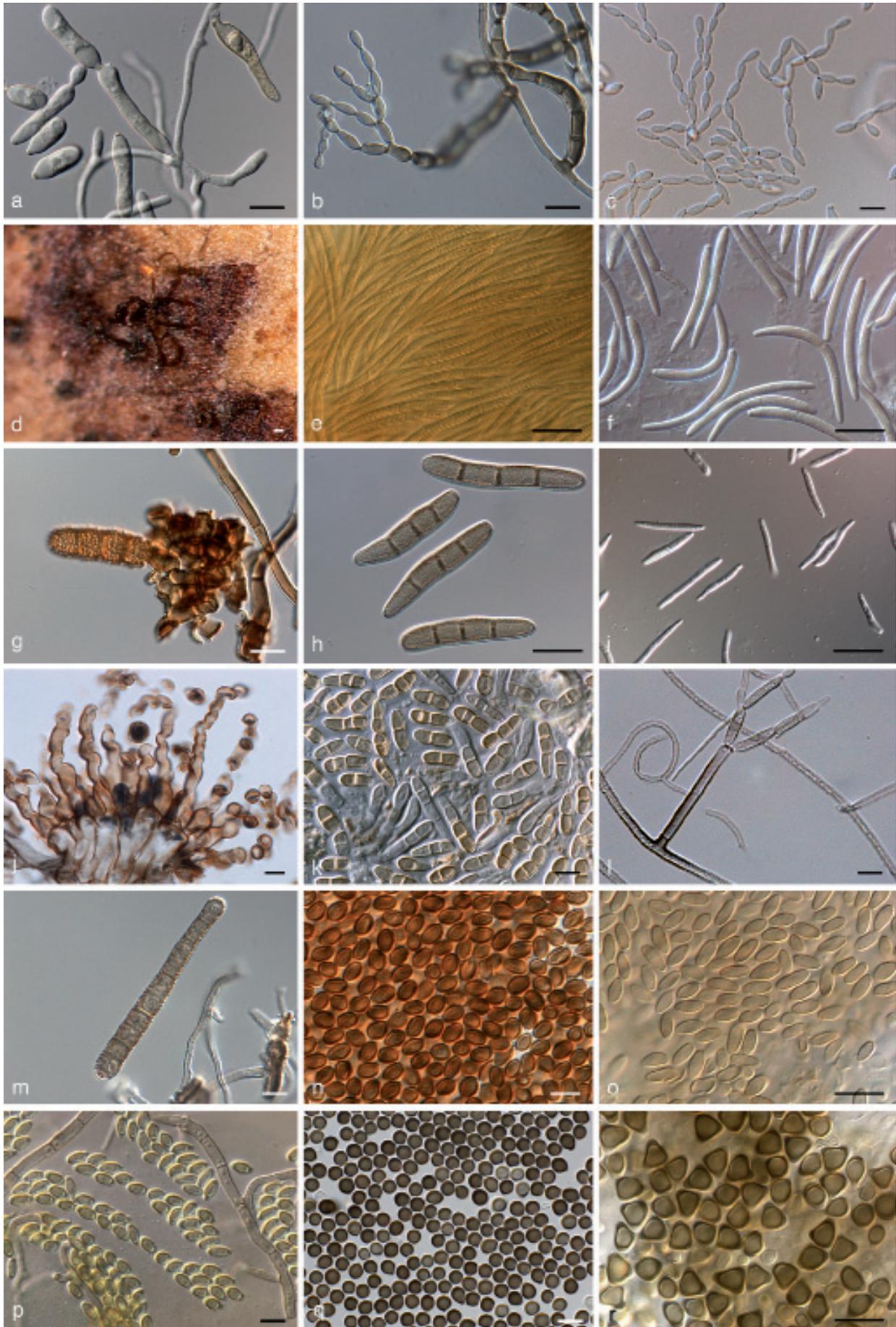
In many instances, species of *Mycosphaerella* with wide host ranges are morphologically indistinguishable, such as those in the *Cercospora apii* complex (Groenewald et al. 2005). In the genus *Cercospora*, however, several species are known that are highly host-specific, and thus there appears to be no general rule regarding this ecological trait (Groenewald et al. 2006a). The fact that many species can co-occur in the same lesion or leaf spot, the so called 'co-occurrence phenomenon' (Crous et al. 2009b), adds a new level of complexity to the isolation of these fungi, suggesting that only those strains that are fertile in culture, can be confirmed as representing the fungus studied on host material.

The majority of the plant pathogenic species of *Mycosphaerella* are thought to be host-specific (Goodwin et al. 2001, Crous & Groenewald 2005, Groenewald et al. 2006a, Stukenbrock et al. 2007), such as *M. fijiensis*, *M. musicola* and *M. eumusae* on banana (Arzanlou et al. 2008) and *M. graminicola* on wheat (Stukenbrock et al. 2009). In contrast, Crous et al. (2009b) reported several species to occur on multiple hosts, namely: *M. communis* (on *Eucalyptus* in South Africa, Spain, New Zealand, *Musa* in Trinidad, *Protea magnifica* in Australia), *M. kонаe* (*Leucospermum* in Hawaii, *Eucalyptus* in Thailand) (Crous et al. 2007c), *M. marksii* (*Eucalyptus*, Australia, Bolivia, China, Ecuador, Ethiopia, Papua New Guinea, New Zealand, South Africa, Spain, Tanzania, Uruguay, *Leucadendron* on the Madeira Islands, and *Musa* in Mozambique) (Arzanlou et al. 2008), *T. associata* (*Eucalyptus* and *Protea* in Australia) (Summerell et al. 2006, Crous et al. 2007c), *T. parva* (*Eucalyptus* in Australia, Chile, Ethiopia, Portugal, South Africa, Spain, and *Protea* in South Africa), *T. nubilosa* (*Eucalyptus* in Australia, New Zealand, Europe, South America, and *Acacia* in Thailand (Crous & Groenewald 2005, Hunter et al. 2008), and *M. citri* (*Musa* in Florida, *Acacia* in Thailand, and *Eucalyptus* in Vietnam, and *Aeglopsis*, *Citrus*, *Fortunella*, *Murraya*, and *Poncirus* in North and South America, as well as Asia (Pretorius et al. 2003, Crous et al. 2004a, b, Crous & Groenewald 2005, Burgess et al. 2007).

The genetics of host-specificity of well-known pathogens such as *M. graminicola* has been studied extensively. For example, Banke et al. (2004) demonstrated that this species infects only



**Fig. 5** Anamorphs associated with the *Mycosphaerella* complex. a–d. Fascicles of *Cercospora zeina*; e. Conidiophore giving rise to conidium of *Cercospora* sp.; f. conidium of *Cercospora* sp.; g. macro and microconidia of *Dissoconium dekkeri* anamorph of *M. lateralis*; h. hyphae with endoconidia of *Phaeothecoidea eucalypti*; i. conidia of *Phaeophleospora eugeniae*; j. conidia of *Batcheloromyces leucadendri*; k. conidia of *Ciblicesia dimorphospora*; l. conidiophore of *Cladosporium sphaerospermum*; m. conidia of *Sonderherenia eucalypticola* anamorph of *M. walkeri*; n. conidiophores of *Pseudocercospora punctata* anamorph of *M. syzygii*; o. conidiophores of *Lecanostictopsis syzygii*; p. conidia of *Nothostrasseria dendritica* anamorph of *T. dendritica*; *Passalora* sp. sporulating in culture; r. pigmented conidia of *Passalora* sp. with thickened hila. — Scale bars = 10 µm, except d = 40 µm.



**Fig. 6** Anamorphs associated with the *Mycosphaerella* complex. a. Conidia of *Passalora fulva*; b. conidiophore of *Penidiella* anamorph of *Teratosphaeria encephalarti*; c. conidia of *Ramularia eucalypti*; d. exuding cirrus of *Kirramyces* anamorph of *T. suttonii*; e. conidia of *Kirramyces destructans*; f. conidia of *K. eucalypti*; g. conidium of *Stigmia eucalypti*; h. conidia of *Sonderhenia eucalyptorum* anamorph of *M. swartii*; i. conidia of *Septoria eucalyptorum*; j. conidiophore of *Polythrincium trifolii*, anamorph of *Cymadothea trifolii*; k. conidia of *Staninwardia suttonii*; l. conidiophore of *Stenella* sp.; m. conidium of *Verrucisporota grevilleae*; n. conidia of *T. verrucosa*; o. conidia of *T. gauchensis*; p. conidia of *Readeriella readeriellophora*; q. conidia of *R. eucalypti*; r. conidia of *R. mirabilis*. — Scale bars = 10  $\mu$ m, except d = 40  $\mu$ m.

**Table 1** Anamorph genera linked to *Mycosphaerellaceae* (M) and *Teratosphaeriaceae* (T)<sup>1</sup>.

Genus	Conidiomata <sup>2</sup>	Synanamorph	Proliferation <sup>3</sup>	Colour <sup>4</sup>	Conidia				Reference
					Conidial septation	Loci <sup>5</sup>	Arrangement <sup>6</sup>	Mycelium <sup>7</sup>	
<i>Batcheloromyces</i> (M)	S	catenulostroma-like	P	P	0–3	I	S,C	I,E	Taylor et al. 2003
<i>Baudoinia</i> (T)	Ph	–	Ph	P	0–1	I	C	E	Scott et al. 2007
<i>Capnobotryella</i> (T)	Ph	Endoconidia	Ph	P	0–1	I	C	E	Sugiyama & Amano 1987
<i>Catenulostroma</i> (T)	A,S,F	–	Ph	P	0-multi	I	S,C	I,E	Crous et al. 2007a
<i>Cercospora</i> (M)	F	–	S	H (conidia)	0-multi	T,D,R	S	I	Crous & Braun 2003
<i>Cercosporella</i> (M)	F	–	S	P (conidioph.) H	multi	T,R	S	I	Braun 1995
<i>Cibiessiae</i> (T)	Ph	readeriella-like	Ph	P	1–3	I	C	I,E	Crous et al. 2007c
<i>Clypeispora</i> (?)	P	–	M	H	0	I	S	I	Ramaley 1991
<i>Colletogloeopsis</i> (T)	A/P	–	P,S	P	0–1	I	S	I,E	Crous & Wingfield 1997
<i>Davisoniella</i> (T)	M	coelomycete	P	P	0	I	S	I	Crous et al. 2006e
<i>Devriesia</i> (T)	Sol	Chlamydo-spores	S	P	0–3	T,D	C	E	Seifert et al. 2004
<i>Dothistroma</i> (M)	A	–	P,S	H	1–5	I	S	I	Barnes et al. 2004
<i>Hortaea</i> (T)	Sol	–	P,M	H (conidia) P (hyphae)	0–2	I	S	I,E	Bonifaz et al. 2008, Plemenitaš et al. 2008
<i>Kirramyces</i> (T)	P	pseudocercospora-like	P,S	P	0-multi	I	S	I	Andjic et al. 2007
<i>Lecanosticta</i> (M)	A	–	P	P	0-multi	I	S	I	Suto & Ougi 1998
<i>Miuraea</i> (M)	F,Sol	–	S	H,P	muriform, multi	I	S	I,E	von Arx 1983
<i>Nothostrasseria</i> (T)	P	–	M	P	0	I	S	I	Crous et al. 2007c
<i>Passalora</i> (M)	F,S,Sol	–	S	P	0-multi	T,D,R	S,C	I,E	Crous & Braun 2003
<i>Penidiella</i> (T)	Sol,F,Syn	–	S	P	0–1	I or T,D	C	I,E	Crous et al. 2007a

**Table 1** (continued) Anamorph genera linked to *Mycosphaerellaceae* (M) and *Teratosphaeriaceae* (T)<sup>1</sup>.

Genus	Conidiomata <sup>2</sup>	Synanamorph	Proliferation <sup>3</sup>	Colour <sup>4</sup>	Conidia				Reference
					Conidial septation	Locis <sup>5</sup>	Arrangement <sup>6</sup>	Mycelium <sup>7</sup>	
<i>Periconiella</i> (M)	Sol	–	S	P	0-multi	T,D	C	I,E	Arzanlou et al. 2007b
<i>Phaeophleospora</i> (M)	P	–	P	P	0-multi	I	S	I	Crous et al. 1997
<i>Phloeospora</i> (M)	A	–	S	H	multi	I	S	I	Sivanesan 1984
<i>Phaeothecoidea</i> (T)	En	–	En	P	0–2	I	S	I,E	Crous et al. 2007c
<i>Pseudocercospora</i> (M)	F,S,Sol,Syn	<i>Stigmina</i>	S	P	1-multi	I	S,C	I,E	Deighton 1976
<i>Pseudocercosporella</i> (M)	F,S,Sol	–	S	H	1-multi	I	S,C	I,E	Braun 1998
<i>Pseudotaeniolina</i> (T)	Sol	–	Ph	P	0–2	I	C	I,E	Crane & Schoknecht 1986
<i>Ramichloridium</i> (M)	Sol	–	S	P	0–1	D	S	I,E	Arzanlou et al. 2007b
<i>Ramularia</i> (M)	F,S,Sol	–	S	H	0–5	T,D,R	S,C	I,E	Braun 1998
<i>Ramulispora</i> (M)	S	Chlamydo-spores	S	H	0-multi	I	S	I,E	Crous et al. 2003
<i>Readeriella</i> (T)	P	–	P,M	P	0	I	S	I	Crous et al. 2007a
<i>Septoria</i> (M)	P/A	–	S	H	1-multi	I	S	I	Von Arx 1983
<i>Sonderhenia</i> (M)	P	–	P	P	0–5	I	S	I	Swart & Walker 1988
<i>Staninwardia</i> (T)	A	–	P	P	1–2	I	C	I	Summerbell et al. 2006
<i>Stenella</i> (T)	F,Sol	scytalidium-like	S	P	0-multi	T,D,R	S,C	I,E	Sivanesan 1984
<i>Trochophora</i> (M)	F,Sol	–	S	P	3	I	S	I,E	Zhao et al. 2007
<i>Verrucisporota</i> (M)	F,Sol	–	S	P	0-multi	T,D,R	S,C	I,E	Crous et al. 2009a
<i>Zasmidium</i> (M)	F,Sol	–	S	P	0-multi	T,D,R	S,C	I,E	Arzanlou et al. 2007b

<sup>1</sup>*Asteromella* spermatial states have also been described in *Ascochyta*, *Asteroma*, *Phyllosticta* and *Phoma*. Excluded genera are *Dissoconium* (= *Uwebraunia*), *Cladosporium* (*Davidiellaceae*), *Thezogonia* (*Helotiales*), *Xenostigmina* (*Pleosporales*). *Mycovellosiella* and *Phaeoramularia* are treated as synonyms of *Passalora*; *Phaeoisariopsis*, *Paracercospora* and *Stigmina* as synonyms of *Pseudocercospora*.

<sup>2</sup>Fasciculate (F), sporodochial (S), solitary (Sol), pycnidial (P), acervular (A), synnematus (Syn), phragmosporous (Ph), hyphae with endoconidia (En), multilocular (M).

<sup>3</sup>Sympodial (S), percurrent (P), monoblastic, determinate (M), phragmospores (Ph), endoconidia (En).

<sup>4</sup>Hyaline (H), pigmented (P).

<sup>5</sup>Thickened (T), darkened (D), refractive (R), protruding (P), inconspicuous (I).

<sup>6</sup>Solitary (S), chains (C).

<sup>7</sup>Internal (I), external (E).

bread wheat and durum wheat. Of interest, however, is the fact that based on certain genes, durum wheat isolates of *Mycosphaerella* clearly separate from bread wheat isolates (Groenewald & Crous, unpubl. data), suggesting that at some stage, these were two distinct species infecting these hosts. Among the *Mycosphaerella* species infecting *Eucalyptus*, some species such as *Teratosphaeria cryptica* (syn. *M. cryptica*) have a broad host range and cause disease on 38 species across the *Eucalyptus* sub-genera *Monocalyptus* and *Symphyomyrtus*, while *T. nubilosa* shows a more narrow host range, infecting only 12 *Eucalyptus* species and a few hybrids within the subgenus *Symphyomyrtus* (Park et al. 2000, Maxwell et al. 2005, Hunter et al. 2008).

To successfully manage and control plant disease epidemics, a thorough understanding of the genetic variation and epidemiology of the causal agent(s) is required. Because *Mycosphaerella* species are morphologically similar, are not necessarily host specific, and several species could co-occur in the same lesion, fungal identification and the choice of subsequent control regimes is not always straight forward. PCR-based techniques have in recent years contributed greatly to disease diagnosis and detection, and have also successfully been employed in the early detection of *Mycosphaerella* infections (Waalwijk et al. 2004, Lievens et al. 2005, Arzanlou et al. 2007a).

## Sex in *Mycosphaerella*

Ascomycetes with both a sexual and asexual reproductive cycle are haploid for the majority of their lifecycle (Heitman 2006). During the short phase of sexual reproduction, they become dikaryotic, and diploid. Sexual reproduction in fungi involves meiosis, which is preceded by the fusion of two cells (plasmogamy), followed by fusion of the two parental nuclei (karyogamy). Sexual reproduction together with mutation, recombination and natural selection are major forces that drive evolution (Heitman 2006, Zhan et al. 2009). It is generally accepted that asexual reproduction generates genetically identical clones, though the role of the parasexual cycle should not be underestimated, as anastomosis between different mycelial types will again influence the genetic makeup of eventual progeny. Conidia can result from fragmentation of hyphal cells (frequently observed in aerial mycelium of *Mycosphaerella* species), or via the production of conidia in naked (hyphomycete) or enclosed (coelomycete) fruiting bodies. Some species of *Mycosphaerella* form several anamorphs (synanamorphs), including hyphomycetes and coelomycetes (Crous et al. 2007a), which enables them to better utilise changing environmental conditions, ensuring optimal spore production and dispersal. Detailed studies on sexual reproduction in fungi may provide better insights into genetic regulation and evolution of closely related taxa (Turgeon 1998, McDonald & Linde 2002, Conde-Ferraz et al. 2007).

In the absence of selection pressure, asexual reproduction dominates populations. Changes in the availability of food resources, environmental conditions and other selection pressures favour a shift towards the sexual reproduction cycle (Heitman 2006, Zhan et al. 2007). In fungi like *Neurospora crassa*, individuals are hermaphrodites, producing both male and female reproductive structures. Sexual exchange of genetic material relies on the existence of simple cell recognition mechanisms that stimulate out-crossing. The term 'mating type' defines sexually compatible individuals. Heterothallism (self-sterility), occurs between two fungal strains with a compatible mating system. In contrast, homothallism (self-fertility) is where a single isolate can complete a successful

sexual cycle. Pseudohomothallism or secondary homothallism occurs in some ascomycetes such as *Neurospora tetrasperma*, *Podospora anserina* and *Gelasinospora tetrasperma* (Merino et al. 1996), where self-fertile ascospores carry nuclei of both mating types.

In fungi sexual development is controlled by mating type loci, which contain a number of genes which occupy a continuous region on a chromosome (Debuchy & Turgeon 2006). In ascomycetes, sexual development is controlled by a single mating type locus (*MAT*). This mating type locus contains one of two forms of dissimilar sequences occupying the same chromosomal position, termed 'idiomorph' in fungal species with a heterothallic mating strategy (Metzenberg & Glass 1990). Complementary idiomorph isolates are referred to as *MAT1-1* and *MAT1-2* mating strains (Turgeon & Yoder 2000).

Although *Mycosphaerella* contains several thousand species, the mating behaviour of most species has not been resolved. Although some species have been observed to be either homo- or heterothallic, pseudohomothallism has not yet been reported for any *Mycosphaerella* species. By continuing the research done on the heterothallic mating system of *M. graminicola*, the mating behaviour of several apparently asexual species of *Cercospora* has been clarified (Groenewald et al. 2006b). Much attention was also devoted to the elucidation of the mating systems active in the *Mycosphaerella* spp. occurring on banana (Conde-Ferraz et al. 2007, Arzanlou et al., in prep.). Using the same approach our knowledge for other *Mycosphaerella* species such as *Passalora fulva* (Stergiopoulos et al. 2007), *Dothistroma septosporum* and *Dothistroma pini* (Groenewald et al. 2007), and *Septoria passerinii* (Ware et al. 2007) has also been extended. From these data it is clear that sex is active in several apparently asexual species of *Mycosphaerella*, and in species where the teleomorph is seldom observed. Furthermore, a study of the genes and ORFs involved in these mating type loci suggest that this is an area of research that will be rewarding to pursue more in depth, and this will also be one of the main focus areas of my research in the coming years. This research will also link to the activities surrounding the whole genome sequences of *M. graminicola* and *M. fijiensis* that are now becoming available for study (<http://genome.jgi-psf.org/Mycf11/Mycf11.home.html>).

## How do we deal with the poly- and paraphyletic nature of *Mycosphaerella* and its anamorphs?

Early phylogenetic trees treating *Mycosphaerella* were based on ITS DNA sequence data, and these suggested that the genus is monophyletic (Crous et al. 1999, 2000, 2001, Stewart et al. 1999, Goodwin et al. 2001). However, once additional loci were included in later analyses, it was shown that *Mycosphaerella* is polyphyletic (Hunter et al. 2006, Crous et al. 2007a). This complex has in recent years been separated into *Davidiella* species with *Cladosporium* anamorphs (*Davidiellaceae*) (Braun et al. 2003, Crous et al. 2007b, Schubert et al. 2007a, b, Zalar et al. 2007, Dugan et al. 2008), *Schizothyrium* species with *Zygothiala* anamorphs (*Schizothyriaceae*) (Batzer et al. 2008), *Teratosphaeria* species with many anamorphs (*Teratosphaeriaceae*) (Crous et al. 2007a, c), and *Mycosphaerella* species, also with numerous anamorph genera (*Mycosphaerellaceae*) (Crous & Braun 2003), all belonging to the *Capnodiales* in the *Dothideomycetes* (Schoch et al. 2006). Although *Davidiella* (*Cladosporium*) and *Schizothyrium* (*Zygothiala*) have a clear one to one relationship with anamorph genera, this is

far from true for *Mycosphaerella* and *Teratosphaeria*, where the teleomorph morphology is relatively conserved throughout the two families. Here the same anamorph morphology has evolved in different clades, and in some cases also outside the families (Crous et al., unpubl. data).

The option of accepting anamorph genera as paraphyletic concepts within the family and order (Arzanlou et al. 2007b, Crous et al. 2007a), has not been widely accepted by the scientific community (see Cortinas et al. 2006, Andjic et al. 2007, Crous et al. 2007a, 2009b). This suggests that new generic names need to be provided for distinct lineages, and novel morphological characters be identified to distinguish them. In order to halt the unnecessary proliferation of generic names, it would thus be preferable to not continue with dual nomenclature, i.e. to use a single generic name per unambiguous phylogenetic lineage. What this would imply, is that in several clades, where anamorph generic names are already available, preference will have to be given to anamorph names to try and achieve a more natural classification among the genera in these families. The greatest challenge, however, is to obtain a workable system, where morphological data can still be used to separate genera in what is presently seen as *Mycosphaerella* s.lat., as *Mycosphaerella* s.str. needs to be confined to those taxa with *Ramularia* anamorphs.

The name *Mycosphaerella* has been confused in the past, and used widely for numerous genera not congeneric with the type species, *M. punctiformis*. If a single generic name is to be used for this '*Mycosphaerella*' clade, the older generic name, *Ramularia* (1833) may be preferable to *Mycosphaerella* (1884); thus *Ramularia endophylla*, not *M. punctiformis*, though both names would remain available, unless this change is implemented via some formal modification of the International Code of Botanical Nomenclature, giving preference to older generic names, irrespective of their sex.

## Conclusions

The genus *Mycosphaerella* is commonly known as the largest genus of *Ascomycetes*, containing over 10 000 taxa if anamorph states are included. This assumption has been shown to be false, as *Mycosphaerella* is now known to be para- and polyphyletic. Furthermore, *Mycosphaerella* s.str has been shown to be confined to taxa with *Ramularia* anamorphs, representing approximately 1000 species. In spite of this narrower circumscription of the genus, major taxonomic challenges remain unresolved. The teleomorph has been shown to be morphologically conserved throughout the family, while minute differences in anamorphs are indicative of different genera, complicated by the phenomenon of synanamorph states that commonly develop in culture, as well as on host material. Although much attention has in recent years been focused on hosts in the *Myrtaceae* and *Proteaceae*, a few preliminary studies on other hosts have indicated that most host plants have a rich representation of underscribed species in the *Mycosphaerella* complex. This aspect is further complicated by the fact that many of these taxa can co-occur on the same lesion, and have the ability to colonise non-host tissue, in an attempt to locate their ideal host to which they are pathogenic. Preliminary studies on their mating types and sexual behaviour have also indicated that some species are having cryptic sex, and that the teleomorph is present, though seldom or not yet observed. These findings are also relevant for the import and export of agricultural and forestry produce, as for some species either one or both mating types have been introduced to different continents, suggesting that quarantine regulations also need to focus below the species level on clones and mating types.

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### Articles included for DSc dissertation

Over the past 20 years I have published 114 papers and two books dealing with the genus *Mycosphaerella* and its associated anamorphs. The papers included in this DSc represents a selection of papers published from 2003 onwards. In 2003 the paper by Braun et al. (2003) separating *Davidiella* (*Cladosporium*) from the *Mycosphaerella* complex was published, representing the onset of a new approach to the taxonomy of this group of organisms. All selected papers deal with species of the *Mycosphaerella*-complex known from culture, and are supported by molecular phylogenetic data. Initially these studies were largely based on phylogenies of the ITS rDNA region to resolve taxa occurring on specific plant hosts. In certain anamorph groups, however, the ITS domain has provided insufficient resolution to enable me distinguish all taxa, and a multi-gene approach needed to be employed. Other papers incorporate sequence data of the LSU and SSU genes to enable the various generic issues within the *Mycosphaerella* complex to be addressed. In recent years I have also developed a focus on the sexual behaviour of *Mycosphaerella*, and the first papers dealing with sex in some prominent sexual and asexual members of the *Mycosphaerella* complex have thus also been included.

1. Arzanlou M, Groenewald JZ, Gams W, Braun U, Shin H-D, Crous PW. 2007. Phylogenetic and morphotaxonomic revision of *Ramichloridium* and allied genera. *Studies in Mycology* 58: 57–93. [own contribution 40%]
2. Arzanlou M, Groenewald JZ, Fullerton RA, Abeln ECA, Carlier J, Zapater M-F, Buddenhagen IW, Viljoen A, Crous PW. 2008. Multiple gene genealogies and phenotypic characters differentiate several novel species of *Mycosphaerella* and related anamorphs on banana. *Persoonia* 20: 19–37. [own contribution 40%]
3. Barnes I, Crous PW, Wingfield BD, Wingfield MJ. 2004. Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma*, *D. septosporum* and *D. pini*. *Studies in Mycology* 50: 551–565. [own contribution 30%]
4. Batzer JC, Mercedes DiazArias M, Harrington TC, Gleason ML, Groenewald JZ, Crous PW. 2008. Four species of *Zygothia* (*Schizothyriaceae*, *Capnodiales*) are associated with the sooty blotch and flyspeck complex on apple. *Mycologia* 100: 246–258. [own contribution 40%]
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7. Conde-Ferrández L, Waalwijk C, Canto-Canché BB, Kema GHJ, Crous PW, James AC, Abeln ECA. 2007. Isolation and characterization of the mating type locus of *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease of banana. *Molecular Plant Pathology* 8: 111–120. [own contribution 20%]
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# Phylogenetic and morphotaxonomic revision of *Ramichloridium* and allied genera

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**Abstract:** The phylogeny of the genera *Periconiella*, *Ramichloridium*, *Rhinocladiella* and *Veronaea* was explored by means of partial sequences of the 28S (LSU) rDNA gene and the ITS region (ITS1, 5.8S rDNA and ITS2). Based on the LSU sequence data, ramichloridium-like species segregate into eight distinct clusters. These include the *Capnodiales* (*Mycosphaerellaceae* and *Teratosphaeriaceae*), the *Chaetothyriales* (*Herpotrichiellaceae*), the *Pleosporales*, and five ascomycete clades with uncertain affinities. The type species of *Ramichloridium*, *R. apiculatum*, together with *R. musae*, *R. biverticillatum*, *R. cerophilum*, *R. verrucosum*, *R. pini*, and three new species isolated from *Strelitzia*, *Musa* and forest soil, respectively, reside in the *Capnodiales* clade. The human-pathogenic species *R. mackenziei* and *R. basitonum*, together with *R. fasciculatum* and *R. anceps*, cluster with *Rhinocladiella* (type species: *Rh. atrovirens*, *Herpotrichiellaceae*, *Chaetothyriales*), and are allocated to this genus. *Veronaea botryosa*, the type species of the genus *Veronaea*, also resides in the *Chaetothyriales* clade, whereas *Veronaea simplex* clusters as a sister taxon to the *Venturiaceae* (*Pleosporales*), and is placed in a new genus, *Veronaeopsis*. *Ramichloridium obovoideum* clusters with *Carpoligna pleurothecii* (anamorph: *Pleurothecium* sp., *Chaetosphaeriales*), and a new combination is proposed in *Pleurothecium*. Other ramichloridium-like clades include *R. subulatum* and *R. epichloë* (*incertae sedis*, *Sordariomycetes*), for which a new genus, *Radulidium* is erected. *Ramichloridium schulzeri* and its varieties are placed in a new genus, *Myrmecridium* (*incertae sedis*, *Sordariomycetes*). The genus *Pseudovirgaria* (*incertae sedis*) is introduced to accommodate ramichloridium-like isolates occurring on various species of rust fungi. A veronaea-like isolate from *Bertia moriformis* with phylogenetic affinity to the *Annulatascaceae* (*Sordariomycetidae*) is placed in a new genus, *Rhodoveronaea*. Besides *Ramichloridium*, *Periconiella* is also polyphyletic. *Thysanorea* is introduced to accommodate *Periconiella papuana* (*Herpotrichiellaceae*), which is unrelated to the type species, *P. velutina* (*Mycosphaerellaceae*).

**Taxonomic novelties:** *Myrmecridium* Arzanlou, W. Gams & Crous, gen. nov., *Myrmecridium flexuosum* (de Hoog) Arzanlou, W. Gams & Crous, comb. et stat. nov., *Myrmecridium schulzeri* (Sacc.) Arzanlou, W. Gams & Crous var. *schulzeri*, comb. nov., *Myrmecridium schulzeri* var. *tritici* (M.B. Ellis) Arzanlou, W. Gams & Crous, comb. nov., *Periconiella arcuata* Arzanlou, S. Lee & Crous, sp. nov., *Periconiella levispora* Arzanlou, W. Gams & Crous, sp. nov., *Pleurothecium obovoideum* (Matsush.) Arzanlou & Crous, comb. nov., *Pseudovirgaria* H.D. Shin, U. Braun, Arzanlou & Crous, gen. nov., *Pseudovirgaria hyperparasitica* H.D. Shin, U. Braun, Arzanlou & Crous, sp. nov., *Radulidium* Arzanlou, W. Gams & Crous, gen. nov., *Radulidium epichloë* (Ellis & Dearn.) Arzanlou, W. Gams & Crous, comb. nov., *Radulidium subulatum* (de Hoog) Arzanlou, W. Gams & Crous, comb. nov., *Ramichloridium australiense* Arzanlou & Crous, sp. nov., *Ramichloridium biverticillatum* Arzanlou & Crous, nom. nov., *Ramichloridium brasilianum* Arzanlou & Crous, sp. nov., *Ramichloridium strelitziae* Arzanlou, W. Gams & Crous, sp. nov., *Rhinocladiella basitona* (de Hoog) Arzanlou & Crous, comb. nov., *Rhinocladiella fasciculata* (V. Rao & de Hoog) Arzanlou & Crous, comb. nov., *Rhinocladiella mackenziei* (C.K. Campb. & Al-Hedaithy) Arzanlou & Crous, comb. nov., *Rhodoveronaea* Arzanlou, W. Gams & Crous, gen. nov., *Rhodoveronaea varioseptata* Arzanlou, W. Gams & Crous, sp. nov., *Thysanorea* Arzanlou, W. Gams & Crous, gen. nov., *Thysanorea papuana* (Aptroot) Arzanlou, W. Gams & Crous, comb. nov., *Veronaea japonica* Arzanlou, W. Gams & Crous, sp. nov., *Veronaeopsis* Arzanlou & Crous, gen. nov., *Veronaeopsis simplex* (Papendorf) Arzanlou & Crous, comb. nov.

**Key words:** *Capnodiales*, *Chaetothyriales*, *Mycosphaerella*, *Periconiella*, phylogeny, *Rhinocladiella*, *Veronaea*.

## INTRODUCTION

The anamorph genus *Ramichloridium* Stahel ex de Hoog 1977 presently accommodates a wide range of species with erect, dark, more or less differentiated, branched or unbranched conidiophores and predominantly aseptate conidia produced on a sympodially proliferating rachis (de Hoog 1977). This heterogeneous group of anamorphic fungi includes species with diverse life styles, viz. saprobes, human and plant pathogens, most of which were classified by Schol-Schwarz (1968) in *Rhinocladiella* Nannf. according to a very broad generic concept. *Ramichloridium* was originally erected by Stahel (1937) with *R. musae* Stahel as type species. However, because his publication lacked a Latin diagnosis, the genus was invalid. Stahel also invalidly described *Chloridium musae* Stahel for a fungus causing leaf spots (tropical speckle disease) on banana. Ellis (1976) validated *Chloridium musae* as *Veronaea musae* M.B. Ellis, and *Ramichloridium musae* as *Periconiella musae* Stahel ex M.B. Ellis.

*Periconiella* Sacc. (1885) [type species *P. velutina* (G. Winter) Sacc.] differs from *Veronaea* Cif. & Montemart. chiefly based on its dark brown, apically branched conidiophores. However, de Hoog (1977) observed numerous specimens of *V. musae* to exhibit branched conidiophores in culture, as did Stahel (1937) for *Ramichloridium musae*. De Hoog (1977) subsequently re-introduced *Ramichloridium*, but typified it with *R. apiculatum* (J.H.

Mill., Giddens & A.A. Foster) de Hoog. He regarded *V. musae* and *P. musae* to be conspecific, and applied the name *R. musae* (Stahel ex M.B. Ellis) de Hoog to both species, regarding *Periconiella musae* as basionym. The circumscription by de Hoog was based on their similar morphology and ecology. Central in his genus concept was the observed presence of more or less differentiated and pigmented conidiophores, with predominantly aseptate conidia produced on a sympodially proliferating rachis. De Hoog (1977) also used some ecological features as additional characters to discriminate *Ramichloridium* from other genera, noting, for instance, that species in *Ramichloridium* were non-pathogenic to humans (de Hoog 1977, Campbell & Al-Hedaithy 1993). This delimitation, however, was not commonly accepted (McGinnis & Schell 1980). De Hoog *et al.* (1983) further discussed the problematic separation of *Ramichloridium* from genera such as *Rhinocladiella*, *Veronaea* and *Cladosporium* Link. It was further noted that the main feature to distinguish *Ramichloridium* from *Rhinocladiella*, was the presence of exophiala-type budding cells in species of *Rhinocladiella* (de Hoog 1977, de Hoog *et al.* 1983, Veerkamp & Gams 1983). The separation of *Veronaea* from this complex is more problematic, as the circumscriptions provided by Ellis (1976) and Morgan-Jones (1979, 1982) overlap with that of *Ramichloridium sensu* de Hoog (1977). *Cladosporium* is more distinct, having very conspicuous, protuberant, darkened and thickened, coronate conidial scars, and catenate conidia (David 1997, Braun *et al.* 2003, Schubert *et al.* 2007 – this volume).

To date 26 species have been named in *Ramichloridium*; they not only differ in morphology, but also in life style. *Ramichloridium mackenziei* C.K. Campb. & Al-Hedaithy is a serious human pathogen, causing cerebral phaeohyphomycosis (Al-Hedaithy *et al.* 1988, Campbell & Al-Hedaithy 1993), whereas *R. musae* causes tropical speckle disease on members of the *Musaceae* (Stahel 1937, Jones 2000). Another plant-pathogenic species, *R. pini* de Hoog & Rahman, causes a needle disease on *Pinus contorta* (de Hoog *et al.* 1983). Other clinically relevant species of *Ramichloridium* are *R. basitonum* de Hoog and occasionally *R. schulzeri* (Sacc.) de Hoog, while the remaining species tend to be common soil saprobes.

No teleomorph has thus far been linked to species of *Ramichloridium*. The main question that remains is whether shared morphology among the species in this genus reflects common ancestry (Seifert 1993, Untereiner & Naveau 1999). To delineate anamorphic genera adequately, morphology and conidial ontogeny alone are no longer satisfactory (Crous *et al.* 2006a, b), and DNA data provide additional characters to help delineate species and genera (Taylor *et al.* 2000, Mostert *et al.* 2006, Zipfel *et al.* 2006). The aim of the present study was to integrate morphological and cultural features with DNA sequence data to resolve the species concepts and generic limits of the taxa currently placed in *Periconiella*, *Ramichloridium*, *Rhinoctadiella* and *Veronaea*, and to resolve the status of several new cultures that were isolated during the course of this study.

## MATERIALS AND METHODS

### Isolates

Species names, substrates, geographical origins and GenBank accession numbers of the isolates included in this study are listed in Table 1. Fungal isolates are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands.

### DNA extraction, amplification and sequence analysis

Genomic DNA was extracted from colonies grown on 2 % malt extract agar (MEA, Difco) (Gams *et al.* 2007) using the FastDNA kit (BIO101, Carlsbad, CA, U.S.A.). The primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including: the 3' end of the 18S rRNA gene, the first internal transcribed spacer region (ITS1), the 5.8S rRNA gene, the second internal transcribed spacer region (ITS2) and the 5' end of 28S rRNA gene. Part of the large subunit 28S rRNA (LSU) gene was amplified with primers LR0R (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990). The ITS region was sequenced only for those isolates for which these data were not available. The ITS analyses confirmed the proposed classification based on LSU analysis for each major clade and are not presented here in detail; but the sequences are deposited in GenBank where applicable. The PCR reaction was performed in a mixture with 0.5 units *Taq* polymerase (Bioline, London, U.K.), 1× PCR buffer, 0.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 5 pmol of each primer, approximately 10–15 ng of fungal genomic DNA, with the total volume adjusted to 25 µL with sterile water. Reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) with cycling conditions consisting of 5 min at 96 °C for primary denaturation, followed by 36 cycles at 96 °C (30 s), 52 °C (30 s), and 72 °C (60 s), with a final 7 min extension step at 72 °C to complete the

reaction. The amplicons were sequenced using BigDye Terminator v. 3.1 (Applied Biosystems, Foster City, CA) or DYEnamicET Terminator (Amersham Biosciences, Freiburg, Germany) Cycle Sequencing Kits and analysed on an ABI Prism 3700 (Applied Biosystems, Foster City, CA) under conditions recommended by the manufacturer. Newly generated sequences were subjected to a Blast search of the NCBI databases, sequences with high similarity were downloaded from GenBank and comparisons were made based on the alignment of the obtained sequences. Sequences from GenBank were also selected for similar taxa. The LSU tree was rooted using sequences of *Athelia epiphylla* Pers. and *Paulliticorticium ansatum* Libertas as outgroups. Phylogenetic analysis was performed with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003), using the neighbour-joining algorithm with the uncorrected ("p"), the Kimura 2-parameter and the HKY85 substitution models. Alignment gaps longer than 10 bases were coded as single events for the phylogenetic analyses; the remaining gaps were treated as missing data. Any ties were broken randomly when encountered. Phylogenetic relationships were also inferred with the parsimony algorithm using the heuristic search option with simple taxon additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm; alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Only the first 5 000 equally most parsimonious trees were saved. Other measures calculated included tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC, respectively). The robustness of the obtained trees was evaluated by 1 000 bootstrap replications. Bayesian analysis was performed following the methods of Crous *et al.* (2006c). The best nucleotide substitution model was determined using MrModeltest v. 2.2 (Nylander 2004). MRBAYES v. 3.1.2 (Ronquist & Huelsenbeck 2003) was used to perform phylogenetic analyses, using a general time-reversible (GTR) substitution model with inverse gamma rates, dirichlet base frequencies and the temp value set to 0.5. New sequences were lodged with NCBI's GenBank (Table 1) and the alignment and trees with TreeBASE ([www.treebase.org](http://www.treebase.org)).

### Morphology

Cultural growth rates and morphology were recorded from colonies grown on MEA for 2 wk at 24 °C in the dark, and colony colours were determined by reference to the colour charts of Rayner (1970). Microscopic observations were made from colonies cultivated on MEA and OA (oatmeal agar, Gams *et al.* 2007), using a slide culture technique. Slide cultures were set up in Petri dishes containing 2 mL of sterile water, into which a U-shaped glass rod was placed, extending above the water surface. A block of freshly growing fungal colony, approx. 1 cm square was placed onto a sterile microscope slide, covered with a somewhat larger, sterile glass cover slip, and incubated in the moist chamber. Fungal sporulation was monitored over time, and when optimal, images were captured by means of a Nikon camera system (Digital Sight DS-5M, Nikon Corporation, Japan). Structures were mounted in lactic acid, and 30 measurements (× 1 000 magnification) determined wherever possible, with the extremes of spore measurements given in parentheses.

**Table 1.** Isolates of *Ramichloridium* and similar genera used for DNA analysis and morphological studies.

Species	Accession number <sup>1</sup>	Source	Origin	GenBank numbers (LSU, ITS)
<i>Myrmecridium flexuosum</i>	CBS 398.76*; IMI 203547	Soil	Suriname	EU041825, EU041768
<i>Myrmecridium schulzeri</i>	CBS 100.54; JCM 6974	Soil	Zaire	EU041826, EU041769
	CBS 134.68; ATCC 16310	Soil	Germany	EU041827, EU041770
	CBS 156.63	<i>Homo sapiens</i>	Netherlands	EU041828, EU041771
	CBS 188.96	Soil	Papua New Guinea	EU041829, EU041772
	CBS 304.73	Wheat straw	South Africa	EU041830, EU041773
	CBS 305.73; JCM 6967	Wheat straw	South Africa	EU041831, EU041774
	CBS 325.74; JCM 7234	<i>Triticum aestivum</i>	Netherlands	EU041832, EU041775
	CBS 381.87	—	Australia	EU041833, EU041776
	CBS 642.76	<i>Malus sylvestris</i>	Switzerland	EU041834, EU041777
	CBS 114996	<i>Cannomois virgata</i>	South Africa	EU041835, EU041778
<i>Periconiella arcuata</i>	CBS 113477*	<i>Ischyrolepsis subverticellata</i>	South Africa	EU041836, EU041779
<i>Periconiella levispora</i>	CBS 873.73*	<i>Turpinia pomifera</i>	Sri Lanka	EU041837, EU041780
<i>Periconiella velutina</i>	CBS 101948*; CPC 2262	<i>Brabejum stellatifolium</i>	South Africa	EU041838, EU041781
	CBS 101949; CPC 2263	<i>Brabejum stellatifolium</i>	South Africa	EU041839, EU041782
	CBS 101950; CPC 2264	<i>Brabejum stellatifolium</i>	South Africa	EU041840, EU041783
<i>Pleurothecium obovoideum</i>	CBS 209.95*; MFC 12477	<i>Pasania edulis</i>	Japan	EU041841, EU041784
<i>Pseudovirgaria hyperparasitica</i>	CBS 121735; CPC 10702	On <i>Phragmidium</i> sp. on <i>Rubus coreanus</i>	Korea	EU041822, EU041765
	CBS 121738; CPC 10704	On <i>Phragmidium</i> sp. on <i>Rubus coreanus</i>	Korea	EU041823, EU041766
	CBS 121739*; CPC 10753	On <i>Pucciniastrum agrimoniae</i> on <i>Agrimonia pilosa</i>	Korea	EU041824, EU041767
<i>Radulidium epichloës</i>	CBS 361.63*; MUCL 3124	<i>Epichloë typhina</i>	U.S.A.	EU041842, EU041785
<i>Radulidium</i> sp.	CBS 115704	<i>Poaceae</i>	Guyana	EU041843, EU041786
<i>Radulidium subulatum</i>	CBS 287.84	<i>Puccinia allii</i>	U.K.	EU041844, EU041787
	CBS 405.76*	<i>Phragmites australis</i>	Czech Republic	EU041845, EU041788
	CBS 912.96	Incubator for cell cultures	Germany	EU041846, EU041789
	CBS 101010	<i>Lasioptera arundinis</i>	Czech Republic	EU041847, EU041790
<i>Ramichloridium apiculatum</i>	CBS 156.59*; ATCC 13211; IMI 100716; JCM 6972; MUCL 7991; MUCL 15753; QM 7716	Forest soil	U.S.A.	EU041848, EU041791
	CBS 390.67	<i>Cucumis sativus</i>	South Africa	EU041849, EU041792
	CBS 391.67; JCM 6966	<i>Aloe</i> sp.	South Africa	EU041850, EU041793
	CBS 400.76; IMI 088021	Soil	Pakistan	EU041851, EU041794
<i>Ramichloridium australiense</i>	CBS 121710	<i>Musa banksii</i>	Australia	EU041852, EU041795
<i>Ramichloridium biverticillatum</i>	CBS 335.36	<i>Musa sapientum</i>	—	EU041853, EU041796
<i>Ramichloridium brasilianum</i>	CBS 283.92*	Forest soil	Brazil	EU041854, EU041797
<i>Ramichloridium cerophilum</i>	CBS 103.59*	<i>Sasa</i> sp.	Japan	EU041855, EU041798
<i>Ramichloridium indicum</i>	CBS 171.96	—	—	EU041856, EU041799
<i>Ramichloridium musae</i>	CBS 190.63; MUCL 9557	<i>Musa sapientum</i>	—	EU041857, EU041800
	CBS 365.36*; JCM 6973; MUCL 9556	<i>Musa sapientum</i>	Surinam	EU041858, EU041801
<i>Ramichloridium pini</i>	CBS 461.82*; MUCL 28942	<i>Pinus contorta</i>	U.K.	EU041859, EU041802
<i>Ramichloridium strelitziae</i>	CBS 121711	<i>Strelitzia</i> sp.	South Africa	EU041860, EU041803
<i>Rhinochlaediella anceps</i>	CBS 157.54; ATCC 15680; MUCL 1081; MUCL 7992; MUCL 15756	<i>Fagus sylvatica</i>	France	EU041861, EU041804
	CBS 181.65*; ATCC 18655; DAOM 84422; IMI 134453; MUCL 8233; OAC 10215	Soil	Canada	EU041862, EU041805
<i>Rhinochlaediella basitona</i>	CBS 101460*; IFM 47593	<i>Homo sapiens</i>	Japan	EU041863, EU041806
<i>Rhinochlaediella fasciculata</i>	CBS 132.86*	Decayed wood	India	EU041864, EU041807
<i>Rhinochlaediella mackenziei</i>	CBS 367.92; NCPF 2738; UTMB 3169	<i>Homo sapiens</i>	Israel	EU041865, EU041808

Table 1. (Continued).

Species	Accession number <sup>1</sup>	Source	Origin	GenBank numbers (LSU, ITS)
	CBS 368.92; UTMB 3170	<i>Homo sapiens</i>	Israel	EU041866, EU041809
	CBS 102590; NCPF 2853	<i>Homo sapiens</i>	United Arab Emirates	EU041867, EU041810
<i>Rhinocladiella phaeophora</i>	CBS 496.78*; IMI 287527	Soil	Colombia	EU041868, EU041811
<i>Rhinocladiella</i> sp.	CBS 264.49; MUCL 9904	Honey	France	EU041869, EU041812
<i>Rhodoveronaea varioseptata</i>	CBS 431.88*	<i>Bertia moriformis</i>	Germany	EU041870, EU041813
<i>Thysanorea papuana</i>	CBS 212.96*	—	Papua New Guinea	EU041871, EU041814
<i>Veronaea botryosa</i>	CBS 121.92	<i>Xanthorrhoea preissii</i>	Australia	EU041872, EU041815
	CBS 254.57*; IMI 070233; MUCL 9821	—	Italy	EU041873, EU041816
	CBS 350.65; IMI 115127; MUCL 7972	Goat dung	India	EU041874, EU041817
<i>Veronaea compacta</i>	CBS 268.75*	—	South Africa	EU041876, EU041819
<i>Veronaea japonica</i>	CBS 776.83*	On dead bamboo culm	Japan	EU041875, EU041818
<i>Veronaeopsis simplex</i>	CBS 588.66*; IMI 203547	<i>Acacia karroo</i>	South Africa	EU041877, EU041820
<i>Zasmidium cellare</i>	CBS 146.36	Wine cellar	—	EU041878, EU041821

<sup>1</sup>ATCC: American Type Culture Collection, Virginia, U.S.A.; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; DAOM: Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; IFM: Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan; IMI: International Mycological Institute, CABI-Bioscience, U.K.; JCM: Japan Collection of Microorganism, RIKEN BioResource Center, Japan; MFC: Matsushima Fungus Collection, Kobe, Japan; MUCL: Mycotheque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; NCPF: The National Collection of Pathogenic Fungi, Holborn, London, U.K.; OAC: Department of Botany and Genetics, University of Guelph, Ont., Canada; QM: Quartermaster Research and Development Center, U.S. Army, MA, U.S.A.; UTMB: University of Texas Medical Branch, Texas, U.S.A.

\*Ex-type cultures.

## RESULTS

### Phylogeny

The manually adjusted alignment of the 28S rDNA data contained 137 sequences (including the two outgroups) and 995 characters including alignment gaps. Of the 748 characters used in the phylogenetic analysis, 373 were parsimony-informative, 61 were variable and parsimony-uninformative, and 314 were constant. Neighbour-joining analysis using the three substitution models on the LSU alignment yielded trees with similar topology and bootstrap values. Parsimony analysis of the alignment yielded 5 000 equally most parsimonious trees, one of which is shown in Fig. 1 (TL = 2 157, CI = 0.377, RI = 0.875, RC = 0.330). The Markov Chain Monte Carlo (MCMC) analysis of four chains started from a random tree topology and lasted 2 000 000 generations. Trees were saved each 1 000 generations, resulting in 2 000 trees. Burn-in was set at 500 000 generations after which the likelihood values were stationary, leaving 1 500 trees from which the consensus tree (Fig. 2) and posterior probabilities (PP's) were calculated. The average standard deviation of split frequencies was 0.043910 at the end of the run. Among the neighbour-joining, Bayesian and parsimony analyses, the trees differed in the hierarchical order of the main families and the support values (data not shown; e.g. the support within of the *Capnodiales* in Figs 1–2).

The phylogenetic trees (Figs 1–2) show that the *Ramichloridium* species segregate into eight distinct clades, residing in the *Capnodiales* (*Mycosphaerellaceae* and *Teratosphaeriaceae*), the *Chaetothyriales* (*Herpotrichiellaceae*), the *Pleosporales*, and five other clades of which the relationships remain to be elucidated. The type species of *Ramichloridium*, *R. apiculatum*, together with *R. musae*, *R. cerophilum* (Tubaki) de Hoog, *R. indicum* (Subram.) de Hoog, *R. pini* and three new species respectively isolated from *Musa banksii*, *Strelitzia nicolai*, and forest soil, reside in different parts of the *Capnodiales* clade (all in the *Mycosphaerellaceae*, except for the

species from forest soil which clusters in the *Teratosphaeriaceae*). The second clade (in the *Chaetothyriomycetes* clade), including the human-pathogenic species *R. mackenziei* and *R. basitonum*, together with *R. fasciculatum* V. Rao & de Hoog and *R. anceps* (Sacc. & Ellis) de Hoog, groups together with *Rhinocladiella* in the *Herpotrichiellaceae*. The third clade (in the *Sordariomycetes* clade) includes *R. obovoideum* (Matsush.) de Hoog, which in a Blast search was found to have affinity with *Carpoligna pleurothecii* F.A. Fernández & Huhndorf (*Chaetosphaeriales*). The fourth clade (in the *Sordariomycetes* clade) includes a veronaea-like isolate from *Bertia moriformis*, with phylogenetic affinity to the *Annulatascaceae* (*Sordariomycetidae*). The fifth clade (in the *Sordariomycetes* clade) includes *R. schulzeri* var. *schulzeri* and *R. schulzeri* var. *flexuosum* de Hoog, the closest relatives being *Thyridium vestitum* (Fr.) Fuckel in the *Thyridiaceae* and *Magnaporthe grisea* (T.T. Hebert) M.E. Barr in the *Magnaporthaceae*. The sixth clade (in the *Incertae sedis* clade) includes *R. subulatum* de Hoog, *R. epichloës* (Ellis & Dearn.) de Hoog and a species isolated from the *Poaceae*. Three ramichloridium-like isolates from *Rubus coreanus* and *Agrimonia pilosa* form another unique clade (in the *Incertae sedis* clade) with uncertain affinity. *Veronaea simplex* Papendorf clusters as sister taxon to the *Venturiaceae* representing the eighth clade (*Dothideomycetes*). The type species of *Periconiella*, *P. velutina*, clusters within the *Mycosphaerellaceae* (*Capnodiales* clade), whereas *P. papuana* Aptroot resides in the *Herpotrichiellaceae* (*Chaetothyriales* clade). *Veronaea botryosa* Cif. & Montemart., the type species of *Veronaea*, also resides in the *Herpotrichiellaceae*.

### Taxonomy

The species previously described in *Ramichloridium* share some morphological features, including erect, pigmented, more or less differentiated conidiophores, sympodially proliferating conidiogenous cells and predominantly aseptate conidia. Other than conidial morphology, features of the conidiogenous apparatus that

appear to be more phylogenetically informative include pigmentation of vegetative hyphae, conidiophores and conidia, denticle density on the rachis, and structure of the scars. By integrating these data

with the molecular data set, more natural genera are delineated, which are discussed below.

### Key to ramichloridium-like genera

1. Conidiogenous cells integrated, terminal and lateral on creeping or ascending hyphae (differentiation between branched vegetative hyphae and conidiophores barely possible); conidiogenous loci bulging, more or less umbonate, apex rounded; occurring on rust pustules ..... ***Pseudovirgaria***
1. Conidiogenous cells integrated in distinct conidiophores; conidiogenous loci non-umbonate (flat, not prominent; subcylindrical or conical denticles; or terminally flat-tipped; or thickened and darkened); rarely occurring on rust pustules, but if so, with a raduliform rachis and distinct denticles ..... 2
2. Conidia 0–2(–3)-septate, conidial base truncate, retaining a marginal frill after liberation [anamorphs of *Sordariomycetes*] ..... ***Rhodoveronaea***
2. Conidial base without marginal frill ..... 3
3. Conidiophores composed of a well-developed erect stalk and a terminal branched head ..... 4
3. Conidiophores unbranched or, if branched, branches loose, irregular or dichotomous, but not distinctly separated into stalk and branched head ..... 5
4. Conidiophores dimorphic, either macronematous, dark brown with a dense apical cluster of branches or micronematous, undifferentiated, resembling vegetative hyphae; both kinds with a denticulate rachis; conidia predominantly 1-septate [anamorph of *Chaetothyriales*] ..... ***Thysanorea***
4. Conidiophores monomorphic; branched head with fewer branches and looser; conidiogenous loci usually flat, non-prominent, less denticle-like; conidia aseptate to pluriseptate [anamorphs of *Capnodiales*] ..... ***Periconiella***
5. Rachis with denticles 1–1.5 µm long, denticles almost cylindrical; conidia at least partly in short chains ..... ***Pleurothecium***
5. Rachis with denticles less than 1 µm long, denticles not cylindrical or denticles lacking, rachis with flat, barely prominent scars ..... 6
6. Conidia predominantly septate ..... 7
6. Conidia predominantly aseptate ..... 8
7. Conidiophores up to 200 µm long; rachis straight, not to slightly geniculate; conidiogenous loci more or less flat, barely prominent, unthickened, slightly darkened [anamorphs of *Chaetothyriales*, *Herpotrichiellaceae*] ..... ***Veronaea***
7. Conidiophores up to 60 µm long; rachis distinctly geniculate; conidiogenous loci denticle-like, prominent, up to 0.5 µm high, slightly thickened and darkened [anamorph of *Pleosporales*, *Venturiaceae*] ..... ***Veronaeopsis***
8. Vegetative mycelium entirely hyaline; rachis long, hyaline, with widely scattered pimple-shaped, terminally pointed, unpigmented denticles ..... ***Myrmecridium***
8. Vegetative mycelium at least partly pigmented; conidiogenous loci distinct, non-denticulate, somewhat darkened-refractive, or denticles, if present, neither pimple-shaped nor pointed ..... 9
9. Rachis distinctly raduliform, with distinct, prominent blunt denticles, 0.5–1 µm long; scars and hila unthickened, but pigmented ..... ***Radulidium***
9. Rachis not distinctly raduliform, at most subdenticulate; scars flat or only slightly prominent (subdenticulate), shorter ..... 10
10. Conidiophores usually poorly differentiated from the vegetative hyphae; conidial apparatus often loosely branched; exophiala-like budding cells usually present in culture [anamorphs of *Chaetothyriales*, *Herpotrichiellaceae*] ..... ***Rhinochlaidiella***
10. Conidiophores usually well differentiated from the vegetative mycelium (macronematous), usually unbranched; without exophiala-like states [anamorphs of *Capnodiales*] ..... ***Ramichloridium***

### ***Capnodiales* (*Mycosphaerellaceae*, *Teratosphaeriaceae*)**

The type species of *Ramichloridium*, *R. apiculatum*, together with *R. indicum* cluster as a sister group to the *Dissoconium* de Hoog, Oorschot & Hijwegen clade in the *Mycosphaerellaceae*. Some other *Ramichloridium* species, including *R. musae*, *R. biverticillatum* Arzanlou & Crous, *R. pini* and *R. cerophilum*, are also allied with members of the *Mycosphaerellaceae*. Three additional new species are introduced for *Ramichloridium* isolates

from *Musa banksii*, *Strelitzia nicolai*, and forest soil. *Periconiella velutina*, the type species of *Periconiella*, which also resides in the *Mycosphaerellaceae*, is morphologically sufficiently distinct to retain its generic status. Two new species of *Periconiella* are introduced for isolates obtained from *Turpinia pomifera* and *Ischyrolepis subverticellata* in South Africa. *Zasmidium cellare* (Pers.) Fr., the type species of *Zasmidium* (Pers.) Fr., is also shown to cluster within the *Mycosphaerellaceae*.

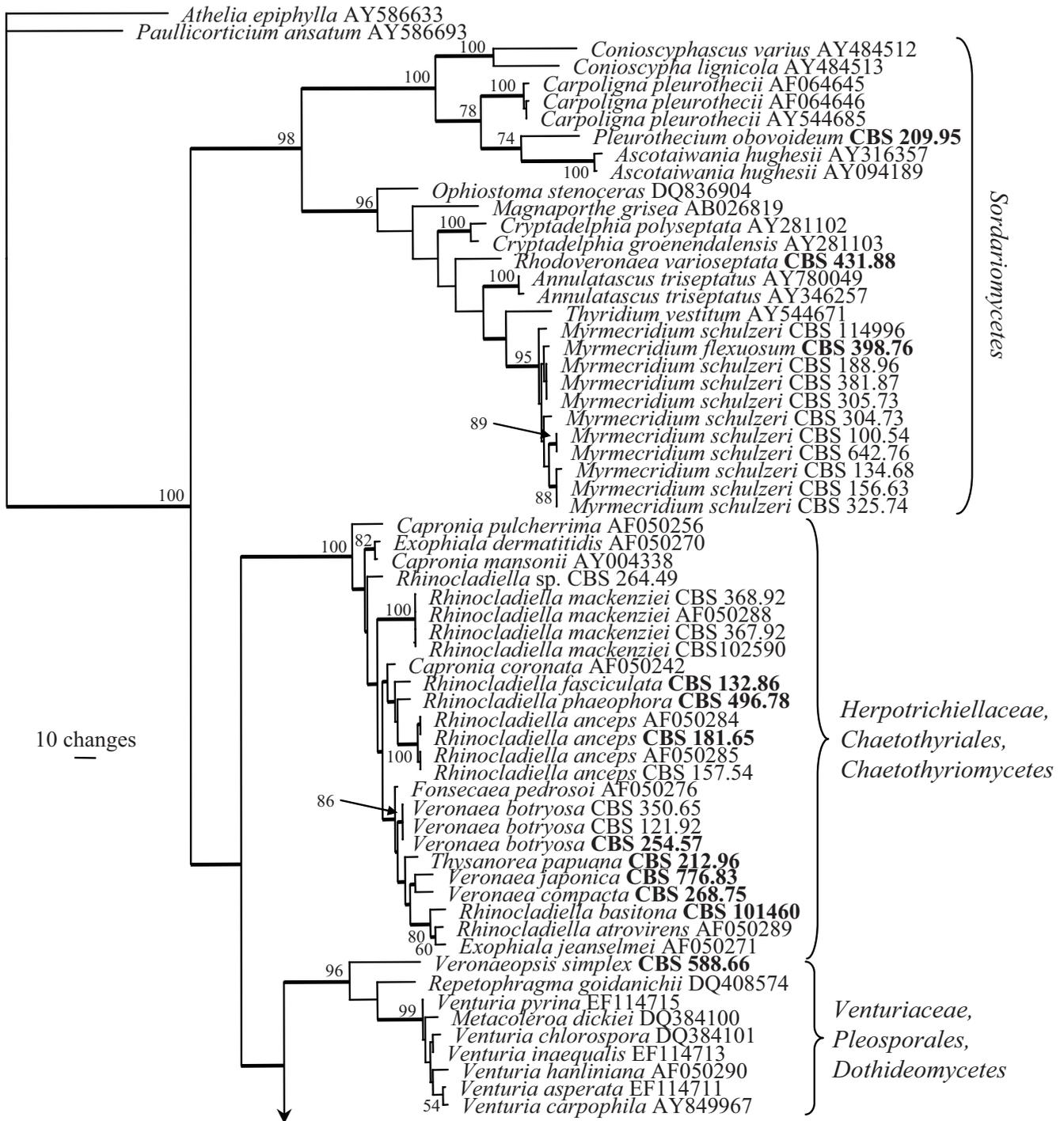


Fig. 1. (Page 62–63). One of 5 000 equally most parsimonious trees obtained from a heuristic search with simple taxon additions of the LSU sequence alignment using PAUP v. 4.0b10. The scale bar shows 10 changes; bootstrap support values from 1 000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and ex-type sequences are printed in bold face. The tree was rooted to two sequences obtained from GenBank (*Athelia epiphylla* AY586633 and *Paulliticium ansatum* AY586693).

*Periconiella* Sacc., in Sacc. & Berlese, Atti Ist. Veneto Sci., Ser. 6, 3: 727. 1885.

*In vitro*: Colonies with entire margin; aerial mycelium rather compact, raised, velvety, olivaceous-grey; reverse olivaceous-black. *Submerged hyphae* verrucose, hyaline, thin-walled, 1–3 µm wide; *aerial hyphae* subhyaline, later becoming dark brown, thick-walled, smooth. *Conidiophores* arising vertically from creeping hyphae, straight or flexuose, up to 260 µm long, dark brown at the base, paler towards the apex, thick-walled; in the upper part bearing short branches. *Conidiogenous cells* terminally integrated, polyblastic, smooth or verrucose, subcylindrical, mostly not or barely

geniculate-sinuous, variable in length, subhyaline, later becoming pale brown, fertile part as wide as the basal part, proliferating sympodially, sometimes becoming septate and forming a short, straight rachis with pigmented, slightly thickened and hardly prominent, more or less flat scars. *Conidia* solitary, occasionally in short chains, 0–multi-septate, subhyaline to rather pale olivaceous or olivaceous-brown, smooth to verrucose, globose, ellipsoidal to obovoid or obclavate, with a slightly darkened and thickened hilum; conidial secession schizolytic.

*Type species*: *P. velutina* (G. Winter) Sacc., *Miscell. mycol.* 2: 17. 1884.

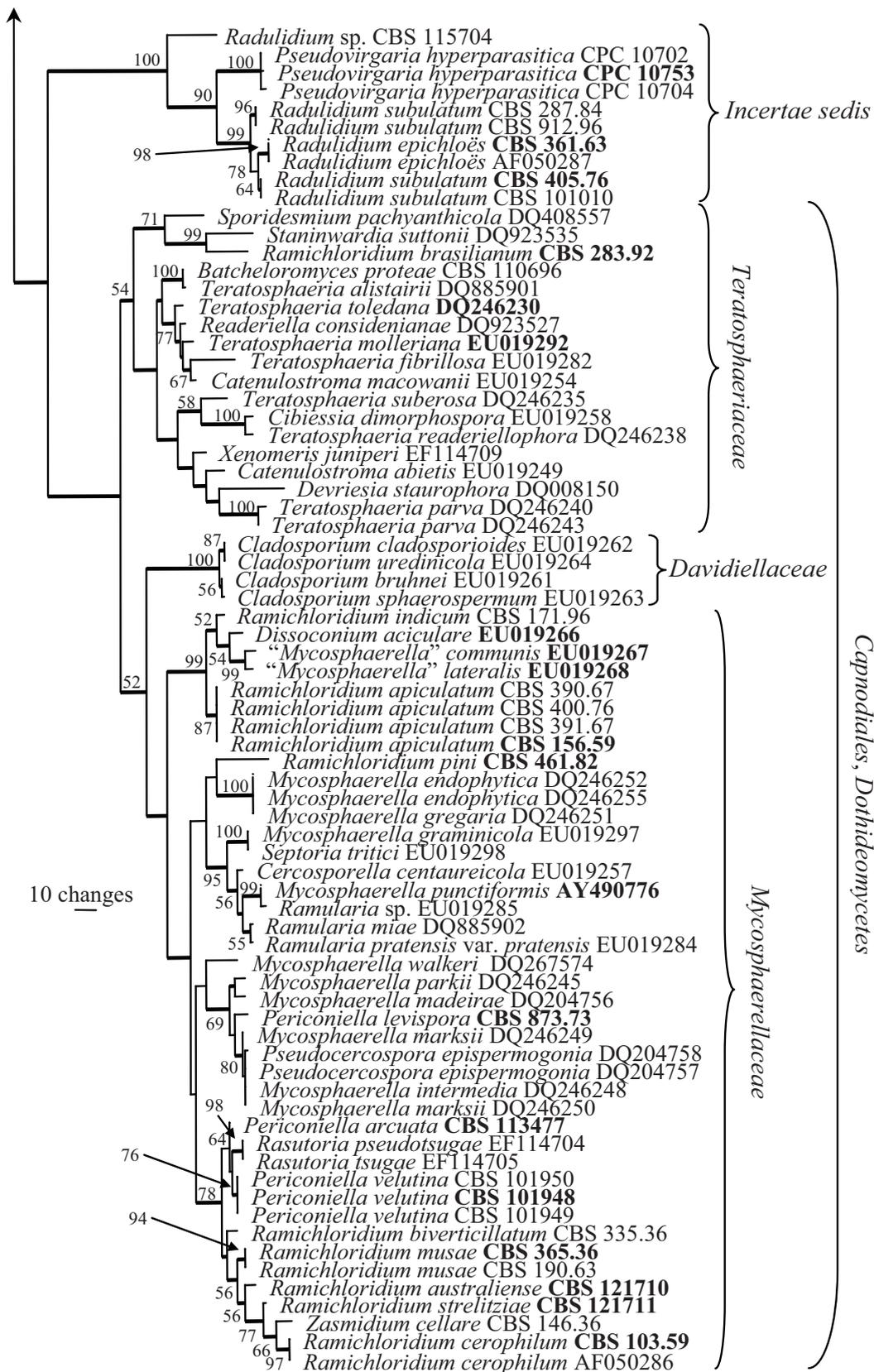
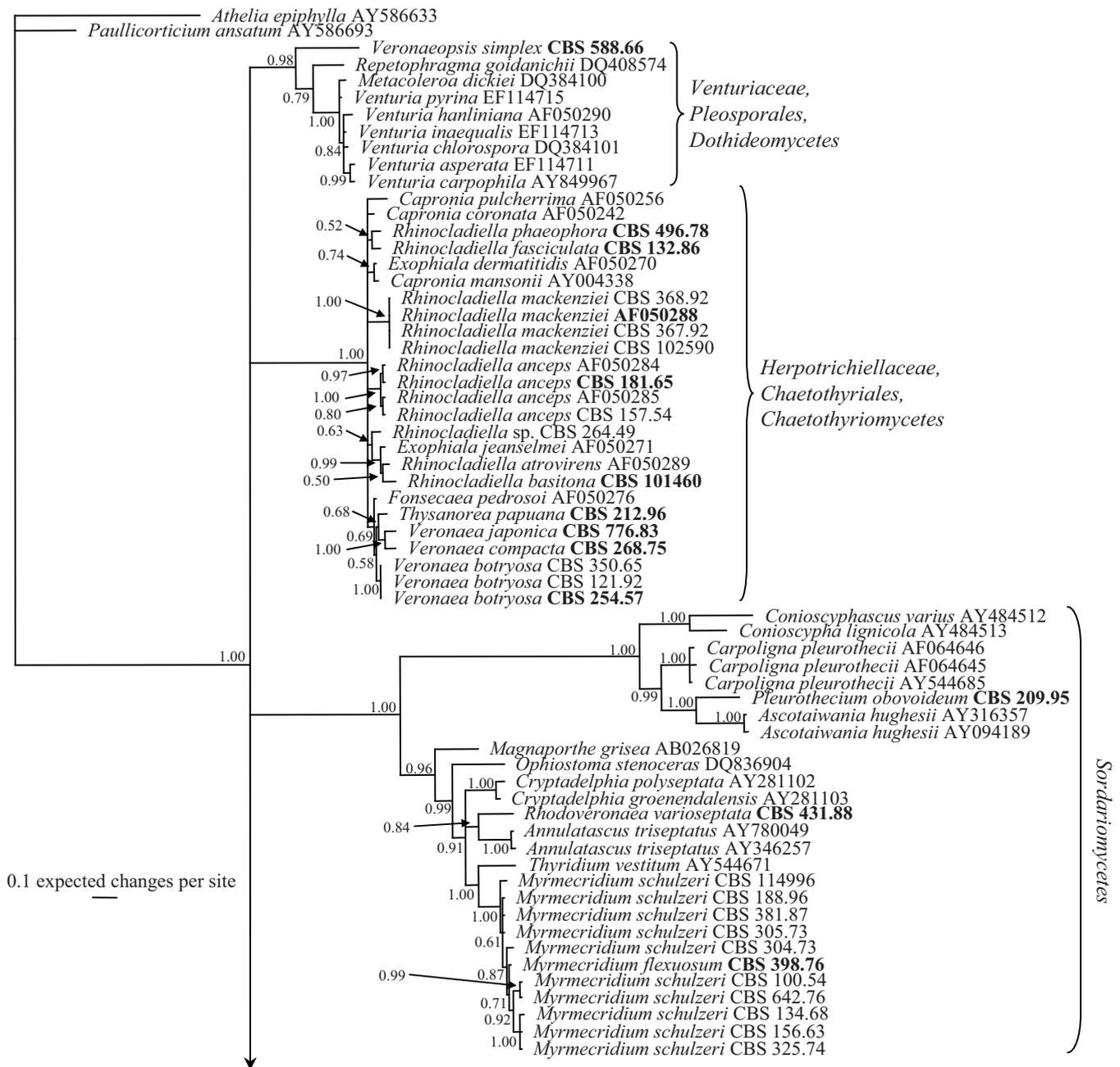


Fig. 1. (Continued).

Notes: *Periconiella* is distinct from other ramichloridium-like genera by its conidiophores that are prominently branched in the upper part, and by its darkened, thickened conidial scars, that are more or less flat and non-prominent. Although conidiophores are also branched in the upper part in *Thysanorea* Arzanlou, W. Gams & Crous, the branching pattern in the latter genus is different from

that of *Periconiella*. *Thysanorea* has a complex head consisting of up to six levels of branches, while in *Periconiella* the branching is limited, with mainly primary and secondary branches. Furthermore, *Thysanorea* is characterised by having dimorphic conidiophores and more or less prominent denticle-like conidigenous loci.



**Fig. 2.** (Page 64–65). Consensus phylogram (50 % majority rule) of 1 500 trees resulting from a Bayesian analysis of the LSU sequence alignment using MrBAYES v. 3.1.2. Bayesian posterior probabilities are indicated at the nodes. Ex-type sequences are printed in bold face. The tree was rooted to two sequences obtained from GenBank (*Athelia epiphylla* AY586633 and *Paullicorticium ansatum* AY586693).

***Periconiella velutina*** (G. Winter) Sacc., *Miscell. mycol.* 2: 17. 1884. Fig. 3.

*Basionym:* *Periconia velutina* G. Winter, *Hedwigia* 23: 174. 1884.

*In vitro:* Submerged hyphae verrucose, hyaline, thin-walled, 1–3 µm wide; aerial hyphae subhyaline, later becoming dark brown, thick-walled, smooth. Conidiophores arising vertically from creeping hyphae, straight or flexuose, up to 260 µm long, dark brown at the base, paler towards the apex, thick-walled; in the upper part bearing short branches, 10–35 µm long. Conidiogenous cells mostly terminally integrated, sometimes discrete, smooth or verrucose, cylindrical, variable in length, subhyaline, later becoming pale brown, fertile part as wide as the basal part, proliferating sympodially, sometimes becoming septate and forming a short, straight rachis with pigmented, slightly thickened and hardly prominent, more

or less flat scars, less than 1 µm diam. Conidia 0(–1)-septate, subhyaline, thin-walled, verrucose or smooth, globose, ellipsoidal to obovoid, (7)–8–9(–11) × (2.5)–3(–4) µm, with a slightly darkened and thickened hilum, 1.5–2 µm diam.

*Cultural characteristics:* Colonies on MEA slow-growing, reaching 4 mm diam after 14 d at 24 °C, with entire margin; aerial mycelium rather compact, raised, velvety, olivaceous-grey; reverse olivaceous-black.

*Specimens examined:* South Africa, Cape Town, on *Brabejum stellatifolium*, P. MacOwan, herb. G. Winter (B), **lectotype selected here**; Cape Town, on leaves of *Brabejum stellatifolium* (= *B. stellatum*), P. MacOwan, PAD, F42165, F462166, **isolectotypes**; Stellenbosch, Jonkershoek Nature Reserve, on *Brabejum stellatifolium*, 21 Jan. 1999, J.E. Taylor, **epitype designated here** CBS H-15612, cultures ex-epitype CBS 101948–101950.

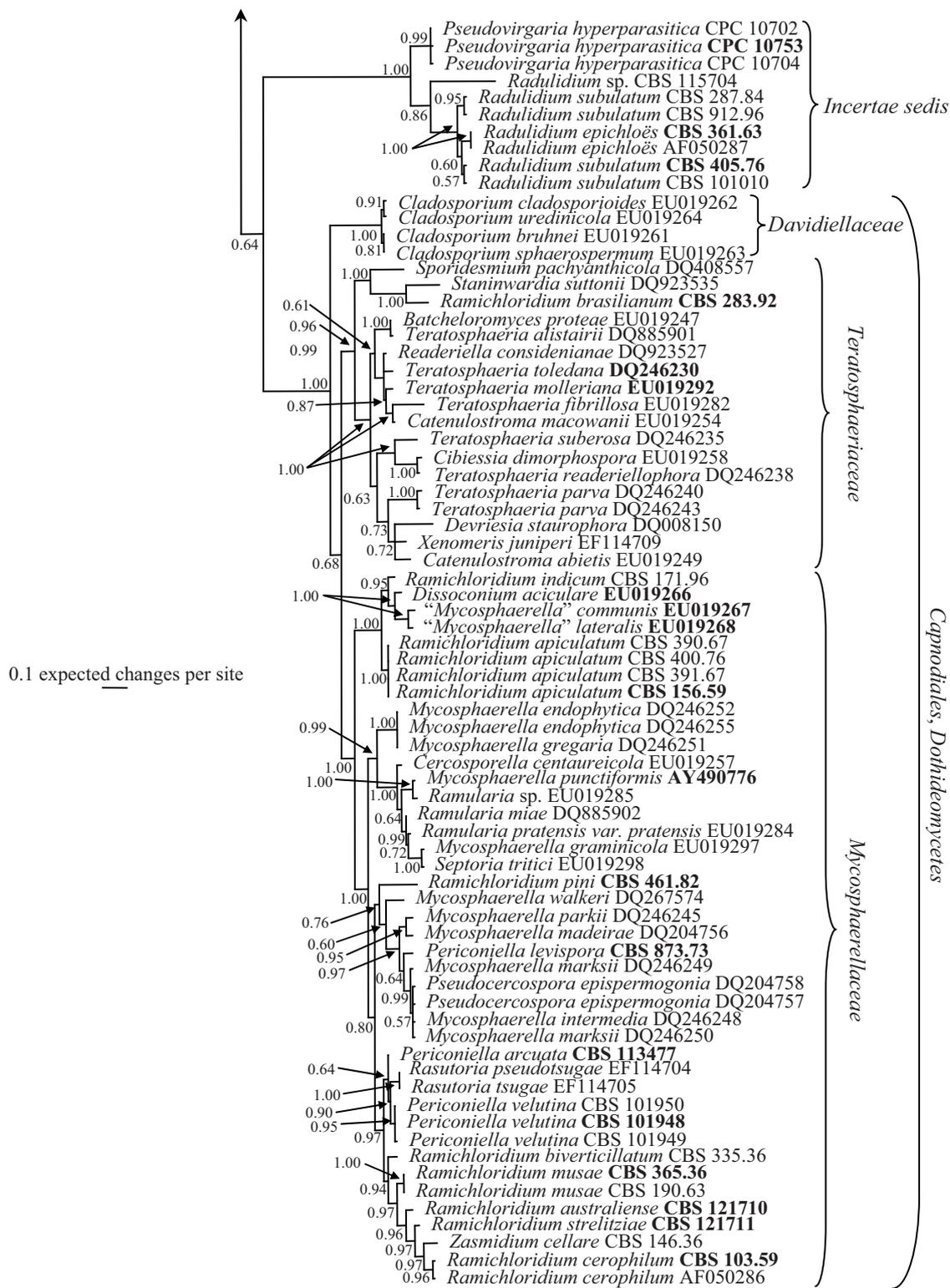


Fig. 2. (Continued).

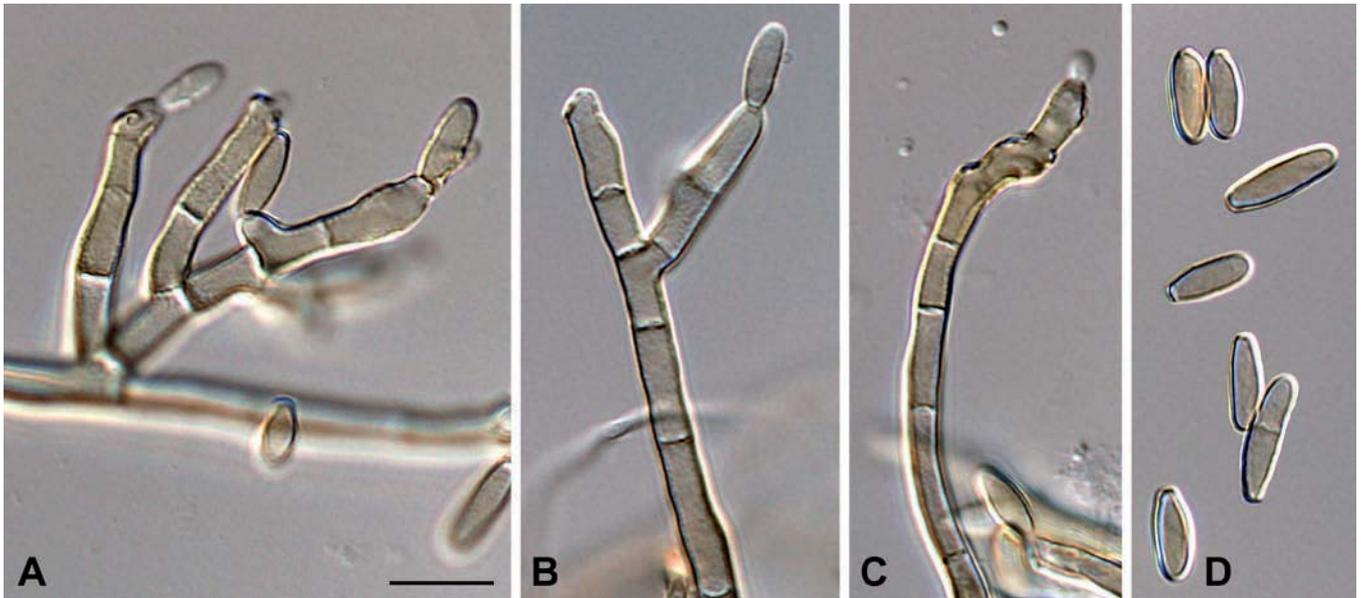
***Periconiella arcuata*** Arzanlou, S. Lee & Crous, **sp. nov.** MycoBank MB504547. Figs 4, 7A.

**Etymology:** Named after its curved conidia.

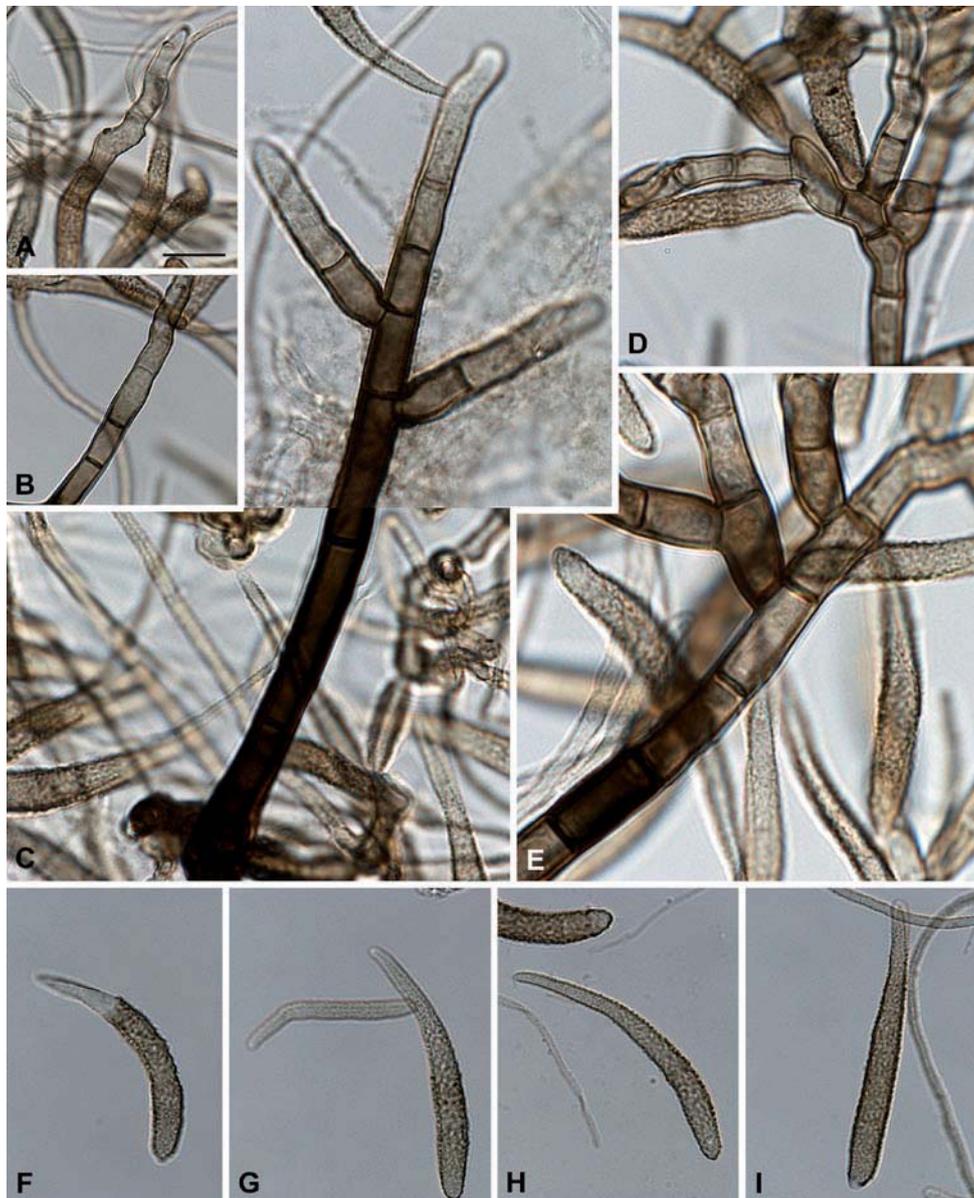
Ab aliis speciebus *Periconiellae* conidiis obclavatis, rectis vel curvatis, (30–)53–61(–79) × (3–)5(–7) µm, distinguenda.

**Submerged hyphae** smooth, hyaline, thin-walled, 2 µm wide; **aerial hyphae** pale brown, smooth or verrucose, slightly narrower. **Conidiophores** arising vertically from creeping hyphae, straight or flexuose, up to 300 µm long, dark brown at the base, paler

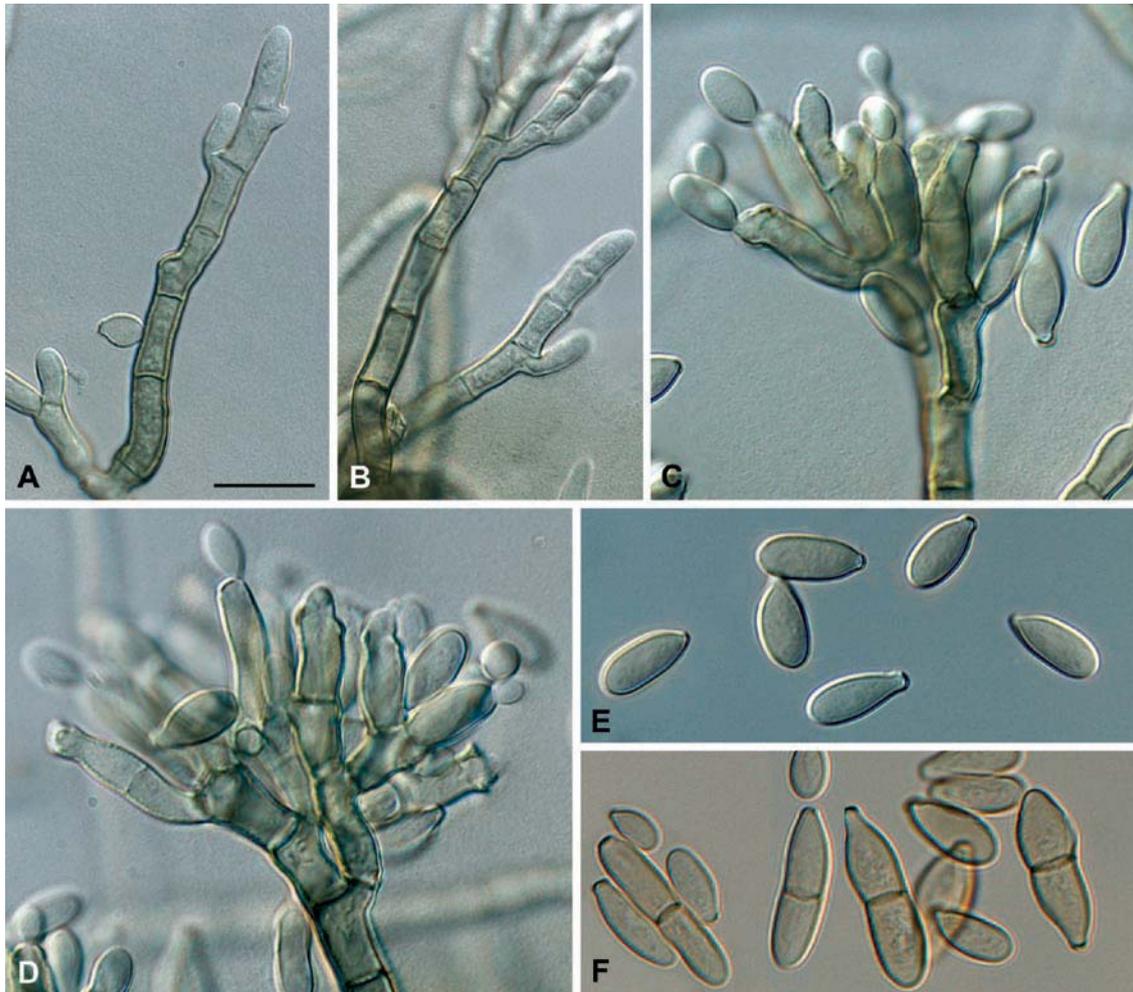
towards the apex, thick-walled; loosely branched in the upper part, bearing short branches. **Conidiogenous cells** integrated, cylindrical, variable in length, 20–50 µm long, subhyaline, later becoming pale brown, fertile part as wide as the basal part, proliferating sympodially, forming a geniculate conidium-bearing rachis with pigmented and thickened, prominent, cone-shaped scars, 1 µm diam. **Conidia** formed singly, obclavate, straight or mostly curved, 0(–4)-septate, coarsely verrucose, pale olive, thin-walled, tapering towards the apex, (30–)53–61(–79) × (3–)5(–7) µm, with a narrowly truncate base and a darkened, hardly thickened hilum, 2 µm diam; microcyclic conidiation observed in culture.



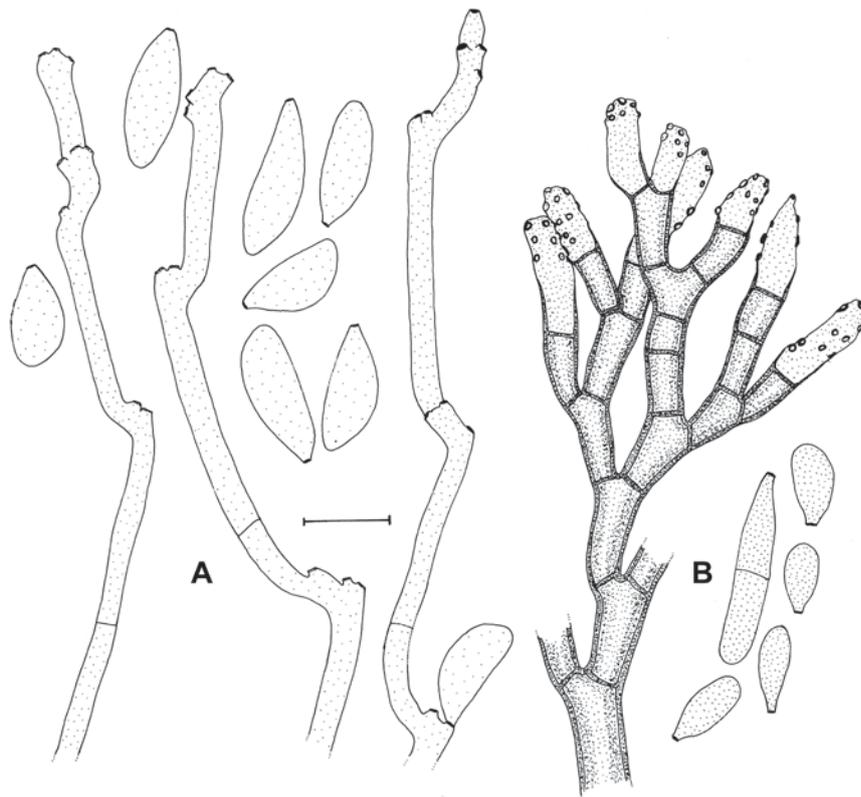
**Fig. 3.** *Periconiella velutina* (CBS 101948). A–B. Macronematous conidiophores with short branches in the upper part. C. Sympodially proliferating conidiogenous cell with darkened and slightly thickened scars. D. Conidia. Scale bar = 10  $\mu$ m.



**Fig. 4.** *Periconiella arcuata* (CBS 113477). A–B. Sympodially proliferating conidiogenous cells with darkened, thickened and cone-shaped scars. C–E. Macronematous conidiophores with loose branches in the upper part. F–I. Conidia. Scale bar = 10  $\mu$ m.



**Fig. 5.** *Periconiella levispora* (CBS 873.73). A–C. Conidial apparatus at different stages of development, which gives rise to macronematous conidiophores with dense branches in the upper part. D. Sympodially proliferating conidiogenous cells with darkened and somewhat protruding scars. E–F. Conidia with truncate base and darkened hilum. Scale bar = 10  $\mu$ m.



**Fig. 6.** A. *Pseudovirgaria hyperparasitica* (CBS 121739 = CPC 10753). B. *Periconiella levispora* (CBS 873.73). Scale bar = 10  $\mu$ m.

**Cultural characteristics:** Colonies on MEA reaching 12 mm diam after 14 d at 24 °C, with entire, smooth, sharp margin; mycelium compacted, becoming hairy, colonies up to 1 mm high; surface olivaceous to olivaceous-grey, reverse dark grey-olivaceous to olivaceous-black.

**Specimen examined:** **South Africa**, Western Cape Province, Kogelberg, on dead culms of *Ischyrolepis subverticillata*, May 2001, S. Lee, **holotype** CBS H-19927, culture ex-type CBS 113477.

***Periconiella levispora*** Arzanlou, W. Gams & Crous, **sp. nov.** MycoBank MB504546. Figs 5–6B.

**Etymology:** (Latin) *levis* = smooth.

A simili *Periconiella velutina* conidiis levibus et maioribus, ad 23 µm longis distinguenda.

**In vitro:** *Submerged hyphae* smooth, hyaline, thin-walled, 2–2.5 µm wide; *aerial hyphae* subhyaline, later becoming dark brown, thick-walled, smooth. *Conidiophores* arising vertically from creeping aerial hyphae, dark brown at the base, paler towards the apex, thick-walled; in the upper part bearing several short branches, up to 120 µm long. *Conidiogenous cells* integrated, occasionally discrete, cylindrical, variable in length, 10–20 µm long, subhyaline, later becoming pale brown, fertile part as wide as the basal part, proliferating sympodially, forming a short rachis with pigmented and slightly thickened, somewhat protruding scars, less than 1 µm diam. *Conidia* solitary, 0(–2)-septate, smooth, pale olivaceous, cylindrical, ellipsoidal, pyriform to clavate, (7–)11–14(–23) × (3–)4–5(–6) µm, with a truncate base and a darkened, slightly thickened hilum, 2 µm diam.

**Cultural characteristics:** Colonies on MEA slow-growing, reaching 5 mm diam after 14 d at 24 °C, with entire margin; aerial mycelium compact, raised, velvety, olivaceous-grey; reverse olivaceous-black.

**Specimen examined:** **Sri Lanka**, Hakgala Botanic Gardens, on dead leaves of *Turpinia pomifera*, Jan. 1973, W. Gams, **holotype** CBS H-15611, culture ex-type CBS 873.73.

***Ramichloridium*** Stahel ex de Hoog, Stud. Mycol. 15: 59. 1977.

**In vitro:** Colonies flat to raised, with entire margin; surface olivaceous-green to olivaceous-black. *Mycelium* consisting of submerged and aerial hyphae; submerged hyphae hyaline to subhyaline, thin-walled, aerial hyphae smooth or verrucose. *Conidiophores* straight, unbranched, rarely branched, thick-walled, dark brown (darker than the subtending hyphae), continuous or with several additional thin septa. *Conidiogenous cells* integrated, terminal, polyblastic, smooth, thick-walled, golden-brown, apical part subhyaline, with sympodial proliferation, straight or flexuose, geniculate or nodose, with conspicuous conidiogenous loci; scars crowded or scattered, unthickened, unpigmented to faintly pigmented, or slightly prominent denticles. *Conidia* solitary, 0–1-septate, subhyaline to pale brown, smooth to coarsely verrucose, rather thin-walled, obovate, obconical or globose to ellipsoidal, fusiform, with a somewhat prominent, slightly pigmented hilum; conidial secession schizolytic.

**Type species:** *R. apiculatum* (J.H. Mill., Giddens & A.A. Foster) de Hoog, Stud. Mycol. 15: 69. 1977.

***Ramichloridium apiculatum*** (J.H. Mill., Giddens & A.A. Foster) de Hoog, Stud. Mycol. 15: 69. 1977. Fig. 8.

**Basionym:** *Chloridium apiculatum* J.H. Mill., Giddens & A.A. Foster, Mycologia 49: 789. 1957.

= *Veronaea apiculata* (J.H. Mill., Giddens & A.A. Foster) M.B. Ellis, in Ellis, *More Dematiaceous Hyphomycetes*: 209. 1976.

[non *Rhinochlaidiella apiculata* Matsush., in Matsushima, *Icon. Microfung. Mats. lect.*: 122. 1975].

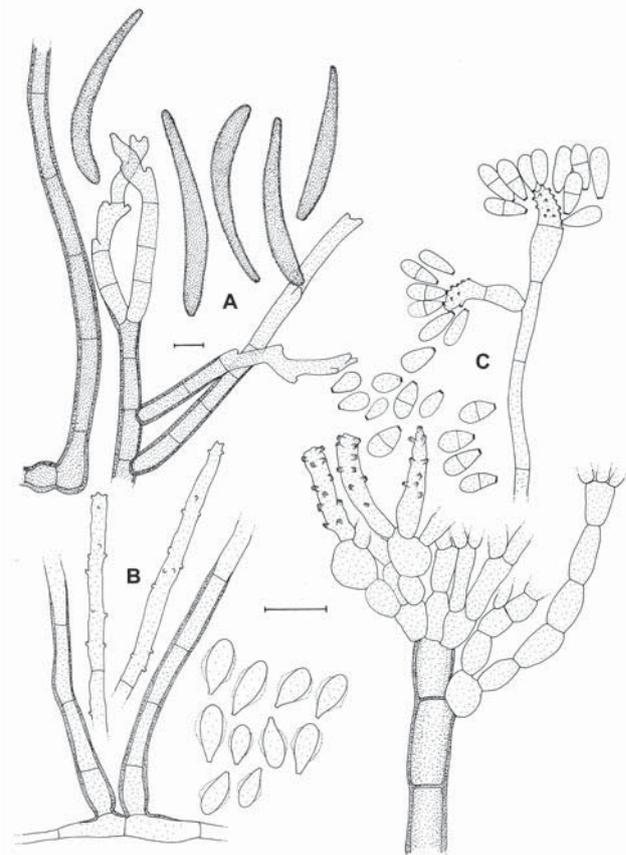
= *Rhinochlaidiella indica* Agarwal, Lloydia 32: 388. 1969.

[non *Chloridium indicum* Subram., Proc. Indian Acad. Sci., Sect. B, 42: 286. 1955].

**In vitro:** *Submerged hyphae* hyaline to subhyaline, thin-walled, 1–2.5 µm wide; *aerial hyphae* slightly darker, smooth-walled. *Conidiophores* generally arising at right angles from creeping aerial hyphae, straight, unbranched, thick-walled, dark brown, continuous or with 1–2(–3) additional thin septa, up to 100 µm long; intercalary cells 10–28 µm long. *Conidiogenous cells* integrated, terminal, smooth, thick-walled, golden-brown, straight, cylindrical, 25–37(–47) × 2–3.5 µm; proliferating sympodially, resulting in a straight rachis with conspicuous conidiogenous loci; scars prominent, crowded, slightly pigmented, less than 1 µm diam. *Conidia* solitary, obovate to obconical, pale brown, finely verrucose, (3–)5–5.5(–7.5) × (2–)2.5–3(–4) µm, hilum conspicuous, slightly pigmented, about 1 µm diam.

**Cultural characteristics:** Colonies on MEA reaching 35 mm diam after 14 d at 24 °C; minimum temperature for growth above 6 °C, optimum 24 °C, maximum 30 °C. Colonies raised, velvety, dense, with entire margin; surface olivaceous-green, reverse olivaceous-black, often with a diffusing citron-yellow pigment.

**Specimens examined:** **Pakistan**, Lahore, from soil, A. Kamal, CBS 400.76 = IMI 088021. **South Africa**, from preserved *Cucumis sativus* in 8-oxyquinoline sulphate, M.C. Papendorf, CBS 390.67; Potchefstroom, from *Aloe* sp., M.C. Papendorf, CBS 391.67. **U.S.A.**, Georgia, isolated from forest soil, CBS 156.59 = ATCC 13211 = IMI 100716 = QM 7716, **ex-type** culture.



**Fig. 7.** A. *Periconiella arcuata* (CBS 113477). B. *Myrmecridium schulzeri* (CBS 325.74). C. *Thysanorea papuana* (CBS 212.96). Scale bars = 10 µm.

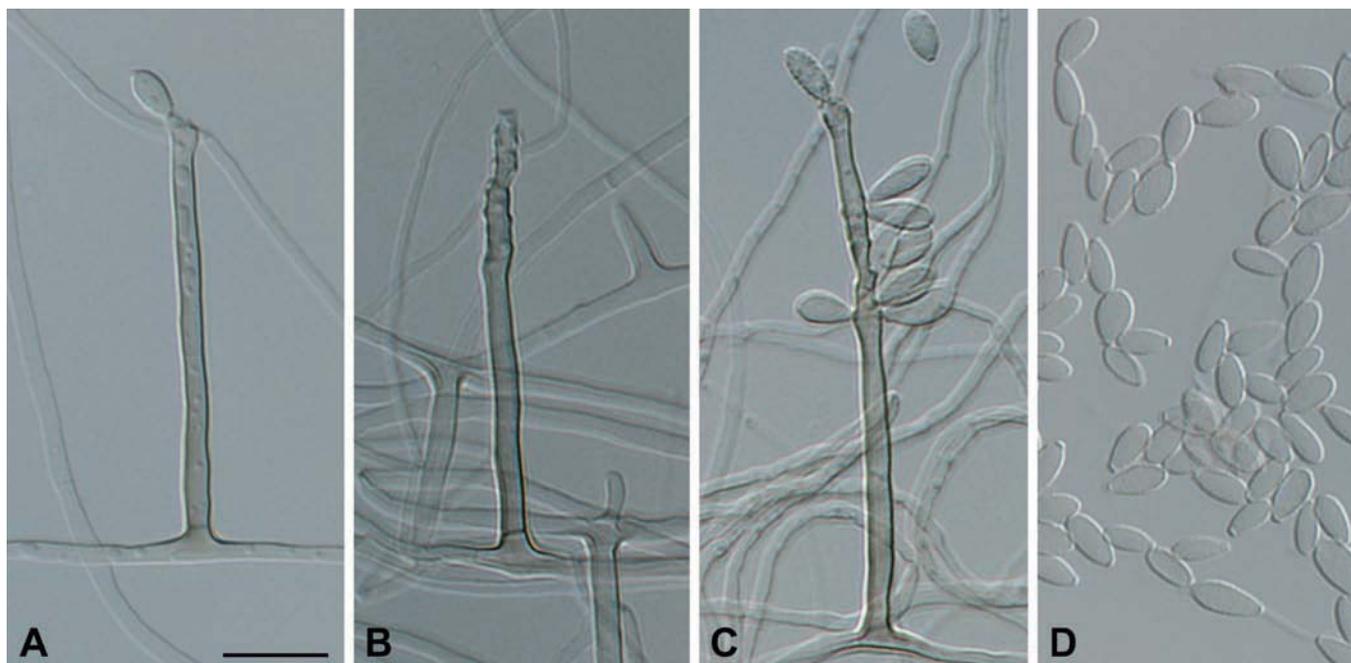


Fig. 8. *Ramichloridium apiculatum* (CBS 156.59). A–C. Macronematous conidiophores with sympodially proliferating conidiogenous cells, which give rise to a conidium-bearing rachis with crowded and prominent scars. D. Conidia. Scale bar = 10  $\mu$ m.

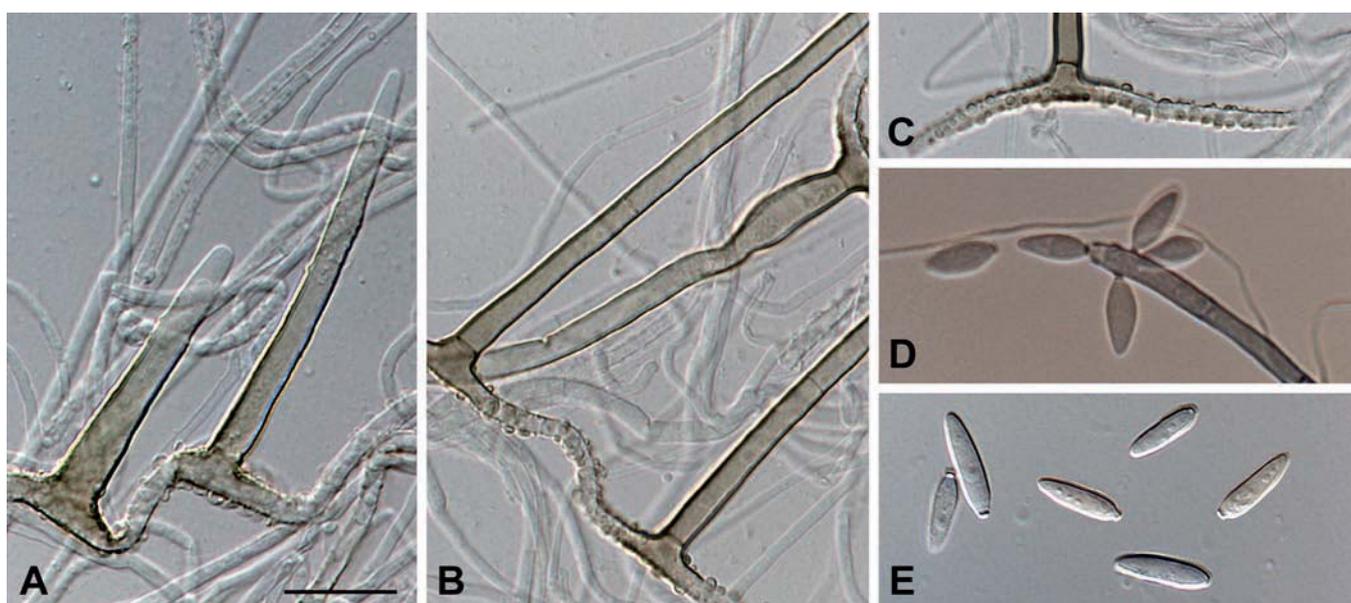


Fig. 9. *Ramichloridium australiense* (CBS 121710). A–C. Macronematous conidiophores with thick-walled and warted subtending hyphae. D. Sympodially proliferating conidiogenous cell, which results in a short rachis with darkened and slightly thickened scars. E. Conidia. Scale bar = 10  $\mu$ m.

***Ramichloridium australiense*** Arzanlou & Crous, **sp. nov.**  
Mycobank MB504548. Figs 9–10A.

**Etymology:** Named after its country of origin, Australia.

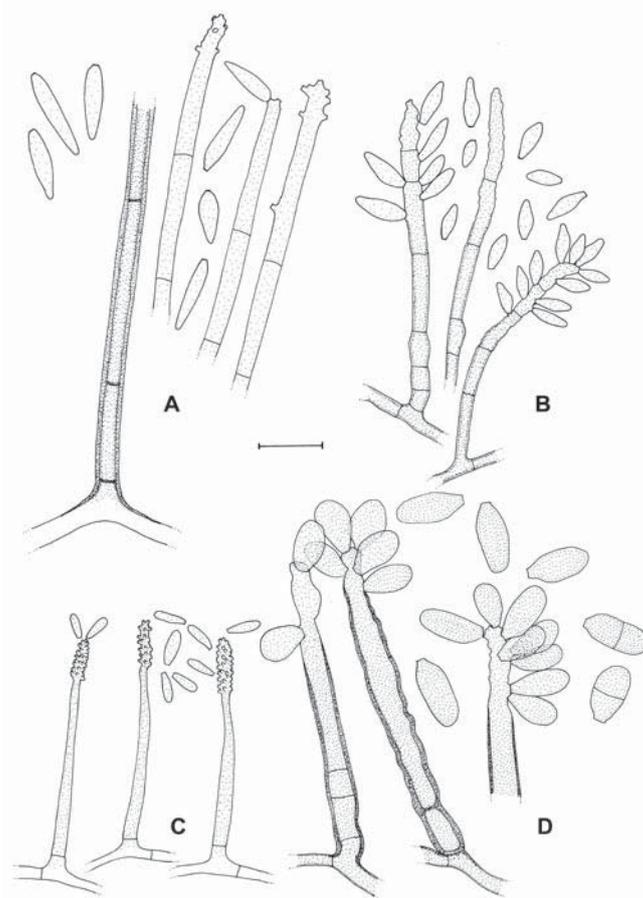
Ab aliis speciebus *Ramichloridii* conidiophoris ex hyphis verrucosis, crassitunicatis ortis distinguendum.

**In vitro:** *Submerged hyphae* hyaline, smooth, thin-walled, 1–2  $\mu$ m wide; *aerial hyphae* pale brown, warted. *Conidiophores* arising vertically and clearly differentiated from creeping aerial hyphae, up to 400  $\mu$ m tall, with several additional thin septa; intercalary cells, 8–40  $\times$  2–5  $\mu$ m, from the broadest part at the base tapering towards the apex, subhyaline, later becoming pale brown and warted in the lower part. Subtending hyphae thick-walled, warted. *Conidiogenous cells* integrated, terminal, 10–18  $\mu$ m long, proliferating sympodially,

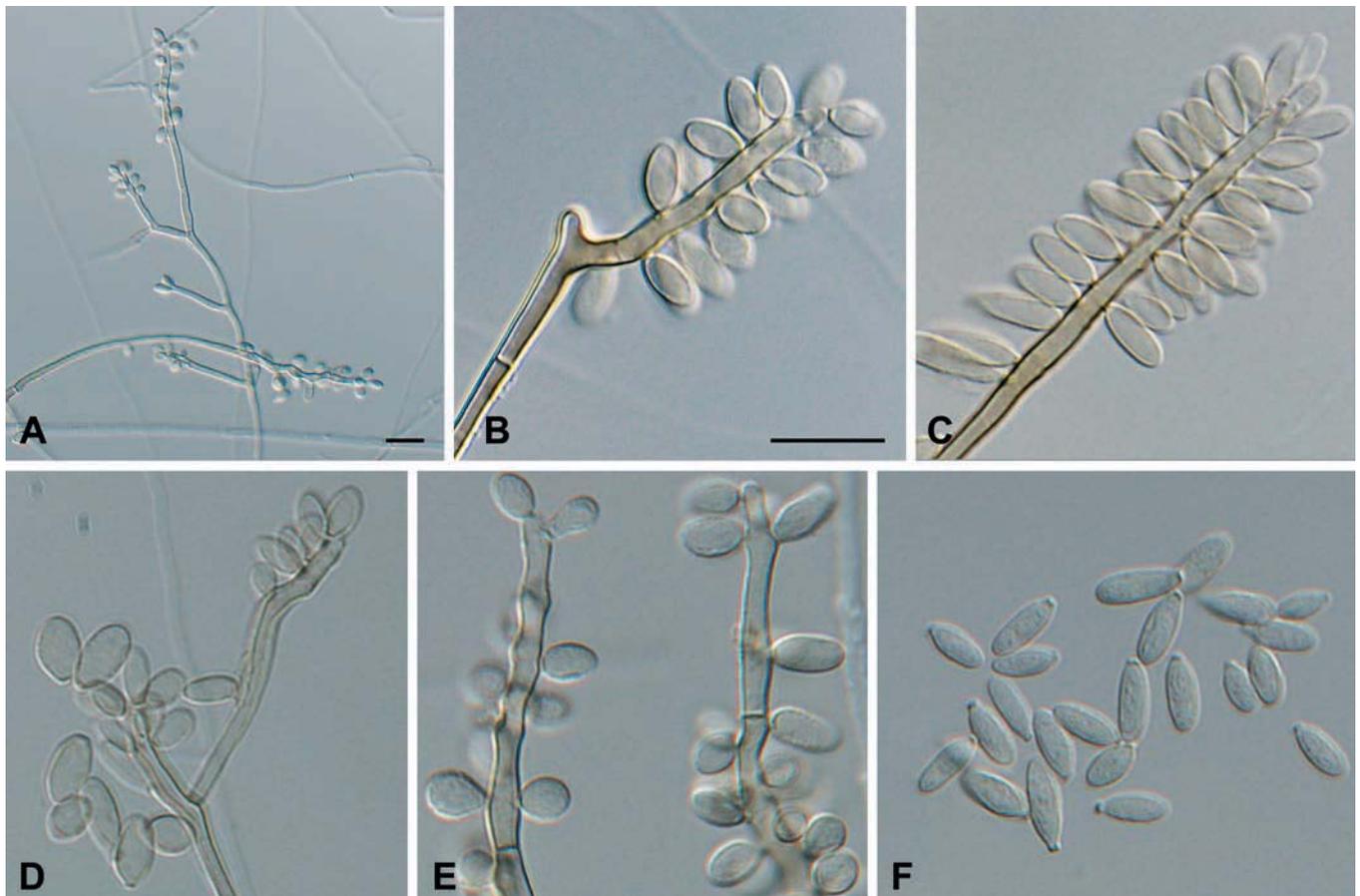
giving rise to a short rachis with conspicuous conidiogenous loci; scars slightly thickened and darkened, about 1  $\mu$ m diam. *Conidia* solitary, aseptate, thin-walled, smooth, subhyaline, subcylindrical to obclavate, (10–)12–15(–23)  $\times$  2.5–3  $\mu$ m, with a truncate base and a slightly darkened and thickened hilum, 1.5–2  $\mu$ m diam, rarely fusing at the basal part.

**Cultural characteristics:** Colonies on MEA rather slow growing, reaching 8 mm diam after 14 d at 24  $^{\circ}$ C, with entire, smooth margin; mycelium flat, olivaceous-grey, becoming granular, with gelatinous droplets at the margin developing with aging; reverse pale olivaceous-grey.

**Specimen examined:** **Australia**, Queensland, Mount Lewis, Mount Lewis Road, 16 $^{\circ}$ 34'47.2" S, 145 $^{\circ}$ 19'7" E, 538 m alt., on *Musa banksii* leaf, Aug. 2006, P.W. Crous and B. Summerell, **holotype** CBS H-19928, culture ex-type CBS 121710.



**Fig. 10.** A. *Ramichloridium australiense* (CBS 121710). B. *Ramichloridium brasilianum* (CBS 283.92). C. *Radulidium subulatum* (CBS 405.76). D. *Rhodoveronea varioseptata* (CBS 431.88). Scale bar = 10  $\mu$ m.



**Fig. 11.** *Ramichloridium musae* (CBS 365.36). A. Conidiophores with loose branches. B–D. Sympodially proliferating conidiogenous cells, resulting in a long conidium-bearing rachis. E. Rachis with hardly prominent, slightly darkened scars. F. Conidia. Scale bars = 10  $\mu$ m.

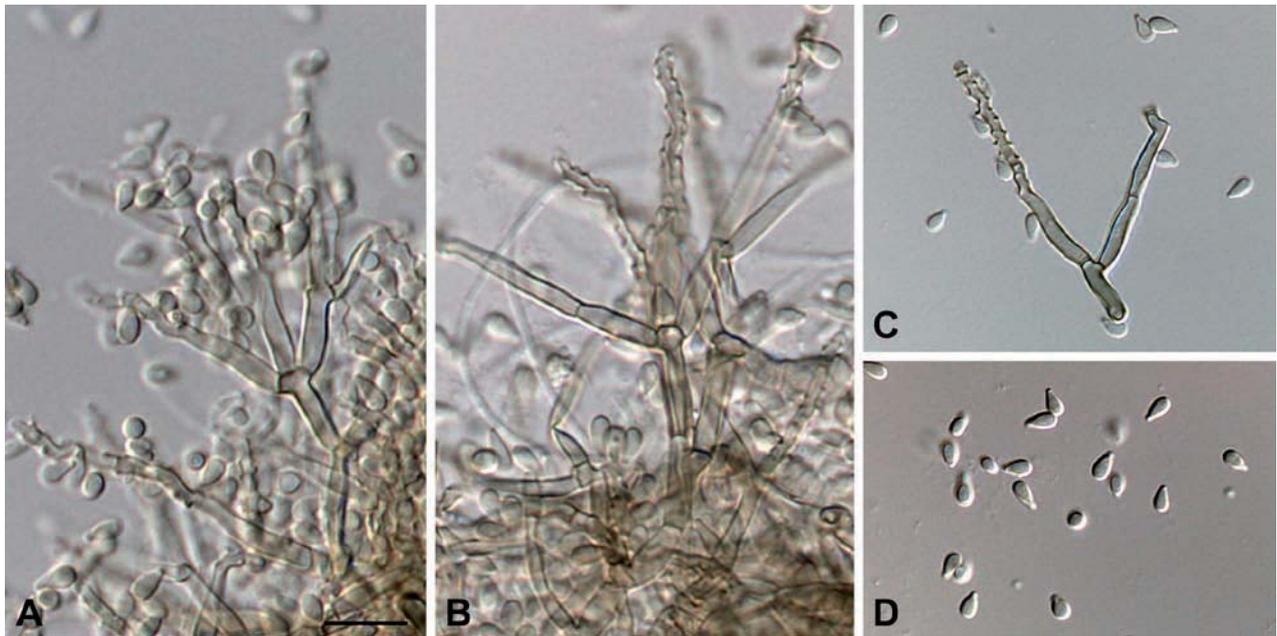


Fig. 12. *Ramichloridium biverticillatum* (CBS 335.36). A–B. Profusely branched and biverticillate conidiophores. C. Sympodially proliferating conidiogenous cells, which give rise to a conidium-bearing rachis with crowded, slightly pigmented and thickened scars. D. Conidia. Scale bar = 10  $\mu$ m.

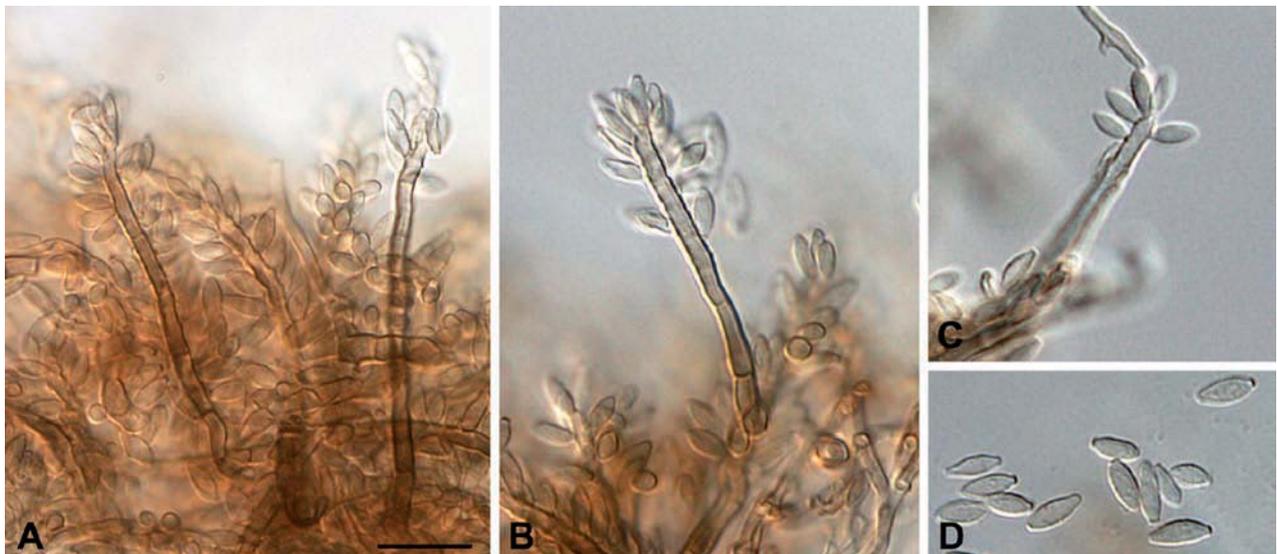


Fig. 13. *Ramichloridium brasilianum* (CBS 283.92). A–B. Macronematous conidiophores with sympodially proliferating conidiogenous cells, resulting in a conidium-bearing rachis. C. Rachis with crowded and slightly pigmented scars. D. Conidia. Scale bar = 10  $\mu$ m.

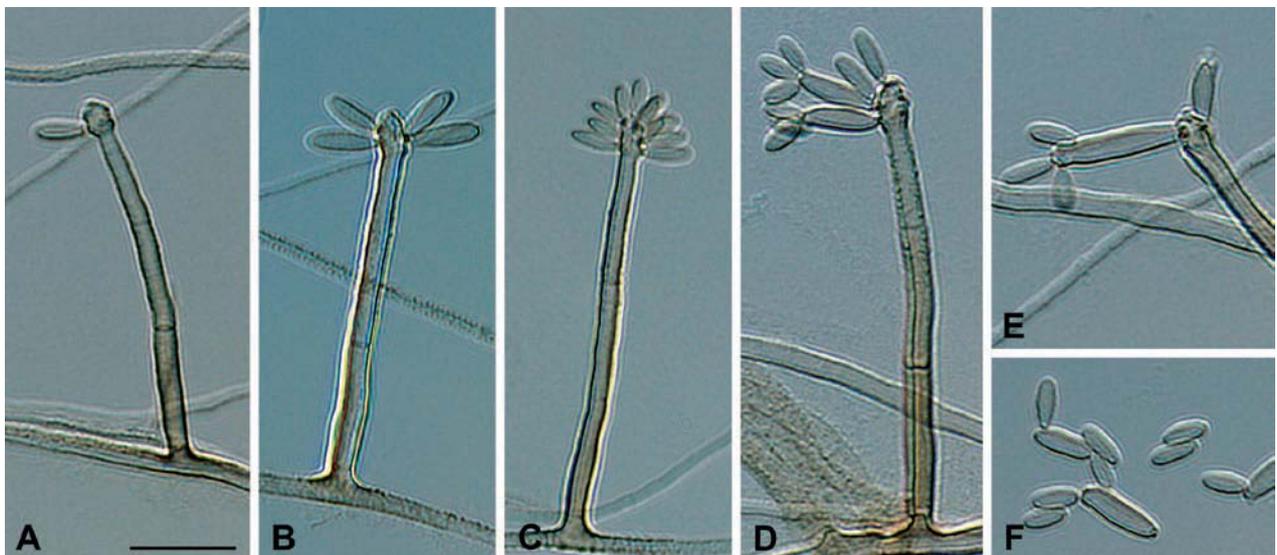


Fig. 14. *Ramichloridium cerophilum* (CBS 103.59). A–C. Conidial apparatus at different stages of development, resulting in macronematous conidiophores and sympodially proliferating conidiogenous cells. D–E. Formation of secondary conidia. F. Conidia. Scale bar = 10  $\mu$ m.

**Ramichloridium musae** (Stahel ex M.B. Ellis) de Hoog, Stud. Mycol. 15: 62. 1977. Fig. 11.

**Basionym:** *Veronaea musae* Stahel ex M.B. Ellis, in Ellis, *More Dematiaceous Hyphomycetes*: 209. 1976.

≡ *Chloridium musae* Stahel, Trop. Agric., Trinidad 14: 43. 1937 (nom. inval. Art. 36).

**Misapplied name:** *Chloridium indicum* Subram., *sensu* Batista & Vital, Anais Soc. Biol. Pernambuco 15: 379. 1957.

**In vitro:** *Submerged hyphae* smooth, hyaline, thin-walled, 1–2 µm wide; *aerial hyphae* subhyaline, smooth. *Conidiophores* arising vertically and mostly sharply differentiated from creeping aerial hyphae, golden-brown; unbranched, rarely branched in the upper part, up to 250 µm tall, with up to 6 additional thin septa, cells 23–40 × 2–2.5 µm, basal cell occasionally inflated. *Conidiogenous cells* terminally integrated, cylindrical, variable in length, 10–40 µm long, golden-brown near the base, subhyaline to pale brown near the end, fertile part as wide as the basal part, later also becoming septate; rachis elongating sympodially, 2–2.5 µm wide, with hardly prominent, scattered, slightly pigmented scars, about 0.5 µm diam. *Conidia* solitary, aseptate, hyaline to subhyaline, ellipsoidal, (4–)7–8(–12) × 2–3 µm, smooth or verruculose, thin-walled, with slightly darkened hilum, about 1 µm diam.

**Cultural characteristics:** Colonies on MEA slow-growing, reaching 27 mm diam after 14 d at 24 °C, with entire, smooth, sharp margin; mycelium mostly submerged, some floccose to lanose aerial mycelium in the olivaceous-grey centre, becoming pale pinkish olivaceous towards the margin; reverse pale orange.

**Specimens examined:** **Cameroon**, from *Musa sapientum*, J.E. Heron, CBS 169.61 = ATCC 15681 = IMI 079492 = DAOM 84655 = MUCL 2689; from *Musa sapientum*, J. Brun, CBS 190.63 = MUCL 9557. **Surinam**, Paramaribo, from *Musa sapientum* leaf, G. Stahel, CBS 365.36 = JCM 6973 = MUCL 9556, **ex-type** strain of *Chloridium musae*; from *Musa sapientum*, G. Stahel, CBS 365.36; dried culture preserved as CBS H-19933.

**Ramichloridium biverticillatum** Arzanlou & Crous, **nom. nov.** MycoBank MB504549. Fig. 12.

**Basionym:** *Periconiella musae* Stahel ex M.B. Ellis, Mycol. Pap. 111: 5. 1967.

[non *Ramichloridium musae* (Stahel ex M.B. Ellis) de Hoog, 1977].  
 ≡ *Ramichloridium musae* Stahel, Trop. Agric., Trinidad 14: 43. 1937 (nom. inval. Art. 36).

= *Ramichloridium musae* (Stahel ex M.B. Ellis) de Hoog, Stud. Mycol. 15: 62. 1977, *sensu* de Hoog, p.p.

**Etymology:** Named after its biverticillate conidiophores.

**In vitro:** *Submerged hyphae* smooth, hyaline, thin-walled, 1–2 µm wide; *aerial hyphae* subhyaline, smooth, slightly darker. *Conidiophores* arising vertically from creeping aerial hyphae, pale brown, profusely branched, biverticillate, with up to three levels of main branches; branches tapering distally, 2–3 µm wide at the base, approx. 2 µm wide in the upper part, up to 250 µm long. *Conidiogenous cells* terminally integrated, cylindrical, variable in length, 15–50 µm long, rachis straight or geniculate, pale brown, as wide as the basal part; elongating sympodially, forming a rachis with crowded, slightly darkened and thickened minute scars, less than 0.5 µm wide. *Conidia* solitary, aseptate, hyaline to subhyaline, dacryoid to pyriform, (2–)3–4(–6) × (1.5–)2(–2.5) µm, smooth, thin-walled, with an inconspicuous hilum.

**Cultural characteristics:** Colonies on MEA slow-growing, reaching 16 mm diam after 14 d at 24 °C, with entire, smooth, sharp margin, rather compact, velvety; surface vinaceous-buff to olivaceous-buff; reverse buff.

**Specimen examined:** **Surinam**, from *Musa sapientum*, Aug. 1936, G. Stahel, CBS 335.36.

**Notes:** *Ramichloridium biverticillatum* is a new name based on *Periconiella musae*. The species is distinct from *R. musae* because of its profusely branched conidiophores, and conidia that are smaller (2–5 × 1.5–2.5 µm) than those of *R. musae* (5–11 × 2–3 µm).

**Ramichloridium brasilianum** Arzanlou & Crous, **sp. nov.** MycoBank MB504550. Figs 10B, 13.

**Etymology:** Named after its country of origin, Brazil.

A simili *Ramichloridio cerophilo* conidiis minoribus, ad 8 µm longis, et conidiis secundariis absentibus distinguendum.

**In vitro:** *Submerged hyphae* pale olivaceous, smooth or slightly rough, 1.5–2 µm wide; *aerial hyphae* olivaceous, smooth or rough, narrower and darker than the submerged hyphae. *Conidiophores* unbranched, arising vertically from creeping aerial hyphae, straight or flexuose, dark brown, with up to 10 additional septa, thick-walled, cylindrical, 2–2.5 µm wide and up to 70 µm long. *Conidiogenous cells* integrated, terminal, 10–30 µm long, proliferating sympodially, giving rise to a long, straight rachis with crowded, slightly darkened minute scars, about 0.5 µm diam. *Conidia* solitary, obovoid to fusiform with the widest part below the middle, thin-walled, verruculose, aseptate, pale brown, slightly rounded at the apex, truncate at the base, (4–)5–6(–8.5) × 2–2.5(–3) µm, with a slightly thickened and darkened hilum, 1–1.5 µm diam.

**Cultural characteristics:** Colonies on MEA slow-growing, reaching 6 mm diam after 14 d at 24 °C, velvety to hairy, colonies with entire margin, surface dark olivaceous-grey; black gelatinous exudate droplets produced on OA.

**Specimen examined:** **Brazil**, São Paulo, Peruibe, Jureia Ecological Reserve, forest soil, Jan. 1991, D. Attili, **holotype** CBS H-19929, culture ex-type CBS 283.92.

**Ramichloridium cerophilum** (Tubaki) de Hoog, Stud. Mycol. 15: 74. 1977. Fig. 14.

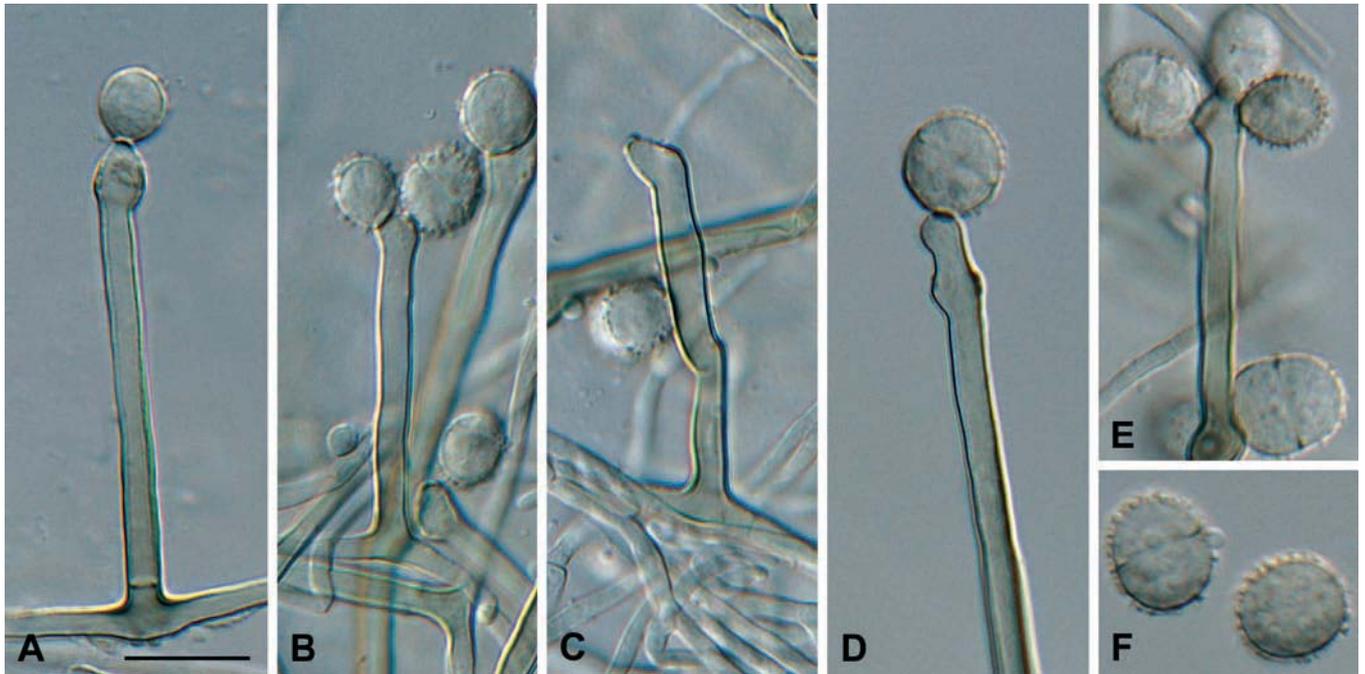
**Basionym:** *Acrotheca cerophila* Tubaki, J. Hattori Bot. Lab. 20: 143. 1958.

≡ *Cladosporium cerophilum* (Tubaki) Matsush., in Matsushima, *Icon. Microfung. Matsush. lect.* (Kobe): 34. 1975.

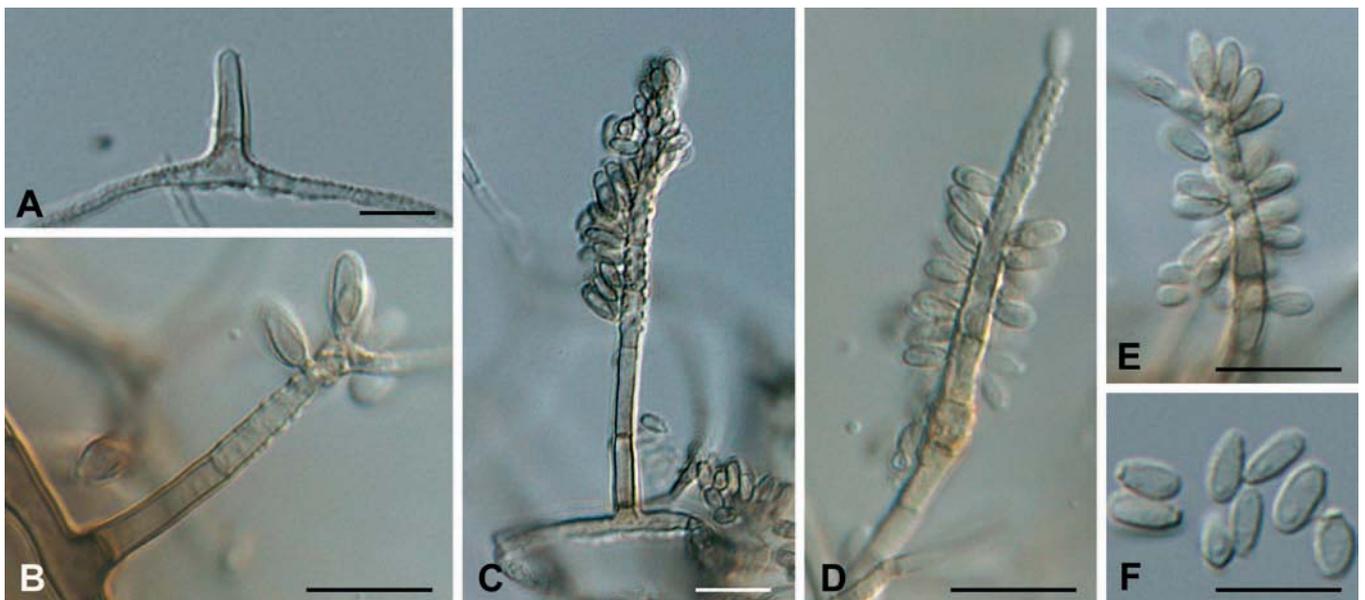
**In vitro:** *Submerged hyphae* pale olivaceous-brown, smooth or slightly rough, 1.5–3 µm wide; *aerial hyphae* olivaceous-brown, smooth or slightly rough, somewhat narrower and darker than the submerged hyphae. *Conidiophores* unbranched, arising vertically from creeping aerial hyphae, dark brown, thick-walled, smooth or verruculose, hardly tapering towards the apex, 2–3 µm wide, up to 50 µm long, with up to 3 additional septa. *Conidiogenous cells* integrated, terminal, proliferating sympodially, rachis short and straight, with crowded, prominent, pigmented unthickened scars, minute, approx. 0.5 µm diam. *Conidia* solitary, fusiform to clavate, thin-walled, smooth, 0(–1)-septate, subhyaline, (4–)6–7(–11) × (2–)2.5(–3) µm, with a conspicuous hilum, about 0.5 µm diam, slightly raised with an inconspicuous marginal frill, somehow resembling those of *Cladosporium*. Conidia sometimes producing 1–3(–4) short secondary conidia.

**Cultural characteristics:** Colonies on MEA rather slow-growing, reaching 12 mm diam after 14 d at 24 °C, velvety to hairy, with entire margin; surface dark olivaceous-grey, with black gelatinous exudate droplets on OA.

**Specimen examined:** **Japan**, isolated from *Sasa* sp., K. Tubaki, CBS 103.59, **ex-type**.



**Fig. 15.** *Ramichloridium indicum* (CBS 171.96). A–B. Macronematous conidiophores. C–E. Sympodially proliferating conidiogenous cells, resulting in a conidium-bearing rachis with pigmented and thickened scars. F. Conidia. Scale bar = 10  $\mu$ m.



**Fig. 16.** *Ramichloridium strelitziae* (CBS 121711). A–C. Conidial apparatus at different stages of development, resulting in macronematous conidiophores and sympodially proliferating conidiogenous cells. D–E. Rachis with crowded, slightly pigmented, thickened, circular scars. F. Conidia. Scale bars = 10  $\mu$ m.

**Notes:** Phylogenetically, this species together with *Ramichloridium apiculatum* and *R. musae* cluster within the *Mycosphaerellaceae* clade. *Ramichloridium cerophilum* can be distinguished from its relatives by the production of secondary conidia and its distinct conidial hila.

***Ramichloridium indicum*** (Subram.) de Hoog, *Stud. Mycol.* 15: 70. 1977. Fig. 15.

**Basionym:** *Chloridium indicum* Subram., *Proc. Indian Acad. Sci., Sect. B*, 42: 286. 1955 [non *Rhinochadiella indica* Agarwal, *Lloydia* 32: 388. 1969].

$\equiv$  *Veronaea indica* (Subram.) M.B. Ellis, in Ellis, *More Dematiaceous Hyphomycetes*: 209. 1976.

= *Veronaea verrucosa* Geeson, *Trans. Brit. Mycol. Soc.* 64: 349. 1975.

**In vitro:** *Submerged hyphae* smooth, thin-walled, hyaline, 1–2.5  $\mu$ m wide, with thin septa; *aerial hyphae* coarsely verrucose, olivaceous-green, rather thick-walled, 2–2.5  $\mu$ m wide, with thin septa. *Conidiophores* arising vertically from creeping hyphae at right angles, straight, unbranched, thick-walled, smooth, dark brown, with up to 10 thin septa, up to 250  $\mu$ m long, 2–4  $\mu$ m wide, often with inflated basal cells. *Conidiogenous cells* terminally integrated, up to 165  $\mu$ m long, smooth, dark brown, sympodially proliferating, rachis straight or flexuose, geniculate or nodose, subhyaline; scars thickened and darkened, clustered at nodes, approx. 0.5  $\mu$ m diam. Microcyclic conidiation observed in culture. *Conidia* solitary, (0–)1-septate, not constricted at the septum, subhyaline to pale brown, smooth or coarsely verrucose, rather thin-walled, broadly ellipsoidal to globose, (5–)7–8(–10)  $\times$  (4–)6–6.5(–9)  $\mu$ m, with truncate base; hilum conspicuous, slightly darkened, not thickened, about 1  $\mu$ m diam.

**Cultural characteristics:** Colonies on MEA reaching 35 mm diam after 14 d at 24 °C. Colonies velvety, rather compact, slightly elevated, with entire, smooth, whitish margin, dark olivaceous-green in the central part.

**Specimen examined:** Living culture, Feb. 1996, L. Marvanová, CBS 171.96.

***Ramichloridium pini*** de Hoog & Rahman, Trans. Brit. Mycol. Soc. 81: 485. 1983.

**Specimen examined:** U.K., Scotland, Old Aberdeen, branch of *Pinus contorta* (*Pinaceae*), 1982, M.A. Rahman, **ex-type** strain, CBS 461.82 = MUCL 28942.

**Note:** The culture examined (CBS 461.82) was sterile. For a full description see de Hoog *et al.* (1983).

***Ramichloridium strelitziae*** Arzanlou, W. Gams & Crous, **sp. nov.** MycoBank MB504551. Figs 16–17A.

**Etymology:** Named after its host, *Strelitzia*.

Ab aliis speciebus *Ramichloridii* conidiophoris brevibus, ad 40 µm longis, et cicatricibus rotundis, paulo protrudentibus distinguendum.

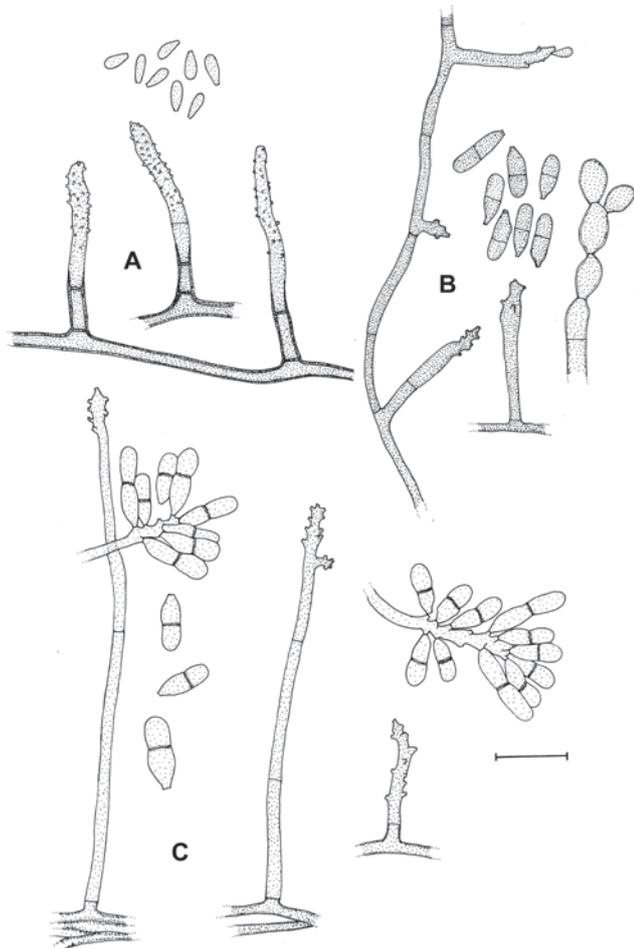
**In vitro:** *Submerged hyphae* smooth, hyaline, thin-walled, 2–2.5 µm wide; *aerial hyphae* pale brown, verrucose. *Conidiophores* arising vertically from creeping aerial hyphae, clearly differentiated from the vegetative hyphae, subhyaline, later becoming pale brown, thick-walled, smooth or verruculose, with 1–3 additional septa; up to 40 µm long and 2 µm wide. *Conidiogenous cells* integrated, terminal, cylindrical, variable in length, 10–35 µm long, subhyaline, later turning pale brown, fertile part as wide as the basal part, proliferating sympodially, forming a straight rachis with slightly thickened and darkened, circular, somewhat protruding scars, approx. 0.5 µm diam. *Conidia* solitary, aseptate, smooth or verruculose, subhyaline, oblong, ellipsoidal to clavate, (3–)4–5(–5.5) × (1–)2(–2.5) µm, with truncate base and unthickened, non-pigmented hilum.

**Cultural characteristics:** Colonies on MEA slow-growing, reaching 5 mm diam after 14 d at 24 °C, with entire margin; aerial mycelium rather compact, raised, dense, olivaceous-grey; reverse olivaceous-black.

**Specimen examined:** South Africa, KwaZulu-Natal, Durban, near Réunion, on leaves of *Strelitzia nicolai*, 5 Feb. 2005, W. Gams & H. Glen, CBS-H 19776, **holotype**, culture ex-type CBS 121711.

***Zasmidium*** Fr., *Summa Veg. Scand.* 2: 407. 1849.

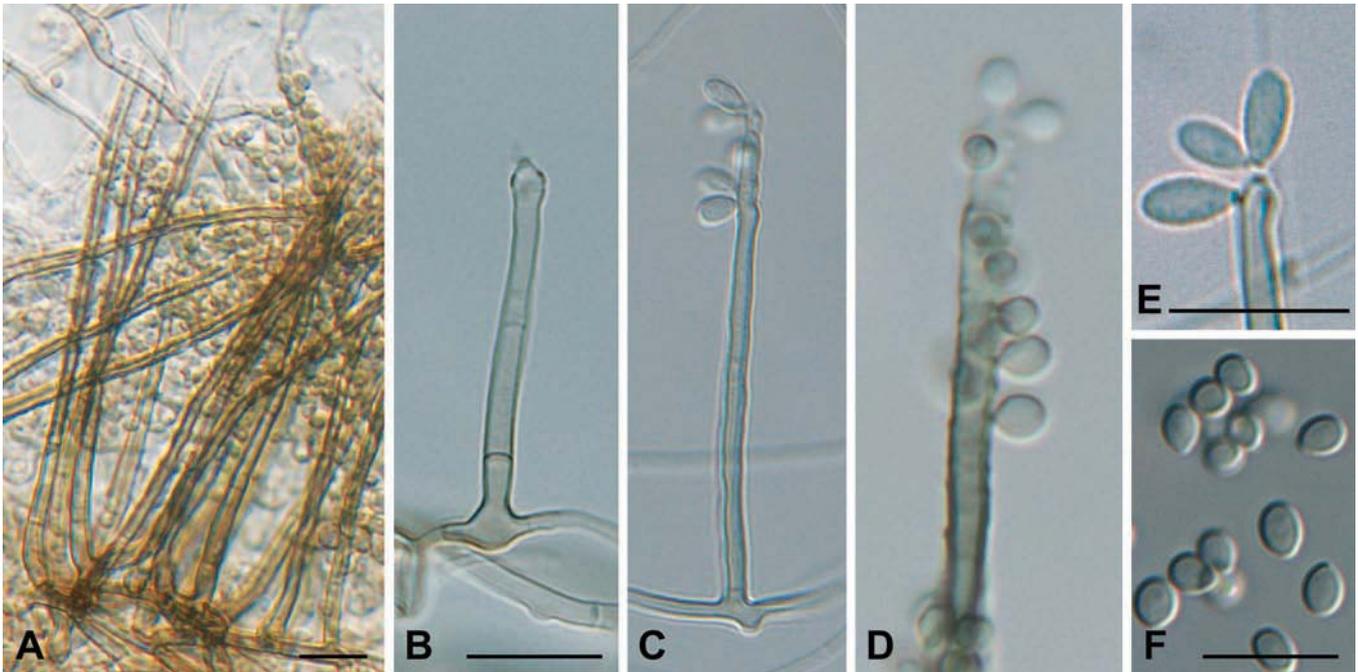
**In vitro:** *Submerged hyphae* smooth, thin-walled, hyaline, with thin septa; *aerial hyphae* coarsely verrucose, olivaceous-green, thick-walled, with thin septa. *Conidiophores* not differentiated from vegetative hyphae, often reduced to conidiogenous



**Fig. 17.** A. *Ramichloridium strelitziae* (CBS 121711). B. *Veronaea japonica* (CBS 776.83). C. *Veronaeopsis simplex* (CBS 588.66). Scale bar = 10 µm.



**Fig. 18.** *Zasmidium cellare* (CBS 146.36). A–D. Micronematous conidiophores with terminal, integrated conidiogenous cells. E. Conidiogenous cell with pigmented, thickened and refractive scars. F–G. Primary and secondary conidia. Scale bar = 10 µm.



**Fig. 19.** *Rhinocladiella anceps* (CBS 181.65). A. Macronematous conidiophores. B–D. Conidial apparatus at different stages of development, resulting in semi-micronematous conidiophores and sympodially proliferating conidiogenous cells. E. Conidiogenous loci. F. Conidia. Scale bars = 10  $\mu$ m.

cells. *Conidiogenous cells* integrated, predominantly terminal, sometimes lateral, arising from aerial hyphae, cylindrical, pale brown; polyblastic, proliferating sympodially producing crowded, conspicuously pigmented, almost flat, darkened, somewhat refractive scars. *Conidia* in short chains, cylindrical to fusiform, verrucose, obovate to obconical, pale brown, base truncate, with a conspicuous, slightly pigmented, thickened and refractive hilum. *Primary conidia* sometimes larger, subhyaline, verrucose or smooth-walled, 0–4-septate, variable in length, fusiform to cylindrical; conidial secession schizolytic.

*Type species:* *Zasmidium cellare* (Pers. : Fr.) Fr., *Summa Veg. Scand.* 2: 407. 1849.

***Zasmidium cellare*** (Pers. : Fr.) Fr., *Summa Veg. Scand.* 2: 407. 1849. Fig. 18.

*Basionym:* *Racodium cellare* Pers., *Neues Mag. Bot.* 1: 123. 1794.

≡ *Antennaria cellaris* (Pers. : Fr.) Fr., *Syst. Mycol.* 3: 229. 1829.

≡ *Cladosporium cellare* (Pers. : Fr.) Schanderl, *Zentralbl. Bakteriol.*, 2. Abt., 94: 117. 1936.

≡ *Rhinocladiella cellaris* (Pers. : Fr.) M.B. Ellis, in Ellis, *Dematiaceous Hyphomycetes*: 248. 1971.

*In vitro:* *Submerged hyphae* smooth, thin-walled, hyaline, 2–3  $\mu$ m wide, with thin septa; *aerial hyphae* coarsely verrucose, olivaceous-green, rather thick-walled, 2–2.5  $\mu$ m wide, with thin septa. *Conidiophores* not differentiated from vegetative hyphae, often reduced to conidiogenous cells. *Conidiogenous cells* integrated, predominantly terminal, sometimes lateral, arising from aerial hyphae, cylindrical, 20–60  $\mu$ m long and 2–2.5  $\mu$ m wide, pale brown, proliferating sympodially producing crowded, conspicuously pigmented scars that are thickened and refractive, about 1  $\mu$ m diam. *Conidia* cylindrical to fusiform, verrucose, obovate to obconical, pale brown, with truncate base, (6–)9–14(–27)  $\times$  2–2.5  $\mu$ m, with a conspicuous, slightly pigmented, refractive hilum, approx. 1  $\mu$ m diam. *Primary conidia* sometimes subhyaline, verrucose or smooth-walled, thin-walled, 0–1(–4)-septate, variable in length, fusiform to cylindrical.

*Cultural characteristics:* Colonies reaching 7 mm diam after 14 d at 24 °C. Colonies velvety, rather compact, slightly elevated with entire margin; surface dark olivaceous-green in the central part, margin smooth, whitish.

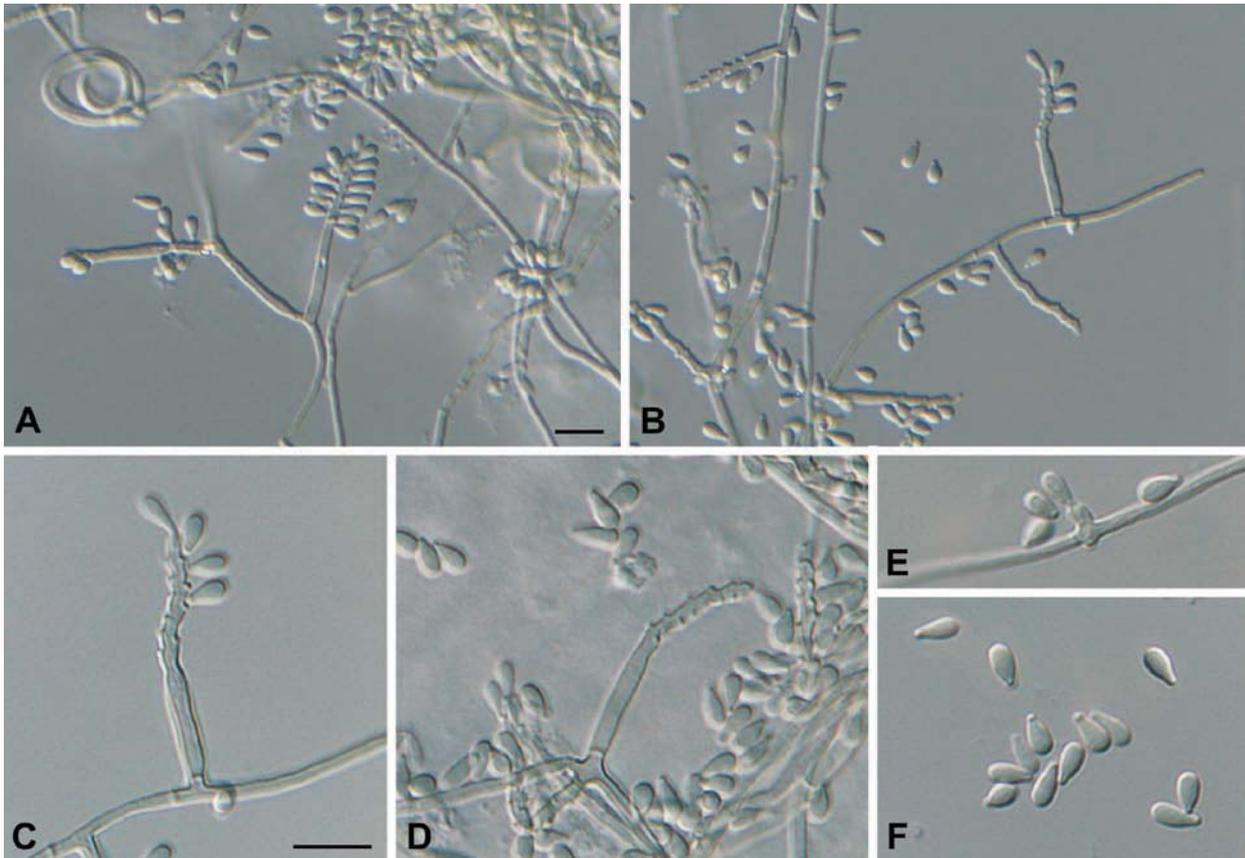
*Specimen examined:* Wall in wine cellar, Jun. 1936, H. Schanderl, ATCC 36951 = IFO 4862 = IMI 044943 = LCP 52.402 = LSHB BB274 = MUCL 10089 = CBS 146.36.

*Notes:* The name *Racodium* Fr., typified by *Ra. rupestre* Pers. : Fr., has been conserved over the older one by Persoon, with *Ra. cellare* as type species. De Hoog (1979) defended the use of *Zasmidium* in its place for the well-known wine-cellar fungus.

Morphologically *Zasmidium* resembles *Stenella* Syd., and both reside in the *Capnodiales*, though the type of *Stenella*, *S. araguata* Syd., clusters in the *Teratosphaeriaceae*, and the type of *Zasmidium*, *Z. cellare*, in the *Mycosphaerellaceae*. When accepting anamorph genera as polyphyletic within an order, preference would be given to the well-known name *Stenella* over the less known *Zasmidium*, even though the latter name is older. Further studies are required, however, to clarify if all *stenella*-like taxa should be accommodated in a single genus, *Stenella*. If this is indeed the case, a new combination for *Zasmidium cellare* will be proposed in *Stenella*, and the latter genus will have to be conserved over *Zasmidium*.

### ***Chaetothyriales (Herpotrichiellaceae)***

The four “*Ramichloridium*” species residing in the *Chaetothyriales* clade do not differ sufficiently in morphology to separate them from *Rhinocladiella* (type *Rh. atrovirens*). Because of the pale brown conidiophores, conidiogenous cells with crowded, slightly prominent scars and the occasional presence of an *Exophiala* J.W. Carmich. synanamorph, *Rhinocladiella* is a suitable genus to accommodate them. These four species chiefly differ from *Ramichloridium* in the morphology of their conidial apparatus, which is clearly differentiated from the vegetative hyphae. The appropriate combinations are therefore introduced for *Ramichloridium anceps*, *R. mackenziei*, *R. fasciculatum* and *R. basitonum*.



**Fig. 20.** *Rhinocladiella basitona* (CBS 101460). A–B. Semi-micronematous conidiophores with verticillate branching pattern. C–D. Sympodially proliferating conidiogenous cells, giving rise to a long rachis with slightly prominent, truncate conidium-bearing denticles. E. Intercalary conidiogenous cell. F. Conidia. Scale bars = 10  $\mu$ m.

The genus *Veronaea* (type species: *V. botryosa*) also resides in the *Chaetothyriales* clade. *Veronaea* can be distinguished from *Rhinocladiella* by the absence of exophiala-type budding cells and its predominantly 1-septate conidia. Furthermore, the conidiogenous loci in *Veronaea* are rather flat, barely prominent.

***Rhinocladiella*** Nannf., Svensk Skogsvårdsfören. Tidskr., Häfte 32: 461. 1934.

*In vitro*: Colonies dark olivaceous-brown, slow-growing, almost moist. *Submerged hyphae* hyaline to pale olivaceous, smooth; *aerial hyphae*, if present, more darkly pigmented. Exophiala-type budding cells usually present in culture. *Conidial apparatus* usually branched, olivaceous-brown, consisting of either slightly differentiated tips of ascending hyphae or septate, markedly differentiated conidiophores. *Conidiogenous cells* intercalary or terminal, polyblastic, cylindrical to acicular, with a sympodially proliferating, subdenticulate rachis; scars unthickened, non-pigmented to somewhat darkened-refractive. *Conidia* solitary, hyaline to subhyaline, aseptate, thin-walled, smooth, subglobose, with a slightly pigmented hilum; conidial secession schizolytic.

*Type species*: *Rh. atrovirens* Nannf., Svenska Skogsvårdsfören. Tidskr. 32: 461. 1934.

***Rhinocladiella anceps*** (Sacc. & Ellis) S. Hughes, Canad. J. Bot. 36: 801. 1958. Fig. 19.

*Basionym*: *Sporotrichum anceps* Sacc. & Ellis, Michelia 2: 576. 1882.

= *Veronaea parvispora* M.B. Ellis, in Ellis, *More Dematiaceous Hyphomycetes*: 210. 1976.

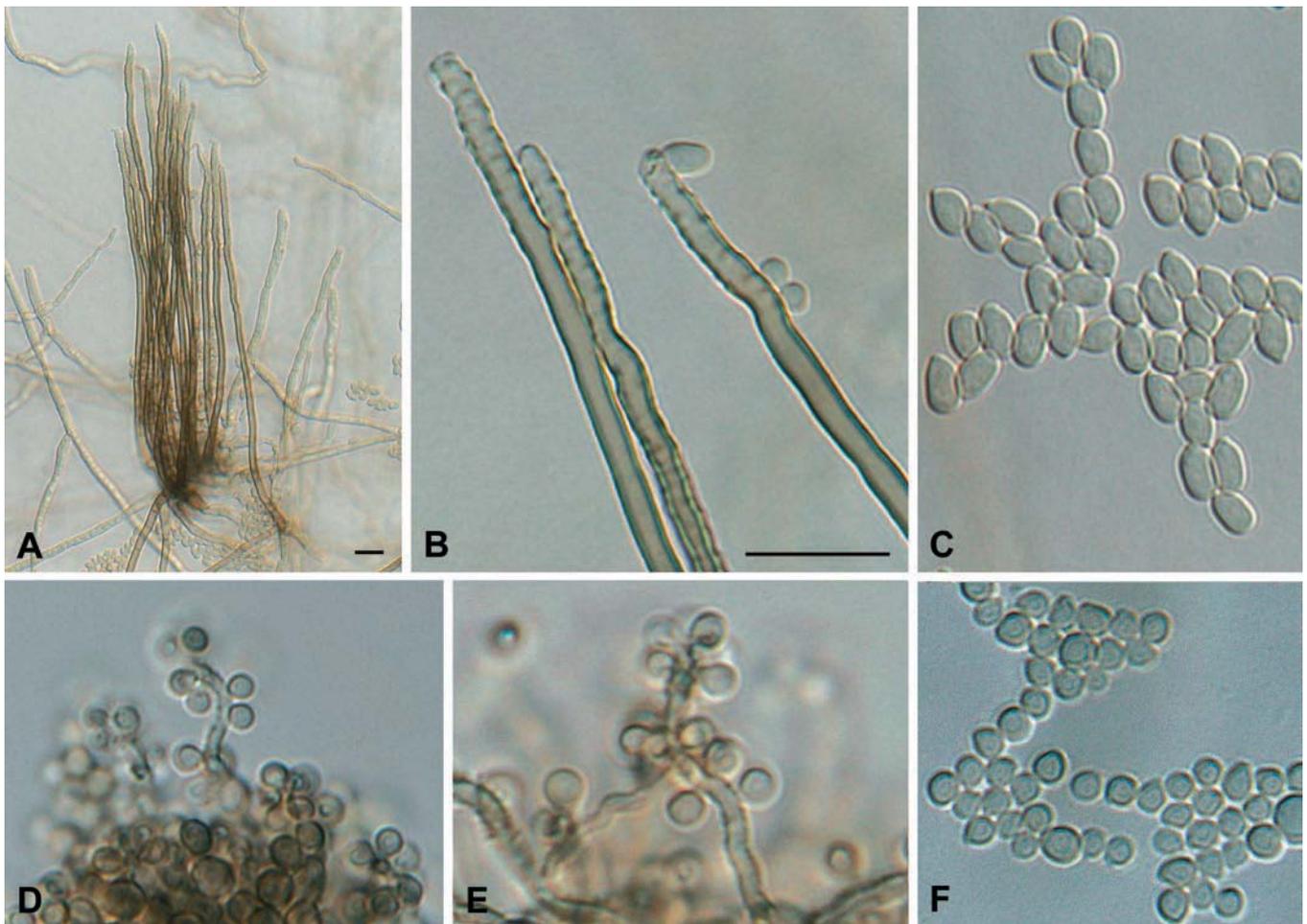
*Misapplied name*: *Chloridium minus* Corda *sensu* Mangenot, Rev. Mycol. (Paris) 18: 137. 1953.

*In vitro*: *Submerged hyphae* subhyaline, smooth, thick-walled, 2–2.5  $\mu$ m wide; *aerial hyphae* pale brown. Swollen germinating cells often present on MEA, giving rise to an *Exophiala* synanamorph. *Conidiophores* slightly differentiated from vegetative hyphae, arising from prostrate aerial hyphae, consisting of either unbranched or loosely branched stalks, thick-walled, golden to dark-brown, up to 350  $\mu$ m tall, which may have up to 15 thin, additional septa, intercalary cells 9–14  $\mu$ m long. *Conidiogenous cells* terminal, rarely lateral, cylindrical, occasionally intercalary, variable in length, smooth, golden to dark brown at the base, paler toward the apex, later becoming inconspicuously septate, fertile part as wide as the basal part, 15–40  $\times$  1.5–2  $\mu$ m; with crowded, slightly prominent, unpigmented, conidium-bearing denticles, about 0.5  $\mu$ m diam. *Conidia* solitary, subhyaline, thin-walled, smooth, subglobose to ellipsoidal, 2.5–4  $\times$  2–2.5  $\mu$ m, with a less conspicuous, slightly darkened hilum, less than 0.5  $\mu$ m diam.

*Cultural characteristics*: Colonies on MEA reaching 6–12 mm diam after 14 d at 24  $^{\circ}$ C, with entire, smooth, sharp margin; mycelium powdery, becoming hairy at centre; olivaceous-green to brown, reverse dark-olivaceous.

*Specimens examined*: **Canada**, Ontario, Campbellville, from soil under *Thuja plicata*, Apr. 1965, G. L. Barron, CBS H-7715 (isoneotype); CBS H-7716 (isoneotype); CBS H-7717 (isoneotype); CBS H-7718 (isoneotype); CBS H-7719 (isoneotype), **ex-type** strain, CBS 181.65 = ATCC 18655 = DAOM 84422 = IMI 134453 = MUCL 8233 = OAC 10215. **France**, from stem of *Fagus sylvatica*, 1953, F. Mangenot, CBS 157.54 = ATCC 15680 = MUCL 1081 = MUCL 7992 = MUCL 15756.

*Notes*: *Rhinocladiella anceps* (conidia 2.5–4  $\mu$ m long) resembles *Rh. phaeophora* Veerkamp & W. Gams (1983) (conidia 5.5–6  $\mu$ m long), but has shorter conidia.



**Fig. 21.** *Rhinocladiella fasciculata* (CBS 132.86). A. Conidiophores. B. Sympodially proliferating conidiogenous cells, which give rise to a long rachis with slightly prominent, unthickened scars. C. Conidia. D–E. Synanamorph consisting of conidiogenous cells with percurrent proliferation. F. Conidia. Scale bars = 10  $\mu$ m.

***Rhinocladiella basitona*** (de Hoog) Arzanlou & Crous, **comb. nov.** MycoBank MB504552. Fig. 20.

*Basionym:* *Ramichloridium basitonum* de Hoog, J. Clin. Microbiol. 41: 4774. 2003.

*In vitro:* *Submerged hyphae* hyaline, smooth, thin-walled, 2  $\mu$ m wide; *aerial hyphae* rather thick-walled, pale brown. *Conidiophores* slightly differentiated from vegetative hyphae, profusely and mostly verticillately branched, straight or flexuose, pale-brown, 2–2.5  $\mu$ m wide. *Conidiogenous cells* terminal, variable in length, 10–100  $\mu$ m long, pale brown, straight or geniculate, proliferating sympodially, giving rise to a long, 2–2.5  $\mu$ m wide rachis, with slightly prominent, truncate conidium-bearing denticles, slightly darkened. *Conidia* solitary, hyaline, thin-walled, smooth, pyriform to clavate, with a round apex, and slightly truncate base, (1–)3–4(–5)  $\times$  1–2  $\mu$ m, hilum conspicuous, slightly darkened and thickened, less than 0.5  $\mu$ m diam.

*Cultural characteristics:* Colonies on MEA reaching 19 mm diam after 14 d at 24  $^{\circ}$ C, with entire, smooth, sharp margin; mycelium rather flat and slightly elevated in the centre, pale olivaceous-grey to olivaceous-grey; reverse olivaceous-black.

*Specimen examined:* **Japan**, Hamamatsu, from subcutaneous lesion with fistula on knee of 70-year-old male, Y. Suzuki, **ex-type** culture CBS 101460 = IFM 47593.

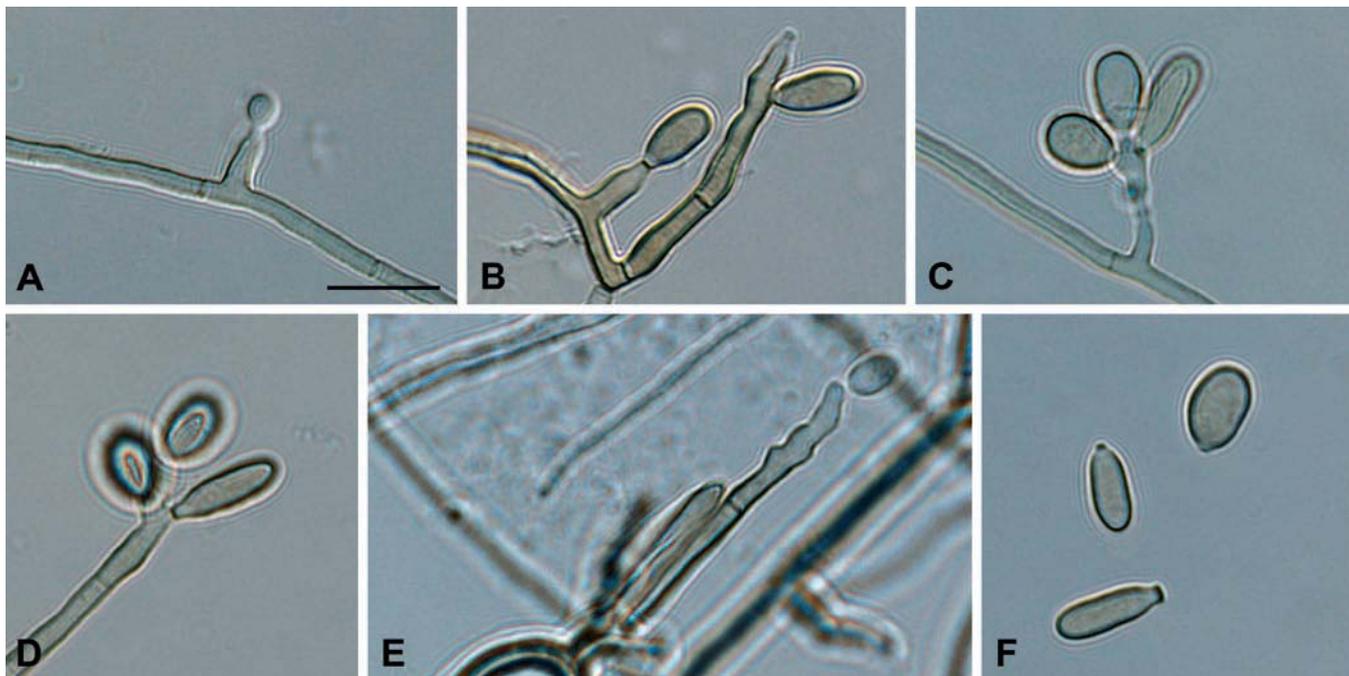
***Rhinocladiella fasciculata*** (V. Rao & de Hoog) Arzanlou & Crous, **comb. nov.** MycoBank MB504553. Fig. 21.

*Basionym:* *Ramichloridium fasciculatum* V. Rao & de Hoog, Stud. Mycol. 28: 39. 1986.

*In vitro:* *Submerged hyphae* subhyaline, smooth, thick-walled, 2–2.5  $\mu$ m wide; *aerial hyphae* pale brown. *Conidiophores* arising vertically from ascending hyphae in loose fascicles, unbranched or loosely branched at acute angles, cylindrical, smooth, brown and thick-walled at the base, up to 220  $\mu$ m long and 2–3  $\mu$ m wide, with 0–5 thin additional septa. *Conidiogenous cells* terminal, cylindrical, 30–100  $\mu$ m long, thin-walled, smooth, pale brown, fertile part as wide as the basal part, up to 2  $\mu$ m wide, proliferating sympodially, giving rise to a rachis with hardly prominent, slightly pigmented, not thickened scars, less than 0.5  $\mu$ m diam. *Conidia* solitary, smooth, thin-walled, subhyaline, ellipsoidal, (2.5–)4–5(–6)  $\times$  2–3  $\mu$ m, with truncate, slightly pigmented hilum, about 0.5  $\mu$ m diam. *Synanamorph* forming on torulose hyphae originating from giant cells; compact heads of densely branched hyphae forming thin-walled, lateral, subglobose cells, on which conidiogenous cells are formed; conidiogenous cells proliferating percurrently, giving rise to tubular annellated zones with inconspicuous annellations, up to 12  $\mu$ m long, 1–1.5  $\mu$ m wide. *Conidia* smooth, thin-walled, aseptate, subhyaline, globose, 2–2.5  $\mu$ m diam.

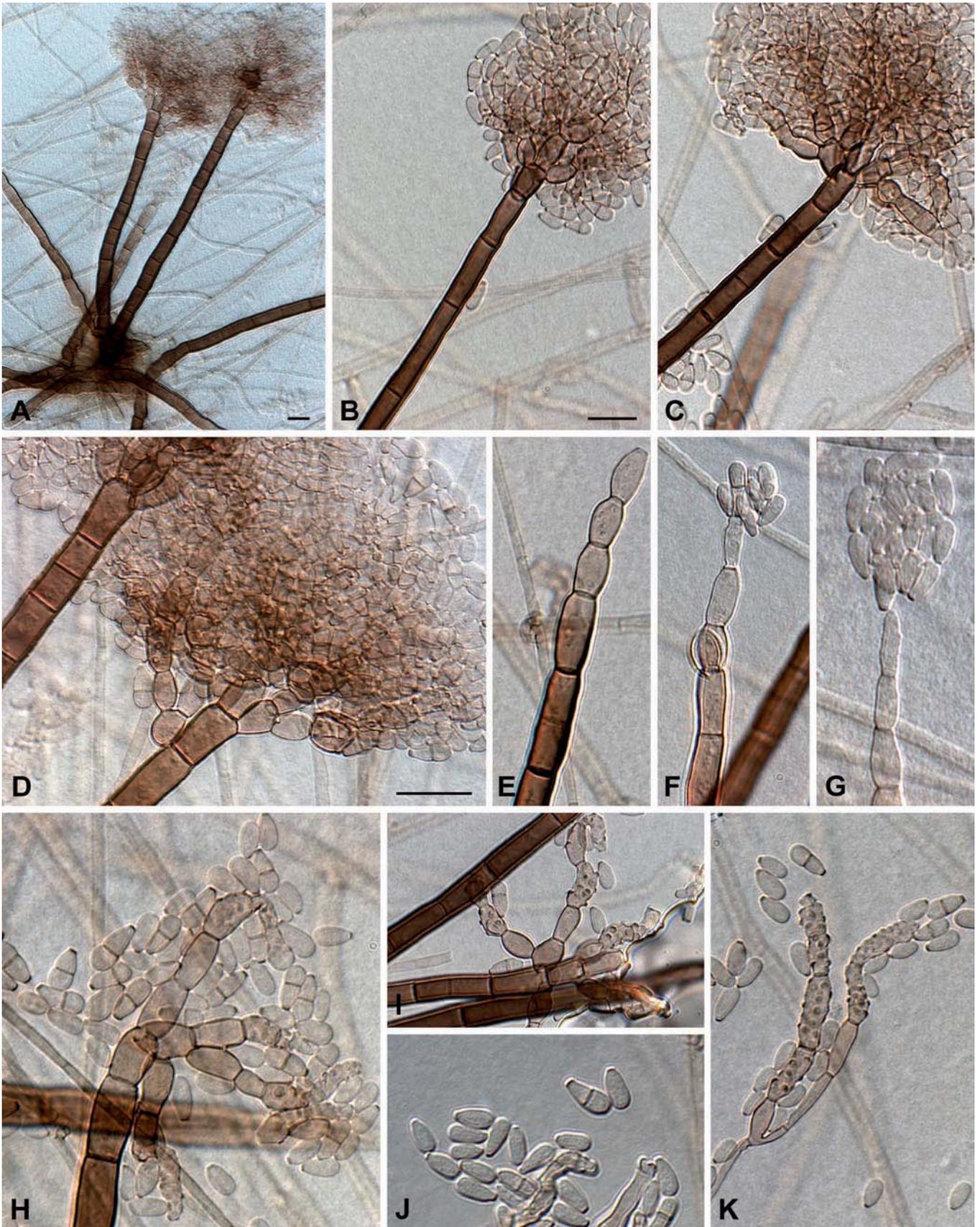
*Cultural characteristics:* Colonies on MEA reaching 8 mm diam after 14 d at 24  $^{\circ}$ C, with entire, smooth, sharp margin; mycelium velvety, becoming farinose in the centre due to abundant sporulation, olivaceous-green to brown, reverse dark olivaceous. Blackish droplets often produced at the centre, which contain masses of *Exophiala* conidia.

*Specimen examined:* **India**, Karnataka, Thirathahalli, isolated by V. Rao from decayed wood, **holotype** CBS-H 3866, culture **ex-type** CBS 132.86.



**Fig. 22.** *Rhinocladiella mackenziei* (CBS 368.92). A. Intercalary conidiogenous cell. B–E. Semi-micronematous conidiophores and sympodially proliferating conidiogenous cells, resulting in a rachis with slightly prominent, unthickened scars. F. Conidia. Scale bar = 10  $\mu$ m.





**Fig. 24.** *Thysanorea papuana* (CBS 212.96), periconiella-like synanamorph. A. Macronematous conidiophores. B–C. Conidiophores with dense apical branches. D. Branches with different levels of branchlets. E–I. Conidiogenous cells at different stages of development; sympodially proliferating conidiogenous cells give rise to a denticulate rachis. J–K. Conidia. Scale bars = 10  $\mu$ m.

**Fig. 23.** (Page 78). *Thysanorea papuana* (CBS 212.96). A. Intercalary conidiogenous cell. B–I. Semi-micronematous conidiophores and sympodially proliferating conidiogenous cells, resulting in a rachis with prominent conidium bearing denticles. J–K. Microcyclic conidiation observed in slide cultures. L. Conidia. Scale bar = 10  $\mu$ m.

**Rhinocladiella mackenziei** (C.K. Campb. & Al-Hedaithy) Arzanlou & Crous, **comb. nov.** MycoBank MB504554. Fig. 22.

*Basionym:* *Ramichloridium mackenziei* C.K. Campb. & Al-Hedaithy, J. Med. Veterin. Mycol. 31: 330. 1993.

*In vitro:* *Submerged hyphae* subhyaline, smooth, thin-walled, 2–3 µm wide; *aerial hyphae* pale brown, slightly narrower. *Conidiophores* slightly or not differentiated from vegetative hyphae, arising laterally from aerial hyphae, with one or two additional septa, often reduced to a discrete or intercalary conidiogenous cell, pale-brown, 10–25 × 2.5–3.5 µm. *Conidiogenous cells* terminal or intercalary, variable in length, 5–15 µm long and 3–5 µm wide, occasionally slightly wider than the basal part, pale brown, rachis with slightly prominent, unpigmented, non-thickened scars, about 0.5 µm diam. *Conidia* golden-brown, thin-walled, smooth, ellipsoidal to obovate, subcylindrical, (5–)8–9(–12) × (2–)3–3.5(–5) µm, with darkened, inconspicuously thickened, protuberant or truncate hilum, less than 1 µm diam.

*Cultural characteristics:* Colonies on MEA reaching 5 mm diam after 14 d at 24 °C, with entire, smooth, sharp margin; mycelium densely lanose and elevated in the centre, olivaceous-green to brown; reverse dark olivaceous.

*Specimens examined:* **Israel**, Haifa, isolated from brain abscess, CBS 368.92 = UTMB 3170; human brain abscess, E. Lefler, CBS 367.92 = NCPF 2738 = UTMB 3169. **Saudi Arabia**, from phaeoerythromycosis of the brain, S.S.A. Al-Hedaithy, **ex-type** strain, CBS 650.93 = MUCL 40057 = NCPF 2808; from brain abscess, Pakistani male who travelled to Saudi Arabia, CBS 102592 = NCPF 7460. **United Arab Emirates**, from fatal brain abscess, CBS 102590 = NCPF 2853.

*Notes:* Morphologically *Rhinocladiella mackenziei* is somewhat similar to *Pleurothecium obovoideum* (Matsush.) Arzanlou & Crous, which was originally isolated from dead wood. However, *P. obovoideum* has distinct conidiophores, and the ascending hyphae are thick-walled, and the denticles cylindrical, up to 1.5 µm long. In contrast, *Rh. mackenziei* has only slightly prominent denticles. *Rhinocladiella mackenziei* is a member of the *Chaetothyriales*, while *P. obovoideum* clusters in the *Chaetosphaeriales*.

**Thysanorea** Arzanlou, W. Gams & Crous, **gen. nov.** MycoBank MB504555.

*Etymology:* (Greek) *thysano* = brush, referring to the brush-like branching pattern, suffix derived from *Veronaea*.

*Veronaeae* similis sed conidiophoris partim *Periconiae* similibus dense ramosis distinguenda.

*In vitro:* *Submerged hyphae* subhyaline, smooth, thin-walled; *aerial hyphae* pale brown, smooth or verrucose. *Conidiophores* dimorphic; *micronematous conidiophores* slightly differentiated from vegetative hyphae, branched or simple, multiseptate. *Conidiogenous cells* terminal, polyblastic, variable in length, smooth, golden- to dark brown at the base, paler towards the apex, later sometimes inconspicuously septate; fertile part often wider than the basal part, clavate to doliiform, with crowded, more or less prominent conidium-bearing denticles, unpigmented, but slightly thickened. *Macronematous conidiophores* consisting of well-differentiated, thick-walled, dark brown stalks; apically repeatedly densely branched, forming a complex head, each branchlet giving rise to a conidium-bearing denticulate rachis with slightly pigmented, thickened scars. *Conidia* of both kinds of conidiophore formed singly, smooth, pale brown, obovoidal to pyriform, (0–)1-septate, with a truncate base and darkened hilum; conidial secession schizolytic.

*Type species:* *Thysanorea papuana* (Aptroot) Arzanlou, W. Gams & Crous, **comb. nov.**

**Thysanorea papuana** (Aptroot) Arzanlou, W. Gams & Crous, **comb. nov.** MycoBank MB504556. Figs 7C, 23–24.

*Basionym:* *Periconiella papuana* Aptroot, Nova Hedwigia 67: 491. 1998.

*In vitro:* *Submerged hyphae* subhyaline, smooth, thin-walled, 1.5–3 µm wide; *aerial hyphae* pale brown, smooth to verrucose, 1.5–2 µm wide. *Conidiophores* dimorphic; *micronematous conidiophores* slightly differentiated from vegetative hyphae, branched or simple, up to 6-septate. *Conidiogenous cells* terminal or intercalary, variable in length, 5–20 µm long, thin-walled, smooth, golden- to dark brown at the base, paler toward the apex, later sometimes becoming inconspicuously septate, fertile part wider than basal part, often clavate, with crowded, more or less prominent conidium-bearing denticles, about 1 µm diam, unpigmented but slightly thickened. *Conidia* solitary, subhyaline, thin-walled, smooth, cylindrical to pyriform, rounded at the apex and truncate at the base, pale brown, (0–)1-septate, (5–)7–8(–11) × (2–)3(–4) µm, with a truncate base and darkened hilum, 1 µm diam. *Macronematous conidiophores* present in old cultures after 1 mo of incubation, consisting of well-differentiated, thick-walled, dark brown stalks, up to 220 µm long, (4–)5–6(–7) µm wide, with up to 15 additional septa, often with inflated basal cells; apically densely branched, forming a complex head, with up to five levels of branchlets, 20–50 µm long, each branchlet giving rise to a denticulate conidium-bearing rachis; scars slightly pigmented, thickened, about 1 µm diam. *Conidia* solitary, thin-walled, smooth, pale brown, obovoidal to pyriform, (0–)1-septate, (4–)5–6(–8) × (2–)3(–4) µm, with a truncate base and darkened hilum, 1–2 µm diam.

*Cultural characteristics:* Colonies on MEA reaching 10 mm diam after 14 d at 24 °C, with entire, sharp margin; mycelium velvety, elevated, with colonies up to 2 mm high, surface olivaceous-grey to iron-grey; reverse greenish black.

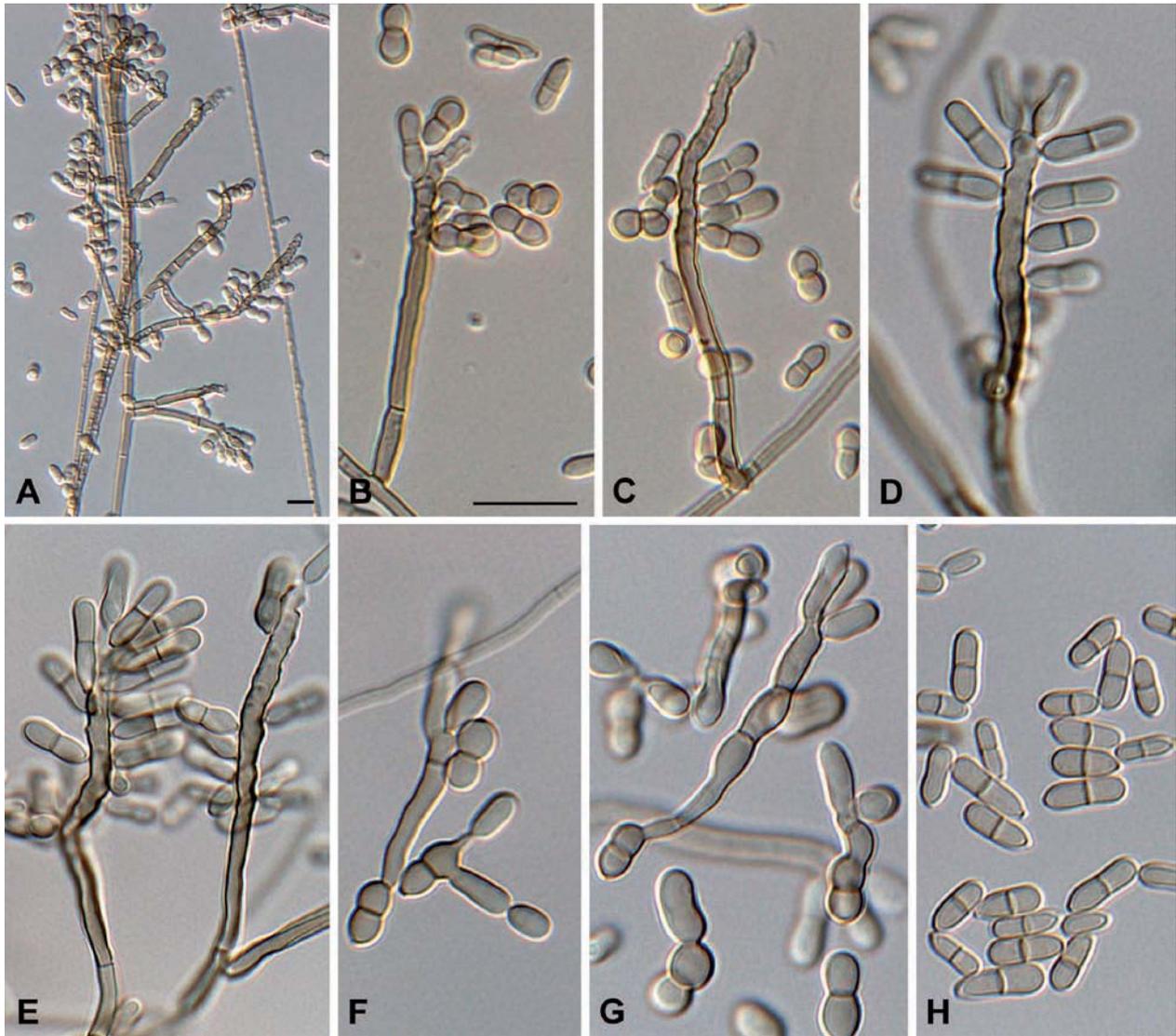
*Specimen examined:* **Papua New Guinea**, Madang Province, foothill of Finisterre range, 40.8 km along road Madang-Lae, alt. 200 m, isolated from unknown stipe, 2 Nov. 1995, A. Aptroot, **holotype** CBS-H 6351, culture ex-type CBS 212.96.

**Veronaea** Cif. & Montemart., Atti Ist. Bot. Lab. Crittog. Univ. Pavia, sér. 5, 15: 68. 1957.

*In vitro:* Colonies velvety, pale olivaceous-brown, moderately fast-growing. *Submerged hyphae* hyaline to pale olivaceous, smooth; *aerial hyphae*, more darkly pigmented. Exophiala-type budding cells absent in culture. *Conidiophores* erect, straight or flexuose, unbranched or occasionally loosely branched, sometimes geniculate, smooth-walled, pale to medium- or olivaceous-brown. *Conidiogenous cells* terminally integrated, polyblastic, occasionally intercalary, cylindrical, pale brown, later often becoming septate, fertile part subhyaline, often as wide as the basal part, rachis with crowded, flat to slightly prominent, faintly pigmented, unthickened scars. *Conidia* solitary, smooth, cylindrical to pyriform, rounded at the apex and truncate at the base, pale brown, 1(–)2-septate; conidial secession schizolytic.

*Type species:* *Veronaea botryosa* Cif. & Montemart., Atti Ist. Bot. Lab. Crittog. Univ. Pavia, sér. 5, 15: 68. 1957.

**Veronaea botryosa** Cif. & Montemart., Atti Ist. Bot. Lab. Crittog. Univ. Pavia, sér. 5, 15: 68. 1957. Fig. 25.



**Fig. 25.** *Veronaea botryosa* (CBS 254.57). A–C. Semi-micronematous conidiophores and sympodially proliferating conidiogenous cells. D–E. Rachis with crowded and flat scars. F–G. Microcyclic conidiation. H. Conidia. Scale bars = 10  $\mu$ m.

*In vitro*: Submerged hyphae hyaline to pale olivaceous, smooth; aerial hyphae more darkly pigmented. Conidiophores erect, straight or flexuose, unbranched or occasionally loosely branched, sometimes geniculate, smooth-walled, pale brown to olivaceous-brown, 2–3  $\mu$ m wide and up to 200  $\mu$ m long. Conidiogenous cells terminal, occasionally intercalary, cylindrical, 10–100  $\mu$ m long, pale brown, later often becoming septate, fertile part subhyaline, often as wide as the basal part, rachis with crowded, flat to slightly prominent, faintly pigmented, unthickened scars. Conidia solitary, smooth, cylindrical to pyriform, (3–)6.5–8.5(–12)  $\times$  (1.5–)2–2.5(–3)  $\mu$ m, rounded at the apex and truncate at the base, pale brown, 1(–2)-septate, with a faintly darkened, unthickened hilum, about 0.5  $\mu$ m diam.

**Cultural characteristics:** Colonies on MEA reaching 30 mm diam after 14 d at 24 °C, with entire, sharp margin; mycelium velvety, slightly elevated in the centre, surface olivaceous-grey to greyish-brown; reverse greenish black.

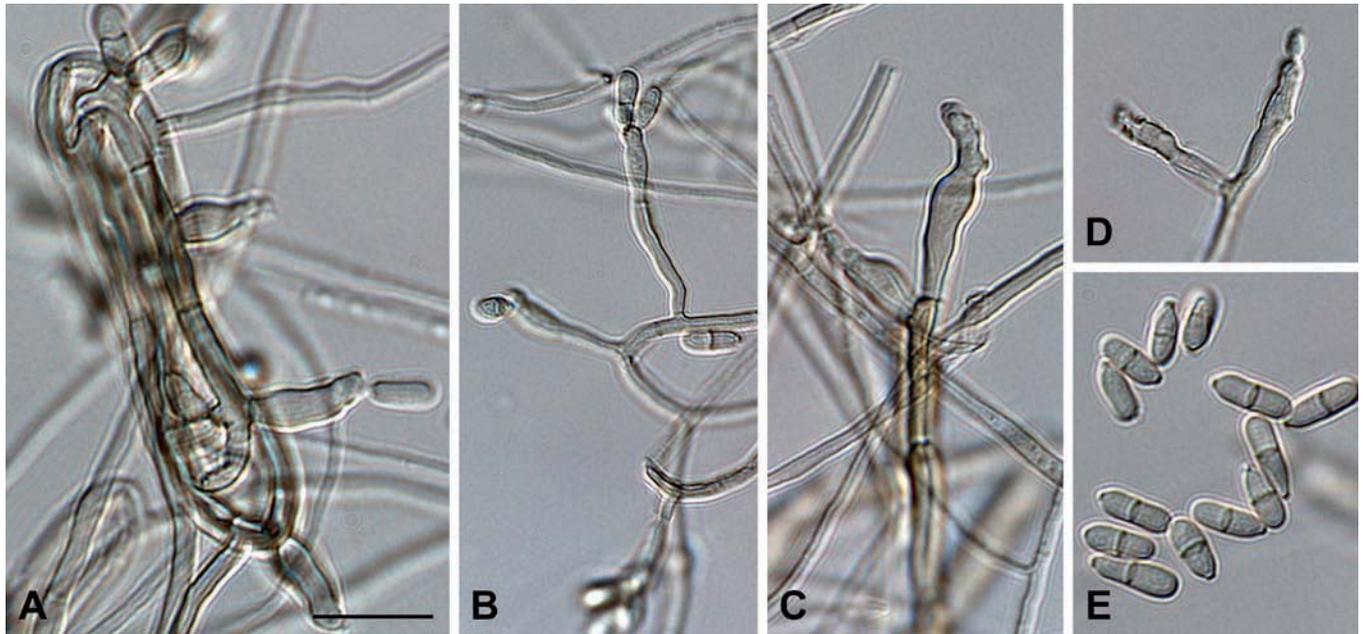
**Specimens examined:** **India**, Ramgarh, about 38 km from Jaipur, isolated from goat dung, 1 Sep. 1963, B.C. Lodha, CBS 350.65 = IMI 115127 = MUCL 7972. **Italy**, Tuscany, Pisa, isolated from Sansa olive slag, 1954, O. Verona, **ex-type** strain, CBS 254.57 = IMI 070233 = MUCL 9821.

***Veronaea compacta*** Papendorf, Bothalia 12: 119. 1976. Fig. 26.

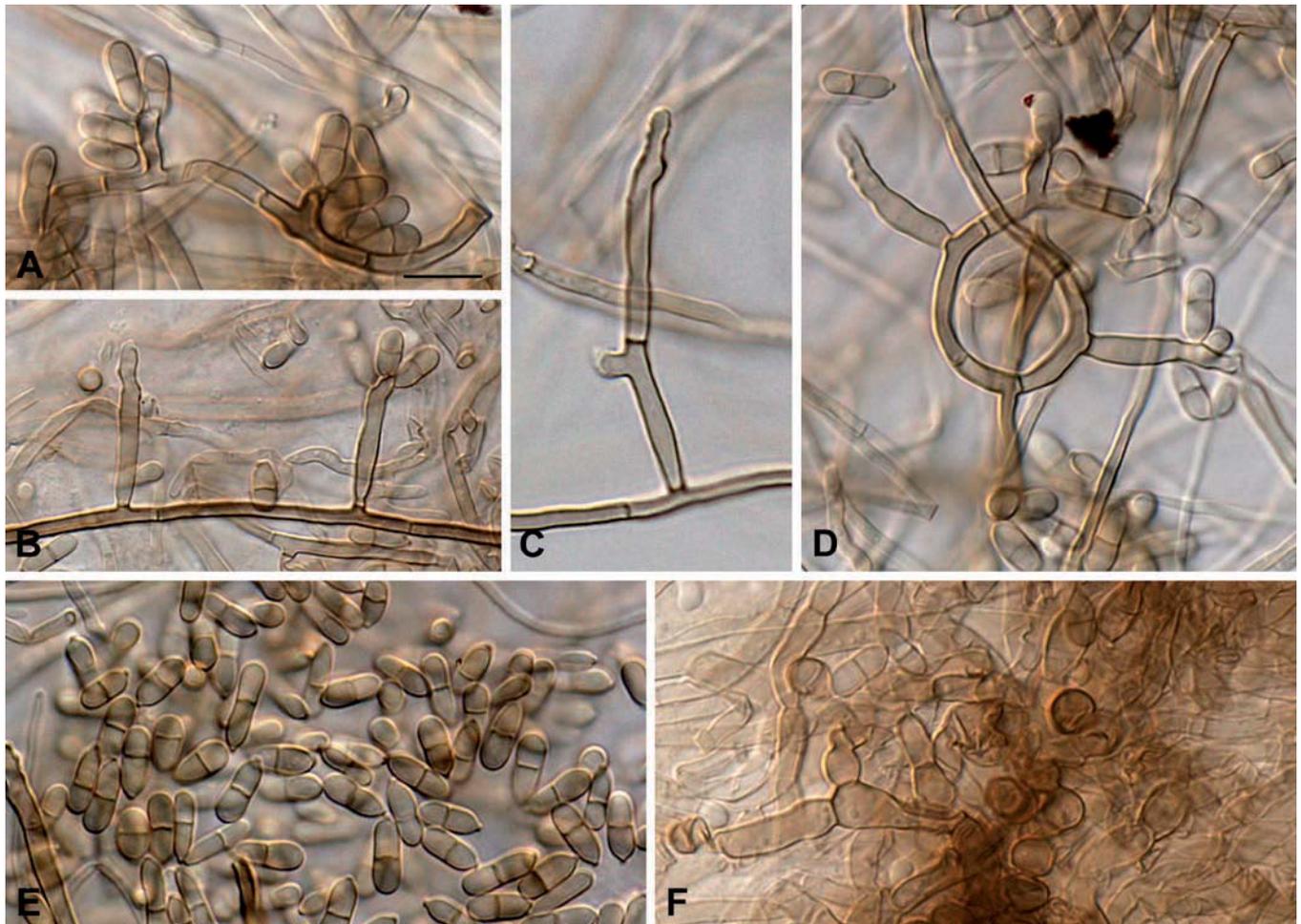
*In vitro*: Submerged hyphae subhyaline, smooth, thin-walled, 1.5–3  $\mu$ m wide; aerial hyphae rather thick-walled, pale brown. Conidiophores slightly differentiated from vegetative hyphae, lateral or occasionally terminal, often wider than the supporting hypha, up to 4  $\mu$ m wide, unbranched or branched at acute angles, with 1–3 additional septa, cells often inflated and flask-shaped, pale-brown, up to 60  $\mu$ m long. Conidiogenous cells terminal, occasionally intercalary, variable in length, up to 10  $\mu$ m long, pale brown, cylindrical to doliiform or flask-shaped, with hardly prominent denticles; scars flat, slightly pigmented, not thickened, about 0.5  $\mu$ m diam. Conidia solitary, pale brown, smooth, thin-walled, ellipsoidal to ovoid, 0–1(–2)-septate, often constricted at the septa, (4–)6–7(–9)  $\times$  2–3  $\mu$ m, with a round apex and truncate base; hilum prominent, slightly darkened, unthickened, about 0.5  $\mu$ m diam.

**Cultural characteristics:** Colonies rather slow growing, reaching 15 mm diam on MEA after 14 d at 24 °C; surface velvety to lanose, slightly raised in the centre, pale grey to pale brownish grey; reverse dark grey.

**Specimen examined:** **South Africa**, soil, M.C. Papendorf, **ex-type** culture CBS 268.75.



**Fig. 26.** *Veronaea compacta* (CBS 268.75). A–B. Semi-micronematous conidiophores and sympodially proliferating conidiogenous cells. C–D. Rachis with hardly prominent denticles. E. Conidia. Scale bar = 10  $\mu$ m.



**Fig. 27.** *Veronaea japonica* (CBS 776.83). A. Intercalary conidiogenous cells. B–D. Semi-micronematous conidiophores and sympodially proliferating conidiogenous cells. E. Conidia. F. Thick-walled, dark brown hyphal cells. Scale bar = 10  $\mu$ m.

***Veronaea japonica*** Arzanlou, W. Gams & Crous, **sp. nov.**  
 MycoBank MB504557. Figs 17B, 27.

**Etymology:** Named after the country of origin, Japan.

*Veronaea compactae* similis, sed cellulis inflatis, aggregatis, crassitunicatis, fuscis *in vitro* formatis distinguenda.

*In vitro:* Submerged hyphae subhyaline, smooth, thin-walled, 1.5–3  $\mu$ m wide; aerial hyphae slightly narrower, pale brown; hyphal cells later becoming swollen, thick-walled, dark brown, often aggregated. Conidiophores slightly differentiated from aerial vegetative hyphae, lateral, or terminal, often wider than the supporting hypha, 2–3  $\mu$ m wide, up to 65  $\mu$ m long, unbranched or occasionally branched,

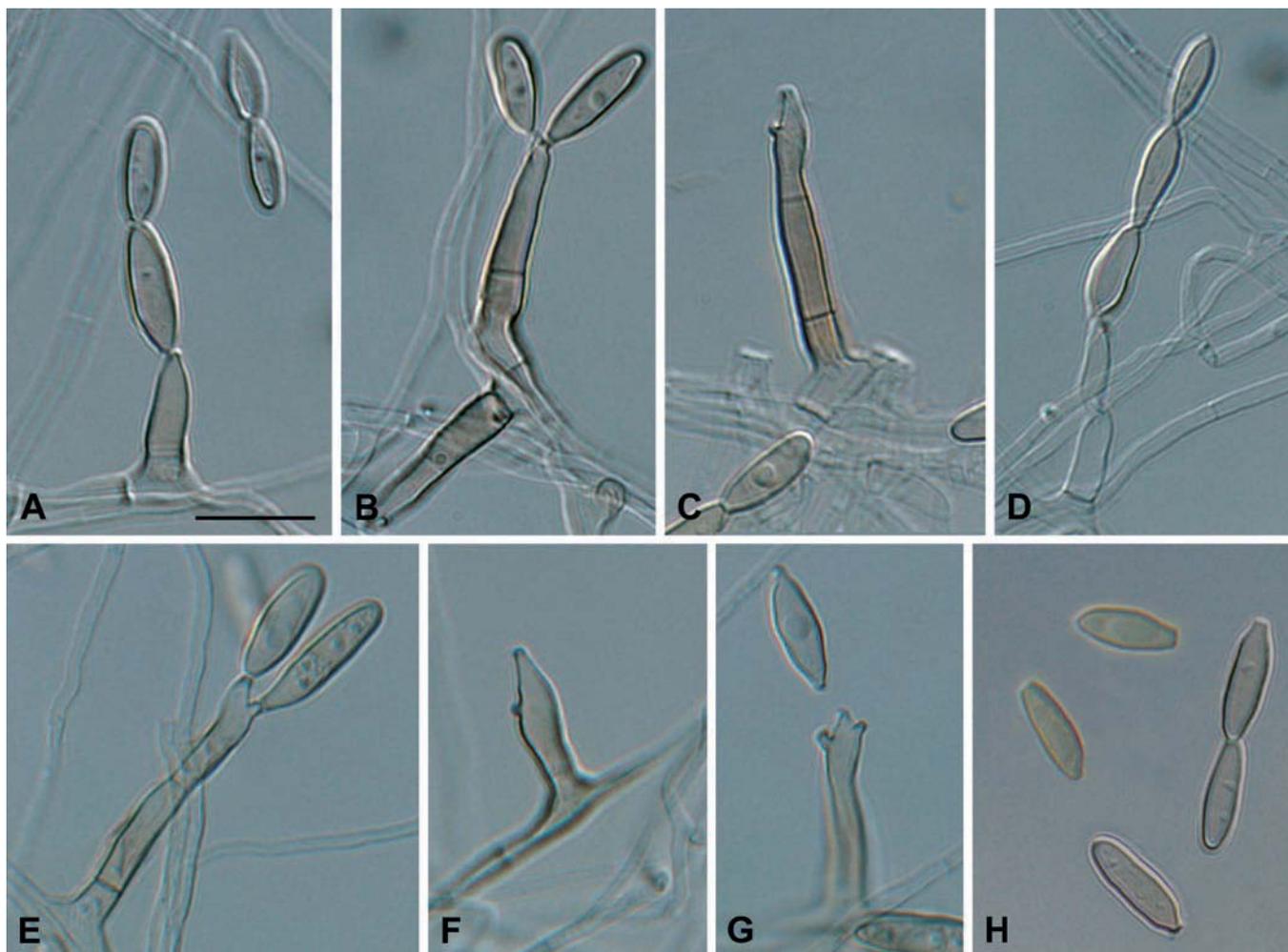


Fig. 28. *Pleurothecium obovoideum* (CBS 209.95). A–C. Conidial apparatus consisting of conidiophores with sympodially proliferating conidiogenous cells as seen in slide cultures of ca. 14 d. D. Short chain of conidia. E–G. Sympodially proliferating conidiogenous cells, resulting in a short rachis with subcylindrical to cylindrical denticles. H. Conidia. Scale bar = 10  $\mu$ m.

pale brown, thin-walled, smooth, with 1–3 additional septa. *Conidiogenous cells* terminal, occasionally intercalary, variable in length, up to 15  $\mu$ m long, pale brown, cylindrical to clavate, with hardly prominent denticles; scars flat, slightly pigmented, not thickened, about 0.5  $\mu$ m diam. *Conidia* solitary, pale brown, smooth, thin-walled, ellipsoidal to ovoid, (0–)1-septate, often constricted at the septum, (6–)7–8(–10)  $\times$  2–2.5(–4)  $\mu$ m, with a round apex and truncate base; hilum unthickened but slightly darkened, about 1  $\mu$ m diam.

**Cultural characteristics:** Colonies rather slow growing, reaching 7.5 mm diam on MEA after 14 d at 24  $^{\circ}$ C; surface velvety to lanose, slightly raised in the centre, olivaceous-brown, with entire margin; reverse dark-olivaceous.

**Specimen examined:** Japan, Kyoto, Daitokuji Temple, Kyoto, inside dead bamboo culm, Dec. 1983, W. Gams, **holotype** CBS-H 3490, ex-type culture CBS 776.83.

**Note:** This species is morphologically similar to *V. compacta* (Papendorf 1976), but can be distinguished based on the presence of dark brown, swollen hyphal cells in culture, which are absent in *V. compacta*.

### *Pleurothecium obovoideum* clade (*Chaetosphaeriales*)

*Ramichloridium obovoideum* was regarded as similar to “*Ramichloridium*” (*Rhinochlaediella*) *mackenziei* by some authors, and subsequently reduced to synonymy (Ur-Rahman *et al.* 1988). However, *R. obovoideum* clusters with *Carpoligna pleurothecii*, the teleomorph of *Pleurothecium* Höhn. Because it is also morphologically similar to other species of *Pleurothecium*, we herewith combine it into that genus.

***Pleurothecium obovoideum* (Matsush.) Arzanlou & Crous, comb. nov.** MycoBank MB504558. Fig. 28.

**Basionym:** *Rhinochlaediella obovoidea* Matsush., *Icones Microfung. Mats. lect.*: 123. 1975.

$\equiv$  *Ramichloridium obovoideum* (Matsush.) de Hoog, *Stud. Mycol.* 15: 73. 1977.

**In vitro:** *Submerged hyphae* smooth, hyaline, thin-walled, 1–2  $\mu$ m wide; *aerial hyphae* hyaline to subhyaline, smooth. *Conidiophores* arising vertically from creeping hyphae, ascending hyphae thick-walled and dark brown; conidiophores 10–35  $\mu$ m long, 1–2-septate, often reduced to a conidiogenous cell, unbranched, thick-walled, smooth, tapering towards the apex, pale brown. *Conidiogenous cells* integrated, cylindrical to ampulliform, 5–20  $\mu$ m long, pale brown, elongating sympodially, with a short rachis giving rise to denticles, 1  $\mu$ m long, slightly pigmented. *Conidia* aseptate, solitary or in short chains of up to 3, smooth, pale brown, ellipsoidal to obovate, (9–)11–12(–14.5)  $\times$  (3–)4(–5)  $\mu$ m, smooth, thin-walled, with a more or less rounded apex, a truncate base and a slightly darkened, unthickened hilum, 1.5  $\mu$ m diam.

**Cultural characteristics:** Colonies slow-growing, reaching 15 diam after 14 d at 24 °C, with entire, smooth margin; surface rather compact, mycelium mainly flat, submerged, some floccose to lanose aerial mycelium in the centre, buff; reverse honey.

**Specimen examined:** Japan, Kobe Municipal Arboretum, T. Matsushima, from dead leaf of *Pasania edulis*, CBS 209.95 = MFC 12477.

## Incertae sedis (Sordariomycetes)

### Ramichloridium schulzeri clade

*Ramichloridium schulzeri*, including its varieties, clusters near *Thyridium* Nitschke and the *Magnaporthaceae*, and is phylogenetically as well as morphologically distinct from the other genera in the *Ramichloridium* complex. To accommodate these taxa, a new genus is introduced below.

**Myrmecridium** Arzanlou, W. Gams & Crous, **gen. nov.** MycoBank MB504559.

**Etymology:** (Greek) *myrmekia* = wart, referring to the wart-like denticles on the rachis, suffix *-ridium* from *Chloridium*.

Genus ab allis generibus *Ramichloridii* similibus rachide recta longa, subhyalina, denticulis distantibus, verruciformibus praedita distinguendum.

**In vitro:** Colonies moderately fast-growing, flat, with mainly submerged mycelium, and entire margin, later becoming powdery to velvety, pale orange to orange. Mycelium rather compact, mainly submerged, in the centre velvety with fertile bundles of hyphae. *Conidiophores* arising vertically and clearly distinct from creeping hyphae, unbranched, straight or flexuose, brown, thick-walled. *Conidiogenous cells* terminally integrated, polyblastic, cylindrical, straight or flexuose, pale brown, sometimes secondarily septate, fertile part subhyaline, as wide as the basal part, with scattered pimple-shaped, apically pointed, unpigmented, conidium-bearing denticles. *Conidia* solitary, subhyaline, smooth or finely verrucose, rather thin-walled, with a wing-like gelatinous sheath, obovoidal or fusiform, tapering towards a narrowly truncate base with a slightly prominent, unpigmented hilum; conidial secession schizolytic.

**Type species:** *Myrmecridium schulzeri* (Sacc.) Arzanlou, W. Gams & Crous, **comb. nov.**

**Notes:** *Myrmecridium schulzeri* was fully described as *Acrotheca acuta* Grove by Hughes (1951). The author discussed several genera, none of which is suitable for the present fungus for various reasons as analysed by de Hoog (1977). Only *Gomphinaría* Preuss is not yet sufficiently documented. Our examination of *G. amoena* Preuss (B!) showed that this is an entirely different fungus, of which no fresh material is available to ascertain its position.

*Myrmecridium* can be distinguished from other ramichloridium-like fungi by having entirely hyaline vegetative hyphae, and widely scattered, pimple-shaped denticles on the long hyaline rachis. The conidial sheath is visible in lactic acid mounts with bright-field microscopy. The *Myrmecridium* clade consists of several subclusters, which are insufficiently resolved based on the ITS sequence data. However, two morphologically distinct varieties of *Myrmecridium* are treated here. The status of the other isolates in this clade will be dealt with in a future study incorporating more strains, and using a multi-gene phylogenetic approach.

**Myrmecridium schulzeri** (Sacc.) Arzanlou, W. Gams & Crous, **comb. nov.** MycoBank MB504560. var. **schulzeri** Figs 7B, 29.

**Basionym:** *Psilobotrys schulzeri* Sacc., *Hedwigia* 23: 126. 1884.

≡ *Chloridium schulzerii* (Sacc.) Sacc., *Syll. Fung.* 4: 322. 1886.

≡ *Rhinochloidiella schulzeri* (Sacc.) Matsush., *Icon. Microfung. Mats. lect.* (Kobe): 124. 1975.

≡ *Ramichloridium schulzeri* (Sacc.) de Hoog, *Stud. Mycol.* 15: 64. 1977 var. *schulzeri*.

= *Acrotheca acuta* Grove, *J. Bot., Lond.* 54: 222. 1916.

≡ *Pleurophragmium acutum* (Grove) M.B. Ellis in Ellis, *More Dematiaceous Hyphomycetes*: 165. 1976.

= *Rhinotrichum multisporum* Doguet, *Rev. Mycol., Suppl. Colon.* 17: 78. 1953 (nom. inval. Art. 36) [non *Acrotheca multispora* (Preuss) Sacc., *Syll. Fung.* 4: 277. 1886].

[non *Acrothecium* (?) *multisporum* G. Arnaud, *Bull. Trimestriell Soc. Mycol. France* 69: 288. 1953 (nom. inval. Art. 36)].

[non *Acrothecium multisporum* G. Arnaud *sensu* Tubaki, *J. Hattori Bot. Lab.* 20: 145. 1958].

**In vitro:** Submerged hyphae hyaline, thin-walled, 1–2 µm wide; aerial hyphae, if present, pale olivaceous-brown. *Conidiophores* arising vertically from creeping aerial hyphae, unbranched, straight, reddish brown, thick-walled, septate, up to 250 µm tall, 2.5–3.5 µm wide, with 2–7 additional septa, basal cell often inflated, 3.5–5 µm wide. *Conidiogenous cells* integrated, cylindrical, variable in length, 15–110 µm long, subhyaline to pale brown, later becoming inconspicuously septate, fertile part subhyaline, as wide as the basal part, forming a straight rachis with scattered, pimple-shaped denticles less than 1 µm long and approx. 0.5 µm wide, apically pointed, unpigmented, slightly thickened scars. *Conidia* solitary, subhyaline, thin-walled, smooth or finely verrucose, surrounded by a wing-like, gelatinous conidial sheath, up to 0.5 µm thick, ellipsoid, obovoid or fusiform, (6–)9–10(–12) × 3–4 µm, tapering to a subtruncate base; hilum unpigmented, inconspicuous.

**Cultural characteristics:** Colonies reaching 29 mm diam after 14 d at 24 °C, pale orange to orange, with entire margin; mycelium flat, rather compact, later becoming farinose or powdery due to sporulation, which occurs in concentric zones when incubated on the laboratory bench.

**Specimens examined:** Germany, Kiel-Kitzeberg, from wheat-field soil, W. Gams, CBS 134.68 = ATCC 16310. The Netherlands, isolated from a man, bronchial secretion, A. Visser, CBS 156.63 = MUCL 1079; Lienden, isolated from *Triticum aestivum* root, C.L. de Graaff, CBS 325.74 = JCM 7234.

**Myrmecridium schulzeri** var. **tritici** (M.B. Ellis) Arzanlou, W. Gams & Crous, **comb. nov.** MycoBank MB504562.

**Basionym:** *Pleurophragmium tritici* M.B. Ellis, in Ellis, *More Dematiaceous Hyphomycetes*: 165. 1976.

≡ *Ramichloridium schulzeri* var. *tritici* (M.B. Ellis) de Hoog, *Stud. Mycol.* 15: 68. 1977.

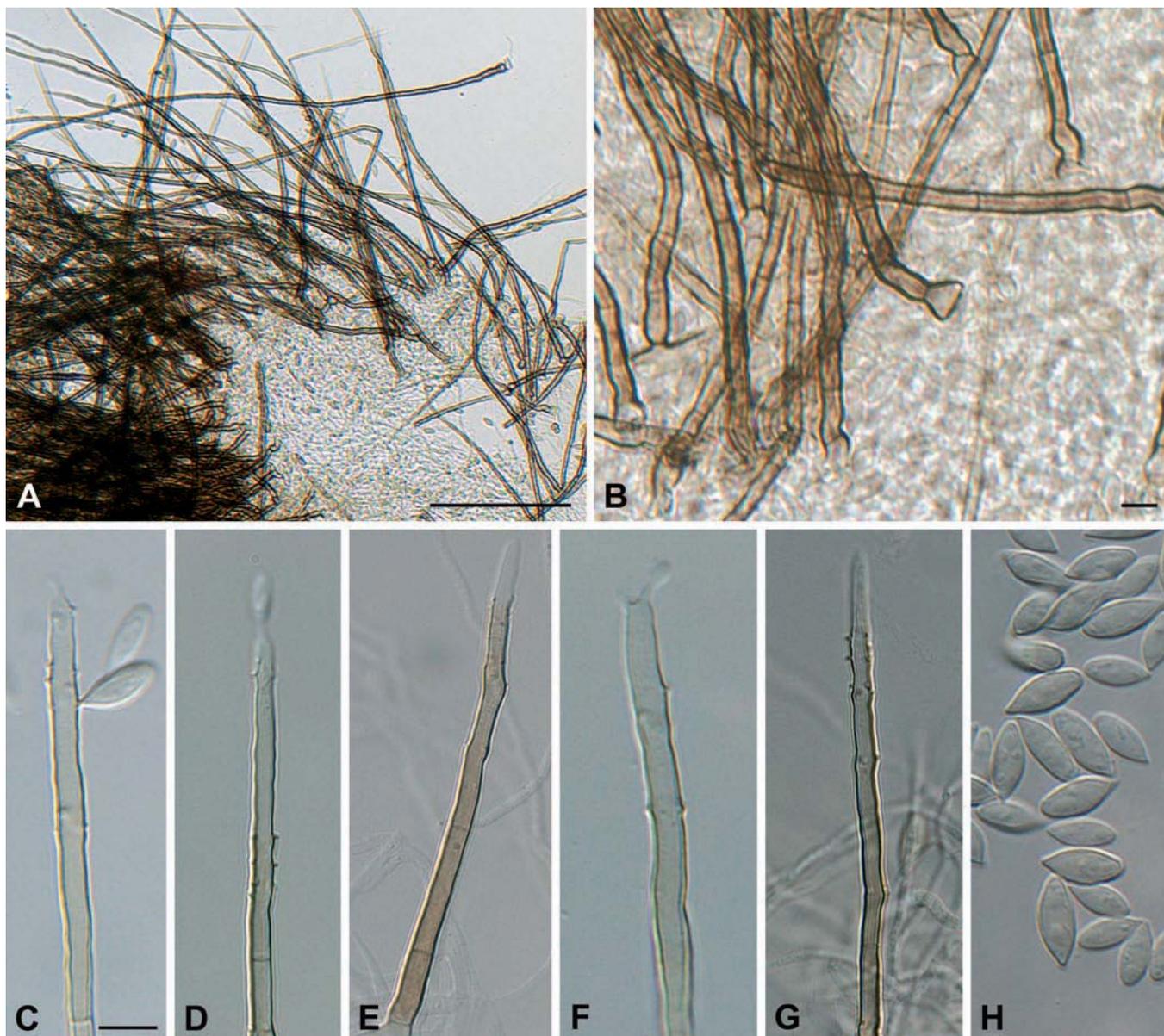
**Specimen examined:** Ireland, Dublin, on wheat stem, Oct. 1960, J.J. Brady, **holotype** IMI 83291.

**Notes:** No reliable living culture is available of this variety. Based on a re-examination of the type specimen in this study, the variety appears sufficiently distinct from *Myrmecridium schulzeri* var. *schulzeri* based on the frequent production of septate conidia.

**Myrmecridium flexuosum** (de Hoog) Arzanlou, W. Gams & Crous, **comb. et stat. nov.** MycoBank MB504563. Fig. 30.

**Basionym:** *Ramichloridium schulzeri* var. *flexuosum* de Hoog, *Stud. Mycol.* 15: 67. 1977.

**In vitro:** Submerged hyphae hyaline, thin-walled, 1–2 µm wide. *Conidiophores* unbranched, flexuose, arising from creeping aerial



**Fig. 29.** *Myrmecridium schulzeri* (CBS 325.74). A. Macronematous conidiophores. B. Inflated basal cells visible in some conidiophores. C–E. Conidial apparatus at different stages of development, resulting in macronematous conidiophores and sympodially proliferating conidiogenous cells. F–G. Rachis with scattered, pimple-shaped denticles. H. Conidia. Scale bars: A = 100  $\mu$ m, B–H = 10  $\mu$ m.

hyphae, pale brown, up to 250  $\mu$ m tall, 3–3.5  $\mu$ m wide, thick-walled, smooth, with up to 24 thin septa, delimiting 8–12  $\mu$ m long cells. *Conidiogenous cells* integrated, elongating sympodially, cylindrical, 20–150  $\mu$ m long, flexuose, brown at the base, subhyaline in the upper part, later becoming inconspicuously septate; rachis slightly flexuose, subhyaline, as wide as the basal part, thick-walled near the base, hyaline and thin-walled in the apical part, with scattered pimple-shaped, unpigmented, approx. 0.5  $\mu$ m long denticles. *Conidia* solitary, subhyaline, thin-walled, finely verrucose, with a wing-like gelatinous sheath, approx. 0.5  $\mu$ m wide, ellipsoid to obovoid, (5–)6–7(–9)  $\times$  3–4  $\mu$ m; hilum slightly prominent, unpigmented, approx. 0.5  $\mu$ m diam.

**Cultural characteristics:** Colonies reaching 40 mm diam after 14 d at 24  $^{\circ}$ C; mycelium submerged, flat, smooth; centrally orange, later becoming powdery to velvety and greyish brown due to sporulation, with sharp, smooth, entire margin; reverse yellowish orange.

**Specimen examined:** Surinam, isolated from soil, J.H. van Emden, ex-type culture CBS 398.76 = JCM 6968.

**Note:** This former variety is sufficiently distinguished from *M. schulzeri* s. str. by its flexuose conidiophores and conidia which lack an acuminate base, to be regarded as a separate species.

***Ramichloridium torvi*** (Ellis & Everh.) de Hoog, Stud. Mycol. 15: 79. 1977.

$\equiv$  *Ramularia torvi* Ellis & Everh., Rep. Missouri Bot. Gard. 9: 119. 1898.

$\equiv$  *Hansfordia torvi* (Ellis & Everh.) Deighton & Piroz., Mycol. Pap. 101: 39. 1965.

$\equiv$  *Acladium biophilum* Cif., Sydowia 10: 164. 1956.

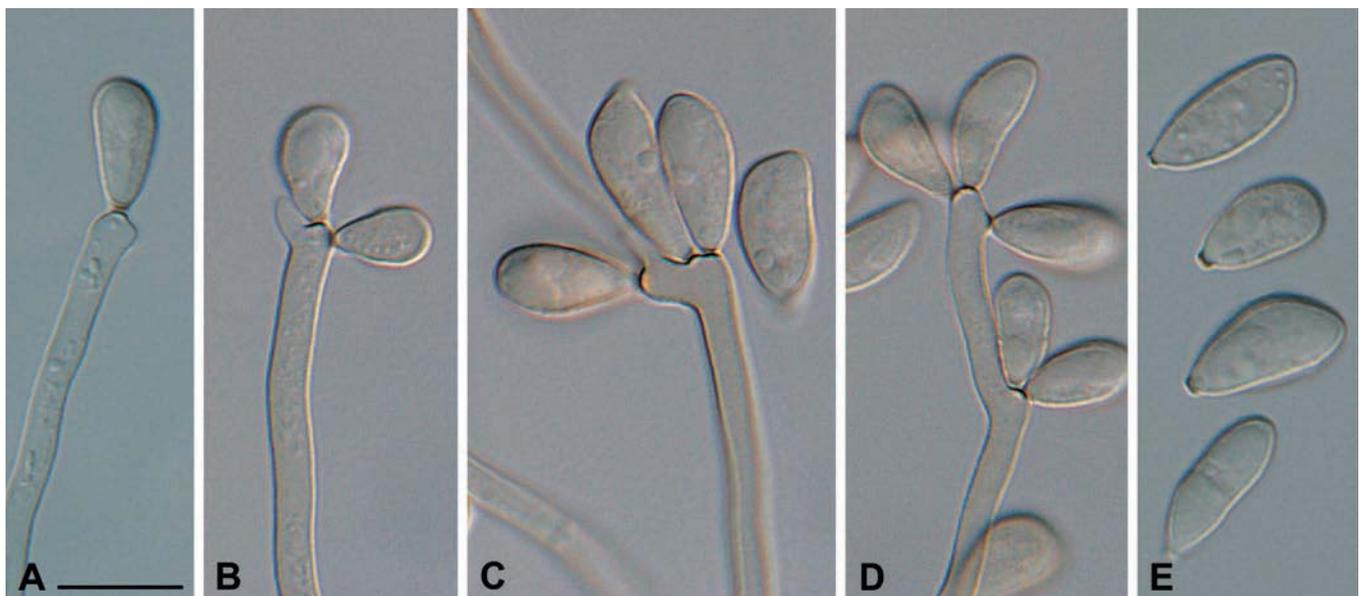
$\equiv$  *Hansfordia biophila* (Cif.) M.B. Ellis, in Ellis, *More Dematiaceous Hyphomycetes*: 199. 1976.

**Specimen:** Jamaica, Port Marant, Dec. 1890, on leaves of *Solanum torvum*, **holotype** of *Ramularia torvi* (NY) (specimen not examined).

**Notes:** According to the description and illustration of *R. torvi* provided by de Hoog (1977), this appears to be an additional species of *Myrmecridium*. Although it is morphologically similar to *M. flexuosum* in having a flexuose rachis, it differs from the other species of the genus by having smooth, clavate conidia. Fresh collections and cultures would be required to resolve its status.



**Fig. 30.** *Myrmecridium flexuosum* (CBS 398.76). A–C. Conidial apparatus at different stages of development, resulting in macronematous conidiophores with sympodially proliferating conidiogenous cells. D–H. Sympodially proliferating conidiogenous cells giving rise to a flexuose conidium-bearing rachis with pimple-shaped denticles. I. Conidia. Scale bar = 10  $\mu$ m.



**Fig. 31.** *Pseudovirgaria hyperparasitica* (CBS 121739). A–D. Conidial apparatus at different stages of development; conidiogenous cells with geniculate proliferation. E. Conidia. Scale bar = 10  $\mu$ m.

***Pseudovirgaria*** H.D. Shin, U. Braun, Arzanlou & Crous, **gen. nov.**  
MycoBank MB504564.

**Etymology:** Named after its morphological similarity to *Virgaria*.

*Hyphomycetes*. Uredinicola. Coloniae *in vivo* pallide vel modice brunneae, ferrugineae vel cinnamomeae, *in vitro* lentissime crescentes, murinae. Mycelium immersum et praecipue externum, ex hyphis ramosis et cellulis conidiogenis integratis compositum, conidiophoris ab hyphis vegetativis vix distinguendis. Hyphae ramosae, septatae, leves, tenuitunicatae, hyalinae vel pallide brunneae. Cellulae conidiogenae integratae in hyphis repentibus, terminales et intercalares, polyblasticae, sympodialiter proliferantes, subcylindricae vel geniculatae, cicatricibus conspicuis, solitariis vel numerosis, dispersis vel aggregatis, subdenticulatis, prominentibus, umbonatis vel apicem versus paulo attenuatis, non inspissatis, non vel parce fuscatis-refringentibus. Conidia solitaria, holoblastica, plus minusve obovoidea, recta vel leniter curvata, asymmetrica, continua, hyalina, subhyalina vel pallidissime olivaceo-brunnea, hilo subconspicuo vel conspicuo, truncato vel rotundato, non inspissato, non vel lenissime fuscato-refringente; secessio schizolytica.

Hyperparasitic on uredosori of rust fungi. Colonies *in vivo* pale to medium brown, rusty or cinnamon, *in vitro* slow-growing, pale to dark mouse-grey. Mycelium immersed and mainly aerial, composed of branched hyphae with integrated conidiogenous cells, differentiation between vegetative hyphae and conidiophores barely possible. Hyphae branched, septate, smooth, thin-walled, hyaline to pale brown. Conidiogenous cells similarly hyaline to pale brown, integrated in creeping threads (hyphae), terminal and intercalary, polyblastic, proliferation sympodial, rachis subcylindrical to geniculate, conidiogenous loci (scars) conspicuous, solitary to numerous, scattered to aggregated, subdenticulate, bulging out, umbonate or slightly attenuated towards a rounded apex, wall unthickened, not to slightly darkened-refractive. Conidia solitary, formation holoblastic, more or less obovoid, straight to somewhat curved, asymmetrical, aseptate, hyaline, subhyaline to very pale olivaceous-brown, with more or less conspicuous hilum, truncate to rounded, unthickened, not or slightly darkened-refractive; conidial secession schizolytic.

**Type species:** *Pseudovirgaria hyperparasitica* H.D. Shin, U. Braun, Arzanlou & Crous, **sp. nov.**

**Notes:** Other ramichloridium-like isolates from various rust species form another unique clade, sister to *Radulidium subulatum* (de Hoog) Arzanlou, W. Gams & Crous and *Ra. epichloës* (Ellis & Dearn.) Arzanlou, W. Gams & Crous in the *Sordariomycetidae*. Although *Pseudovirgaria* is morphologically similar to *Virgaria* Nees, it has hyaline to pale brown hyphae, conidia and conidiogenous cells. The conidiogenous cells are integrated in creeping threads (hyphae), terminal and intercalary, and the proliferation is distinctly sympodial. The subdenticulate conidiogenous loci are scattered, solitary, at small shoulders of geniculate conidiogenous cells, caused by sympodial proliferation, or aggregated, forming slight swellings of the rachis, i.e., a typical raduliform rachis as in *Virgaria* is lacking. Furthermore, the conidiogenous loci of *Pseudovirgaria* are bulging, convex, slightly attenuated towards the rounded apex, in contrast to more cylindrical denticles in *Virgaria* (Ellis 1971). The scar type of *Pseudovirgaria* is peculiar due to its convex, papilla-like shape and reminiscent of conidiogenous loci in plant-pathogenic genera like *Neoovularia* U. Braun and *Pseudodidymaria* U. Braun (Braun 1998). The superficially similar genus *Veronaea* is quite distinct from *Pseudovirgaria* by having erect conidiophores with a typical rachis and crowded conidiogenous loci which are flat or only slightly prominent and darkened. *Pseudovirgaria* is characterised by its mycelium which is composed of branched hyphae with integrated, terminal and intercalary conidiogenous cells. A differentiation between branched hyphae and “branched

conidiophores” is difficult and barely possible. It remains unclear if the “creeping threads” and terminal branches of hyphae are to be interpreted as “creeping conidiophores”. In any case, the mycelium forms complex fertile branched hyphal structures in which individual conidiophores are barely discernable. These structures and difficulties in discerning individual conidiophores remind one of some species of *Pseudocercospora* Speg. and other cercosporoid genera with abundant superficial mycelium *in vivo*.

***Pseudovirgaria hyperparasitica*** H.D. Shin, U. Braun, Arzanlou & Crous, **sp. nov.** MycoBank MB504565. Figs 6A, 31.

**Etymology:** Named after its hyperparasitic habit on rust fungi.

Hyphae 1.5–4 µm latae, tenuitunicatae, ≤ 0.5 µm crassae. Cellulae conidiogenae 15–50 × 2–5 µm, tenuitunicatae (≤ 0.5 µm), cicatricibus (0.5–)1.0(–1.5) µm diam, 0.5–1 µm altis. Conidia saepe obovoidea, interdum subclavata, 10–20 × 5–9 µm, apice rotundato vel paulo attenuato, basi truncata vel rotundata, hilo ca 1 µm diam.

*In vivo:* Colonies on rust sori, thin to moderately thick, loose, cobwebby, to dense, tomentose, pale to medium brown, rusty or cinnamon. Mycelium partly immersed in the sori, but mainly superficial, composed of a system of branched hyphae with integrated conidiogenous cells (fertile threads), distinction between conidiophores and vegetative hyphae difficult and barely possible. Hyphae 1.5–4 µm wide, hyaline, subhyaline to pale yellowish, greenish or very pale olivaceous, light brownish in mass, thin-walled (≤ 0.5 µm), smooth, pluriseptate, occasionally slightly constricted at the septa. Conidiogenous cells integrated in creeping fertile threads, terminal or intercalary, 15–50 µm long, 2–5 µm wide, subcylindrical to geniculate, subhyaline to very pale brownish, wall thin, ≤ 0.5 µm, smooth, proliferation sympodial, with a single to usually several conidiogenous loci per cell, often crowded, causing slight swellings, up to 6 µm wide, subdenticulate loci, formed by the slightly bulging wall, convex, slightly narrowed towards the rounded apex, (0.5–)1.0(–1.5) µm diam and 0.5–1 µm high, wall of the loci unthickened, not or slightly darkened-refractive, in surface view visible as minute circle (only rim visible and dark). Conidia solitary, obovoid, often slightly curved with ± unequal sides, 10–20 × 5–9 µm, aseptate, subhyaline, pale yellowish greenish to very pale olivaceous, wall ≤ 0.5 µm thick, smooth, apex slightly attenuated to usually broadly rounded, base rounded to somewhat attenuated towards a more or less conspicuous hilum, (0.5–)1(–1.5) µm diam, convex to truncate, unthickened, not to slightly darkened-refractive.

*In vitro:* Submerged hyphae hyaline to subhyaline, smooth; aerial hyphae smooth, subhyaline, up to 4 µm wide. Conidiogenous cells arising imperceptibly from aerial vegetative hyphae, terminal, occasionally intercalary, holoblastic, proliferating sympodially in a geniculate pattern, with more or less long intervals between groups of scars; loci slightly darkened, unthickened, approx. 0.5 µm diam. Conidia hyaline to subhyaline, aseptate, ovoid, often somewhat curved, (10–)13–15(–17) × (5–)6–7(–8) µm, with truncate base and acutely rounded apex; hila unthickened, slightly darkened-refractive.

**Cultural characteristics:** Colonies on MEA rather slow-growing, reaching 11 mm diam after 14 d at 24 °C, pale to dark mouse-grey, velvety, compacted, with colonies being up to 1 mm high.

**Specimens examined:** Korea, Seoul, on uredosori of *Frommeëlla* sp., on *Duchesnea chrysantha*, 17 Sep. 2003, H.D. Shin, **paratype**, 4/10, CPC 10702–10703 = CBS 121735–121736, HAL 2053 F; Chunchon, on *Phragmidium griseum* on *Rubus crataegifolius*, 20 Jul. 2004, H.D. Shin, **paratype**, 2/8, HAL 2057 F; Suwon,



**Fig. 32.** *Radulidium subulatum* (CBS 405.76). A–B. Macronematous conidiophores with symphyally proliferating conidiogenous cells, resulting in a conidium-bearing rachis. C–D. Rachis with crowded, blunt conidium-bearing denticles. E. Conidia. Scale bar = 10  $\mu$ m.



**Fig. 33.** *Radulidium epichloës* (CBS 361.63). A–C. Conidial apparatus at different stages of development, resulting in semi-micronematous conidiophores and symphyally proliferating conidiogenous cells. D. Rachis with crowded, blunt conidium-bearing denticles. E–F. Conidiophores with acute branches in the lower part. G. Conidia. Scale bar = 10  $\mu$ m.

on *Phragmidium pauciloculare* on *Rubus parvifolius*, 14 Oct. 2003, H.D. Shin, **paratype**, 23/10, HAL 2055 F; Hongchun, on *Phragmidium rosae-multiflorae* on *Rosa multiflora*, 11 Aug. 2004, H.D. Shin, **paratype**, 23/8, HAL 2056 F; Yangpyong, on *Phragmidium* sp. on *Rubus coreanus*, 30 Sep. 2003, H.D. Shin, **paratype**, 11/10-1, CPC 10704–10705 = CBS 121737–121738, HAL 2052 F, and the same locality, 23 Jul. 2004, HAL 2058 F; Chuncheon, on *Pucciniastrum agrimoniae* on *Agrimonia pilosa*, 7 Oct. 2002, H.D. Shin, **holotype**, HAL 2054 F, culture ex-type CPC 10753–10755 = CBS 121739–121741.

### ***Radulidium subulatum* and *Ra. epichloës* clade**

*Ramichloridium subulatum* and *R. epichloës* form a distinct, well-supported clade with uncertain affinity. This clade is morphologically distinct and a new genus is introduced below to accommodate it.

***Radulidium*** Arzanlou, W. Gams & Crous, **gen. nov.** MycoBank MB504566.

**Etymology:** Latin *radula* = A flexible tongue-like organ in gastropods, referring to the radula-like denticles on the rachis.

Genus ab aliis generibus *Ramichloridii* similibus denticulis densissimis, prominentibus, hebetibus in rachide e cellula conidiogena aculeata orta distinguendum.

**Type species:** *Radulidium subulatum* (de Hoog) Arzanlou, W. Gams & Crous, **comb. nov.**

**In vitro:** Colonies fast-growing, velvety, floccose near the margin, centrally with fertile hyphal bundles up to 10 mm high, about 2 mm diam, with entire but vague margin; *mycelium* whitish, later becoming greyish brown. *Submerged hyphae* smooth, thin-walled. *Conidiophores* usually reduced to polyblastic conidiogenous cells arising from undifferentiated or slightly differentiated aerial hyphae, terminally integrated or lateral, rarely a branched conidiophore present, smooth, slightly thick-walled, pale brown, cylindrical to acicular, widest at the base and tapering towards the apex; apical part forming a pale brown, generally straight rachis, with crowded, prominent, blunt denticles, suggesting a gastropod radula; denticles 0.5–1 µm long, apically pale brown. *Conidia* solitary, subhyaline, thin- or slightly thick-walled, smooth or verrucose, obovoidal, fusiform to subcylindrical, base subtruncate and with a slightly prominent, conspicuously pigmented hilum; conidial secession schizolytic.

**Notes:** *Radulidium* can be distinguished from other ramichloridium-like fungi by its slightly differentiated conidiophores and prominent, blunt, very dense conidium-bearing denticles. Although the *Radulidium* clade consists of several subclusters that correlate with differences in morphology, the ITS sequence data appear insufficient to resolve this species complex. Therefore, only two species of *Radulidium* with clear morphological and molecular differences are treated here. The phylogenetic situation of other taxa in this clade will be treated in a further study employing a multi-gene approach.

***Radulidium subulatum*** (de Hoog) Arzanlou, W. Gams & Crous, **comb. nov.** MycoBank MB504567. Figs 10C, 32.

**Basionym:** *Ramichloridium subulatum* de Hoog, Stud. Mycol. 15: 83. 1977.

**Misapplied name:** *Rhinochlaeniella elatior* Mangelot *sensu* dal Vesco & B. Peyronel, Allionia 14: 38. 1968.

**In vitro:** *Submerged hyphae* hyaline, thin-walled, 1–2.5 µm wide; *aerial hyphae* brownish. *Conidiogenous cells* arising laterally from vegetative hyphae, pale brown, smooth, thick-walled, sometimes without a basal septum, cylindrical to aculeate, tapering gradually

towards the apex, widest at the base, 25–40 × 2–3 µm; proliferating sympodially, forming a pale brown rachis, with densely crowded, prominent, blunt conidium-bearing denticles, with pale brown apex. *Conidia* solitary, subhyaline, thin-walled, smooth, ellipsoidal to almost clavate, 5–7 × 1.5–2 µm, with a slightly pigmented, non-refractive hilum, about 1 µm diam.

**Cultural characteristics:** Colonies on MEA rather fast growing, reaching 50 mm diam after 14 d at 24 °C, with entire but vague margin, velvety, floccose near the margin, centrally with fertile hyphal bundles up to 10 mm high, about 2 mm diam; mycelium whitish, later becoming greyish brown; reverse grey, zonate.

**Specimens examined:** **Czech Republic**, on *Phragmites australis*, A. Samšňáková, **ex-type** culture CBS 405.76; Opatovický pond, from *Lasiptera arundinis* (gall midge) mycangia on *Phragmites australis*, M. Skuhřavá, CBS 101010.

***Radulidium epichloës*** (Ellis & Dearn.) Arzanlou, W. Gams & Crous, **comb. nov.** MycoBank MB504568. Fig. 33.

**Basionym:** *Botrytis epichloës* Ellis & Dearn., Canad. Record Sci. 9: 272. 1893.

= *Ramichloridium epichloës* (Ellis & Dearn.) de Hoog, Stud. Mycol. 15: 81. 1977.

**In vitro:** *Submerged hyphae* hyaline, thin-walled, 1–2.5 µm wide; *aerial hyphae* somewhat darker. *Conidiogenous cells* arising laterally or terminally from undifferentiated or slightly differentiated aerial hyphae, occasionally acutely branched in the lower part, smooth, thick-walled, pale brown, more or less cylindrical, later with thin septa, 25–47 µm long; proliferating sympodially, forming a rather short, pale brown, straight or somewhat geniculate rachis, with crowded, prominent, blunt denticles with pale brown apex. *Conidia* solitary, subhyaline, rather thin-walled, verrucose, obovoidal to fusiform, (4.5–)7–8(–11) × 2–3 µm, with a pigmented hilum, 1–1.5 µm diam.

**Cultural characteristics:** Colonies reaching 45 mm diam after 14 d at 24 °C, with smooth, rather vague, entire margin; velvety, centrally floccose and elevated up to 2 mm high; surface mycelium whitish, later becoming greyish brown; reverse pale ochraceous.

**Specimen examined:** **U.S.A.**, Cranberry Lake, Michigan, isolated from *Epichloë typhina* on *Glyceria striata*, G.L. Hennebert, CBS 361.63 = MUCL 3124; specimen in MUCL designated here as **epitype**.

### **Veronaea-like clade, allied to the *Annulatasceae***

A veronaea-like isolate from *Bertia moriformis* clusters near the *Annulatasceae*, and is morphologically distinct from other known anamorph genera in the *Ramichloridium* complex, and therefore a new genus is introduced to accommodate it.

***Rhodoveronaea*** Arzanlou, W. Gams & Crous, **gen. nov.** MycoBank MB504569.

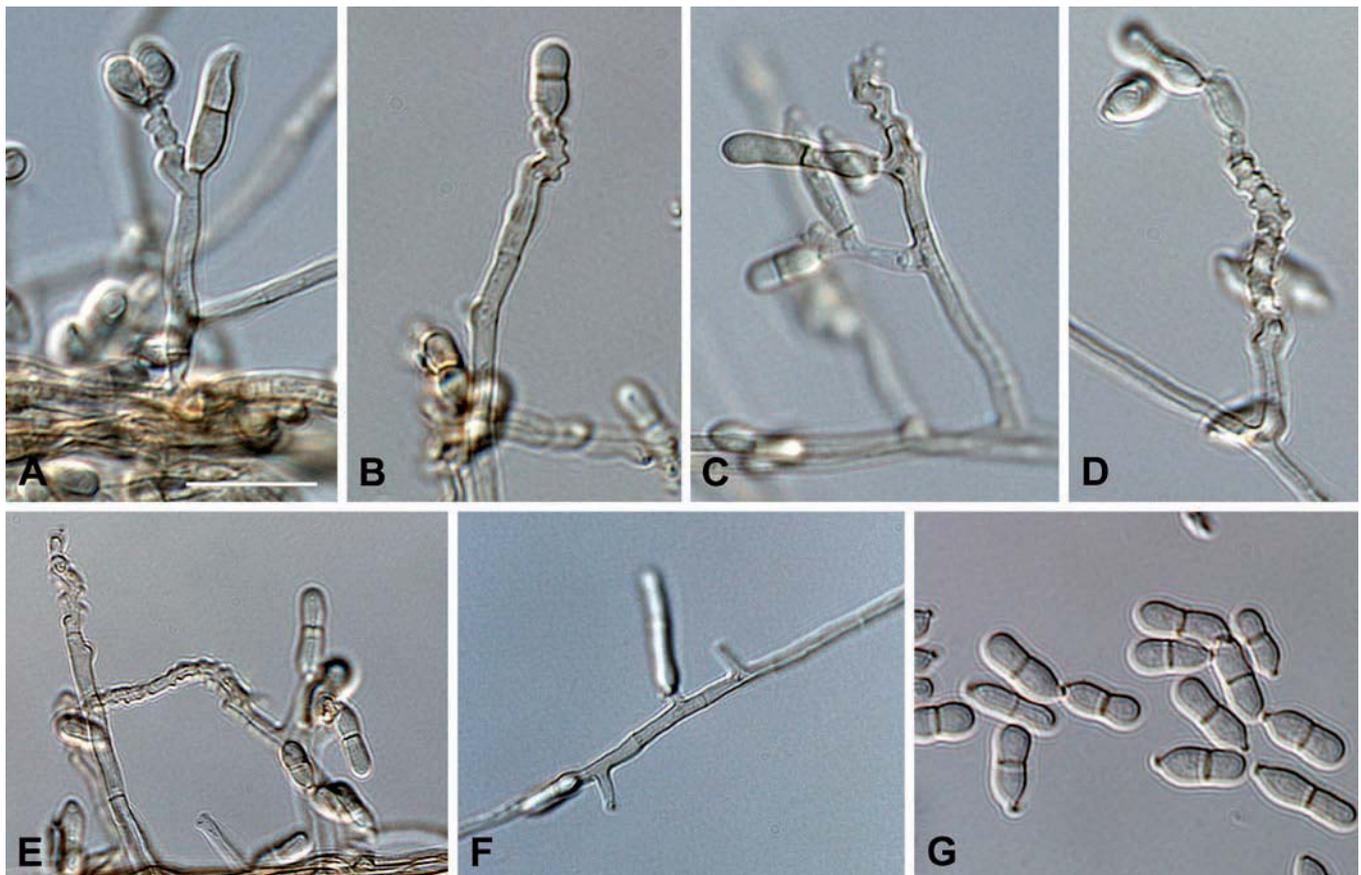
**Etymology:** (Greek) *rhodon* = the rose, referring to the red-brown conidiophores, suffix *-veronaea* from *Veronaea*.

Genus ab aliis generibus *Ramichloridii* similibus basi condiorum late truncata et marginata distinguenda.

**In vitro:** Colonies slow-growing, velvety, floccose; surface olivaceous-grey to dark olivaceous-green; reverse olivaceous-black. *Hyphae* smooth, thin-walled, pale olivaceous. *Conidiophores* arising vertically from creeping hyphae, straight or flexuose, simple, thick-walled, red-brown, with inflated basal cell. *Conidiogenous cells* terminally integrated, polyblastic, sympodial, smooth, thick-



**Fig. 34.** *Rhodoveronaea varioseptata* (CBS 431.88). A–D. Macronematous conidiophores with sympodially proliferating conidiogenous cells, resulting in conidium bearing rachis with slightly prominent conidium-bearing denticles. E–F. Conidia with minute marginal frill. Scale bar = 10  $\mu$ m.



**Fig. 35.** *Veronaeopsis simplex* (CBS 588.66). A–C. Conidial apparatus at different stages of development, resulting in semi-micronematous conidiophores and sympodially proliferating conidiogenous cells. D–E. Rachis with crowded, prominent denticles. F. Intercalary conidiogenous cells. G. Conidia. Scale bar = 10  $\mu$ m.

walled, pale brown, rachis straight, occasionally geniculate, with crowded, slightly prominent conidium-bearing denticles; denticles flat-tipped, slightly pigmented. *Conidia* solitary, pale brown, thin- or slightly thick-walled, smooth, ellipsoidal to obovoidal, 0–multi-septate, with a protruding base and a marginal basal frill; conidial secession schizolytic.

*Type species:* *Rhodoveronaea varioseptata* Arzanlou, W. Gams & Crous, sp. nov.

*Notes:* *Rhodoveronaea* differs from other ramichloridium-like fungi by the presence of a basal, marginal conidial frill, and variably septate conidia.

**Rhodoveronea varioseptata** Arzanlou, W. Gams & Crous, **sp. nov.** MycoBank MB504570. Figs 10D, 34.

**Etymology:** Named for its variably septate conidia.

Hyphae 2–3 µm latae. Conidiophora ad 125 µm longa et 3–5 µm lata. Cellulae conidiogenae 30–70 µm longae et 3–5 µm latae. Conidia 0–2(–3)-septata, (8–)11–13(–15) × (2–)3–4(–6) µm.

**In vitro:** Submerged hyphae smooth, thin-walled, pale olivaceous, 2–3 µm wide; aerial hyphae smooth, brownish and slightly narrower. Conidiophores arising vertically from creeping hyphae, straight or flexuose, simple, smooth, thick-walled, red-brown, up to 125 µm long, 3–5 µm wide, often with inflated basal cell. Conidiogenous cells terminally integrated, smooth, thick-walled, pale brown at the base, paler towards the apex, straight, variable in length, 30–70 µm long and 3–5 µm wide, rachis straight, occasionally geniculate; slightly prominent conidium-bearing denticles, crowded, with slightly pigmented apex, about 1 µm diam. Conidia solitary, pale brown, thin- or slightly thick-walled, smooth, ellipsoid to obovoid, 0–2(–3)-septate, (8–)11–13(–15) × (2–)3–4(–6) µm with a protruding base, 1.5 µm wide, and marginal frill.

**Cultural characteristics:** Colonies reaching 12 mm diam after 14 d at 24 °C, velvety, floccose; surface olivaceous-grey to dark olivaceous-green; reverse olivaceous-black.

**Specimen examined:** Germany, Eifel, Berndorf, on *Bertia moriformis*, Sep. 1987, W. Gams, **holotype** CBS-H 19932, culture ex-type CBS 431.88.

### Venturiaceae (Pleosporales)

The ex-type strain of *Veronaea simplex* (Papendorf 1969) did not cluster with the genus *Veronaea* (*Herpotrichiellaceae*), but is allied to the *Venturiaceae*. *Veronaea simplex* is distinct from species of *Fusicladium* Bonord. by having a well-developed rachis with densely aggregated scars. A new genus is thus introduced to accommodate this taxon.

**Veronaeopsis** Arzanlou & Crous, **gen. nov.** MycoBank MB504571.

**Etymology:** The suffix *-opsis* refers to its similarity with *Veronaea*.

Genus *Veronaeae* simile sed conidiophoris brevioribus (ad 60 µm longis) et rachide dense denticulata distinguendum.

**In vitro:** Colonies moderately fast-growing; surface velvety, floccose, greyish sepia to hazel, with smooth margin; reverse mouse-grey to dark mouse-grey. Conidiophores arising vertically from aerial hyphae, lateral or intercalary, simple or branched, occasionally reduced to conidiogenous cells, pale brown. Conidiogenous cells terminally integrated on simple or branched conidiophores, polyblastic, smooth, thin-walled, pale brown; rachis commonly straight, geniculate, with densely crowded, prominent denticles, and slightly pigmented scars. Conidia solitary, subhyaline to pale brown, thin- or slightly thick-walled, smooth, oblong-ellipsoidal to subcylindrical, (0–)1-septate, with a slightly darkened, thickened, hilum; conidial secession schizolytic.

**Type species:** *Veronaeopsis simplex* (Papendorf) Arzanlou & Crous, **comb. nov.**

**Veronaeopsis simplex** (Papendorf) Arzanlou & Crous, **comb. nov.** MycoBank MB504572. Figs 17C, 35.

**Basionym:** *Veronaea simplex* Papendorf, Trans. Brit. Mycol. Soc. 52: 486. 1969.

**In vitro:** Submerged hyphae smooth, thin-walled, pale brown; aerial hyphae aggregated in bundles. Conidiophores arising vertically from aerial hyphae, lateral or intercalary, simple or branched, occasionally reduced to conidiogenous cells, pale brown, rather short, up to 60 µm long, 1.5–2 µm wide. Conidiogenous cells terminally integrated in the conidiophores, smooth, thin-walled, pale brown, variable in length, 5–25 µm long, rachis generally straight or irregularly geniculate, with crowded, prominent denticles, about 0.5 µm long, flat-tipped, with slightly pigmented apex. Conidia solitary, subhyaline to pale brown, thin- or slightly thick-walled, smooth, oblong-ellipsoidal to subcylindrical, (0–)1-septate, slightly constricted at the septum, (6–)10–12(–15) × (2–)2.5–3(–4) µm; hilum slightly darkened and thickened, not refractive, about 1 µm diam.

**Cultural characteristics:** Colonies reaching 25 mm diam after 14 d at 24 °C; surface velvety, floccose, greyish sepia to hazel, with smooth margin; reverse mouse-grey to dark mouse-grey.

**Specimen examined:** South Africa, Potchefstroom, on leaf litter of *Acacia karroo*, 1966, M.C. Papendorf, **holotype**, CBS H-7810; culture ex-type CBS 588.66 = IMI 203547.

**Notes:** The presence of 1-septate conidia in *Veronaeopsis* overlaps with *Veronaea*. However, *Veronaeopsis* differs from *Veronaea* based on its conidiophore and conidiogenous cell morphology. *Veronaea* has much longer, macronematous conidiophores than *Veronaeopsis*. Furthermore, *Veronaea* has a more or less straight rachis, whereas in *Veronaeopsis* the rachis is often geniculate. The conidiogenous loci in *Veronaea* are less prominent, i.e., less denticle-like.

## DISCUSSION

The present study was initiated chiefly to clarify the status of *Ramichloridium musae*, the causal organism of tropical speckle disease of banana (Jones 2000). Much confusion surrounded this name in the past, relating, respectively, to its validation, species and generic status. As was revealed in the present study, however, two species are involved in banana speckle disease, namely *R. musae* and *R. biverticillatum*. Even more surprising was the fact that *Ramichloridium* comprises anamorphs of *Mycosphaerella* Johanson (*Mycosphaerellaceae*), though no teleomorphs have thus far been conclusively linked to any species of *Ramichloridium*. By investigating the *Ramichloridium* generic complex as outlined by de Hoog (1977), another genus associated with leaf spots, namely *Periconiella*, was also shown to represent an anamorph of *Mycosphaerella*. Although no teleomorph connections have been proven for ramichloridium-like taxa, de Hoog *et al.* (1983) refer to the type specimen of *Wentomyces javanicus* Koord. (*Pseudoperisporiaceae*), on the type specimen of which (PC) some ramichloridium-like conidiophores were seen. Without fresh material and an anamorph-teleomorph connection proven in culture, however, this matter cannot be investigated further. It is interesting to note, however, that *Wentomyces* Koord. shows a strong resemblance to *Mycosphaerella*, except for the external perithecial appendages.

The genus *Mycosphaerella* is presently one of the largest genera of ascomycetes, containing close to 3 000 names (Aptroot 2006), to which approximately 30 anamorph genera have already been linked (Crous *et al.* 2006a, b, 2007). By adding two additional anamorph genera, the *Mycosphaerella* complex appears to be expanding

even further, though some taxa have been shown to reside in other families in the *Capnodiales*, such as *Davidiella* Crous & U. Braun (*Davidiellaceae*) and *Teratosphaeria* (*Teratosphaeriaceae*) (Braun *et al.* 2003, Crous *et al.* 2007, Schubert *et al.* 2007 – this volume).

Another family, which proved to accommodate several ramichloridium-like taxa, is the *Herpotrichiellaceae* (*Chaetothyriales*). Members of the *Chaetothyriales* are regularly encountered as causal agents of human mycoses (Haase *et al.* 1999, de Hoog *et al.* 2003), whereas species of the *Capnodiales* are common plant pathogens, or chiefly associated with plants. Species in the *Chaetothyriales* have consistently melanized thalli, which is a factor enabling them to invade humans, and cause a wide diversity of mycoses, such as chromoblastomycosis, mycetoma, brain infection and subcutaneous phaeohyphomycosis (de Hoog *et al.* 2003). The only known teleomorph connection in this genus is *Capronia* Sacc. (Untereiner & Naveau 1999).

*Rhinocladiella* and *Veronaea* were in the past frequently confused with the genus *Ramichloridium*. However, *Rhinocladiella*, as well as *Veronaea* and *Thysanoreia*, were shown to cluster in the *Chaetothyriales*, while *Ramichloridium* clusters in the *Capnodiales*. *Rhinocladiella mackenziei*, which causes severe cerebral phaeohyphomycosis in humans (Sutton *et al.* 1998), has in the past been confused with *Pleurothecium obovoideum* (Ur-Rahman *et al.* 1988). Data presented here reveal, however, that although morphologically similar, these species are phylogenetically separate, with *P. obovoideum* belonging to the *Sordariales*, where it clusters with sexual species of *Carpoligna* F.A. Fernández & Huhndorf that have *Pleurothecium* anamorphs (Fernández *et al.* 1999).

In addition to the genera clustering in the *Capnodiales* and *Chaetothyriales*, several ramichloridium-like genera are newly introduced to accommodate species that cluster elsewhere in the ascomycetes, namely *Pseudovirgaria*, *Radulidium* and *Myrmecridium*, *Veronaeopsis*, and *Rhodoveronaea*. Although the ecological role of these taxa is much less known than that of taxa in the *Capnodiales* and *Chaetothyriales*, some exhibit an interesting ecology. For instance, the fungicolous habit of *Pseudovirgaria*, as well as some species in *Radulidium*, which are found on various rust species, suggests that these genera should be screened further to establish if they have any potential biocontrol properties. Furthermore, these two genera share a common ancestor, and further work is required to determine whether speciation was shaped by co-evolution with the rusts. A further species of “*Veronaea*” that might belong to *Pseudovirgaria* is *Veronaea harunganae* (Hansf.) M.B. Ellis, which is known to occur on *Hemileia harunganae* Cummins on *Harungana* in Tanzania and Uganda (Ellis 1976). The latter species, however, is presently not known from culture, and needs to be recollected to facilitate further study.

The genera distinguished here represent homogeneous clades in the phylogenetic analysis. Only the species of *Rhinocladiella* are dispersed among others morphologically classified in *Exophiala* or other genera.

By integrating the phylogenetic data generated here with the various morphological data sets, we were able to resolve eight clades for taxa formerly regarded as representative of the *Ramichloridium* complex. According to the phylogeny inferred from 28S rDNA sequence data, the genera *Ramichloridium* and *Periconiella* were heterogeneous, requiring the introduction of several novel genera. Although the present 11 odd genera can still be distinguished based on their morphology, it is unlikely that morphological identifications without the supplement of molecular data would in the future be able to accurately identify all the novel

isolates that undoubtedly await description. The integration of morphology with phylogenetic data not only helps to resolve generic affinities, but it also assists in discriminating between the various cryptic species that surround many of these well-known names that are presently freely used in the literature. To that end it is interesting to note that for the majority of the taxa studied here, the ITS domain (Table 1) provided good species resolution. However, more genes will have to be screened in future studies aimed at characterising some of the species complexes where the ITS domain provided insufficient phylogenetic signal (data not shown) to resolve all of the observed morphological species.

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# Multiple gene genealogies and phenotypic characters differentiate several novel species of *Mycosphaerella* and related anamorphs on banana

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## Key words

*Mycosphaerella*  
phylogeny  
Sigatoka disease complex  
taxonomy

**Abstract** Three species of *Mycosphaerella*, namely *M. eumusae*, *M. fijiensis*, and *M. musicola* are involved in the Sigatoka disease complex of bananas. Besides these three primary pathogens, several additional species of *Mycosphaerella* or their anamorphs have been described from *Musa*. However, very little is known about these taxa, and for the majority of these species no culture or DNA is available for study. In the present study, we collected a global set of *Mycosphaerella* strains from banana, and compared them by means of morphology and a multi-gene nucleotide sequence data set. The phylogeny inferred from the ITS region and the combined data set containing partial gene sequences of the actin gene, the small subunit mitochondrial ribosomal DNA and the histone H3 gene revealed a rich diversity of *Mycosphaerella* species on *Musa*. Integration of morphological and molecular data sets confirmed more than 20 species of *Mycosphaerella* (incl. anamorphs) to occur on banana. This study reconfirmed the previously described presence of *Cercospora apii*, *M. citri* and *M. thailandica*, and also identified *Mycosphaerella communis*, *M. lateralis* and *Passalora loranthi* on this host. Moreover, eight new species identified from *Musa* are described, namely *Dissoconium musae*, *Mycosphaerella mozambica*, *Pseudocercospora assamensis*, *P. indonesiana*, *P. longispora*, *Stenella musae*, *S. musicola*, and *S. queenslandica*.

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## INTRODUCTION

The genus *Mycosphaerella* is phylogenetically heterogeneous (Crous et al. 2007a), contains more than 3000 names (Aptroot 2006), and has been linked to more than 30 well-known anamorphic genera (Crous et al. 2006a, b, 2007a, b, Arzanlou et al. 2007a). Species of *Mycosphaerella* inhabit different ecological niches as saprobes, plant pathogens or endophytes (Farr et al. 1995, Verkley & Starink-Willemse 2004, Crous et al. 2004b, 2006a, 2007a, b), and have a worldwide distribution from tropical and subtropical to warm and cool regions (Crous 1998, Crous et al. 2000, 2001). Plant-pathogenic species of *Mycosphaerella* are among the most common and destructive plant pathogens occurring on a wide range of hosts including trees, herbaceous plants, and plantation crops. The invasion of leaf and stem tissue and concomitant distortion of the host plant physiology cause considerable economic losses (Park

et al. 2000, Goodwin et al. 2001, Maxwell et al. 2005, Cortinas et al. 2006, Crous et al. 2006a, b, Hunter et al. 2006).

The Sigatoka disease complex, which is the most serious and economically important leaf spot disease of banana, is attributed to species of *Mycosphaerella*. *Mycosphaerella musicola* (anamorph *Pseudocercospora musae*) which causes (yellow) Sigatoka disease, *M. fijiensis* (anamorph *P. fijiensis*) which causes the black Sigatoka disease, and *M. eumusae* (anamorph *P. eumusae*), which causes eumusae leaf spot disease (reviewed in Jones 2000, 2003, Crous & Mourichon 2002) are the major constituents of the Sigatoka disease complex. The disease reduces the photosynthetic capacity of the plant as a consequence of necrotic leaf lesions, and induces physiological alterations of the plant, resulting in reduced crop yield and fruit quality. All three species emerged on bananas during the last century and became major constraints to commercial production worldwide. The chronology of disease records around the world and genetic structure of pathogen population suggests that South-East Asia, where the host genus *Musa* is indigenous, is the centre of origin for all three fungal species (Mourichon & Fullerton 1990, Carlier et al. 1996, Hayden et al. 2003, Rivas et al. 2004).

Yellow Sigatoka disease was first reported on banana in Java in 1902. The disease spread rapidly to all banana-growing regions during the following 20 years, and has since reached the limits of its distribution worldwide (reviewed in Jones 2000, 2003). The fungus responsible for the disease was described as *Cercospora musae*. In 1941 Leach established the connection between *C. musae* and its teleomorph, *Mycosphaerella*

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**Table 1** Isolates of *Mycosphaerella* or its anamorphs used for DNA analysis and morphological studies.

Species	Accession number <sup>1</sup>	Source	Origin	GenBank numbers (ITS, ACT, HIS, mtSSU)	
<i>Cercospora apii</i>	CPC 12682; CBS 119395	<i>Musa</i> cv. Cavendish	Western Bangladesh	EU514222, —, —, —	
	CPC 12683	<i>Musa</i> cv. Cavendish	Western Bangladesh	EU514223, —, —, —	
	CPC 12684	<i>Musa</i> cv. Cavendish	Western Bangladesh	EU514224, —, —, —	
<i>Dissoconium musae</i>	X1021; CBS 122453	<i>Musa</i> cv. Nendran (Plantain) AAB	India	EU514225, EU514296, EU514349, EU514402	
	X1022; CBS 122454	<i>Musa</i> cv. Nendran (Plantain) AAB	India	EU514226, EU514297, EU514350, EU514403	
<i>Mycosphaerella citri</i>	X126; CBS 122455	<i>Citrus</i> sp.	USA, Florida	EU514227, —, —, —	
	X742; CBS 122456	<i>Musa</i> cv. SH 3436 AAAA	Tonga	EU514228, —, —, —	
	X743	<i>Musa</i> cv. SH 3362 AA	Tonga	EU514229, —, —, —	
<i>Mycosphaerella colombiensis</i>	X24	<i>Musa</i> cultivar	Mozambique	EU514230, —, —, —	
	X215	—	South Africa	EU514231, EU514298, EU514351, EU514404	
<i>Mycosphaerella communis</i>	X1023	<i>Musa</i> cv. Valery: Cavaendish	Trinidad	EU514232, EU514299, EU514352, EU514405	
<i>Mycosphaerella eumusae</i>	S1030B	<i>Musa</i> cultivar	Mauritius	EU514233, EU514300, EU514353, EU514406	
	S1037B	<i>Musa</i> cultivar	Mauritius	EU514234, EU514301, EU514354, EU514407	
<i>Mycosphaerella fijiensis</i>	S1037C	<i>Musa</i> cultivar	Mauritius	EU514235, EU514302, EU514355, EU514408	
	S1037G	<i>Musa</i> cultivar	Mauritius	EU514236, —, —, —	
	S1037H	<i>Musa</i> cultivar	Mauritius	EU514237, —, —, —	
	X208; CIRAD 1156; CPC 4579	<i>Musa</i> cultivar	Mauritius	EU514238, —, —, —	
	X209; CBS 114825; CIRAD 1157; CPC 4580	<i>Musa</i> cultivar	Mauritius	EU514239, —, —, —	
	X865; CIRAD 535	<i>Musa</i> cultivar	India	EU514240, EU514303, EU514356, EU514409	
	X866; CBS 121377; CIRAD 458	<i>Musa</i> cultivar	Malaysia	EU514241, EU514304, EU514357, EU514410	
	X867; CBS 121378; CIRAD 459	<i>Musa</i> cultivar	Malaysia	EU514242, EU514305, EU514358, EU514411	
	X869; CBS 121380; CIRAD 563	<i>Musa</i> cultivar	Sri Lanka	EU514243, EU514306, EU514359, EU514412	
	X870; CBS 121381; CIRAD 485	<i>Musa</i> cultivar	Thailand	EU514244, EU514307, EU514360, EU514413	
	X873; CBS 121382; CIRAD 487	<i>Musa</i> cultivar	Thailand	EU514245, EU514308, EU514361, EU514414	
	X875; CIRAD 671	<i>Musa</i> cultivar	Vietnam	EU514246, EU514309, EU514362, EU514415	
	X876; CBS 121383; CIRAD 744	<i>Musa</i> cultivar	Mauritius	EU514247, EU514310, EU514363, EU514416	
	<i>Mycosphaerella fijiensis</i>	CIRAD 86; CBS 120258; C86a	<i>Musa</i> cultivar	Cameroon	EU514248, —, —, —
		X84	<i>Musa</i> cultivar	Colombia	EU514249, EU514311, EU514364, EU514417
		X92	<i>Musa</i> cultivar	Colombia	EU514250, EU514312, EU514365, EU514418
		X104	<i>Musa</i> cultivar	Colombia	EU514251, EU514313, EU514366, EU514419
		X110	<i>Musa</i> cultivar	Colombia	EU514252, EU514314, EU514367, EU514420
		X843; CIRAD 11	<i>Musa</i> cultivar	Honduras	EU514253, EU514315, EU514368, EU514421
		X847; CBS 121362; CIRAD 364	<i>Musa</i> cultivar	Taiwan	EU514254, EU514316, EU514369, EU514422
X850; CIRAD 355		<i>Musa</i> cultivar	Ivory Coast	EU514255, EU514317, EU514370, EU514423	
<i>Mycosphaerella lateralis</i>		S1024	<i>Musa</i> cultivar	Mauritius	EU514256, —, —, —
		<i>Mycosphaerella mozambica</i>	X34; CBS 122464	<i>Musa</i> cultivar	Mozambique
X884; CBS 121391; UQ438	<i>Musa</i> cultivar		Australia	EU514258, EU514319, EU514372, EU514425	
<i>Mycosphaerella musae</i>	X398; CBS 122458	<i>Musa</i> cv. Cavendish AAA	Tonga	EU514259, EU514320, EU514373, EU514426	
	X813	<i>Musa</i> cultivar	Malawi	EU514260, EU514321, EU514374, EU514427	
	X814; CBS 122459	<i>Musa</i> cultivar	Malawi	EU514261, EU514322, EU514375, EU514428	
	X818; CBS 122460	<i>Musa</i> cultivar	Malawi	EU514262, —, —, —	
	X819; CBS 122461	<i>Musa</i> cultivar	Malawi	EU514263, —, —, —	
	X879; CBS 121386; CIRAD 64	<i>Musa</i> cultivar	Malawi	EU514264, EU514323, EU514376, EU514429	
<i>Mycosphaerella musicola</i>	X42; CBS 116634; IMI 123823	<i>Musa</i> cultivar	Cuba	EU514265, —, —, —	
	X63	<i>Musa</i> cultivar	Windward Islands	EU514266, EU514324, EU514377, EU514430	
	X67	<i>Musa</i> cultivar	Windward Islands	EU514267, EU514325, EU514378, EU514431	
	X588	<i>Musa</i> cv. Williams	Australia	EU514268, EU514326, EU514379, EU514432	
	X589	<i>Musa</i> cv. Williams	Australia	EU514269, EU514327, EU514380, EU514433	
	X596	<i>Musa</i> cv. SH-3362 AA	Australia	EU514270, EU514328, EU514381, EU514434	
	X602	<i>Musa</i> cv. Lakatan	Australia	EU514271, EU514329, EU514382, EU514435	
	X857; CBS 121371; UQ430	<i>Musa</i> cultivar	Australia	EU514272, EU514330, EU514383, EU514436	

	X858; CBS 121372; UQ433	<i>Musa</i> cultivar	Australia	EU514273, EU514331, EU514384, EU514437
	X860; CBS 121374; UQ2003	<i>Musa</i> cultivar	Australia	EU514274, EU514332, EU514385, EU514438
<i>Mycosphaerella thailandica</i>	X22	<i>Musa</i> cultivar	Windward Islands	EU514275, EU514333, EU514386, EU514439
	X53	<i>Musa</i> cultivar	Australia	EU514276, EU514334, EU514387, EU514440
<i>Passalora loranthi</i>	X882; CBS 121389; CIRAD 81	<i>Musa</i> cultivar	Mozambique	EU514277, EU514335, EU514388, EU514441
	X883; CBS 121390; CIRAD 1165	<i>Musa</i> cultivar	Brazil	EU514278, EU514336, EU514389, EU514442
	X28; CBS 122465	<i>Musa</i> cultivar	Cameroon	EU514279, EU514337, EU514390, EU514443
	X138; CBS 122466	<i>Cifrus</i> sp.	Mozambique	EU514280, EU514338, EU514391, EU514444
<i>Pseudocercospora assamensis</i>	X988; #9; CBS 122467	<i>Musa</i> cultivar	India, Assam	EU514281, EU514339, EU514392, EU514445
<i>Pseudocercospora indonesiana</i>	X991; #11-5; CBS 122473	<i>Musa</i> cultivar	Indonesia, Western Sumatra	EU514282, EU514340, EU514393, EU514446
<i>Pseudocercospora longispora</i>	X992; #11-6; CBS 122474	<i>Musa</i> cultivar	Indonesia, Western Sumatra	EU514283, —, —, —
	X474; CBS 122469	<i>Musa</i> cv. Pisang Mas AA	Malaysia	EU514284, EU514341, EU514394, EU514447
<i>Pseudocercospora</i> sp.	X475; CBS 122470	<i>Musa</i> cv. Pisang Mas AA	Malaysia	EU514285, EU514342, EU514395, EU514448
<i>Stenella musae</i>	X1083; CBS 122468	<i>Ravenala madagascariensis</i>	India	EU514286, —, —, —
	X45	<i>Musa</i> cultivar	Windward Islands	EU514287, EU514343, EU514396, EU514449
	X47; CBS 122476	<i>Musa</i> cultivar	Windward Islands	EU514288, EU514344, EU514397, EU514450
	X55	<i>Musa</i> cultivar	Windward Islands	EU514289, —, —, —
	X70; CBS 122478	<i>Musa</i> cultivar	Windward Islands	EU514290, EU514345, EU514398, EU514451
	X745; CBS 122477	<i>Musa</i> cv. T8 AAAAA	Tonga	EU514291, EU514346, EU514399, EU514452
	X877; CBS 121384; CIRAD 41	<i>Musa</i> cultivar	Martinique	EU514292, EU514347, EU514400, EU514453
	X878; CBS 121385; CIRAD 56	<i>Musa</i> cultivar	Martinique	EU514293, EU514348, EU514401, EU514454
<i>Stenella musicola</i>	X1019; CBS 122479	<i>Musa</i> cv. Grand Nain AAA (Cav.)	India	EU514294, —, —, —
<i>Stenella queenslandica</i>	X1084; CBS 122475	<i>Musa</i> <i>benkissii</i>	Australia	EU514295, —, —, —

<sup>1</sup> CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CIRAD: Centre de coopération internationale en recherche agronomique pour le développement, Montpellier, France; CPC: Culture collection of Pedro Crous, housed at CBS; IMI: International Mycological Institute, CAB International, Egham, Bakenham Lane, UK; UQ: University of Queensland, Australia; X: S: Culture collection of Mahdi Arzanlou, housed at CBS.

*musicola*. Mulder (in Mulder & Stover 1976) validated the species descriptions, while the anamorph was transferred to *Pseudocercospora* as *P. musae* (Deighton 1976). In the early 1960s, another, even more severe leaf spot disease on banana appeared in the Fiji Islands, which Rhodes (1964) described as black leaf streak disease and later became known as the black Sigatoka disease. Morelet (1969) validated the species name as *M. fijiensis*, while Deighton (1976) placed its anamorph in *Pseudocercospora* as *P. fijiensis*. In 1974 a new variety of *M. fijiensis* was described from Honduras and named as *M. fijiensis* var. *difformis*. Deighton (1979) placed both varieties in the genus *Paracercospora*, based on the slight thickening observed on the rims of scars and conidial hila. However, this feature was not supported by DNA phylogeny, and as there were many intermediate morphological forms, the genus *Paracercospora* was again reduced to synonymy under *Pseudocercospora* by Crous et al. (2001). *Mycosphaerella eumusae* (*Pseudocercospora eumusae*), was recognised as a new constituent of the Sigatoka complex of banana in the mid-1990s (Carlier et al. 2000, Crous & Mourichon 2002, Jones 2003). Presently, *M. eumusae* is known from parts of South-East Asia, Indian Ocean Islands and Nigeria, where it could co-exist with the other two species. Besides the three primary agents of the Sigatoka disease complex, several additional species of *Mycosphaerella* (or their anamorphs) have been described from *Musa*, but for the majority of these species no culture or herbarium specimen is available, and the pathological relevance of those species remains unclear (reviewed in Jones 2000, Crous et al. 2003, Aptroot 2006).

The identity and distribution of the various *Mycosphaerella* species associated with leaf spots of banana are not yet fully understood, which is mainly due to the difficulties experienced by scientists who have to identify them by conventional methods and without specialist taxonomic support. Furthermore, because these species are morphologically highly similar and frequently co-occur on the same lesion, pathogen recognition and subsequent disease management have proven to be rather difficult. To enable the development of specific molecular-based diagnostic tools for pathogen recognition, all related species present on the same host have to be considered. Recently, Arzanlou et al. (2007a) developed a highly sensitive set of Taqman probes to distinguish *M. fijiensis* from *M. musicola* and *M. eumusae* in leaf material. Little attention has been given to date, however, to other species of *Mycosphaerella* that occur on *Musa* spp. Because several *Mycosphaerella* species can co-occur in the same lesion (Crous 1998), it is quite possible that there may be other species of *Mycosphaerella* associated with the Sigatoka disease complex. The aim of the present study was, therefore, to employ a multi-gene DNA sequence typing approach on a global set of *Mycosphaerella* isolates to distinguish the various species occurring on banana. To this end morphological and cultural growth data were integrated with DNA sequence data from the internal transcribed spacer region of the rDNA operon, and partial actin, histone H3, and small subunit mitochondrial ribosomal DNA gene sequences.

## MATERIALS AND METHODS

### Isolates

Isolates (Table 1) were obtained by isolation from infected symptomatic banana leaves, or supplied as pure cultures by the following departments and institutes: The Horticulture and Food Research Institute of New Zealand, Auckland, New Zealand; Centre de coopération internationale en recherche agronomique pour le développement (CIRAD, Montpellier, France); University of Florida, Tropical Research & Education Centre (USA); Forestry and Agricultural Biotechnology Institute (FABI, Pretoria, South Africa). Isolates were recovered from infected banana leaves as single ascospores or conidia. Germinating spores were examined 24 h after germination on 2 % malt extract agar (MEA; Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) plates under a stereomicroscope, and single-spore cultures were established on fresh MEA plates following the protocol of Crous (1998).

### DNA phylogeny

Genomic DNA was isolated from fungal mycelia grown on MEA, using the FastDNA kit (BIO101, Carlsbad, CA, USA) according to the manufacturer's protocol. The primers ITS1 and ITS4 (White et al. 1990) were used to amplify part of the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3' end of the 18S rRNA gene, the first ITS region, the 5.8S rRNA gene, the second ITS region, and the 5' end of the 28S rRNA gene. A part of the actin gene (ACT) was amplified with primers ACT-512F and ACT-783R (Carbone & Kohn 1999), a part of the small subunit mitochondrial ribosomal DNA (mtSSU) with primers MNS1 and MNS2 (Li et al. 1994), and a part of the histone H3 (HIS) gene with primers CYLH3F and CYLH3R (Crous et al. 2004b). Amplification reactions were performed with each primer set in a total reaction volume of 25  $\mu$ l, which was composed of 1  $\times$  PCR Buffer (Applied Biosystems, Foster City, USA), variable  $MgCl_2$  concentrations, 60  $\mu$ M dNTPs, 0.2  $\mu$ M of each forward and reverse primer, 1.5 U of *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, USA) and 1–10 ng of genomic DNA. PCR cycle conditions were 5 min of 95 °C, followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final elongation at 72 °C for 7 min. Amplicons were sequenced using both PCR primers with a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, the Netherlands) according to the manufacturer's recommendations, and sequences were analysed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, CA).

The resulting nucleotide sequences were analysed and automatically aligned using BioNumerics v. 4.5 (Applied Maths, Kortrijk, Belgium) followed by manual improvement by eye where necessary. Phylogenetic analyses were performed with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003), using the neighbour-joining algorithm with the uncorrected ("p"), the Kimura 2-parameter and the HKY85 substitution models. Alignment gaps longer than 10 bases were coded as single events for the phylogenetic analyses; the remaining gaps were treated as missing data. Any encountered ties were randomly broken. Phylogenetic relationships were also inferred

with the parsimony algorithm using the heuristic search option with simple (ITS alignment) or 100 random taxa additions (combined alignment) and tree bisection and reconstruction (TBR) as the branch-swapping algorithm; alignment gaps were treated as missing (combined alignment) or as a fifth character state (ITS alignment) and all characters were unordered and of equal weight. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Other measures calculated included tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC, respectively). The robustness of the obtained trees was evaluated by 10 000 000 fast stepwise (ITS alignment) or 1000 bootstrap heuristic bootstrap replications (combined alignment). Sequences were deposited in GenBank (Table 1) and the alignments in TreeBASE ([www.treebase.org](http://www.treebase.org)).

### Morphology

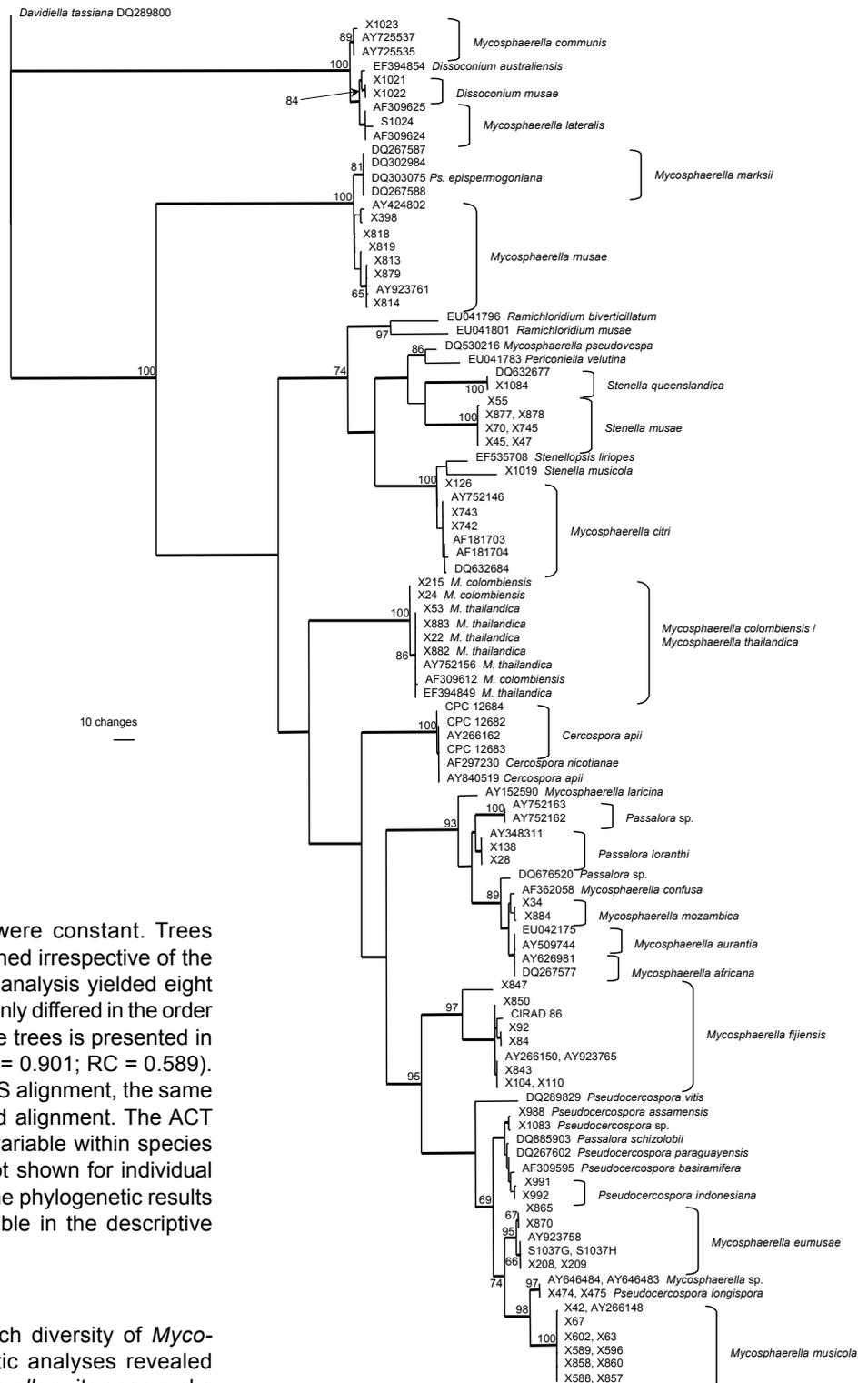
Growth rates and colony morphology were recorded from colonies grown on MEA plates after 30 d incubation in darkness at 24 °C. Colony colours (surface and reverse) were assessed after growth on MEA and oatmeal agar (OA, Gams et al. 2007) using the colour charts of Rayner (1970). Microscopic observations were made from colonies cultivated on MEA and OA. Preparations were mounted in lactic acid and studied under a light microscope ( $\times$  1000 magnification). The 95 % confidence intervals were derived from 30 observations of spores formed on MEA or OA, with extremes given in parentheses. All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands or the working collections of Pedro Crous (CPC) or Mahdi Arzanlou (X, S numbers) at CBS (Table 1). Nomenclatural novelties and descriptions were deposited in MycoBank ([www.MycoBank.org](http://www.MycoBank.org)) (Crous et al. 2004a).

## RESULTS

### DNA phylogeny

Two alignments of DNA sequences were subjected to phylogenetic analyses. The first alignment consisted of ITS sequences generated in this study as well as sequences obtained from the NCBI GenBank nucleotide sequence database. The ITS alignment consisted of a total number of 113 sequences (including one outgroup); 508 characters including alignment gaps were subjected to the analyses. Of these characters, 224 were parsimony-informative, 42 variable and parsimony-uninformative, and 242 were constant. Trees supporting the same clades were obtained irrespective of the analysis method used. The parsimony analysis yielded 11 780 equally most parsimonious trees that mainly differed in the order of taxa at the terminal nodes; one of the trees is presented in Fig. 1 (TL = 861 steps; CI = 0.569; RI = 0.934; RC = 0.532).

The sequence data in the second alignment were analysed as one combined set consisting of 1648 characters (incl. alignment gaps) (number of included characters: ITS: 509, ACT: 188, HIS: 375, mtSSU: 576). This second alignment included 54 sequences (including the outgroup) and of the 1648 characters, 517 were parsimony-informative, 93 were variable and

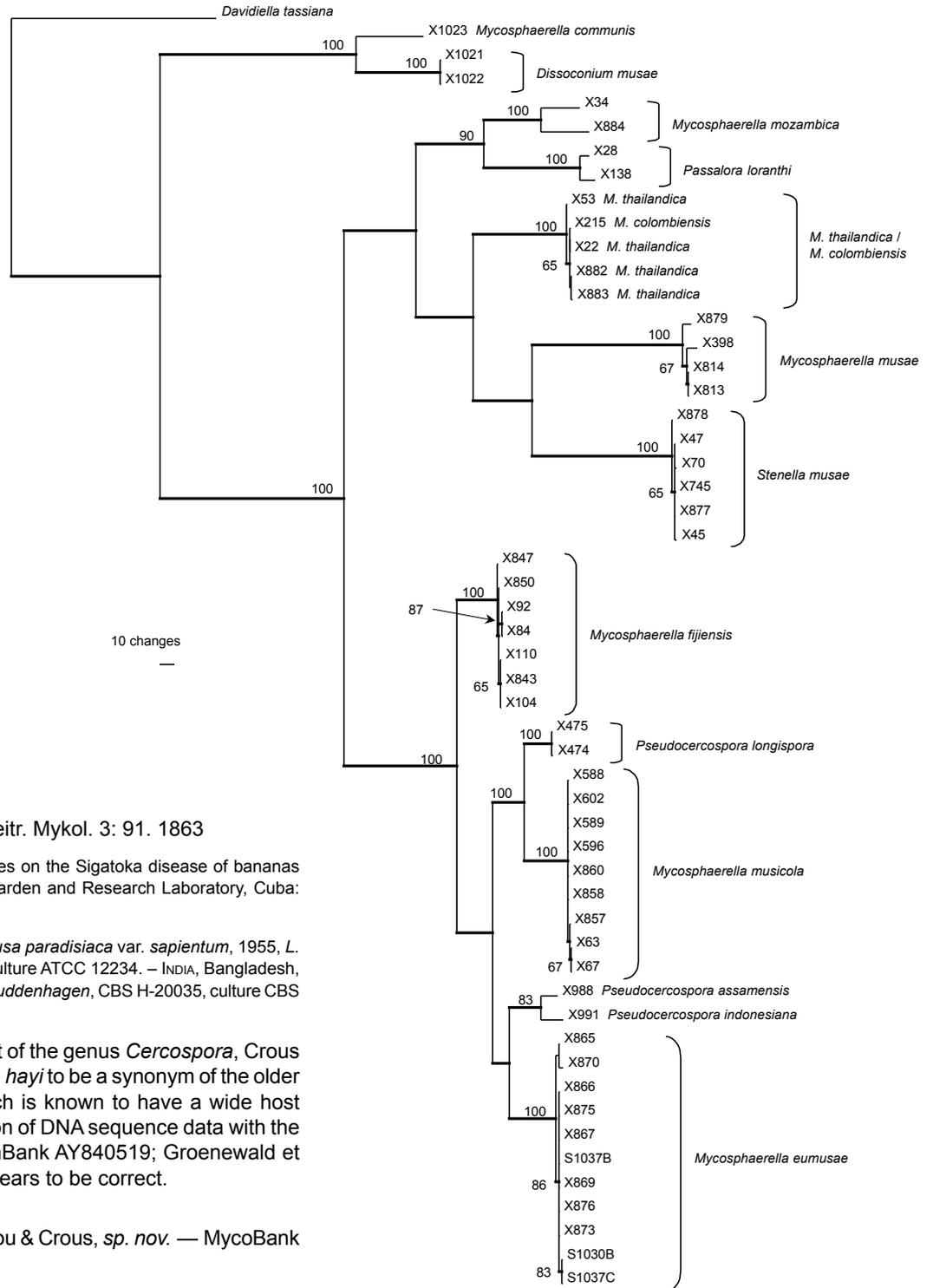


parsimony-uninformative, and 1038 were constant. Trees supporting the same clades were obtained irrespective of the analysis method used. The parsimony analysis yielded eight equally most parsimonious trees that mainly differed in the order of taxa at the terminal nodes; one of the trees is presented in Fig. 2 (TL = 1513 steps; CI = 0.654; RI = 0.901; RC = 0.589). Similar to the results obtained for the ITS alignment, the same lineages were found with the combined alignment. The ACT and HIS data were found to be more variable within species than the ITS and mtSSU data (data not shown for individual loci, variation within clades in Fig. 2). The phylogenetic results obtained are discussed where applicable in the descriptive notes below.

### Taxonomy

The results of this study showed a rich diversity of *Mycosphaerella* spp. on *Musa*. Phylogenetic analyses revealed that more than 20 species of *Mycosphaerella* or its anamorphs occur on banana, including species known from hosts other than banana, namely *Cercospora apii*, *Mycosphaerella citri*, *M. communis*, *M. lateralis*, *M. thailandica*, and *Passalora loranthi* (Fig. 1). Furthermore, eight species proved to be morphologically and phylogenetically distinct from the species presently known from banana. These new species are described below.

**Fig. 1** One of 11 780 equally most parsimonious trees obtained from a heuristic search with simple taxon additions of the ITS sequence alignment. The scale bar shows 10 changes, and bootstrap support values (65 % and higher) from 10 000 000 fast stepwise replicates are shown at the nodes. Thickened lines indicate the strict consensus branches. The tree was rooted to sequences of *Davidiella tassiana* strain CPC 11600 (GenBank accession number DQ289800). *M.* = *Mycosphaerella* and *Ps.* = *Pseudocercospora*.



**Fig. 2** One of eight equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined (ITS, ACT, HIS, mtSSU) sequence alignment. The scale bar shows 10 changes, and bootstrap support values (65 % and higher) from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches. The tree was rooted to sequences of *Davidiella tassiana* strain CPC 11600 (GenBank accession number DQ289800, DQ289867, EF679665, EU514455, respectively). *M.* = *Mycosphaerella*.

***Cercospora apii* Fresen., Beitr. Mykol. 3: 91. 1863**

= *Cercospora hayi* Calp., Studies on the Sigatoka disease of bananas and its fungus pathogen, Atkins Garden and Research Laboratory, Cuba: 63. 1955.

*Specimens examined.* CUBA, *Musa paradisiaca* var. *sapientum*, 1955, L. Calpouzos, holotype FH, ex-type culture ATCC 12234. – INDIA, Bangladesh, *Musa* cv. Cavendish, Oct. 2005, I. Buddenhagen, CBS H-20035, culture CBS 119395.

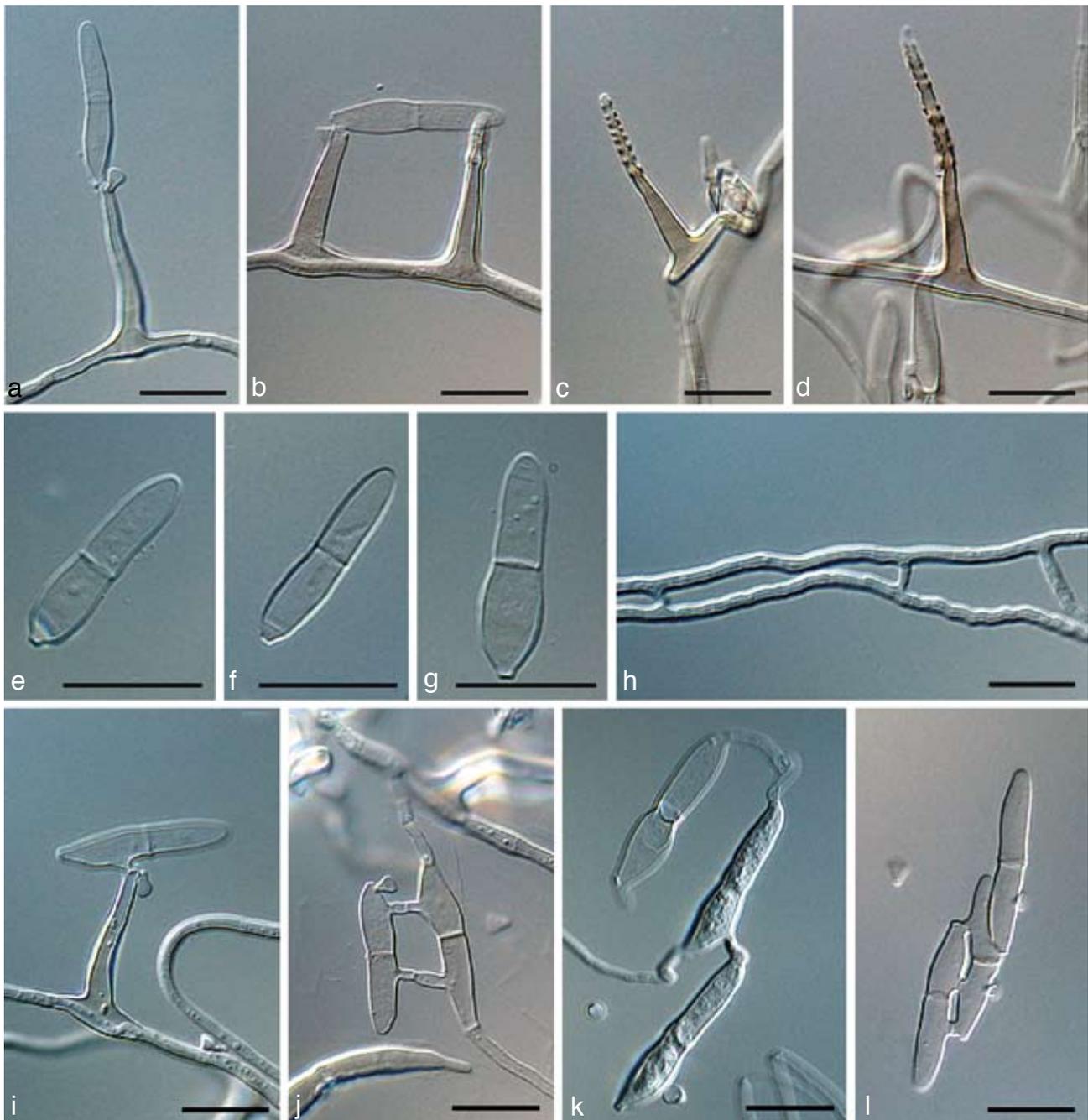
**Notes** — In their treatment of the genus *Cercospora*, Crous & Braun (2003) considered *C. hayi* to be a synonym of the older name, *Cercospora apii*, which is known to have a wide host range. Based on a comparison of DNA sequence data with the ex-type strain of *C. apii* (GenBank AY840519; Groenewald et al. 2006), this synonymy appears to be correct.

***Dissoconium musae* Arzanlou & Crous, sp. nov. — MycoBank MB505972; Fig. 3, 4**

*Dissoconio communi* simile, sed coloniis in vitro tarde crescentibus (usque ad 10 mm diam post 30 dies ad 24 °C in agar maltoso).

**Etymology.** Named after its host plant, *Musa*.

In vitro on MEA: *Mycelium* submerged and superficial; submerged hyphae hyaline to subhyaline, thin-walled, smooth, forming a dense network with numerous anastomoses, 2–3 µm wide; aerial hyphae subhyaline, smooth, 2–3 µm wide.



**Fig. 3** *Dissoconium musae* (CBS 122453). a–d. Conidiophores with sympodially proliferating conidiogenous cells, which produce primary and secondary conidia in pairs; e–g. primary conidia with truncate base; h–l. anastomoses between hyphae, primary and secondary conidia and primary conidia. — Scale bar = 10  $\mu$ m.

*Conidiophores* arising orthotropically from vegetative hyphae, often reduced to conidiogenous cells and continuous with supporting hyphae, thin-walled, smooth, pale brown, unbranched, straight, subulate to lageniform, tapering towards the apex,  $(10\text{--}19\text{--}25\text{--}53) \times (2.5\text{--}3\text{--}5)$   $\mu$ m. *Conidiogenous cells* terminal, proliferating sympodially (but appearing as annellides under the light microscope), giving rise to a short conidium-bearing rachis, loci somewhat darkened and thickened. *Conidia* form-

ing in sympodial order in pairs on a conidiogenous cell; the primary conidium is 2-celled, while the secondary conidium is aseptate; primary conidia pale olivaceous-brown, thin-walled, smooth, ellipsoidal to obclavate, 1-septate, apex obtuse, base obconically-truncate,  $(11\text{--}22\text{--}26\text{--}35) \times (3\text{--}4\text{--}5)$   $\mu$ m, hilum unthickened; about 1  $\mu$ m diam. Secondary conidia 1-celled, pale olivaceous-brown, pyriform to turbinate,  $4\text{--}5 \times 3\text{--}4$   $\mu$ m, base truncate, flat, unthickened, about 0.5  $\mu$ m diam. Both conidial

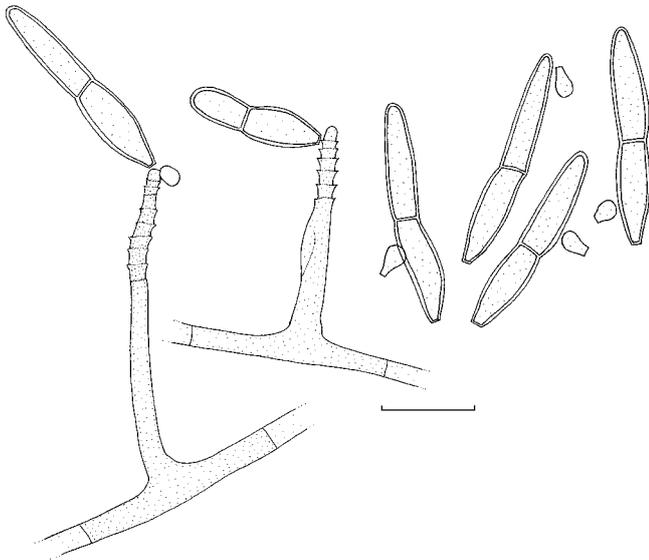


Fig. 4 *Dissoconium musae* (CBS 122453). — Scale bar = 10  $\mu$ m.

types are discharged forcibly in pairs and then anastomose on the agar surface. Anastomosis between primary conidia occurs as well and primary conidia may show multiple anastomoses. Primary conidia germinate from both ends and produce several conidiogenous cells and conidia (microcyclic conidiation). Germination of secondary conidia was not observed.

**Cultural characteristics** — Colonies on MEA slow-growing, reaching 10 mm diam after 30 d at 24 °C, erumpent, unevenly folded, with sparse aerial mycelium, colonies with granulate margin; surface hazel to isabelline in centre, and vinaceous-buff in outer region; brown-vinaceous in reverse. Colonies on OA reaching 25 mm diam after 30 d at 24 °C, effuse, with moderate aerial mycelium, later become powdery in centre, surface hazel; olivaceous in reverse.

**Specimen examined.** INDIA, Tamil Nadu, Tiruchirapally, *Musa* cv. Nendran (Plantain) AAB, 2005, I. Buddenhagen, holotype CBS H-20036, culture ex-type X1021 = CBS 122453.

**Notes** — The genus *Dissoconium* is characterised by producing pairs of forcibly discharged primary and secondary conidia on sympodially proliferating conidiogenous cells. Sympodial proliferation of the conidiogenous cells gives rise to a conidium-bearing rachis, which resembles that encountered in the genus *Ramichloridium*. The recent revision of the genus *Ramichloridium* and allied genera (Arzanlou et al. 2007b) revealed that *R. apiculatum*, the type species of the genus, is phylogenetically close to the species in the genus *Dissoconium*. However, *Dissoconium* is morphologically distinct from *Ramichloridium* by producing two types of forcibly discharged conidia. So far, seven species of *Dissoconium* have been described from different substrates (de Hoog et al. 1991, Jackson et al. 2004). *Dissoconium musae* is phylogenetically distinct from the other species of this genus, but morphologically similar to *D. commune* and *D. dekkeri* (teleomorph: *Mycosphaerella lateralis*), from which it differs based on its slower growth rate in culture.

***Mycosphaerella eumusae* Crous & Mour., Sydowia 54: 36. 2002**

**Anamorph.** *Pseudocercospora eumusae* Crous & Mour., Sydowia 54: 36. 2002.

**Specimen examined.** REUNION, on leaves of *Musa* sp., 2001, J. Carlier, PREM 57314 (holotype of teleomorph), PREM 57315 (holotype of anamorph), cultures ex-type (CIRAD 1156, 1157 = CPC 4579, 4580 = CBS 114824, CBS 114825).

**Notes** — Based on the DNA sequence data obtained in this study (Fig. 2), it appears that *M. eumusae* is heterogeneous as presently circumscribed. Further studies would be required to determine if the phylogenetic variation also correlates with differences in morphology.

***Mycosphaerella fijiensis* M. Morelet, Ann. Soc. Sci. Nat. Archéol. Toulon Var 21: 105. 1969**

= *Mycosphaerella fijiensis* var. *difformis* J.L. Mulder & R.H. Stover, Trans. Brit. Mycol. Soc. 67: 82. 1976.

**Anamorph.** *Pseudocercospora fijiensis* (M. Morelet) Deighton, Mycol. Pap. 140: 144. 1976.

**Basionym.** *Cercospora fijiensis* M. Morelet, Ann. Soc. Sci. Nat. Archéol. Toulon Var 21: 105. 1969.

= *Paracercospora fijiensis* (M. Morelet) Deighton, Mycol. Pap. 144: 51. 1979.

= *Cercospora fijiensis* var. *difformis* J.L. Mulder & R.H. Stover, Trans. Brit. Mycol. Soc. 67: 82. 1976.

= *Paracercospora fijiensis* var. *difformis* (J.L. Mulder & R.H. Stover) Deighton, Mycol. Pap. 144: 52. 1979.

**Specimens examined.** HAWAII, on leaves of *Musa* sp., D.S. Meredith & J.S. Lawrence, holotype IMI 136696. — CAMEROON, date and collector unknown, epitype designated here CBS H-20037, culture ex-epitype CIRAD 86 = CBS 120258.

**Note** — The specimen and associated strain designated here as epitype, represent the strain that was selected by the *Mycosphaerella* consortium to obtain the full genome sequence of *M. fijiensis* ([www.jgi.doe.gov/sequencing/why/CSP2006/mycosphaerella.html](http://www.jgi.doe.gov/sequencing/why/CSP2006/mycosphaerella.html)).

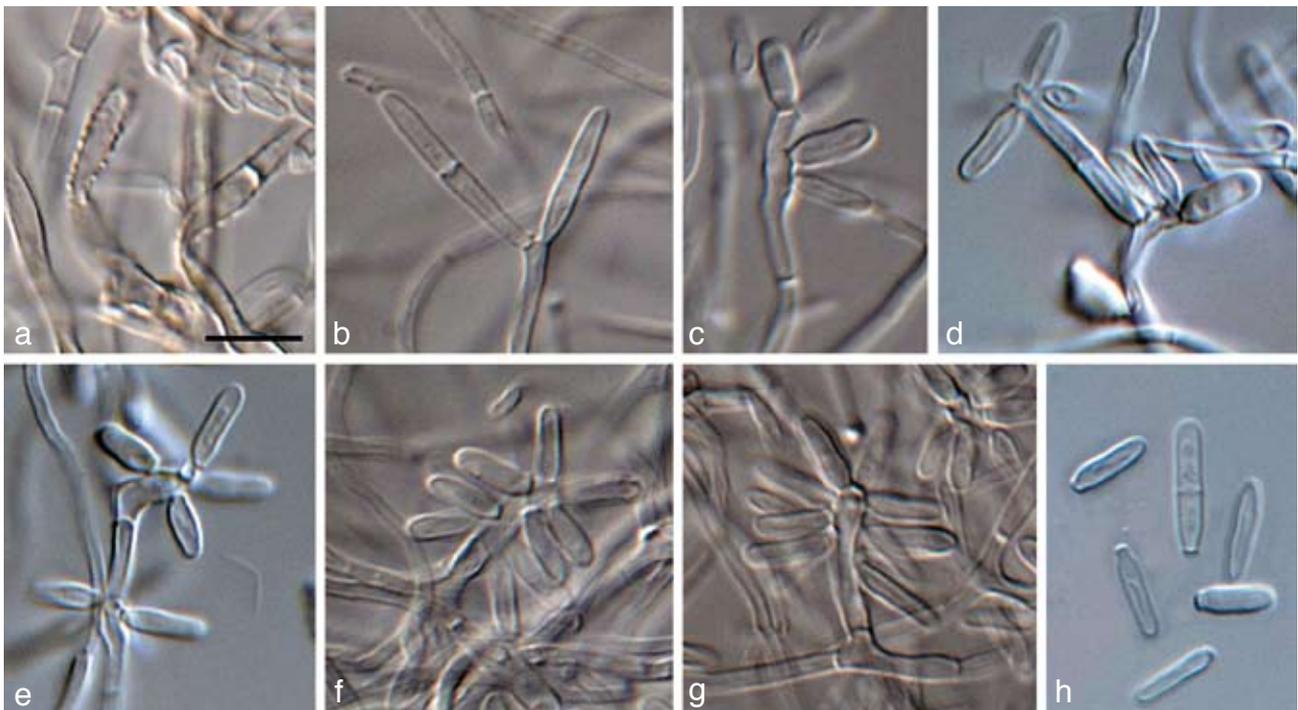
***Mycosphaerella mozambica* Arzanlou & Crous, sp. nov. — MycoBank MB505973; Fig. 5, 6**

**Anamorph.** *Ramichloridium*-like.

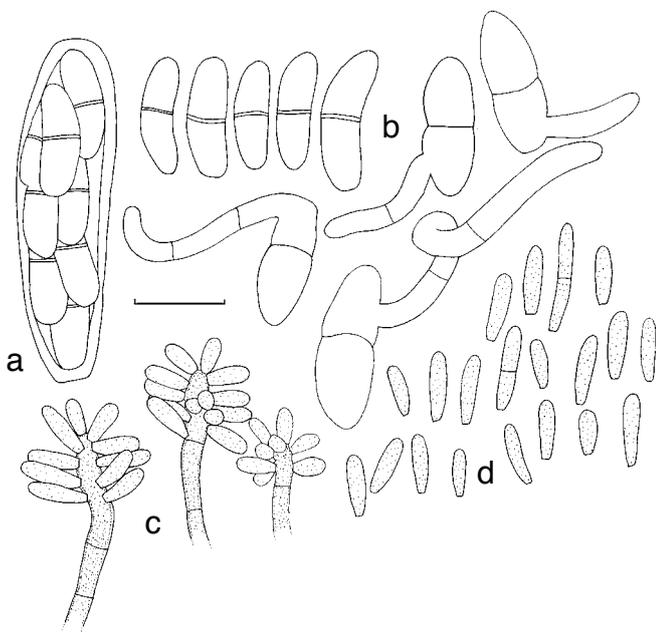
*Ascosporae* rectae vel curvatae, fusoido-ellipsoideae utrinque obtusae, ad septum medianum vix constrictae, (9–)10–11(–12)  $\times$  3–3.5(–4)  $\mu$ m.

**Etymology.** Named after the country of origin, Mozambique.

**In vivo:** *Leaf spots* amphigenous, irregular to subcircular, 1–7 mm diam, grey to pale brown on adaxial surface, grey on abaxial surface, with dark brown margins. *Ascomata* amphigenous, intermingled among those of *M. musicola*, dark brown, subepidermal, becoming erumpent, globose, 70–90  $\mu$ m diam; wall consisting of 2–3 layers of medium brown textura angularis. *Asci* paraphysate, fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoid, straight to slightly curved, 8-spored, 28–35  $\times$  7–9  $\mu$ m. *Ascospores* bi- to tri-seriate, overlapping, hyaline, non-guttulate, thin-walled, straight to curved, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, medianly 1-septate, not to slightly constricted at the septum, tapering



**Fig. 5** *Mycosphaerella mozambica* (CBS 122464). a. Verruculose hyphae; b–e. unbranched or loosely branched conidiophores with sympodially proliferating conidiogenous cells; f–g. sympodially proliferating conidiogenous cells give rise to short conidium-bearing rachis; h. conidia with truncate base. — Scale bars = 10  $\mu$ m.



**Fig. 6** *Mycosphaerella mozambica* (CBS 122464). a. Ascus with biseriolate ascospores; b. ascospore germination pattern; c. conidiophores with sympodially proliferating conidiogenous cells, which give rise to short conidium-bearing rachis; d. conidia. — Scale bar = 10  $\mu$ m.

towards both ends, but more prominently towards the lower end, (9–)10–11(–12)  $\times$  3–3.5(–4)  $\mu$ m; ascospores becoming distorted upon germination after 24 h on MEA, becoming constricted at the septum, 6–7  $\mu$ m wide with irregular, wavy germ tubes, growing 90° to the long axis, and not arising from the polar ends of the spore.

In vitro on MEA: *Mycelium* submerged and superficial; submerged hyphae hyaline to subhyaline, thin-walled, smooth or slightly rough, 2–4  $\mu$ m wide; aerial hyphae pale olivaceous, smooth or finely verruculose. *Conidiophores* arising from unbranched or loosely branched hyphae, occasionally reduced to conidiogenous cells or integrated, hyaline, subcylindrical, 2–2.5  $\mu$ m wide and up to 35  $\mu$ m long. *Conidiogenous cells* integrated, terminal, polyblastic, sympodial, loci aggregated, flat, not protuberant (not denticle-like), unthickened, but somewhat darkened. *Conidia* solitary, obovoid, ellipsoidal, obclavate 0(–1)-septate, hyaline, thin-walled, smooth, (5–)9–12(–22)  $\times$  2–2.5(–3)  $\mu$ m; hilum truncate, flat, broad, unthickened, slightly darkened, about 1  $\mu$ m diam. Although rarely observed, older conidia can become elongated, obclavate, and up to 4-septate.

Cultural characteristics — Colonies on MEA reaching 45 mm diam after 30 d at 24 °C; erumpent, folded, with moderate velvety to hairy aerial mycelium, with smooth, entire margins; surface pale vinaceous to mouse-grey; brown-vinaceous in reverse. Colonies on OA reaching 51 mm diam after 30 d at 24 °C; effuse, with sparse aerial mycelium and entire edge; surface vinaceous-buff to vinaceous, and pale vinaceous in reverse.

*Specimens examined.* MOZAMBIQUE, Chimoio, Bairro, on leaf of *Musa* cv. 2003, A. Viljoen, holotype CBS H-20039, culture ex-type X34 = CBS 122464; CBS H-20040, CBS H-20041, CBS H-20042.

**Notes** — Sympodially proliferating conidiogenous cells are somewhat confusing with other morphologically similar genera such as *Ramichloridium* and *Veronaea*. The type species and most of the taxa referred to these genera are dematiaceous. The scars in *Ramichloridium* are subhyaline and slightly prominent. *Veronaea* has pigmented, truncate, flat loci and conidia with truncate bases. A recent revision of *Ramichloridium* and allied genera (Arzanlou et al. 2007b) revealed the type species of *Ramichloridium*, *R. apiculatum*, to be allied to the *Dissoconium* clade in Capnodiales, while the type species of *Veronaea*, *V. botryosa*, resides in Chaetothiales. *Mycosphaerella mozambica* appeared to occur quite commonly on the banana samples investigated from Mozambique. Based on DNA sequence data, the ex-type strain appears similar to an isolate collected in Australia (CBS 121391 = X884). Unfortunately, however, the latter strain was sterile, so this could not be confirmed based on morphology.

***Mycosphaerella musae*** (Speg.) Syd. & P. Syd., Philipp. J. Sci., C 8: 482. 1913

*Basionym.* *Sphaerella musae* Speg., Anales Mus. Nac. Hist. Nat. Buenos Aires 19: 354. 1909.

= *Sphaerella musae* Sacc., Atti Accad. Sci. Veneto-Trentino-Istriana, Ser. 3, 10: 67. 1917, homonym.

*Specimen examined.* ARGENTINA, Jujuy, Orán, on leaves of *Musa sapientum*, Mar. 1905, holotype LPS, slide ex-type IMI 91165.

**Notes** — *Mycosphaerella musae* is reported to be the causal organism of *Mycosphaerella* speckle disease. However, as shown in the present study (Fig. 1), several distinct species appear to be able to induce these symptoms. Further collections would thus be required to recollect this species. All cultures examined in the present study were sterile.

***Mycosphaerella musicola*** R. Leach ex J.L. Mulder, Trans. Brit. Mycol. Soc. 67: 77. 1976

*Basionym.* *Mycosphaerella musicola* R. Leach, Trop. Agric. (Trinidad) 18: 92. 1941 (nom. nud.).

*Anamorph.* *Pseudocercospora musae* (Zimm.) Deighton, Mycol. Pap. 140: 148. 1976.

*Basionym.* *Cercospora musae* Zimm., Centralbl. Bakteriol. Parasitenk. 2. Abt. 8: 219. 1902.

= *Cercospora musae* Massee, Bull. Misc. Inform. Kew 28: 159. 1914.

*Specimens examined.* JAMAICA, on leaves of *Musa sapientum*, Jan. 1959, R. Leach, holotype IMI 75804a. — CUBA, on leaves of *Musa* sp., epitype designated here CBS H-20038, culture ex-epitype IMI 123823 = CBS 116634.

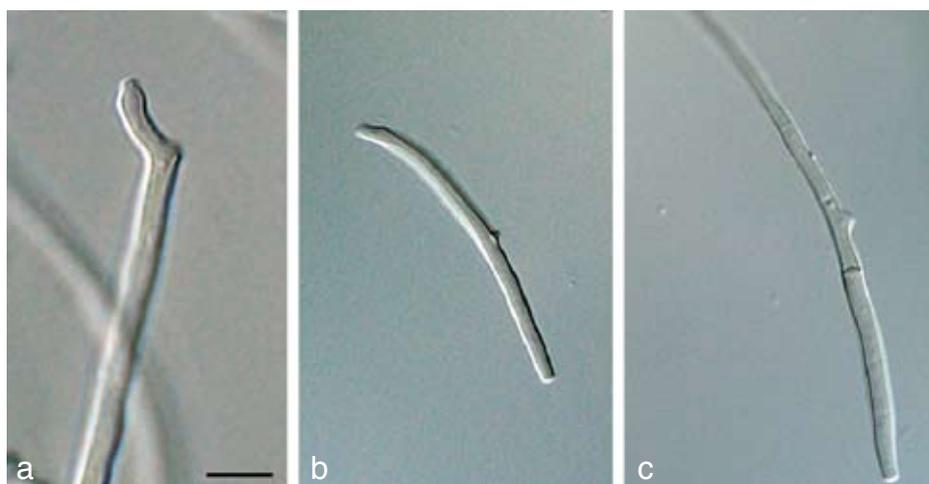
***Pseudocercospora assamensis*** Arzanlou & Crous, *sp. nov.*  
— MycoBank MB505974; Fig. 7, 8

*Pseudocercosporae musae* similis, sed conidiis longioribus et angustioribus, (30–)59–70(–83) × 2–3 µm.

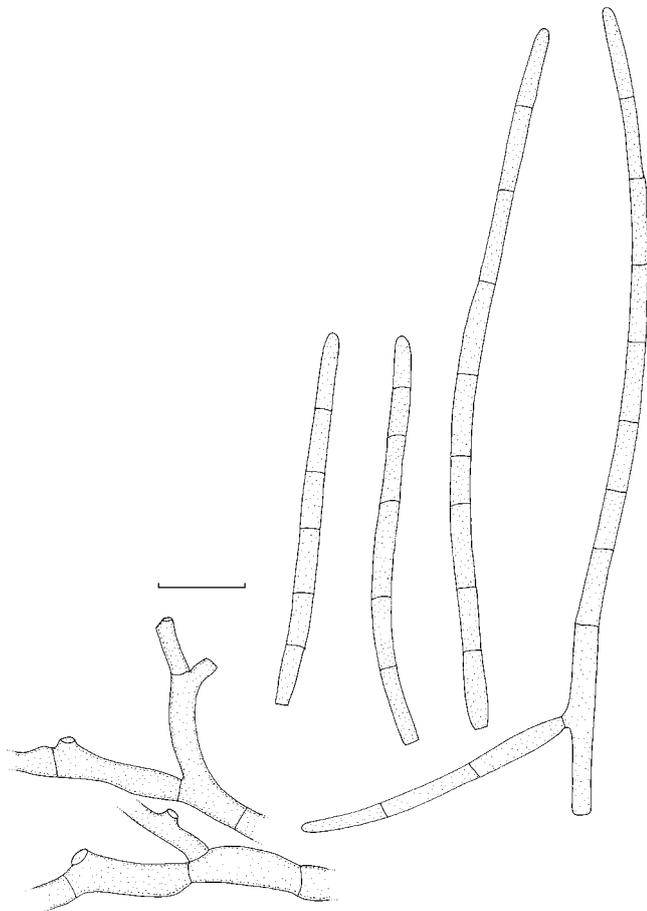
*Etymology.* Named after the locality of origin, India, Assam.

In vitro on MEA: *Mycelium* submerged and superficial; submerged hyphae smooth, branched, septate, medium brown, 2.5–4 µm wide; aerial hyphae thin-walled, smooth, medium brown. *Conidiophores* solitary, arising from superficial hyphae, medium brown, thin-walled, smooth, unbranched or branched above, 0–1-septate, subcylindrical, straight, up to 20 µm long, 2–3 µm wide. *Conidiogenous cells* integrated, terminal, or conidiophores reduced to conidiogenous cells, subcylindrical, tapering to truncate or bluntly rounded apices, medium brown, smooth, proliferating sympodially; conidial scars inconspicuous. *Conidia* solitary, pale brown, smooth, subcylindrical, with truncate bases and bluntly rounded apices, thin-walled with irregular swellings in older conidia, straight or curved, pluri-septate, (30–)59–70(–83) × 2–3 µm; hila about 1 µm wide, neither thickened nor darkened-refractive; microcyclic conidiation observed.

**Cultural characteristics** — Colonies on MEA reaching 47 mm diam after 30 d at 24 °C. Colonies elevated at the centre, with



**Fig. 7** *Pseudocercospora assamensis* (CBS 122467). a. Conidiophore with sympodial and percurrent growth of conidiogenous cell; b–c. conidia. — Scale bar = 10 µm.



**Fig. 8** *Pseudocercospora assamensis* (CBS 122467). — Scale bar = 10  $\mu$ m.

abundant aerial mycelium, and entire, smooth margin; surface pale mouse-grey to mouse-grey, olivaceous in reverse. Colonies on OA reaching 35 mm diam after 30 d at 24 °C; effuse, with moderate, velvety aerial mycelium, and entire, smooth margins; surface pale mouse-grey, and iron-grey in reverse.

*Specimen examined.* INDIA, Assam, Naojan, on leaf of *Musa* cv. Nanderan (Plantain), 2005, I. Buddenhagen, holotype CBS H-20044, culture ex-type X988 = CBS 122467.

*Notes* — Based on its characteristic conidial shape and dimensions, *P. assamensis* appears distinct from those species presently known from this host. *Pseudocercospora musae* conidia are shorter and above all wider (10–80  $\times$  2–6  $\mu$ m; Carlier et al. 2000) than in *P. longispora*. *Pseudocercospora longispora* has much longer and somewhat wider conidia.

***Pseudocercospora indonesiana*** Arzanlou & Crous, *sp. nov.*  
 — MycoBank MB505975; Fig. 9, 10

*Pseudocercosporae longisporae* similis, sed conidiis modice brunneis, hyphis tenuitunicatis, modice brunneis, non inflatis et non monilioidibus-muriformibus, coloniis in vitro celeriter crescentibus (usque ad 27 mm diam post 30 dies ad 24 °C in agar maltoso).

*Etymology.* Named after its country of origin, Indonesia.

In vitro on MEA: *Mycelium* submerged and superficial; submerged hyphae thin-walled, smooth, branched, septate, medium brown, 2.5–4  $\mu$ m wide; aerial hyphae, thin-walled, smooth, medium brown. *Conidiophores* solitary, arising from superficial hyphae, medium brown, smooth, unbranched, 0–2-septate, subcylindrical, straight, up to 30  $\mu$ m long, 2–2.5  $\mu$ m wide. *Conidiogenous cells* integrated, terminal, subcylindrical, tapering to truncate or bluntly rounded apices, medium brown, smooth, proliferating sympodially, frequently reduced to conidiogenous loci; conidial scars inconspicuous. *Conidia* solitary, pale brown, smooth, subcylindrical, bases truncate, apices bluntly rounded, thin-walled, straight or curved, guttulate, 3–7-septate,



**Fig. 9** *Pseudocercospora indonesiana* (CBS 122473). a–d. Conidia; e. intercalary conidiogenous cell. — Scale bar = 10  $\mu$ m.

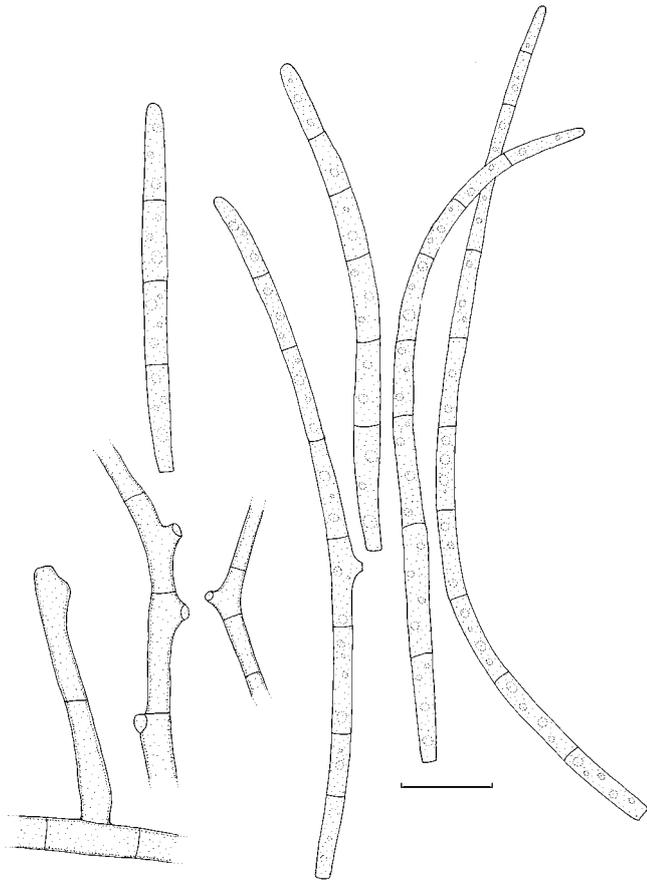


Fig. 10 *Pseudocercospora indonesiana* (CBS 122473). — Scale bar = 10  $\mu$ m.

(40–)78–95(–120)  $\times$  2–3  $\mu$ m; hila unthickened, neither darkened nor refractive.

Cultural characteristics — Colonies on MEA reaching 27 mm diam after 30 d at 24 °C. Colonies low convex, with abundant aerial mycelium, and entire, smooth margin; surface pale mouse-grey to mouse-grey; in reverse dark mouse-grey. Colonies on OA reaching 35 mm diam after 47 d at 24 °C; effuse, with moderate aerial mycelium, and entire, smooth margins; surface pale mouse-grey; in reverse olivaceous-black.

*Specimen examined.* INDONESIA, Western Sumatra, Kumango, on leaf of *Musa* cv. Buai, 2004, I. Buddenhagen, holotype CBS H-20045, culture ex-type X992 = CBS 122473.

Notes — *Pseudocercospora indonesiana* is phylogenetically distinct from the other species of *Pseudocercospora* occurring on *Musa*. Morphologically it has longer conidia than *P. musae* (teleomorph *M. musicola*) and *P. assamensis*, though they are very similar to those of *P. longispora*; it can, however, be distinguished from the latter by having medium brown conidia (those of *P. longispora* being pale brown), and its faster growth rate on MEA and OA.

***Pseudocercospora longispora*** Arzanlou & Crous, sp. nov.  
— MycoBank MB505976; Fig. 11, 12

*Pseudocercosporae musae* similis, sed conidiis longioribus, 82–120  $\times$  2.5–4  $\mu$ m.

*Etymology.* Named after its characteristically long conidia.

In vitro on OA: *Mycelium* submerged and superficial; submerged hyphae smooth, branched, septate, medium brown, thin-walled, 2–3  $\mu$ m wide; aerial hyphae smooth, medium brown; hyphal cells become thick-walled, swollen, forming dark-brown moniloid, muriform cells, 5–17  $\times$  7–12  $\mu$ m. *Conidiophores* solitary, arising from superficial hyphae; conidiophores medium brown, smooth, unbranched or branched above, 0–2-septate, subcylindrical, straight, up to 30  $\mu$ m long, 2–3  $\mu$ m wide. *Conidiogenous cells* integrated, terminal, subcylindrical, tapering to truncate or

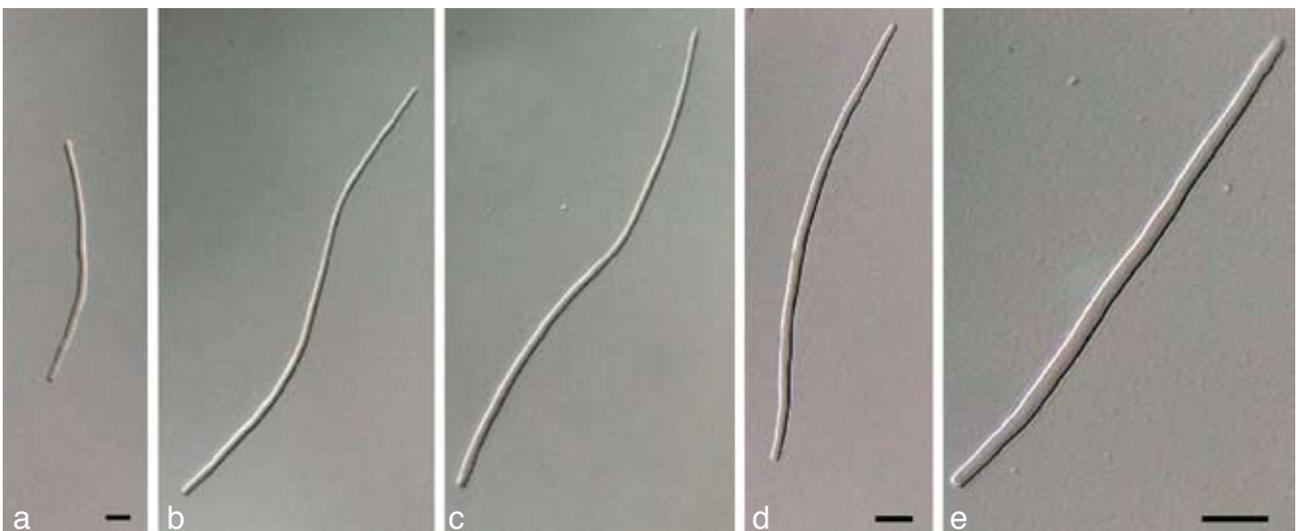
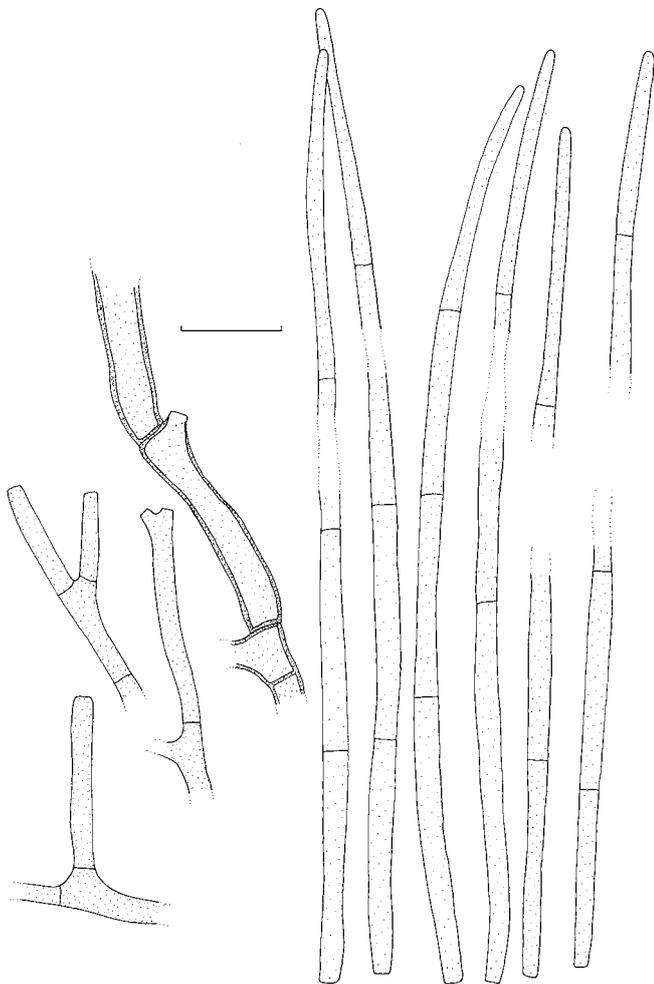


Fig. 11 *Pseudocercospora longispora* (CBS 122469). a–e. Conidia. — Scale bar = 10  $\mu$ m.



**Fig. 12** *Pseudocercospora longispora* (CBS 122469). — Scale bar = 10  $\mu$ m.

bluntly rounded apices, medium brown, smooth, forming conidia by sympodial proliferation, rarely by means of percurrent proliferation; conidial scars inconspicuous. *Conidia* solitary, pale brown, thin-walled, smooth, cylindrical to subcylindrical, widest in the middle of conidium, tapering towards the apex, bases truncate, straight, multi-septate, 82–120  $\times$  2.5–4  $\mu$ m; hila about 1  $\mu$ m diam, neither thickened nor darkened-refractive.

Cultural characteristics — Colonies reaching 15 mm diam after 30 d at 24 °C. Colonies erumpent, with moderate aerial mycelium, and entire, smooth edges; surface buff to rosy-buff, mouse-grey to dark grey; in reverse dark mouse-grey. Colonies on OA reaching 15 mm diam after 30 d at 24 °C, effuse, with abundant aerial mycelium, and entire, smooth margins; surface pale mouse-grey; in reverse dark mouse-grey.

*Specimen examined.* MALAYSIA, Felcra Plantation, Melaka, *Musa* cv. Pisang Byok AAA/AAB, July 1988, D.R. Jones, holotype CBS H-20043, culture ex-type X475 = CBS 122470.

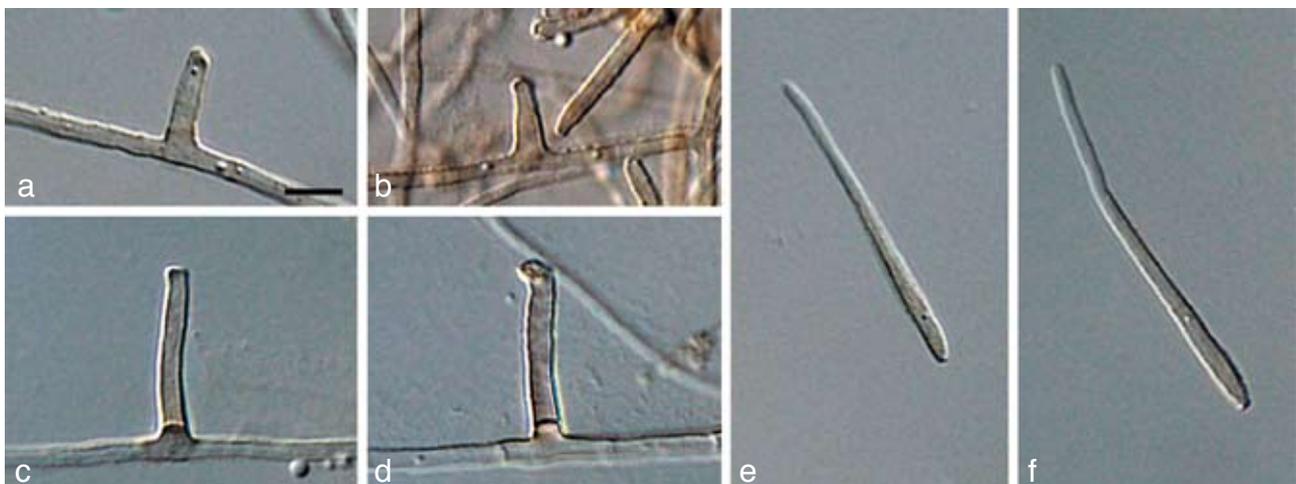
*Notes* — *Pseudocercospora longispora* resembles *P. musae* (teleomorph *Mycosphaerella musicola*) in its colony morphology on MEA and OA. However, in *P. musae* conidia are much shorter (10–80  $\times$  2–6  $\mu$ m; Carlier et al. 2000) than in *P. longispora*.

***Stenella musae*** Arzanlou & Crous, *sp. nov.* — MycoBank MB505977; Fig. 13, 14a

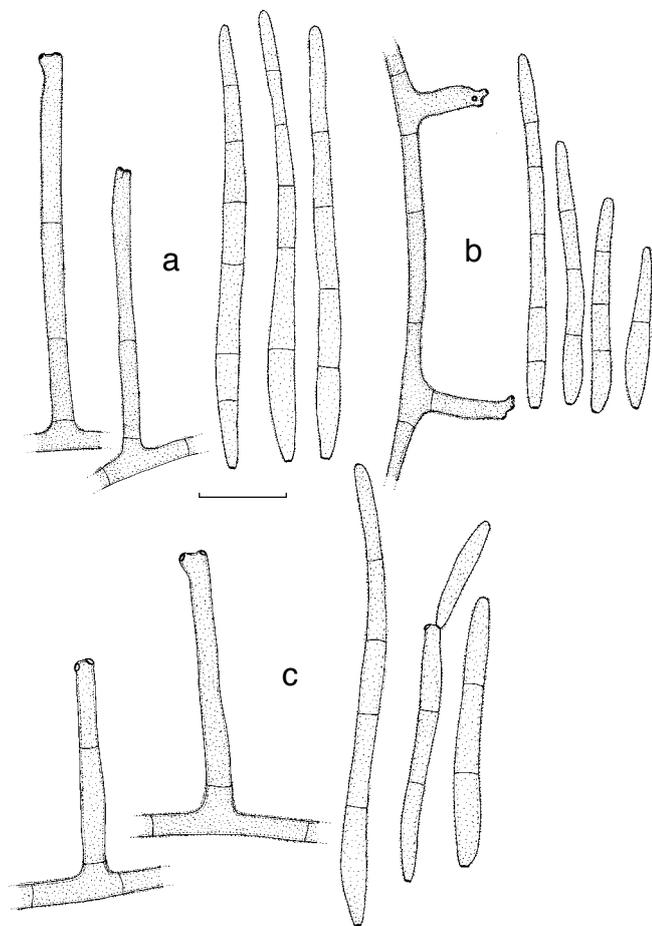
Conidiophora ex hyphis superficialibus oriunda, modice brunnea, tenuitunicata, verruculosa vel verrucosa, 0–3-septata, subcylindrica, recta vel geniculata-sinuosa, non ramosa, ad 30  $\mu$ m longa et 2–2.5  $\mu$ m lata. Cellulae conidiogenae integratae, terminales, interdum intercalares, modice brunneae, verruculosae, subcylindricae, apicem versus attenuatae, sympodiales, locis truncatis, subdenticulatis, 1–1.5  $\mu$ m diam, inspissatis et fuscatis-refringentibus praeditae. Conidia solitaria, dilute brunnea, verruculosa, tenuitunicata, subcylindrica vel obclavata, recta vel curvata, 0–7-septata, (7–)27–40(–70)  $\times$  1.5–3  $\mu$ m, hilo inspissato obscuriore refringente, 1–1.5  $\mu$ m diam praedita.

*Etymology.* Named after its host, *Musa*.

In vitro on MEA: *Mycelium* submerged and superficial; submerged hyphae smooth to verrucose, thin-walled, subhyaline to medium brown, 2–3  $\mu$ m wide, with thin septa; aerial hyphae coarsely verrucose, olivaceous-brown to medium brown, rather



**Fig. 13** *Stenella musae* (CBS 122477). a–d. Conidiophores with sympodially proliferating conidiogenous cells; e–f. conidia. — Scale bar = 10  $\mu$ m.



**Fig. 14** a. *Stenella musae* (CBS 122477); b. *Stenella queenslandica* (CBS 122475); c. *Stenella musicola* (CBS 122479). — Scale bar = 10  $\mu$ m.

thick-walled, 2–2.5  $\mu$ m wide, with thin septa. *Conidiophores* arising from superficial hyphae, medium brown, rather thick-walled, finely verrucose to verruculose, 0–3-septate, subcylindrical, straight to geniculate-sinuous, unbranched, up to 30  $\mu$ m long, 2–2.5  $\mu$ m wide. *Conidiogenous cells* integrated, terminal, sometimes intercalary, unbranched, medium brown, finely verruculose, subcylindrical, tapering towards flat-tipped, subdentate apical loci, 1–1.5  $\mu$ m diam, proliferating sympodially; loci thickened, darkened, refractive. *Conidia* solitary, thin-walled, pale brown, finely verrucose, subcylindrical to obclavate, with subobtuse apex, and long obconically subtruncate

to obconically subtruncate base, straight to curved, 0–7-septate, (7–)27–40(–70)  $\times$  1.5–3  $\mu$ m; hilum thickened, darkened, refractive, 1–1.5  $\mu$ m diam.

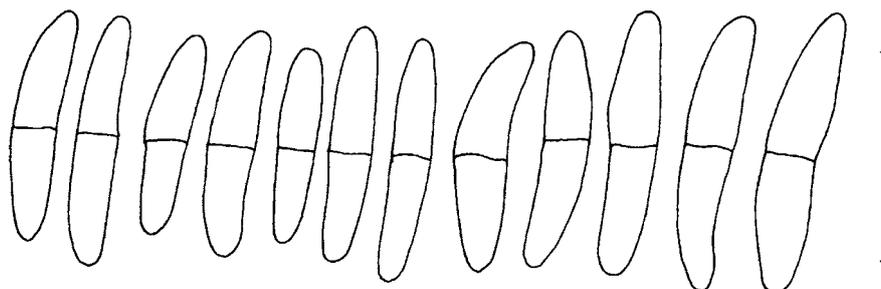
**Cultural characteristics** — Colonies on MEA reaching 30 mm diam after 30 d at 24  $^{\circ}$ C. Colonies erumpent, unevenly folded, with moderate aerial mycelium, and entire, smooth margin; surface pale mouse-grey to mouse-grey; in reverse dark mouse-grey. Colonies on OA reaching 48 mm diam after 30 d at 24  $^{\circ}$ C; effuse, with moderate aerial mycelium, and entire margins; surface pale mouse-grey to mouse-grey, and dark mouse-grey in reverse.

**Specimens examined.** TONGA, ACIAR Plot, Tongatapu, *Musa* cv. TU8 AAAA, Mar. 1990, R.A. Fullerton, holotype CBS H-20047, culture ex-type X745 = CBS 122477. — WINDWARD ISLANDS, St Lucia, on *Musa* cv., 2003, E. Reid, culture X47 = CBS 122476.

**Notes** — Stover (1994) discussed and illustrated a *Stenella* sp. from banana, and named it '*Cercospora non-virulentum*', which was considered as a prevalent co-inhabitant with Black Leaf Streak and Sigatoka. *Mycosphaerella musae* is the causal agent of Mycosphaerella Speckle disease of banana (Carlier et al. 2000). A comparison made between strains isolated from Mycosphaerella Speckle disease symptoms (presumed *M. musae*), and '*Cercospora non-virulentum*' isolates in culture, suggested that the two species are identical, both producing brown, verruculose conidia with thickened scars on agar medium (Stover 1994). An inoculation assay carried out by using a mixture of conidia and mycelium of '*Cercospora non-virulentum*' on banana 'Cavendish Valery' leaves resulted in leaf spot symptoms after 70 d incubation, resembling those obtained using ascospores derived from '*M. musae*' strains.

Because '*Cercospora non-virulentum*' was never validly published, it is difficult to make a comparison with *Stenella musae*. However, based on the description provided by Stover (1994), *S. musae* has shorter conidia (7–70  $\times$  1.5–3  $\mu$ m) than '*Cercospora non-virulentum*' (55–200  $\times$  2.6–3.2  $\mu$ m).

A further complication lies in the fact that several phylogenetically distinct species of *Mycosphaerella* have in the past been isolated from Mycosphaerella Speckle disease symptoms of banana. All the '*M. musae*' isolates examined in this study were sterile, and thus could not be used for morphological comparison. *Mycosphaerella musae* was originally described from *Musa sapientum* leaves collected in Argentina. An examination of the type (IMI 91165) shows ascospores to be straight to slightly curved, fusoid-ellipsoidal with narrowly obtuse ends, being widest at the median septum (Fig. 15). Further collections would thus be required to clarify the identity of this species.



**Fig. 15** Ascospores of *Mycosphaerella musae* (IMI 91165). — Scale bar = 10  $\mu$ m.

***Stenella musicola*** Arzanlou & Crous, *sp. nov.* — MycoBank MB505978; Fig. 14c, 16

*Stenellae musae* similis, sed conidiophoris leviter longioribus et latioribus, (18–)30–36(–45) × (2–)2.5–3(–4) μm, conidiis saepe longioribus, (7–)37–57(–120) × 2–4 μm. A *Stenellae queenslandica* conidiophoris 0–2-septatis et conidiis 2–4 μm latis differt.

*Etymology.* Named after its host, *Musa*.

In vitro on MEA: *Mycelium* submerged and superficial; submerged hyphae smooth to verrucose, thin-walled, subhyaline to olivaceous brown, 2–3 μm wide, with thin septa; aerial hyphae coarsely verrucose, olivaceous-brown, rather thick-



**Fig. 16** *Stenella musicola* (CBS 122479). a–e. Conidiophores with sympodially proliferating conidiogenous cells and darkened, thickened loci; f–g. hyphal anastomoses; h–i. conidia. — Scale bar = 10 μm.

walled, 2–2.5 µm wide, with thin septa. *Conidiophores* arising from superficial hyphae, pale brown, rather thick-walled, finely verruculous, 0–2-septate, occasionally continuous with supporting hyphae, subcylindrical, straight to geniculate-sinuous, unbranched, (18–)30–36(–45) × (2–)2.5–3(–4) µm. *Conidiogenous cells* integrated, terminal, sometimes intercalary, unbranched, pale brown, smooth or finely verruculose, cylindrical to subcylindrical, sometimes swollen at the apex, with flat-tipped apical loci, proliferating sympodially; 1–1.5 µm diam, loci thickened, darkened, refractive. *Conidia* solitary, rarely in unbranched chains, medium brown, thin-walled, finely verruculose subcylindrical to obclavate, with subobtuse apex, and long obconically subtruncate to obconically subtruncate base, straight to curved, 0–pluri-septate, (7–)37–57(–120) × 2–4 µm; hilum thickened, darkened, refractive, 1–1.5 µm wide.

**Cultural characteristics** — Colonies on MEA reaching 28 mm diam after 30 d at 24 °C; effuse, slightly raised at the centre, with moderate, velvety to hairy aerial mycelium; folded, with entire smooth margin; surface pale mouse-grey to mouse-grey; in reverse dark mouse-grey. Colonies on OA reaching 39 mm diam after 30 d at 24 °C; effuse, with moderate velvety to hairy aerial mycelium, and entire, smooth margins; surface pale mouse-grey to mouse-grey, and olivaceous in reverse.

**Specimen examined.** INDIA, Tamil Nadu, Tiruchirapally, on leaf of *Musa* cv. Grand Nain AAA (Cav.), 2005, *I. Buddenhagen*, holotype CBS H-20046, culture ex-type X1019 = CBS 122479.

**Notes** — *Stenella musicola* morphologically also resembles *S. citri-grisea* (teleomorph *Mycosphaerella citri*), which is known from *Citrus* (Pretorius et al. 2003). It differs from the later species, however, based on its conidial dimensions. In *S. musicola* conidia range from (7–)37–57(–120) × 2–4 µm, while in *S. citri-grisea* conidia are longer and narrower, namely 25–200 × 1.5–3 µm. The three new *Stenella* species on *Musa* spp. are morphologically very similar and only gradually differentiated in the size and septation of the conidiophores and conidia.

***Stenella queenslandica* Arzanlou & Crous, sp. nov.** — MycoBank MB505979; Fig. 14b, 17

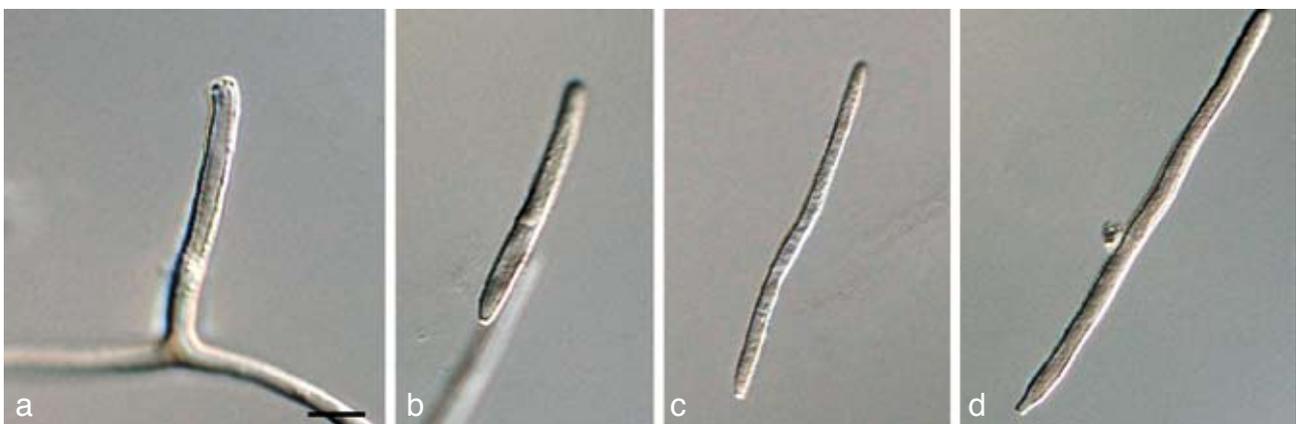
*Stenellae musae* similis, sed conidiis longioribus, 51–83 × 2–2.5 µm. A *Stenella musicola* conidiophoris 1–4-septatis et conidiis saepe longioribus et angustioribus, 51–83 × 2–2.5 µm, differt.

**Etymology.** Named after Queensland, the state in Australia where this fungus was collected.

**In vitro on MEA:** *Mycelium* submerged and superficial; submerged hyphae smooth, thin-walled, subhyaline to olivaceous-brown, 2–3 µm wide, with thin septa; aerial hyphae coarsely verrucose, olivaceous-brown, rather thick-walled, 2–2.5 µm wide, with thin septa. *Conidiophores* arising from superficial hyphae, pale brown, thin-walled, finely verrucose, 1–4-septate, occasionally reduced to conidiogenous cells, subcylindrical, straight to geniculate-sinuous, unbranched, up to 40 µm long and 2–3 µm wide. *Conidiogenous cells* integrated, terminal, sometimes intercalary, unbranched, pale brown, smooth or finely verruculose, cylindrical, tapering to a bluntly rounded apex with flat-tipped apical loci that proliferate sympodially; loci thickened, darkened, refractive about 1 µm diam. *Conidia* solitary, medium brown, thin-walled, verruculose, subcylindrical to obclavate, with subobtuse to obtuse apex and long obconically subtruncate to obconically subtruncate base, straight to curved, 0–multi-septate, 51–83 × 2–2.5 µm; hilum thickened, darkened, refractive, 0.5–1 µm wide.

**Cultural characteristics** — Colonies on MEA reaching 24 mm diam after 30 d at 24 °C. Colonies effuse, slightly elevated at the centre with abundant aerial mycelium, and entire, smooth margins; surface mouse-grey to dark mouse-grey; dark mouse-grey in reverse. Colonies on OA reaching 41 mm diam after 30 d at 24 °C, colonies effuse, with moderate aerial mycelium, and entire, smooth margin; surface olivaceous-grey; iron-grey in reverse.

**Specimen examined.** AUSTRALIA, Queensland, Mount Lewis, Mount Lewis Road, 16° 34' 47.2" S, 145° 19' 7" E, 538 m alt., on *Musa banksii* leaf, Aug. 2006, *P.W. Crous, W. Gams & B. Summerell*, holotype CBS H-20050, culture ex-type CBS 122475.



**Fig. 17** *Stenella queenslandica* (CBS 122475). a. Conidiophore with terminal conidiogenous cell; b–d. conidia. — Scale bar = 10 µm.

Notes — The ITS sequence of *Stenella queenslandica* is identical to that of *Mycosphaerella obscuris* (Burgess et al. 2007), a pathogen of *Eucalyptus* known from Vietnam and Indonesia. However, the latter fungus is a species of *Teratosphaeria* with a *Readeriella* anamorph (CBS 119973), which appears to be a synonym of *T. suttonii* (Crous & Wingfield 1997, Crous et al. 2007a, b), and the deposited sequences (DQ632676, DQ632677) belong to another species.

## DISCUSSION

The present study is the first multi-gene DNA phylogenetic study of a global set of *Mycosphaerella* isolates associated with the Sigatoka disease complex of banana. Considering that Sigatoka diseases are the economically most important diseases of banana and the main constraint for banana production worldwide (reviewed in Jones 2000), there was a huge paucity of knowledge relating to the identity of other *Mycosphaerella* species occurring on banana. Even though several species of *Mycosphaerella* have in the past been described from *Musa*, the majority has never been known from culture (Pont 1960, Stover 1963, 1969, 1977, 1980, 1994, Mulder & Stover 1976, Pons 1987, Crous et al. 2003, Aptroot 2006, Arzanlou et al. 2007a). The integration of DNA analyses and morphology in the present study revealed more than 20 species of *Mycosphaerella* to occur on banana. Five of these species were shown to have wider host ranges than banana only, and we described a further eight new species of *Mycosphaerella* from various *Musa* collections.

The three primary agents of the Sigatoka disease complex, *M. eumusae*, *M. fijiensis*, and *M. musicola* can be distinguished based on their conidial morphology and ascospore germination patterns (reviewed in Jones 2000, Crous & Mourichon 2002). Conidia of *M. fijiensis* are medium brown, and have a characteristic thickening along the basal rim of the hilum, which is absent in *M. musicola* and *M. eumusae*. These two species have medium and pale brown conidia, respectively. Ascospores of *M. fijiensis* and *M. musicola* germinate from both polar ends, do not become distorted (4–5 µm wide), with a germ tube parallel to the long axis of the spore. However, in *M. musicola* a mucoid sheath surrounds the germinating ascospores, and the germ tubes are more irregular in width than in *M. fijiensis*. Ascospores of *M. eumusae* show some distortion upon germination (5–6 µm wide), and frequently germinate by means of 3–4 germ tubes, which grow parallel or lateral to the long axis of the spore (Fig. 18, 19). Thus, all of these species can be identified based on a combination of morphology and cultural characteristics, but proper identification remains problematic to the non-specialist. Hence the DNA barcodes generated in this study, along with the Taqman probes (Arzanlou et al. 2007a) is an alternative method of identification.

Besides the three primary agents of the Sigatoka complex disease, which have *Pseudocercospora* anamorphs, three additional *Pseudocercospora* species were described from *Musa* in the present study. One of these, *Pseudocercospora longispora*, has in the past been confused with *P. musae* (teleomorph *M. musicola*) and has been isolated from similar Sigatoka disease lesions. Although these species can be distinguished

based on differences in conidial size and shape, these characters overlap among the various *Pseudocercospora* species, making explicit identification solely possible by means of additional markers such as DNA sequence data (Fig. 2).

Much confusion still surrounds the identity of *M. musae*. According to Stover (1994), *M. musae* is identical to a *Stenella* species called '*Cercospora non-virulentum*'. This species was considered as a prevalent co-inhabitant with black Sigatoka and yellow Sigatoka. A comparison made between isolates isolated from *Mycosphaerella* Speckle disease symptoms, revealed several phylogenetically distinct species to be associated with this disease. In the present study we treated four *Stenella* species, three of which proved to be new on banana. None of these three new species fit with the description provided for '*Cercospora non-virulentum*' by Stover (1994), which appears to represent yet another undescribed species of *Stenella*. Further collections would thus be required to resolve the status of '*Cercospora non-virulentum*' and *M. musae*.

Data obtained in the present study revealed three species of *Dissoconium* on *Musa*, of which one is described as new. The recent revision of the genus *Ramichloridium* and allied genera (Arzanlou et al. 2007b) revealed that *R. apiculatum*, type spe-

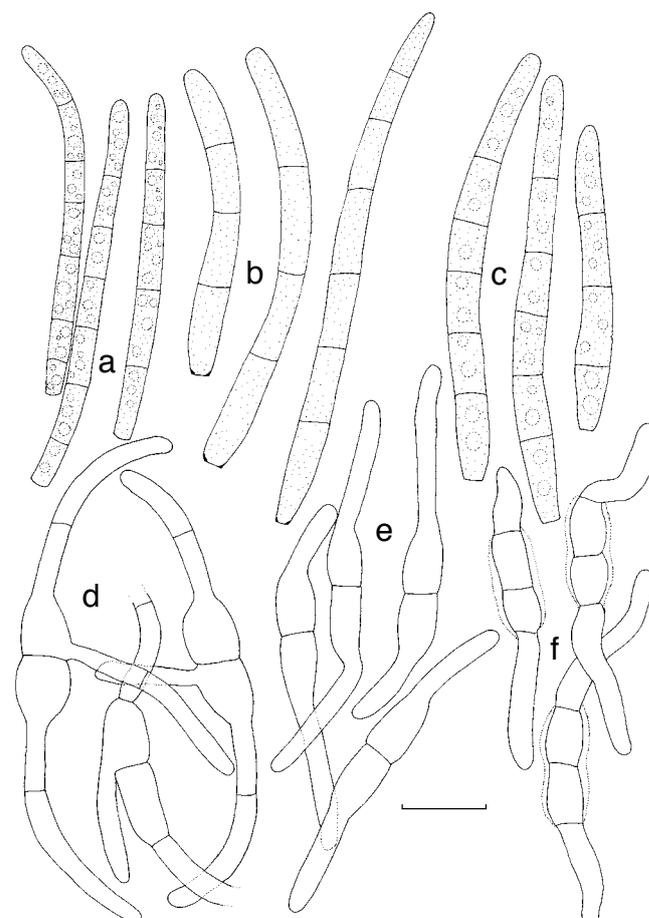


Fig. 18 a–c. Conidia in *Pseudocercospora eumusae*, *P. fijiensis*, and *P. musae*, respectively; d–f. ascospore germination pattern in *M. eumusae*, *M. fijiensis* and *M. musicola*, respectively. — Scale bar = 10 µm.



**Fig. 19** *Pseudocercospora fijiensis*. a–c. Conidiophores with sympodially proliferating conidiogenous cells; d–f. obclavate conidia with darkened hilum. — Scale bar = 10 µm.

cies of *Ramichloridium*, has phylogenetic affinity with the genus *Dissoconium*. However, the latter genus is morphologically distinct from *Ramichloridium* by producing forcibly discharged pairs of primary and secondary conidia. Thus far seven species of *Dissoconium* have been described from different substrates, and as in the *Pseudocercospora* species occurring on *Musa*, identification is best achieved by means of molecular sequence data.

It is interesting to note that up to six species have been reported during the course of the present study as occurring on hosts other than *Musa*. Although our present data suggest the causal agents of Sigatoka to be highly specific to banana, no information is presently available to elucidate the ecology and possible pathology of the wide host range species, and inoculation studies would now be required to fully resolve their status as foliar pathogens of banana. The possibility exists that some of the species described here as new have been described previously on hosts other than banana. However, none of the sequences presently in GenBank, or in the MycoBank database, match any known comparable species.

From the data presented in this study, it is clear that the Sigatoka disease complex is caused by a multitude of *Mycosphaerella* species. However, the exact contribution of each of these species to the disease complex remains unclear. The multi-locus DNA sequence data set established in this study can be used to develop species-specific molecular detection tools, which is a good alternative for traditional diagnostics. These tools can subsequently be implemented in disease management programmes.

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# Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma*, *D. septosporum* and *D. pini*

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**Abstract:** The red band needle blight fungus, *Dothistroma septosporum* is a widely distributed pathogen of many pine species. Three morphological varieties of this pathogen have been described based on differences in conidial length. However, controversy exists as to whether spore size represents an adequate characteristic to distinguish between forms of *D. septosporum*. The aim of this investigation was to consider the phylogenetic relationships between *D. septosporum* isolates from different countries. An additional objective was to determine whether comparisons of DNA sequence data support the morphological varieties recognized for this species. DNA from portions of the nuclear ribosomal internal transcribed spacer (ITS),  $\beta$ -tubulin and elongation factor 1- $\alpha$  genes were sequenced and analysed for isolates from 13 different countries representing five continents. Results show that isolates of the pathogen encompass two divergent lineages representing distinct phylogenetic species. One phylogenetic species (Lineage I) is found worldwide, while the other (Lineage II), is restricted to the North-Central U.S.A. The names *D. pini* and *D. septosporum* are available for these species. The former name should apply to the phylogenetic species currently known only from the United States. The latter fungus has a worldwide distribution and is the causal agent of the serious disease known as red band needle blight that has damaged exotic plantations of *Pinus radiata* in the Southern Hemisphere. A PCR-restriction fragment length polymorphism (RFLP) diagnostic protocol is described that distinguishes between all the currently known *Dothistroma* species. The previous classification of *D. septosporum* isolates into different varieties based on morphology is inconsistent and not supported by our DNA analyses. We therefore reject further use of varietal names in *Dothistroma*.

**Key words:** *Dothistroma pini*, *D. septosporum*, *Mycosphaerella pini*, needle cast disease, PCR-RFLP, phylogenetic species, red band needle blight.

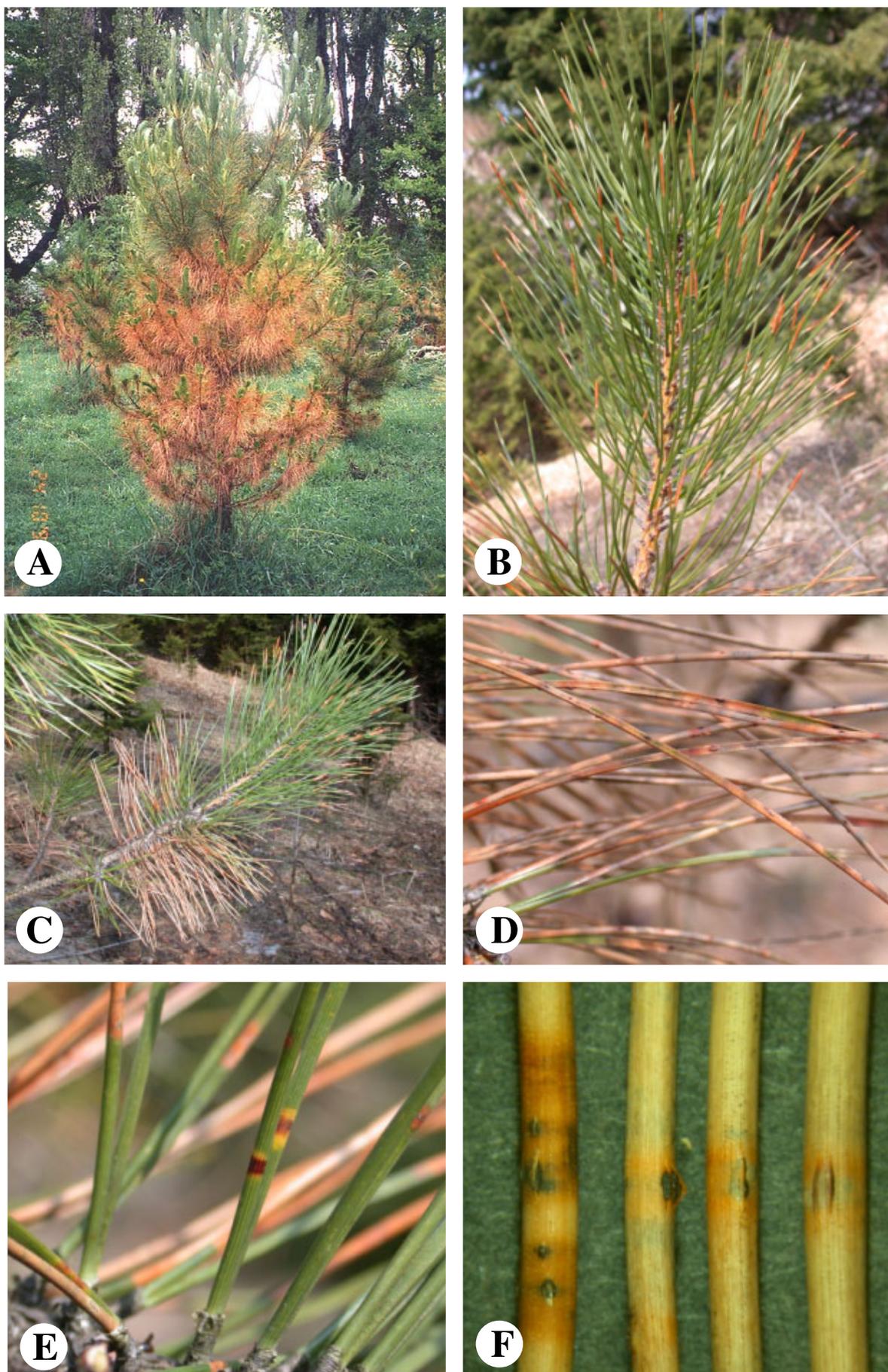
## INTRODUCTION

*Dothistroma septosporum* (Dorog.) M. Morelet, an ascomycetous pine needle pathogen, is the causal agent of the notorious red band needle blight disease. This fungus is known to infect over 60 different pine species (Ivory 1994). In situations where favourable conditions and high infection pressures exist, *D. septosporum* has also been reported infecting *Pseudotsuga menziesii* (Mirbel) Franco (Dubin & Walper 1967), *Larix decidua* P. Mill. (Bassett 1969), *Picea abies* (L.) Karst. (Lang 1987), *Picea sitchensis* (Bong.) Carr. (Gadgil 1984) and *Picea omorika* (Purkyne) (Karadži 1994), though no data exist to confirm that these incidents were caused by *D. septosporum*.

After the fungus infects via the stomata, initial symptoms appear as water-soaked lesions on the needles. Black conidiomata develop at these infection sites, which are characteristically surrounded by a red band, hence the common name of the fungus. Infected needles become necrotic and are cast (Fig. 1). In severe cases, complete defoliation occurs, leading to

growth retardation and tree death (Gibson *et al.* 1964). Red band needle blight is one of the most important diseases of pines, which has seriously damaged plantation forestry in many countries.

The red band needle blight pathogen has a cosmopolitan distribution, having been reported from more than 44 different countries in Eurasia, Africa, Oceania and the Americas (Data sheets on Quarantine pests: *Mycosphaerella dearnessi* and *Mycosphaerella pini* [http://www.eppo.org/QUARANTINE/QP\\_fungi.htm](http://www.eppo.org/QUARANTINE/QP_fungi.htm), Ivory 1994). The severity of the disease appears to be related to a favourable climate in the Southern Hemisphere and to the exotic planting of susceptible host species such as *Pinus radiata* D. Don and *P. ponderosa* Laws. Thus, countries such as Chile, New Zealand and Kenya, where plantations are primarily monocultures of susceptible hosts, have experienced huge economic losses (Gibson 1974, van der Pas 1981). Control is limited to sanitary silvicultural practices, copper sprays and the planting of resistant tree species, families and clones (Carson & Carson 1989, Dick 1989, Chou 1991).



**Fig. 1.** Symptoms of *Dothistroma septosporum* infection on *Pinus* spp. A. 50–75 % infection on *P. radiata* in Chile. B. Tip die-back of infected *P. nigra* needles. C. Characteristically, needles from the lower branches show the first signs of disease. D. Severely infected needles showing complete necrosis and distinct red bands bearing mature conidiomata. E. Symptoms first appear as water soaked lesions followed by necrotic bands that turn reddish in colour. F. Mature conidiomata erupting through the epidermal tissue of pine needles.



The taxonomic history of *D. septosporum* is beset with confusion. The species concept has two independent roots of origin: one stems from Europe and the other from the U.S.A. In Europe, Dorogin (1911) first described this fungus as *Cytosporina septosporum* Dorog. from Russia. *Cytosporina septosporum* was later transferred to the genus *Septoriella* Oudem. as *S. septosporum* (Dorog.) Sacc. (Trotter 1931).

In the U.S.A., the species became involved in taxonomic confusion stemming from a failure to distinguish between the red band fungus and the brown spot fungus, *Lecanosticta acicola* (Thüm.) Syd. Initially, Saccardo (1920) described the red band fungus found on *P. ponderosa* in Idaho as *Actinothyrium marginatum* Sacc. Both Dearness (1928) and Hedgcock (1929) believed that the red band fungus was conspecific with *L. acicola*, although Dearness referred to it as *Cryptosporium acicola* Thüm., and Hedgcock used the name *Septoria acicola* (Thüm.) Sacc. Sydow & Petrak (1942) later recognised that *A. marginatum* represented a *nomen confusum* and referred to the fungus as *L. acicola*. Independently, Hulbary (1941) described the red band fungus occurring on *Pinus nigra* Arn. var. *austriaca* Aschers. & Graebn, collected in Illinois, and erected the name *Dothistroma pini* Hulbary for it. Siggers (1944) discovered that the material previously referred to as *L. acicola*, *C. acicola*, *S. acicola* and *A. marginatum* on *P. nigra* var. *austriaca* was not conspecific with the type specimen of *L. acicola*, but rather with that of *Dothistroma pini*.

The connection between the American and European fungi was made when Gremmen (1968) and Morelet (1968) realized that the fungus described in Europe as *C. septosporum* was the same as *D. pini* causing red band needle disease in the U.S.A. Morelet (1968) synonymized all collections associated with red band needle blight and made a new combination in *Dothistroma* for the species epithet “*septosporum*” (as “*septospora*”), which is now widely accepted for the red band needle blight fungus.

Three different varieties of *D. septosporum* have been described based on differences in the average conidial length. *Dothistroma septosporum* var. *septosporum* ( $\equiv$  *D. pini* var. *pini*) and *D. septosporum* var. *lineare* ( $\equiv$  *D. pini* var. *lineare*), proposed by Thyr & Shaw (1964), are respectively the varieties with short (15.4–28  $\times$  2.6–4  $\mu$ m) and long (23–42  $\times$  1.8–2.9  $\mu$ m) conidia. *Dothistroma septosporum* var. *keniense* ( $\equiv$  *D. pini* var. *keniense*), proposed by Ivory (1967), accommodates collections of the fungus with conidia of intermediate (15–47.5  $\times$  1.5–3.5  $\mu$ m) size. There has, however, been considerable debate as to whether conidial size represents an appropriate character by which to distinguish among forms or varieties of *D. septosporum* (Gadgil 1967, Funk & Parker 1966, Sutton 1980). Evans (1984) studied a large number of

collections of these fungi from many parts of the world and found considerable differences in both anamorph and teleomorph morphology. He contested the validity of varieties in *Dothistroma*, but acknowledged that morphotypes or ecotypes probably exist.

The aim of the present investigation was to consider the phylogenetic relationships of *D. septosporum* isolates from different countries, and further to determine whether morphotypes or ecotypes might exist for the fungus. An additional aim was to determine whether DNA sequence data reflect the separation of *D. septosporum* into different varieties.

## MATERIALS AND METHODS

### Isolates

A total of 32 isolates from various locations in 13 countries were chosen to represent a global distribution of *D. septosporum* (Table 1). We also included sufficient material to reflect the three varieties that have been described for the fungus. Further isolates, representing the species *Mycosphaerella dearnessii* M.E. Barr (the brown spot needle blight fungus, *L. acicola*), *D. rhabdoclinis* Butin and *Botryosphaeria ribis* Grossenb. & Duggar were included in this study.

Isolates were obtained either directly from culture collections (Table 1), or from isolations made from infected needles. Infected needles collected from the field were first deposited in  $-70$  °C freezers (minimum 1 h), in brown paper bags to kill possible contaminant insects or mites. Mature conidiomata from the needles were scraped from the needle surfaces and rolled across the surface of 2 % malt extract agar (MEA, Biolab, Midrand, Johannesburg) plates to release the conidia. Blocks of agar were cut from the plates in areas where there were many conidia but no contaminating debris. These blocks were then lifted and transferred to new MEA plates. Cultures were incubated at 20 °C until colonies formed. All cultures used in this study are stored in the culture collection (CMW), of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Duplicates of representative isolates have been deposited in the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands (Table 1).

### DNA extraction, amplification and sequencing

Spores of representative cultures were spread onto 2 % MEA plates and incubated at 20 °C until colonies had formed (approx. 4 wk, 10–15 mm diam). Colonies were scraped from the plates, excess agar removed and placed directly into Eppendorf tubes. The colonies (constituting mycelium and spores) were freeze-dried and crushed with the aid of liquid nitrogen and a glass rod.

Table 1. Isolates of *Dothistroma* and related species examined in this study.

Fungus	Culture number <sup>a</sup>	Other culture numbers <sup>a</sup>	Country	Extra location information	Suggested variety <sup>b</sup>	Host	Collector	Date collected
<i>Dothistroma septosporum</i>	CMW 684	–	South Africa	Eastern Cape	var. <i>keniense</i>	<i>Pinus radiata</i>	M.H. Ivory	1984
	CMW 8658	–	South Africa	Hogsback, Eastern Cape	var. <i>keniense</i>	<i>P. radiata</i>	J. Roux	2001
	CMW 11372	CBS 116489	South Africa	Tzaneen, Limpopo	var. <i>keniense</i>	<i>P. radiata</i>	I. Barnes	2002
	CMW 10622	–	Kenya	Napkoi	var. <i>keniense</i>	<i>P. radiata</i>	J. Roux	2001
	CMW 10722	–	Kenya	Napkoi	var. <i>keniense</i>	<i>P. radiata</i>	J. Roux	2002
	CMW 9937	–	New Zealand	Karioi	var. <i>pini</i>	<i>P. contorta</i>	M. Dick	2001
	CMW 9939	–	New Zealand	Rotorua	var. <i>pini</i>	<i>P. radiata</i>	M. Dick	2001
	CMW 9943	–	New Zealand	Rotorua	var. <i>pini</i>	<i>P. radiata</i>	M. Dick	2002
	CMW 6841	–	Australia	Canberra, Australia Capital Territory (A.C.T.)	var. <i>pini</i>	<i>Pinus sp.</i>	K. Old	2000
	CMW 6845	–	Australia	Canberra, A.C.T.	var. <i>pini</i>	<i>Pinus sp.</i>	K. Old	2000
	CMW 6846	–	Australia	Canberra, A.C.T.	var. <i>pini</i>	<i>Pinus sp.</i>	K. Old	2001
	CMW 10247	–	Chile	Bio Bio, VIII Region	var. <i>pini</i>	<i>P. radiata</i>	M.J. Wingfield	2001
	CMW 9304	–	Chile	Valdivia, X Region	var. <i>pini</i>	<i>P. radiata</i>	M.J. Wingfield	2001
	CMW 8611	–	Chile	Valdivia, X Region	var. <i>pini</i>	<i>Pinus sp.</i>	M.J. Wingfield	2001
	CMW 9920	–	Ecuador	Lasso Highlands, Cotopaxi	var. <i>pini</i>	<i>P. muricata</i>	M.J. Wingfield	2001
	CMW 9992	CBS 383.74	France	Arboretum d' Amance, Amance, Meurthe et Moselle prefecture	var. <i>lineare</i>	<i>P. coulteri</i>	M. Morelet	–
	CMW 13004	CBS 116488	Poland	Miechów Forest District, Cracow	–	<i>P. nigra</i>	T. Kowalski	2003
	CMW 13007	–	Poland	Miechów Forest District, Cracow	–	<i>P. nigra</i>	T. Kowalski	2003
	CMW 13010	–	Poland	Miechów Forest District, Cracow	–	<i>P. nigra</i>	T. Kowalski	2003
	CMW 13123	ATCC MYA-603	Slovakia	–	–	<i>P. sylvestris</i>	–	–
CMW 13122	ATCC MYA-604	Germany	Bavarian Alps	var. <i>lineare</i>	<i>P. mugo</i>	–	–	
CMW 14903	–	Austria	Vienna	–	<i>P. peuce</i>	T. Kirisits	2004	
CMW 14904	–	Austria	Thenneberg	–	<i>P. nigra</i>	T. Kirisits	2004	
CMW 14823	ATCC MYA-602	Canada	Goldstream River, British Columbia	var. <i>lineare</i>	<i>P. contorta</i>	–	1997	
CMW15077	–	U.S.A.	Lochsa Historical Ranger Station, Idaho	var. <i>lineare</i>	var. <i>latifolia</i>	L.M. Carris	2004	
CMW 14822	ATCC MYA-610	U.S.A.	Bandon, Oregon	var. <i>lineare</i>	<i>P. ponderosa</i>	–	1983	
CMW 10930	CBS 116485	U.S.A.	Crystal Lake, Crystal Township, Montcalm County, Michigan	var. <i>pini</i>	<i>P. nigra</i>	G. Adams	2001	
CMW 10951	CBS 116487	U.S.A.	Stanton, Evergreen Township, Montcalm County, Michigan	var. <i>pini</i>	<i>P. nigra</i>	G. Adams	2001	

CMW 6400	–	U.S.A.	Stanton, Michigan	var. <i>pini</i>	<i>P. nigra</i>	G. Adams	–
CMW 14905	CBS 116483	U.S.A.	McBain, Riverside Township, Massauke County, Michigan	var. <i>pini</i>	<i>P. nigra</i>	G. Adams	2001
CMW 14820	ATCC MYA-609	U.S.A.	Central, Minnesota	var. <i>pini</i>	<i>P. nigra</i>	–	1970
CMW 14821	ATCC MYA-606	U.S.A.	Lincoln, Nebraska	var. <i>pini</i>	<i>P. nigra</i>	–	1964
–	ILLS 27093 T	U.S.A.	DeKalb County, Illinois	var. <i>pini</i>	<i>P. nigra</i> var. <i>austrica</i>	J.C. Carter	1938
–	WSP 48361	U.S.A.	Meadow Creek, Clearwater Ranger District, Idaho	var. <i>lineare</i>	<i>P. ponderosa</i>	F. Matzner	1957
CMW 9985	CBS 871.95	France	Le-Teich, Gironde prefecture (Aquitaine)	–	<i>P. radiata</i>	M. Morelet	1995
CMW 13119	ATCC 200602	China	Fujie	–	<i>P. elliotii</i>	Z.Y. Huang	–
CMW 12519	CBS 102195	Germany	Wolfenbüttel	–	<i>Pseudotsuga menziesii</i>	H. Butin	1998
CMW 7773	–	U.S.A.	New York	–	<i>Ribes</i> sp.	G. Hudler	2000

*Actinothyrium marginatum*  
(T of *D. septosporum* var. *lineare*)

*Mycosphaerella dearnessii*

*Dothistroma rhabdoclinis*

*Botryosphaeria ribis*

<sup>a</sup>Abbreviations: ATCC, American Type Culture Collection; Virginia, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW, Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. ILLS, Illinois Natural History Survey, Illinois, U.S.A.; WSP, Washington State University, Washington, U.S.A. <sup>b</sup>Varieties suggested are assigned based on conidial dimensions and/or origin as defined by Thyr & Shaw (1964) and Ivory (1967). T = ex-type.



Before DNA was extracted using the method described by Barnes *et al.* (2001), 800  $\mu$ L of extraction buffer was added to the tubes, which were then incubated in a heating block for 15 min at 85 °C followed by another 1 h at 60 °C. DNA concentrations were measured with a NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, Delaware, U.S.A.). DNA from herbarium material was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). A single conidioma was scraped from a needle and excess plant material removed. The conidioma was then crushed between two slides before DNA extraction was continued. The success of this method, using one conidioma, was first tested on the Idaho material (CMW 15077) before attempting to extract DNA from the herbarium specimens.

Primers ITS1 and ITS4 (White *et al.* 1990), were used to amplify the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S gene of the ribosomal RNA operon. Parts of the  $\beta$ -tubulin gene were amplified using the primer pairs Bt2a/Bt2b and Bt1a/Bt1b (Glass & Donaldson 1995). The translation elongation factor (EF1- $\alpha$ ) gene was amplified using the forward EF1-728F and reverse primer EF1-986R (Carbone & Kohn 1999).

PCR was performed in total volumes of 25  $\mu$ L. The reaction mixtures consisted of  $\pm$  5 ng DNA template, 200 nM of the forward and reverse primers, 0.2 mM of each dNTP, 1U Taq DNA Polymerase with 10 $\times$  buffer (Roche Molecular Biochemicals, Mannheim, Germany) and 1.5 mM MgCl<sub>2</sub>. The PCR cycling profile was as follows: 96 °C for 2 min, 10 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. A further 30 cycles were included with the annealing time altered to 40 s and a 5 s extension after each cycle. Ten min at 72 °C completed the programme. PCR amplicons were visualized on 2 % agarose (Roche) gels stained with ethidium bromide under UV illumination. Amplicons were purified using Sephadex G-50 columns (SIGMA-Aldrich, Steinheim, Germany).

PCR amplicons were cycle-sequenced using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) following the manufacturer's protocol. The same primers used for the PCR reactions were used to sequence the amplicons in both directions. Sequence reactions were run on an ABI PRISM™ 377 Autosequencer (Applied Biosystems) and sequence electropherograms were analyzed using Sequence Navigator version 1.0.1 (Applied Biosystems).

### Phylogenetic analysis

Sequences were aligned using Clustal X (Thompson 1997) and checked visually before analyses were run using PAUP v. 4.0 (Phylogenetic Analysis Using

Parsimony) (Swofford 2002). Intron and exon positions were identified using the original sequences from which each primer set was designed. The *Neurospora crassa* sequence (GenBank M13630) was used for the  $\beta$ -tubulin gene regions and the *Puccinia graminis* sequence (GenBank X73529) for the EF1- $\alpha$  region. The random sequence (GenBank AJ544253) of *Saccharomyces cerevisiae* was used to identify the ITS1, 5.8S and ITS2 regions in our sequences.

The heuristic search option, based on parsimony, with random stepwise addition of 1000 replicates and tree bisection reconnection (TBR) as the swapping algorithm, was used to construct the phylogram. Gaps were treated as "new state" and, therefore, all characters were given equal weight. Confidence levels of the branching points were determined using 1000 bootstrap replicates. *Botryosphaeria ribis* (GenBank accession numbers AY236936, AY236878, AY236907) was used as the outgroup and was treated as a monophyletic sister group to the ingroup. A partition homogeneity test (PHT), was performed in PAUP with 100 replicates to determine the combinability of the four data sets. All sequences derived in this study have been deposited in the GenBank database with accession numbers AY808275–AY808308 (ITS), AY808170–AY808204 ( $\beta$ -tubulin 1), AY808205–AY808239 ( $\beta$ -tubulin 2) and AY808240–AY808274 (EF1- $\alpha$ ). Sequence alignments and trees have been deposited in TreeBASE, accession number S1209, M2088–M2091. Percentage divergence within *D. septosporum* (other species were excluded) was calculated by dividing the number of variable positions in the aligned sequence by the total length of the consensus sequence.

### Morphology

All cultures for growth rate studies were grown on 2 % MEA supplemented with 0.2 % yeast extract. Isolates CMW 13004 from Poland, CMW 11372 from South Africa and CMW 10951 from the U.S.A. were used for growth rate studies at 5 ° intervals from 5–30 °C. The growth rates were determined by taking 2 mm plugs of actively growing cultures and placing a single plug the centre of 35 mm, 2 % MEA Petri dishes. Three repeats of each culture were incubated at the above temperature and the average colony diameter measured every seventh day for 6 wk.

Descriptions and measurements of morphological characters were done directly from the fungal material obtained from the host tissue. Fungal structures were mounted in clear lactophenol or lactic acid, and observations were made using a Carl Zeiss (Carl Zeiss Ltd., Mannheim, West Germany) microscope. Spore lengths and widths from cultures and herbarium material were measured electronically using a Zeiss Axio Vision (Carl Zeiss) camera system.

## PCR-restriction fragment length polymorphism (RFLP) diagnostic procedure

Potential restriction enzymes for species identification, i.e., enzymes interacting with three or fewer restriction sites on the ITS sequences, were identified using Webcutter 2.0 (<http://rna.lundberg.gu.se/cutter2/>). PCR-RFLP patterns were generated using the ITS PCR amplicons of CMW6841, CMW14822, CMW14820 and CMW12519. Amplicons (~10 µL) were digested with 5 units *AluI* (Roche 10 U/µL) restriction enzyme in 20 µL reaction mixtures containing 2 µL 10× SuRE/Cut Buffer A and 7.5 µL water. CMW14822 was left undigested as a control. Reaction mixtures were incubated overnight at 37 °C followed by heat inactivation of the enzyme at 65 °C for 20 min. PCR-RFLP profiles were visualized on an ethidium bromide-stained agarose gel (3 %), under UV illumination.

## RESULTS

### Isolates

The technique by which conidiomata are rolled across the surface of an agar plate was an effective means of easily obtaining pure cultures of *D. septosporum*. This method significantly reduces, and in some cases completely eliminates, contamination by the faster growing secondary pathogens that normally complicate isolation of this fungus.

### DNA extraction, amplification and sequencing

Amplicons of the ITS region were ~520 bp long, the β-tubulin 1 region ~470 bp, the β-tubulin 2 region ~430 bp and the EF1-α region ~310 bp. Occasionally, for some isolates, an extra primer set of elongation factor primers (EF1F – 5'TGCGGTGGTATCGA CAAGCGT3' and EF1R- 5'AGCATGTTGTCGCC GTTG AAG3', Jacobs *et al.* 2004) was used to generate sequences. Amplicons using this primer set were then ~760 bp in length.

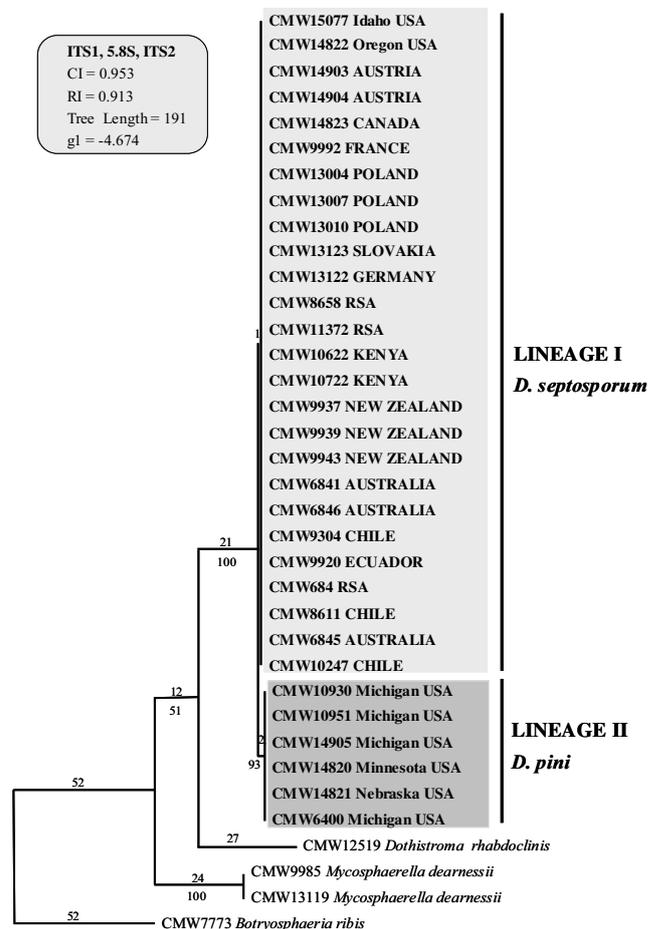
The extraction of DNA using the DNeasy Plant Mini Kit, and subsequent PCR from one conidioma from the Idaho material (less than 1-yr-old) was successful, and was thus attempted on herbarium specimens ILLS 27093 and WSP 48361. PCR of the type of *D. septosporum* var. *lineare* (*Actinothyrium marginatum*, WSP 48361), although successful, gave faint bands and contained smears. Only the ITS sequence was recovered. Poor PCR could be the result of degraded DNA associated with the fact that the material was 47-yr-old. PCR of the type of *Dothistroma pini* (ILLS 27093) from Illinois, which was 66-yr-old, was not successful.

### Phylogenetic analysis

Intron and exon positions were easily identified using the respective sequences of the gene regions from

GenBank. Two introns were present in the ITS sequence and the aligned data set was 473 bp in length. None of the sequences of the β-tubulin-1 gene region contained introns and thus, no alignment was necessary. The amino acid alignment of the β-tubulin-2 gene region was somewhat different to that of *N. crassa*. Exon 3 and 6 were identified and intron C was absent. Only part of exon 4 was similar, but the rest of the sequence up to exon 6 was not comparable with the corresponding section of the *N. crassa* sequence. In total, the aligned sequences were 418 bp long. The EF1-α gene resulted in an aligned dataset of 346 bp in length and contained one intron.

Significant incongruence ( $P = 0.03$ ) in the PHT was found among the four data sets of aligned sequences and thus they were not combinable. Phylograms for each gene region are thus represented individually (Figs 2–5). Only one most parsimonious tree is represented for data sets that produced multiple trees.

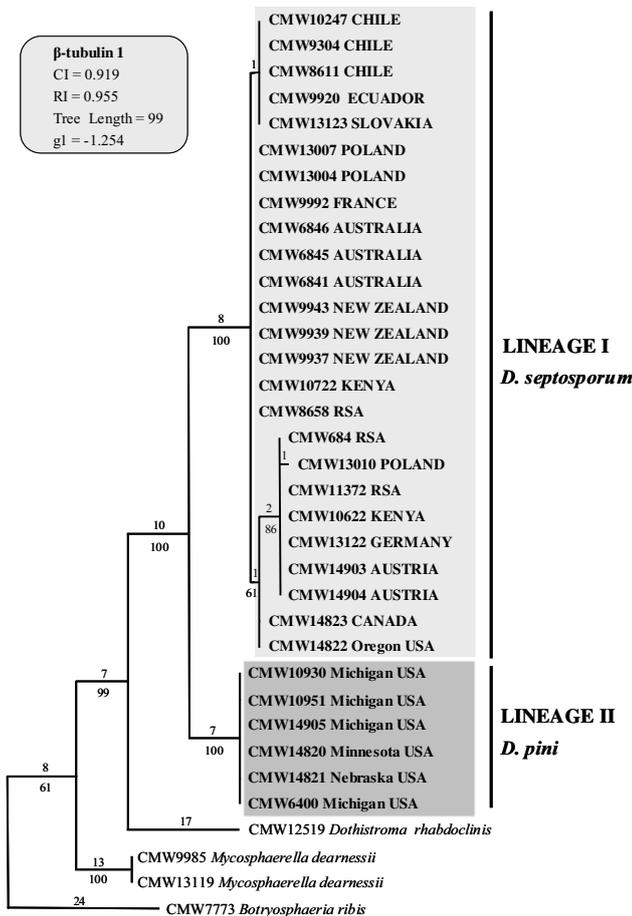


**Fig. 2.** One of 9 most parsimonious trees inferred from nuclear ribosomal internal transcribed spacer (ITS)1, 5.8S and ITS2 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 473 characters, 90 variable characters were parsimony-uninformative and 57 were parsimony-informative. No variation within either lineage is observed. Bootstrap values are indicated above the branches while branch lengths are indicated below. *Botryosphaeria ribis* was used as the outgroup.

Parsimony data and scores obtained from the heuristic search and analyses using PAUP are presented on each tree (Figs 2–5).

All four phylograms had very similar topology. The isolates of *D. septosporum* were resolved into two very distinct lineages, consistently supported with a 100 % bootstrap value (Figs 2–5). Lineage I included the majority of the isolates in this study, including isolates from all 13 countries represented in the data set.

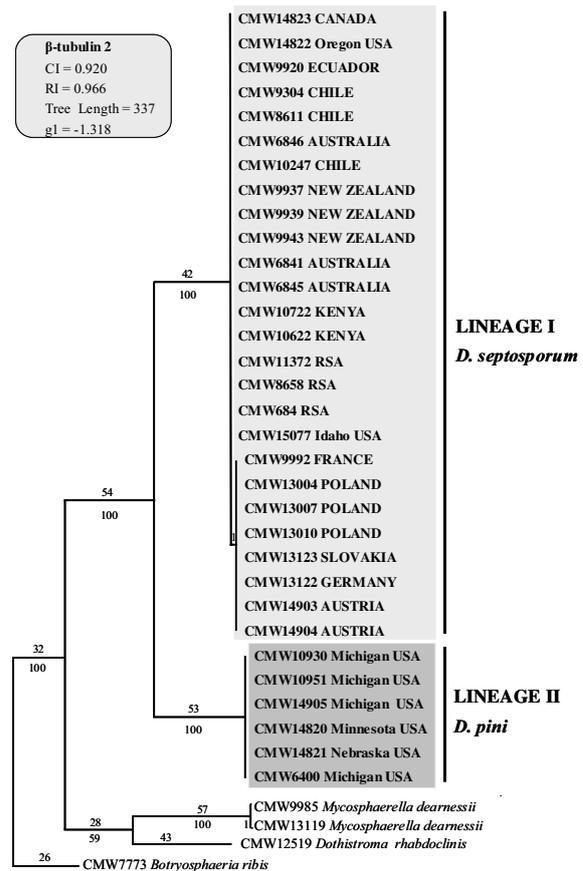
The sequence obtained from the type material of *D. septosporum* var. *lineare* (WSP 48361), was also included in this clade (Fig. 2). The ITS sequences in this lineage were identical while slight variation was observed randomly in the  $\beta$ -tubulin 1 (5 bp differences),  $\beta$ -tubulin 2 (1 bp differences), and EF-1 $\alpha$  gene (2 bp differences) regions. Lineage II was limited to isolates originating from the North Central U.S.A. (Minnesota, Nebraska and Michigan). No variation among these isolates was evident for the four gene regions sequenced.



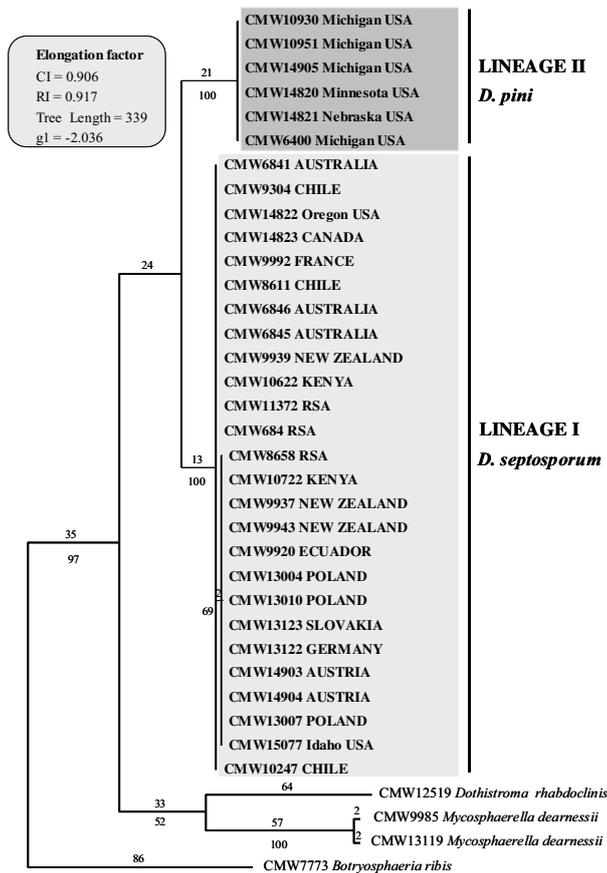
**Fig. 3.** Phylogeny of the red band needle blight fungi based on the  $\beta$ -tubulin-1 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 367 characters, 28 variable characters were parsimony-uninformative and 45 were parsimony-informative. Within-species variation is observed for Lineage I. Bootstrap values are indicated above the branches while branch lengths are indicated below. *Botryosphaeria ribis* was used as the outgroup.

From a total of 1508 bp of aligned sequences using only *D. septosporum* isolates, there were 147 bp polymorphisms distinguishing the two lineages. Most of the variation observed between the two lineages was in the conserved exon positions. Although the ITS had only 3 bp differences between the lineages, the  $\beta$ -tubulin-1 region contained 15 polymorphisms, the  $\beta$ -tubulin-2 showed 95 polymorphisms, and the EF-1 $\alpha$  gene-regions had 34 polymorphisms. Percentage divergence between the two lineages was thus significant at 9.7 %, indicating the presence of a species boundary. Sufficient variation between the two lineages exists for the recognition of two separate taxa.

There was no evidence in the sequence data to justify recognizing the three varieties described based on morphological differences. Isolates from South Africa and Kenya, that might have been considered to represent the variety “*keniense*”, were identical in sequence to those from Idaho and France, representing the variety “*lineare*”. These isolates could also not be distinguished from those from New Zealand and Chile that might have represented the variety “*pini*”. All these isolates resided in Lineage I.



**Fig. 4.** Phylogeny of the red band needle blight fungi based on the  $\beta$ -tubulin-2 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Slight variation is observed within Lineage I while no variation is observed within Lineage II. Of 418 characters, 30 variable characters were parsimony-uninformative and 170 were parsimony-informative. Bootstrap values are indicated above the branches while branch lengths are indicated below. *Botryosphaeria ribis* was used as the outgroup.



**Fig. 5.** One of 12 most parsimonious trees inferred from the EF1- $\alpha$  sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 346 characters, 87 variable characters were parsimony-uninformative and 130 were parsimony-informative. Bootstrap values are indicated above the branches while branch lengths are indicated below. *Botryosphaeria ribis* was used as the outgroup.

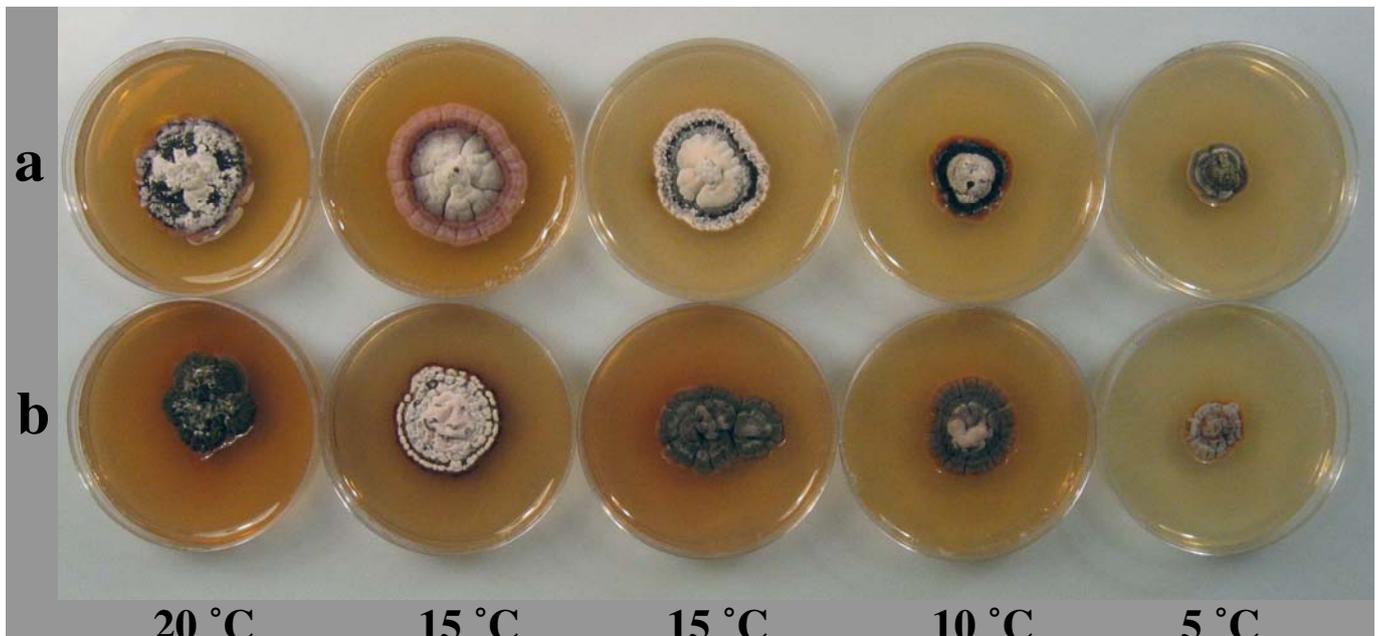
## Morphology

In an attempt to find morphological differences between the two phylogenetic species distinguished within *D. septosporum sensu lato*, differences in growth rates, culture morphology and spore dimensions were investigated. Growth rates for the phylogenetic Lineage I represented by isolates CMW 13004 and CMW 13010 from Poland, and CMW 11372 from South Africa were 1, 3.2, 2.2, 1.9 and 1.4 mm per week at 25, 20, 15, 10 and 5 °C respectively. The growth rates for the Central U.S.A. isolates CMW 10930, CMW 10951 and CMW 14905, representing phylogenetic lineage II, were 0.9, 3.6, 2.7, 1.6 and 1.3 mm per week at 25, 20, 15, 10 and 5 °C. Optimum growth for isolates in both lineages was at 20 °C, while no isolate of either lineage grew at 30 °C.

Substantial variability in culture morphology was observed among isolates from different countries, isolates obtained within a single country and even subcultures of the same isolate inoculated onto replica plates (Fig. 6).

In some cases, zones of red or blue pigment were observed in the agar surrounding the cultures. Pigment production was, however, not consistent within individual isolates and not observed at all in some isolates.

*Dothistroma septosporum* isolates chosen for spore measurements were selected 1) to represent isolates from all three varieties proposed in the literature (Table 1) and 2) from the two phylogenetic lineages revealed in this study (Figs 2–5).



**Fig. 6.** Culture morphology of *Dothistroma* isolates from Lineages I (*D. septosporum s. str.*) and II (*D. pini*). Cultures, grown on 2 % MEA, have approximately the same amount of growth at their respective temperatures after a six week period. Cultures vary considerably in morphology and colour within the same isolate at both the same (15 °C), and at different temperatures; a) Lineage I and b) Lineage II.

Conidial length showed extreme variation, ranging from 12–50 µm in isolates belonging to Lineage I (Fig. 7). Even spores from different conidiomata from the same tree differed in average measurement (data not shown).

There was considerable overlap in size ranges for those isolates labeled as var. *lineare*, *keniense* and *pini*, and no clear distinction between the isolates could be made. There was also no correlation between isolates from different continents, although conidia from the Southern Hemisphere tended to be shorter while those from the Northern Hemisphere were longer.

Although it was not immediately obvious, slight variation in morphology between isolates for the two lineages could be observed. The range of conidial dimension for isolates from Lineage II was smaller than that seen in Lineage I, and in general, there was a tendency for the isolates from the Central U.S.A. to have relatively short conidia, which were slightly wider than those produced by members of Lineage I (Fig. 8). Conidial septation was also more clearly defined and obvious in Lineage II isolates than in Lineage I isolates (Figs 7, 8). The conidial dimensions of the type specimen of *Dothistroma pini* from Illinois (ILLS 27093) closely matched those of other collections from the North Central U.S.A., i.e. relatively short and wide conidia.

Based on these observations we propose that isolates in the two phylogenetically distinct lineages be recognized as two discrete species. This separation is based on fixed nucleotide differences between isolates in the two lineages and variation in conidial dimensions. For isolates associated with red band needle blight belonging to Lineage I, the name *Dothistroma septosporum* is retained, and *Dothistroma pini* is resurrected for isolates belonging to Lineage II.

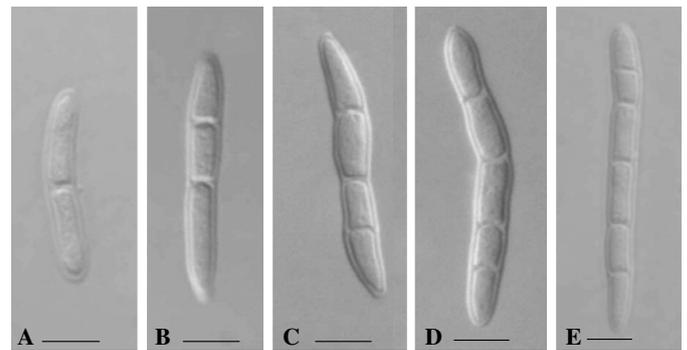
***Dothistroma pini*** Hulbary, Bull. Ill. St. nat. Hist. Surv. 21: 235. 1941. Figs 2–5, 8, 10, 12.

*Conidiomata* predominantly occurring in red bands on the upper and lower needle surfaces, separate to aggregated, sub-epidermal, becoming erumpent, and splitting the needle surface with one or two longitudinal slits; at maturity acervular, black, up to 1 mm in length, lined internally with pseudoparenchymatous cells giving rise to conidiophores; these cells brown, becoming paler at the point of conidiophore attachment. *Conidiophores* pale brown to hyaline, smooth, densely aggregated, subcylindrical to irregular, 1–4-septate, branched or simple, 15–27 × 2–3 µm. *Conidiogenous cells* integrated, hyaline, smooth, subcylindrical, tapering towards the bluntly rounded apices, proliferating sympodially or percurrently near the apex, 7–12 × 2–3 µm. *Conidia* aggregated in cream to pale brown, slimy masses; smooth, thin-walled, hyaline, subcylindrical to narrowly obclavate or irregular,

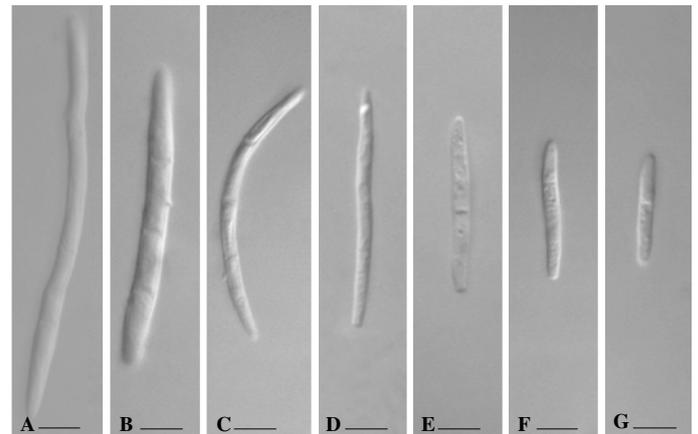
subobtuse at the apices, truncate to obconically subtruncate at the bases, (1–)3(–5) septate, (18–)25–35 (–45) × 3–5 µm (av. 30 × 3.5 µm) *in vivo*, (11–)20–25(–27) × (2–)2.5–3(–3.5) µm (av. 22 × 3 µm) *in vitro*.

*Notes:* Amplification of the ITS/5.8S/ITS2 region using primers ITS1 and ITS4 elucidates three polymorphisms distinct from those seen in *D. septosporum sensu stricto* at positions 68, 115 and 318. The polymorphism at position 318 results in the addition of an *AluI* restriction site in *D. pini* isolates. Upon digestion of the PCR product, this yields distinctive fragments of 170 and 350 base pairs in length.

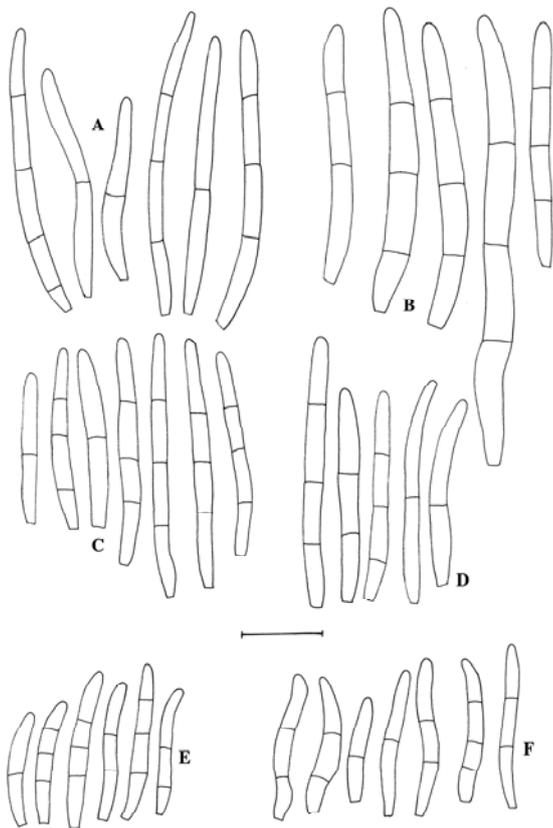
*Other specimens examined:* U.S.A., Michigan, Massaukee County, McBain, Riverside Township, isolated from *Pinus nigra*, Aug. 2001, G. Adams, herb. CBS 12203, culture CMW 14905 = CBS 116483; Michigan, Montcalm County, Stanton, Evergreen Township, from *Pinus nigra*, 2001, G. Adams, herb. CBS 12211, culture CMW 10951 = CBS 116487.



**Fig. 8.** Variation observed in conidial dimensions and number of septa within isolate CBS 116487 (Michigan, U.S.A.), from Lineage II (*D. pini*). Scale bars = 5 µm.



**Fig. 7.** Variation in conidial dimensions found within isolates from Lineage I (*D. septosporum s. str.*). Conidia obtained directly from infected hosts. A–C. Austria. D, E. New Zealand. F, G. Ecuador. Scale bars = 5 µm.



**Fig. 9.** Variation in conidial morphology of *Dothistroma septosporum* s. str. on needles. A. Idaho, WSP 48361, type of *Actinothyrium marginatum*. B. Idaho, herb. CBS 12204. C. Chile, herb. CBS 12206. D. Austria, herb. CBS 12205. E. Ecuador, herb. CBS 12207. F. New Zealand, herb. CBS 12208. Scale bar = 10  $\mu$ m.

***Dothistroma septosporum*** (Dorog.) M. Morelet (as “*septospora*”), Bull. Soc. Sci. nat. Archéol. Toulon Var 177: 9. 1968. Figs 2–5, 7, 9, 11, 12.

≡ *Cytosporina septospora* Dorog., Bull. Trimest. Soc. Mycol. Fr. 27: 106. 1911.

≡ *Septoriella septospora* (Dorog.) Sacc. apud Trotter, Syll. Fung. 25: 480. 1931.

= *Actinothyrium marginatum* Sacc., Nuovo Giorn. Bot. Ital. 27: 83. 1920.

= *Dothistroma pini* var. *lineare* Thyr & C.G. Shaw, Mycologia 56: 107. 1964.

≡ *Dothistroma septosporum* var. *lineare* (Thyr & C.G. Shaw) B. Sutton, The Coelomycetes. Fungi imperfecti with pycnidia acervuli and stromata (Kew): 173. 1980.

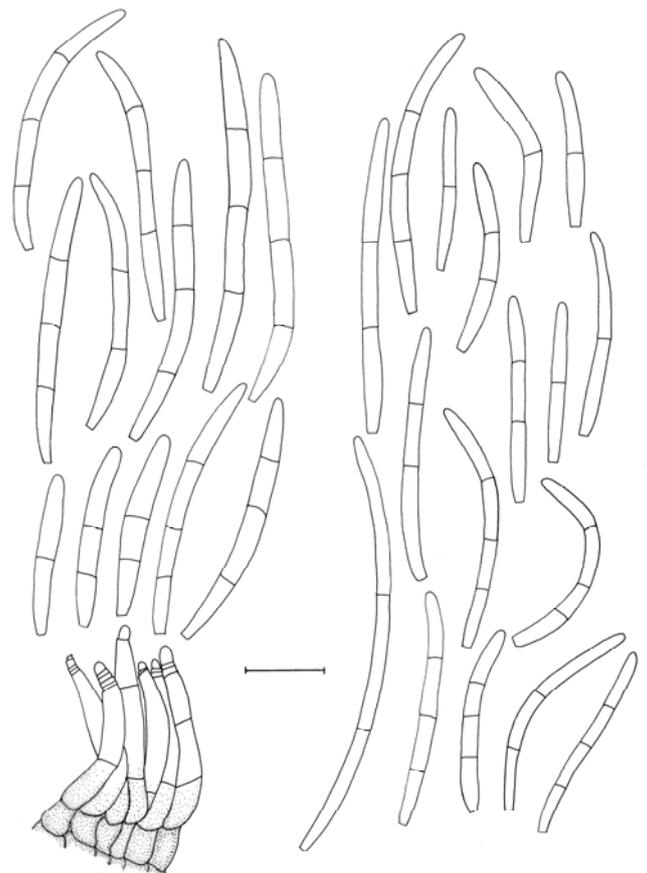
= *Dothistroma pini* var. *keniense* M.H. Ivory (as “*keniensis*”), Trans. Br. mycol. Soc. 50: 294. 1967.

≡ *Dothistroma septosporum* var. *keniense* (M.H. Ivory) B. Sutton, The Coelomycetes. Fungi imperfecti with pycnidia acervuli and stromata (Kew): 174. 1980.

*Teleomorph: Mycosphaerella pini* E. Rostr., Dansk bot. Ark. 17(1): 312. 1957.

≡ *Eruptio pini* (Rostr.) M.E. Barr, Mycotaxon 60: 438. 1996.

= *Scirrhia pini* A. Funk & A.K. Parker, Canad. J. Bot. 44: 1171. 1966.

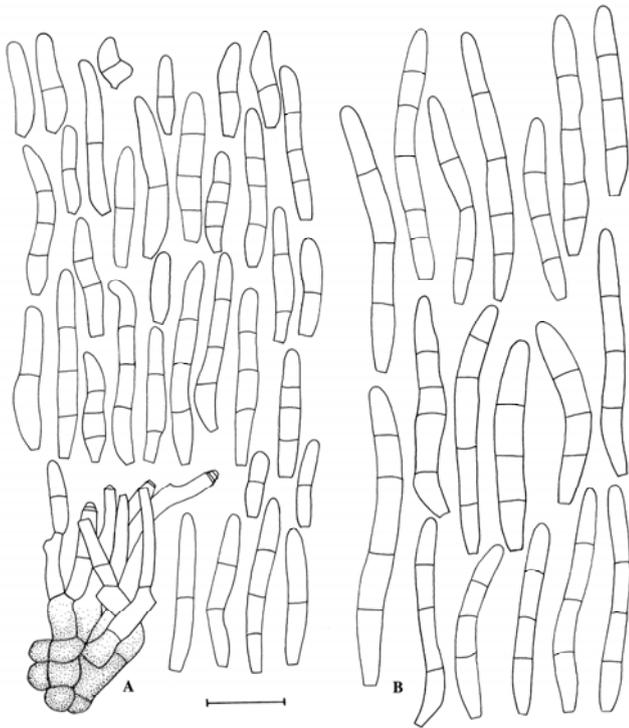


**Fig. 10.** Conidia and conidiogenous cells of *Dothistroma pini* from Michigan on *Pinus nigra* (herb. CBS 12211). On needles (left), and on oatmeal agar (right). Scale bar = 10  $\mu$ m.

≡ *Mycosphaerella pini* (A. Funk & A.K. Parker) Arx, Proc. K. Ned. Akad. Wet., Ser. C 86(1): 33 (1983) (nom. illegit., Art. 53).

*Conidiomata* predominantly occurring in red bands on the upper and lower needle surface, separate to aggregated, sub-epidermal, becoming erumpent, and splitting the needle surface with one or two longitudinal slits; at maturity acervular, black, up to 1 mm in length, lined internally with pseudoparenchymatous cells giving rise to conidiophores; these cells brown, becoming paler at the point of conidiophore attachment. *Conidiophores* pale brown to hyaline, smooth, densely aggregated, subcylindrical to irregular, 0–4-septate, branched or simple, 7–25  $\times$  2–3.5  $\mu$ m. *Conidiogenous cells* integrated, hyaline, smooth, subcylindrical, tapering towards flattened apices, proliferating percurrently or rarely sympodially near the apex, 7–15  $\times$  2–3  $\mu$ m. *Conidia* aggregated in cream to pale brown, slimy masses; smooth, thin-walled, hyaline, subcylindrical to narrowly obclavate, long subobtuse at the apices, truncate to obconically subtruncate at the bases, (1–)3(–5)-septate, (18–)26–30(–40)  $\times$  2(–2.5)  $\mu$ m (av. 28  $\times$  2  $\mu$ m) *in vivo*, (15–)25–30(–40)  $\times$  1.5–2(–2.5)  $\mu$ m (av. 28  $\times$  2  $\mu$ m) *in vitro*.

**Notes:** Amplification of the ITS1/5.8S/ITS2 region using primers ITS1 and ITS4 results in three polymorphisms distinct from those seen in *D. pini* at positions 68, 115 and 318. The polymorphism at position 318 does not result in the addition of an *AluI* restriction site, and thus, upon exposure of the PCR product to *AluI*, the fragment retains its original length of 520 base pairs.



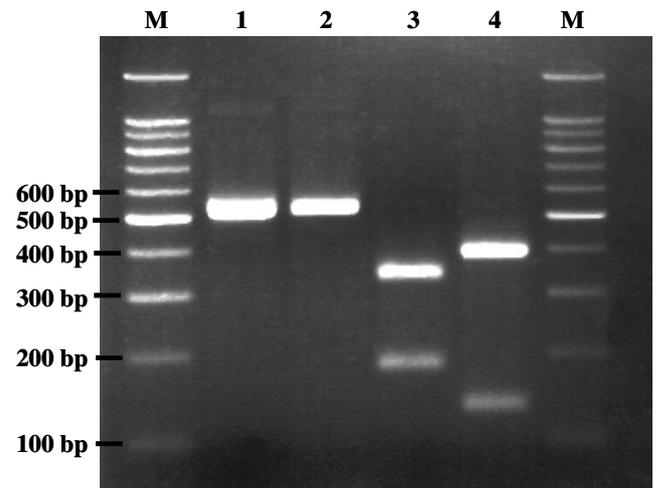
**Fig. 11.** Conidia and conidiogenous cells of *Dothistroma septosporum* from Poland on *P. nigra* (herb. CBS 12209). A. on needles. B. on oatmeal agar. Scale bar = 10 µm.

**Specimens examined:** **Austria**, Thenneberg, from *Pinus nigra*, Apr. 2004, T. Kirisits, herb. CBS 12205. **Chile**, near Valdivia, from *Pinus radiata*, 2001, M.J. Wingfield, herb. CBS 12206. **Ecuador**, Lasso Highlands, *Pinus muricata* D. Don., 2001, M.J. Wingfield, herb. CBS 12207. **New Zealand**, Rotorua, FRI nursery, from *Pinus radiata*, 2001, M. Dick, herb. CBS 12208. **Poland**, Miechów Forest District, Goszcza Forest Unit, Compartment 71 h, approx. 20 km from Cracow, isolated from 19-yr-old *Pinus nigra* in a seed plantation, Jun. 2003, Tadeusz Kowalski, herb. CBS 12209, culture CMW 13004 = CBS 116488, culture CMW 13010. **South Africa**, Tzaneen, from 6-yr-old *Pinus radiata*, 2002, M.J. Wingfield, herb. CBS 12210, culture CMW 11372 = CBS 116489. *Actinothyrium marginatum* Sacc., **U.S.A.**, Meadow Creek, Clearwater Ranger District, Idaho, isolated from *Pinus ponderosa*, Jun. 1957, Fred Matzner, WSP 48361; Idaho, Lochsa Historical Ranger Station, *Pinus ponderosa*, Jun. 2004, L.M. Carris, herb. CBS 12204. *Cytosporina septospora* Dorog., **Ukraine**, Kiev Guberniya, Smiela, *Pinus sylvestris* L., 25 Mar. 1914, L. Kaznowski, LE 116244, herb. CBS 11381.

#### PCR-RFLP diagnostic procedure

The ITS regions were selected for the construction of a simple diagnostic RFLP test to distinguish between *Dothistroma pini* and *D. septosporum* s. str. This

gene region was chosen because it showed no variation within the two lineages. This lack of variation suggests that this method will remain robust even if other isolates from different countries are to be tested. At position 319 of the ITS GenBank sequences (GenBank sequences are shorter than the PCR products here obtained due to the splicing off of sequence ends for alignment purposes), the transition from A to G creates an *AluI* restriction site in *D. pini*, producing fragments of ~170 and ~350 base pairs in length. This restriction site is not present in *D. septosporum* s. str. The only other recognised *Dothistroma* species, *D. rhabdoclinis*, has a restriction site for *AluI* at base pair position 371, giving it an RFLP profile distinguishable from those of the red band fungi (Fig. 12).



**Fig. 12.** PCR-restriction fragment length polymorphism (RFLP) pattern of the three *Dothistroma* species digested with the restriction enzyme *AluI*. A 100 bp marker (M) is on either side of the gel. Lane 1: uncut PCR amplicon (CMW 14822) used as a control; Lane 2: *D. septosporum* (CMW 6841) from Lineage I is not digested by *AluI*, Lane 3: the digested product of *D. pini* (CMW 14820) from Lineage II producing 2 bands of ~170 and ~350 in length; Lane 4: digested product of *D. rhabdoclinis* (CMW 12519) producing 2 bands of ~120 and ~400 bp in length.

## DISCUSSION

Comparisons of DNA sequence data for four regions of the genome have shown clearly that the very serious pine disease known as red band needle blight, also referred to as *Dothistroma* needle blight, is caused by two distinct fungi. These fungi, *D. septosporum* and *D. pini*, make up two distinct phylogenetic lineages. *Dothistroma septosporum* has a worldwide distribution and it is the causal agent of the disease that has severely damaged plantations of *P. radiata*, grown as an exotic in the Southern Hemisphere. In contrast, *D. pini* is a serious pathogen of pines that currently appears to be restricted in distribution to the North Central United States.

DNA sequence comparisons provide no support for separating the red band needle blight fungus into three varieties based on conidial dimensions. Isolates from



Idaho representing the variety “*linearis*” have the same DNA sequence as isolates from Africa representing the variety “*keniense*” as do those from Chile and New Zealand thought to be of the variety “*pini*”. We, therefore, support the views of Sutton (1980) and Evans (1984) rejecting the use of varietal names in *Dothistroma*. Although various morphotypes and ecotypes of *Dothistroma* have been suggested by Ivory (1967) and Evans (1984), no evidence of these was observed in the current study based on sequence data.

Species delimitations for a global collection of red band needle blight fungi were identified using multiple gene genealogies in this study. The 9.7 % divergence between these lineages, compiling polymorphisms in all four gene regions investigated, corresponds with what has been accepted as significantly different in previous species descriptions based on phylogenetic characters. For example, Couch & Kohn (2002) described a new species, *Magnaporthe oryzae*, based on a 9.7 % divergence observed within multilocus gene genealogies. Likewise, O’Donnell *et al.* (2004) recently presented formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade, based on fixed nucleotide characters observed in multiple gene phylogenies.

An important aspect of this study is that it incorporated a large number of isolates and sequences from four different gene regions. Bradshaw *et al.* (2002) compared several isolates of *D. septosporum* based on a small portion of the ITS region. Their results revealed only two nucleotide polymorphisms differing between North Central U.S.A. isolates and isolates from other parts of the world, and they therefore concluded that the fungi were conspecific. Goodwin *et al.* (2001), considered the phylogenetic relationships among *Mycosphaerella* species, and happened to include two *D. septosporum* sequences obtained from GenBank in their analyses. Although they were not aware of it, these two sequences coincidentally came from each of the distinct lineages recognised in the present study. The distinction between these isolates, and their differing placement in the larger *Mycosphaerella* group, can clearly be seen in the ITS ribosomal DNA phylogram in that paper. Although Goodwin *et al.* (2001) focussed on *Mycosphaerella* and did not discuss *Dothistroma*, their results support those presented here.

Recognition that two species cause the single disease known as red band needle blight has important consequences for disease control and quarantine. Our choice has been to retain the names that have been most closely associated with the red-band fungus and to amend the description of *D. septosporum* to exclude the genetically distinct isolates from Central U.S.A. We have consequently also restored the use of *D. pini* to represent this distinctly different fungus that occurs in the North Central United States, including Illinois,

where the type specimen of *D. pini* was collected. This specimen, described by Hulbary in 1941, could not be analysed based on sequence data but is morphologically consistent with isolates in phylogenetic Lineage II/*D. pini*. All other isolates associated with red band needle blight, including those from Western North America and Europe, are in Lineage I. They should be referred to as *D. septosporum* as proposed by Morelet (1968).

*Dothistroma pini*, as opposed to *D. septosporum*, has a limited host and geographical range. Within its range in Minnesota, Nebraska, Illinois, and Michigan, however, the exotic species, *P. nigra* is severely damaged by it, particularly in Christmas tree plantations (Peterson 1974). Our interpretation of the observations of Thyr & Shaw (1964) is that collections from Kansas and Kentucky assigned to the variety “*pini*” probably represent *D. pini*. If this were the case, then the host range of *D. pini* would be broadened to include the tree species considered in that study, *P. mugo* Turra (as *P. montana* Mill.).

The teleomorph *Mycosphaerella pini*, associated with the red band fungus, was not observed in the current study. So far, it has been reported only from Central America (Evans 1984), the western U.S.A. (Peterson 1974), western Canada (Funk & Parker 1966) and Europe (Kowalski & Jankowiak 1998). The original description of *M. pini* was from needles of *Pinus sylvestris* collected in Denmark. *Scirrha pini*, a synonym (Evans 1984), was described from needles of *Pinus contorta* Douglas ex Loudon from British Columbia, Canada (Funk & Parker 1966), and has been linked taxonomically to the anamorph *D. septospora* var. *lineare* (Ivory 1967). This dictates that *M. pini* is connected to the fungus reflected by phylogenetic Lineage I with the anamorph *D. septosporum*. The separation of *M. pini* into a separate genus, *Eruptio* M.E. Barr (Barr 1996), was refuted by Crous *et al.* (2001), who showed that *Eruptio* is a synonym of *Mycosphaerella*.

In this study, we have been able to provide a simple and relatively rapid method to distinguish between *D. pini* and *D. septosporum*. This should be particularly useful because the fungi are similar in morphology and ecology, and cause similar symptoms on hosts in the genus *Pinus*. DNA sequencing facilities are not always available for comparison of fungi and the more accessible PCR-RFLP technique may facilitate correct identification.

The only other species of *Dothistroma* is *D. rhabdoclinis*. This fungus is associated with *Rhabdocline pseudotsugae* Syd. as a hyperparasite on *Pseudotsuga menziesii* (Butin 2000). Although *D. rhabdoclinis* is clearly distinguishable from *D. septosporum* and *D. pini* based on morphological and cultural as well as symptom and host differences (Butin 2000), it can also be distinguished with this PCR-RFLP test and with sequence data.

Dothistroma or red band needle blight is one of the most important diseases of pines in the world. Some of the most serious damages caused by this disease have been seen in plantations of exotic species such as those of *P. radiata* in the Southern Hemisphere and plantations of native species, such as *P. ponderosa*, and exotics, such as *P. nigra*, in the United States. Recognition that two different fungi are associated with this disease has substantial implications for global tree health. Accidental introduction of *D. pini*, clearly a serious pathogen of *P. nigra*, could have very significant negative consequences in areas of Europe where this tree is native. Whether *P. radiata* and other species widely planted as exotics in the tropics and Southern Hemisphere are susceptible to *D. pini* is unknown but its accidental introduction into new areas could be catastrophic. Likewise, its introduction into temperate areas where as yet unelucidated, vulnerable hosts may grow, might have very severe consequences. The global distribution of *D. septosporum* implies that these fungi are easily moved into new environments, most probably with seeds. The potential threat of *D. pini* to pine forestry worldwide clearly deserves serious consideration.

## ACKNOWLEDGEMENTS

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## Four species of *Zygothiala* (Schizothyriaceae, Capnodiales) are associated with the sooty blotch and flyspeck complex on apple

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**Abstract:** Sooty blotch and flyspeck (SBFS) is a complex of fungi that cause late-season blemishes of apple and pear fruit that cosmetically damage the cuticle, which result in fruit that are unacceptable to consumers. Previous studies reported that a single, wide-host-range species, *Schizothyrium pomi* (presumed anamorph *Zygothiala jamaicensis*), caused flyspeck on apple. In the present study we compared morphology and DNA phylogeny (ITS, LSU) of 139 fungal strains isolated from flyspeck signs from 39 apple orchards in 14 midwestern and eastern states (USA). Parsimony analysis, supported by cultural characteristics and morphology in vitro, provided support to delimit the flyspeck isolates into four species of *Zygothiala*, two of which are known to be sexual. Three of these species are described as new. Based on DNA phylogeny, species of *Schizothyrium* were shown to cluster with members of the genus *Mycosphaerella* in the Capnodiales, having similar asci and ascospores but morphologically distinct ascospores. These data question the value of ascospore morphology at the ordinal level, although it still appears to be relevant at the family level, delimiting the thyrothecial Schizothyriaceae from other families in the Capnodiales.

**Key words:** anamorph, plant pathology, SBFS, *Schizothyrium pomi*, *Zygothiala jamaicensis*

### INTRODUCTION

Sooty blotch and flyspeck (SBFS) are late-season blemishes on the cuticle of apples and pears in humid regions worldwide, resulting in produce that is unacceptable to fresh market consumers. Fungi in the

SBFS complex grow superficially on the epicuticular wax, do not penetrate the cuticle (Belding 2000) and may use exuded nutrients present on the apple surface (Baker 1977, Nasu and Kunoh 1987b, Wrona 2004, Wrona and Gleason 2005, Le Corronc et al 2006). The term “flyspeck” designates colonies in the SBFS complex that develop clusters of shiny, black, round to ovoid, sclerotium-like bodies and have no visible mycelial mat. *Schizothyrium pomi* (Mont. & Fr.) Von Arx (presumed anamorph *Zygothiala jamaicensis* E.W. Mason) has been described as the cause of flyspeck (Baines 1940, Baker 1977). In contrast the term “sooty blotch” designates fungi in the complex that form a dark mycelial mat with or without sclerotium-like bodies. Several newly described SBFS fungi, referred to as compact speck and discrete speck, closely resemble flyspeck, but they can be distinguished from flyspeck by the absence of ring-like remnants of the sclerotium-like bodies on the apple cuticle when the bodies are removed and by size and density of sclerotium-like bodies (Batzer et al 2005).

What is now recognized as the SBFS complex initially was described from apples collected in Pennsylvania, USA, as *Dothidea pomigena* Schwein. (Schweinitz 1834). Diverse colony morphologies on blemished fruit were thought to be caused by a single species, and flyspeck and sooty blotch were presumed to be developmental stages of the same fungus (Montagne 1834, Sprague 1856, Duggar 1909). Colby (1920) however concluded that sooty blotch and flyspeck were caused respectively by separate fungi, *Gloeodes pomigena* (Schwein.) Colby and *Leptothyrium pomi* A. Selby. The name *L. pomi* was synonymized with *Mycothyriella rubi* Petr. (Baines 1940), but it later was recognized as *Schizothyrium pomi* (Mont. & Fr.) Von Arx (Baker et al 1977). In the past 10 y, the SBFS complex has been further expanded to include as many as 30 species based on a combination of genetic and morphological evidence (Johnson and Sutton 1994; Johnson et al 1996, 1997; Batzer et al 2005).

*Schizothyrium pomi* was linked to its presumed anamorph, *Z. jamaicensis*, when immature apple fruit inoculated with ascospores produced both the sexual and asexual stages (Durbin et al 1953). Numerous hosts of *Z. jamaicensis* subsequently have been identified, including 120 species in 44 families of seed plants throughout temperate and tropical

regions (Baines 1940, Baker et al 1977, Sutton et al 1988, Nasu and Kunoh 1987a). Although isolates from these diverse hosts were morphologically similar, they were observed to differ in their cultural characteristics (Durbin et al 1953). However cross-inoculation studies gave no evidence for host specialization (Baker et al 1977, Nasu and Kunoh 1987b), and Nasu and Kunoh (1993) conjectured that *Z. jamaicensis* might be able to survive on all plants whose surfaces are covered by a waxy bloom, unless antifungal substances or inadequate nutritional sources prevent fungal growth.

Several *Schizothyrium* species were named for the host from which they were isolated but subsequently were found to be morphologically similar. For example *S. acerinum*, *S. gaultheria* and *S. reticulatum* were shown to be synonymous with the flyspeck fungus *S. pomi* (von Arx 1959). Although 12 *Schizothyrium* species were recognized by von Arx and Müller (1975), only a single anamorph species, *Z. jamaicensis*, has been reported.

Conidiophores of *Zygothiala* arising from superficial hyphae have a distinctive conidiophore morphology, namely a foot cell that gives rise to a twisted, or curved, dark brown, smooth-walled stipe, which tends to be widest in the middle, an angular, subhyaline, finely verruculose terminal cell and at its apex, two (rarely three) laterally divergent, pale brown, finely verruculose, ovate to ampulliform to elongated subcylindrical conidiogenous cells that bear one to several prominently thickened, circular, darkened and somewhat refractive conidial scars. Conidia are produced in pairs, have a slightly granular surface, are medianly or unevenly 1-septate (rarely multiseptate), ellipsoidal to ovate (rarely obclavate), constricted at septa, with prominently thickened, darkened, refractive scars. Although the morphology of diverse *Zygothiala* isolates has been compared, these observations have not been used to distinguish additional species. Nasu et al (1985) distinguished two isolates based on differing growth patterns, colony color, numbers of sclerotium-like body produced, optimal temperature and pH ranges. Lerner (2000) also grouped 30 isolates from six eastern states in the USA based on growth rate and colony morphology.

During a survey in 2000 of nine apple orchards in five states in the midwestern USA, four putative species of *Zygothiala* were delineated based on their morphology on the host and cultural growth characteristics. These isolates and other flyspeck isolates collected during a survey in 2005 covering 30 apple orchards in 10 eastern states were used for taxonomic study. The aim of the present study was to identify and describe species of flyspeck fungi based on DNA phylogeny and phenotype.

## MATERIALS AND METHODS

*Sources of isolates.*—Three isolates of *Schizothyrium pomi* were obtained from the CBS collection (TABLE I). Three isolates identified as *S. pomi* were also kindly provided by Dr Turner B. Sutton of North Carolina State University (NCSU). All other isolates were obtained from orchards surveyed in the eastern and midwestern USA (TABLE I). In autumn 2000 isolates were obtained from SBFS colonies on 40 apples harvested from each of nine orchards in Iowa, Illinois, Missouri and Wisconsin. In autumn 2005 a similar survey was conducted from 30 orchards in 10 eastern states (Georgia, North Carolina, Virginia, Kentucky, Tennessee, New York, Massachusetts, Pennsylvania, Ohio and Michigan). Approximately 12 flyspeck colonies were selected arbitrarily from apples sampled from each orchard. Isolations were made as described by Batzer et al (2005). A total of 139 flyspeck isolates were purified and stored in glycerol at  $-80^{\circ}\text{C}$ . Segments of apple peels with flyspeck signs were preserved by pressing the thallus and supporting peel between paper towels until dry. Representative cultures were deposited at the Centraalbureau voor Schimmelcultures (CBS), Fungal Biodiversity Centre, Utrecht, The Netherlands, and specimens on apple peels were deposited at the Iowa State University Herbarium, Ames, Iowa, and at CBS.

*Polymerase chain reaction and sequencing.*—The internal transcribed spacer region of the ribosomal DNA (ITS1, 5.8S rDNA gene, ITS2) of 130 isolates from flyspeck-like colonies was sequenced. A portion of the 28S (large subunit, LSU) rDNA gene was sequenced for representative isolates of each clade identified by parsimony analysis of the ITS region.

For isolates obtained in 2000, template DNA for polymerase chain reaction (PCR) was obtained by scraping mycelia with a pipette tip from 4- to 6 wk old cultures grown on PDA (Harrington and Wingfield 1995). For the isolates obtained in 2005, DNA was extracted from mycelia with Prepman Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California). Primer pairs used for amplification and sequencing of the ITS region were ITS-1F/ITS4 (White et al 1990), and primer pairs used for amplification and sequencing of LSU were respectively LR0R/LR5 and LR0R/LR3 (Vilgalys and Hester 1990). Amplification reactions consisted of 4 mM  $\text{MgCl}_2$ , 5% DMSO, 1 $\times$  Sigma buffer, 200  $\mu\text{M}$  dNTPs, 0.5  $\mu\text{M}$  of the forward and reverse primers, and 3 units of *Taq* polymerase (Sigma Chemical Co., St Louis, Missouri). Cycling conditions (MJ Research Inc. thermocycler, PTC-100 Waltham, Massachusetts) for amplifications were an initial denaturation at 94 C for 95 s followed by 35 cycles of denaturation at 94 C for 35 s, annealing at 49 C for LSU and at 52 C for ITS for 60 s, and extension at 72 C for 2 min. The PCR product was purified with a QIAquick DNA Purification Kit (QIAGEN, Valencia, California) and quantified on a Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech, San Francisco, California). Automated sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility.

TABLE I. Accession numbers from Centraalbureau voor Schimmelcultures (CBS), Iowa State University Herbarium and GenBank for partial rDNA sequences of *Zygothiala* spp. occurring on apple fruit

Species	Strain CBS Accession No.	Herbarium Accession No.	GenBank Accession		
			LSU	ITS	
<i>Schizothyrium pomi</i>	CUA1a, CBS 118957	438789, CBS-H19787	AY598895	EF164898	
	ZJ001		AY598894	AY598848	
	ZJ002			AY598849	
	ZJ003			AY598850	
	AHA2a			AY598851	
	GTA1a			AY598852	
	CBS 228.57			EF134947	EF134947
	CBS 406.61			EF134949	EF134949
	CBS 486.50			EF134948	EF134948
<i>Zygothiala cryptogama</i>	FVA2a, CBS 118949	438791, CBS-H19785	AY598896	AY598854	
	MWA8a			EF164899	
	KYI 1.2A1c			EF164902	EF164900
<i>Zygothiala tardicrescens</i>	MWA1a, CBS 118946	438792, CBS-H19788	EF164901	AY598856	
<i>Zygothiala wisconsinensis</i>	MSTA8a, CBS 118950	438790, CBS-H19786	AY598897	AY598853	
	GTA4b			AY598855	

*Sequence alignment and phylogenetic analysis.*—Sequences were imported into BioEdit (Hall 1999), and the 5'- and the 3'- ends were trimmed to aid alignment. Length of the ITS sequences analyzed was approximately 485 base pairs. Preliminary alignments of the ITS sequences were generated with Clustal X (Thompson et al 1997) with gap opening and gap extension parameters of 50:5, and these alignments were optimized manually. Isolates with redundant ITS and LSU sequences obtained from the same orchard were eliminated from the dataset, reducing the number of isolates in the analyses from 130 to 82 and 45 to 13 respectively. Maximum parsimony (MP) analysis was performed with PAUP v.4.0b10 (Swofford 2002). Heuristic searches were conducted with a 1000 random sequence additions and tree bisection-reconnection (TBR) branch swapping algorithms, collapsing zero-length branches, and saving all minimal length trees. MAXTREES was set at 10 000. Alignable gaps were treated as a "fifth base". All characters were given equal weight. To assess the robustness of clades and internal branches, a strict consensus of the most parsimonious trees was generated and a bootstrap analysis of 1000 replications was performed. We rooted the LSU tree to four species from the Chaetothiales (*Ceramothyrium carniolicum* [Rehm] Petr., *Exophiala dermatitidis* [Kano] de Hoog, *Rhinocladiella atrovirens* Nannf. and *Ramichloridium anceps* [Sacc. & Ellis] de Hoog). Outgroup for ITS phylogenetic analysis was *Mycosphaerella marksii* Carnegie & Keane. MP analysis, treating gaps as missing data, also was conducted on the LSU alignment because of concerns that gaps could be over-weighted in the analysis where gaps were treated as a fifth character. Alignments and the representative trees (FIGS. 1, 2) were deposited in TreeBASE SN3221.

*Morphology of SBFS isolates on apple and in vitro.*—Signs of SBFS on preserved apple peels were described, including mycelial growth patterns and fruiting body size and density.

Colony descriptions were made after 1 mo growth on oatmeal agar (OA) at 21–24 C under intermittent ambient light. Fungal structures were mounted in clear lactic acid and examined at 1000× magnification. Thirty measurements were determined for each structure. For conidial measurements, the 95% percentiles are presented and extremes given in brackets.

## RESULTS

*Phylogenetic analysis.*—The ITS alignment contained 83 taxa (including outgroup), and 481 characters were used for the analyses. Of these characters, 33 were parsimony informative, 101 were variable and parsimony uninformative and 347 were constant. The 24 equally parsimonious trees obtained from ITS analysis delimited four putative species of *Zygothiala* (FIG. 1). The largest clade (86% bootstrap support) consisted of 102 isolates and included isolates from all 14 states surveyed and from 30 of the 39 orchards. This clade contained three strains from the CBS culture collection and was identified as *S. pomi*. Three other clades, representing previously undescribed species, also were delimited in the ITS analysis. The first of these was poorly supported but appeared sister of the *S. pomi* clade. Isolates from this clade were obtained from Iowa, Ohio, Michigan and Kentucky, and the species is described as *Zygothiala cryptogama* sp. nov. A well supported clade (89% bootstrap support) contained isolates obtained from Wisconsin, Ohio, Michigan, Virginia and Missouri and is described as *Zygothiala wisconsinensis* sp. nov. Isolates from the last clade (100% bootstrap support and sister of *Z. wisconsinensis*) were obtained from a

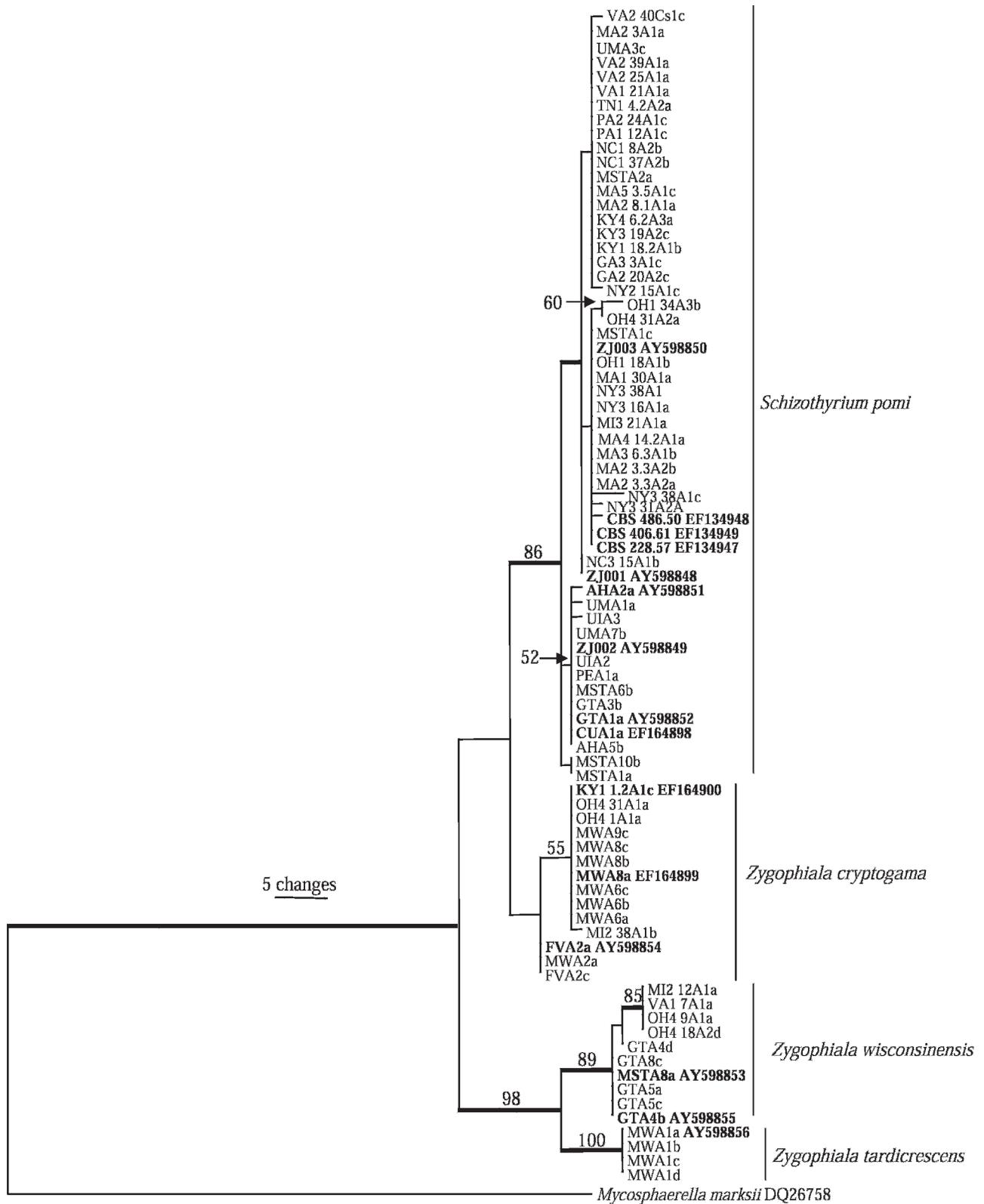


FIG. 1. One of 24 equally most parsimonious trees determined from ITS sequences obtained from isolates taken from flyspeck signs on apple fruit from eastern and midwestern orchards. Bootstrap support values (>50%) based on 1000 replicates are shown at the nodes, and strict consensus branches are thickened. The tree is rooted to *Mycosphaerella marksii* and new sequences deposited in GenBank are printed in boldface. Tree length = 167, consistency index = 0.898, retention index = 0.969, rescaled consistency index = 0.867.

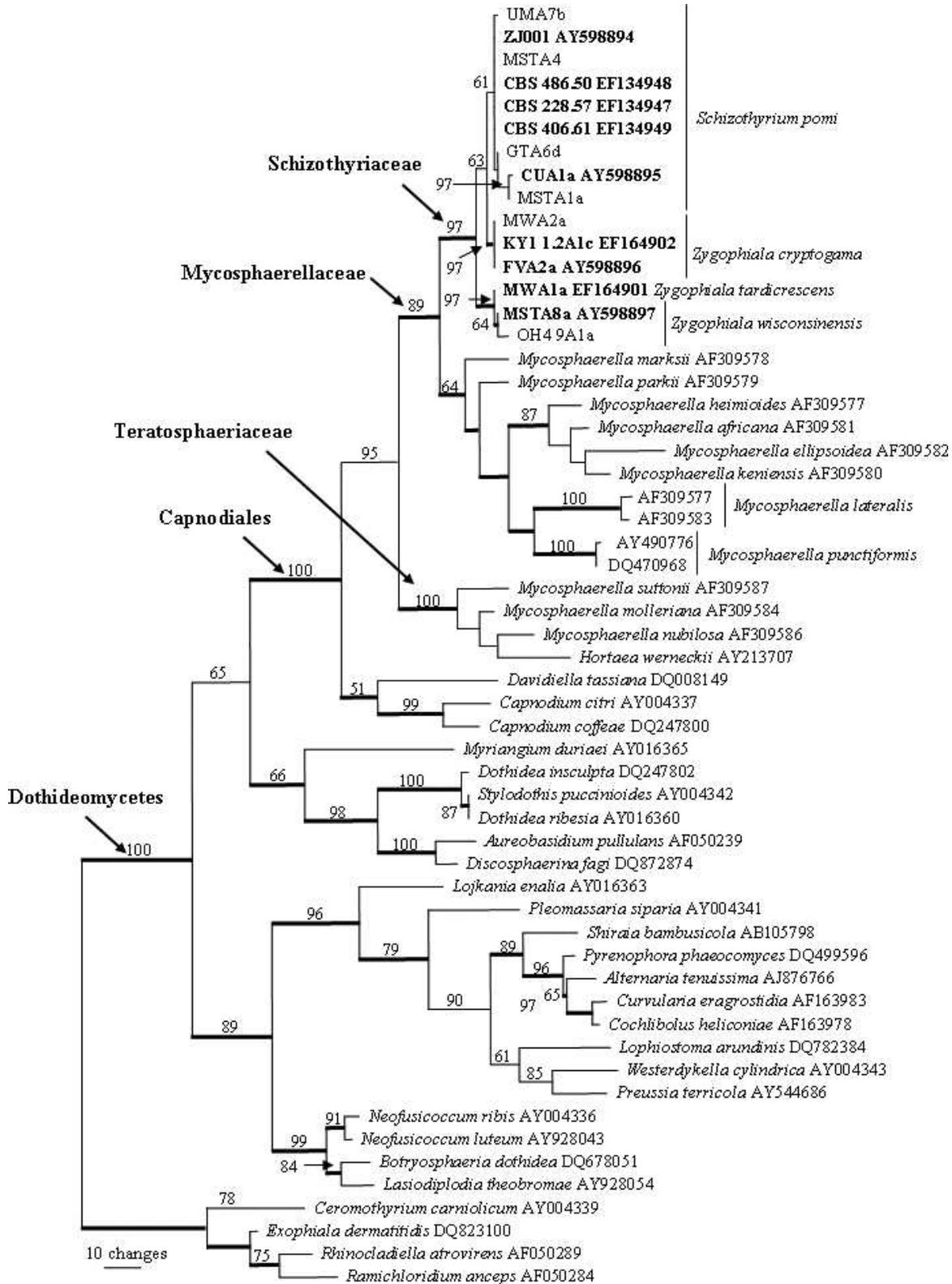


FIG. 2. One of 10 equally most parsimonious trees of partial sequences of the 28S large subunit (LSU) region of rDNA from flyspeck isolates on apple fruit from eastern and midwestern orchards and other ascomycetes. Bootstrap support values (>50%) based on 1000 replicates are shown at the nodes, and strict consensus branches are thickened. The tree is rooted to four species from the Chaetothyriales (*Ceromathyrium carniolicum*, *Exophiala dermatitidis*, *Rhinochadiella atrovirens* and

single Iowa orchard and are described as *Zygophiala tardicrescens* sp. nov.

The LSU alignment contained 56 taxa (including the four outgroup taxa) and 554 characters were used for the analyses. Of these characters 215 were parsimony informative, 42 were variable and parsimony uninformative and 297 were constant. Maximum parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (FIG. 2). Parsimony analysis grouped the *Zygophiala* species within the Capnodiales (Schoch et al 2006) with bootstrap support value of 100%. The Schizothyriaceae formed a well supported (97% bootstrap support) clade within the Mycosphaerellaceae (95% bootstrap support) clade when gaps were treated as a fifth character. When gap treatment was altered to missing data, bootstrap support of the Mycosphaerellaceae was reduced to 63%. However the overall topology of the trees was almost identical when gaps were treated as missing characters.

**Taxonomy.**—Isolates could be grouped into four species based on their morphology on cultural media, growth characteristics and DNA phylogeny. Sclerotium-like bodies of *Schizothyrium pomi* on apple were round, 250(155–480)  $\mu\text{m}$  diam and with a density of 2.4/mm<sup>2</sup>. Sclerotium-like bodies of *Zygophiala cryptogama* were also round but slightly smaller, 230(150–364)  $\mu\text{m}$  diam, and more densely arranged, averaging of 3.6 sclerotium-like bodies/mm<sup>2</sup>. *Zygophiala wisconsinensis* sclerotium-like bodies were ovoid, larger, 380(300–450)  $\times$  500(425–600)  $\mu\text{m}$  and were more sparsely arranged with a density of 0.8/mm<sup>2</sup>. Sclerotium-like bodies of *Zygophiala tardicrescens* were similar to *S. pomi*, 260(250–270)  $\mu\text{m}$  diam and were arranged at a density of 2.8/mm<sup>2</sup>.

Three new species of *Zygophiala* were distinguished and are described below.

**Schizothyrium pomi** (Mont. & Fr.) Von Arx, Proc. K. Ned. Akad. Wet., Ser. C, Biol. Med. Sci. 62:336. 1959. FIGS. 3, 4.

$\equiv$  *Labrella pomi* Mont. (Fr. in litt.), Ann. Sci. Nat., Sér. 2, Bot. 1:347. 1834.

**Anamorph.** *Zygophiala* sp. (non *Z. jamaicensis* E.W. Mason).

**Ascomata** black, shiny, dimidiate, in random clusters, but frequently in circles, superficial on leaves, stems or fruit, appressed to the cuticle, 150–375  $\mu\text{m}$  diam, 30–50  $\mu\text{m}$  high, with irregular margins; upper

layer consisting of interwoven mycelium, forming 2–4 layers of thick-walled, brown, pseudoparenchymatal cells, 4–8  $\mu\text{m}$  thick; ostiole central, but upper layer splitting at maturity via irregular ruptures from the elevated center; ascomata situated on a thin, hyaline, basal stroma. *Hamathecium* hyaline, consisting of branched, septate, pseudoparaphysoid-like filaments, 3–5  $\mu\text{m}$  wide. *Asci* bitunicate, 8-spored, ovoid to subglobose or ellipsoid to clavate, apical chamber present but inconspicuous at maturity, 20–45  $\times$  8–16  $\mu\text{m}$ ; formed in a single layer in the hamathecial tissue. *Ascospores* hyaline, guttulate, thick-walled, medianly 1-septate, constricted at septum, fusoid-ellipsoidal, widest in the middle of the apical cell, which is acutely rounded, while the lower cell is subobtusely rounded, (10–)12–13(–14)  $\times$  (3–)3.5–4(–5)  $\mu\text{m}$ . Ascospores germinating after 24 h on MEA, becoming brown and verruculose, with a visible mucoid sheath surrounding the spore on the agar surface, slightly or not constricted at the septum, 4–5  $\mu\text{m}$  wide, not distorting, germinating from both ends, with 2–3 germ tubes; cultures are homothallic.

*Conidiophores* arising from superficial hyphae, 2–3  $\mu\text{m}$  wide, erect, scattered, 3–4-septate, subcylindrical, rarely straight, mostly flexuous, consisting of a hyaline to subhyaline supporting cell that gives rise to a smooth, dark brown stipe, 25–35  $\times$  7–8  $\mu\text{m}$  (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, 6–7  $\times$  6–7  $\mu\text{m}$ , that gives rise to two (rarely three) medium brown, finely verruculose, doliform to ellipsoid or subcylindrical, polyblastic conidiogenous cells, 8–12  $\times$  6–7  $\mu\text{m}$ ; scars prominent, apical, darkened, thickened, somewhat refractive, with 1(–2) per conidiogenous cell, 2  $\mu\text{m}$  wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, transversely 1(–7)-septate, prominently constricted at septa, (20–)22–25(–30)  $\times$  5–7(–8)  $\mu\text{m}$  if 1-septate but up to 110  $\mu\text{m}$  long if 7-septate; apex subobtuse, base subtruncate, with a darkened, thickened hilum, 2  $\mu\text{m}$  wide.

**Cultural characteristics.** Colonies after 2 wk on OA in the dark flat, spreading with sparse aerial mycelium and smooth, regular margins; pale olivaceous gray to olivaceous gray in the center, becoming cream to pale luteous toward the margin; developing erumpent ascomatal initials in older cultures.

**Specimen examined.** USA. ILLINOIS: Rockford, on apple fruit, Sep 2000, J. Batzer, 438789, CBS-H19787, cultures CUA1 = CBS 118957, GenBank: AY598895.

←

*Ramichloridium anceps*) and new sequences deposited in GenBank are printed in boldface. Tree length = 1002, consistency index = 0.438, retention index = 0.805, rescaled consistency index = 0.374.

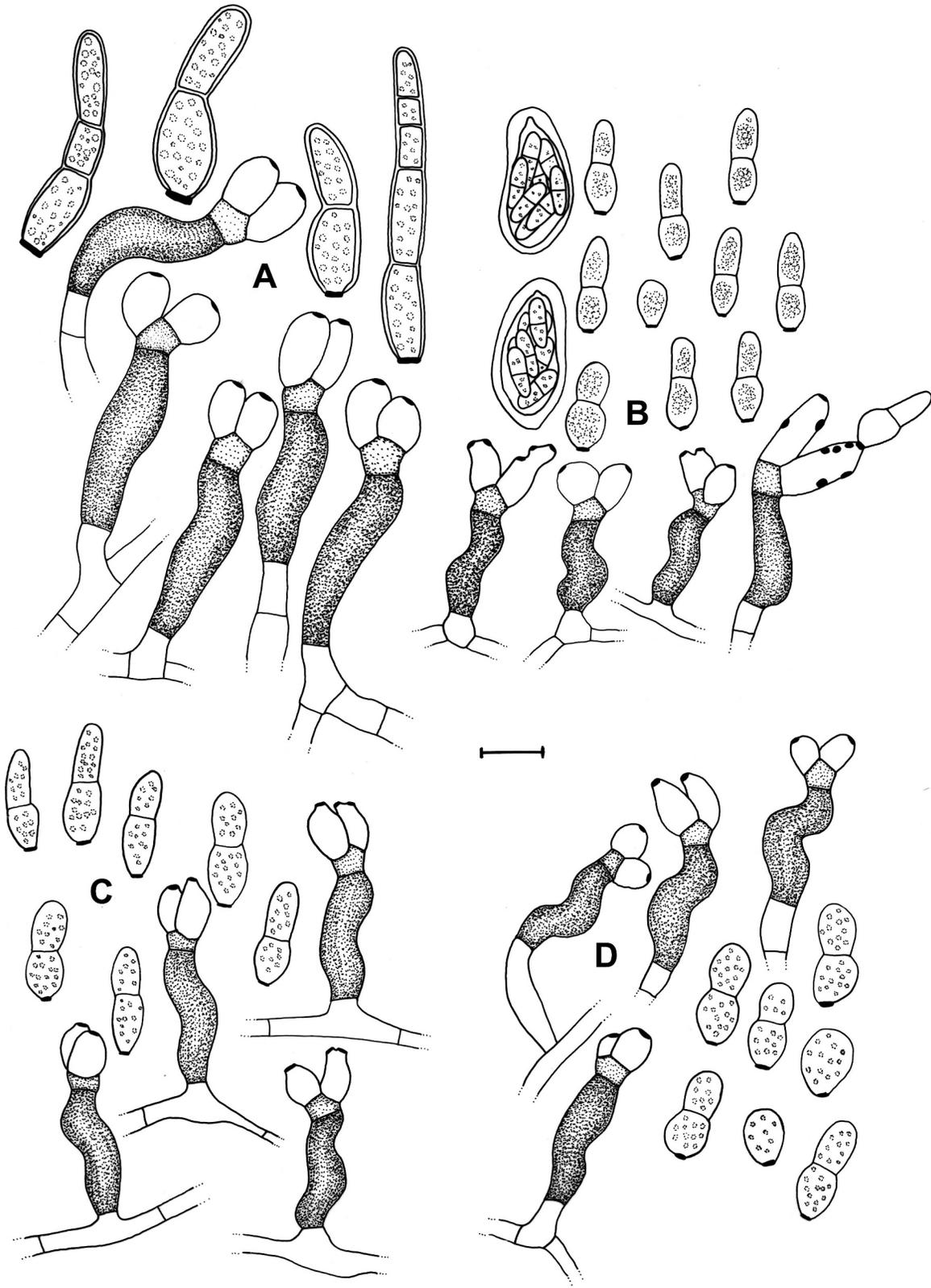


FIG. 3. *Zygophiala* spp. sporulation on oatmeal agar. A. Conidiophores and conidia of the *Zygophiala* anamorph of *Schizothyrium pomi* (CBS 118957). B. Asci, conidiophores and conidia of *Z. cryptogama* (CBS 118949). C. Conidiophores and conidia of *Z. tardicrescens* (CBS 118946). D. Conidiophores and conidia of *Z. wisconsinensis* (CBS 118950). Bar = 10  $\mu$ m.

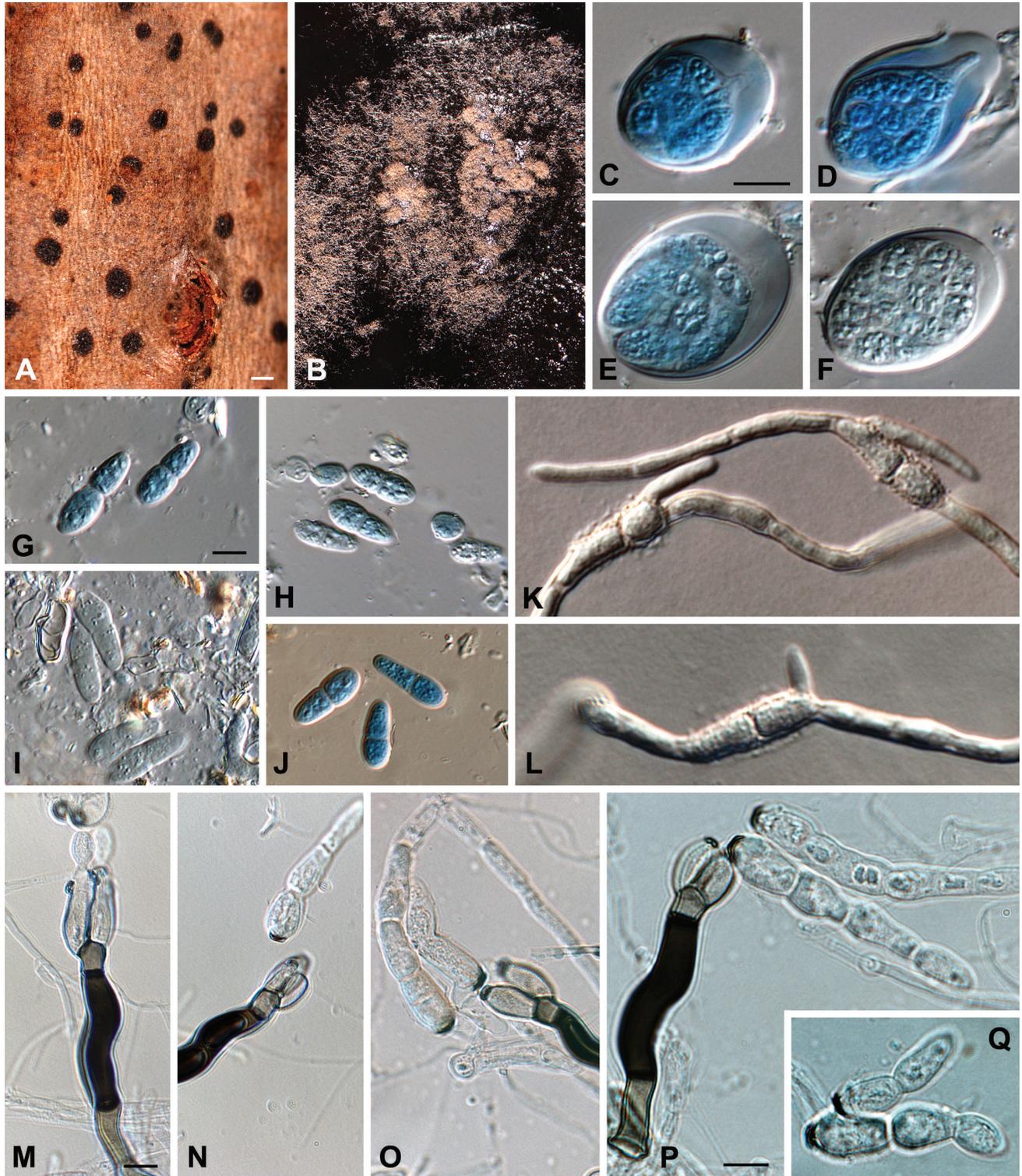


FIG. 4. *Schizothyrium pomi* and its *Zygothiala* anamorph. A. Thyrothecia occurring on a *Rhus* stem. B. Ascomatal initials forming on oatmeal agar. C-F. Asci. G-J. Ascospores. K-L. Germinating ascospores. M-Q. Conidiophores and conidia in vitro. Bars: C = 6, G = 5, M, P = 8  $\mu$ m.

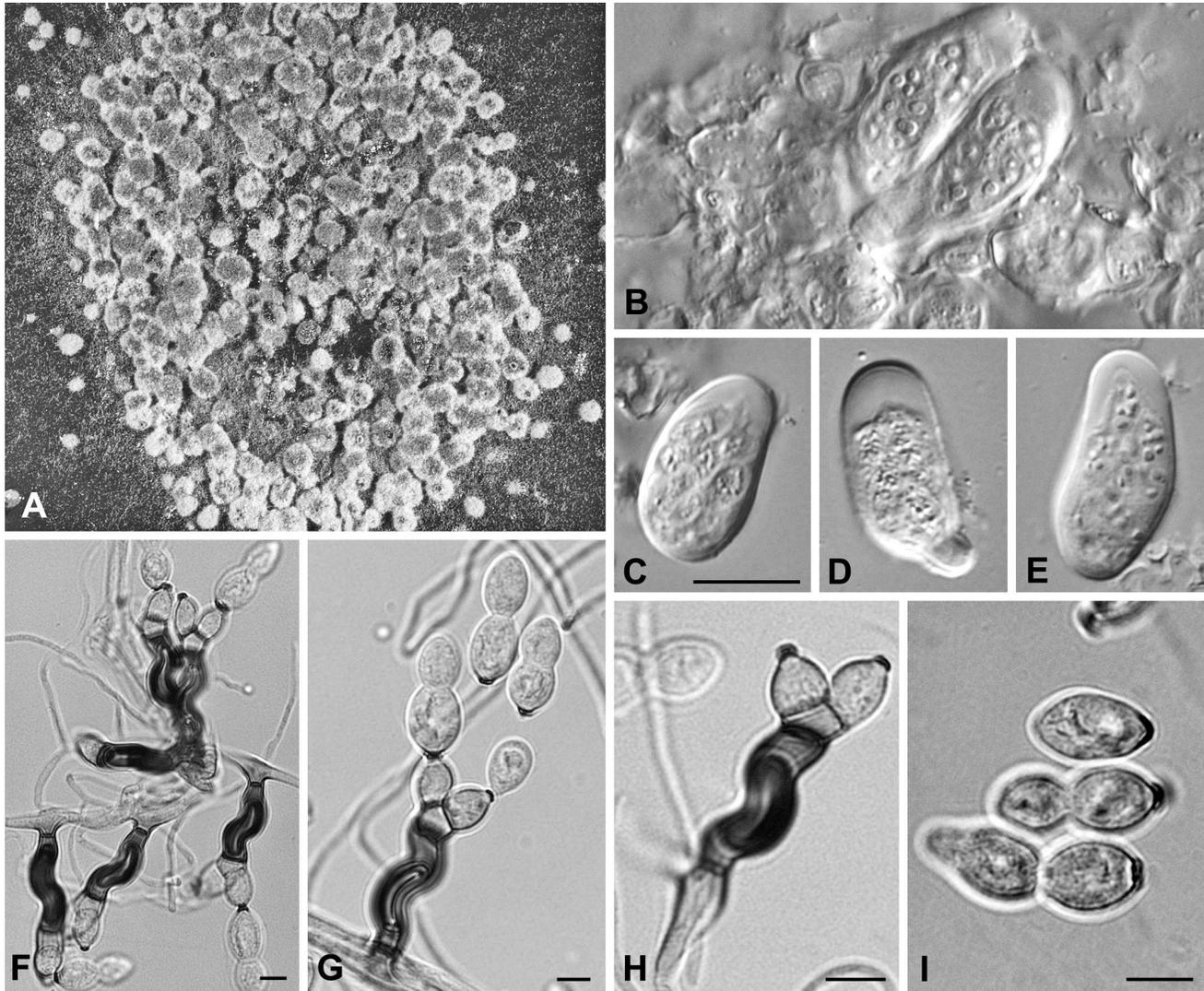


FIG. 5. *Zygophiala cryptogama* on oatmeal agar (CBS 118949). A. Ascomatal initials. B–E. Asci. F–H. Conidiophores. I. Conidia. Bars: C = 13, F = 4, G, H = 5, I = 6  $\mu\text{m}$ .

*Notes.* The link between *Schizothyrium pomi* and *Zygophiala jamaicensis* was established by Durbin et al (1953), who inoculated apple fruit with ascospores, which resulted in both the teleomorph and anamorph states developing. This relationship has been observed numerous times subsequently and has not yet been questioned. However, when Martyn (1945) described *Z. jamaicensis* from banana leaves collected in Jamaica, conidiophores were observed to be  $16\text{--}24 \times 4\text{--}5 \mu\text{m}$  and conidia  $15\text{--}18 \times 4\text{--}5 \mu\text{m}$ . In the present study we found that neither of these measurements overlapped with those of the *Zygophiala* anamorph of *S. pomi*. Although the relationship between *Schizothyrium* and *Zygophiala* is correct, our data suggest that the anamorph of *S. pomi* is an unnamed species of *Zygophiala* and not *Z. jamaicensis*.

***Zygophiala cryptogama* Batzer & Crous, sp. nov.**

FIGS. 3, 5.

Mycobank MB501243.

*Etymology.* Named after a hidden sexual cycle observed only in culture.

*Zygophialae jamaicensis* similis, sed conidiis latoribus,  $(12\text{--}14\text{--}18\text{--}20) \times (4\text{--}5\text{--}6\text{--}8) \mu\text{m}$ , distinguenda.

*Conidiophores* arising from superficial hyphae,  $1.5\text{--}3 \mu\text{m}$  wide, erect, scattered, 3-septate, subcylindrical, irregularly flexuous, consisting of a hyaline supporting cell that gives rise to a smooth, dark brown stipe,  $17\text{--}22 \times 4\text{--}5 \mu\text{m}$  (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell,  $3\text{--}4 \times 4\text{--}5 \mu\text{m}$ , that gives rise to two medium brown, finely verruculose, doliiform to elongated subcylindrical, polyblastic conidiogenous

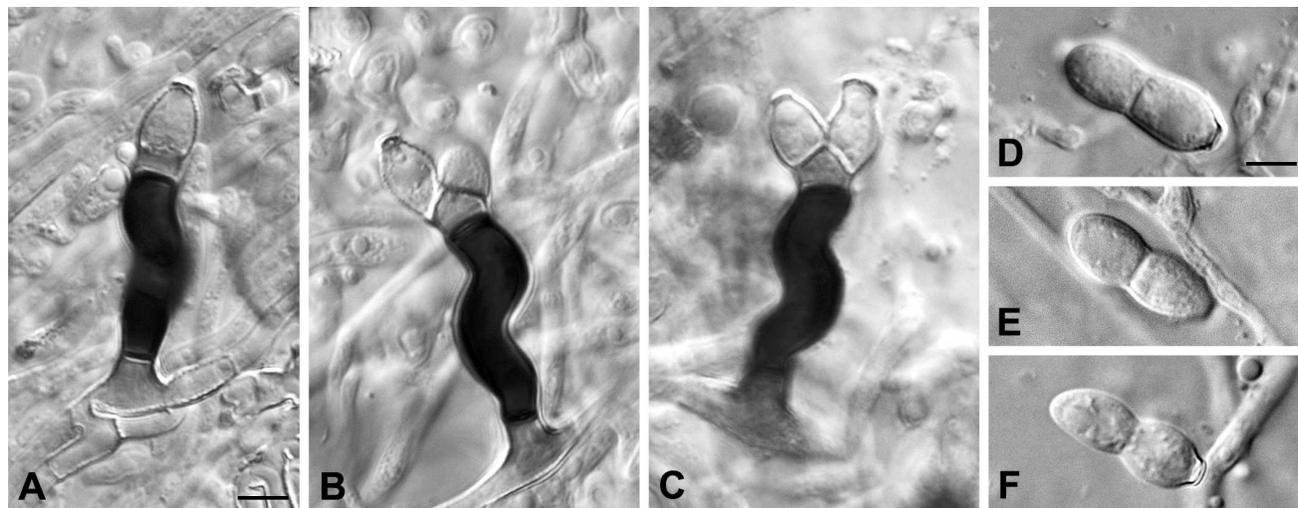


FIG. 6. *Zygophiala tardicrescens* on oatmeal agar (CBS 118946). A–C. Conidiophores. D–F. Conidia. Bars: A = 6, D = 7  $\mu$ m.

cells with 1–10 loci, 6–15  $\times$  5–6  $\mu$ m; scars prominent, apical and lateral, darkened, thickened, somewhat refractive, 1–2  $\mu$ m wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, transversely (0–)1(–2)-septate; aseptate, 6–7(–9)  $\times$  5–6(–7)  $\mu$ m, 1-septate, (12–)14–18(–20)  $\times$  (4–)5–6(–8)  $\mu$ m, 2-septate, 19–24(–30)  $\times$  5–6(–7)  $\mu$ m, prominently constricted at septa; apex subobtuse, base subtruncate, with a darkened, thickened hilum, 1–2  $\mu$ m wide. Forming fertile, globose ascomata on the surface of OA plates. *Asci* 8-spored, obovoid to ellipsoid, bitunicate, with an apical chamber (note that this is inconspicuous in *S. pomi*), 20–25  $\times$  12–13  $\mu$ m. *Ascospores* multiseriate, hyaline, smooth, fusoid-ellipsoidal, medianly 1-septate, 7–8  $\times$  3  $\mu$ m.

*Cultural characteristics.* Colonies after 2 wk on OA in the dark flat, spreading, aerial mycelium absent, margins smooth, regular; olivaceous gray throughout; developing submerged to erumpent, globose ascomatal initials.

*Specimen examined.* USA. IOWA: Iowa Falls, on apple fruit, Sep 2000, J. Batzer, HOLOTYPE 438791, ISOTYPE CBS-H19785, cultures ex-type FVA2a = CBS 118949, GenBank: AY598896, AY598854.

*Notes.* The globose structures observed embedded and on the surface of OA plates became fertile and were shown to be ascomata. It is interesting to note that all four species form ascomatal initials, although ascospore production was only confirmed in vitro in *Z. cryptogama*.

***Zygophiala tardicrescens* Batzer & Crous, sp. nov.**

FIGS. 3, 6. MycoBank MB501244.

*Etymology.* Named after its slow growth.

*Zygothialae jamaicensi* similis, sed coloniis lentius crescentibus et conidiis 20  $\mu$ m vel magis longis, 6  $\mu$ m vel magis latis distinguenda.

*Conidiophores* arising from superficial hyphae, 2–3  $\mu$ m wide, erect, scattered, 3-septate, subcylindrical, irregularly flexuous, consisting of a hyaline supporting cell that gives rise to a smooth, dark brown stipe, 14–16  $\times$  5–6  $\mu$ m (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, 3–4  $\times$  4–6  $\mu$ m, that gives rise to two medium brown, finely verruculose, doliform to ellipsoidal, polyblastic conidiogenous cells, 7–10  $\times$  5–6  $\mu$ m, with 1–2 prominent scars, apical and lateral, darkened, thickened, somewhat refractive, 2  $\mu$ m wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, granular, transversely 1-septate (rarely median), (13–)16–20(–23)  $\times$  (6–)7–8  $\mu$ m, prominently constricted at the septum; apex obtuse, base subtruncate, with a darkened, thickened hilum, 2  $\mu$ m wide.

*Cultural characteristics.* Colonies after 2 wk on OA in the dark flat, spreading, aerial mycelium absent, margins smooth, and somewhat irregular; olivaceous gray in the center, with a thin, white outer margin, and a reddish pigment that diffuses into the agar.

*Specimen examined.* USA. IOWA: Indianola, on apple fruit, Sep 2000, J. Batzer, HOLOTYPE 438792, ISOTYPE CBS-H19788, cultures ex-type MWA1a = CBS 118946, GenBank: AY598856.

*Notes.* *Zygophiala tardicrescens* is morphologically distinct from other species of *Zygophiala* by having conidia intermediate in size between those of *S. pomi* and *Z. jamaicensis* (see key below).

***Zygophiala wisconsinensis* Batzer & Crous, sp. nov.**

FIGS. 3, 7. MycoBank MB501245.

*Etymology.* Named after its type locality, Wisconsin, USA.

*Zygothialae jamaicensi* similis, sed coloniis celerius

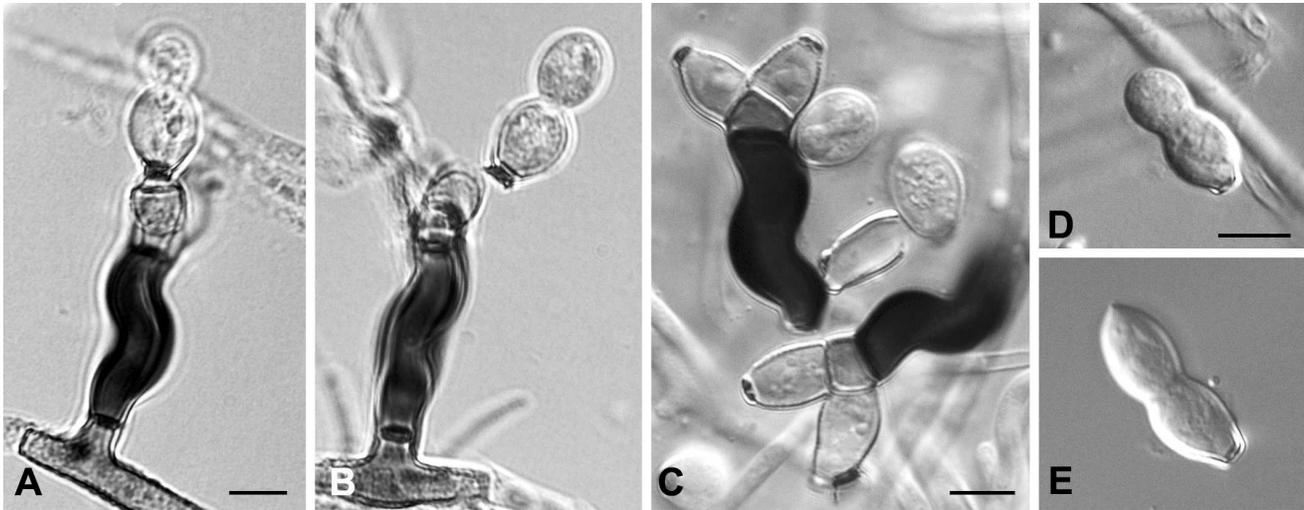


FIG. 7. *Zygophiala wisconsinensis* on oatmeal agar (CBS 118950). A–C. Conidiophores. D–E. Conidia. Bars: A, C = 6, D = 7  $\mu$ m.

crescentibus et conidiis 20  $\mu$ m vel magis longis, 6  $\mu$ m vel magis latis distinguenda.

*Conidiophores* arising from superficial hyphae, 2–3  $\mu$ m wide, erect, scattered, 3–4-septate, subcylindrical, irregularly flexuous, consisting of a hyaline supporting cell that gives rise to a smooth, dark brown stipe, 15–20  $\times$  4–7  $\mu$ m (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, 3–4  $\times$  4–5  $\mu$ m, that gives rise to two medium brown, finely verruculose, dolii-form to ellipsoidal, polyblastic conidiogenous cells, 7–11  $\times$  5–6  $\mu$ m, with 1–2 prominent scars, apical and lateral, darkened, thickened, somewhat refractive, 2  $\mu$ m wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, granular, aseptate, 6–8  $\times$  6–8  $\mu$ m, or transversely 1-septate (rarely median), (13–)15–18(–23)  $\times$  (6–)7–8  $\mu$ m, prominently constricted at the septum; apex obtuse, base subtruncate, with a darkened, thickened hilum, 2–3  $\mu$ m wide.

*Cultural characteristics.* Colonies after 2 wk on OA in the dark flat, spreading with moderate aerial mycelium and smooth, regular margins; pale olivaceous gray in the middle, with a large, dirty white to cream outer zone.

*Specimen examined.* USA. WISCONSIN: New Munster, on apple fruit, Sep 2000, J. Batzer, HOLOTYPE 438790, ISOTYPE CBS-H19786, cultures ex-type MSTA8a = CBS 118950, GenBank: AY598897, AY598853.

*Notes.* Morphologically *Z. wisconsinensis* is similar to *Z. tardicrescens*. However the two species can be distinguished easily in culture because *Z. wisconsinensis* grows relatively rapidly, reaching 13.5–22.5 mm diam on MEA after 2 wk at 25 C, while *Z. tardicrescens*, reached only 2.5–4.5 mm.

#### DISCUSSION

The present study has revealed several novel findings. First, flyspeck can be caused by at least four species of *Zygophiala*. Although several papers have commented on cultural variation among isolates of *Zygophiala* (Durbin et al 1953, Baker et al 1977), the genus until now has been accepted as monotypic, having a wide host range and geographic distribution. The fact that several species are involved strongly questions reports on host and geographic distribution of *Z. jamaicensis*. However all strains of *S. pomi* available in the CBS culture collection appear to be a single species, conspecific with the many apple isolates included in this study. It appears therefore that the majority of records reporting *S. pomi* from different hosts could be correct, but that records reporting *Z. jamaicensis* should be considered with care. *Z. jamaicensis* originally was described from banana leaves collected in Jamaica, with conidia cited as being 15–18  $\times$  4–5  $\mu$ m (Martyn 1945). Ellis (1971) reported conidia to be 13–20  $\times$  5–6  $\mu$ m, while Williamson and Sutton (2000) cited them as 13–20  $\times$  4–6  $\mu$ m, whereas the present study found conidia of *S. pomi* to be 1(–7)-septate, prominently constricted at septa, (20–)22–25(–30)  $\times$  5–7(–8)  $\mu$ m if 1-septate, but up to 110  $\mu$ m long if 7-septate. Thus it is likely that there are additional *Zygophiala* species associated with flyspeck signs.

The genus *Mycosphaerella* currently is characterized by pseudothecial ascomata that vary in wall thickness (Crous 1998, Crous et al 2004a), position on or in the host substrate (Crous 1998) and superficial stromatal development, which usually gives rise to an associated cercosporoid anamorph (Crous et al 2004b, 2006).

Although reports have shown that some species of *Mycosphaerella* may form ascospores that are 3-septate (*Sphaerulina* s. str.) (Crous et al 2003), taxa placed in *Mycosphaerella* generally have 1-septate, hyaline to pale brown ascospores, with or without a sheath, and lack any pseudoparaphyses, although some taxa do have remnants of the hamothecium that still could be visible among asci (Crous et al 2004b, 2006). As far as we are aware however ours is the first report of a fungus with a thyrothecial ascoma that is phylogenetically closely related to *Mycosphaerella*. The genus *Schizothyrium*, which is based on *S. pomi*, traditionally has been placed in the family Schizothyriaceae of the Dothideales (von Arx and Müller 1975). The Dictionary of Fungi (Kirk et al 2001) placed *Schizothyrium* (Schizothyriaceae) in the Microthyriales, characterized by strongly flattened, crustose, rounded or elongated ascomata, opening by irregular splits, with bitunicate asci lacking an apical chamber (but see descriptions above), and some interascal tissue composed of remnants of stromatal cells, and transversely 1-septate, hyaline to pale brown ascospores. In Myconet Eriksson (2006) placed *Schizothyrium* (Schizothyriaceae) in the Dothideomycetes, which agrees with phylogenetic data.

Our findings that *Mycosphaerella* was paraphyletic was unexpected. As part of the Fungal Tree of Life project Schoch et al (2006) used a data matrix consisting of 4 loci (nuc SSU rDNA, nuc LSU rDNA, tef1, RPB2), showing that the genus *Mycosphaerella* resides in the Dothideomycetes, subclass Dothideomycetidae, order Capnodiales. *Schizothyrium* appears to be within the Mycosphaerellaceae in our rDNA analyses, but other gene trees need to be examined to confirm this relationship.

Our findings provide the first evidence that one part of the SBFS complex, flyspeck, is caused by at least four species of fungi rather than a single species. Because only a small portion of the geographic range of SBFS fungi was examined in our surveys it is likely that additional flyspeck species remain to be discovered. As the full range of genetic diversity in SBFS causing organisms is revealed the environmental biology and geographic range of each species must be clarified to improve the effectiveness of SBFS management practices.

KEY TO SPECIES OF *ZYGOPHIALA*

1. Conidia (0–)1 to multiseptate on OA . . . . . 2
1. Conidia (0–)1-septate on OA . . . . . 3
2. One-septate conidia (20–)22–25(–30) × 5–7(–8) µm. . . . . *Schizothyrium pomi*
2. One-septate conidia (12–)14–18(–20) × (4–)5–6(–8) µm . . . . . *Zygothiala cryptogama*

3. One-septate conidia shorter than 20 µm, and narrower than 6 µm; conidia 15–18 × 4–5 µm . . . . . *Zygothiala jamaicensis*
3. One-septate conidia 20 µm or longer, and 6 µm or wider; conidia 13–23 × 6–8 µm . . . . . 4
4. Colonies fast-growing, reaching 13.5–22.5 mm diam on MEA after 2 wk at 25 C. . . . . *Zygothiala wisconsinensis*
4. Colonies slow-growing, reaching 2.5–4.5 mm diam on MEA after 2 wk at 25 C. . . . . *Zygothiala tardicrescens*

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## Phylogeny and taxonomy of *Cladosporium*-like hyphomycetes, including *Davidiella* gen. nov., the teleomorph of *Cladosporium s. str.*

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A phylogenetic study employing sequence data from the internal transcribed spacers (ITS1, ITS2) and 5.8S gene, as well as the 18S rRNA gene of various *Cladosporium*-like hyphomycetes revealed *Cladosporium s. lat.* to be heterogeneous. The genus *Cladosporium s. str.* was shown to represent a sister clade to *Mycosphaerella s. str.*, for which the teleomorph genus *Davidiella* is proposed. The morphology, phylogeny and taxonomy of the cladosporioid fungi are discussed on the basis of this phylogeny, which consists of several clades representing *Cladosporium*-like genera. *Cladosporium* is confined to *Davidiella* (Mycosphaerellaceae) anamorphs with coronate conidiogenous loci and conidial hila. *Pseudocladosporium* is confined to anamorphs of *Caproventuria* (Venturiaceae). *Cladosporium*-like anamorphs of the *Venturia* (conidia catenate) are referred to *Fusicladium*. Human-pathogenic *Cladosporium* species belong in *Cladophialophora* (*Capronia*, Herpotrichiellaceae) and *Cladosporium fulvum* is representative of the *Mycosphaerella/Passalora* clade (Mycosphaerellaceae). *Cladosporium malorum* proved to provide the correct epithet for *Pseudocladosporium kellermanianum* (syn. *Phaeoramularia kellermaniana*, *Cladophialophora kellermaniana*) as well as *Cladosporium porophorum*. Based on differences in conidiogenesis and the structure of the conidiogenous loci, further supported by molecular data, *C. malorum* is allocated to *Alternaria*.

**Taxonomic novelties:** *Alternaria malorum* (Ruehle) U. Braun, Crous & Dugan, *Alternaria malorum* var. *polymorpha* Dugan, *Davidiella* Crous & U. Braun, *Davidiella tassiana* (De Not.) Crous & U. Braun, *Davidiella allii-cepae* (M. M. Jord., Maude & Burchill) Crous & U. Braun, *Davidiella dianthi* (C. C. Burt) Crous & U. Braun, *Davidiella macrospora* (Kleb.) Crous & U. Braun, *Davidiella ornithogali* (J. E. Jacques) Crous & U. Braun

The genus *Cladosporium* was described by LINK (1816) with *Cladosporium herbarum* as type species. Surveys of the generic history of *Cladosporium* were given by DE VRIES (1952) and DAVID (1997). Early descriptions of *Cladosporium* were rather vague and the delimitations from similar genera obscure (NEES 1817, CORDA 1837, 1842, FRIES 1832, 1849, SACCARDO 1886, LINDAU 1907, etc.). Since its introduction, more than five hundred taxa have been attributed to *Cladosporium*. Due to the imprecise circumscription of *Cladosporium*, it is not surprising that numerous superficially similar but unrelated hyphomycetes have been assigned to this genus, making it very heterogeneous. DE VRIES (1952) and ELLIS (1971, 1976) maintained broad concepts of *Cladosporium* and did not contribute towards a reduction of its heterogeneity, which was later discussed in detail by VON

ARX (1983), MORGAN-JONES & JACOBSEN (1988), MCKEMY & MORGAN-JONES (1990), MORGAN-JONES & MCKEMY (1990), and DAVID (1997).

There are two ways to treat anamorphic genera, viz. the maintenance of broad, unnatural circumscriptions, based on superficial morphological similarities, implying that such genera need not be naturally classified (KENDRICK 1980), or, on the other hand, the restriction of anamorph genera to characterise natural fungal groups. The second option is desirable, but in reality often only theoretical since most anamorphic taxa are only known and examined by classical morphological methods. As far as possible, anamorphs should reflect monophyletic holomorphic taxa, but this approach is only applicable satisfactorily when the connection of anamorphs and teleomorphs has been proved experimentally or by molecular studies, so that the taxa concerned become established as holomorphs (REYNOLDS 1993).

Anamorphs are increasingly important for the classification of fungi, above all in ascomycetes (SUTTON & HENNEBERT 1994). In several groups, the diversity of anamorphs is often more important for a natural classification than that of the teleomorphs (e.g. *Erysiphales*; BRAUN & TAKAMATSU 2000). In other cases, the morphological variation in the anamorphs is much greater than in the teleomorphs, e.g. in *Mycosphaerella*.

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*sphaerella* (CROUS et al. 2000, CROUS, KANG & BRAUN 2001), *Venturia* (RITSCHEL 2001, SCHUBERT 2001), *Botryosphaeria* (DENMAN et al. 2000), and *Calonectria* (CROUS 2002).

The present study resulted from our trying to find a suitable genus for *Cladosporium malorum*, a widespread and relatively common, mostly saprobic hyphomycete isolated from different substrata including soil, grain, fruits, and grass litter. MARASAS & BREDELL (1974) described this fungus from South Africa as *Phaeoramularia kellermaniana*, and MATSUSHIMA (1975) treated it as *C. porophorum*. BRAUN & FEILER (1995) excluded *P. kellermaniana* from *Phaeoramularia*, and assigned it to *Cladophialophora*, which contains morphologically similar human-pathogenic hyphomycetes. Later BRAUN (1998) placed it in *Pseudocladosporium*, a genus introduced for anamorphs of *Caproventuria*. HO et al. (1999) recognized *C. malorum*, *C. porophorum* and *P. kellermaniana* as conspecific. Detailed morphological investigations of cultures of *C. malorum*, above all of the conidiogenesis and the structure of the conidiogenous loci, raised doubts concerning the correct placement of this species in either *Cladosporium* or *Pseudocladosporium*. The first aim of the present paper, therefore, was to resolve the generic affinity of *C. malorum*. Previous studies employing rDNA ITS sequence data (CROUS et al. 2000, 2001) have shown *Mycosphaerella* to be monophyletic, and *Cladosporium*-like taxa to form a sister clade to the main *Mycosphaerella* clade. A further aim was, therefore, to resolve the identity of *Cladosporium s. str.* in relation to *Mycosphaerella*.

## Material and methods

### DNA isolation, amplification and phylogeny

The isolation protocol of CROUS et al. (2000) was used to isolate genomic DNA from fungal mycelia grown on 2 % malt extract agar (MEA; Biolab, Midrand, Johannesburg) plates. The primers ITS1 (5'-TTT CCG TAG GTG AAC CTG C-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (WHITE et al. 1990) were used to amplify part of the nuclear rRNA operon spanning the 3' end of the 18S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS (ITS2) region and the 5' end of the 28S (large subunit) of the rRNA gene. The reaction mixture contained 5 µL of diluted sample, 1 x buffer, 8 mM MgCl<sub>2</sub>, 500 µM of each of the dNTPs, 2.5 U (Bioline) Taq polymerase and 10 pM of each primer and made up to a total volume of 25 µl with sterile water. The cycling conditions comprised denaturing at 96 °C for 5 min, followed by 30 cycles of denaturation at 96 °C (30 s), annealing 50 °C (30 s) and elongation at 72 °C (90 s). A final elongation step at 72 °C for 7 min was included. The 5' end of the 18S rRNA gene was amplified with primers NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') and NS4 (5'-CTT CCG TCA ATT CCT TTA AG-3') (WHITE et al. 1990). PCR conditions were the same for this region, except for the MgCl<sub>2</sub> concentration, which was lowered to 1.5

mm. PCR products were separated by electrophoresis at 75 V for 1 h in a 0.8 % (w/v) agarose gel in 0.5 x TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK) following ethidium bromide staining.

The amplification products were purified by using a GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Europe Freiburg, Germany). The cycle sequencing reaction with 20 to 40 ng of purified PCR products and 10 pmol primer in a total volume of 10 µl was carried out with an ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) containing AmpliTaq DNA Polymerase. The reaction was set up as denaturing at 94 °C for 5 min, followed by 25 cycles of 96 °C for 10 s, 55 °C for 10 s, and 60 °C for 4 min, with a final incubation of 30 s at 60 °C. The resulting products were analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, CN).

The nucleotide sequences generated in this study were added to the ITS outgroup, *Phomopsis vaccinii* AF317578, the 18S outgroup, *Fusarium oxysporum* f. sp. *fragariae* E17083, and other sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov>). The alignments were assembled using Sequence Alignment Editor version 2.0a11 (RAMBAUT 2002). Adjustments for improvement were made by eye where necessary. Phylogenetic analyses with neighbour joining (using the uncorrected ('p') substitution model) were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (SWOFFORD 2000). Alignment gaps were treated as missing data and all characters were unordered and of equal weight. The robustness of the trees was evaluated by 1000 bootstrap replications (HILLIS & BULL 1993). Resulting trees were printed with TreeView Version 1.6.6 (PAGE 1996).

### Morphology

Slide cultures (RIDDELL 1950) were examined at 100–1000 x to record branching patterns of conidial chains and other characters. Cultures were also transferred to half strength V8 agar to enhance sporulation (STEVENS 1981). Plates were incubated under alternating cool white fluorescent light and darkness (12 h cycles) at 25 °C. Morphological observations were made from structures mounted in lactic acid after wetting with Et-OH, and photographs were taken under an Olympus BH-2 microscope with a DP-11 digital camera.

## Results

### Phylogenetic analysis

For ITS, approximately 530 bases were determined for each isolate (spanning the 3' end of 18S, ITS1, the 5.8S rRNA gene, ITS2 and the 5' end of the 28S rRNA gene) and added to the alignment. The manually adjusted alignment of the ITS

nucleotide sequences contained 72 taxa and 575 characters including alignment gaps (data not shown). Approximately 1075 bases of the 5' end of the SSU gene were determined for each isolate and the manually adjusted alignment of the nucleotide sequences contained 59 taxa and 1394 characters including alignment gaps (data not shown). The SSU sequence of *Mycosphaerella juvenis* (STE-U 1004) contained an insertion spanning bases 514 to 838, which was excluded from the analysis. New sequences were deposited in GenBank (Tab. 1), and the alignments in TreeBASE (S872, M1413, M1414).

The NJ tree for the ITS sequencing data (Fig. 1) contains isolates from five main groups (Herpotrichiellaceae, Amorphythaceae, Mycosphaerellaceae, Pleosporaceae and Venturiaceae). The Herpotrichiellaceae formed a well-supported clade (100 % bootstrap support) comprising species of *Cladophialophora* and *Phialophora*. The Amorphythaceae clade was also well-supported with a bootstrap support value of 100 % and contained isolates of *Amorphytheca resiniae* (anamorph *Sorocybe resiniae*) and '*Cladosporium*' *breviramosum*. The Herpotrichiellaceae and Amorphythaceae clades were grouped together with a bootstrap support value of 75 %. The Mycosphaerellaceae consisted of isolates of *Mycosphaerella* and a strongly supported clade (100 %) of *Davidiella* containing *Cladosporium* anamorphs. *Mycosphaerella* isolates were represented in two separate groups, one of which consisted of '*Cladosporium*' *staurophorum* AF393723 and '*Phaeoramularia hachijoensis*' (STE-U 5121) (88 % bootstrap support), and the other well-supported (100 %) clade contained *Passalora arachidicola* AF297224, isolates of *P. fulva*, *P. henningsii* AF284389, *P. dissiliens* AF222835, *P. vaginiae* AF222832 and *P. bellynckii* AF222831. The clade for the Pleosporaceae was also well-supported (100 %) and contained isolates of *Alternaria malorum* and additional species of *Alternaria* and *Lewia*. An isolate of '*Mycosphaerella iridis*' (CBS 281.49) grouped with 100 % bootstrap support outside the Pleosporaceae clade. The Venturiaceae clade consisted of '*Phaeoramularia hachijoensis*' (STE-U 3679) (60 % bootstrap support) and a well-supported (100 %) clade containing *Fusicladium convolvulorum* (STE-U 3884), *Pseudocladosporium hachijoense* (STE-U 5391) and species of *Venturia* as well as isolates of *Fusicladium effusum*. *Anungitopsis amoena* (CBS 254.95) AF393682 grouped with 81 % bootstrap support outside the Venturiaceae clade.

The NJ tree for the SSU sequencing data (Fig. 2) contained isolates from the Mycosphaerellaceae, Pleosporaceae, Venturiaceae, as well as Dothioraceae, Dothideaceae, Botryosphaeriaceae, Leptosphaeriaceae and Pleosporales *inc. sed.* The Mycosphaerellaceae isolates consisted of isolates of *Mycosphaerella* and a strongly supported clade (90 %) of *Davidiella* containing *Cladosporium* spp. and a single isolate of *Sphaerulina polyspora* (STE-U 4301). *Mycosphaerella* isolates were present in a poorly supported (55 %) group, and contained, amongst others, '*Cladophialophora hachijoensis*' (STE-U 5121), *Passalora fulva* (STE-U 3688), '*Cladosporium*' *staurophorum* (STE-U 3687) and *Mycosphaerella* spp.

The Dothideaceae clade was well-supported (100 %) and was grouped inside a clade with a 98 % bootstrap support value that contained a single isolate of the 'Dothioraceae'. The Venturiaceae clade (100 % bootstrap support) consisted of *Pseudocladosporium hachijoense* (STE-U 5391), *Fusicladium convolvulorum* (STE-U 3884), as well as isolates of *Fusicladium effusum*. *Anungitopsis amoena* (CBS 254.95) grouped with 99 % bootstrap support outside the Venturiaceae clade. The Pleosporaceae clade consisted of *Pleospora betae* U43465 (100 % bootstrap support) and a well-supported (100 %) clade containing *Pleospora herbarum* (U43458), isolates of *Alternaria malorum* and species of *Alternaria* and *Lewia*. The Paraphaeosphaeriaceae clade was well supported (100 %), and was grouped inside a clade that also contained a single isolate of the Leptosphaeriaceae (100 % bootstrap support).

## Morphology

### *Cladosporium malorum* (Pleosporaceae) clade

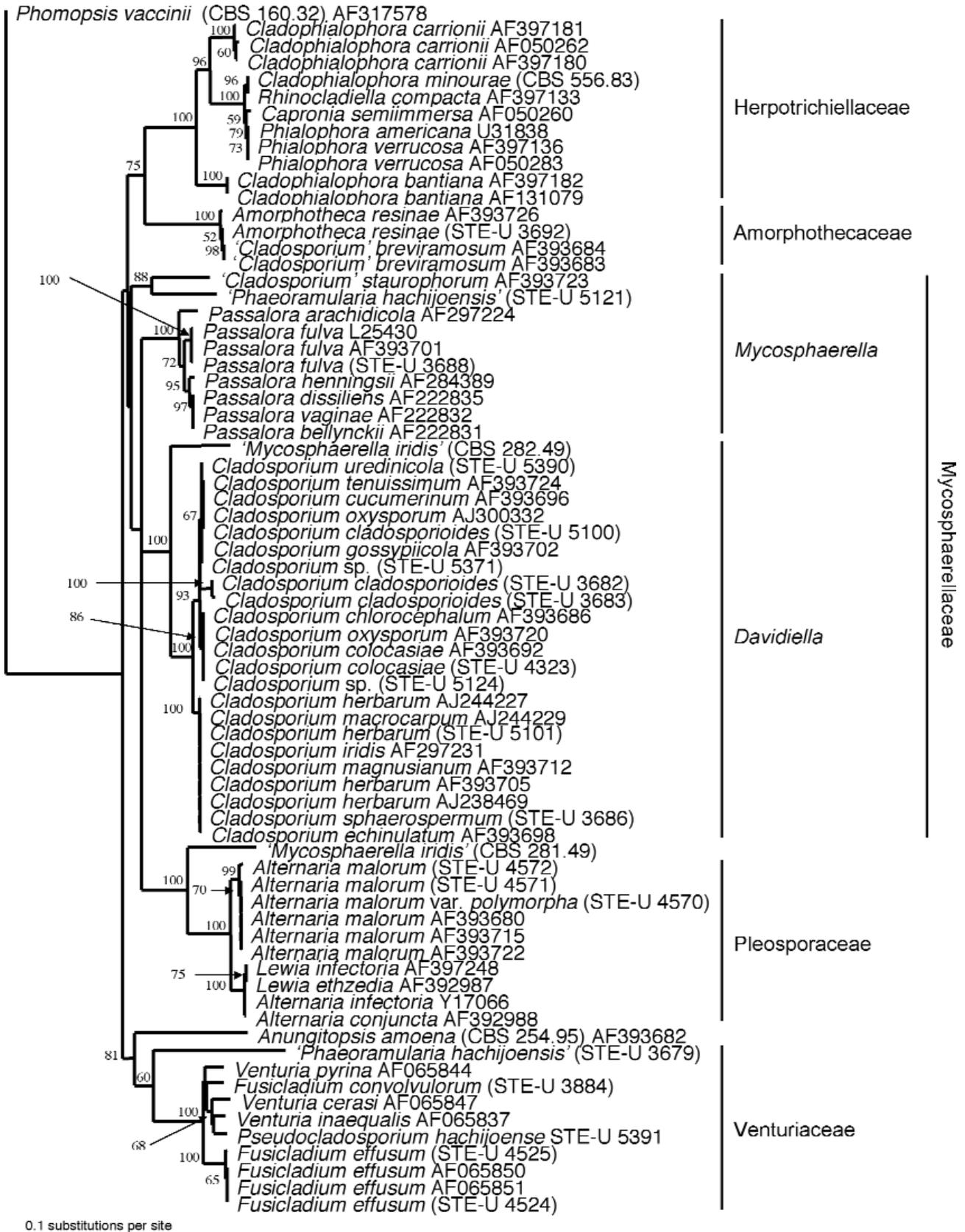
Strains of *Cladosporium malorum*, *C. porophorum* and *Phaeoramularia kellermaniana* are morphologically identical. Conidiogenous cells of *C. malorum* possess minute, but rather conspicuous pores (Fig. 3). Conidia, therefore, can be classified as poroconidia, the product of tretic conidiogenesis. Due to the distinctly tretic nature of the conidiogenous loci, *C. malorum* has to be excluded from *Cladosporium*, *Cladophialophora* as well as *Pseudocladosporium*. Its conidiogenesis is similar to that of the genus *Alternaria*, and other species in the Pleosporaceae/Pleosporales. Furthermore, the formation of alternarioid conidia (Figs. 9–10) in the new variety of *C. malorum* described below is also reminiscent of *Alternaria* (teleomorph: *Lewia*) and allied genera with tretic conidiogenesis and catenulate conidia. Its unique mode of conidiogenesis, as well as its DNA phylogeny, support assignment of *C. malorum* to *Alternaria*:

### *Alternaria malorum* (Ruehle) U. Braun, Crous & Dugan, **comb. nov.**

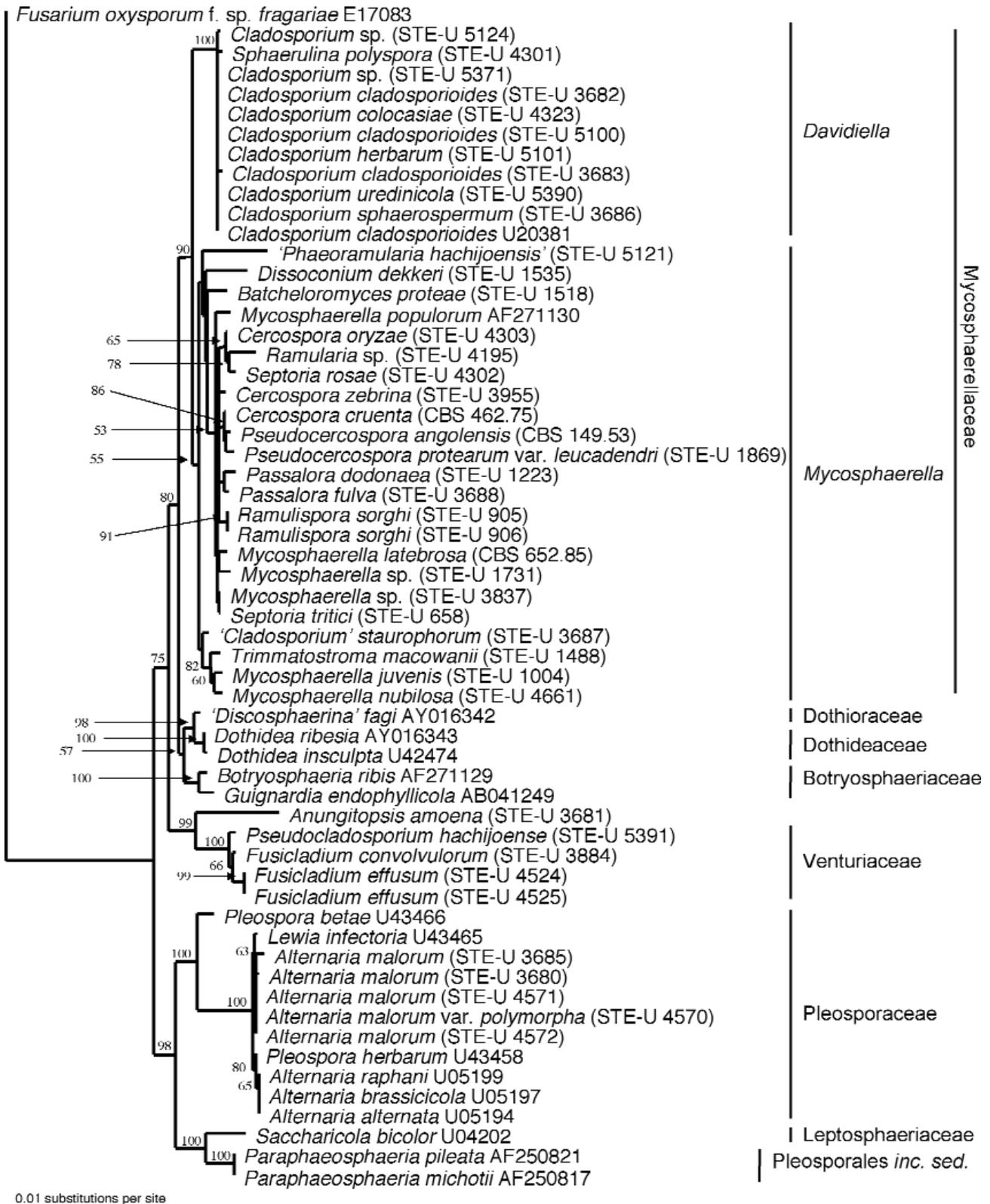
Basionym: *Cladosporium malorum* Ruehle, *Phytopathology* 21: 1146, 1931.

Synonyms: *Phaeoramularia kellermaniana* Marasas & Bredell, *Botanical* 11: 217, 1974. *Cladophialophora kellermaniana* (Marasas & Bredell) U. Braun & Feiler, *Microbiol. Res.* 150: 83, 1995. *Pseudocladosporium kellermaninum* (Marasas & Bredell) U. Braun, *A Monogr. Cercosporella, Ramularia and allied gen.* 2: 393, 1998. *Cladosporium porophorum* Matsush., *Icones Microf. Matsushima Lect.*: 36, 1975.

Colonies effuse, floccose, velvety to woolly, olivaceous-grey to deep olivaceous-green, reverse olivaceous to blackish olive. Hyphae of two types: sterile hyphae branched, sometimes forming strands, occasionally anastomosing, smooth to faintly rough-walled, septate, occasionally constricted at the septa, subhyaline to pale olivaceous, slender, usually 1–4  $\mu\text{m}$  wide; fertile hyphae with conidiophores (Fig. 3) sometimes darker, brown, to 7  $\mu\text{m}$  wide, hyphal cells in old cultures sometimes swollen, becoming thick-walled, darker brown, subglobose,



**Fig. 1.** Phylogram of neighbour joining tree obtained from ITS sequencing data using the uncorrected 'p' model of substitution. Bootstrap support values from 1000 replicates are shown at nodes. Due to the fact that not all nodes are clearly visible, some bootstrap values are not shown. The GenBank sequence *Phomopsis vaccinii* AF317578 was used as outgroup.



**Fig. 2.** Phylogram of neighbor joining tree obtained from small subunit rRNA gene sequencing data using the uncorrected 'p' model of substitution. Bootstrap support values from 1000 replicates are shown at nodes. Due to the fact that not all nodes are clearly visible, some bootstrap values are not shown. The GenBank sequence *Fusarium oxysporum* f.sp.*fragariae* E17083 was used as outgroup.



intercalary or terminal, chlamydospore-like (Fig. 4). Conidiophores pleurogenous and terminal, erect, straight, subcylindrical or somewhat attenuated towards the apex, slightly geniculate-sinuous, unbranched or rarely branched, 5–50 x 2–5(7)  $\mu\text{m}$ , 0–2(3)-septate, pale olivaceous to olivaceous-brown, thin-walled, smooth or almost so; conidiogenous cells integrated, terminal or conidiophores reduced to conidiogenous cells, 5–15  $\mu\text{m}$  long, monotretic, determinate or polytretic, sympodial, usually with 1–2 conspicuous loci, 0.5–1.5  $\mu\text{m}$  wide, unthickened, with minute central pori, 0.5–1  $\mu\text{m}$  wide, usually surrounded by a narrow darker margin, dark brown. *Conidia* in long acropetal chains (Fig. 5), simple or branched, narrowly ellipsoid-ovoid, cylindrical or fusiform, aseptate, 6–14(17) x 2–4  $\mu\text{m}$ , ramoconidia 0–2(3)-septate, very rarely to 6-septate, to 35 x 7  $\mu\text{m}$ , subhyaline, pale olivaceous to olivaceous-brown, thin-walled, smooth, apex and base rounded to truncate, with 1–3(4) inconspicuous or conspicuous distal hila, 0.5–1  $\mu\text{m}$  diam, unthickened, composed of a minute central pore, 0.3–0.5  $\mu\text{m}$  wide, and a narrow darker margin or margin sometimes lacking.

**Substrata and distribution:** Generally saprobic, isolated from soil, grass litter (*Bromus inermis*, *Hordeum* spp., *Triticum aestivum*), stored grains, Bing cherry fruit, fruits of *Malus domestica*, *Prunus persica*, and an old polypore on *Picea* sp., Canada, Lebanon, Libya, Pakistan, South Africa, Syria, Turkey, and the USA. Pathogenic in ripe apples (RUEHLE 1931) and ripe cherries (DUGAN & ROBERTS 1994). Once recorded as the principal fungal contaminant in market wheat in Washington state, USA (SCHNELHARDT & HEALD 1936).

**Material examined:** CANADA, Saskatchewan, Matador, from grass litter, 27 May 1968, G. C. Bhatt 255 (IMI 144487, ATCC 38025 as *C. malorum*); from (?) soil, 18 Sept. 1973, H. A. H. Wallace (IMI 179345, as *P. kellermaniana*). Alberta, from *Bromus inermis*, 1994, R. J. Howad 397 (IMI 360655, HAL, as *P. malorum*). – PAKISTAN, Karachi, from stored grains, 5 Jan. 1967, S. S. Hussain (IMI 124270, as *P. kellermaniana*). – LEBANON, from soil, July 1987, F. Seigle-Murandi (ATCC 200938, CBS 900.87, as *C. porophorum*). – LIBYA, from *Prunus persica*, April 1975, Casay (IMI 194863, as *P. kellermaniana*). – SOUTH AFRICA, Western Cape Province, Kogvat, Calvinia, from wheat stubble, Feb. 1972, W. F. O. Marasas OP-76 (PREM 44703, holotype of *P. kellermaniana*, IMI 165252, isotype; ATCC 28332 and CBS 266.75 ex-type cultures). – SYRIA, from agricultural soil, Febr. 1980, M. I. A. Abdel-Kader (ATCC 200939, CBS 173.80, as *C. porophorum*). – TURKEY, Manisa, from *Hordeum* sp., 16 June 1971, Maksu & Selçuc (IMI 159198, as *P. kellermaniana*). – USA, New Mexico, Red River, from a polypore on *Picea* sp., 4 Sept. 1996, D. Wicklow (IMI 386094, as *P. malorum*). Washington State, from Bing cherry fruit, June 1992, F. Dugan (ATCC 96020, as *C. malorum*); from fruits of *Malus domestica*, F. D. Heald (ATCC 36953, authentic for *C. malorum*).

*Alternaria malorum* var. *polymorpha* Dugan, var. nov.

Figs. 3–12

Differt a var. *malorum* conidiis latioribus, ca 3.5–6  $\mu\text{m}$  latis, atrioribus, leviter crassitunicatis, interdum longitudine septatis, raro alternarioidibus intermixtis.

**Etymology:** Referring to its variable conidial shape.

**Typus:** USA, Washington State, Roza Canal near Prosser, isolated from dormant buds (overwintered) of *Vitis vinifera*, Mar. 2001, F. M. Dugan. Holotype WSP 70286; STE-U 4570, FMD V5#19, CBS 112048, ex-type cultures).

#### *Cladosporium* s. str. (*Mycosphaerellaceae*) clade

The genus *Cladosporium* s. str. (incl. *Heterosporium*) is distinguished from other *Mycosphaerella* anamorphs by its unique scars and conidial hila. DAVID (1997) examined *Cladosporium* and *Heterosporium* by means of scanning electron microscopy and demonstrated both genera to have coronate conidiogenous loci (scars) and conidial hila of the ‘*Cladosporium*-type’, e.g. protuberant with a central dome surrounded by a raised rim. Based on these results, DAVID (1997) placed *Heterosporium* in *Cladosporium* as *Cladosporium* subgen. *Heterosporium*. He proposed to confine *Cladosporium* to saprobic and phytopathogenic (rarely mycoparasitic) hyphomycetes with coronate scars and hila. The peculiar and separate phylogenetic position of *Cladosporium* in relation to *Mycosphaerella* was already shown in previous studies (CROUS et al. 2000, 2001). This distant position is further supported by the ITS as well as 18S data sets derived in the present study (Figs. 1–2), where the *Cladosporium* clade clustered separately from *Mycosphaerella*. Based on the unique ‘*Cladosporium*-type’ scars and conidial hila, as well as distinct phylogeny according to ITS and 18S sequences, we therefore propose a new teleomorphic genus for those ‘*Mycosphaerella*’ species with *Cladosporium* anamorphs sensu DAVID (1997).

#### *Davidiella* Crous & U. Braun, gen. nov.

*Mycosphaerella* sect. *Tassiana* M. E. Barr, Contr. Univ. Michigan Herb. 9: 601, 1972.

Ascomata ut in *Mycosphaerella* sect. *Tassiana* (asci non numerosi, saccati; ascosporae obovatae, utrinque rotundatae). Differt a *Mycosphaerella* statu conidiali, i.e. *Cladosporium* sensu DAVID (1997).

**Etymology:** Named in honour of the British mycologist, John C. David, who has significantly contributed to our knowledge of this group of fungi.

**Typus:** *Davidiella tassiana* (De Not.) Crous & U. Braun 2003; status anamorphosis *Cladosporium herbarum*.

Ascomata morphologically identical to those of *Mycosphaerella* (sect. *Tassiana*), but distinct in having *Cladosporium* anamorphs sensu DAVID (1997).

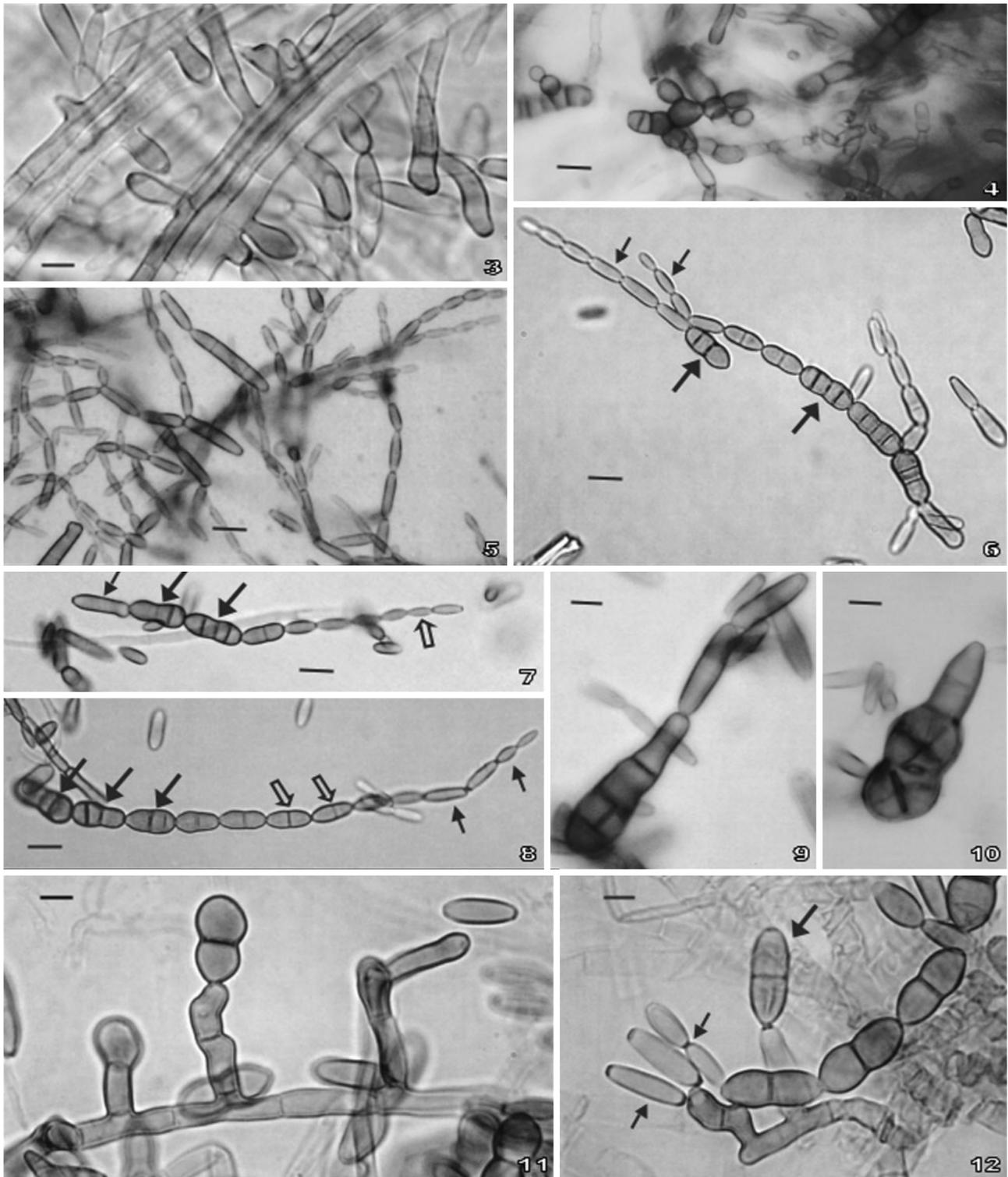
#### *Davidiella tassiana* (De Not.) Crous & U. Braun, comb. nov.

Basionym: *Sphaerella tassiana* De Not., Sferiacei Italici 1: 87, 1863.

*Mycosphaerella tassiana* (De Not.) Johanson, Öfvers. Förh. Kongl. Svenska Vetensk.-Akad. 9: 167, 1884.

Anamorph: *Cladosporium herbarum* (Pers.: Fr.) Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 7: 37, 1816.

Basionym: *Dematium herbarum* Pers., Annl. Bot. (Usteri), 11 Stück: 32, 1794: Fr., Syst. Mycol. 3: 370, 1832.



**Figs. 3–12.** *Alternaria malorum* var. *polymorpha*. **Fig. 3.** Conidiophores borne on aggregated hyphae. **Fig. 4.** Chlamydospore-like cells. **Fig. 5.** Chains of conidia characteristic of the species. **Fig. 6.** Conidial chains containing conidia characteristic of the species (small arrows) and conidia characteristic of var. *polymorpha* (large arrows). **Fig. 7.** A chain containing conidia typical of the species (open arrow) and typical of the new variety (large arrows), subtended by a ramoconidium (small arrow) typical of the species. **Fig. 8.** A chain in which basal conidia typical of var. *polymorpha* (large arrows) are connected to distal conidia typical of the species (small arrows) by conidia intermediate in morphology (open arrows). **Fig. 9.** A typical *Alternaria* conidium, basal to two conidia typical of *A. malorum*. **Fig. 10.** An *Alternaria* conidium irregular in outline. **Fig. 11.** A 1-septate conidium typical of the var. *polymorpha*, borne on a conidiophore typical of the species. **Fig. 12.** Small aseptate conidia typical of the species (small arrows) and a larger, 1-septate conidium typical of var. *polymorpha* (large arrow) borne on a single, branched conidiophore. Bars: Figs. 3, 9–12 = 5 µm, Figs. 4–8 = 10 µm.



***Davidiella allii-cepae*** (M. M. Jord., Maude & Burchill) Crous & U. Braun, **comb. nov.**

Basionym: *Mycosphaerella allii-cepae* M. M. Jord., Maude & Burchill, Trans. Br. mycol. Soc. 86: 392, 1986.

Anamorph: *Cladosporium allii-cepae* (Ranoj.) M. B. Ellis, More Dermat. Hyphom.: 337, 1976.

Basionym: *Heterosporium allii-cepae* Ranoj., Annls Mycol. 8: 399, 1910.

***Davidiella dianthi*** (C. C. Burt) Crous & U. Braun, **comb. nov.**

Basionym: *Didymellina dianthi* C. C. Burt, Trans. Br. mycol. Soc. 20: 214, 1936.

*Mycosphaerella dianthi* (C. C. Burt) Jørst., Meld. Statens Plantepatol. Inst. 1: 17, 1945.

Anamorph: *Cladosporium echinulatum* (Berk.) G. A. de Vries, Contr. Gen. *Cladosporium*: 49, 1952.

Basionym: *Helminthosporium echinulatum* Berk., Gdnrs' Chron. 1870: 382, 1870.

***Davidiella macrospora*** (Kleb.) Crous & U. Braun, **comb. nov.**

Basionym: *Didymellina macrospora* Kleb., Ber. dt. bot. Ges. 42: 60, 1924 (1925).

*Mycosphaerella macrospora* (Kleb.) Jørst., Meld. Statens. Plantepatol. Inst. 1: 20, 1945.

Anamorph: *Cladosporium iridis* (Fautrey & Roum.) G. A. de Vries, Contr. Gen. *Cladosporium*: 49, 1952.

Basionym: *Scolecotrichum iridis* Fautrey & Roum., Revue Mycol. 13: 82, 1891.

***Davidiella ornithogali*** (J. E. Jacques) Crous & U. Braun, **comb. nov.**

Basionym: *Didymellina ornithogali* J.E. Jacques, Contr. Inst. Bot. Univ. Montréal 39: 35, 1941.

Anamorph: *Cladosporium ornithogali* (Klotzsch ex Cooke) G. A. de Vries, Contr. Gen. *Cladosporium*: 491, 1952.

Basionym: *Heterosporium ornithogali* Klotzsch ex Cooke, Grevillea 5: 123, 1877.

The link between the teleomorph and anamorph has not been clearly established for *Davidiella ornithogali* and *Cladosporium ornithogali*, though the discussion provided by DAVID (1997) suggests that DE VRIES (1952) was correct in stating the teleomorph to be representative of 'Mycosphaerella'. Another species that needs clarification is *Didymellina intermedia*, and its presumed anamorph *Cladosporium allii* (David 1997). Fresh collections are required to resolve this possible anamorph-teleomorph association.

When the genus *Mycosphaerella* was treated by VON ARX (1949), he divided it into three sections, including *Didymellina* (with *Cladosporium* and *Heterosporium* spp.), for which he chose *Mycosphaerella tassiana* as type. As pointed out by DAVID (1997), this was erroneous as *Didymellina* was formerly described at the generic level by VON HÖHNEL (1918), having *Dothidea iridis* (syn. *Didymellina iridis*) as type, with *Sphae-*

*rella iridis* (syn. *Mycosphaerella iridis*) as proposed synonyms. The literature is filled with erroneous links between *C. iridis* and a fungus initially identified as *Mycosphaerella iridis*, but later described as *M. macrospora*. This confusing situation is explained by DAVID (1997). We have examined the type specimen of *Dothidea iridis* in PC, which is a species of *Mycosphaerella* and not of *Dothidea*; it is morphologically distinct from *M. iridis* (CBS 281.49, herb. CBS 4933; CBS 282.49, herb. CBS 4907). Further, no link between *Dothidea iridis* and a *Cladosporium* has ever been established. The fungus present on the two specimens from CBS represents *M. iridis*. The cultures, however, represent two different fungi, neither of which appear to be *M. iridis*. Further studies are therefore presently underway to resolve the *Mycosphaerella* spp. occurring on *Iris*. In conclusion, we were unable to find any evidence linking a *Cladosporium* state to either *Mycosphaerella iridis* or *Dothidea iridis*, and have therefore decided not to choose the name *Didymellina* as teleomorph genus for *Cladosporium*.

## Discussion

This study has provided further evidence for the separation of *Cladosporium s. str.* anamorphs from the main *Mycosphaerella* clade, and has provided the basis for the introduction of a new teleomorph genus, *Davidiella*, for this group of fungi. Furthermore, it has also shown that several *Cladosporium*-like fungi are clearly not congeneric with *Cladosporium s. str.*, and that the relatively minor differences in the scars and conidial loci, are supportive of their different phylogenetic affinities. Similarly, *C. malorum* appears to be best assigned to *Alternaria* based on its ITS and SSU phylogenetic placement, and such placement is also supported by its unique mode of conidiogenesis. As in other hyphomycetes in this complex (CROUS, KANG & BRAUN 2001), conidial septation, and the presence of oblique septa, are of less importance at the generic level. HÖLLER, GLOER & WICKLOW (2002) identified various metabolites produced by an undetermined *Cladosporium*-like hyphomycete, which was isolated from a resupinate polypore in the USA. These metabolites, which included altersolanol and macrosporin, are commonly produced by *Alternaria* spp. A culture derived from this isolate, and which was deposited at IMI, was examined by U. Braun, and identified as *C. malorum*. The taxonomic decision to place this species in *Alternaria* is thus further supported by these metabolite data from HÖLLER, GLOER & WICKLOW (2002).

The phylograms derived in the present study delineate several clades (families) in which *Cladosporium*-like taxa are presently accommodated. These are discussed below:

### *Herpotrichiellaceae* and *Venturiaceae*

Of particular interest in the *Herpotrichiellaceae* are those species pathogenic to humans, which are presently placed in *Cladophialophora* (Fig. 1). Human-pathogenic cladosporioid

hyphomycetes have previously been placed in *Cladosporium s. lat.* and confused with true *Cladosporium* species. There is a large number of publications dealing with all aspects of these fungi, including morphology, biology/ecology, physiology and molecular data (MASCLAUX et al. 1995, UNTEREINER 1997, GERRITS VAN DEN ENDE & DE HOOG 1999, UNTEREINER & NAVEAU 1999, UNTEREINER, GERRITS VAN DEN ENDE & DE HOOG 1999, DE HOOG et al. 2000). It has been clearly demonstrated in all phylogenetic analyses that the truly human-pathogenic *Cladosporium* species are *Capronia* anamorphs belonging to the *Herpotrichiellaceae*, and all species concerned have been placed in *Cladophialophora*. The morphological distinction between *Cladophialophora* and *Cladosporium s. str.* has also been demonstrated by several authors (BRAUN & FEILER 1995, BRAUN 1998, DE HOOG et al. 2000). *Cladophialophora* species are characterised by truncate, unthickened, barely darkened, often somewhat denticle-like conidiogenous loci, whereas *Cladosporium* loci are 'coronate' (DAVID 1997), e.g. protuberant and with raised periclinal rims that surround a central convex dome. True *Cladosporium* species also differ from *Cladophialophora* physiologically in their ability to liquefy gelatine (DE HOOG et al. 1995).

The morphological distinction between *Cladophialophora* and *Pseudocladosporium* is rather difficult, but the two genera are ecologically and phylogenetically clearly distinct, viz. species of *Pseudocladosporium* are saprobic fungi, usually isolated from leaf litter, and anamorphs of *Caproventuria* (*Venturiaceae*), whereas *Cladophialophora* spp. are true human-pathogenic fungi connected with *Capronia* (*Herpotrichiellaceae*).

Anamorphs of the *Venturiaceae* have recently been monographed by RITSCHEL (2001) and SCHUBERT (2001), including molecular examinations (rDNA ITS) of numerous taxa in which *Venturia* species and their anamorphs formed a single monophyletic clade. Some *Fusicladium* species with catenate conidia have often been confused with *Cladosporium*, e.g., *C. carpophilum* (syn. *Fusicladium carpophilum*), *C. cerasi* (syn. *F. cerasi*) and *C. caryigenum* (syn. *F. effusum*). As already discussed by MORGAN-JONES & JACOBSEN (1988), these anamorphs should rather be referred to *Fusicladium* (*Venturia* anamorphs), a conclusion supported by the present molecular data. Furthermore, the structures of the conidiogenous loci in *Fusicladium* species with solitary as well as catenate conidia are very uniform, and quite distinct from those of *Cladosporium s. str.* In *Fusicladium* the conidiogenous loci are more or less denticle-like, apically truncate to slightly convex, unthickened or almost so, and not or only slightly darkened. These loci, therefore, more closely resemble those of some saprobic genera, like *Anungitea* and *Pseudocladosporium*. The form genus *Fusicladium* is also associated with various other genera of the *Venturiaceae*, viz. *Acantharia*, *Apiosporina* and *Venturia*.

Several authors have dealt with *Phaeoramularia hachijoensis* (MATSUSHIMA 1975), but all reassessments of this species were based on non-type material, since type material and strains were not available and are possibly not extant any lon-

ger. Cultures assigned to this species are undoubtedly heterogeneous. BRAUN & FEILER (1995) considered CBS 462.82 and ATCC 96019 to be representative of *P. hachijoensis* and placed the species in *Cladophialophora*. DUGAN, ROBERTS & HANLIN (1995) found the teleomorph of ATCC 96019, and described it as *Capronia hystricoides*. A German strain was similar, but differed by having paler structures, finer conidia and a distinct habit of the colonies (BRAUN & FEILER 1995). UNTEREINER & NAVEAU (1999) provided 28S rDNA sequence data to support the fact that the BBA strain was not conspecific with *P. hachijoensis* sensu BRAUN & FEILER (1995) and DUGAN et al. (1995), but even quite unrelated. Of the three isolates of *P. hachijoensis* studied, it appears that each isolate represents a different species in distinct genera. Hence, the application of the name *P. hachijoensis* must be based on an interpretation. We propose to follow the treatment and application of this name by DUGAN, ROBERTS & HANLIN (1995) as anamorph of *Capronia hystricoides* (Syn. *Caproventuria hystricoides*). UNTEREINER (1997) reduced the latter species to synonymy with *Capronia hanliniana* (anamorph *Cladophialophora brevicatenata*), assigned it to the *Venturiaceae* and proposed the combination *Venturia hanliniana*. In the present study, the isolate of *P. hachijoensis* used by DUGAN, ROBERTS & HANLIN (1995) (ATCC 96019 = STE-U 5391) also clustered in *Venturia*, thus supporting the conclusion by UNTEREINER (1997). BRAUN (1998) recognised UNTEREINER'S (1997) exclusion of this species from *Capronia*. He discussed some distinctive features supporting *C. hanliniana* and *C. hystricoides*, which are well-distinguished by their anamorphs, and also from true *Venturia* species. BRAUN (1998) therefore introduced the new genus *Caproventuria* for the teleomorphs, and *Pseudocladosporium* for the anamorphs. In the present phylogram, it can be seen that *Caproventuria/Pseudocladosporium* is unrelated to the *Herpotrichiellaceae* (*Capronia/Cladophialophora*), but rather clusters within *Venturiaceae* (Figs. 1–2). The genus *Pseudocladosporium* is tentatively maintained and confined to anamorphs of *Caproventuria*, awaiting the treatment of more taxa.

### *Amorphothecaceae*

*Sorocybe resinae* (syn.: *Hormoconis resinae*; teleomorph *Amorphotheca resinae*) belongs to a group of hyphomycetes characterised by having more or less distinctly denticulate, pigmented conidiogenous cells and 0–2-septate, pigmented conidia formed in long, often branched chains. This assemblage of anamorphs can be considered as a counterpart to the *Dactylaria* (DE HOOG 1985) complex distinguished by catenate conidia. The delimitations of these genera and some allied ones, e.g., *Anungitea*, *Pleurotheciopsis* and *Polyscytalum*, is difficult and partly vague, since morphology and conidiogenesis are very similar to each other. It is still unclear in this complex which characteristics are appropriate for a generic delimitation. PARTRIDGE & MORGAN-JONES (2002) reduced *Hormoconis* (VON ARX 1973) to synonymy with *Sorocybe*.



They considered *H. resinae* to be the mononematous form of *S. resinae*, and noted that the connection between *Amorphotheca* (PARBERY 1969) as teleomorph and *S. resinae* as anamorph, remains to be resolved. *Sorocybe resinae*, the type species of this genus, differs from species of allied genera in having rather inconspicuous, not distinctly denticle-like conidiogenous loci (DE VRIES 1952; PARTRIDGE & MORGAN-JONES 2002). The clustering of two isolates of '*Cladosporium*' *breviramosum* (AF393683, 393684) in the *Amorphothecaceae* is unusual, and the original strains will have to be re-examined to resolve their identity and position.

#### *Incertae sedis*

The status of *Anungitopsis amoena* (syn. *Cladosporium amoenum*) (HO et al. 1999) is unclear, and the correct placement of this species in *Anungitopsis* is not certain. The type species of the latter genus and the other species assigned to it have long rachis-like conidiogenous cells with numerous, dense, rather inconspicuous conidiogenous loci. The loci in *A. amoena* are less numerous, scattered and more distinct, partly almost denticle-like.

#### *Pleosporaceae*

This study has shown that *Cladosporium malorum* belongs to *Alternaria* (Figs. 1–2). Conidiogenesis and the structure of the conidiogenous loci of this fungus were undoubtedly misinterpreted by all previous mycologists, who placed this fungus in *Cladosporium*, *Cladophialophora*, *Phaeoramularia* or *Pseudocladosporium*, suggesting that the conidiogenesis was holoblastic. These treatments were undoubtedly influenced by the cladosporioid habit of this fungus, e.g., pigmented, 0–2-septate conidia formed in long acropetal chains (Fig. 5). However, the conidiogenous cells possess minute, but conspicuous pores, and should rather be regarded as poroconidia. Within the genus *Alternaria*, however, *A. malorum* is not totally unique in having largely aseptate, cylindrical conidia, as this is also found in other species of *Alternaria*, e.g. *A. cetera* (SIMMONS 1996).

*Alternaria malorum* var. *polymorpha* is distinguished from var. *malorum* by the production of an additional class of 1(–3)-septate conidia which differ from normal *A. malorum* conidia largely by the degree of septation, greater width, deeper colour and somewhat thicker walls (Figs. 6–8). In addition, these alternative conidia could become longitudinally septate and, in rare instances, distinctly alternarioid (Figs. 9–10). The alternative conidia are borne on the same kinds of conidiophores as those bearing regular conidia (Fig. 11), and sometimes from a single, branched conidiophore (Fig. 12). The alternative forms of conidia could occur together with the regular conidia in the same chain (Figs. 6–8) and could be subtended by normal ramo-conidia within the chain (Fig. 7). That the division between the regular conidia and those with alternative morphologies is not absolute can be seen by occasional production of intermediate types (Fig. 8). A small minority of the

dictyoconidia were regularly (Fig. 9) or irregularly (Fig. 10) alternarioid in shape. Conidiogenesis is the same for normal conidia and those characterising var. *polymorpha*, and the alternative conidia occur mixed together with normal *P. malorum* conidia, so that classification as a variety seems to be appropriate. The two varieties appeared similar, however, based on the molecular data presented here.

#### *Mycosphaerella* (*Mycosphaerellaceae*)

This clade contains *Mycosphaerella* species and cercosporioid anamorphs that are now placed in *Passalora s.lat.* (incl. *Fulvia*, *Mycovellosiella* and *Phaeoramularia*). Comprehensive morphological and molecular analyses of this fungal group were recently conducted (CROUS et al. 2000, 2001), in which it was shown that *Mycosphaerella* isolates form a single large monophyletic clade, with species of *Mycosphaerella* with *Cladosporium s. str.* anamorphs in a distinct subclade. These molecular data further showed that *Passalora fulva* [= *Fulvia fulva*, *Cladosporium fulvum*, *Mycovellosiella fulva*] is also a part of the *Mycosphaerella* clade, clustering together with other taxa with *Passalora s.lat.* anamorphs. Furthermore, the conidiogenous loci of *P. fulva* are quite distinct from *Cladosporium s. str.* scars, and agree better with cercosporioid scar types (BRAUN 1995).

Various authors confused *Cladosporium* with *Biharia*, *Fulvia*, *Mycovellosiella* and *Stenella*. For instance, VON ARX (1981) reduced these names to synonymy with *Cladosporium*. ELLIS (1971) listed *Biharia* as a synonym of *Mycovellosiella*, but since the superficial hyphae of the type species, *B. vanguardiae*, are verruculose, DEIGHTON (1979) reduced *Biharia* to synonymy with *Stenella*. VON ARX (1983) recognised *Mycovellosiella*, including *Fulvia*, but maintained *Biharia* and *Stenella* as synonyms of *Cladosporium*. However, *Passalora s.lat.* and *Stenella* are easily distinguishable from *Cladosporium s. str.* by their distinct conidiogenous loci (scars) and conidial hila, which are truncate to pileate, barely protuberant, somewhat thickened and darkened, but always without a raised periclinal rim. Furthermore, the separation of *Cladosporium*, *Passalora s.lat.* and *Stenella* is also supported by molecular data (CROUS et al. 2000, 2001, Crous unpubl.).

#### *Davidiella* (*Mycosphaerellaceae*)

*Cladosporium herbarum*, the lectotype species of *Cladosporium* (CLEMENS & SHEAR 1931), is the anamorph of *Davidiella tassiana* (VON ARX 1950, BARR 1958), which has also been confirmed by molecular examinations (MASCLAUX et al. 1995, DE HOOG et al. 1999). All species of *Cladosporium s. str.* examined represent a monophyletic clade (DE HOOG et al. 1999, UNTEREINER & NAVEAU 1999, CROUS et al. 2000, 2001) (Figs. 1–2).

True *Cladosporium* species are easily separable from all other *Cladosporium*-like hyphomycetes by their distinctive conidiogenous loci, which were described in detail by DAVID (1997), who pointed out that this scar type is a significant ge-

neric character. The first detailed examinations of *Cladosporium* scars were published by ROQUEBERT (1981). The conidiogenous loci (scars) and conidial hila are usually distinctly protuberant, thickened, darkened and composed of a raised periclinal rim that surrounds a central convex part (dome or mound, DAVID 1997). This type of scar has been called 'coronate' (DAVID 1997) or it may simply be described as 'Cladosporium-type', since it is so characteristic and unique. *Cladosporium s. str.* should be confined to *Davidiella* anamorphs with coronate conidiogenous loci. The first clear circumscription in this sense, including a clear description of the peculiar scars has been published by DAVID (1997).

The genus *Heterosporium* was reduced to synonymy with *Cladosporium* by DE VRIES (1952), a view endorsed by HUGHES (1958) and ELLIS (1971, 1976). VON ARX (1981, 1983) reinstated *Heterosporium* and various authors followed his decision. DAVID (1997) examined the conidiogenous loci (scars) and conidial hila of *Cladosporium* and *Heterosporium* species, showed that these structures are generally uniform in all species of the two 'genera', and so reduced *Heterosporium* to synonymy with *Cladosporium*. DAVID'S (1997) taxonomic decisions are fully supported by our study, in which several *Heterosporium* species that have *Davidiella* teleomorphs, cluster within the *Cladosporium* clade.

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**Tab. 1:** Isolates of *Cladosporium* and allied genera studied

Sequence data	Species	Accession no. <sup>a</sup>	GenBank accession no.	Substrate	Origin
SSU	<i>Alternaria alternata</i>	–	U05194	<i>Brassica rapa</i> ssp. <i>oleifera</i>	Alberta, Canada
SSU	<i>Alternaria brassicicola</i> (syn. <i>Helminthosporium brassicicola</i> )	–	U05197	<i>Brassica oleracea</i> ssp. <i>capitata</i>	British Columbia, Canada
ITS	<i>Alternaria conjuncta</i>	'EGS' 37-139	AF392988	<i>Pastinaca sativa</i>	Switzerland
ITS	<i>Alternaria ethzedia</i> / <i>Lewia ethzedia</i>	'EGS' 37-143	AF392987	<i>Brassica</i> sp.	Switzerland
ITS	<i>Alternaria infectoria</i> / <i>Lewia infectoria</i>		Y17066	Linseed	–
SSU	<i>Alternaria infectoria</i> / <i>Lewia infectoria</i>	IMI 303186	U43465	<i>Triticum</i> sp.	UK
ITS	<i>Alternaria infectoria</i> / <i>Lewia infectoria</i>	STE-U 4271	AF397248	<i>Triticum</i> sp.	UK
ITS	<i>Alternaria malorum</i> (syn. <i>Cladosporium porophorum</i> )	ATCC 36953 CBS 135.31	AF393715	Fruit of <i>Malus sylvestris</i>	USA
ITS; SSU	<i>Alternaria malorum</i> (syn. <i>Cladosporium porophorum</i> )	ATCC 200939 CBS 173.80 STE-U 3685	AF393722; AY251128	Agricultural soil	Syria
ITS; SSU	<i>Alternaria malorum</i> (syn. <i>Phaeoramularia kellermaniana</i> )	ATCC 28332 CBS 266.75 STE-U 3680	AF393680; AY251127	Straw of <i>Triticum aestivum</i>	Western Cape, South Africa
ITS; SSU	<i>Alternaria malorum</i>	STE-U 4572	AY251079; AY251131	<i>Festuca idahoensis</i>	Washington, USA
ITS; SSU	<i>Alternaria malorum</i>	STE-U 4571	AY251081; AY251130	<i>Bromus tectorum</i>	Washington, USA
ITS; SSU	<i>Alternaria malorum</i> var. <i>polymorpha</i>	STE-U 4570 CBS 112048 <sup>b</sup>	AY251080; AY251129	<i>Vitis vinifera</i>	Washington, USA
SSU	<i>Alternaria raphani</i>	–	U05199	<i>Brassica rapa</i> ssp. <i>oleifera</i>	Saskatchewan, Canada
ITS; SSU	<i>Anungitopsis amoena</i> (syn. <i>Cladosporium amoenum</i> )	ATCC 200947 CBS 254.95 STE-U 3681	AF393682; AY251122	<i>Eucalyptus grandis</i>	Cuba
SSU	<i>Batcheloromyces proteae</i>	STE-U 1518	AY251102	<i>Protea cynaroides</i>	Western Cape, Stellenbosch, South Africa
ITS	<i>Capronia semiimmersa</i>	MUCL 39979	AF050260	Rotten wood, <i>Acer</i> sp.	USA
SSU	<i>Cercospora zebrina</i>	STE-U 3955	AY251104	<i>Trifolium pratense</i>	Canada
ITS	<i>Cladophialophora bantiana</i> (syn. <i>Xylohypha bantiana</i> )	WC 2907	AF397182	–	USA
ITS	<i>Cladophialophora bantiana</i> (syn. <i>Xylohypha bantiana</i> )	UTHSC 94-986	AF131079	–	–
ITS	<i>Cladophialophora carrionii</i> (syn. <i>Cladosporium carrionii</i> )	ATCC 16264 CBS 160.54	AF050262	Man, chromoblastomycosis	Australia
ITS	<i>Cladophialophora carrionii</i> (syn. <i>Cladosporium carrionii</i> )	FMC 248	AF397181	–	Venezuela
ITS	<i>Cladophialophora carrionii</i> (syn. <i>Cladosporium carrionii</i> )	IMTSP 690	AF397180	–	USA
ITS	<i>Cladophialophora minourae</i> (syn. <i>Cladosporium minourae</i> )	ATCC 52853 CBS 556.83 <sup>b</sup>	AY251087	Decaying wood	Japan
ITS	' <i>Cladosporium</i> ' <i>breviramosum</i>	ATCC 64696	AF393684	Vinyl wallpaper	Georgia, USA
ITS	' <i>Cladosporium</i> ' <i>breviramosum</i>	ATCC 76215	AF393683	Discolored wallpaper	Georgia, USA
ITS	<i>Cladosporium chlorocephalum</i> (syn. <i>Periconia chlorocephala</i> )	ATCC 38011	AF393686	<i>Paeonia suffruticosa</i> leaf	Japan
SSU	<i>Cladosporium cladosporioides</i>	–	U20381	–	–
ITS; SSU	<i>Cladosporium cladosporioides</i>	CBS 109.21 ATCC 11277 STE-U 3682	AY251073; AY251093	<i>Hedera helix</i>	UK
ITS; SSU	<i>Cladosporium cladosporioides</i>	CBS 401.80 ATCC 200941 STE-U 3683	AY251074; AY251091	<i>Triticum aestivum</i>	Netherlands
ITS; SSU	<i>Cladosporium cladosporioides</i>	ATCC 66669 STE-U 5100	AY251070; AY251094	Creosote-treated southern pine pole	Binghamton, New York, USA
ITS	<i>Cladosporium colocasiae</i>	ATCC 38014	AF393692	<i>Colocasia esculenta</i> leaf	Japan

**Tab. 1:** Isolates of *Cladosporium* and allied genera studied (continued)

Sequence data	Species	Accession no. <sup>a</sup>	GenBank accession no.	Substrate	Origin
ITS; SSU	<i>Cladosporium colocasiae</i>	STE-U 4323	AY251075; AY251092	<i>Colocasia esculenta</i>	Fiji islands
ITS	<i>Cladosporium cucumerinum</i>	ATCC 26211	AF393696	<i>Cucumis sativa</i>	–
ITS	<i>Cladosporium echinulatum</i> / <i>Davidiella dianthi</i> (syn. <i>Mycosphaerella dianthi</i> )	ATCC 56129	AF393698	<i>Dianthus caryophyllus</i> leaves	Portugal
ITS	<i>Cladosporium gossypicola</i>	ATCC 38026 CBS 674.82	AF393702	Seed of <i>Gossypium</i> sp.	Jaffa, Israel
ITS	<i>Cladosporium herbarum</i> / <i>Davidiella tassiana</i> (syn. <i>Mycosphaerella tassiana</i> )	ATCC 201090	AF393705	Asymptomatic cherry fruits, <i>Prunus avium</i> cv. 'Bing'	Wenatchee, Washington, USA
ITS	<i>Cladosporium herbarum</i> / <i>Davidiella tassiana</i> (syn. <i>Mycosphaerella tassiana</i> )	CBS 399.80	AJ244227	Skin of man, foot	Geleen, Netherlands
ITS	<i>Cladosporium herbarum</i> / <i>Davidiella tassiana</i> (syn. <i>Mycosphaerella tassiana</i> )	CBS 111.82	AJ238469	<i>Arctostaphylos uva-ursi</i>	Alvaneu, Graubünden, Switzerland
ITS; SSU	<i>Cladosporium herbarum</i> / <i>Davidiella tassiana</i> (syn. <i>Mycosphaerella tassiana</i> )	ATCC 66670 STE-U 5101	AY251078; AY251096	CCA-treated Douglas-fir pole	Geneva, New York, USA
ITS	<i>Cladosporium iridis</i> / <i>Davidiella macrospora</i> (syn. <i>Mycosphaerella macrospora</i> )	–	AF297231	<i>Iris germanica</i>	Indiana, USA
ITS	<i>Cladosporium macrocarpum</i>	CBS 175.62	AJ244229	Grain of <i>Hordeum vulgare</i>	Netherlands
ITS	<i>Cladosporium magnusianum</i> (syn. <i>Heterosporium magnusianum</i> )	ATCC 200946 CBS 842.91	AF393712	Green leaf of <i>Narthecium ossifragum</i>	Bjerkreim County, Norway
ITS	<i>Cladosporium oxysporum</i>	CBS 125.80	AJ300332	Seedcoat of <i>Cirsium vulgare</i>	Netherlands
ITS	<i>Cladosporium oxysporum</i>	ATCC 76499	AF393720	Decayed leaf, <i>Lespedeza bicolor</i>	Lee Co., Alabama, USA
ITS; SSU	<i>Cladosporium</i> sp.	STE-U 5371	AY251072; AY251099	<i>Spinacia</i> sp.	Gaborone, Botswana
ITS; SSU	<i>Cladosporium</i> sp.	STE-U 5124	AY251076; AY251090	<i>Apium graveolens</i>	New Zealand
ITS; SSU	<i>Cladosporium sphaerospermum</i>	ATCC 11290 CBS 188.54 STE-U 3686	AY251077; AY251098	–	–
ITS; SSU	<i>Cladosporium staurophorum</i> (syn. <i>Hormodendrum staurophorum</i> )	ATCC 200934 CBS 375.81 STE-U 3687	AF393723; AY251121	Soil	Cruz Verde, Cundinamarca, Colombia
ITS	<i>Cladosporium tenuissimum</i>	ATCC 38027	AF393724	Soil	New Caledonia
ITS; SSU	<i>Cladosporium uredinicola</i>	ATCC 46649 STE-U 5390	AY251071; AY251097	Hyperparasite on <i>Cronartium fusiforme</i> f. sp. <i>quercum</i> on <i>Quercus nigra</i> leaves	Alabama, USA
SSU	' <i>Discosphaerina</i> ' <i>fagi</i> (syn. <i>Guignardia fagi</i> )	CBS 171.93 IMI 189460A	AY016342	Leaf of <i>Populus</i> sp.	UK
SSU	<i>Dissoconium dekkeri</i> / <i>Mycosphaerella lateralis</i>	CBS 567.89 STE-U 1535	AY251101	<i>Juniperus chinensis</i> , 'Old Gold'	Maarsse, Netherlands
SSU	<i>Dothidea insculpta</i>	–	U42474	–	–
SSU	<i>Dothidea ribesia</i>	CBS 195.58	AY016343	<i>Ribes</i> sp.	Gunzgen, Kt. Solothurn, Switzerland
ITS	<i>Fusicladium effusum</i> (syn. <i>Cladosporium caryigenum</i> )	–	AF065850	Pecan nuts	Georgia, USA
ITS; SSU	<i>Fusicladium effusum</i> (syn. <i>Cladosporium caryigenum</i> )	STE-U 4524	AY251084; AY251125	Pecan nuts	Georgia, USA
ITS; SSU	<i>Fusicladium effusum</i> (syn. <i>Cladosporium caryigenum</i> )	STE-U 4525	AY251085; AY251126	Pecan nuts	Georgia, USA

**Tab. 1:** Isolates of *Cladosporium* and allied genera studied (continued)

Sequence data	Species	Accession no. <sup>a</sup>	GenBank accession no.	Substrate	Origin
ITS	<i>Fusicladium effusum</i> (syn. <i>Cladosporium caryigenum</i> )		AF065851	Pecan nuts	Louisiana, USA
ITS; SSU	<i>Fusicladium convolvulorum</i>	IMI 383037 STE-U 3884	AY251082; AY251124	–	New Zealand
ITS	<i>Fusicladium pomi</i> (syn. <i>Spilocaea pomi</i> ) / <i>Venturia inaequalis</i>	–	AF065837	<i>Malus</i> sp.	–
ITS	<i>Fusicladium pyrorum</i> / <i>Venturia pyrina</i>	–	AF065844	Pear	Israel
SSU	<i>Fusicoccum</i> sp. / <i>Botryosphaeria ribis</i>	–	AF271129	–	–
SSU	<i>Guignardia endophyllicola</i>	CBS 398.80 IFO 33062	AB041249	–	New Zealand
SSU	<i>Saccharicola bicolor</i> (syn. <i>Leptosphaeria bicolor</i> )	ATCC 42652	U04202	<i>Saccharum officinarum</i>	Kenya
ITS	<i>Mycosphaerella iridis</i> (syn. <i>Sphaerella iridis</i> )	CBS 282.49	AY251088	Leaf spot in <i>Iris pseudacorus</i>	Baarn, Netherlands
ITS	<i>Mycosphaerella iridis</i> (syn. <i>Sphaerella iridis</i> )	CBS 281.49	AY251089	Leaf spot in <i>Iris pseudacorus</i>	Glattfelden, Zürich, Switzerland
SSU	<i>Mycosphaerella latebrosa</i> (syn. <i>Sphaerella latebrosa</i> )	CBS 652.85	AY251114	Leaf spot in <i>Acer pseudoplatanus</i>	Baarn, Netherlands
SSU	<i>Mycosphaerella nubilosa</i> (syn. <i>Sphaerella nubilosa</i> )	STE-U 4661	AY251120	<i>Eucalyptus globulus</i>	Ponte Areas, Spain
SSU	<i>Mycosphaerella populorum</i>	–	AF271130	–	–
SSU	<i>Mycosphaerella</i> sp.	STE-U 1731	AY251115	<i>Protea</i> sp.	Drakensberg, Kwazulu-Natal, South Africa
SSU	<i>Mycosphaerella</i> sp.	STE-U 3837	AY251116	<i>Acacia</i> sp.	Venezuela
SSU	<i>Paraphaeosphaeria michotii</i> (syn. <i>Sphaeria michotii</i> )	CBS 591.73	AF250817	<i>Juncus squarrosus</i>	France
SSU	<i>Paraphaeosphaeria pilleata</i>	CBS 102207	AF250821	<i>Juncus roemerianus</i>	North Carolina, USA
ITS	<i>Passalora arachidicola</i> (syn. <i>Cercospora arachidis</i> ) / <i>Mycosphaerella arachidis</i>	–	AF 297224	<i>Arachis hypogaea</i>	USA
ITS	<i>Passalora bellynckii</i> (syn. <i>Mycovellosiella bellynckii</i> )	CBS 150.49 STE-U 3635	AF222831	<i>Cynanchum vincetoxicum</i>	Switzerland
ITS	<i>Passalora dissiliens</i> (syn. <i>Phaeoramularia dissiliens</i> )	CBS 219.77	AF222835	Living leaf of <i>Vitis vinifera</i>	Basrah Province, Iraq
SSU	<i>Passalora dodonaeae</i>	STE-U 1223 <sup>b</sup>	AY251108	<i>Dodonaea</i> sp.	Western Cape, South Africa
ITS	<i>Passalora fulva</i> (syn. <i>Cladosporium fulvum</i> )	ATCC 44960	AF393701	Tomato	Netherlands
ITS	<i>Passalora fulva</i> (syn. <i>Cladosporium fulvum</i> )	IMI 050487	L25430	<i>Lycopersicon esculentum</i>	Zimbabwe
ITS; SSU	<i>Passalora fulva</i> (syn. <i>Cladosporium fulvum</i> )	CBS 119.46 STE-U 3688	AY251069; AY251109	<i>Lycopersicon esculentum</i>	Netherlands
ITS	<i>Passalora henningsii</i> (syn. <i>Cercospora henningsii</i> )	–	AF284389	<i>Manihot esculenta</i>	Pernambuco, Brazil
SSU	<i>Passalora janseana</i> (syn. <i>Napicladium janseanum</i> )	CBS 145.37 IMI 303642 STE-U 4303	AY251103	<i>Oryza sativa</i>	Arkansas, USA
ITS	<i>Passalora vaginae</i> (syn. <i>Mycovellosiella vaginae</i> )	CBS 140.34	AF222832	<i>Saccharum officinarum</i>	Taiwan
ITS; SSU	' <i>Phaeoramularia hachijoensis</i> '	ATCC 96545 STE-U 5121	AY251068; AY251100	Air	Long Island, New York, USA
ITS	' <i>Phaeoramularia hachijoensis</i> '	CBS 462.82 STE-U 3679	AY251086	<i>Pinus</i> sp	De Vuursche, Baarn, Netherlands
ITS	<i>Phialophora americana</i>	CDC 10	U31838	Paper pulp	Wisconsin, USA



**Tab. 1:** Isolates of *Cladosporium* and allied genera studied (continued)

Sequence data	Species	Accession no. <sup>a</sup>	GenBank accession no.	Substrate	Origin
ITS	<i>Phialophora americana</i>	FMC 2214	AF397136	–	Colombia
ITS	<i>Phialophora americana</i>	CBS 840.69 MUCL 15537	AF050283	Decaying timber	Helsinki, Finland
SSU	<i>Pleospora betae</i>	IMI 156653	U43466	Seed of <i>Beta</i> sp.	UK
SSU	<i>Pseudocercospora cruenta</i> (syn. <i>Cercospora cruenta</i> )	CBS 462.75	AY251105	<i>Phaseolus</i> sp.	Labasa, Fiji
SSU	<i>Pseudocercospora protearum</i> var. <i>leucadendri</i> (syn. <i>Cercospora</i> <i>protearum</i> var. <i>leucadendri</i> )	STE-U 1869	AY251107	<i>Leucadendron</i> sp.	Western Cape, Stellenbosch, South Africa
ITS; SSU	<i>Pseudocladosporium hachijoense</i>	ATCC 96019 STE-U 5391	AY251083; AY251123	<i>Prunus avium</i>	Wenatchee, Washington, USA
SSU	<i>Pseudocercospora angolensis</i> (syn. <i>Cercospora angolensis</i> )	ATCC 11669 CBS 149.53	AY251106	Leaf of <i>Citrus sinensis</i>	Bié, Angola
SSU	<i>Ramularia</i> sp.	STE-U 4195	AY251112	–	–
SSU	<i>Ramulispora sorghi</i>	STE-U 905	AY251110	<i>Sorghum</i> sp.	KwaZulu-Natal, South Africa
SSU	<i>Ramulispora sorghi</i>	STE-U 906	AY251111	<i>Sorghum</i> sp.	KwaZulu-Natal, South Africa
ITS	<i>Rhinocladiella compacta</i> (syn. <i>Hormodendrum compactum</i> )	IMTSP 373	AF397133	–	–
SSU	<i>Septoria rosae</i>	ATCC 24311 CBS 355.58 STE-U 4302	AY251113	Leaf of <i>Rosa</i> sp.	–
SSU	<i>Septoria tritici</i>	STE-U 658	AY251117	<i>Triticum</i> sp.	Western Cape, South Africa
SSU	<i>Sphaerulina polyspora</i>	CBS 354.29 STE-U 4301	AY251095	–	–
SSU	<i>Stemphyllium herbarum</i> / <i>Pleospora herbarum</i>	ATCC 11681	U43458	Onion leaf	Colorado
ITS	<i>Sorocybe resinae</i> (syn. <i>Hormodendrum resinae</i> ) / <i>Amorphotheca resinae</i>	ATCC 200942 CBS 406.68	AF393726	Soil	UK
ITS	<i>Sorocybe resinae</i> (syn. <i>Hormodendrum resinae</i> ) / <i>Amorphotheca resinae</i>	ATCC 11841 CBS 184.54 STE-U 3692	AY251067	Creosote-treated wooden pole	St Louis, Missouri, USA
SSU	<i>Trimmatostroma macowanii</i>	STE-U 1488	AY251118	<i>Protea</i> sp.	Hermanus, Western Cape, South Africa
SSU	<i>Uwebraunia juvenis</i> / <i>Mycosphaerella juvenis</i>	STE-U 1004 <sup>b</sup>	AY251119	Leaves of <i>Eucalyptus grandis</i>	Hazyview, Gauteng, South Africa
ITS	<i>Venturia cerasi</i>	ATCC 12119 CBS 444.54	AF065847	<i>Prunus cerasus</i>	East Germany

<sup>a</sup> ATCC: American Type Culture Collection, Virginia, U.S.A.;  
IMI: International Mycological Institute, CABI-Bioscience, Egham, Basingstoke, U.K.;  
E.G.S.: E. Simmons, 717 Thornwood Road, Crawfordsville, Indiana U.S.A.;  
STE-U: Department of Plant Pathology, University of Stellenbosch, South Africa;  
CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands;  
MUCL: Université Catholique de Louvain, Louvain-la-Neuve, Belgium;  
WC: Wadsworth Center for Laboratories and Research Collection (New York State Department of Health);  
UTHSC: University of Texas Health Science Centre, U.S.A.;  
FMC: Venezuelan School of Medicine;  
IMTSP: Institute of Tropical Medicine of São Paulo;  
CDC: Centre for Disease Control and Prevention, U.S. Department of Health and Human Services.  
IFO: Institute for Fermentation, Osaka, Japan.

<sup>b</sup> Ex-type isolates.



## Species of *Mycosphaerella* and related anamorphs on *Eucalyptus* leaves from Thailand

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### Key words

*Eucalyptus*  
*Mycosphaerella*  
*Mycosphaerella* leaf disease  
*Penidiella*  
*Pseudocercospora*  
taxonomy

**Abstract** Species of *Mycosphaerella* and their related anamorphs represent potentially serious foliar pathogens of *Eucalyptus*. The fungi treated in the present study were isolated from symptomatic *Eucalyptus* leaves collected in Thailand during June–October 2007. Species were initially identified based on morphological and cultural characteristics. Identifications were confirmed using comparisons of DNA sequence data of the internal transcribed spacers (ITS1, 5.8S nrDNA, ITS2) and the 28S nrDNA (LSU) regions. To help distinguish species of *Pseudocercospora*, the dataset was expanded by generating partial sequences of the translation elongation factor 1- $\alpha$  and actin genes. By integrating the morphological and molecular datasets, five new taxa were distinguished, namely *Mycosphaerella irregulari*, *M. pseudomarksii*, *M. quasiparkii*, *Penidiella eucalypti* and *Pseudocercospora changmaiensis*, while *M. vietnamensis* represents a new record for Thailand.

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### INTRODUCTION

Species of *Eucalyptus* (Myrtaceae) are hosts to a wide range of fungal pathogens (Sankaran et al. 1995, Crous et al. 2004a, 2006b, 2007a, Summerell et al. 2006). *Eucalyptus* spp. are commonly planted as exotics in commercial plantations for fuel wood, timber, and the paper and pulp industries in various tropical and subtropical regions (Ball 1995). *Eucalypts* have been cultivated extensively because of their fast growth rates and high adaptability to different soil types and climates (Turnbull 2000). In 1995, the total *Eucalyptus* plantation area in South-East Asia already exceeded 2 million ha (Old et al. 2003) and this number has continually increased over the years. In Thailand alone, a total of 443 000 ha *Eucalyptus* plantations was established by 2005 (Barney 2005). In spite of these huge areas already afforested to *Eucalyptus*, this crop is still increasingly being planted in Thailand and other Asian countries to meet rising global timber demand.

Plant pathogenic microfungi associated with *Eucalyptus* spp. can substantially decrease timber yield (Park et al. 2000, Old et al. 2003). In particular, species of *Mycosphaerella* (*Mycosphaerellaceae*) and *Teratosphaeria* (*Teratosphaeriaceae*) have proven to be serious pathogens of *Eucalyptus*, causing severe leaf spot formation, defoliation, shoot die-back and stem cankers (Crous 1998, Maxwell et al. 2003, Crous et al. 2004a, 2006a, c, 2007b, Hunter et al. 2004, 2006b, Jackson et al. 2005, Cortinas et al. 2006, Carnegie 2007). Nonetheless, information about the diversity of *Mycosphaerella* and related anamorph species on *Eucalyptus* from Thailand is generally lacking, and presently only five species have been reported,

namely *Pseudocercospora basiramifera* and *Ps. flavomarginata* (Crous 1998, Hunter et al. 2006a), *Mycosphaerella heimii*, *M. konae* and *M. thailandica* (Crous et al. 2007b). Species of this pathogen complex reported from *Eucalyptus* in Asia include *Dissoconium aciculare*, *Kirramyces destructans*, *K. eucalypti*, *M. crystallina*, *M. eucalyptorum*, *M. gracilis*, *M. heimioides*, *M. marksii*, *M. obscuris*, *M. parkii*, *M. robusta*, *M. stramenticola*, *M. sumatrensis*, *M. verrocossiafricana*, *M. vietnamensis*, *M. yunnanensis*, *Ps. deglupta*, *Ps. eucalyptorum*, *Ps. fatouae*, *Ps. paraguayensis*, *Ps. robusta*, *Septoria eucalyptorum*, *S. xenoparkii*, *Teratosphaeria fimbriata*, *T. gamsii*, *T. suberosa* and *T. suttonii* (Crous & Alfenas 1995, Crous & Wingfield 1997, Hunter et al. 2004, 2006a, Crous 1998, Crous & Braun 2003, Crous et al. 2004a, 2006c, 2007c, Burgess et al. 2007). Many *Eucalyptus* leaf pathogens originally described as related species of *Mycosphaerella* (i.e. *M. cryptica*, *M. gamsii*, *M. pseudocryptica*, *M. pseudosuberosa* and *M. suttonii*) have been re-classified into the genus *Teratosphaeria* (*Teratosphaeriaceae*) after substantial taxonomic revisions based on novel morphological characters integrated with their DNA phylogeny obtained by using the 28S nrDNA gene (Crous et al. 2007c, 2008).

DNA sequencing of the ITS nrDNA gene has in the past proven highly effective to distinguish among species of *Mycosphaerella* (Crous et al. 2000, 2006a, b, 2007b, Cortinas et al. 2006). The main objective of the present study was to identify species of *Mycosphaerella* and related anamorphs associated with *Eucalyptus* leaves collected from plantations in Thailand, and to resolve their taxonomy and DNA phylogeny.

### MATERIAL AND METHODS

#### Isolates

Symptomatic *Eucalyptus* leaves were collected at various locations in Thailand (Table 1). Lesions with ascomata were removed, soaked in distilled water for 2 h, and then placed in

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**Table 1** Isolates of *Mycosphaerella* and related anamorphs used for DNA analysis and morphological studies.

Species	Accession number <sup>1</sup>	Host	Location	GenBank number			
				ITS	LSU	EF	ACT
<i>Mycosphaerella heimii</i>	CPC 15428	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882123	–	–	–
	CPC 15429	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882122	EU882141	–	–
	CPC 15430	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882121	EU882140	–	–
<i>Mycosphaerella irregulari</i>	CPC 15408, CBS 123242	<i>Eucalyptus</i> sp.	Udonthani, Thailand	EU882110	–	–	–
	CPC 15431	<i>Eucalyptus</i> sp.	Udonthani, Thailand	EU882111	–	–	–
	CPC 15432	<i>Eucalyptus</i> sp.	Udonthani, Thailand	EU882112	–	–	–
<i>Mycosphaerella pseudomarksii</i>	CPC 15410, CBS 123241	<i>Eucalyptus</i> sp.	Chiang Mai, Thailand	EU882113	EU882137	–	–
	CPC 15435	<i>Eucalyptus</i> sp.	Chiang Mai, Thailand	EU882114	–	–	–
	CPC 15436	<i>Eucalyptus</i> sp.	Chiang Mai, Thailand	EU882115	–	–	–
<i>Mycosphaerella quasiparkii</i>	CPC 15409, CBS 123243	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882125	EU882143	–	–
	CPC 15433	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882126	–	–	–
	CPC 15434	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882127	–	–	–
<i>Mycosphaerella</i> sp.	CPC 15446	<i>E. camaldulensis</i>	Burirum, Thailand	EU882108	EU882136	–	–
	CPC 15447	<i>E. camaldulensis</i>	Burirum, Thailand	EU882109	–	–	–
	CPC 15448	<i>E. camaldulensis</i>	Ubonratchathani, Thailand	EU882124	EU882142	–	–
<i>Mycosphaerella thailandica</i>	CPC 15437	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882120	–	–	–
	CPC 15438	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882119	–	–	–
	CPC 15439	<i>E. camaldulensis</i>	Mahasarakam, Thailand	EU882118	EU882139	–	–
	CPC 15440	<i>E. camaldulensis</i>	Ubonratchathani, Thailand	EU882117	EU882138	–	–
	CPC 15441	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882116	–	–	–
<i>Mycosphaerella vietnamensis</i>	CPC 15442	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882104	–	–	–
	CPC 15443	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882107	EU882135	–	–
	CPC 15444	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882106	EU882134	–	–
	CPC 15445	<i>E. camaldulensis</i>	Udonthani, Thailand	EU882105	–	–	–
<i>Penidiella eucalypti</i>	CPC 15411, CBS 123246	<i>Eucalyptus</i> sp.	Mahasarakam, Thailand	EU882131	EU882145	–	–
	CBS 123245	<i>E. camaldulensis</i>	Burirum, Thailand	EU882132	–	–	–
	CPC 15449	<i>E. camaldulensis</i>	Burirum, Thailand	EU882133	EU882146	–	–
<i>Pseudocercospora chiangmaiensis</i>	CPC 15412, CBS 123244	<i>E. camaldulensis</i>	Chiang Mai, Thailand	EU882128	EU882144	EU882147	EU882150
	CPC 15450	<i>E. camaldulensis</i>	Chiang Mai, Thailand	EU882129	–	EU882148	EU882151
	CPC 15451	<i>E. camaldulensis</i>	Chiang Mai, Thailand	EU882130	–	EU882149	EU882152

<sup>1</sup> CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.

the bottom of the Petri dish lids, with the top half of the dish containing 2 % malt extract agar (MEA; Oxoid, Gams et al. 2007). Dishes were incubated at room temperature in the dark. After 24 h ascospore germination patterns from discharged ascospores on MEA were examined under a compound microscope. Single ascospore cultures were established on fresh MEA dishes as described by Crous (1998). Symptomatic leaves were also incubated in moist chambers (Petri dishes containing moist filter paper). Leaves were inspected daily for microfungi, and single conidial colonies of hyphomycetes and coelomycetes established on MEA (Crous 2002). Cultures were plated onto fresh MEA, oatmeal agar (OA; Gams et al. 2007) and pine needle agar (Slippers et al. 2006), and subsequently incubated at 25 °C in dark, to promote sporulation. Reference strains are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and BCC, BIOTEC, Thailand (Table 1).

#### DNA isolation, amplification and analyses

Genomic DNA was extracted from mycelia of fungal colonies cultivated on MEA using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). The Primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990) were used to amplify part of the nuclear rDNA operon spanning the 3' end of the 18S rRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region (ITS2) and the 5' end of the 28S rRNA gene (LSU). The primers ITS4 (White et al. 1990) and LR0R (Rehner & Samuels 1994) were used as internal sequence primers to ensure good quality sequences over the entire length of the amplicon. To help resolve species of *Pseudocercospora*, the ITS region was supplemented with sequences of the translation elongation factor 1- $\alpha$  gene (EF-1 $\alpha$ ) using the primers EF1-728F and EF1-986R (Carbone & Kohn 1999) and the actin gene (ACT) using the primers ACT-512F and ACT-783R (Carbone & Kohn 1999). The PCR amplifications were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in a total volume of 12.5  $\mu$ L solution containing 10–20 ng of template DNA, 1  $\times$  PCR buffer, 2.5 mM MgCl<sub>2</sub>, 15 pmol for each primer, 60  $\mu$ M of each dNTP and 0.75 U *Taq* DNA polymerase (Bioline GmbH, Luckenwalde, Germany). PCR amplification conditions were set as follows: an initial denaturation temperature of 94 °C for 5 min, followed by 40 cycles of denaturation temperature of 94 °C for 45 s, primer annealing at 48 °C for 30 s, primer extension at 72 °C for 90 s and a final extension step at 72 °C for 7 min. The primer annealing temperature for EF-1 $\alpha$  and ACT was at 55 °C. The resulting fragments were sequenced using the PCR primers together with a BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA) and analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, CN).

The generated sequences were compared with other fungal DNA sequences from NCBI's GenBank sequence database using a blast search; sequences with high similarity were added to the alignments. The additional GenBank sequences were manually aligned using Sequence Alignment Editor v. 2.0a11 (Rambaut 2002). The phylogenetic analyses of the aligned sequence data were performed using PAUP (Phylogenetic

Analysis Using Parsimony) v. 4.0b10 (Swofford 2003) and consisted of neighbour-joining analyses with the uncorrected ('p'), the Kimura 2-parameter and the HKY85 substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analyses, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed using the heuristic search option with 100 random simple taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993). Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated and the resulting trees were printed with TreeView v. 1.6.6 (Page 1996). New sequences were lodged in GenBank and the alignments and phylogenetic trees in TreeBASE (www.treebase.org).

#### Morphology

Preparations from cultured fungal colonies were mounted on glass slides with clear lactic acid for microscopic examination. Sections of ascomata were made by hand for examination purposes. Measurements of all taxonomically relevant parameters were made at 1 000  $\times$  magnification, with 30 measurements per structure where possible. Colony colours on MEA (surface and reverse) were determined using the colour charts of Rayner (1970) after 15 d at 25 °C in the dark.

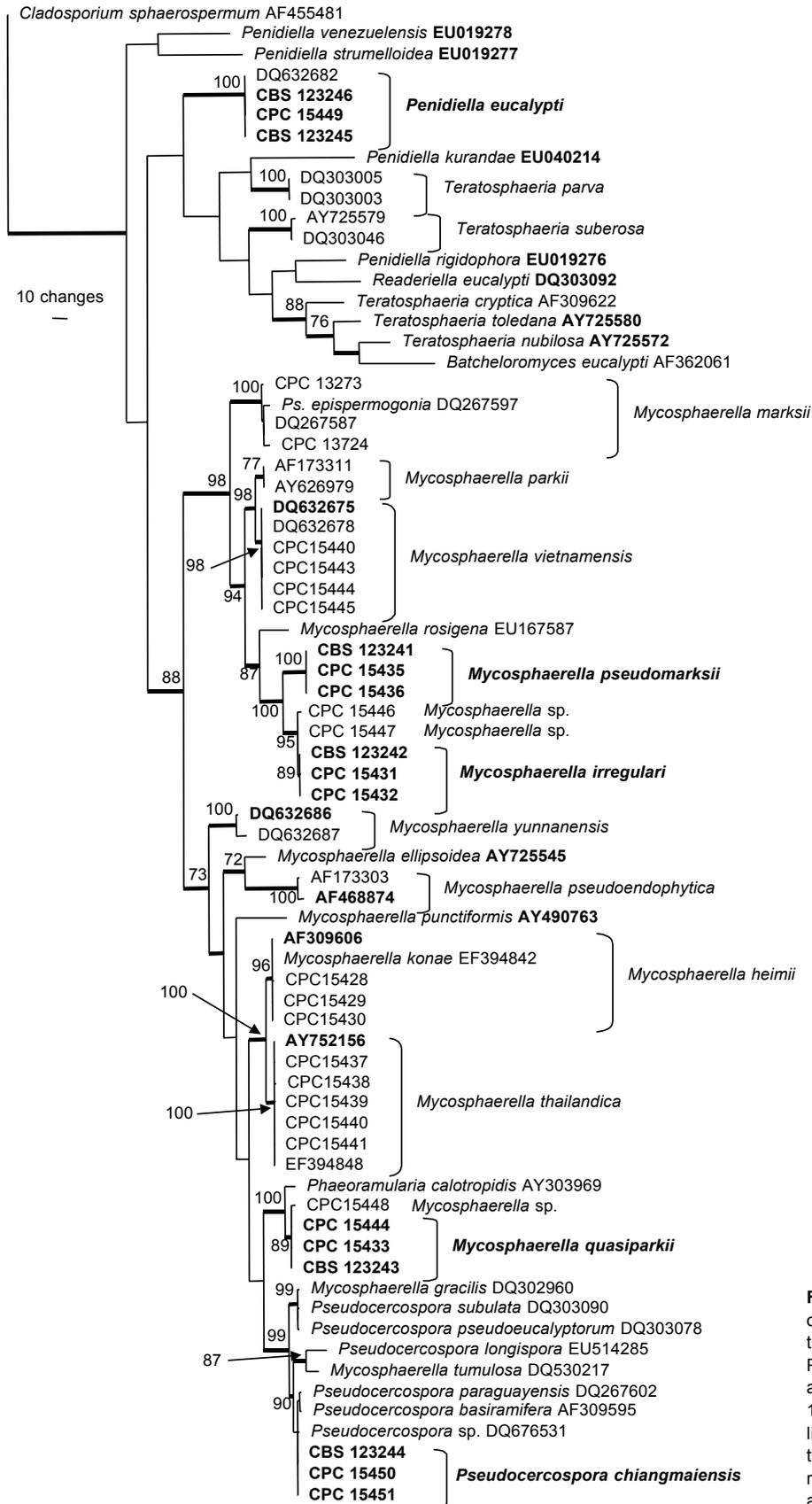
## RESULTS

#### Phylogenetic analysis

Approximately 1 700 bases, spanning the ITS and LSU regions, were obtained for isolates listed in Table 1. These two regions were analysed separately; ITS to determine species level relationships and LSU for the generic placement. Approximately 300 and 220 bases were determined for EF-1 $\alpha$  and ACT, respectively, and these were concatenated with the corresponding ITS sequences for a combined analysis of the *Pseudocercospora* clade.

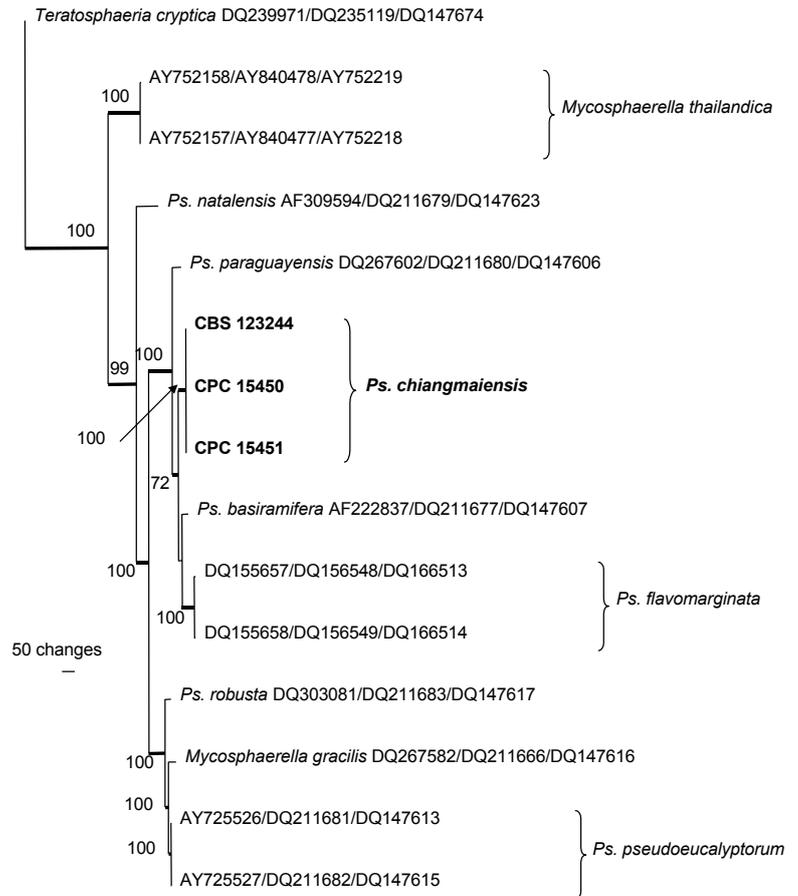
The manually adjusted ITS alignment contained 73 taxa (including the outgroup sequence) and, of the 533 characters used in the phylogenetic analysis, 228 were parsimony-informative, 59 were variable and parsimony-uninformative and 246 were constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values; 1 100 equally most parsimonious trees were obtained from the heuristic search, one of which is shown in Fig. 1 (TL = 1386, CI = 0.444, RI = 0.797, RC = 0.354). The phylogenetic tree derived from the ITS region (Fig. 1) showed that some of the isolates belong to known species, whereas others appeared to be new to science.

The manually adjusted LSU alignment contained 35 taxa (including the outgroup sequence) and, of the 797 characters used in the phylogenetic analysis, 121 were parsimony-informative,



**Fig. 1** One of 1 100 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment using PAUP v. 4.0b10. The scale bar shows 10 changes and bootstrap support values higher than 70 % from 1 000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and ex-type sequences are printed in bold face. The tree was rooted to *Cladosporium sphaerospermum* (GenBank accession AF455481).





**Fig. 3** Single most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined ITS, EF and ACT sequence alignment using PAUP v. 4.0b10. The scale bar shows 100 changes and bootstrap support values from 1 000 replicates are shown at the nodes. The tree was rooted to *Teratosphaeria cryptica* (GenBank accessions DQ239971, DQ235119 and DQ147674, respectively). *Ps.* = *Pseudocercospora*.

**Taxonomy**

Several taxonomic novelties, namely three *Mycosphaerella*, one *Pseudocercospora* and one *Penidiella* species, were found. These species do not match any species presently described from these genera, or any linked to sequences available in GenBank, and are thus described as new below.

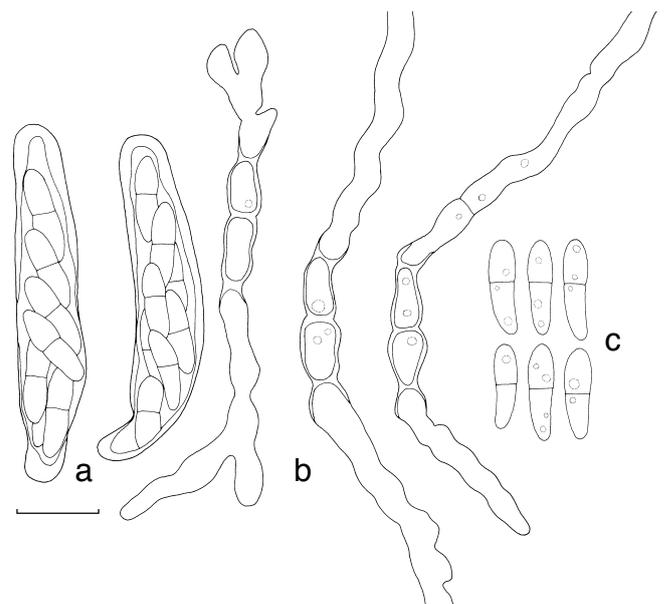
***Mycosphaerella irregulari*** Cheewangkoon, K.D. Hyde & Crous, *sp. nov.* — MycoBank MB507001; Fig. 4, 5

*Anamorph.* Unknown.

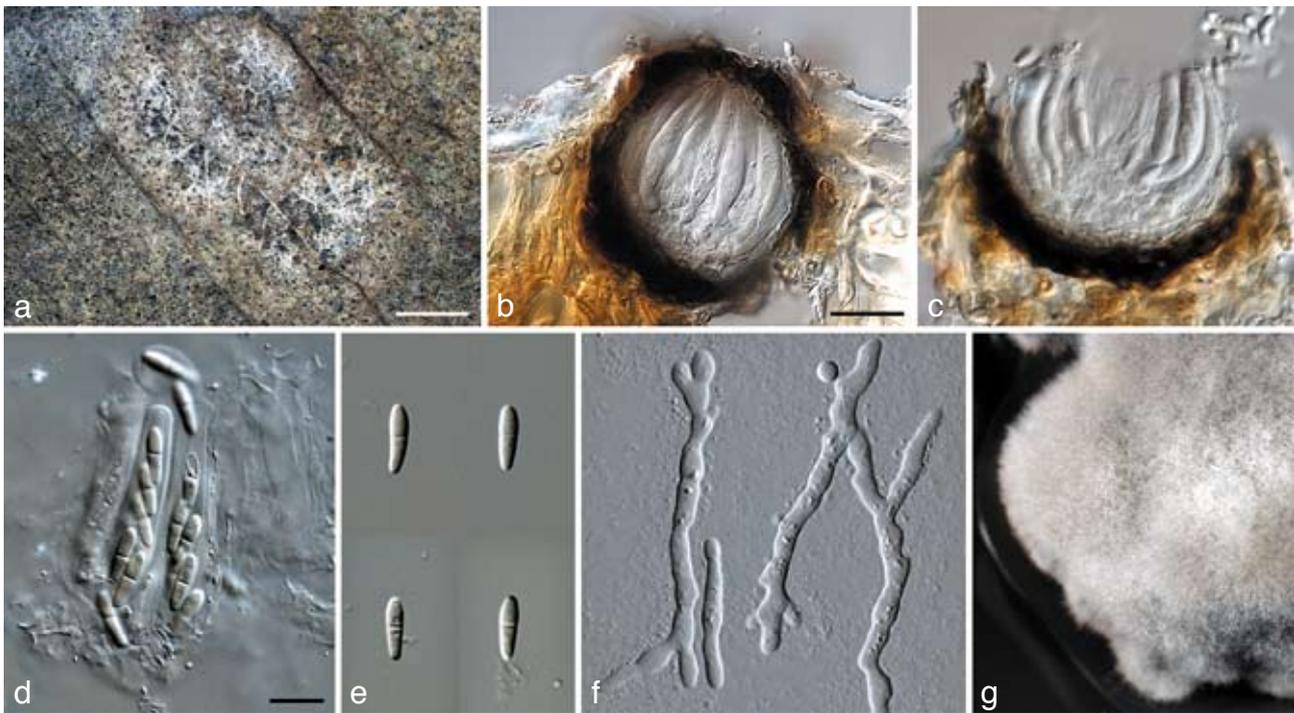
*Mycosphaerellae tasmaniensis* similis, sed ascosporis cum tubis germinabilibus irregulariter latis.

*Etymology.* Named after the irregular width of its ascospore germ tubes.

*Leaf spots* amphigenous, subcircular to oval, pale brown with grey centres, 5–12 mm diam, surrounded by a thin, medium brown margin. *Mycelium* external, smooth, septate, branch, medium brown, (2–)2.5–3(–4) µm wide hyphae. *Ascomata* epiphyllous, black, subepidermal to erumpent, ovoid to subglobose, 45–83 × 45–78 µm; apical ostiole 20–25 µm wide; wall thick (6.5–)7–10(–12) µm, consisting of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, subsessile, subcylindrical to narrowly obovoid, straight to



**Fig. 4** *Mycosphaerella irregulari*. a. Asci; b. germinating ascospores; c. ascospores. — Scale bar = 10 µm.



**Fig. 5** *Mycosphaerella irregulari*. a. Leaf spot; b, c. sections through ascomata; d. asci; e. ascospores; f. germinating ascospores; g. colony on MEA. — Scale bars: a = 1 mm; b, c = 20  $\mu$ m; d–f = 10  $\mu$ m.

slightly curved, 8-spored, (25–)35–40(–45)  $\times$  (6–)7–8(–10)  $\mu$ m. *Ascospores* bi- to tri-seriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, widest just above the septum, or in the middle of the apical cell, medianly 1-septate or slightly longer in the basal cell, slightly constricted at septum, tapering toward both ends, but with more prominent taper towards lower end, at times with a mucous-like coating, (8–)9–11(–13)  $\times$  2.5–3(–3.5)  $\mu$ m.

*Ascospore germination* — Germinating from both ends, remaining hyaline; germ tubes grow parallel to the long axis of the spore, with lateral branches parallel or perpendicular to the long axis of the spore; germination tubes irregular in width, constrict at the median septum of the spore, becoming (11–)13–15(–16.5)  $\times$  4–5(–5.5)  $\mu$ m, slightly distorting (Type I; Crous 1998).

*Cultural characteristics* — Colonies reach 15 mm diam on MEA after 15 d at 25  $^{\circ}$ C in the dark; circular, convex, with a slightly undulate, smooth margin, and medium aerial mycelium; pale greenish grey to pale olivaceous-grey (surface); olivaceous-black (reverse).

*Specimen examined*. THAILAND, Udonthani, on living leaves of *Eucalyptus* sp., July 2007, R. Cheewangkoon, holotype CBS H-20135, cultures ex-type CBS 123242 = CPC 15408, CPC 15431, CPC 15432.

*Notes* — The ascospore morphology of *M. irregulari* is similar to that of *M. tasmaniensis* (Crous et al. 1998), *M. flexuosa* (Crous 1998), *M. ellipsoidea* (Crous & Wingfield 1996) and *M. heimii* (Crous & Swart 1995). However, *M. irregulari* can be distinguished from these species by its irregular germ tubes and germination pattern. Phylogenetically, it is also not closely

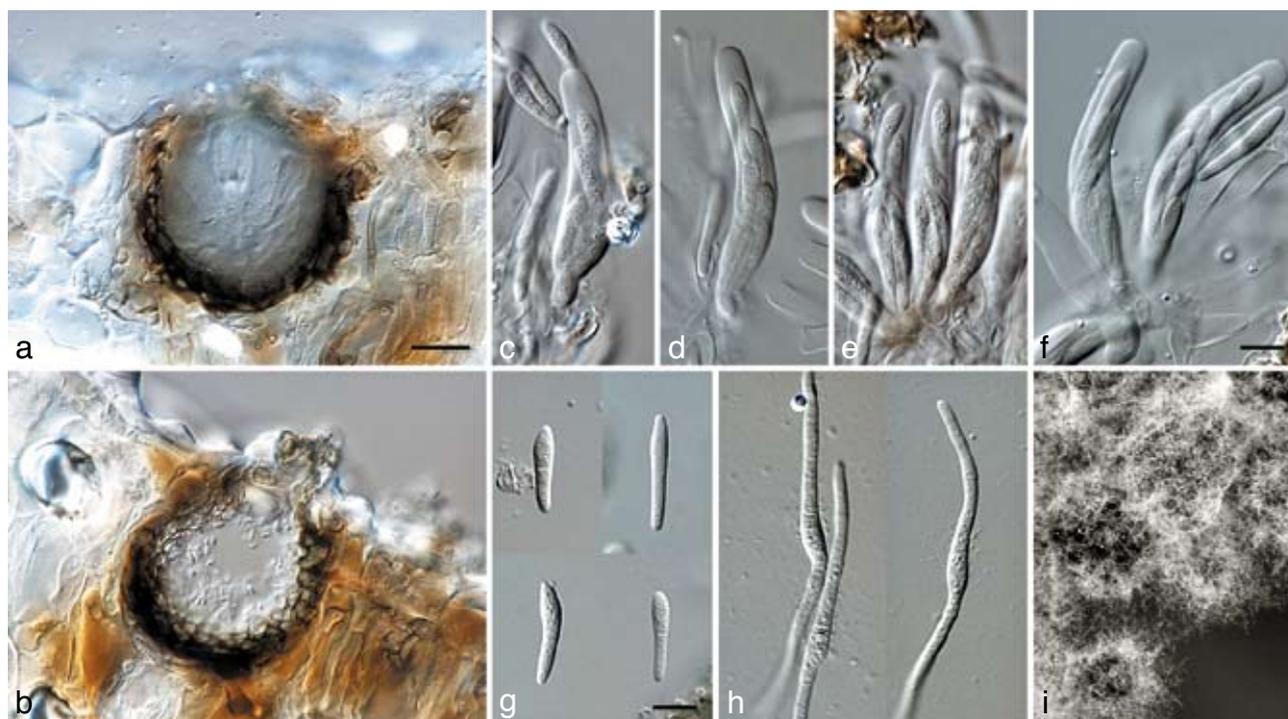
related to any of the species cited above, but clusters near to *M. pseudomarksii* (100 % bootstrap, Fig. 1), which has a distinct morphology.

***Mycosphaerella pseudomarksii*** Cheewangkoon, K.D. Hyde & Crous, sp. nov. — MycoBank MB507003; Fig. 6, 7

*Mycosphaerellae marksii* similis, sed ascosporis majoribus, (12–)14–17(–18.5)  $\times$  (2.5–)3(–3.5)  $\mu$ m.

*Etymology*. Named after *Mycosphaerella marksii* to which it is morphologically similar.

*Leaf spots* not observed. *Ascomata* amphigenous in apparently healthy tissue (endophyte?), occurring on greenish brown part of the leaf after incubation in moist chambers for 2 d, black, subepidermal to erumpent, globose to subglobose, 42–60  $\times$  45–80  $\mu$ m; apical ostiole 20–35  $\mu$ m wide; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* paraphysate, fasciculate, bitunicate, sessile, subcylindrical to narrowly ovoid, slightly curved, 8-spored, (40–)42–45(–48)  $\times$  (6.5–)7–8(–8.5)  $\mu$ m. *Ascospores* bi- to tri-seriate overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid with obtuse ends, widest in the middle of the asymmetrical apical cell, medianly 1-septate or with slightly longer basal cell; tapering toward both ends, but with more prominent taper towards lower end, (12–)14–17(–18.5)  $\times$  (2.5–)3(–3.5)  $\mu$ m. *Spermatogonia* well developed, amphigenous, dark brown, subepidermal to erumpent, globose to subglobose, up to 90  $\mu$ m diam. *Spermatia* hyaline, smooth, rod-shaped, with obtuse ends, (3.5–)4–5(–5.5)  $\times$  1.5–2  $\mu$ m.



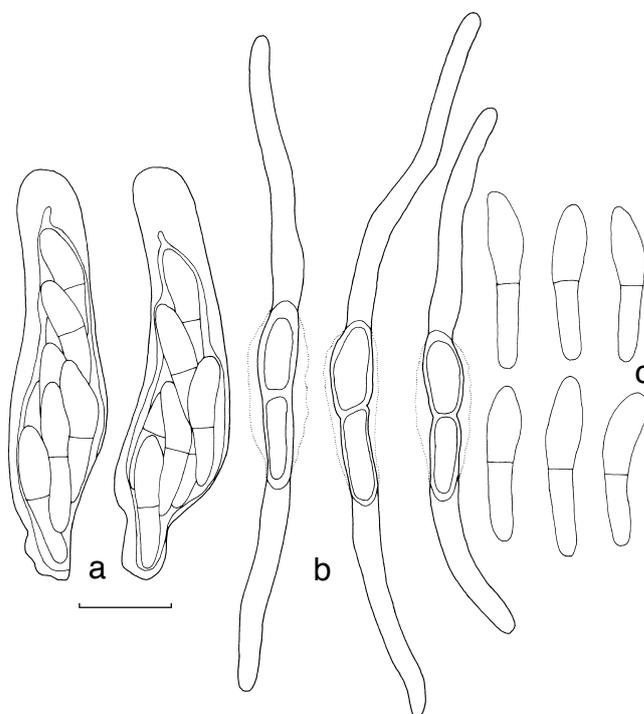
**Fig. 6** *Mycosphaerella pseudomarksii*. a. Vertical section through an ascoma; b. vertical section through a spermatogonium; c–f. asci; g. ascospores; h. germinating ascospores; i. colonies on MEA. — Scale bars: a, b = 10  $\mu$ m; c–h = 10  $\mu$ m.

**Ascospore germination** — Germinating from both ends, with a thin, mucous-like coat visible surrounding ascospores on agar; germ tube growing parallel to the long axis of the spore, regular in width, remaining hyaline, not distorting or becoming constricted at septum (Type B; Crous 1998).

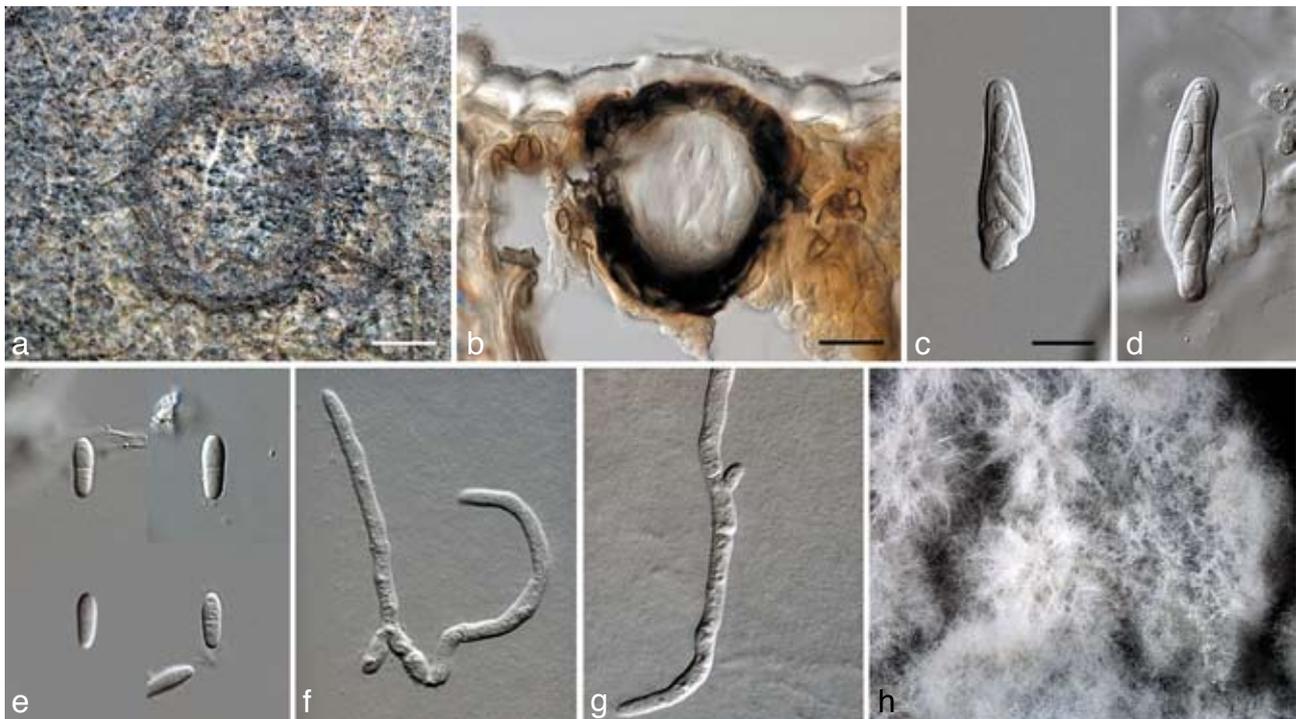
**Cultural characteristics** — Colonies reach 15 mm diam on MEA after 15 d at 25 °C in the dark; subcircular, convex, with even margin, slightly folded, with sparse aerial mycelium; pale olivaceous-grey to olivaceous-grey (surface); olivaceous-black (reverse).

**Specimen examined.** THAILAND, Chiang Mai, Mae Tang, on living leaves of *Eucalyptus* sp., June 2007, R. Cheewangkoon, holotype CBS H-20134, cultures ex-type CBS 123241 = CPC 15410, CPC 15435, CPC 15436.

**Notes** — *Mycosphaerella pseudomarksii* was most similar to *M. marksii* (Carnegie & Keane 1994) based on its asymmetrical apical ascospore cells and ascospore germination patterns. However, germinating ascospores of *M. pseudomarksii* have a visible mucilaginous sheath, which was not observed in germinating ascospores of *M. marksii*. Furthermore, ascospores of *M. pseudomarksii* were slightly longer and wider (12–)14–17(–18.5)  $\times$  (2.5–)3(–3.5)  $\mu$ m than those of *M. marksii* (11–)12–14(–16)  $\times$  2–2.5(–3)  $\mu$ m. Crous & Wingfield (1996) observed considerable variation in ascospore dimensions of several collections of *M. marksii* (12.5–22.5  $\times$  2.5–5  $\mu$ m) commenting that this may represent a species complex. Phylogenetically the two species are also distinct (Fig. 1).



**Fig. 7** *Mycosphaerella pseudomarksii*. a. Asci; b. germinating ascospores; c. ascospores. — Scale bar = 10  $\mu$ m.



**Fig. 8** *Mycosphaerella quasiparkii*. a. Leaf spot; b. section through an ascoma; c, d. asci; e. ascospores; f, g. germinating ascospores; h. colony on MEA. — Scale bars: a = 1 mm; b, = 20 µm; c–g = 10 µm.

***Mycosphaerella quasiparkii*** Cheewangkoon, K.D. Hyde & Crous, *sp. nov.* — MycoBank MB507002; Fig. 8, 9

*Anamorph.* Unknown.

*Mycosphaerellae parkii* similis, sed ascosporis ellipsoideis et coloniis bubalinis in agar MEA.

*Etymology.* Named after its similarity to *Mycosphaerella parkii*.

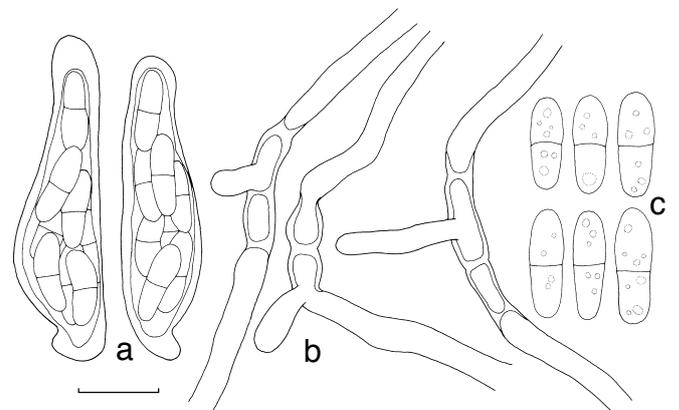
**Leaf spots** amphigenous, round to irregular, separate, becoming confluent, 5–15 mm diam, medium brown on adaxial surface, pale brown on abaxial surface, surrounded by a raised border, which is dark brown on the adaxial surface and paler brown on the abaxial surface. **Ascomata** epiphyllous, black, subepidermal to erumpent, subglobose, 40–60 × 40–55 µm; apical ostiole 10–15 µm wide; wall consisting of 3–4 layers of medium to dark brown *textura angularis*. **Asci** paraphysate, fasciculate, bitunicate, sessile, broadly ellipsoid to obclavate, straight to slightly curved, 8-spored, (30–)45–50(–57) × (7–)8.5–9(–9.5) µm. **Ascospores** bi- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, ellipsoidal to obovoid with obtuse ends, widest in the middle of the apical cell, medianly 1-septate, not constricted at the septum, tapering toward both ends, with a thin mucilaginous sheath, (9–)10–11(–12.5) × (2.5–)3–3.5(–4.5) µm.

**Ascospore germination** — Germinating with more than one germ tube per cell. Initial germ tubes originating from polar ends, growing parallel to the long axis of the spore, with perpendicular germ tubes developing later; ascospores remain hyaline, but become constricted at the median septum, and distorting, (2.8–)3.5–4(–4.5) µm wide (Type D; Crous 1998).

**Cultural characteristics** — Colonies reach 27 mm diam on MEA after 15 d at 25 °C in the dark; circular, low convex, with entire edge and sparse aerial mycelium; buff (surface); vinaceous-buff (reverse).

**Specimen examined.** THAILAND, Burirum, on living leaves of *Eucalyptus* sp., July 2007, P. Suwannawong, holotype CBS H-20132, cultures ex-type CBS 123243 = CPC 15409, CPC 15433, CPC 15434.

**Notes** — *Mycosphaerella quasiparkii* is morphologically similar to *M. parkii* (Crous et al. 1993, 2006c) with which it also shares the same ascospore germination pattern. However, *M. quasiparkii* has more ellipsoid ascospores and paler colonies on MEA. Phylogenetically, *M. quasiparkii* clusters close to *Phaeoramularia calotropidis* (AY303969, Fig. 1).



**Fig. 9** *Mycosphaerella quasiparkii*. a. Asci; b. germinating ascospores; c. ascospores. — Scale bar = 10 µm.



**Fig. 10** *Penidiella eucalypti*. a. Colony on MEA; b–h. catenulate conidia; i. conidiogenous cell and primary ramoconidia; j, primary ramoconidium (left) and secondary ramoconidium (right); k. conidia with mucilaginous sheath. — Scale bars: b = 60  $\mu$ m; c, d = 80  $\mu$ m; e = 30  $\mu$ m; f, g = 20  $\mu$ m; h, i = 15  $\mu$ m; j, k = 10  $\mu$ m.

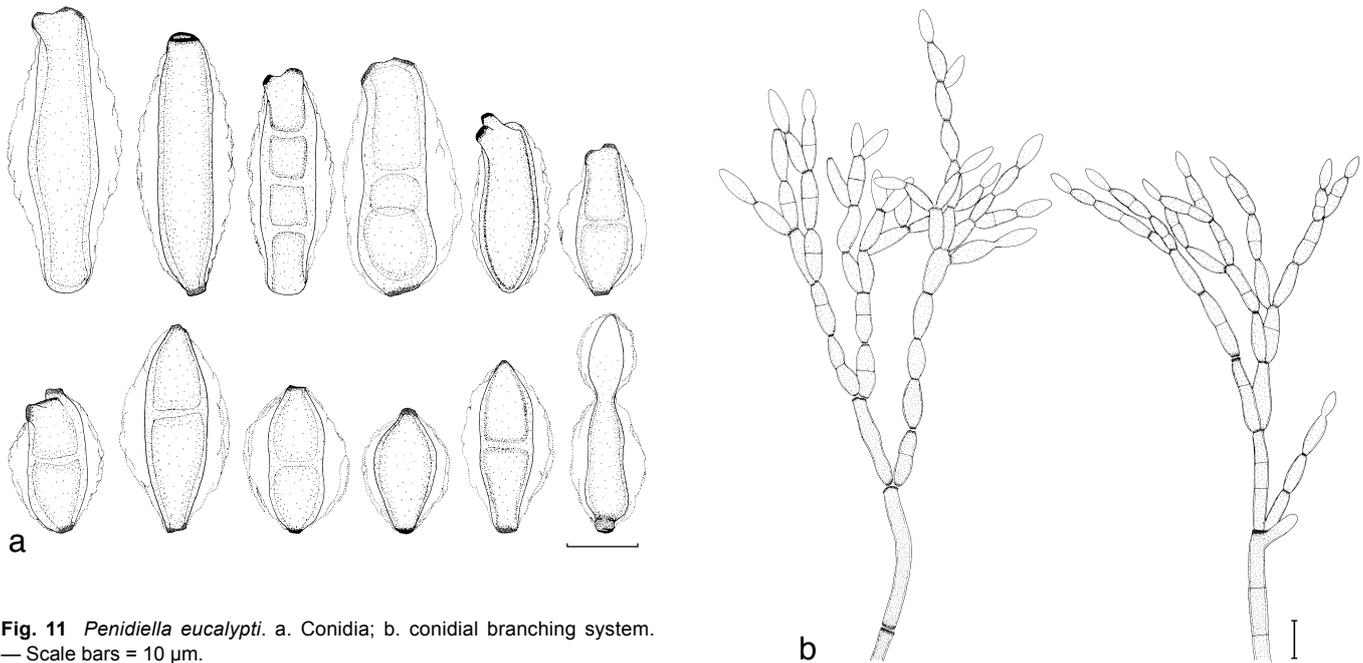
***Penidiella eucalypti*** Cheewangkoon, K.D. Hyde & Crous,  
*sp. nov.* — MycoBank MB507004; Fig. 10, 11

*Teleomorph.* Unknown.

Differt a omnibus speciebus *Penidiellae* conidiis mucosis in vitro (MEA) formantibus.

*Etymology.* Named after its host genus, *Eucalyptus*.

*Leaf spots* not observed. *Mycelium* consisting of branched, septate, smooth to slightly verruculose, pale to medium brown, (2.5–)3–4(–5)  $\mu$ m wide hyphae. *Conidiophores* macro-nematous, occasionally micronematous, arising from superficial mycelium, solitary, erect, straight to slightly curved, branched laterally or not, medium to dark brown, slightly thick-walled, wall  $\leq$  1  $\mu$ m wide, smooth to finely verruculose, (30–)150–



**Fig. 11** *Penidiella eucalypti*. a. Conidia; b. conidial branching system. — Scale bars = 10 µm.

200(–220) × (4–)4.5–5.5(–6.5) µm, wider at the base, (5.5–)6–7(–8.5) µm, (0–)4–6(–8) septate. *Conidiogenous cells* terminal, cylindrical to subcylindrical, tapering to a flattened apical region, smooth to finely verruculose, medium brown, (10–)13–17(–25) × (3–)4–5(–6) µm; scars thickened, somewhat darkened, 2–2.5 µm wide. *Ramoconidia*: primary ramoconidia subcylindrical or obovoid, (0–)1(–3)-septate, base truncate, with (1–)2–3(–4) apical hila, pale olivaceous to pale brown, smooth to finely verruculose, wall < 1 µm wide, (25–)35–40(–48) × (3.5–)4–5(–6.5) µm; scars thickened and slightly darkened, 2–2.5 µm wide; giving rise to chains of up to 12 conidia; secondary ramoconidia obovoid, 0–1-septate, some constricted at septum, base truncate, with 2–3(–4) apical hila, pale olivaceous, smooth, (13–)15–20(–28) × (4.5–)5–6(–7.5) µm; intercalary conidia obovoid, 0–1(–2)-septate, some constricted at septa, base truncate, with 2–3 apical hila, pale olivaceous, smooth, (10–)12–15(–18) × (3.5–)4–5(–5.5) µm. *Conidia* in branched acropetal chains, broadly fusiform to obovoid, 0–1-septate, pale olivaceous, paler towards the apex, (9–)12–15(–19) × (4–)5–6(–7) µm; terminal conidia ovoid, 0-septate, pale olivaceous to hyaline, paler towards the apex, base truncate, conidia have a wing-like mucilaginous sheath in culture, extending up to 5 µm wide on each side, tapering towards the polar ends.

**Cultural characteristics** — Colonies on MEA reaching 20 mm diam after 15 d at 25 °C; margin feathery, colonies erumpent, spreading, with moderate aerial mycelium. Surface grey-olivaceous, reverse olivaceous-black.

**Specimens examined.** THAILAND, Payakpoompisai, Mahasarakam, on leaves of *Eucalyptus camaldulensis*, July 2007, *P. Suwannawong*, holotype CBS H-20136, cultures ex-type CBS 123246 = CPC15411, AGI064.1, AGI064.2 (occurring on a lesion in association with *Harknessia* sp.); Satuk, Burirum, on leaves of *Eucalyptus camaldulensis*, July 2007, *R. Cheewang-*

*koon*, cultures CBS 123245, CPC15449 (occurring on a lesion in association with several microfungi).

**Notes** — *Penidiella eucalypti* is a typical species of *Penidiella* by having solitary conidiophores with a branching system consisting of ramoconidia that form secondary ramoconidia and conidia, with slightly thickened, darkened scars (Crous et al. 2007c). Other than being phylogenetically distinct (Fig. 1, 2), *P. eucalypti* is distinct from most other species of *Penidiella* by having a prominent branching system which develops on a single terminal conidiogenous cell. Another character that has not previously been reported in the genus is the distinct mucilaginous sheath observed on conidia formed on MEA.

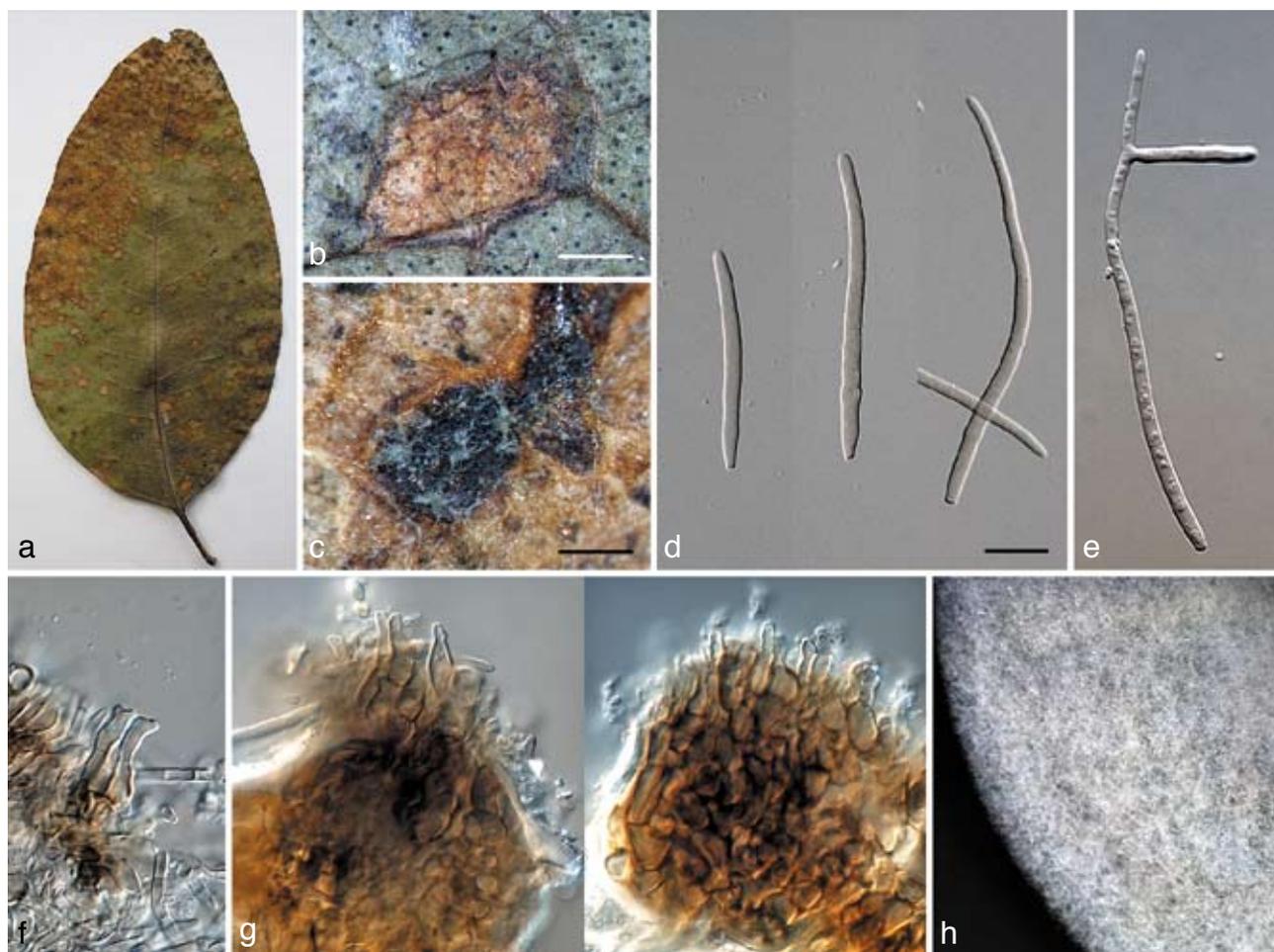
***Pseudocercospora chiangmaiensis*** Cheewangkoon, K.D. Hyde & Crous, *sp. nov.* — MycoBank MB507005; Fig. 12, 13

*Teleomorph.* Unknown.

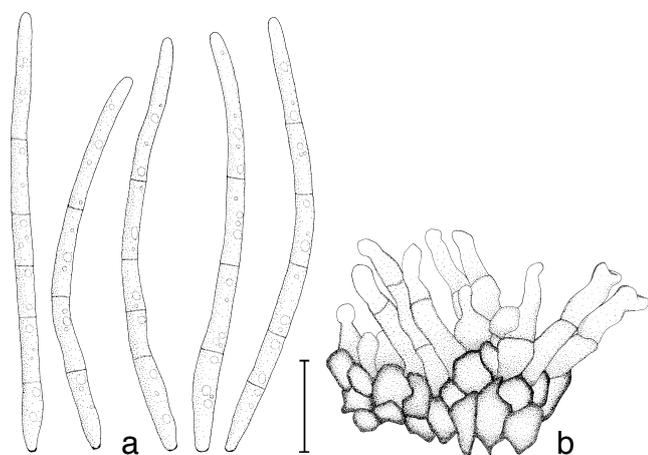
*Pseudocercosporae basiramiferae* similis, sed cellulis conidiogenis terminalibus et intercalariibus et stromatibus bene evolutis.

**Etymology.** Named after Chiang Mai, the province in Thailand from where it was collected.

**Leaf spots** amphigenous, subcircular to angular, 2–6 mm diam, pale to medium brown, surrounded by a slightly raised, dark-brown border, becoming confluent with age, leading to leaf blight from the leaf tip. *Mycelium* predominantly internal, consisting of smooth, septate, branched (1.5–)2–3(–4) µm wide hyphae. *Caespituli* amphigenous, more prominent on abaxial leaf surface, pale grey on leaves, often on dark brown to black and thickened leaf tissue, 25–50 × 50–100 µm. *Stromata* immersed, becoming erumpent, medium brown, 25–70 × 30–70 µm wide. *Conidiophores* reduced to conidiogenous



**Fig. 12** *Pseudocercospora chiangmaiensis*. a. Leaf spots; b, c. close up of leaf spot; d, e. conidia; f, g. conidiogenous cells; h. colony on MEA. — Scale bars: b = 1 mm; c = 300  $\mu$ m; d–g = 10  $\mu$ m.



**Fig. 13** *Pseudocercospora chiangmaiensis*. a. Conidia; b. conidiophores with conidiogenous cells. — Scale bar = 10  $\mu$ m.

cells or one supporting cell, occasionally arising from upper cells of stroma, subcylindrical, 1–3(–6)-septate with intercalary conidiogenous cells, (13–)20–25(–60)  $\times$  3–4(–4.5)  $\mu$ m, situated on the superficial part of the stroma. *Conidiogenous cells* terminal or intercalary, subcylindrical to obclavate, medium brown, becoming paler toward apex, straight to geniculate-sinuous, tapering to a truncate or bluntly rounded apex, at times subdenticulate, smooth, medium to thick-walled, variable in length, (5–)10–11(–12)  $\times$  (2–)3(–5)  $\mu$ m, unbranched, proliferating sympodially; conidial scars thickened at the rim, not darkened, inconspicuous. *Conidia* solitary, subcylindrical to narrowly obclavate, tapering toward the subobtuse apex; base obconic-subtruncate, (2–)3–5(–10)-septate, straight to slightly curved, pale to medium brown, smooth, thin-walled, guttulate, (40–)50–60(–100)  $\times$  (2–)2.5–3(–3.5)  $\mu$ m (up to 140  $\mu$ m long in moist chambers); hilum thickened and somewhat darkened at the rim (paracercospora-like), not refractive, at times inconspicuous; microcyclic conidiation observed when incubated in a moist chamber.

**Cultural characteristics** — Colonies reaching 18 mm diam on MEA after 15 d at 25  $^{\circ}$ C in the dark; colonies circular, convex, with entire margin and medium aerial mycelium; pale greenish grey (surface), fuscous-black (reverse).

*Specimen examined.* THAILAND, Chiang Mai, Doi Lor, on leaves of *Eucalyptus camaldulensis*, June 2007, P. Suwannawong, holotype CBS H-20133, cultures ex-type CBS 123244 = CPC 15412, CPC 15450, CPC 15451.

**Notes** — Of the *Pseudocercospora* species known from *Eucalyptus* (Crous 1998, Braun & Dick 2002, Crous et al. 2004a, 2006c, 2007b, Hunter et al. 2006a, Carnegie et al. 2007), *Ps. chiangmaiensis* is morphologically most similar to *Ps. basiramifera* (Crous 1998) in conidium morphology (dimensions, shape, microcyclic conidiation and scar thickening along the rim). *Pseudocercospora chiangmaiensis* is distinct from *Ps. basiramifera* based on its terminal and intercalary conidigenous cells in vivo, and by its conidiophores occurring on well-developed stomata. Phylogenetically it is closely related to *Ps. basiramifera* (Fig. 3) but differs from it with 5 nucleotide positions on ITS, 34 on EF-1 $\alpha$  and 12 on ACT.

## DISCUSSION

Although numerous species of *Mycosphaerella* have been associated with *Eucalyptus* leaf diseases in tropical regions around the world (Crous 1998, Crous et al. 2000, 2006c, 2007b, c, 2008), only a few of these species have been documented from Asia. The number of novel species found in the present study from sampled *Eucalyptus* leaves collected in Thailand, was thus not totally unexpected. The three new *Mycosphaerella* spp. (i.e. *M. irregulari*, *M. pseudomarksii* and *M. quasiparkii*) identified here were difficult to distinguish from other species of *Mycosphaerella* based on their ascospore morphology and germination patterns alone, which are the characters that have been commonly used in the past for taxonomic classification of *Mycosphaerella* spp. (Park & Keane 1982, Crous 1998). Therefore, the DNA sequencing data generated here proved particularly helpful in distinguishing these species.

*Mycosphaerella pseudomarksii* has ascospores with asymmetrical apical cells similar to *M. marksii*, the only difference being ascospore dimensions and the presence of a mucilaginous ascospore sheath in *M. pseudomarksii*. Ascospores of *M. irregulari* are fusoid-ellipsoidal, thus being similar as those of *M. ellipsoidea*, *M. flexuosa*, *M. heimii* and *M. tasmaniensis*. Although these species have similar ascospore dimensions, *M. irregulari* is characterised by the distinctive irregular width of its ascospore germination tubes. *Mycosphaerella quasiparkii* has an ascospore morphology and germination pattern almost identical to *M. parkii*, but the presence of a thin mucous-like layer on ascospores of *M. quasiparkii* and its buff colonies, distinguish it from *M. parkii*, which lacks an ascospore sheath and has olivaceous-grey colonies. *Pseudocercospora chiangmaiensis*, which is also newly described from *Eucalyptus*, shares some morphological features (conidial dimensions, hilum thickening and microcyclic conidiation) with *Ps. basiramifera*. It is distinct, however, by having terminal and intercalary conidigenous cells in vivo and having conidiophores arising from a well-defined stroma. Phylogenetic analyses of the ITS and ACT genes showed limited differences (only 1 nucleotide) between *Ps. chiangmaiensis* and *Ps. assamensis*, which Arzanlou et al. (2008) recently described from banana. Morphologically, however, they differ in the basal conidial cell shape and marginal thickening along the hilum. These differences were supported

by analyses based on the EF gene, which indicated these two species to differ by 38 nucleotides.

Other than new species of *Mycosphaerella* and *Pseudocercospora*, the present study also led to the discovery of a novel species of *Penidiella*. *Penidiella eucalypti* is distinct in that it has a distinctive branching system developed chiefly on a single conidiogenous locus, and conidia with a persistent, characteristic mucilaginous sheath in vitro. Results from the phylogenetic analyses also indicated that this species belongs to a clade represented by an undescribed *Teratosphaeria* sp. (DQ632682) which could represent its teleomorph.

Two strains that were phylogenetically closely related to *M. irregulari* (95 % bootstrap), namely CPC 15446 and CPC 15447, represent two undescribed species of *Mycosphaerella*. *Mycosphaerella* sp. CPC 15446 differs from *M. irregulari* by having ascospores with more obtuse apical cells and germination tubes that are regular in width. Although insufficient material was available of *Mycosphaerella* sp. CPC 15447, it occurred in lesions in association with *M. thailandica* and *M. heimii*. Another undescribed *Mycosphaerella* species, CPC 15448, was closely related to *M. quasiparkii* (89 % bootstrap). More collections are required to resolve their status. In the present study, three known species were also identified from the diseased *Eucalyptus* leaf samples, namely *M. heimii*, *M. thailandica* and *M. vietnamensis*. Although *M. thailandica* and *M. heimii* were previously reported on *Eucalyptus* in Thailand (Crous et al. 2007b), *M. vietnamensis* represents a record for this country.

This study has again demonstrated that morphological characters and molecular techniques are complementary, and necessary, to uncover the diversity and geographical range of *Mycosphaerella* and *Teratosphaeria* species occurring on *Eucalyptus*. Although five new fungal species have been identified and one species represents a new record from diseased *Eucalyptus* leaves from Thailand, it is still unknown whether these species are native or exotic. We expect that more unidentified disease-causing microfungi await discovery in Thailand, because the expanding area of *Eucalyptus* plantations allow fungal pathogens to cross geographical barriers to infect new hosts (i.e. from exotic *Eucalyptus* to other native trees) more easily, and also increase the chance of infection by native fungi to the exotic plantations (Slippers et al. 2005). Some examples of introduced pathogens from exotic *Eucalyptus* are *T. cryptica*, *T. nubilosa* (Park & Keane 1982, Wingfield et al. 1995), and *T. suttonii* (Chipompha 1987, Crous et al. 1989, 1997). These species were described from Australia where *Eucalyptus* is native, but were found later in other countries where this host has been planted as an exotic. In addition, the study of *Mycosphaerella* spp. on exotic *Acacia* in the tropics (Crous et al. 2004b) again revealed examples of host sharing of *Mycosphaerella citri* on *Citrus*, *Acacia* and *Musa*. More extensive research should thus be carried out to provide information concerning the fungal diversity of exotic *Eucalyptus* plantations in Thailand and other Asian countries to promote the understanding of the evolution of new pathogens and the movement of fungi between continents.

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# Isolation and characterization of the mating type locus of *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease of banana

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## SUMMARY

Idiomorphs *mat1-1* and *mat1-2* from *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease of banana, were isolated. Degenerate oligos were used to amplify the HMG box of the *mat1-2* idiomorph from *M. fijiensis*, showing homology with the HMG box of *Mycosphaerella graminicola*. Using a DNA walking strategy, anchored on the DNA lyase gene towards the HMG box, a 9-kb-long region of *mat1-2* was obtained. A 5-kb fragment from the *mat1-1* region was obtained by long-range PCR using primers on the flanking regions, which have close to 100% identity between both idiomorphs. High-identity (77–89%), inverted regions within both idiomorphs were found, which suggest unique inversion events, which have not been found before, and that could have been significant in the evolution of this species. The predicted genes showed the conserved introns in both idiomorphs as well as an additional intron within the alpha box. The implications for the evolution of species in the *Mycosphaerella* complex on banana are discussed.

## INTRODUCTION

Bananas (*Musa* spp.) are grown in all tropical regions of the world and play a key role in the economies of many developing countries. World consumption during 1998–2000 in developing countries was 21 kg per capita (mostly domestically produced), while the total value of the international banana trade ranges between US\$4.5 and 5 billion per year (Arias *et al.*, 2003).

The crop is affected by several diseases and pests such as the foliar fungal pathogens *Mycosphaerella fijiensis*, *M. musicola* and *M. eumusae*, which all share similar morphologies and symptom development. *M. fijiensis* (anamorph *Pseudocercospora fijiensis*; Mycosphaerellaceae) is the causal agent of Black Sigatoka or black leaf streak disease (BLS), which rapidly became the most devastating disease of banana production world-wide. It decreases photosynthesis, reduces fruit size and induces premature maturation. The cost of controlling the disease in large plantations is about US\$1000 per hectare (Arias *et al.*, 2003), but it is higher in smaller plantations where fungicides cannot be applied by air and in which crop losses can be up to 50% (Mobambo *et al.*, 1993). Repercussions of the frequent and high input of fungicides include the development of both reduced sensitivity and resistance to these active compounds (Romero and Sutton, 1997; Sierotzki *et al.*, 2000). This was recently exemplified by the rapid development and spread of resistance to strobilurin fungicides in Central America (Marin *et al.*, 2003).

Owing to the fact that *Mycosphaerella* is one of the largest genera of plant pathogenic fungi, with more than 3000 *Mycosphaerella* species (Aptroot, 2006), Goodwin *et al.* (2004) proposed *Mycosphaerella graminicola* as the working model for the Dothideomycetes. As a result, the genomes of both *M. graminicola* and *M. fijiensis* are currently being sequenced within the DOE-JGI Community Sequencing Program (see <http://www.jgi.doe.gov/sequencing/cspseqplans2006.html>).

The sexual cycle of the fungus plays an important role in BLS epidemiology (Gauhl *et al.*, 2000; Hayden *et al.*, 2003). Apart from the generation of air-borne inoculum, sexual reproduction results in genetic variation and contributes to evolution. In heterothallic ascomycetes, such as *M. fijiensis* (Mourichon and Zapater, 1990), mating can only occur between strains with opposite mating types. These mating types are determined by highly dissimilar sequences, called idiomorphs, that are embedded in regions common to all isolates of a given species, and from which

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the only conserved regions are designated as the alpha box (for *mat1-1*) and the HMG box (for *mat1-2*) (Turgeon and Yoder, 2000). In addition, the structure of the *mat* idiomorphs has aided in understanding the evolution of heterothallic and homothallic species (Pöggeler, 1999; Turgeon, 1998; Yun *et al.*, 1999).

As *Mycosphaerella* leaf spot disease in banana is caused by a complex of at least three species (M. Arzanlou, personal communication), knowledge of the *mat* genes sequences in *M. fijiensis* is a starting point to a better understanding of the relevance of reproduction and recombination, in relation to the epidemiology of these important pathogens and the interaction with other species.

We presumed substantial synteny between *M. graminicola* and *M. fijiensis* as a basis to isolate the *mat* genes of the latter. Indeed, a PCR-based strategy using the DNA lyase gene, which flanks the idiomorphs in *M. graminicola*, allowed cloning the *mat1-2* idiomorph. In turn, the flanks of the *mat1-2* idiomorph were used to clone the *mat1-1* idiomorph by long-range PCR. Comparative analyses showed that both idiomorphs contain a highly unusual inversion not previously observed in idiomorphs in other ascomycetes.

## RESULTS

### PCR amplification of the HMG box and flanking genes

To amplify the HMG box from *mat1-2* isolates, primers reported for *M. graminicola* (Waalwijk *et al.*, 2002) and *Septoria passerinii* (Goodwin *et al.*, 2003) were unsuccessfully assayed. However, the degenerate primer pair KIKRP-F + SEKKR-R (-F for forward and -R for reverse) (Table 1) produced amplicons with the expected size of around 300 bp in some isolates of *M. fijiensis*. Therefore, these bands were cloned and sequenced. TBLASTx analysis revealed homology with the HMG box from *M. graminicola*

( $E = 6e-10$ ) and *S. passerinii* ( $E = 1e-09$ ), showing 71.9% identity in a predicted 82-amino-acid sequence. A multiple alignment of the predicted amino acid sequences is presented in Fig. 1a.

In addition, DNA lyase and *sla2* homologues in *M. fijiensis* were both amplified by degenerate PCR. A 1200-bp amplicon showed homology to the putative SLA2 protein (involved in cytoskeleton assembly, with no known function in mating) in *Aspergillus fumigatus* ( $E = 6e-85$ , 87% identity; GenPept accession no. EAL92953), *Magnaporthe grisea* ( $E = 6e-85$ , 78% identity; GenPept accession no. EAL92953) and *Neurospora crassa* ( $E = 6e-85$ , 73% identity; GenPept accession no. EAA35004).

The 850-bp amplicon showed homology to DNA lyase from several other fungi. Further on, a DNA walking strategy was employed using the DNA lyase as initial anchor. This strategy resulted in a 9817-bp genomic sequence containing the complete DNA lyase gene, a gene encoding the anaphase promoting complex (APC) and the *mat1-2* idiomorph (Fig. 2), which was deposited in GenBank with accession number DQ787016.

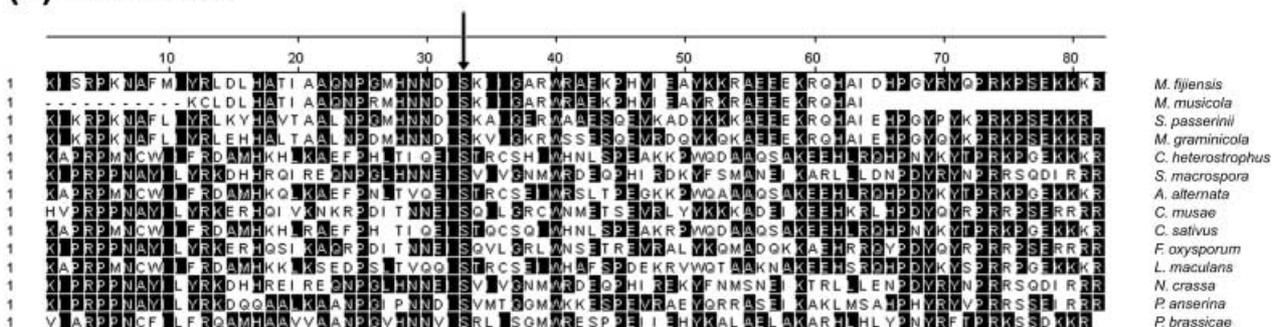
The DNA lyase sequence obtained is 1868 bp long; the predicted gene has a single exon and 622 amino acids. Local alignments showed up to 70% identity in a 106-amino-acid stretch ( $E = 2e-158$ ; 82% similarities) and up to 75% identity on a 65-amino-acid portion ( $E = 1e-95$ , 78% similarities) with the DNA lyase sequence from *M. graminicola*. ClustalW global multiple alignments showed an overall identity of 40% with *M. graminicola* and *Cordyceps militaris*.

Tblastx analysis on the *mat1-2* idiomorph showed only homology with the HMG box region of *mat1-2* from *M. graminicola*, with expected value  $E = 6e-69$ , on a 40-amino-acid stretch (67% identities and 77% similarities). The next hit was *mat1-2* from *S. passerinii*, with an expected value of  $E = 4e-27$  (70% identities, 77% similarities). No other region with similarity was found within the *mat1-2* idiomorph.

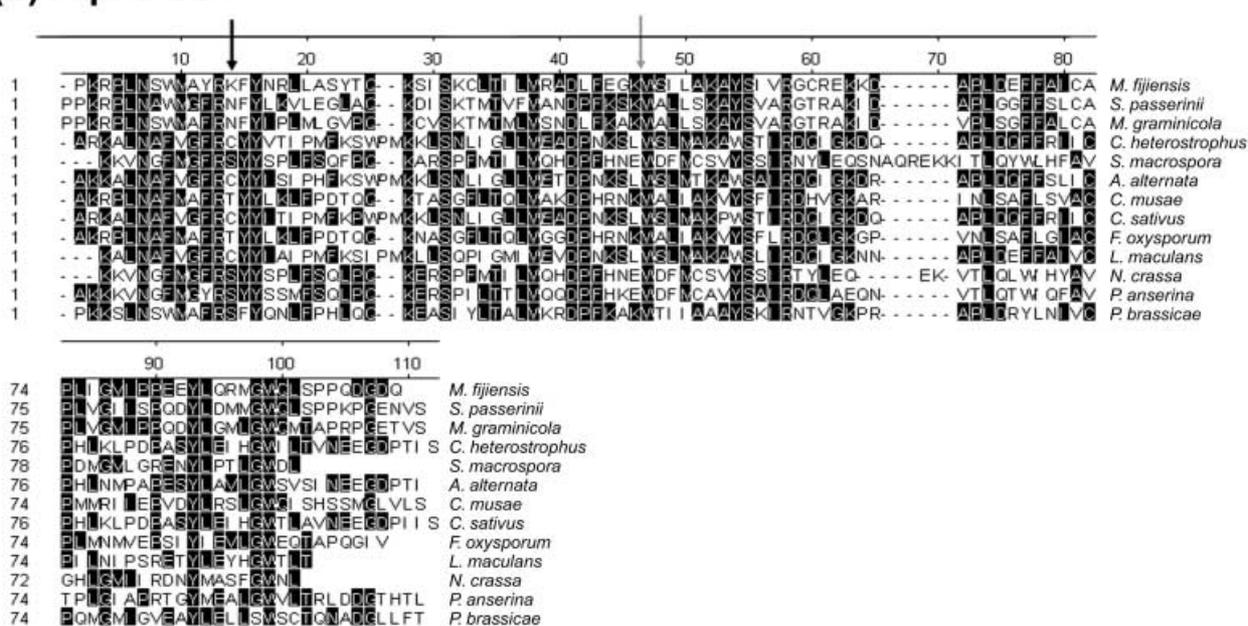
**Table 1** Primers used to amplify regions of the mating type idiomorphs, the DNA lyase gene and the *sla2* gene.

Primer	Sequence	Based on
KIKRP-F	5'-AAGATCAAGCGYCCAAG-3'	<i>mat1-2</i> from <i>M. graminicola</i> and <i>S. passerinii</i>
SEKKR-R	5'-ATGCGRCGTTTCTTCTCSG-3'	<i>mat1-2</i> from <i>M. graminicola</i> and <i>S. passerinii</i>
DNAlydegF3	5'-CGCGCCGATCAGAGCNGARGARGG-3'	DNA lyase from <i>M. graminicola</i> , <i>N. crassa</i> , <i>F. graminearum</i> , <i>M. grisea</i> and <i>A. nidulans</i>
DNAlydegR3	5'-CATTCTTCTCGTGTCCCAARYRNGTRT-3'	DNA lyase from <i>M. graminicola</i> , <i>N. crassa</i> , <i>F. graminearum</i> , <i>M. grisea</i> and <i>A. nidulans</i>
DNAlyaseMfF	5'-TCTCACGTCGGTCCAGATG-3'	DNA lyase from <i>M. fijiensis</i>
DNAlyaseMfR	5'-TGAGGTTTCGTATCCGATGG-3'	DNA lyase from <i>M. fijiensis</i>
SLA2degF1	5'-CATCAACGACCCCAAYGARGGNTAYGA-3'	<i>sla2</i> from <i>A. nidulans</i> , <i>N. crassa</i> and <i>M. grisea</i>
SLA2degR1	5'-CCCGCTCVCGGATCATRTCNGC-3'	<i>sla2</i> from <i>A. nidulans</i> , <i>N. crassa</i> and <i>M. grisea</i>
E56	5'-CCCTCTGGACCAGGATACC-3'	Flanking region upstream of <i>M. fijiensis</i> idiomorphs
E66	5'-TCGAAAATGAGTTGAAACG-3'	Flanking regions downstream of <i>M. fijiensis</i> idiomorphs
flan4739-F	5'-GCGGTTTTGGAGCGGTGACG-3'	Flanking regions upstream of <i>M. fijiensis</i> idiomorphs
inver5656-R	5'-GAAGCTCTGGGTATCTCAGCACAGG-3'	Inverted sequence within idiomorphs (upstream <i>mat1-2</i> )
inver8486-F	5'-GCACCTCAGGGAGGCATTGG-3'	Inverted sequence within idiomorphs (downstream <i>mat1-2</i> )
flan9352-R	5'-TGATGCATCTGCCGAGACC-3'	Flanking regions downstream of <i>M. fijiensis</i> idiomorphs

### (a) HMG box



### (b) Alpha box



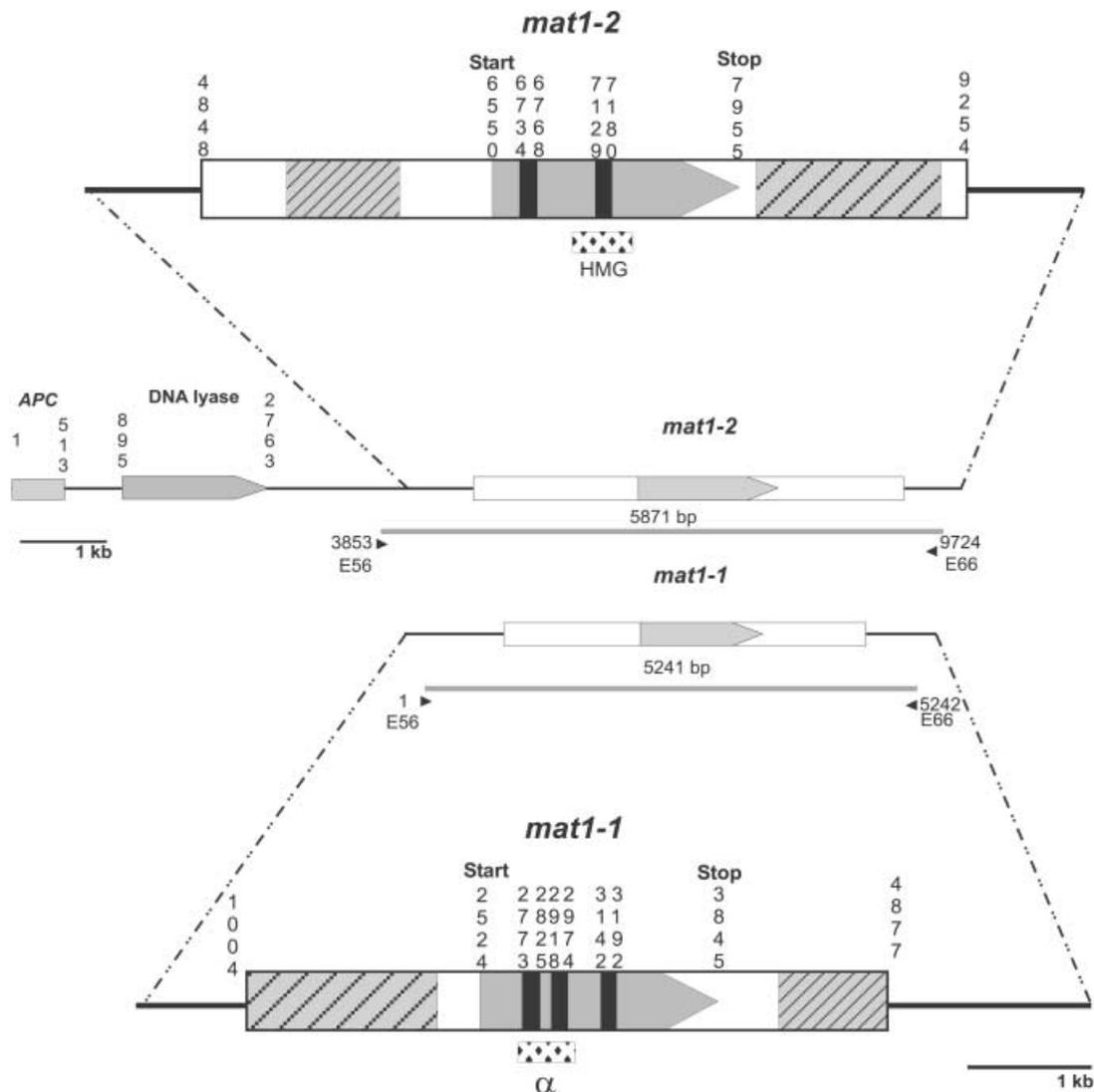
**Fig. 1** Multiple sequence alignments of predicted amino acids from (a) the HMG box and (b) the alpha box of *Mycosphaerella fijiensis*, *Mycosphaerella musicola*, *Septoria passerinii*, *Cochliobolus heterostrophus*, *Sordaria macrospora*, *Alternaria alternata*, *Colletotrichum musae*, *Cochliobolus sativus*, *Fusarium oxysporum*, *Leptosphaeria maculans*, *Neurospora crassa*, *Podospora anserina* and *Pyrenopeziza brassicae*. Black arrows indicate the positions of the conserved introns; the grey arrow indicates another intron present only in the *Mycosphaerellaceae fijiensis* alpha box. Shaded amino acids are in agreement with the consensus.

#### Long-range PCR isolation of the *mat1-1* idiomorph

Long-range PCRs using primers anchored on the flanking regions of the *mat1-2* idiomorph (primers E56 and E66, Table 1) resulted in a 5.8-kb fragment corresponding to *mat1-2* in some isolates, and a slightly smaller fragment (5.2 kb) in others. This 5.2-kb fragment was identified as containing the *mat1-1* idiomorph. The sequence was deposited in the GenBank database with accession number DQ787015. Tblastx analysis of the *mat1-1* sequence of *M. fijiensis* showed homology with *mat1-1* of *M. graminicola* and *S. passerinii* as first hits ( $E = 3e-75$  and  $E = 5e-68$ , respectively). MegAlign and ClustalW multiple alignment of the amino-acid sequence showed a particularly conserved region, which corresponds to the alpha box of *mat1-1* (Fig. 1b).

#### Characterization of the idiomorphs

Primers that were developed on the flanking regions enabled the amplification of the complete idiomorphs (see Fig. 2). To define the flanking regions of the idiomorphs, the nucleotide sequences of *mat1-1* and *mat1-2* of *M. fijiensis* were aligned in ClustalW. The sequence similarity between the flanking regions, which had 95.2–98.4% identity, differed significantly from that of the idiomorphs (Fig. 3). We predicted the upstream ends of the idiomorphs *mat1-1* and *mat1-2* in positions 1004 bp and 4848 bp, respectively, and the downstream ends were determined on nucleotide 4877 of the *mat1-1* sequence, and nucleotide 9254 of the *mat1-2* sequence. Hence, the length of the *mat1-1* idiomorph is 3873 bp and that of *mat1-2* is 4406 bp.



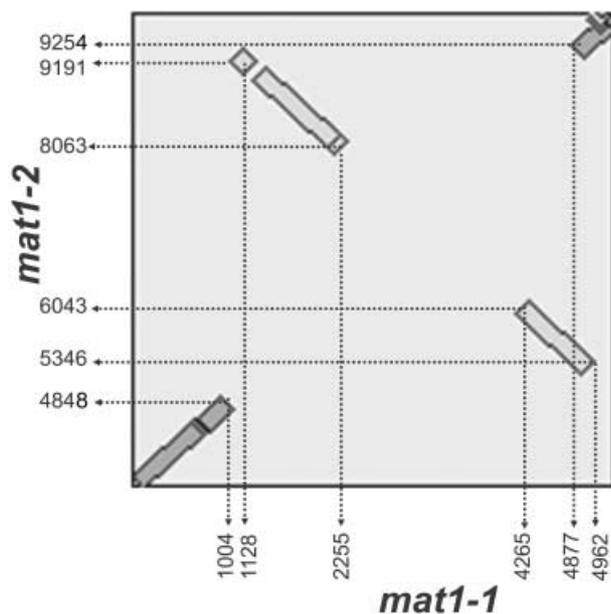
**Fig. 2** Diagram of the *mat* locus organization in *Mycosphaerella fijiensis*. The boxes labelled *mat1-1* and *mat1-2* represent the idiormorphs; the adjacent genes are labelled *APC* (Anaphase Promoting Complex) and *DNA lyase*. Numbers indicate positions in the sequences of *mat1-1* (GenBank DQ787015) and *mat1-2* (GenBank DQ787016) from *M. fijiensis*. Numbered triangles represent primers used in long-range PCR. In expanded diagrams, the arrow-shaped boxes represent the predicted genes interrupted by introns (black boxes); the dashed boxes are inverted regions with high percentage identity between the plus (+) strand of one idiormorph and the minus (–) strand of the other.

Surprisingly, the alignment showed two regions with a high percentage of identity but in reverse orientation compared with the flanking sequences. These regions of inverted homology were located close to the ends of both idiormorphs (Fig. 3). In *M. fijiensis* a portion of 1127 bp in the 5' end of the *mat1-1* idiormorph shows 77% identity (in nucleotide sequence) to a portion of the 3' end of the *mat1-2* idiormorph, but in reverse orientation. The same is observed on the 3' end of *mat1-1*, in which a 697-bp portion is highly similar (89% identity) to the 5' end of *mat1-2* in the minus chain. Interestingly, this phenomenon was not observed when the idiormorphs of *M. graminicola*, *S. passerinii*, *Leptosphaeria maculans* or *Phaeosphaeria nodorum* were aligned among each

other (data not shown). To rule out the possibility of assembly artefacts, we confirmed the presence of these inverted regions by PCR using primers on the flanking regions and others designed on the sequence of one of the idiormorphs. This approach allowed us to observe the amplification in one of the mating types and absence of an amplicon in the opposite mating type, and the reciprocal situation was observed when combining two reverse or two forward primers to produce amplicons from the opposite mating type (Fig. 4).

*ORF finding and gene prediction*

The predicted *M. fijiensis mat1-1* gene has three introns (Fig. 2); the conserved alpha region is included in a predicted protein of



**Fig. 3** Dot-plot graph of both idiomorphs of *M. fijiensis* generated with Blast2: x-axis, sequence of *mat1-1*; y-axis, sequence of *mat1-2*. The flanking regions draw two lines from bottom left to top right. The high-identity regions within the idiomorphs appear from top left to bottom right (between + and – strands). The numbers correspond to nucleotide positions on each sequence.

388 amino acids. In comparison, in *M. graminicola* the predicted MAT1-1 protein has 297 amino acids (Waalwijk *et al.*, 2002); the orthologous gene in *S. passerinii* (Goodwin *et al.*, 2003) has two introns as well, and the predicted protein has 310 residues. It is important to notice that two of the predicted introns are located in the alpha box. The first (2774–2824) is located in a conserved position shared by all ascomycetes (Fig. 1b). The second intron (in position 2919–2973) is unique to *Mycosphaerellaceae fijiensis* and has not been reported in other fungi (Figs 1b and 2). The conserved sequence of the alpha box would be interrupted if this intron was not excised. The last intron is located in position 3143–3191 (Fig. 2). The excision of the last intron was confirmed by comparison with the *mat1-1* cDNA sequence (S. Zhong, personal communication). The predicted coding region of *mat1-1* of *M. fijiensis* is 1167 bp, which is in good agreement with those reported for *M. graminicola* and *S. passerinii*.

By contrast, the *M. fijiensis mat1-2* predicted gene has two introns and the protein has 441 amino acids. The intron positions are 6735–6767 bp and 7130–7179 bp (Figs 1a and 2).

### Comparative analysis

When comparing the idiomorphs of *M. fijiensis* with those from *M. graminicola* and *S. passerinii*, using Blast2, the alignments showed that *mat1-1* sequences are closer to the diagonal than *mat1-2* (data not shown). This indicates a

generally higher similarity between the *mat1-1* sequences, which is remarkable as it has been observed in ascomycetes that the HMG box shows a stronger conservation than the alpha box (Arie *et al.*, 1997). Nevertheless, in a short 39-amino-acid stretch of the HMG box, the comparison with *M. graminicola* was as high as 66% identity and 73% similarity, whilst *mat1-1* showed a lower percentage of similarity but in a longer stretch of 157 amino acids (36% identity, 46% similarity). ClustalW analysis of the conserved alpha and HMG amino acid sequences of several ascomycetes resulted in two phylogenetic trees (Fig. 5).

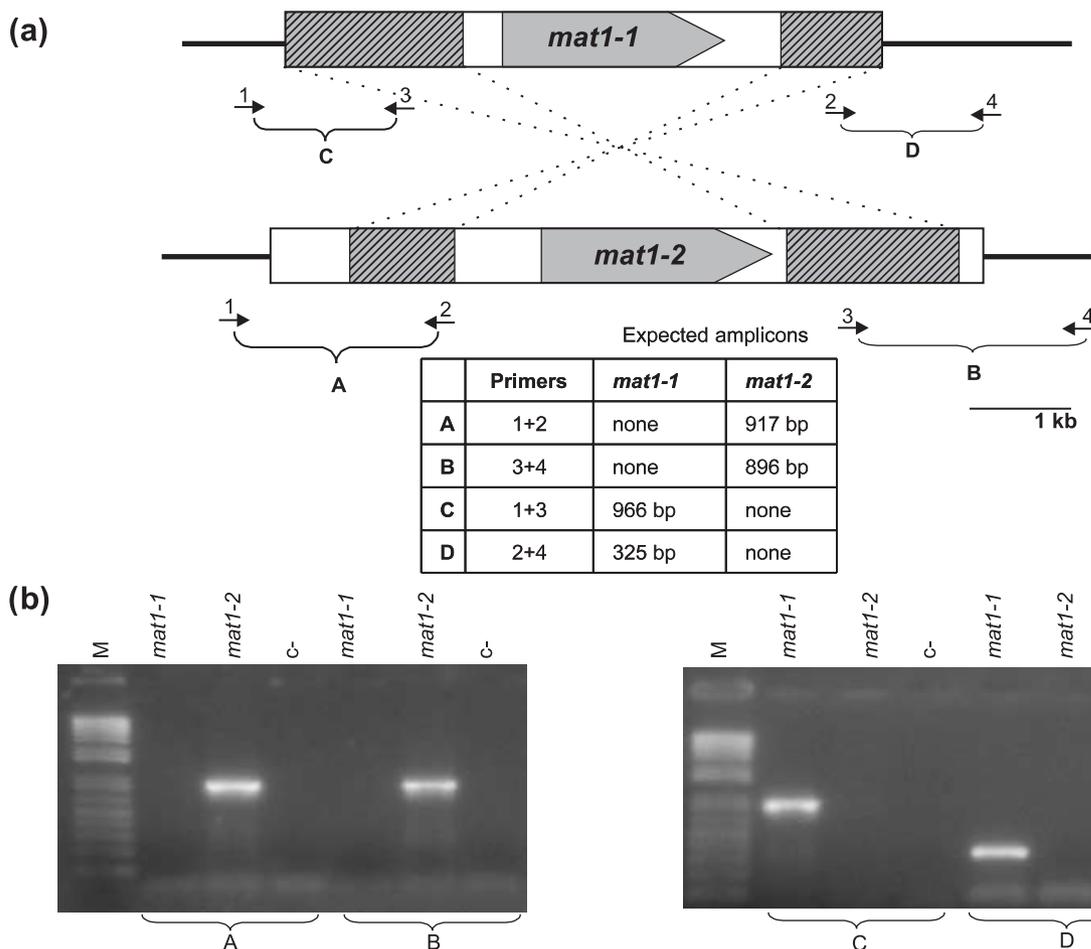
## DISCUSSION

### Amplification of the HMG box

We successfully used postulated synteny between *M. graminicola* and *M. fijiensis* to clone the *mat1-1* and *mat1-2* idiomorphs from the banana black leaf streak pathogen *M. fijiensis*. The HMG box of the *mat1-2* locus from several distantly related fungi has been obtained by PCR (Arie *et al.*, 1997), but reported primers did not amplify the HMG box in *M. fijiensis*. The HMG box has been used as an indicator to differentiate *Fusarium* species (O'Donnell *et al.*, 2004), and it has been proposed as a candidate to construct reliable phylogenetic trees (Yun *et al.*, 2000). Based on internal transcribed spacer (ITS) sequences, *M. graminicola* and *M. fijiensis* appeared to be closely related (Goodwin *et al.*, 2001). However, primers reported for *M. graminicola* and *S. passerinii* also failed to amplify the HMG box of *M. fijiensis*. Our sequence analyses of the *mat* orthologues and the flanking regions suggest that *M. fijiensis* and *M. graminicola* seem to be more distantly related than previously expected.

We did expect similarity between the two banana pathogens *M. fijiensis* and *M. musicola*, and indeed primers based on the *M. fijiensis* HMG box sequence successfully produced an amplicon in *M. musicola*. tBLASTx analysis of the *M. musicola* amplicon sequences (212 bp) showed homology with *mat1-2* from *S. passerinii* ( $E = 4e-04$ , 68% identities). Overall nucleotide sequences of the HMG box of *M. fijiensis* and *M. musicola* showed a high percentage of identities (85%) on pair-wise alignments. Predicted amino-acid sequence alignment of the HMG box of *M. fijiensis* and the fragment of the HMG box of *M. musicola* (53 amino acids) showed 92% identity (Fig. 1a). The similarities found between these banana pathogens are in agreement with the findings obtained with ITS analysis (Goodwin and Zismann, 2001), according to which *M. fijiensis* and *M. musicola* are closely related (*Pseudocercospora* anamorphs) as are *S. passerinii* and *M. graminicola* (*Septoria* anamorphs).

As seen in all fungi reported so far, a serine is found in the conserved intron position of the HMG box, which is present as well in *M. fijiensis* (Fig. 1a).



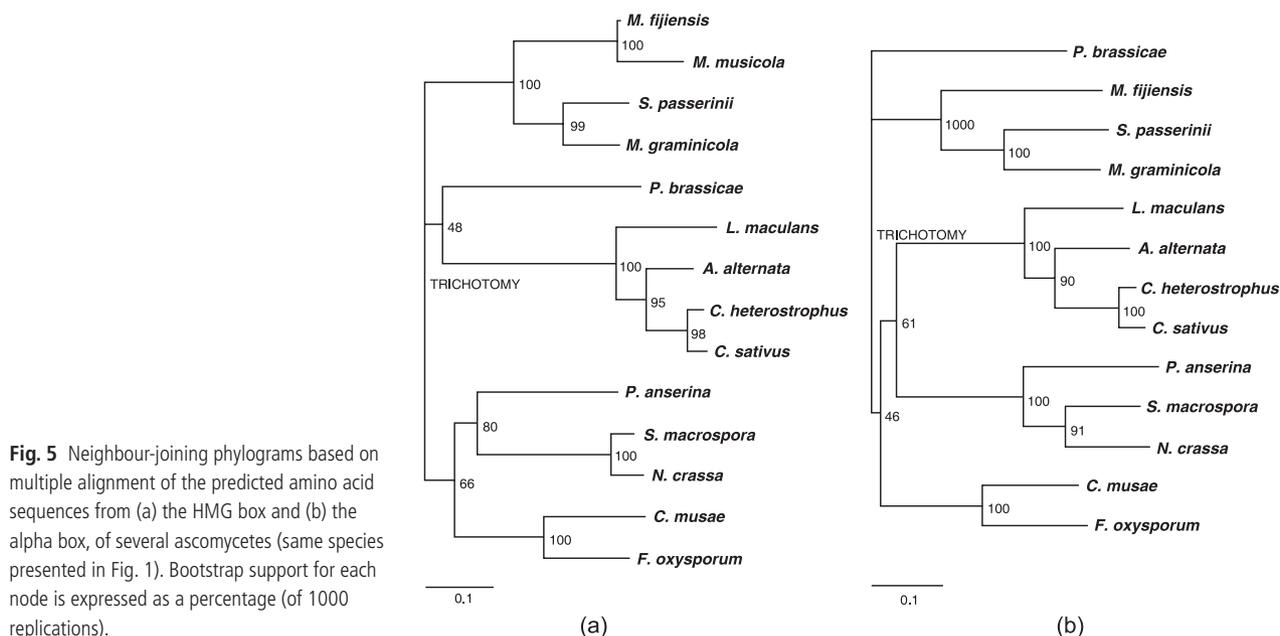
**Fig. 4** PCR confirmation of the inverted regions found within the idiomorphs of *M. fijiensis*. (a) Location of the primers and predicted PCR results if inversions occur (inserted table). Solid boxes represent the idiomorphs, the dashed boxes are inverted regions with high percentage identity between the plus (+) strand of one idiomorph and the minus (–) strand of the other; numbered arrows represent primers; primer 1 is flan4739, primer 2 is inver5656, primer 3 is inver 8486 and primer 4 is flan9352, which were used in four different combinations (A, B, C and D). (b) Agarose gel showing PCR results with primer pairs A, B, C and D. Primers 1 and 4 work as ‘forward’ and ‘reverse’ primers respectively for both idiomorphs, while primer 2 is ‘reverse’ for *mat1-2* but ‘forward’ for the inverted sequence in *mat1-1*; and primer 3 is ‘forward’ for *mat1-2* but ‘reverse’ for the inverted sequence in *mat1-1*. Therefore, if the inversions are present, in *mat1-2* the amplification only occurs if forward and reverse primers are combined (e.g. 1+2, 3+4); while in *mat1-1* the amplification only occurs if two forward or two reverse primers are combined (1+3, 2+4). DNAs employed were from strains 86 (mating type *mat1-1*) and 89 (mating type *mat1-2*); marker (M) is 1 kb Plus (Invitrogen®).

### Characterization of the idiomorphs

We hypothesize that the inverted sequences in the *M. fijiensis* idiomorphs might have originated from an inversion event in a recent ancestor, given that independent blast analyses of these regions did not result in significant homologies in the databases, including the *M. graminicola* genome. The only exception was a hit with a small fragment of *mat1-1* of *S. passerinii* ( $E = 2e-04$ , 58%, 20/34 identities), on a non-coding region of the upstream end of the idiomorph. To our knowledge, the only two reports in which large portions of sequences of one idiomorph are found in the opposite is in *Cordyceps takaomontana* (anamorph: *Paecilomyces tenuipes*), where a *mat1-1-1* pseudogene is found in the *mat1-2*

idiomorph (Yokoyama *et al.*, 2003); and *Aspergillus fumigatus* (Paoletti *et al.*, 2005), where a fragment of *mat1-2-1* is found within the flanking region of the *mat1-1* idiomorph. In addition, small fragments of common sequences were found in *Cochliobolus* spp., which were called ‘islands of identity’ of 8–9 bp, and may indicate recombination spots (Yun *et al.*, 1999).

It is not known how such dissimilar sequences of the idiomorphs can occupy the same locus in the genome, but it is thought that they were initially identical, but diverged through successive rearrangements and deletion/insertion events (Turgeon, 1998). It has also been suggested that the small fragments of identical sequence that have been found in both idiomorphs are probably remnants, explaining their common origin (Coppin *et al.*, 1997).



**Fig. 5** Neighbour-joining phylograms based on multiple alignment of the predicted amino acid sequences from (a) the HMG box and (b) the alpha box, of several ascomycetes (same species presented in Fig. 1). Bootstrap support for each node is expressed as a percentage (of 1000 replications).

In *Stemphylium* spp. it was found that an inversion-fusion event gave rise to selfing species from outcrossing ancestors, resulting in a *mat* locus with both idiomorphs, one of which was inverted with respect to the heterothallic ancestor (Inderbitzin *et al.*, 2005). Conversely, the inverted regions found in *M. fijiensis* correspond to non-coding sequences. Whether a homothallic ancestor gave rise to *M. fijiensis* as a heterothallic species with this particular feature, as proposed for *Aspergillus* spp. (Paoletti *et al.*, 2005), is a question that can be addressed with the cloning of the mating type genes of other close *Mycosphaerella* relatives. These inverted regions represent an important finding, which can be the basis for further evolutionary studies on homo- and heterothallic species that are related to *M. fijiensis*.

### Comparative analysis

As seen in *M. graminicola* (Waalwijk *et al.*, 2002) and other fungi (such as *Rhynchosporium secalis* and *Cochliobolus heterostrophus*) (Foster and Fitt, 2004; Turgeon *et al.*, 1993), the boundaries of the idiomorphs in *M. fijiensis* are defined by highly similar flanking regions, but in other species (e.g. *Phaeosphaeria nodorum* and *Neurospora crassa*) there is only a gradual transition from the flanking region to the idiomorphs (Bennett *et al.*, 2003; Randall and Metzenberg, 1998).

*M. fijiensis* idiomorphs (3873 bp for *mat1-1* and 4406 bp for *mat1-2*) are longer than the orthologues in *M. graminicola* (2839 bp for *mat1-1*, AF440399, and 2772 bp for *mat1-2*, AF440398), *S. passerinii* (3048 bp for *mat1-1*, AF483193, and 2897 bp for *mat1-2*, AF483194) and *Xanthoria polycarpa* in which the *mat1-1* idiomorph is 3270 bp (AJ884599) and *mat1-2* is 3150 bp

(AJ884598). The *M. fijiensis* idiomorphs seem to be more similar in size to more distantly related species such as *P. nodorum*, in which *mat1-1* is 4282 bp (AY212018) and *mat1-2* is 4505 bp (AY212019); *Fusarium oxysporum* (*mat1-1* is 4618 bp, AB011379, and *mat1-2* is 3849 bp, AB011378) or *R. secalis* (*mat1-1* is 4049 bp, EMBL:AJ537511 and *mat1-2* is 3153 bp, EMBL:AJ549759). Conversely, other single-gene idiomorphs can be much shorter, for example *C. heterostrophus* idiomorph *mat1-1* is 1297 bp (AF029913) and *mat1-2* is 1171 bp (AF027687).

### Synteny among ascomycetes

We used synteny to isolate the idiomorphs of *M. fijiensis* by DNA walking anchored on a flanking gene. An extraordinary level of synteny has recently been reported in the mating type region of distantly related fungi such as *Aspergillus nidulans*, *M. grisea*, *N. crassa*, *Fusarium proliferatum* and *Gibberella zeae* (Waalwijk *et al.*, 2004). It has been proposed that the conservation in gene order in the vicinity of the *mat* locus is due to suppressed recombination that is caused by the dissimilarity of the idiomorph sequences. The *mat* locus of several ascomycete species has been characterized in detail. In yeast species such as *Saccharomyces cerevisiae*, *S. castellii*, *Candida glabrata* and *Kluyveromyces delphensis*, the genes encoding BUD5 and HO endonuclease, which are required for mating type switching, are located in the region of the idiomorphs; in other species such as *S. kluyveri*, *K. lactis*, *Pichia angusta* and *Yarrowia lipolytica*, the *mat* locus is located next to the *sla2* gene (Butler *et al.*, 2004; Debuchy and Turgeon, 2006). This gene is also located near the *mat* locus in filamentous fungi such as *N. crassa* (AABX01000036) and

several species of *Fusarium* (Waalwijk *et al.*, 2004). The DNA lyase and APC genes flank the *mat* idiomorphs in *M. graminicola* (Waalwijk *et al.*, 2002). In *L. maculans* an orthologue of the DNA lyase gene was also found in the same location (Cozijnsen and Howlett, 2003). In fact, data mining performed during this work showed that orthologues of the DNA lyase gene are found near the *mat* locus in *Glomerella cingulata* (AY357890), *Sordaria macrospora* (Y10616), *P. tenuipes* (AB084921) and *C. militaris* (AB084257). Other examples of fungi in which both the DNA lyase and the *sla2* genes flank the idiomorphs are *Y. lipolytica* (CR382129), *Xanthoria parietina* and *X. polycarpa* (AJ884600 and AJ884598).

However, in *M. fijiensis*, it is unlikely that the *sla2* gene is located near the *mat* locus. The 1200-bp *sla2* amplicon that we obtained in our study was used as a probe against a *M. fijiensis* BAC library (90 kb mean insert size) at high stringency conditions (D.K. Guillén-Maldonado *et al.*, unpublished data). The *sla2* homologue was present in at least six different BAC clones (data not shown), but hybridizations with different heterologous and homologous *mat* probes suggested that *sla2* and the *mat* idiomorphs are not physically linked in *M. fijiensis*, which is supported by the draft sequence of the *M. graminicola* genome (DOE-JGI Community Sequencing Program).

### The use of the idiomorphs for the study of populations and evolution

The synteny observed in gene order and intron position, in contrast to the high sequence divergence within the idiomorphs, reflects the importance of the *mat* locus in determining a species barrier. Because of its polymorphism, the *mat* locus can be used as a marker for population studies, and it is currently being used for genetic mapping (G. Manzo-Sánchez *et al.*, unpublished data).

Population genetic studies support the hypothesis that in *M. fijiensis* sexual reproduction is random and frequent (Carlier *et al.*, 1996; Hayden *et al.*, 2003; Rivas *et al.*, 2004). The identifi-

cation of the *mat* genes will provide further evidence for this observation, because populations with a high occurrence of sexual reproduction would have strains of opposite mating type distributed in a 1 : 1 ratio (Zhan *et al.*, 2002).

The highly similar inverted regions that we identified within the idiomorphs of *M. fijiensis*, and the additional intron found within the alpha box are unique features, which will be useful in evolution studies. Ongoing studies have shown that both are present in the *mat* genes from *M. musicola*, suggesting that these events occurred before speciation (L. Conde-Ferráez, unpublished results). Future work focusing on these characteristics would give additional information about the evolution and ecology of the genus *Mycosphaerella*. As the *Mycosphaerella* pathogens of banana constitute a complex of species that have coexisted and interacted on their common host, the analysis of their mating type loci would give insights for a better understanding on their relationship and evolution.

## EXPERIMENTAL PROCEDURES

### Fungal isolates and DNA extraction

Monoascoporic strains of *M. fijiensis* were obtained from diverse sources (Table 2) and were grown at 26 °C in potato dextrose broth (PDB) with continuous shaking (100 r.p.m.) under continuous light. DNA was extracted from mycelium collected after filtration by grinding under liquid nitrogen according to the protocol described by Johanson (1997). We used DNAs from *M. graminicola* and *S. passerinii* isolates of known mating type as comparisons in degenerate PCRs. The list of DNAs used in this study is summarized in Table 2.

### PCR and DNA walking strategies

We aligned the *mat* genes and flanking sequences of *M. graminicola* and its close relative *S. passerinii*, and the DNA lyase and the *sla2*

**Table 2** DNAs used in this work. Grand Naine is a cultivar of *Musa acuminata*, which is highly susceptible to *M. fijiensis*.

Isolate	Species	Host	Mating type	Geographical origin	Reference
IPO323	<i>M. graminicola</i>	Wheat	<i>mat1-1</i>	The Netherlands	Kema <i>et al.</i> (2002)
IPO94269	<i>M. graminicola</i>	Wheat	<i>mat1-2</i>	The Netherlands	Kema <i>et al.</i> (2002)
P64	<i>S. passerinii</i>	Barley	<i>mat1-1</i>	USA	Goodwin <i>et al.</i> (2003)
P76	<i>S. passerinii</i>	Barley	<i>mat1-2</i>	USA	Goodwin <i>et al.</i> (2003)
86	<i>M. fijiensis</i>	Grand Naine	A*	Cameroon	Mourichon and Zapater (1990)
89	<i>M. fijiensis</i>	Grand Naine	a*	Cameroon	Mourichon and Zapater (1990)
139a	<i>M. fijiensis</i>	Grand Naine	a*	Colombia	Mourichon and Zapater (1990)
138	<i>M. fijiensis</i>	Grand Naine	A*	Colombia	Mourichon and Zapater (1990)
1231	<i>M. fijiensis</i>	Grand Naine	<i>mat1-2</i>	Tabasco, Mexico	This work
1233	<i>M. fijiensis</i>	Grand Naine	<i>mat1-1</i>	Tabasco, Mexico	This work

\*The mating type tester strains have been traditionally designated as 'A' and 'a'; in this work it was determined that 'A' mating type corresponds to *mat1-1*, while 'a' corresponds to *mat1-2*.



genes from several other ascomycetes, to develop degenerate primers using the Codehop program (<http://nar.oxfordjournals.org/cgi/content/full/31/13/3763>). Specific primers were developed using the Primer3 program (Rozen and Skaletsky, 2000). Only those primers that yielded amplicons are detailed in Table 1.

PCRs were performed in an Eppendorf thermal cycler. General cycling conditions were 94 °C for 1 min, 30 cycles of 94 °C for 1 min, annealing at 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension of 7 min at 72 °C. For degenerate PCR, with primers based on the DNA lyase, *sla2* and the idiomorphs from other fungi, a touchdown PCR was used, from 60 to 55 °C for the annealing temperature (−1 °C per cycle), with 30 additional cycles at an annealing temperature of 55 °C.

To obtain the *mat1-2* idiomorph, a DNA walking strategy was performed with the DNAwalking SpeedUp™ Premix Kit (Seegene, Seoul, Republic of Korea), using the DNA lyase gene as anchor according to the specifications given in the DNA walking manual. The full length of the *mat1-1* idiomorph was obtained by long-range PCR with primers E56 and E66 (Table 1), which were designed based on the *mat1-2* sequence, and are located in a region outside the idiomorph. The annealing temperature was 55 °C for 40 s; extension was at 72 °C for 3.5 min. The resulting 5.5-kb PCR product was sonicated, followed by treatment with T4 DNA polymerase (Promega Benelux b.v., Leiden, The Netherlands) to obtain blunt ends and treated with kinase (T4 polynucleotide kinase, NEB) for 30 s. The fragments were then ligated into the pUC19 vector linearized with *Sma*I (Promega). Clones with insert sizes between 300 and 1200 bp were selected to be sequenced. Other amplicons were either cloned into the pCR2.1-TOPO vector (Invitrogen®), according to the manufacturer's instructions or were purified and sequenced directly. Sequencing was performed using Big Dye® Terminator technology (Applied Biosystems, Foster City, USA).

### PCR confirmation of inverted sequences within the idiomorphs

Primers flan4739-F, inver5656-R, inver8486-F and flan9352-R (Table 1) were designed on the predicted inverted regions and on the flanking regions of the idiomorphs; the orientations (forward or reverse) correspond to the sequence of *mat1-2*. These primers were used in four different combinations (A, B, C and D), which are specified in the table inserted in Fig. 4. Annealing was at 62 °C for 40 s, and extension at 72 °C for 40 s.

### Bioinformatic analyses

Sequences were assembled and edited in the SeqMan and Edit-Seq programs (DNASTar, Lasergene™) and analysed using Blast (<http://www.ncbi.nlm.nih.gov>). Sequence alignments were performed by using ClustalW (<http://www.ebi.ac.uk/clustalw/>), Blast2-

sequences (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>) and MegAlign (Lasergene DNASTar™). Neighbour-joining trees were constructed based on alignments of the conserved alpha and HMG domains, respectively, using the tree-drawing application in Clustal X. Bootstrap analysis (1000 replicates) was performed to evaluate the degree of support for each group in the tree.

Identification of open reading frames (ORFs) and gene predictions were performed using FGENESH and FGENESH+ software (Softberry™, <http://www.softberry.com/berry.phtml>) with the codon usage of *M. grisea*. The results were compared with those obtained with the GenScan (<http://genes.mit.edu/GENSCAN.html>) and GenomeScan (<http://genes.mit.edu/genomescan.html>) programs, using as homologue models the MAT proteins of *M. graminicola* and *S. passerinii*. Conserved intron boundaries (GT/AG) and branching signals (RCURAY) most commonly found in other fungi (Kupfer *et al.*, 2004) were also identified in the predicted genes.

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## *Mycosphaerella* is polyphyletic

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**Abstract:** *Mycosphaerella*, one of the largest genera of ascomycetes, encompasses several thousand species and has anamorphs residing in more than 30 form genera. Although previous phylogenetic studies based on the ITS rDNA locus supported the monophyly of the genus, DNA sequence data derived from the LSU gene distinguish several clades and families in what has hitherto been considered to represent the *Mycosphaerellaceae*. Several important leaf spotting and extremotolerant species need to be disposed to the genus *Teratosphaeria*, for which a new family, the *Teratosphaeriaceae*, is introduced. Other distinct clades represent the *Schizothyriaceae*, *Davidiellaceae*, *Capnodiales*, and the *Mycosphaerellaceae*. Within the two major clades, namely *Teratosphaeriaceae* and *Mycosphaerellaceae*, most anamorph genera are polyphyletic, and new anamorph concepts need to be derived to cope with dual nomenclature within the *Mycosphaerella* complex.

**Taxonomic novelties:** *Batcheloromyces eucalypti* (Alcorn) Crous & U. Braun, comb. nov., *Catenulostroma* Crous & U. Braun, gen. nov., *Catenulostroma abietis* (Butin & Pehl) Crous & U. Braun, comb. nov., *Catenulostroma chromoblastomycosum* Crous & U. Braun, sp. nov., *Catenulostroma elginense* (Joanne E. Taylor & Crous) Crous & U. Braun, comb. nov., *Catenulostroma excentricum* (B. Sutton & Ganap.) Crous & U. Braun, comb. nov., *Catenulostroma germanicum* Crous & U. Braun, sp. nov., *Catenulostroma macowanii* (Sacc.) Crous & U. Braun, comb. nov., *Catenulostroma microsporium* (Joanne E. Taylor & Crous) Crous & U. Braun, comb. nov., *Catenulostroma protearum* (Crous & M.E. Palm) Crous & U. Braun, comb. nov., *Penidiella* Crous & U. Braun, gen. nov., *Penidiella columbiana* Crous & U. Braun, sp. nov., *Penidiella cubensis* (R.F. Castañeda) U. Braun, Crous & R.F. Castañeda, comb. nov., *Penidiella nectandrae* Crous, U. Braun & R.F. Castañeda, nom. nov., *Penidiella rigidophora* Crous, R.F. Castañeda & U. Braun, sp. nov., *Penidiella strumelloidea* (Milko & Dunaev) Crous & U. Braun, comb. nov., *Penidiella venezuelensis* Crous & U. Braun, sp. nov., *Readeriella blakelyi* (Crous & Summerell) Crous & U. Braun, comb. nov., *Readeriella brunneotingens* Crous & Summerell, sp. nov., *Readeriella consideniana* (Crous & Summerell) Crous & U. Braun, comb. nov., *Readeriella destructans* (M.J. Wingf. & Crous) Crous & U. Braun, comb. nov., *Readeriella dimorpha* (Crous & Carnegie) Crous & U. Braun, comb. nov., *Readeriella epicoccoides* (Cooke & Masee) Crous & U. Braun, comb. nov., *Readeriella gauchensis* (M.-N. Cortinas, Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Readeriella molleriana* (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Readeriella nubilosa* (Ganap. & Corbin) Crous & U. Braun, comb. nov., *Readeriella pulcherrima* (Gadgil & M. Dick) Crous & U. Braun, comb. nov., *Readeriella stellenboschiana* (Crous) Crous & U. Braun, comb. nov., *Readeriella toledana* (Crous & Bills) Crous & U. Braun, comb. nov., *Readeriella zuluensis* (M.J. Wingf., Crous & T.A. Cout.) Crous & U. Braun, comb. nov., *Teratosphaeria africana* (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Teratosphaeria alistairii* (Crous) Crous & U. Braun, comb. nov., *Teratosphaeria associata* (Crous & Carnegie) Crous & U. Braun, comb. nov., *Teratosphaeria bellula* (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Teratosphaeria cryptica* (Cooke) Crous & U. Braun, comb. nov., *Teratosphaeria dentritica* (Crous & Summerell) Crous & U. Braun, comb. nov., *Teratosphaeria excentrica* (Crous & Carnegie) Crous & U. Braun, comb. nov., *Teratosphaeria fimbriata* (Crous & Summerell) Crous & U. Braun, comb. nov., *Teratosphaeria flexuosa* (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Teratosphaeria gamsii* (Crous) Crous & U. Braun, comb. nov., *Teratosphaeria jonkershoekensis* (P.S. van Wyk, Marasas & Knox-Dav.) Crous & U. Braun, comb. nov., *Teratosphaeria maxii* (Crous) Crous & U. Braun, comb. nov., *Teratosphaeria mexicana* (Crous) Crous & U. Braun, comb. nov., *Teratosphaeria molleriana* (Thüm.) Crous & U. Braun, comb. nov., *Teratosphaeria nubilosa* (Cooke) Crous & U. Braun, comb. nov., *Teratosphaeria ohnowa* (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Teratosphaeria parkiiifinis* (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Teratosphaeria parva* (R.F. Park & Keane) Crous & U. Braun, comb. nov., *Teratosphaeria perpendicularis* (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Teratosphaeria pluritubularis* (Crous & Mansilla) Crous & U. Braun, comb. nov., *Teratosphaeria pseudoafricana* (Crous & T.A. Cout.) Crous & U. Braun, comb. nov., *Teratosphaeria pseudocryptica* (Crous) Crous & U. Braun, comb. nov., *Teratosphaeria pseudosuberosa* (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Teratosphaeria quasircospora* (Crous & T.A. Cout.) Crous & U. Braun, comb. nov., *Teratosphaeria readeriellophora* (Crous & Mansilla) Crous & U. Braun, comb. nov., *Teratosphaeria secundaria* (Crous & Alfenas) Crous & U. Braun, comb. nov., *Teratosphaeria stramenticola* (Crous & Alfenas) Crous & U. Braun, comb. nov., *Teratosphaeria suberosa* (Crous, F.A. Ferreira, Alfenas & M.J. Wingf.) Crous & U. Braun, comb. nov., *Teratosphaeria suttonii* (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov., *Teratosphaeria toledana* (Crous & Bills) Crous & U. Braun, comb. nov., *Teratosphaeriaceae* Crous & U. Braun, fam. nov.

**Key words:** Ascomycetes, *Batcheloromyces*, *Colletogloeopsis*, *Readeriella*, *Teratosphaeria*, *Trimmatostroma*, DNA sequence comparisons, systematics.

## INTRODUCTION

The genus *Mycosphaerella* Johanson as presently circumscribed contains close to 3000 species (Aptroot 2006), excluding its anamorphs, which represent thousands of additional species (Crous *et al.* 2000, 2001, 2004a, b, 2006a, b, 2007b, Crous & Braun 2003). Crous (1998) predicted that *Mycosphaerella* would eventually be split according to its anamorph genera, and Crous *et al.* (2000) recognised six sections, as originally defined by Barr (1972). This was followed by a set of papers (Crous *et al.* 2001, Goodwin *et al.* 2001), where it was concluded, based on ITS DNA sequence data, that *Mycosphaerella* was monophyletic. A revision of the various coelomycete and hyphomycete anamorph concepts led Crous & Braun (2003) to propose a system whereby the asexual morphs could be allocated to various form genera affiliated with *Mycosphaerella* holomorphs.

In a recent study that formed part of the US "Assembling the Fungal Tree of Life" project, Schoch *et al.* (2006) were able to show that the *Mycosphaerellaceae* represents a family within *Capnodiales*. Furthermore, some variation was also depicted within

the family, which supported similar findings in other recent papers employing LSU sequence data, such as Hunter *et al.* (2006), and Batzer *et al.* (2007). To further elucidate the phylogenetic variation observed within the *Mycosphaerellaceae* in these studies, a subset of isolates was selected for the present study, representing the various species recognised as morphologically distinct from *Mycosphaerella s. str.*

The genus *Mycosphaerella* has in recent years been linked to approximately 30 anamorph genera (Crous & Braun 2003, Crous *et al.* 2007b). Many of these anamorph genera resulted from a reassessment of cercosporoid forms. Chupp (1954) was of the opinion that they all represented species of the genus *Cercospora* Fresen., although he clearly recognised differences in their morphology. In a series of papers by Deighton, as well as others such as Sutton, Braun and Crous, the genus *Cercospora* was delimited based on its type species, *Cercospora penicillata* (Ces.) Fresen., while taxa formerly included in the genus by Chupp (1954) but differing in conidiophore arrangement, conidiogenesis, pigmentation, conidial catenulation, septation, and scar/hilum structure were allocated to other genera. Similar studies in which the type species were recollected and subjected to DNA sequence

Table 1. Isolates for which new sequences were generated.

Anamorph	Teleomorph	Accession number <sup>1</sup>	Host	Country	Collector	GenBank Accession number
<i>Batcheloromyces eucalypti</i>		CBS 313.76; CPC 3632	<i>Eucalyptus tessellaris</i>	Australia	J.L. Alcorn	EU019245
<i>Batcheloromyces leucadendri</i>		CBS 110892; CPC 1837	<i>Leucadendron</i> sp.	South Africa	L. Swart	EU019246
<i>Batcheloromyces proteae</i>		CBS 110696; CPC 1518	<i>Protea cynaroides</i>	South Africa	L. Viljoen	EU019247
<i>Capnobotryella renispora</i>		CBS 214.90*; CBS 176.88; IAM 13014; JCM 6932	<i>Capnobotrys neessii</i>	Japan	J. Sugiyama	EU019248
<i>Catenulostroma abietis</i>		CBS 290.90	Man, skin lesion	Netherlands	R.G.F. Wintermans	EU019249
<i>Catenulostroma castellanii</i>		CBS 105.75*; ATCC 24788	Man, <i>tinea nigra</i>	Venezuela	—	EU019250
<i>Catenulostroma chromoblastomycosum</i>		CBS 597.97	Man, chromoblastomycosis	Zaire	V. de Brouwere	EU019251
<i>Catenulostroma elginense</i>		CBS 111030; CPC 1958	<i>Protea grandiceps</i>	South Africa	J.E. Taylor	EU019252
<i>Catenulostroma germanicum</i>		CBS 539.88	Stone	Germany	—	EU019253
<i>Catenulostroma macowanii</i>		CBS 110756; CPC 1872	<i>Protea nitida</i>	South Africa	J.E. Taylor	EU019254
<i>Catenulostroma microsporium</i>	<i>Teratosphaeria microspora</i>	CBS 110890; CPC 1832	<i>Protea cynaroides</i>	South Africa	L. Swart	EU019255
<i>Catenulostroma</i> sp.	<i>Teratosphaeria pseudosuberosa</i>	CBS 118911; CPC 12085	<i>Eucalyptus</i> sp.	Uruguay	M.J. Wingfield	EU019256
<i>Cercosporella centaureicola</i>		CBS 120253	<i>Centaurea solstitialis</i>	Greece	D. Berner	EU019257
<i>Cibiessia dimorphospora</i>		CBS 120034; CPC 12636	<i>Eucalyptus nitens</i>	Australia	—	EU019258
<i>Cibiessia minutispora</i>		CPC 13071*	<i>Eucalyptus henryii</i>	Australia	A.J. Carnegie	EU019259
<i>Cibiessia nontingens</i>	<i>Teratosphaeria</i> sp.	CBS 120725*; CPC 13217	<i>Eucalyptus tereticornis</i>	Australia	B. Summerell	EU019260
<i>Cladosporium bruhnei</i>	<i>Davidiella allicina</i>	CBS 115683; ATCC 66670; CPC 5101	CCA-treated Douglas-fire pole	U.S.A., New York	C.J. Wang	EU019261
<i>Cladosporium cladosporioides</i>		CBS 109.21; ATCC 11277; ATCC 200940; IFO 6368; IMI 049625	Sooty mould on <i>Hedera helix</i>	U.K.	—	EU019262
<i>Cladosporium sphaerospermum</i>		CBS 188.54; ATCC 11290; IMI 049638	—	—	—	EU019263
<i>Cladosporium uredinicola</i>		ATCC 46649	Hyperparasite on <i>Cronartium fusiforme</i> f. sp. <i>quercum</i>	U.S.A., Alabama	—	EU019264
<i>Coccodinium bartschii</i>		CBS 121708; CPC 13861–13863	Sooty mould on unidentified tree	Canada	K.A. Seifert	EU019265
<i>Dissoconium aciculare</i>		CBS 342.82*; CPC 1534	<i>Erysiphe</i> , on <i>Medicago lupulina</i>	Germany	T. Hijwegen	EU019266
<i>Dissoconium commune</i>	<i>"Mycosphaerella" communis</i>	CBS 114238*; CPC 10440	<i>Eucalyptus globulus</i>	Spain	J.P.M. Vazquez	EU019267
<i>Dissoconium dekkeri</i>	<i>"Mycosphaerella" lateralis</i>	CBS 567.89*; CPC 1535	<i>Juniperus chinensis</i>	Netherlands	T. Hijwegen	EU019268
<i>Fumagospora capnodioides</i>	<i>Capnodium salicinum</i>	CBS 131.34	Sooty mould on <i>Bursaria spinosa</i>	Indonesia	—	EU019269
<i>Hortaea werneckii</i>		CBS 107.67*	Man, <i>tinea nigra</i>	Portugal	—	EU019270
<i>Nothostrasseria dendritica</i>	<i>Teratosphaeria dendritica</i>	CPC 12820	<i>Eucalyptus nitens</i>	Australia	A.J. Carnegie	EU019271
<i>"Passalora" zambiae</i>		CBS 112970*; CPC 1228	<i>Eucalyptus globulus</i>	Zambia	T. Coutinho	EU019272
		CBS 112971*; CMW 14782; CPC 1227	<i>Eucalyptus globulus</i>	Zambia	T. Coutinho	EU019273
<i>Penidiella columbiana</i>		CBS 486.80	<i>Paepalanthus columbianus</i>	Colombia	W. Gams	EU019274

Anamorph	Teleomorph	Accession number <sup>1</sup>	Host	Country	Collector	GenBank Accession number
<i>Penidiella nectandrae</i>		CBS 734.87*; ATCC 200932; INIFAT 87/45	<i>Nectandra coriacea</i>	Cuba	R.F. Castañeda & G. Arnold	EU019275
<i>Penidiella rigidophora</i>		CBS 314.95*	Leaf litter of <i>Smilax</i> sp.	Cuba	R.F. Castañeda	EU019276
<i>Penidiella strumelloidea</i>		CBS 114484*; VKM F-2534	<i>Carex</i> leaf, from stagnant water	Russia	S. Ozerskaya	EU019277
<i>Penidiella venezuelensis</i>		CBS 106.75*	Man, <i>tinea nigra</i>	Venezuela	D. Borelli	EU019278
<i>Phaeothea triangularis</i>		CBS 471.90*	Wet surface of humidifier of airconditioning	Belgium	H. Beguin	EU019279
<i>Phaeothea eucalypti</i>		CPC 13010	<i>Corymbia henryii</i>	Australia	B. Summerell	EU019280
		CPC 12918*	<i>Eucalyptus botryooides</i>	Australia	B. Summerell	EU019281
<i>Pleurophoma</i> sp.	<i>Teratosphaeria fibrillosa</i>	CPC 1876	<i>Protea nitida</i>	South Africa	J.E. Taylor	EU019282
<i>Pseudotaeniolina globosa</i>		CBS 109889*	Rock	Italy	C. Urzi	EU019283
<i>Ramularia pratensis</i> var. <i>pratensis</i>		CPC 11294	<i>Rumex crispus</i>	Korea	H.D. Shin	EU019284
<i>Ramularia</i> sp.		CBS 324.87	On <i>Mycosphaerella</i> sp., leaf spot on <i>Brassica</i> sp.	Netherlands	—	EU019285
<i>Readeriella brunneotogens</i>		CPC 13303	<i>Eucalyptus tereticornis</i>	Australia	P.W. Crous	EU019286
<i>Readeriella destructans</i>		CBS 111369*; CPC 1366	<i>Eucalyptus grandis</i>	Indonesia	M.J. Wingfield	EU019287
<i>Readeriella epicoccoides</i>	<i>Teratosphaeria suttonii</i>	CPC 12352	<i>Eucalyptus</i> sp.	U.S.A.,Hawaii	W. Gams	EU019288
<i>Readeriella eucalypti</i>		CPC 11186	<i>Eucalyptus globulus</i>	Spain	M.J. Wingfield	EU019289
<i>Readeriella gauchensis</i>		CBS 120303*; CMW 17331	<i>Eucalyptus grandis</i>	Uruguay	M.J. Wingfield	EU019290
<i>Readeriella mirabilis</i>		CBS 116293; CPC 10506	<i>Eucalyptus fastigata</i>	New Zealand	W. Gams	EU019291
<i>Readeriella molleriana</i>	<i>Teratosphaeria molleriana</i>	CBS 111164*; CMW 4940; CPC 1214	<i>Eucalyptus globulus</i>	Portugal	M.J. Wingfield	EU019292
<i>Readeriella ovata</i> complex		CPC 18	<i>Eucalyptus cladocalyx</i>	South Africa	P.W. Crous	EU019293
		CBS 111149; CPC 23	<i>Eucalyptus cladocalyx</i>	South Africa	P.W. Crous	EU019294
<i>Readeriella stellenboschiana</i>		CBS 116428; CPC 10886	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	EU019295
<i>Readeriella zuluensis</i>		CBS 120301*; CMW 17321	<i>Eucalyptus grandis</i>	South Africa	M.J. Wingfield	EU019296
<i>Septoria tritici</i>	<i>Mycosphaerella graminicola</i>	CBS 100335; IPO 69001.61	<i>Triticum aestivum</i>	—	G.H.J. Kema	EU019297
		CBS 110744; CPC 658	<i>Triticum</i> sp.	South Africa	P.W. Crous	EU019298
<i>Trimmatostroma betulinum</i>		CBS 282.74	<i>Betula verrucosa</i>	Netherlands	W.M. Loerakker	EU019299
<i>Trimmatostroma salicis</i>		CPC 13571	<i>Salix alba</i>	Germany	U. Braun	EU019300
	<i>Teratosphaeria bellula</i>	CBS 111700; CPC 1821	<i>Protea eximia</i>	South Africa	J.E. Taylor	EU019301
	<i>Teratosphaeria mexicana</i>	CPC 12349	<i>Eucalyptus</i> sp.	U.S.A.,Hawaii	W. Gams	EU019302
	<i>Teratosphaeria nubilosa</i>	CBS 114419; CPC 10497	<i>Eucalyptus globulus</i>	New Zealand	—	EU019303
		CBS 116005*; CMW 3282; CPC 937	<i>Eucalyptus globulus</i>	Australia	A. Carnegie	EU019304



Table 1. (Continued).

Anamorph	Teleomorph	Accession number <sup>1</sup>	Host	Country	Collector	GenBank Accession number
	<i>Teratosphaeria ohnowa</i>	CBS 112896 <sup>*</sup> ; CMW 4937; CPC 1004	<i>Eucalyptus grandis</i>	South Africa	M.J. Wingfield	EU019305
	<i>Teratosphaeria secundaria</i>	CBS 115608; CPC 504	<i>Eucalyptus grandis</i>	Brazil	A.C. Alfenas	EU019306
	<i>Teratosphaeria</i> sp.	CBS 208.94; CPC 727	<i>Eucalyptus grandis</i>	Indonesia	A.C. Alfenas	EU019307

<sup>1</sup>ATCC: American Type Culture Collection, Virginia, U.S.A.; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; CMW: Culture collection of Mike Wingfield, housed at FAO, Pretoria, South Africa; IAM: Institute of Applied Microbiology, University of Tokyo, Institute of molecular and cellular bioscience, Tokyo, Japan; IFO: Institute For Fermentation, Osaka, Japan; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bokerham Lane, U.K.; INIFAT: Alexander Humboldt Institute for Basic Research in Tropical Agriculture, Ciudad de La Habana, Cuba; JCM: Japan Collection Of Microorganisms, RIKEN BioResource Center, Japan; VKM: All-Russian Collection of Microorganisms, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia.

<sup>\*</sup>Ex-type cultures.

analysis were undertaken to characterise *Mycosphaerella* (Verkley *et al.* 2004), and anamorph genera such as *Pseudocercospora* Speg., *Stigmina* Sacc., *Phaeoisariopsis* Ferraris (Crous *et al.* 2006a), *Ramulispora* Miura (Crous *et al.* 2003), *Batcheloromyces* Marasas, P.S. van Wyk & Knox-Dav. (Taylor *et al.* 2003), *Phaeophleospora* Rangel and *Dothistroma* Hulbary (Crous *et al.* 2000, 2001, Barnes *et al.* 2004).

To assess the phylogeny of the species selected for the present study, DNA sequences were generated of the 28S rRNA (LSU) gene. In a further attempt to address monophyletic groups within this complex, these data were integrated with their morphological characteristics. To further resolve pleomorphism among the species studied, isolates were examined on a range of cultural media to induce possible synanamorphs.

## MATERIALS AND METHODS

### Isolates

Chosen isolates represent various species previously observed to be morphologically distinct from *Mycosphaerella* s. str. (Crous 1998, Crous *et al.* 2004a, b, 2006a, b, 2007b). In a few cases, specifically *Teratosphaeria fibrillosa* Syd. & P. Syd. and *Coccolodinium bartschii* A. Massal., fresh material had to be collected from South Africa and Canada, respectively. Excised tissue pieces bearing ascospores were soaked in water for approximately 2 h, after which they were placed in the bottom of Petri dish lids, with the top half of the dish containing 2 % malt extract agar (MEA) (Gams *et al.* 2007). Ascospore germination patterns were examined after 24 h, and single-ascospore and conidial cultures established as described by Crous (1998). Colonies were sub-cultured onto synthetic nutrient-poor agar (SNA), potato-dextrose agar (PDA), oatmeal agar (OA), MEA (Gams *et al.* 2007), and incubated at 25 °C under continuous near-ultraviolet light to promote sporulation.

### DNA phylogeny

Fungal colonies were established on agar plates, and genomic DNA was isolated following the CTAB-based protocol described in Gams *et al.* (2007). The primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990) were used to amplify part of the nuclear rDNA operon spanning the 3' end of the 18S rRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region (ITS2) and the 5' end of the 28S rRNA gene (LSU). The primers ITS4 (White *et al.* 1990), LR0R (Rehner & Samuels 1994), LR3R (www.biology.duke.edu/fungi/mycolab/primers.htm), and LR16 (Moncalvo *et al.* 1993), were used as internal sequence primers to ensure good quality sequences over the entire length of the amplicon. The ITS1, ITS2 and 5.8S rRNA gene (ITS) were only sequenced for isolates of which these data were not available. The ITS data were not included in the analyses but deposited in GenBank where applicable. The PCR conditions, sequence alignment and subsequent phylogenetic analysis using parsimony, distance and Bayesian analyses followed the methods of Crous *et al.* (2006c). Gaps longer than 10 bases were coded as single events for the phylogenetic analyses; the remaining gaps were treated as new character states. Sequence data were deposited in GenBank (Table 1) and alignments in TreeBASE (www.treebase.org).

### Taxonomy

Wherever possible, 30 measurements (× 1 000 magnification)

were made of structures mounted in lactic acid, with the extremes of spore measurements given in parentheses. Ascospores were frequently also mounted in water to observe mucoid appendages and sheaths. Colony colours (surface and reverse) were assessed after 1–2 mo on MEA at 25 °C in the dark, using the colour charts of Rayner (1970). All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands (Table 1). Nomenclatural novelties and descriptions were deposited in MycoBank (www.MycoBank.org).

## RESULTS

### DNA phylogeny

Amplification products of approximately 1 700 bases were obtained for the isolates listed in Table 1. The LSU region of the sequences was used to obtain additional sequences from GenBank which were added to the alignment. The manually adjusted alignment contained 97 sequences (including the two outgroup sequences) and 844 characters including alignment gaps. Of the 844 characters used in the phylogenetic analysis, 308 were parsimony-informative, 105 were variable and parsimony-uninformative, and 431 were constant.

The parsimony analysis of the LSU region yielded 1 135 equally most parsimonious trees (TL = 1 502 steps; CI = 0.446; RI = 0.787; RC = 0.351), one of which is shown in Fig. 1. Three orders are represented by the ingroup isolates, namely *Chaetothyriales* (100 % bootstrap support), *Helotiales* (100 % bootstrap support) and *Capnodiales* (100 % bootstrap support). These are discussed in detail in the Taxonomy and Discussion sections. A new collection of *Coccodinium bartschii* A. Massal clusters (100 % bootstrap support) with members of the *Herpotrichiellaceae* (*Chaetothyriales*), whereas the type species of the genus *Trimmatostroma* Corda, namely *T. salicis* Corda, as well as *T. betulinum* (Corda) S. Hughes, are allied (99 % bootstrap support) with the *Dermateaceae* (*Helotiales*). The *Capnodiales* encompasses members of the *Capnodiaceae*, *Trichosphaeriaceae*, *Davidiellaceae*, *Schizothyriaceae* and taxa traditionally placed in the *Mycosphaerellaceae*, which is divided here into the *Teratosphaeriaceae*, (65 % bootstrap support), and

the *Mycosphaerellaceae* (76 % bootstrap support), which contains several subclades. Also included in the *Capnodiales* are *Devriesia staurophora* (W.B. Kendr.) Seifert & N.L. Nick., *Staninwardia suttonii* Crous & Summerell and *Capnobotryella renispora* Sugiy. as sister taxa to *Teratosphaeriaceae* s. str. Neighbour-joining analysis using three substitution models on the sequence data yielded trees supporting the same topologies, but differed from the parsimony tree presented with regard to the order of the families and orders at the deeper nodes, e.g., the *Helotiales* and *Chaetothyriales* are swapped around, as are the *Capnodiaceae* and the *Trichosphaeriaceae* / *Davidiellaceae* (data not shown). Using neighbour-joining analyses, the *Mycosphaerellaceae* s. str. clade obtained 71 %, 70 % and 70 % bootstrap support respectively with the uncorrected “p”, Kimura 2-parameter and HKY85 substitution models whereas the *Teratosphaeriaceae* clade obtained 74 %, 79 % and 78 % bootstrap support respectively with the same models. The *Schizothyriaceae* clade appeared basal in the *Capnodiales*, irrespective of which substitution model was used.

Bayesian analysis was conducted on the same aligned LSU dataset using a general time-reversible (GTR) substitution model with inverse gamma rates and dirichlet base frequencies. The Markov Chain Monte Carlo (MCMC) analysis of 4 chains started from a random tree topology and lasted 23 881 500 generations. Trees were saved each 100 generations, resulting in 238 815 saved trees. Burn-in was set at 22 000 000 generations after which the likelihood values were stationary, leaving 18 815 trees from which the consensus tree (Fig. 2) and posterior probabilities (PP's) were calculated. The average standard deviation of split frequencies was 0.011508 at the end of the run. The same overall topology as that observed using parsimony was obtained, with the exception of the inclusion of *Staninwardia suttonii* in the *Mycosphaerellaceae* (PP value of 0.74) and not in the *Teratosphaeriaceae*. The *Mycosphaerellaceae* s. str. clade, as well as the *Teratosphaeriaceae* clade, obtained a PP value of 1.00.

### Taxonomy

Based on the dataset generated in this study, several well-supported genera could be distinguished in the *Mycosphaerella* complex (Figs 1–2), for which we have identified morphological characters. These genera, and a selection of their species, are treated below.

## Key to *Mycosphaerella*, and *Mycosphaerella*-like genera treated

1. Ascómata thyrothecial; anamorph *Zygosporium* ..... ***Schizothyrium***
1. Ascómata pseudothecial ..... 2
2. Ascospores with irregular, angular lumens typical of *Davidiella*; anamorph *Cladosporium* s. str. .... ***Davidiella***
2. Ascospores guttulate or not, lacking angular lumens; anamorph other than *Cladosporium* ..... 3
3. Ascómata frequently linked by superficial stroma; hamathecial tissue, ascospore sheath, multi-layered endotunica, prominent periphysoids, and ascospores turning brown in asci frequently observed ..... ***Teratosphaeria***
3. Ascómata not linked by superficial stroma; hamathecial tissue, ascospore sheath, multi-layered endotunica, prominent periphysoids, ascospores turning brown in asci not observed ..... 4
4. Conidiophores solitary, pale brown, giving rise to primary and secondary, actively discharged conidia; anamorph *Dissoconium* ..... teleomorph *Mycosphaerella*-like
4. Conidiomata variable from solitary conidiophores to sporodochia, fascicles to pycnidia, but conidia not actively discharged ..... ***Mycosphaerella* s. str.**

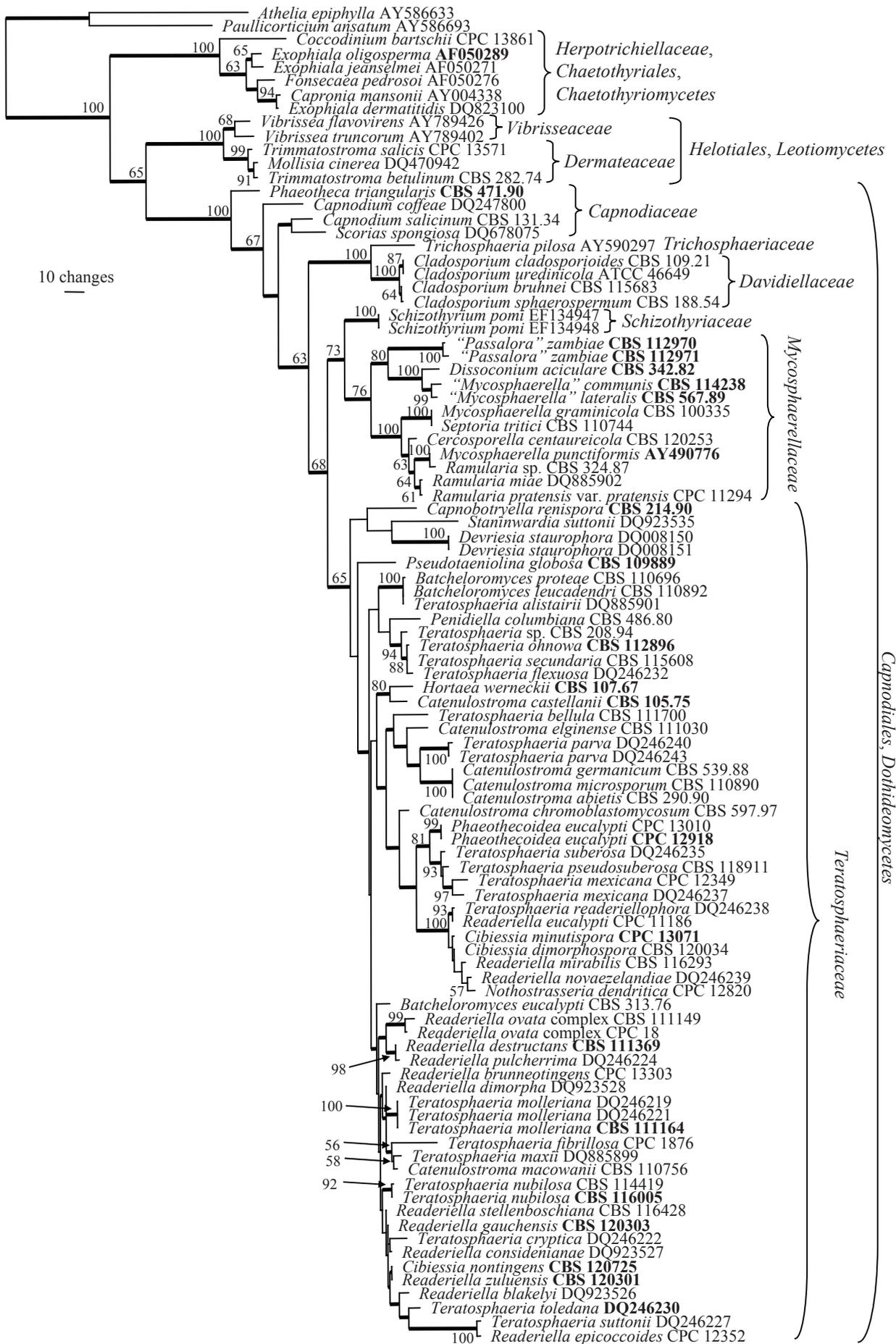
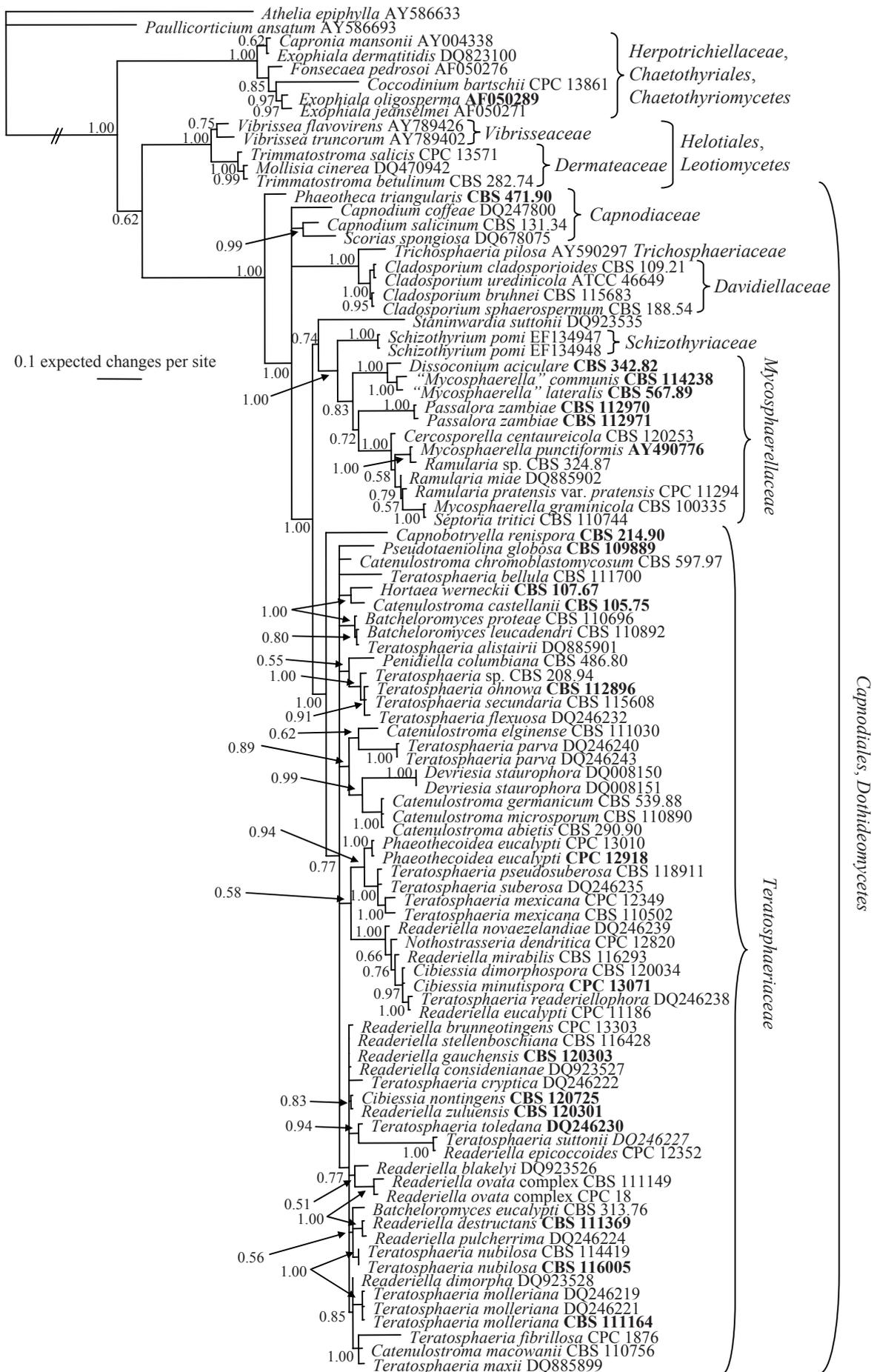


Fig. 1. One of 1 135 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the LSU sequence alignment using PAUP v. 4.0b10. The scale bar shows 10 changes, and bootstrap support values from 1 000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and ex-type sequences are printed in bold face. The tree was rooted to two sequences obtained from GenBank (*Athelia epiphylla* AY586633 and *Paulliacortium ansatum* AY586693).



**Fig. 2.** Consensus phylogram (50 % majority rule) of 18 815 trees resulting from a Bayesian analysis of the LSU sequence alignment using MrBAYES v. 3.1.2. Bayesian posterior probabilities are indicated at the nodes. Ex-type sequences are printed in bold face. The tree was rooted to two sequences obtained from GenBank (*Athelia epiphylla* AY586633 and *Paulllicorticium ansatum* AY586693).

## Treatment of phylogenetic clades

### Davidiellaceae clade

**Davidiella** Crous & U. Braun, Mycol. Progr. 2: 8. 2003.

*Type species: Davidiella tassiana* (De Not.) Crous & U. Braun, Mycol. Progr. 2: 8. 2003.

*Basionym: Sphaerella tassiana* De Not., Sferiacei Italici 1: 87. 1863.

*Description:* Schubert *et al.* (2007 – this volume).

*Anamorph: Cladosporium* Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 7: 37. 1816.

*Type species: Cladosporium herbarum* (Pers. : Fr.) Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 7: 37. 1816.

*Basionym: Dematium herbarum* Pers., Ann. Bot. (Usteri), 11 Stück: 32. 1794: Fr., Syst. Mycol. 3: 370. 1832.

*Description:* Schubert *et al.* (2007 – this volume).

*Notes:* The genus *Davidiella* (Davidiellaceae) was recently introduced for teleomorphs of *Cladosporium* s. str. (Braun *et al.* 2003). The genus *Cladosporium* is well-established, and contains around 772 names (Dugan *et al.* 2004), while *Davidiella* presently has 33 names (www.Mycobank.org), of which only around five have acknowledged *Cladosporium* states.

### Teratosphaeriaceae clade

**Teratosphaeria** Syd. & P. Syd., Ann. Mycol. 10: 39. 1912.

*Type species: Teratosphaeria fibrillosa* Syd. & P. Syd., Ann. Mycol. 10: 40. 1912. Fig. 3.

*Description:* Crous *et al.* (2004a; figs 182–185).

*Notes:* Although similar in morphology, the genus *Teratosphaeria* was separated from *Mycosphaerella* based on its ascomatal arrangement, and periphysate ostioles (Müller & Oehrens 1982). It was later synonymised under *Mycosphaerella* by Taylor *et al.* (2003), who showed that the type species clustered within *Mycosphaerella* based on ITS DNA sequence data. The LSU sequence data generated in the present study, has clearly shown that *Mycosphaerella* is polyphyletic, thus contradicting earlier reports of monophyly by Crous *et al.* (2000) and Goodwin *et al.* (2001), which were based on ITS data.

A re-examination of *T. fibrillosa*, the type species of *Teratosphaeria*, revealed several morphological features that characterise the majority of the taxa clustering in the clade, though several characters have been lost in some of the small-spored species. These characters are discussed below:

1. *Teratosphaeria fibrillosa* has a superficial stroma linking ascomata together, almost appearing like a spider's web on the leaf surface. Although this feature is not seen in other taxa in this clade, some species, such as *M. suberosa* Crous, F.A. Ferreira, Alfenas & M.J. Wingf. and *M. pseudosuberosa* Crous & M.J. Wingf. have a superficial stroma, into which the ascomata are inbedded (Crous 1998, Crous *et al.* 2006b).

2. Ascospores of *Teratosphaeria* become brown and

verruculose while still in their asci. This feature is commonly observed in species such as *M. jonkershoekensis* P.S. van Wyk, Marasas & Knox-Dav., *M. alistairii* Crous, *M. mexicana* Crous, *M. maxii* Crous and *M. excentricum* Crous & Carnegie (Crous 1998, Crous & Groenewald 2006a, b, Crous *et al.* 2007b).

3. A few ascomata of *T. fibrillosa* were found to have some pseudoparaphysoidal remnants (cells to distinguish pseudoparaphyses), though they mostly disappear with age. This feature is rather uncommon, though pseudoparaphyses were observed in ascomata of *M. eucalypti* (Wakef.) Hansf.

4. Ascospores of *Teratosphaeria* were found to be covered in a mucous sheath, which is commonly observed in other taxa in this clade, such as *M. bellula* Crous & M.J. Wingf., *M. pseudocryptica* Crous, *M. suberosa*, *M. pseudosuberosa*, *M. associata* Crous & Carnegie, *M. dendritica* Crous & Summerell and *M. fimbriata* Crous & Summerell (Crous *et al.* 2004b, 2006b, 2007b). Re-examination of fresh collections also revealed ascospores of *M. cryptica* (Cooke) Hansf. and *M. nubilosa* (Cooke) Hansf. to have a weakly definable sheath. Germinating ascospores of species in this clade all exhibit a prominent mucoid sheath.

5. Asci of *T. fibrillosa* were observed to have a multi-layered endotunica, which, although not common, can be seen in species such as *M. excentrica*, *M. maxii*, *M. alistairii*, *M. pseudosuberosa*, *M. fimbriata* (Crous *et al.* 2006b, 2007b, Crous & Groenewald 2006a, b), and also *M. nubilosa*.

6. Finally, ascomata of *T. fibrillosa* and *T. proteae-arboreae* P.S. van Wyk, Marasas & Knox-Dav. have well-developed ostiolar periphyses, which are also present in species such as *M. suberosa*, *M. pseudosuberosa*, *M. maxii* and *T. microspora* Joanne E. Taylor & Crous (Crous 1998, Crous *et al.* 2004a, b, 2006b). Morphologically thus, the *Teratosphaeria* clade is distinguishable from *Mycosphaerella* s. str., though these differences are less pronounced in some of the smaller-spored species. Based on these distinct morphological features, as well as its phylogenetic position within the *Capnodiales*, a new family is herewith proposed to accommodate species of *Teratosphaeria*:

**Teratosphaeriaceae** Crous & U. Braun, **fam. nov.** MycoBank MB504464.

Ascomata pseudotheciales, superficiales vel immersa, saepe in stromate ex cellulis brunneis pseudoparenchymatibus disposita, globulares, uniloculares, papillata, apice ostiolato, periphysata, saepe cum periphysoidibus; tunica multistratosa, ex cellulis brunneis angularibus composita, strato interiore ex cellulis applanatis hyalinis; saepe cum pseudoparaphysibus subcylindricis, ramosis, septatis, anastomosibus. Asci fasciculati, octospori, bitunicati, saepe cum endotunica multistratosa. Ascospores ellipsoideae-fusiformes vel obovoideae, 1-septatae, hyalinae, deinde pallide brunneae et verruculosae, saepe mucosae.

*Ascomata* pseudothecial, superficial to immersed, frequently situated in a stroma of brown pseudoparenchymatal cells, globose, unilocular, papillate, ostiolate, canal periphysate, with periphysoids frequently present; wall consisting of several layers of brown *textura angularis*; inner layer of flattened, hyaline cells. *Pseudoparaphyses* frequently present, subcylindrical, branched, septate, anastomosing. *Asci* fasciculate, 8-spored, bitunicate, frequently with multi-layered endotunica. *Ascospores* ellipsoid-fusoid to obovoid, 1-septate, hyaline, but becoming pale brown and verruculose, frequently covered in mucoid sheath.

*Typus: Teratosphaeria* Syd. & P. Syd., Ann. Mycol. 10: 39. 1912.

**Teratosphaeria africana** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504466.



Fig. 3. *Teratosphaeria fibrillosa* (epitype material). A. Leaf spots. B. Subepidermal asci linked by means of stromatic tissue. C. Paraphyses among asci. D. Periphysoids. E. Ascospores becoming brown in asci. F–G. Multi-layered endotunica. H–K. Ascospores, becoming brown and verruculose. L–M. Germinating ascospores. Scale bars = 10  $\mu$ m.

*Basionym:* *Mycosphaerella africana* Crous & M.J. Wingf., *Mycologia* 88: 450. 1996.

***Teratosphaeria associata*** (Crous & Carnegie) Crous & U. Braun, **comb. nov.** MycoBank MB504467.

*Basionym:* *Mycosphaerella associata* Crous & Carnegie, *Fungal Diversity* 26: 159. 2007.

***Teratosphaeria alistairii*** (Crous) Crous & U. Braun, **comb. nov.** MycoBank MB504468.

*Basionym:* *Mycosphaerella alistairii* Crous, in Crous & Groenewald, *Fungal Planet*, No. 4. 2006.

*Anamorph:* *Batcheloromyces* sp.

***Teratosphaeria bellula*** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504469.

*Basionym:* *Mycosphaerella bellula* Crous & M.J. Wingf., *Mycotaxon* 46: 20. 1993.

***Teratosphaeria cryptica*** (Cooke) Crous & U. Braun, **comb. nov.** MycoBank MB504470.

*Basionym:* *Sphaerella cryptica* Cooke, *Grevillea* 20: 5. 1891.

≡ *Mycosphaerella cryptica* (Cooke) Hansf., *Proc. Linn. Soc. New South Wales* 81: 35. 1956.

*Anamorph:* ***Readeriella nubilosa*** (Ganap. & Corbin) Crous & U. Braun, **comb. nov.** MycoBank MB504471.

*Basionym:* *Colletogloeum nubilosum* Ganap. & Corbin, *Trans. Brit. Mycol. Soc.* 72: 237. 1979.

≡ *Colletogloeopsis nubilosum* (Ganap. & Corbin) Crous & M.J. Wingf., *Canad. J. Bot.* 75: 668. 1997.

***Teratosphaeria dendritica*** (Crous & Summerell) Crous & U. Braun, **comb. nov.** MycoBank MB504472.

*Basionym:* *Mycosphaerella dendritica* Crous & Summerell, *Fungal Diversity* 26: 161. 2007.

*Anamorph:* ***Nothostrasseria dendritica*** (Hansf.) Nag Raj, *Canad. J. Bot.* 61: 25. 1983.

*Basionym:* *Spilomyces dendriticus* Hansf., *Proc. Linn. Soc. New South Wales* 81: 32. 1956.

***Teratosphaeria excentrica*** (Crous & Carnegie) Crous & U. Braun, **comb. nov.** MycoBank MB504473.

*Basionym:* *Mycosphaerella excentrica* Crous & Carnegie, *Fungal Diversity* 26: 164. 2007.

*Anamorph:* ***Catenulostroma excentricum*** (B. Sutton & Ganap.) Crous & U. Braun, **comb. nov.** MycoBank MB504475.

*Basionym:* *Trimmatostroma excentricum* B. Sutton & Ganap., *New Zealand J. Bot.* 16: 529. 1978.

***Teratosphaeria fibrillosa*** Syd. & P. Syd., *Ann. Mycol.* 10: 40. 1912.

≡ *Mycosphaerella fibrillosa* (Syd. & P. Syd.) Joanne E. Taylor & Crous, *Mycol. Res.* 107: 657. 2003.

*Specimens examined:* South Africa, Western Cape Province, Bains Kloof near Wellington, on living leaves of *Protea grandiflora*, 26 Feb. 1911, E.M. Doidge, **holotype** PREM; Stellenbosch, Jonkershoek valley, S33° 59' 44.7", E18° 58' 50.6", 1 Apr. 2007, on leaves of *Protea* sp., P.W. Crous & L. Mostert, **epitype designated here** CBS H-19913, culture ex-epitype CBS 121707 = CPC 13960.

***Teratosphaeria fimbriata*** (Crous & Summerell) Crous & U. Braun, **comb. nov.** MycoBank MB504476.

*Basionym:* *Mycosphaerella fimbriata* Crous & Summerell, *Fungal Diversity* 26: 166. 2007.

***Teratosphaeria flexuosa*** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504477.

*Basionym:* *Mycosphaerella flexuosa* Crous & M.J. Wingf., *Mycol. Mem.* 21: 58. 1998.

***Teratosphaeria gamsii*** (Crous) Crous & U. Braun, **comb. nov.** MycoBank MB504478.

*Basionym:* *Mycosphaerella gamsii* Crous, *Stud. Mycol.* 55: 113. 2006.

***Teratosphaeria jonkershoekensis*** (P.S. van Wyk, Marasas & Knox-Dav.) Crous & U. Braun, **comb. nov.** MycoBank MB504479.

*Basionym:* *Mycosphaerella jonkershoekensis* P.S. van Wyk, Marasas & Knox-Dav., *J. S. African Bot.* 41: 234. 1975.

***Teratosphaeria maxii*** (Crous) Crous & U. Braun, **comb. nov.** MycoBank MB504480.

*Basionym:* *Mycosphaerella maxii* Crous, in Crous & Groenewald, *Fungal Planet* No. 6. 2006.

***Teratosphaeria mexicana*** (Crous) Crous & U. Braun, **comb. nov.** MycoBank MB504481.

*Basionym:* *Mycosphaerella mexicana* Crous, *Mycol. Mem.* 21: 81. 1998.

***Teratosphaeria microspora*** Joanne E. Taylor & Crous, *Mycol. Res.* 104: 631. 2000.

≡ *Mycosphaerella microspora* (Joanne E. Taylor & Crous) Joanne E. Taylor & Crous, *Mycol. Res.* 107: 657. 2003.

*Anamorph:* ***Catenulostroma microsporum*** (Joanne E. Taylor & Crous) Crous & U. Braun, **comb. nov.** MycoBank MB504482.

*Basionym:* *Trimmatostroma microsporum* Joanne E. Taylor & Crous, *Mycol. Res.* 104: 631. 2000.

***Teratosphaeria molleriana*** (Thüm.) Crous & U. Braun, **comb. nov.** MycoBank MB504483.

*Basionym:* *Sphaerella molleriana* Thüm., *Revista Inst. Sci. Lit. Coimbra* 28: 31. 1881.

≡ *Mycosphaerella molleriana* (Thüm) Lindau, *Nat. Pflanzenfam.* 1: 424. 1897.

= *Mycosphaerella vespa* Carnegie & Keane, *Mycol. Res.* 102: 1275. 1998.

= *Mycosphaerella ambiphyllo* A. Maxwell, *Mycol. Res.* 107: 354. 2003.

*Anamorph:* ***Readeriella molleriana*** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504484.

*Basionym:* *Colletogloeopsis molleriana* Crous & M.J. Wingf., *Canad. J. Bot.* 75: 670. 1997.

***Teratosphaeria nubilosa*** (Cooke) Crous & U. Braun, **comb. nov.** MycoBank MB504485.

*Basionym:* *Sphaerella nubilosa* Cooke, *Grevillea* 19: 61. 1892.

≡ *Mycosphaerella nubilosa* (Cooke) Hansf., *Proc. Linn. Soc. New South Wales* 81: 36. 1965.

= *Mycosphaerella juvenis* Crous & M.J. Wingf., *Mycologia* 88: 453. 1996.

***Teratosphaeria ohnowa*** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504486.

*Basionym:* *Mycosphaerella ohnowa* Crous & M.J. Wingf., *Stud. Mycol.* 50: 206. 2004.

***Teratosphaeria parkii*** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504487.

*Basionym:* *Mycosphaerella parkii* (Crous & M.J. Wingf.), *Fungal Diversity* 26: 168. 2007.

***Teratosphaeria parva*** (R.F. Park & Keane) Crous & U. Braun, **comb. nov.** MycoBank MB504488.

*Basionym:* *Mycosphaerella parva* R.F. Park & Keane, *Trans. Brit. Mycol. Soc.* 79: 99. 1982.

= *Mycosphaerella grandis* Carnegie & Keane, *Mycol. Res.* 98: 414. 1994.

***Teratosphaeria perpendicularis*** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504489.

*Basionym:* *Mycosphaerella perpendicularis* Crous & M.J. Wingf., *Stud. Mycol.* 55: 113. 2006.

***Teratosphaeria pluritubularis*** (Crous & Mansilla) Crous & U. Braun, **comb. nov.** MycoBank MB504490.

*Basionym:* *Mycosphaerella pluritubularis* Crous & Mansilla, *Stud. Mycol.* 55: 114. 2006.

*Teratosphaeria pseudaficana* (Crous & T.A. Cout.) Crous & U. Braun, **comb. nov.** MycoBank MB504491.

*Basionym:* *Mycosphaerella pseudaficana* Crous & T.A. Cout., Stud. Mycol. 55: 115. 2006.

*Teratosphaeria pseudocryptica* (Crous) Crous & U. Braun, **comb. nov.** MycoBank MB504492.

*Basionym:* *Mycosphaerella pseudocryptica* Crous, Stud. Mycol. 55: 116. 2006.

*Anamorph:* *Readeriella* sp.

*Teratosphaeria pseudosuberosa* (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504493.

*Basionym:* *Mycosphaerella pseudosuberosa* Crous & M.J. Wingf., Stud. Mycol. 55: 118. 2006.

*Anamorph:* *Catenulostroma* sp.

*Teratosphaeria quasercospora* (Crous & T.A. Cout.) Crous & U. Braun, **comb. nov.** MycoBank MB504494.

*Basionym:* *Mycosphaerella quasercospora* Crous & T.A. Cout., Stud. Mycol. 55: 119. 2006.

*Teratosphaeria readeriellophora* (Crous & Mansilla) Crous & U. Braun, **comb. nov.** MycoBank MB504495.

*Basionym:* *Mycosphaerella readeriellophora* Crous & Mansilla, Stud. Mycol. 50: 207. 2004.

*Anamorph:* *Readeriella readeriellophora* Crous & Mansilla, Stud. Mycol. 50: 207. 2004. Fig. 18.

*Teratosphaeria secundaria* (Crous & Alfenas) Crous & U. Braun, **comb. nov.** MycoBank MB504496.

*Basionym:* *Mycosphaerella secundaria* Crous & Alfenas, Stud. Mycol. 55: 122. 2006.

*Teratosphaeria stramenticola* (Crous & Alfenas) Crous & U. Braun, **comb. nov.** MycoBank MB504497.

*Basionym:* *Mycosphaerella stramenticola* Crous & Alfenas, Stud. Mycol. 55: 123. 2006.

*Teratosphaeria suberosa* (Crous, F.A. Ferreira, Alfenas & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504498.

*Basionym:* *Mycosphaerella suberosa* Crous, F.A. Ferreira, Alfenas & M.J. Wingf., Mycologia 85: 707. 1993.

*Teratosphaeria suttonii* (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504499.

*Basionym:* *Mycosphaerella suttonii* Crous & M.J. Wingf. (*suttoniae*), Canad. J. Bot. 75: 783. 1997.

*Anamorph:* *Readeriella epicoccoides* (Cooke & Masee) Crous & U. Braun, **comb. nov.** MycoBank MB504500.

*Basionym:* *Cercospora epicoccoides* Cooke & Masee apud Cooke, Grevillea 19: 91. 1891.

≡ *Phaeophleospora epicoccoides* (Cooke & Masee) Crous, F.A. Ferreira & B. Sutton, S. African J. Bot. 63: 113. 1997.

≡ *Kirramyces epicoccoides* (Cooke & Masee) J. Walker, B. Sutton & Pascoe, Mycol. Res. 96: 919. 1992.

= *Hendersonia grandispora* McAlp., Proc. Linn. Soc. New South Wales 28: 99. 1903.

= *Phaeoseptoria eucalypti* Hansf., Proc. Linn. Soc. New South Wales 82: 225. 1957.

= *Phaeoseptoria luzonensis* T. Kobayashi, Trans. Mycol. Soc. Japan 19: 377. 1978.

*Synanamorph:* *Pseudocercospora* sp.

*Teratosphaeria toledana* (Crous & Bills) Crous & U. Braun, **comb. nov.** MycoBank MB504501.

*Basionym:* *Mycosphaerella toledana* Crous & Bills, Stud. Mycol. 50: 208. 2004.

*Anamorph:* *Readeriella toledana* (Crous & Bills) Crous & U. Braun, **comb. nov.** MycoBank MB504502.

*Basionym:* *Phaeophleospora toledana* Crous & Bills, Stud. Mycol. 50: 208. 2004.

## Key to treated anamorph genera of *Teratosphaeria* (*Teratosphaeriaceae*)

1. Hyphae submerged to superficial, disarticulating into arthroconidia ..... 2
1. Hyphae not disarticulating into arthroconidia ..... 3
2. Mature, brown hyphae disarticulating into thick-walled, spherical, smooth to verruculose 0(–2) transversely septate, brown conidia ..... *Pseudotaeniolina* (= *Friedmanniomyces*)
2. Hyphae superficial, brown to green-brown, smooth, disarticulating to form pale brown, cylindrical, 0–3-septate conidia with subtruncate ends, frequently with a *Readeriella* synanamorph ..... *Cibiessia*
3. Hyphal ends forming endoconidia; hyphae pale to medium brown, verruculose, end cells dividing into several brown, verruculose, thick-walled, ellipsoid to obovoid endoconidia ..... *Phaeothecoidea*
3. Endoconidia absent..... 4
4. Conidiogenous cells integrated in hyphae; well-developed conidiomata or long, solitary, macronematous, terminally penicillate conidiophores absent ..... 5
4. Conidiomata well-developed or with long, solitary, terminally penicillate conidiophores ..... 7
5. Conidia in chains, holoblastic, pseudocladosprium-like in morphology, but scars and hila not excessively thickened, nor refractive, producing chlamydospores in culture; species are mostly heat resistant ..... *Devriesia*
5. Conidia solitary on indistinct to well defined phialides on hyphae ..... 6
6. Conidiogenous cells integrated in the distal ends of hyphae; conidia thick-walled, brown, smooth, 1-septate ..... *Capnobotryella*

6. Conidiophores short and frequently reduced to conidiogenous cells that proliferate percurrently via wide necks, giving rise to hyaline, 0(–2)-septate, broadly ellipsoidal conidia ..... **Hortaea**
7. Conidia brown, with hyaline basal appendages; conidiomata pycnidial, conidiogenous cells phialidic, but also percurrent, subhyaline ..... **Nothotrasseria**
7. Conidia brown, but basal appendages lacking, amero- to scolecospores ..... 8
8. Conidiomata pycnidial to acervular ..... 9
8. Conidiomata not enclosed by host tissue, fasciculate to sporodochial or solitary, hyphomycetous ..... 10
9. Conidia solitary, dry, without mucilaginous sheath ..... **Readeriella**
9. Conidia catenulate, with persistent mucilaginous sheath ..... **Staninwardia**
10. Conidiophores usually solitary, rarely densely fasciculate to synnematosus (*in vivo*), penicillate, with a branched, apical conidiogenous apparatus giving rise to ramoconidia and branched chains of secondary conidia; scars not to slightly thickened and darkened-refractive ..... **Penidiella**
10. Conidiophores not penicillate, without a branched conidiogenous apparatus, *in vivo* fasciculate to sporodochial ..... 11
11. Biotrophic; fruiting composed of sporodochia and radiating layers of hyphae arising from the stromata, conidiophores arising from superficial sporodochia and radiating hyphae, conidiogenous cells unilocal, with conspicuous annellations, conidia solitary or in fragile disarticulating chains, aseptate or transversely 1–3-septate, usually with distinct frills, secession rhexolytic ..... **Batcheloromyces**
11. Biotrophic, leaf-inhabiting, with distinct, subepidermal to erumpent, well-developed sporodochia, or saxicolous, saprobic, sometimes causing opportunistic human infections; radiating layers of hyphae arising from sporodochia; conidiogenous cells without annellations; conidia in true simple or branched basipetal chains, transversely 1- to pluriseptate or with longitudinal and oblique septa (dictyosporous), occasionally distoseptate ..... **Catenulostroma**

To explain the arguments behind the selection and synonymies of some of these anamorphic genera, they are briefly discussed below:

**Acidomyces** Baker *et al.*, Appl. Environ. Microbiol. 70: 6270. 2004. (nom. inval.)

*Type species: Acidomyces richmondensis* Baker *et al.*, Appl. Environ. Microbiol. 70: 6270. 2004. (nom. inval.)

*Notes:* The genus presently clusters among isolates in the *Teratosphaeria* clade based on sequences deposited in GenBank. *Acidomyces* lacks a Latin description and holotype specimen, and is thus invalidly described. The genus, which was distinguished from other taxa based on its DNA phylogeny (*Dothideomycetes*), forms filamentous hyphae with disarticulating cells. It is unclear how it differs from *Friedmanniomyces* Onofri and *Pseudotaeniolina* J.L. Crane & Schokn.

**Batcheloromyces** Marasas, P.S. van Wyk & Knox-Dav., J. S. African Bot. 41: 41. 1975.

*Type species: Batcheloromyces proteae* Marasas, P.S. van Wyk & Knox-Dav., J. S. African Bot. 41: 43. 1975.

*Description:* Crous *et al.* (2004a; figs 4–26).

*Notes:* *Batcheloromyces* is presently circumscribed as a genus that forms emergent hyphae, giving rise to superficial sporodochial plates, forming brown, verrucose, erect conidiophores that proliferate holoblastically, with ragged percurrent proliferations that become visible with age. Conidia are produced singly or in fragile, disarticulating chains, are brown, thick-walled, 0–3 transversely euseptate (though at times they appear as distoseptate). The genus *Batcheloromyces* has in recent years been confused with *Stigmina* (Sutton & Pascoe 1989) on the basis that some collections showed conidiophores to give rise to solitary conidia only, though conidial catenulation was clearly illustrated by Taylor *et al.* (1999). In culture colonies tend to sporulate in a slimy mass (on OA), though a

synanamorph can be seen (in *B. leucadendri*, Fig. 4) to sporulate via holoblastic conidiogenesis on hyphal tips of the aerial mycelium, forming elongate-globose to ellipsoid, muriformly septate, thick-walled conidia, that occur in clusters.

The finding that *Stigmina s. str.* [based on *S. platani* (Fuckel) Sacc., the type species] is a generic synonym of *Pseudocercospora* Speg. (Crous *et al.* 2006a), and that the type species of *Trimmatostroma* (*T. salicis*, Fig. 5) belongs to the *Helotiales* (Fig. 1), raises the question of where to place *stigmina*- and *trimmatostroma*-like anamorphs that reside in the *Teratosphaeria* clade. Although the *stigmina*-like species can be accommodated in *Batcheloromyces* (see Sutton & Pascoe 1989), a new genus is required for *Teratosphaeria* anamorphs that have a *trimmatostroma*-like morphology. The recognition of *Batcheloromyces* and the introduction of a new anamorph genus for *trimmatostroma*-like anamorphs of *Teratosphaeria* are also morphologically justified. *Batcheloromyces* is easily distinguishable from *Stigmina s. str.* by its special structure of the fruiting body, composed of sporodochia and radiating layers of hyphae arising from the sporodochia and the conidia often formed in delicate disarticulating chains. *Trimmatostroma*-like anamorphs of *Teratosphaeria* are morphologically also sufficiently distinct from *Trimmatostroma s. str.* (see notes under *Catenulostroma* Crous & U. Braun) as well as *Batcheloromyces* (see key above).

**Batcheloromyces eucalypti** (Alcorn) Crous & U. Braun, **comb. nov.** MycoBank MB504503.

*Basionym:* *Stigmina eucalypti* Alcorn, Trans. Brit. Mycol. Soc. 60: 151. 1973.

**Capnobotryella** Sugiy., in Sugiyama, *Pleomorphic Fungi: The Diversity and its Taxonomic Implications* (Tokyo): 148. 1987.

*Type species: Capnobotryella renispora* Sugiy., in Sugiyama, *Pleomorphic Fungi: The Diversity and its Taxonomic Implications* (Tokyo): 148. 1987.

*Description:* Sugiyama & Amano (1987, figs 7.5–7.8).

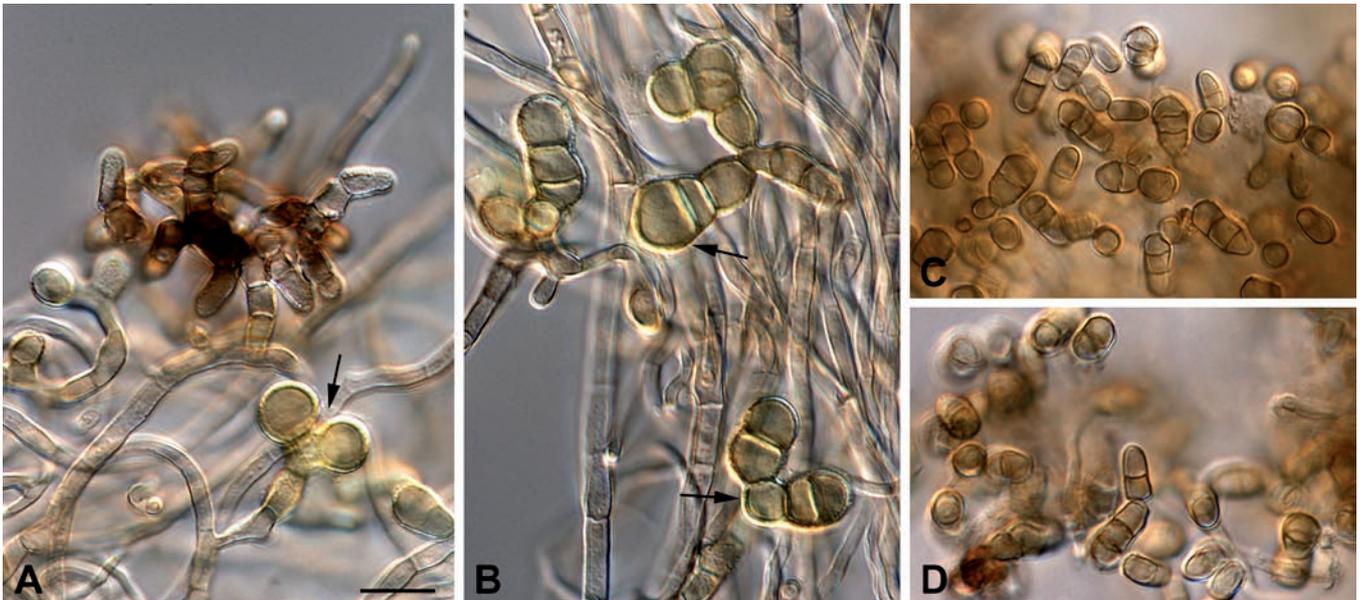


Fig. 4. *Batcheloromyces leucadendri* in vitro. A–B. *Batcheloromyces* state with synanamorph (arrows). C–D. Conidia occurring solitary or in short chains. Scale bar = 10  $\mu$ m.

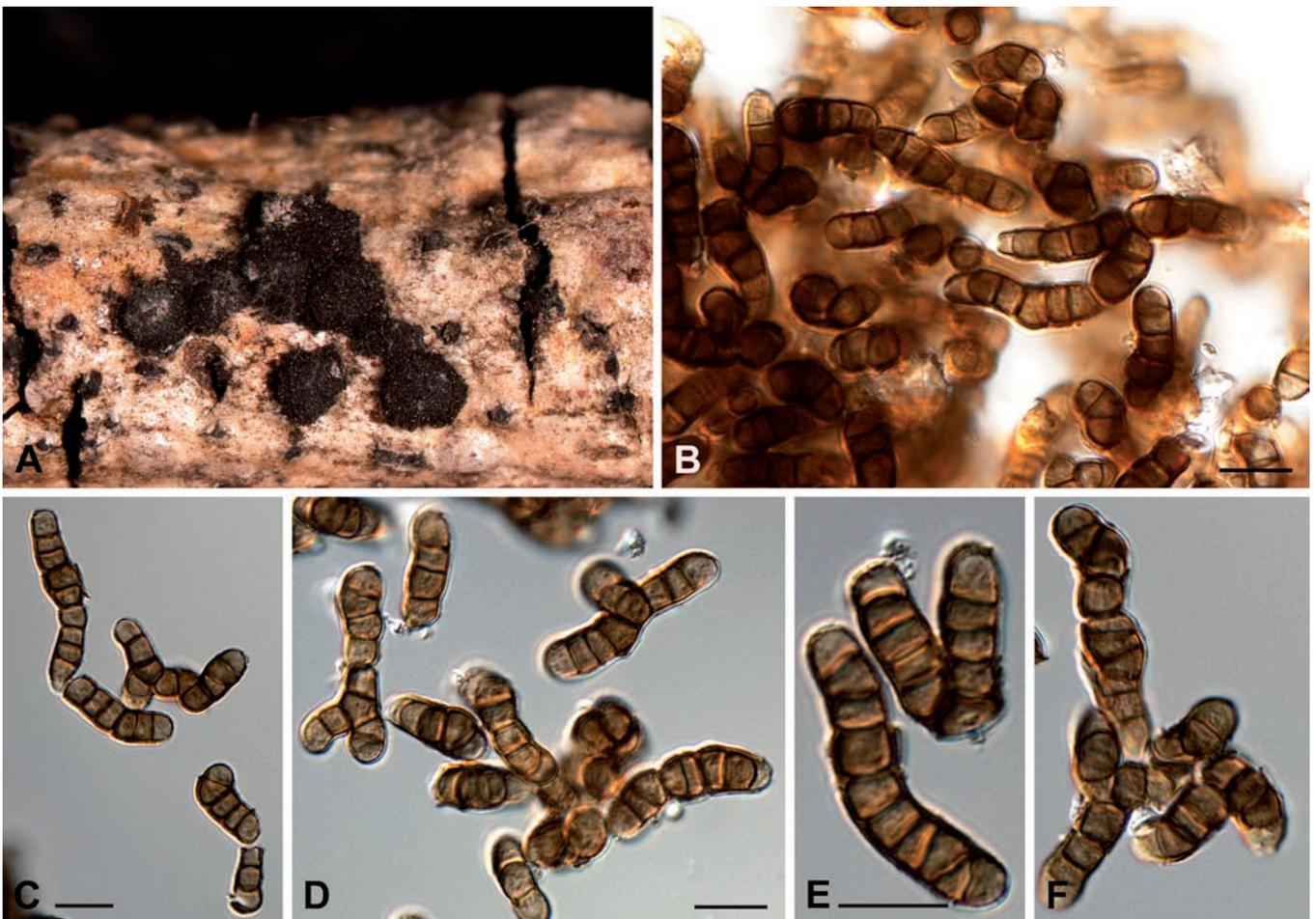


Fig. 5. *Trimmatostroma salicis*. A. Sporodochia on twig. B–E. Chains of disarticulating conidia. Scale bars = 10  $\mu$ m.

*Notes:* The genus forms brown, septate, thick-walled hyphae, with ellipsoidal, 0–1-septate conidia forming directly on the hyphae, via minute phialides. Hambleton *et al.* (2003) also noted the occurrence of endoconidiation.

***Catenulostroma*** Crous & U. Braun, **gen. nov.** MycoBank MB504474.

*Etymology:* Named after its catenulate conidia, and stromata giving rise to sporodochia.

Hyphomycetes. Differt a *Trimmatostromate* habitu phytoparasitico, maculis formantibus, conidiophoris saepe fasciculatis, per stoma emergentibus vel habitu saxiphilo-saprophytico, interdum sejunctis ex mycosibus humanis.

*Habit* plant pathogenic, leaf-spotting or saxicolous-saprobic, occasionally isolated from opportunistic human mycoses. *Mycelium* internal and external; hyphae dark brown, septate, branched. *Conidiomata in vivo* vary from acervuli to sporodochia or fascicles of conidiophores arising from well-developed or reduced, pseudoparenchymatal stromata. *Setae* and *hyphopodia* absent. *Conidiophores* arising from hyphae or stromata, solitary, fasciculate to sporodochial, in biotrophic, plant pathogenic species emerging through stomata, little differentiated, semimacronematous, branched or not, continuous to septate, brown, smooth to verruculose. *Conidiogenous cells* integrated, terminal or conidiophores reduced to conidiogenous cells, holoblastic-thalloblastic, meristematic, unilocal, delimitation of conidium by a single septum with retrogressive delimitation of next conidium giving an unconnected chain of conidia, brown, smooth to verruculose, conidiogenous scars (conidiogenous loci) inconspicuous, truncate, neither thickened nor darkened. *Conidia* solitary or usually forming simple to branched basipetal chains of transversely to muriformly eu- or distoseptate, 1- to multiseptate, brown, smooth, verruculose to verrucose conidia, conidial secession schizolytic.

*Type species: Catenulostroma protearum* (Crous & M.E. Palm) Crous & U. Braun, comb. nov.

*Description:* Crous & Palm (1999), Crous *et al.* (2004a; figs 364–365).

*Notes:* *Catenulostroma* contains several plant pathogenic species previously placed in *Trimmatostroma*, a morphologically similar but, based on its type species, phylogenetically distinct genus belonging to *Helotiales* (Fig. 1). *Trimmatostroma s. str.* is well-distinguished from most *Catenulostroma* species by being saprobic, living on twigs and branches of woody plants, or occasionally isolated from leaf litter, i.e., they are not associated with leaf spots. The conidiomata of *Trimmatostroma* species are subepidermal, acervular-sporodochial with a well defined wall of *textura angularis*, little differentiated, micronematous conidiophores giving rise to long chains of conidia that disarticulate at the surface to form a grey-black to brown powdery mass. The generic affinity of other species assigned to *Trimmatostroma*, e.g. those having a lichenicolous habit, is unresolved.

*Trimmatostroma abietis* Butin & Pehl (Butin *et al.* 1996) clusters together with the plant pathogenic *Catenulostroma* species, but

differs from these species in having a more complex ecology. *Trimmatostroma abietis* is usually foliicolous on living or necrotic conifer needles on which characteristic acervuli to sporodochia with densely arranged, fasciculate fertile hyphae are formed, comparable to the fasciculate conidiomata of the plant pathogenic species of *Catenulostroma* (Butin *et al.* 1996: 205, fig. 1). Although not discussed by Butin *et al.* (1996), *T. abietis* needs to be compared to *T. abietina* Doherty, which was originally described from *Abies balsamea* needles collected in Guelph, Canada (Doherty 1900). Morphologically the two species appear to be synonymous, except for reference to muriformly septate conidia, which is a feature not seen *in vivo* in the type of *T. abietis*. Furthermore, as this is clearly a species complex, this matter can only be resolved once fresh Canadian material has been collected to serve as epitype for *T. abietina*.

Isolates from stone, agreeing with *T. abietis* in cultural, morphological and physiological characteristics, have frequently been found (Wollenzien *et al.* 1995, Butin *et al.* 1996, Gorbushina *et al.* 1996, Kogej *et al.* 2006, Krumbein *et al.* 1996). Furthermore, isolates from humans (ex skin lesions and ex chronic osteomyelitis of human patients) and *Ilex* leaves are known (Butin *et al.* 1996). De Hoog *et al.* (1999) included strains of *T. abietis* from stone, man and *Ilex* leaves in molecular sequence analyses and demonstrated their genetical identity based on 5.8S rDNA and ITS2 data, but strains from conifer needles were not included. Furthermore, we consider *T. abietis*, as presently defined, to represent a species complex, with Dutch isolates from *Pinus* again appearing distinct from German *Abies* isolates, suggesting that different conifer genera could harbour different *Catenulostroma* species. Isolates from stone form stromatic, durable microcolonies, which are able to grow under extreme xerophilic environmental conditions. Cultural growth resembles that of other meristematic black yeasts (Butin *et al.* 1996, Kogej *et al.* 2006). Another fungus isolated from stone in Germany is *in vitro* morphologically close to *C. abietis*, but differs in forming conidia with oblique septa. Furthermore, a human pathogenic isolate from Africa clusters together with other *Catenulostroma* species. The habit and origin of this human pathogenic fungus in nature and its potential morphology on “natural” substrates, which typically deviates strongly from the growth *in vitro*, are still unknown. However, *C. abietis*, usually growing as a foliicolous and saxicolous fungus, has already shown the potential ability of *Catenulostroma* species to cause opportunistic human infections.

## Key to *Catenulostroma* species

1. Conidia formed in basipetal chains, smooth, 4-celled, consisting of two basal cells with truncate lateral sides, each giving rise to a secondary globose apical cell, that can extend and develop additional septa, appearing as two lateral arms ..... **C. excentricum**
1. Conidia variable in shape, but without two basal cells giving rise to two lateral arms ..... 2
2. Conidia smooth or almost so, at most very faintly rough-walled; usually foliicolous on conifer needles or saxicolous, forming stromatic, xerophilic durable microcolonies on stone, occasionally causing opportunistic human infections ..... 3
2. Conidia distinctly verruculose to verrucose; plant pathogenic, forming leaf spots ..... 5
3. Conidia (8–)20–35(–60) × 4–5(–7) μm, 1–10-septate ..... **C. chromoblastomycosum**
3. Conidia much shorter, 8–20 μm long, 0–5-septate ..... 4
4. Conidia 0–5 times transversely septate, mostly two-celled; usually foliicolous on conifer needles or saxicolous ..... **C. abietis**
4. Conidia 2–4 times transversely septate and often with 1–2 oblique septa; isolated from stone ..... **C. germanicum**
5. Conidia rather broad, usually wider than 10 μm ..... 6

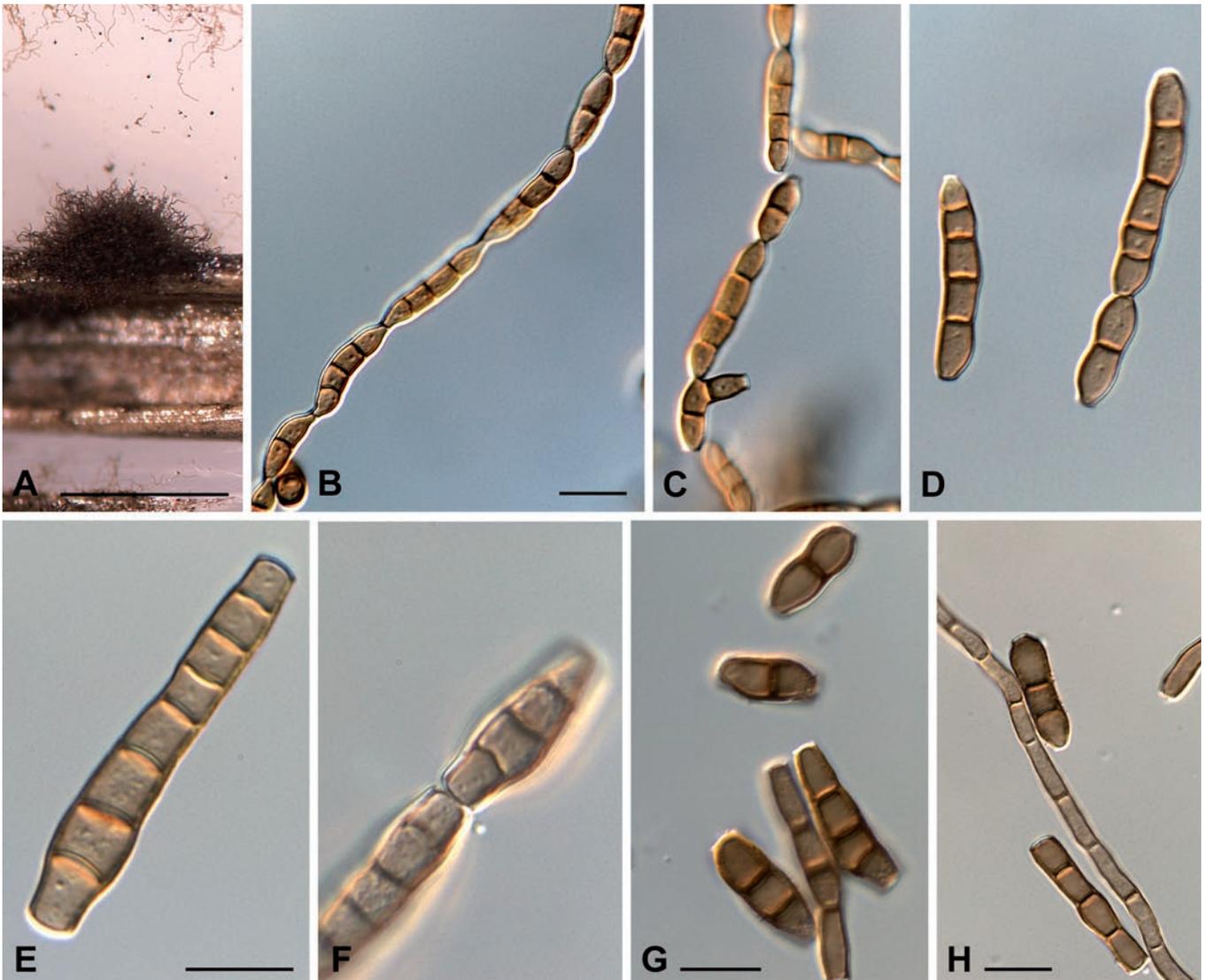


Fig. 6. *Catenulostroma chromoblastomycosum* (type material). A. Sporodochium on pine needle *in vitro*. B–H. Chains of disarticulating conidia. Scale bars: A = 350, B, E, G, H = 10  $\mu$ m.

5. Conidia narrower, width below 10  $\mu$ m ..... 7
6. Conidia distoseptate, rather long, (12–)25–35(–45)  $\times$  (7–)10–15(–25)  $\mu$ m; conidiomata large, up to 250  $\mu$ m diam, on *Protea anceps* ..... ***C. protearum***
6. Conidia euseptate, shorter, (9–)16–20(–36)  $\times$  (10–)14–18(–27)  $\mu$ m; sporodochia 90–100  $\times$  40–80  $\mu$ m; on *Protea grandiceps* ..... ***C. elginense***
7. Conidia 1- to multiseptate, (10–)15–17(–23)  $\times$  (5–)6.5–7(–9)  $\mu$ m; on various *Proteaceae* ..... ***C. macowanii***
7. Conidia *in vivo* predominantly 1-septate, (8–)13–15(–21)  $\times$  (3.5–)5.5–6(–8)  $\mu$ m; on *Protea cynaroides* ..... ***C. microsporum* (*Teratosphaeria microspora*)**

***Catenulostroma abietis*** (Butin & Pehl) Crous & U. Braun, **comb. nov.** MycoBank MB504504.

*Basionym:* *Trimmatostroma abietis* Butin & Pehl, Antonie van Leeuwenhoek 69: 204. 1996.

*Notes:* *Catenulostroma abietis* needs to be compared to *Trimmatostroma abietina* Doherty (*Abies balsamea* needles Canada), which is either an older name for this species, or a closely related taxon. Presently *T. abietina* is not known from culture, and needs to be recollected.

***Catenulostroma chromoblastomycosum*** Crous & U. Braun, **sp. nov.** MycoBank MB504505. Fig. 6.

*Etymology:* Named after the disease symptoms observed due to opportunistic human infection.

Differt a *C. abietis* et *C. germanico* conidiis longioribus, (8–)20–35(–60)  $\times$  4–5(–7)  $\mu$ m, 1–10-septatis.

Description based on cultures sporulating on WA supplemented with sterile pine needles. *Mycelium* consisting of branched, septate, smooth to finely verruculose, medium to dark brown, thick-walled, 3–4  $\mu$ m wide hyphae. *Conidiomata* brown, superficial,



Fig. 7. *Catenulostroma germanicum* (type material). A–D. Chains of disarticulating conidia *in vitro*. Scale bars = 10  $\mu\text{m}$ .

sporodochial, up to 350  $\mu\text{m}$  diam. *Conidiophores* reduced to inconspicuous conidiogenous loci on hyphae, 2–4  $\mu\text{m}$  wide, neither darkened nor thickened or refractive. *Conidia* occurring in branched chains, that tend to remain attached to each other, subcylindrical with subtruncate ends, straight to slightly curved, (8–)20–35(–60)  $\times$  4–5(–7)  $\mu\text{m}$ , 1–10-septate, medium brown, smooth to finely verruculose.

*Cultural characteristics*: Colonies on PDA erumpent, spreading, slow growing, with sparse to moderate aerial mycelium and smooth, irregular, submerged margins; greenish black (surface).

*Specimen examined*: Zaire, Pawa, isolated from man with chromoblastomycosis, Mar. 1997, V. de Brouwere, *holotype* CBS H-19935, culture ex-type CBS 597.97.

*Notes*: *Catenulostroma chromoblastomycosum* was originally identified as an isolate of *Stenella araguata* Syd. The latter fungus is morphologically distinct, however, having much shorter and narrower conidia, formed in acropetal chains, as well as quite different conidiogenous loci and conidial hila which are small, thickened and darkened.

*Catenulostroma elginense* (Joanne E. Taylor & Crous) Crous & U. Braun, *comb. nov.* MycoBank MB504506.

*Basionym*: *Trimmatostroma elginense* Joanne E. Taylor & Crous, Mycol. Res. 104: 633. 2000.

*Catenulostroma excentricum*, see *Teratosphaeria excentrica*.

*Catenulostroma germanicum* Crous & U. Braun, *sp. nov.* MycoBank MB504507. Fig. 7.

*Etymology*: Named after the geographic location of its type strain in Germany.

Differt a *C. abietis* conidiis 1–2 oblique septatis.

*Mycelium* consisting of branched, septate, smooth, pale to medium brown, 2–4  $\mu\text{m}$  wide hyphae, giving rise to conidial chains. *Conidiophores* integrated, subcylindrical, branched or not, septate, little differentiated, micronematous, 3–5  $\mu\text{m}$  wide, 3- to multiseptate, medium brown, thick-walled; conidiogenous cells integrated, terminal, inconspicuous, unilocal, conidiogenous loci

inconspicuous. *Conidia* in simple or branched basipetal chains, subcylindrical, straight to flexuous, (8–)10–15(–20) × 4–5(–6) μm, 2–4 transversely septate or with 1–2 oblique septa, medium to dark brown, thick-walled, smooth.

**Cultural characteristics:** Colonies on OA erumpent, spreading, with even, smooth margins and sparse to moderate aerial mycelium; olivaceous-grey, with iron-grey margins (surface). Colonies reaching 12 mm diam after 1 mo at 25 °C in the dark; colonies fertile.

**Specimen examined:** **Germany** (former West-Germany), isolated from stone, Oct. 1988, J. Kuroczkin, **holotype** CBS H-19936, culture ex-type CBS 539.88.

**Notes:** *Catenulostroma germanicum* was originally deposited as *Taeniolina scripta* (P. Karst.) P.M. Kirk. It is clearly distinct, however, as the latter fungus forms intricate, branched, brown conidia (Kirk 1981), unlike those of *C. germanicum*. Phylogenetically *C. germanicum* forms part of the *C. abietis* species complex.

***Catenulostroma macowanii*** (Sacc.) Crous & U. Braun, **comb. nov.** MycoBank MB504508.

**Basionym:** *Coniothecium macowanii* Sacc., Syll. Fung. 4: 512. 1886.

≡ *Coniothecium punctiforme* G. Winter, Hedwigia 24: 33. 1885, non *C. punctiforme* Corda, *Icones Fungorum* (Prague) 1: 2. 1837.  
≡ *Trimmatostroma macowanii* (Sacc.) M.B. Ellis, *More Dematiaceous Hyphomycetes*: 29. 1976.

*Catenulostroma microsporum*, see ***Teratosphaeria microspora***.

***Catenulostroma protearum*** (Crous & M.E. Palm) Crous & U. Braun, **comb. nov.** MycoBank MB504509.

**Basionym:** *Trimmatostroma protearum* Crous & M.E. Palm, Mycol. Res. 103: 1303. 1999.

***Cibiessia*** Crous, Fungal Diversity 26: 151. 2007.

**Type species:** *Cibiessia dimorphospora* Crous & C. Mohammed, Fungal Diversity 26: 151. 2007.

**Description:** Crous *et al.* (2007b; figs 3–5).

**Notes:** The genus *Cibiessia* was introduced to accommodate species with chains of disarticulating conidia (arthroconidia). Some species have been shown to have a *Readeriella* synanamorph.

***Devriesia*** Seifert & N.L. Nick., Can. J. Bot. 82: 919. 2004.

**Type species:** *Devriesia staurophora* (W.B. Kendr.) Seifert & N.L. Nick., Canad. J. Bot. 82: 919. 2004.

**Description:** Seifert *et al.* (2004; figs 2–42).

**Notes:** The genus is characterised by producing chains of pale brown, subcylindrical to fusiform, 0–1-septate conidia with somewhat thickened, darkened hila, forming chlamydospores in culture, and being heat resistant. Morphologically they resemble taxa placed in *Pseudocladosporium* U. Braun (= *Fusicladium* Bonord.; *Venturiaceae*), though phylogenetically *Devriesia* is not allied to this family.

***Hortaea*** Nishim. & Miyaji, Jap. J. Med. Mycol. 26: 145. 1984.

**Type species:** *Hortaea werneckii* (Horta) Nishim. & Miyaji, Jap. J. Med. Mycol. 26: 145. 1984.

**Description:** de Hoog *et al.* (2000, illust. p. 721).

**Notes:** The genus forms brown, septate, thick-walled hyphae, with ellipsoidal, 0–1-septate (becoming muriformly septate), hyaline to pale brown conidia forming directly on the hyphae, via phialides with percurrent proliferation. Isolates of *H. werneckii* are restricted to tropical or subtropical areas, where they occur as halophilic saprobes, frequently being associated with *tinea nigra* of humans (de Hoog *et al.* 2000). The generic distinction with *Capnobotryella* is less clear, except that the latter tends to have darker, thick-walled conidia, and reduced, less prominent phialides.

***Penidiella*** Crous & U. Braun, **gen. nov.** MycoBank MB504463.

**Etymology:** Named after its penicillate conidiophores.

Differt a Periconiellae conidiophoris apice penicillato ex cellulis conidiogenis et ramoconidiis compositis, cellulis conidiogenis saepe 1–3(–4) locis conidiogenis, terminalibus vel subterminalibus, subdenticulatis, non vel subincrassatis, non vel leviter fuscatis-refractivis, ramoconidiis praesentibus, saepe numerosis, conidiis ramificatis.

**Mycelium** consisting of branched, septate, smooth to verruculose, subhyaline to pale brown hyphae. *Conidiophores* macronematous, occasionally also with some micronematous conidiophores; macronematous conidiophores arising from superficial mycelium or stromata, solitary, fasciculate or in synnemata, erect, brown, thin- to thick-walled, smooth to finely verruculose; terminally penicillate, branched terminal part consisting of a conidiogenous apparatus composed of a series of conidiogenous cells and/or ramoconidia. *Conidiogenous cells* integrated, terminal, intercalary or pleurogenous, unbranched, pale to medium brown, smooth to finely verruculose, tapering to a flattened or rounded apical region or tips slightly inflated, polyblastic, sympodial, giving rise to a single or several sets of ramoconidia on different levels; with relatively few conidiogenous loci, 1–3(–4), terminal or subterminal, subdenticulate, denticle-like loci usually conical, terminally truncate, usually unthickened or at most very slightly thickened, not to slightly darkened or somewhat refractive. *Conidia* in branched acropetal chains. *Ramoconidia* 0–1-septate, pale to medium brown, smooth to verruculose, thin-walled, ellipsoidal, obovoid, fusiform, subcylindrical to obclavate; conidia subcylindrical, fusiform to ellipsoid-ovoid, 0–1-septate, pale olivaceous to brown, smooth to verruculose, thin-walled, catenate; hila truncate, unthickened or almost so, barely to somewhat darkened-refractive.

**Type species:** *Penidiella columbiana* Crous & U. Braun, sp. nov.

**Notes:** Three ramichloridium-like genera cluster within *Capnodiales*, namely *Periconiella* Sacc. [type: *P. velutina* (G. Winter) Sacc.], *Ramichloridium* Stahel ex de Hoog [type: *R. apiculatum* (J.H. Mill., Giddens & A.A. Foster) de Hoog] and *Penidiella* [type: *P. columbiana* Crous & U. Braun]. All three genera have brown, macronematous conidiophores with similar conidial scars. Within this complex, *Ramichloridium* is distinct in having a prominent rachis giving rise to solitary conidia. *Periconiella* and *Penidiella* are branched in the apical part of their conidiophores, and lack a rachis. In *Periconiella* conidia are solitary or formed in short, mostly simple chains, ramoconidia are lacking. The apical conidiogenous apparatus is composed of conidiogenous cells or branches with integrated, usually terminal conidiogenous cells, which are persistent. The conidiogenous cells are subcylindrical to somewhat clavate, usually not distinctly attenuated towards the tip, and have several, often numerous loci, aggregated or spread over the whole cell, terminal to usually lateral, flat, non-protuberant, not denticle-like, usually distinctly thickened and darkened, at least at the rim. In contrast, *Penidiella* has a quite distinct branching

system, consisting of a single terminal conidiogenous cell giving rise to several ramoconidia that form secondary ramoconidia, etc., or the branched apparatus is composed of several terminal and sometimes lateral conidiogenous cells giving rise to sequences of ramoconidia (conidiogenous cells and ramoconidia are often barely distinguishable, with conidiogenous cells disarticulating, becoming ramoconidia). The branched apparatus may be loose to dense, metula-like. The conidiogenous cells have only few, usually 1–3 (–4), terminal or subterminal subdenticulate loci, and ramoconidia are prominent and numerous, giving rise to branched chains of secondary conidia with flat-tipped hila. Some species of *Penidiella* with compact, metula-like branched apices are morphologically close to *Metulocladosporiella* Crous, Schroers, J.Z. Groenew., U. Braun & K. Schub. (Crous *et al.* 2006d). This genus encompasses

two species of banana diseases belonging to *Herpotrichiellaceae* (*Chaetothyriales*), characterised by having conidiophore bases with rhizoid hyphal appendages and abundant micronematous conidiophores. *Penidiella* species with less pronounced penicillate apices, e.g. *P. strumelloidea* (Milko & Dunaev) Crous & U. Braun, are comparable with species of the genus *Pleurotheciopsis* B. Sutton (see Ellis 1976). The latter genus is distinct in having unbranched, often percurrently proliferating conidiophores, lacking ramoconidia and colourless conidia formed in simple chains.

*Cladosporium helicosporum* R.F. Castañeda & W.B. Kendr. (Castañeda *et al.* 1997) is another penidiella-like fungus with terminally branched conidiophores, subdenticulate conidiogenous loci and conidia in long acropetal chains, but its affinity to *Penidiella* has still to be proven.

## Key to *Penidiella* species

1. Conidiophores *in vivo* in well-developed, dense fascicles and distinct synnemata arising from a basal stroma; on fallen leaves of *Ficus* sp., Cuba ..... ***P. cubensis***
1. Conidiophores solitary, at most loosely aggregated ..... 2
2. Conidiophores with a terminal conidiogenous cell, often somewhat swollen, giving rise to several ramoconidia (on one level) that form chains of straight to distinctly curved conidia; isolated from leaf of *Carex* sp., Russia ..... ***P. strumelloidea***
2. Penicillate apex of the conidiophores composed of a system of true branchlets, conidiogenous cells and ramoconidia or at least a sequence of ramoconidia on several levels; conidia usually straight ..... 3
3. Mycelium verruculose; long filiform conidiophores ending with a subdenticulate cell giving rise to sets of penicillate conidiogenous cells or ramoconidia which are barely distinguishable and turn into each other; ramoconidia and conidia consistently narrow, (1.5–)2(–2.5)  $\mu\text{m}$  wide, and aseptate, ramoconidia sometimes heterochromous; on living leaves of *Nectandra coriacea*, Cuba ..... ***P. nectandrae***
3. Mycelium more or less smooth; penicillate apex at least partly with true branchlets; conidia wider, 2–5  $\mu\text{m}$ , at least partly septate, uniformly pigmented ..... 4
4. Hyphae, conidiophores and conidia frequently distinctly constricted at the septa; penicillate apex of the conidiophores sparingly developed, branchlets more or less divergent; isolated from leaf litter of *Smilax* sp., Cuba ..... ***P. rigidophora***
4. Hyphae and conidia without distinct constrictions at the septa; penicillate apex of the conidiophores usually well-developed, with abundant branchings ..... 5
5. Conidiophores short, up to 120  $\times$  3–4  $\mu\text{m}$ , frequently with intercalary conidiogenous cell, swollen at the conidiogenous portion just below the upper septum which render the conidiophores subnodulose to distinctly nodulose, apex  $\pm$  loosely penicillate; conidia (4–)5–7(–8)  $\mu\text{m}$  long; occasionally with micronematous conidiophores; isolated from man with *tinea nigra*, Venezuela ..... ***P. venezuelensis***
5. Conidiophores much longer, up to 800  $\mu\text{m}$ , 7–9  $\mu\text{m}$  wide at the base, not distinctly nodulose, penicillate apex loose to often more compact, tight, metula-like; conidia longer, 7–25  $\times$  2–5  $\mu\text{m}$ ; micronematous conidiophores lacking; isolated from dead leaf of *Paepalanthus columbianus*, Colombia ..... ***P. columbiana***

***Penidiella columbiana*** Crous & U. Braun, **sp. nov.** MycoBank MB504510. Figs 8–9.

**Etymology:** Named after its country of origin, Colombia.

Mycelium ex hyphis ramosis, septatis, levibus, pallide brunneis, 2–3  $\mu\text{m}$  latis compositum. Conidiophora ex hyphis superficialibus oriunda, penicillata, erecta, brunnea, crassitunicata, minute verruculosa, ad 800  $\mu\text{m}$  longa, ad basim 7–9  $\mu\text{m}$  lata, ad apicem pluriramosa, ex ramibus diversibus et cellulis conidiogenis composita, ramibus primariis (–2) subcylindraceis, 1–7-septatis, 50–120  $\times$  4–6  $\mu\text{m}$ ; ramibus secundariis (–2) subcylindraceis, 1–5-septatis, 40–60  $\times$  4–6  $\mu\text{m}$ ; ramibus tertiariis et subsequentibus 1–4-septatis, 10–30  $\times$  3–5  $\mu\text{m}$ . Cellulae conidiogenae terminales vel laterales, non ramosae, 5–15  $\times$  3–5  $\mu\text{m}$ , modice brunneae, minute verruculosae, apicem versus attenuatae, truncatae vel rotundatae, polyblasticae, sympodiales, cicatrices conidiales incrassatae, sed leviter fuscatae et non refractivae. Ramoconidia 0–1-septata, modice brunnea, levia, ellipsoidea, obclavata vel obovoidea, cum 1–3 hiliis terminalibus, 10–20  $\times$  3–5  $\mu\text{m}$ ; conidia subcylindrica vel ellipsoidea, 0–1-septata, pallide brunnea, catenata (–10), hila truncata, non incrassata, vix vel leviter fuscata.

*Mycelium* consisting of branched, septate, smooth, pale brown, 2–3  $\mu\text{m}$  wide hyphae. *Conidiophores* arising from superficial mycelium, terminally penicillate, erect, brown, wall up to 1  $\mu\text{m}$  wide, almost smooth to finely verruculose, up to 800  $\mu\text{m}$  tall, 7–9  $\mu\text{m}$  wide at the base; conidiogenous region consisting of a series of branches composed of true branchlets, conidiogenous cells and ramoconidia, branched portion usually rather compact, even metula-like, but also looser, with divergent branches; primary branches (–2), subcylindrical, 1–7-septate, 50–120  $\times$  4–6  $\mu\text{m}$ ; secondary branches (–2), subcylindrical, 1–5-septate, 40–60  $\times$  4–6  $\mu\text{m}$ ; tertiary and additional branches 1–4-septate, 10–30  $\times$  3–5  $\mu\text{m}$ . *Conidiogenous cells* terminal, intercalary or lateral, unbranched, 5–15  $\times$  3–5  $\mu\text{m}$ , medium brown, finely verruculose, tapering to a flattened or rounded (frequently swollen) apical region, scars thickened, but only somewhat darkened, not refractive. *Ramoconidia* 0–1-septate,

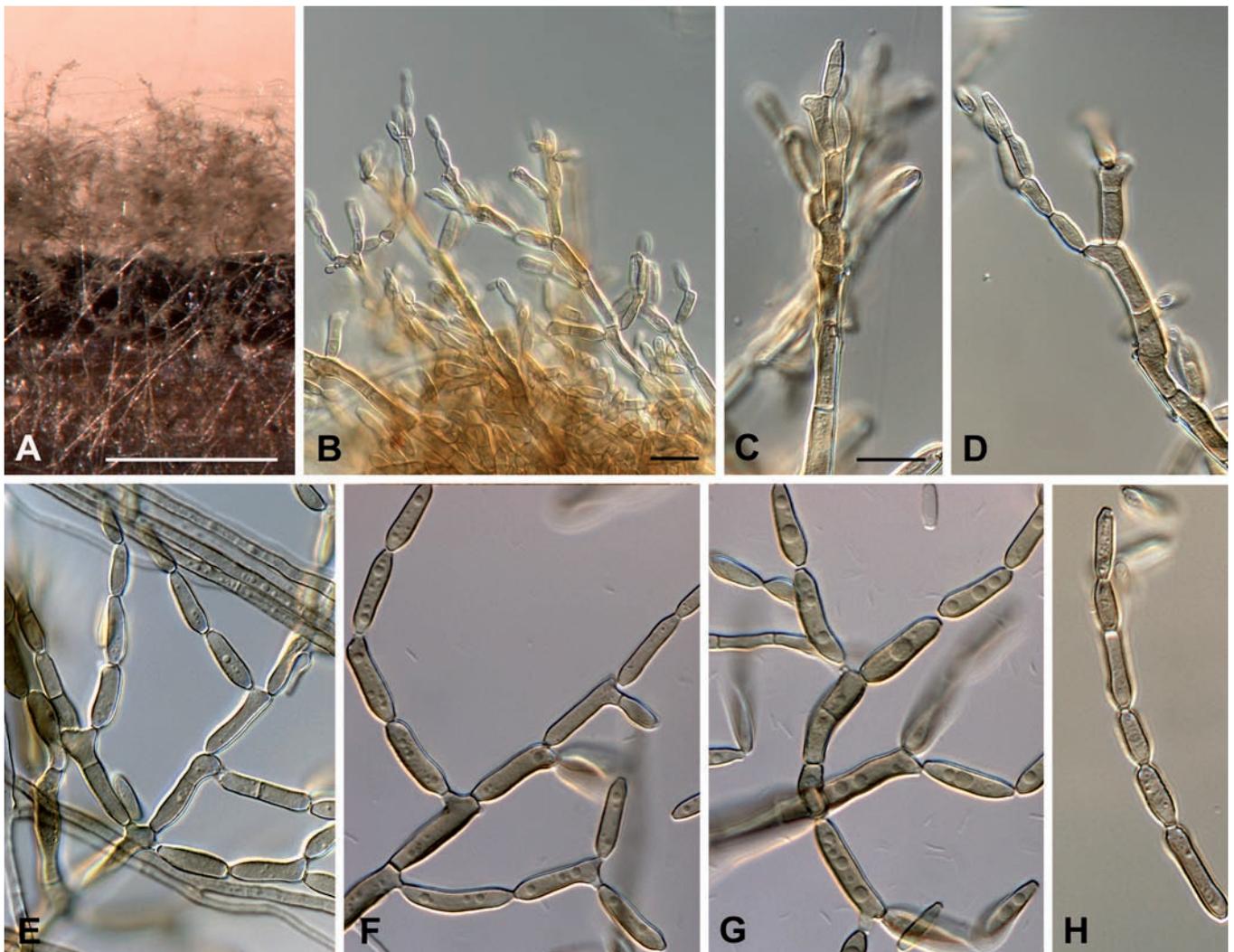


Fig. 8. *Penidiella columbiana* (type material). A. Conidiophores on pine needle *in vitro*. B–H. Conidiophores with chains of disarticulating conidia. Scale bars: A = 450, B–C = 10  $\mu$ m.

medium brown, smooth, wall  $\leq 1 \mu$ m wide, ellipsoidal to obclavate or obovoid, with 1–3 apical hila, 10–25  $\times$  3–5  $\mu$ m, ramoconidia with broadly truncate base, not or barely attenuated, up to 4  $\mu$ m wide, or at least somewhat attenuated at the base, hila 1.5–3  $\mu$ m wide. *Conidia* subcylindrical to ellipsoid, 0(–1)-septate, pale brown, in chains of up to 10, 7–15  $\times$  2–3  $\mu$ m, hila truncate, unthickened, barely to somewhat darkened, 1–2  $\mu$ m wide.

**Cultural characteristics:** Colonies on PDA erumpent, spreading, with moderate aerial mycelium and smooth, even, submerged margins; olivaceous-grey in central part, iron-grey in outer region (surface); colonies fertile.

**Specimen examined:** Colombia, Páramo de Guasca, 3400 m alt., isolated from dead leaf of *Paepalanthus columbianus* (*Eriocaulaceae*), Aug. 1980, W. Gams, holotype CBS H-19937, culture ex-type CBS 486.80.

**Notes:** This isolate was originally identified as belonging to the *Stenella araguata* species complex. The latter name has been somewhat confused in the literature, and has been incorrectly applied to isolates associated with opportunistic human infections (de Hoog *et al.* 2000). The “*araguata*” species complex is treated elsewhere in the volume (see Crous *et al.* 2007a – this volume).

***Penidiella cubensis*** (R.F. Castañeda) U. Braun, Crous & R.F. Castañeda, **comb. nov.** MycoBank MB504511. Fig. 10.

**Basionym:** *Cladosporium cubense* R.F. Castañeda, *Fungi Cubenses II (La Habana)*: 4. 1987.

***In vivo:*** Colonies on fallen leaves, amphigenous, effuse, pilose, brown. ***Mycelium*** usually external, superficial, but also internal, composed of branched, septate, brown, thin-walled, smooth to rough-walled hyphae, 2–3  $\mu$ m wide. ***Stromata*** present, 40–80  $\mu$ m diam, brown, immersed. ***Conidiophores*** densely fasciculate or in distinct synnemata, arising from stromata, erect, synnemata up to about 1000  $\mu$ m long and (10–)20–40(–50)  $\mu$ m wide, individual threads filiform, pluriseptate throughout, brown, thin-walled ( $\leq 0.5 \mu$ m), smooth or almost so to distinctly verruculose, apically penicillate. ***Conidiogenous cells*** integrated, terminal and intercalary, 10–30  $\mu$ m long, subcylindrical, terminal conidiogenous cells often slightly enlarged at the tip, with (1–)2–3(–4) terminal or subterminal subdenticulate conidiogenous loci, short conically truncate, 1–2  $\mu$ m diam, unthickened or almost so, but often slightly refractive or darkened-refractive, intercalary conidiogenous cells usually with a single lateral locus just below the upper septum, conidiogenous cells giving rise to a single set of primary ramoconidia, or a sequence of ramoconidia at different levels. ***Ramoconidia*** cylindrical to ellipsoid-fusoid, 8–18(–25)  $\times$  2–3  $\mu$ m, aseptate, pale olivaceous, olivaceous-brown to brown, thin-walled, smooth or almost so to

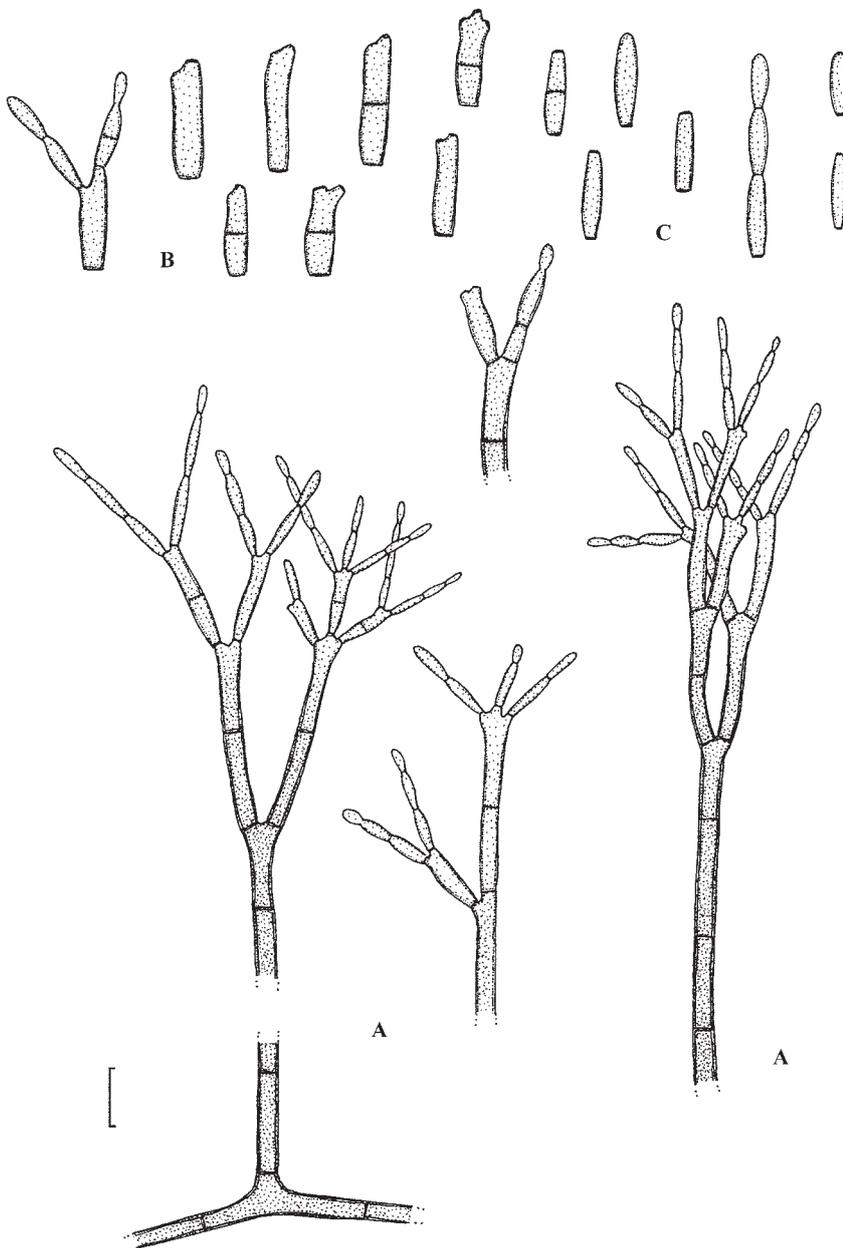


Fig. 9. *Penidiella columbiana* (type material). A. Conidiophores. B. Ramoconidia. C. Secondary conidia. Scale bar = 10  $\mu$ m. U. Braun del.

faintly verruculose, ramoconidia with broadly truncate base, barely narrowed, or ramoconidia more or less attenuated at the base, hila 1–2  $\mu$ m wide, unthickened or almost so, but often slightly refractive or darkened-refractive. *Conidia* in long acropetal chains, narrowly ellipsoid-ovoid, fusiform, 5–12(–15)  $\times$  (1–)1.5–3  $\mu$ m, aseptate, pale olivaceous to brownish, thin-walled, smooth to faintly rough-walled, ends attenuated, hila 1–1.5  $\mu$ m wide, unthickened, not darkened, at most somewhat refractive.

*Specimen examined*: Cuba, Guantánamo, Maisí, on fallen leaves of *Ficus* sp., 24 Apr. 1986, M. Camino, holotype INIFAT C86/134 (HAL 2019 F, ex holotype).

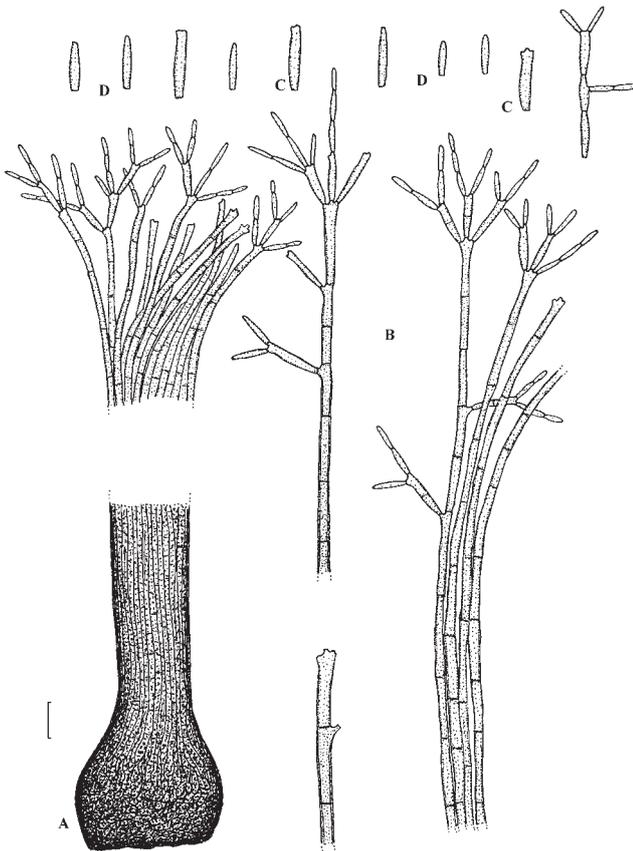
*Notes*: *Cladosporium cubense* was not available in culture and molecular sequence data are not available, but type material could be re-examined and revealed that this species is quite distinct from *Cladosporium* s. str., but agreeing with the concept of the genus *Penidiella*. *Penidiella cubensis* differs from all other species of this genus in having densely fasciculate conidiophores to synnematus conidiomata, arising from stromata.

***Penidiella nectandrae*** Crous, U. Braun & R.F. Castañeda, nom. nov. MycoBank MB504512. Fig. 11.

*Basionym*: *Cladosporium ferrugineum* R.F. Castañeda, *Fungi*

*Cubenses* II (*La Habana*): 4. 1987, homonym, non *C. ferrugineum* Allesch., 1895.

*In vivo*: Colonies amphigenous, brown. *Mycelium* internal and external, superficial, composed of sparingly branched hyphae, septate, 1–3  $\mu$ m wide, pale olivaceous-brown or brown, thin-walled ( $\leq$  0.5  $\mu$ m), smooth or almost so to distinctly verruculose, fertile cells giving rise to conidiophores somewhat swollen at the branching point, up to 5  $\mu$ m diam, and somewhat darker. *Stromata* lacking. *Conidiophores* erect, straight, filiform, up to 350  $\mu$ m long, 2.5–4  $\mu$ m wide, pluriseptate throughout, brown, darker below and paler above, thin-walled, smooth, apex penicillate, terminal cell of the conidiophore with 2–4 short denticle-like loci giving rise to sets of conidiogenous cells or ramoconidia that then form a sequence of new sets of ramoconidia on different levels, i.e., the loose to dense, metula-like branching system is composed of conidiogenous cells and ramoconidia which are often barely distinguishable and turn into each other; *conidiogenous loci* terminal or subterminal, usually 1–3(–4), subdenticulate, 1–2  $\mu$ m diam, conical, apically truncate, unthickened or almost so, not to somewhat darkened-refractive. *Ramoconidia* with truncate base, barely attenuated, or ramoconidia distinctly attenuated at the truncate base, up to 20  $\times$  2  $\mu$ m, aseptate, at the apex with 2–3(–4) subdenticulate hila, subcylindrical,



**Fig. 10.** *Penidiella cubensis* (type material). A. Swollen stromatic base of synnema. B. Conidiophores. C. Ramoconidia. D. Secondary conidia. Scale bar = 10 µm. U. Braun del.

very pale olivaceous, olivaceous-brown to brown, sometimes with different shades of brown (heterochromatous), thin-walled ( $\leq 0.5 \mu\text{m}$ ), smooth to faintly verruculose. *Conidia* in long acropetal chains, narrowly ellipsoid-ovoid, fusiform to cylindrical,  $5\text{--}16 \times (1.5\text{--})2\text{--}(2.5) \mu\text{m}$ , aseptate, very pale olivaceous, olivaceous-brown to brown, thin-walled, smooth to very faintly rough-walled, primary conidia with rounded apex and truncate base, somewhat attenuated, secondary conidia truncate at both ends, hila  $1\text{--}1.5 \mu\text{m}$  diam, unthickened or almost so, at most slightly darkened-refractive.

**Cultural characteristics:** Colonies on PDA slimy, smooth, spreading; aerial mycelium absent, margins smooth, irregular; surface black with patches of cream. Colonies reaching 20 mm diam after 1 mo at  $25^\circ\text{C}$  in the dark; colonies sterile on PDA, SNA and OA.

**Specimen examined:** Cuba, Matanzas, San Miguel de los Baños, isolated from living leaves of *Nectandra coriacea* (Lauraceae), 24 Jan. 1987, R.F. Castañeda and G. Arnold, **holotype** INIFAT C87/45, culture ex-type CBS 734.87, and HAL 2018 F (ex-holotype).

**Notes:** Although the ex-type strain of *Cladosporium ferrugineum* is sterile, its LSU DNA phylogeny reveals it to be unrelated to *Cladosporium* s. str. (see Fig. 1 in Crous *et al.* 2007a – this volume). Based on a re-examination of the type material it could clearly be shown that the morphology of this species fully agrees with the concept of the new genus *Penidiella*, which is supported by its phylogenetic position within *Capnodiales*.

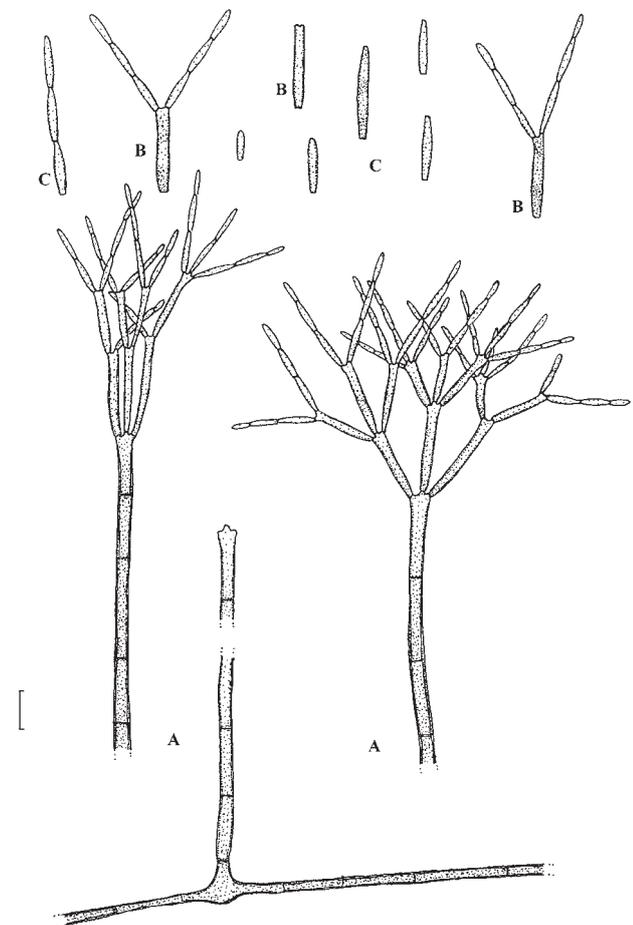
***Penidiella rigidophora*** Crous, R.F. Castañeda & U. Braun, **sp. nov.** MycoBank MB504513. Figs 12–13.

= *Cladosporium rigidophorum* R.F. Castañeda, nom. nud. (herbarium name).

Differt a specibus *Penidiellae* conidiophoris dimorphosis, hyphis et conidiis ad septa saepe distincte constrictis.

*Mycelium* consisting of strongly branched, septate, smooth or almost so, pale olivaceous to medium brown, guttulate, commonly constricted at septa,  $2\text{--}6 \mu\text{m}$  wide hyphae, swollen cells up to  $8 \mu\text{m}$  wide, wall up to  $1\text{--}(1.5) \mu\text{m}$  wide. *Conidiophores* dimorphic. *Macronematous conidiophores* separate, erect, subcylindrical, predominantly straight to slightly curved, terminally loosely penicillate, up to  $120 \mu\text{m}$  long, and  $4\text{--}5 \mu\text{m}$  wide at the base, which is neither lobed nor swollen, and lacks rhizoids, up to 10-septate, medium to dark brown, wall up to  $1\text{--}(1.5) \mu\text{m}$  wide. *Micronematous conidiophores* erect, subcylindrical, up to  $40 \mu\text{m}$  tall,  $3\text{--}4 \mu\text{m}$  wide, 1–3-septate, pale to medium brown (concolorous with hyphae). *Conidiogenous cells* predominantly terminal, rarely intercalary, medium brown, smooth, subcylindrical, but frequently swollen at apex,  $10\text{--}20 \times 5\text{--}6 \mu\text{m}$ , loci (predominantly single in micronematous conidiophores, but up to 4 in macronematous conidiophores) flat-tipped, sub-denticulate or not,  $1\text{--}1.5 \mu\text{m}$  wide, barely to slightly darkened and thickened-refractive. *Conidia* in branched chains, medium brown, verruculose, (appearing like small spines under light microscope), ellipsoid to cylindrical-oblong, up to  $1\text{--}(1.5) \mu\text{m}$  wide, frequently constricted at septa, which turn dark with age; ramoconidia  $(10\text{--})13\text{--}17\text{--}(25) \times 3\text{--}4\text{--}(5) \mu\text{m}$ , 1(–3)-septate; secondary conidia  $(7\text{--})8\text{--}10\text{--}(12) \times 3\text{--}4\text{--}(5)$ ; hila unthickened to very slightly thickened and darkened, not refractive,  $(0.5\text{--})1\text{--}(1.5) \mu\text{m}$ .

**Cultural characteristics:** Colonies on PDA erumpent, spreading, with lobate margins and moderate aerial mycelium; iron-grey (surface), with a greenish black margin; reverse greenish black. Colonies reaching 20 mm diam after 1 mo at  $25^\circ\text{C}$  in the dark; colonies fertile.



**Fig. 11.** *Penidiella nectandrae* (type material). A. Conidiophores. B. Ramoconidia. C. Secondary conidia. Scale bar = 10 µm. U. Braun del.

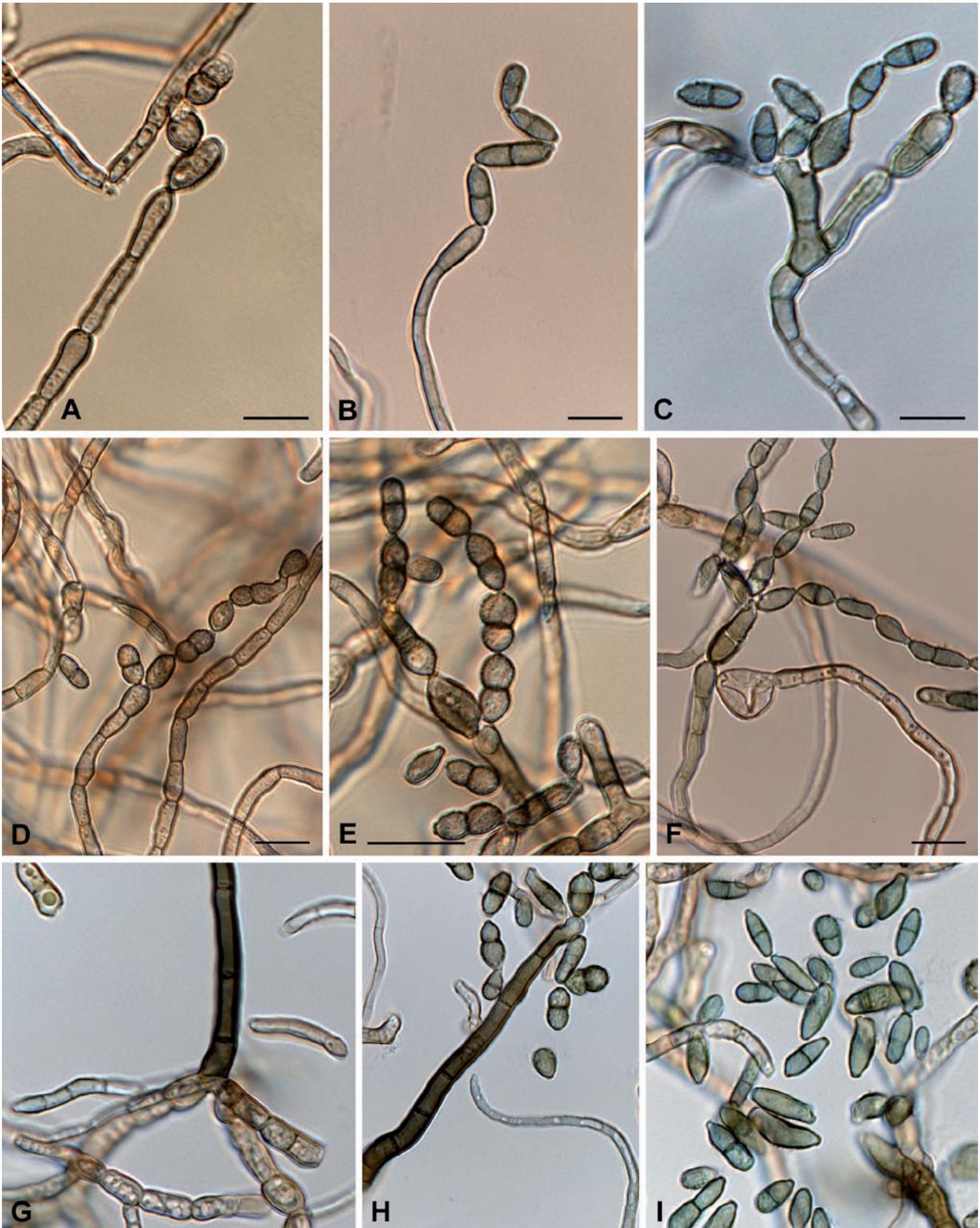


Fig. 12. *Penidiella rigidophora* (type material). A–F. Micronematous conidiophores giving rise to chains of conidia. G–H. Macronematous conidiophores (note base in G, and apex in H). I. Conidia. Scale bars = 10 µm.

Specimen examined: Cuba, isolated from leaf litter of *Smilax* sp. (*Smilacaceae*), 6. Nov. 1994, R.F. Castañeda, holotype CBS H-19938, culture ex-type CBS 314.95.

Notes: *Cladosporium rigidophorum* is a herbarium name, which was never validly published. The ex-type strain, however, represents a new species of *Penidiella*, for which a valid name with Latin diagnosis is herewith provided. This species is easily distinguishable from all

other taxa of *Penidiella* by forming distinct constrictions at hyphal and conidial septa as well as micronematous conidiophores (except for *P. venezuelensis* in which a few micronematous conidiophores have been observed). It is also phylogenetically distinct from the other taxa of *Penidiella* (see Fig. 1 in Crous et al. 2007a – this volume).

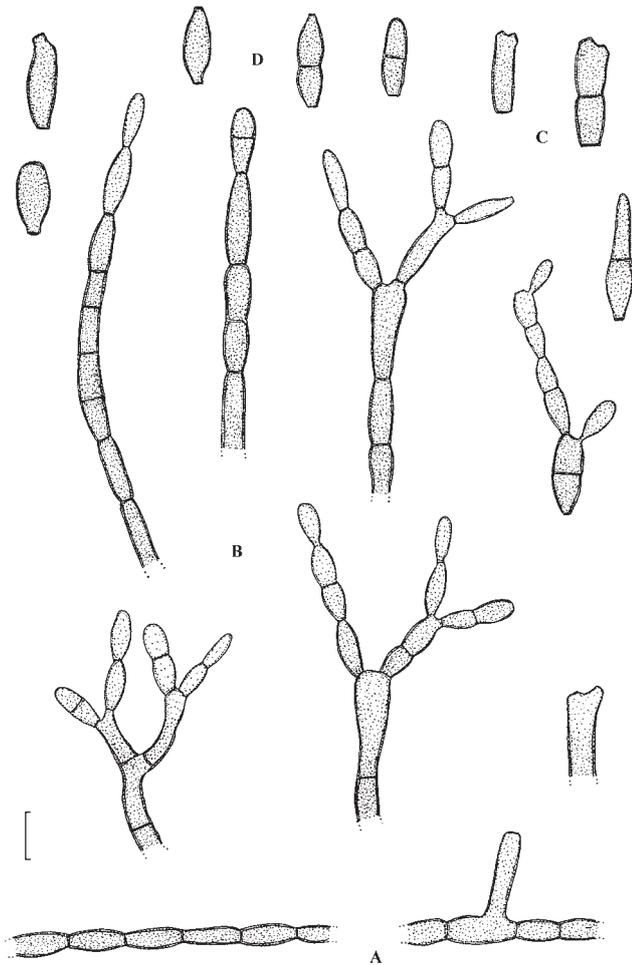


Fig. 13. *Penidiella rigidophora* (type material). A. Hyphae. B. Conidiophores. C. Ramoconidia. D. Secondary conidia. Scale bar = 10  $\mu$ m. U. Braun del.

*Penidiella strumelloidea* (Milko & Dunaev) Crous & U. Braun, **comb. nov.** MycoBank MB504514. Figs 14–15.

*Basionym:* *Cladosporium strumelloideum* Milko & Dunaev, Novosti Sist. Nizsh. Rast. 23: 134. 1986.

*Mycelium* consisting of branched, septate, smooth, hyaline to pale olivaceous, 1–4  $\mu$ m wide hyphae, sometimes constricted at somewhat darker septa. *Conidiophores* solitary, erect, arising from superficial mycelium, micronematous, i.e., reduced to conidiogenous cells, or macronematous, subcylindrical, straight to slightly curved, subcylindrical throughout or often somewhat attenuated towards the apex, 12–80  $\times$  (2–)2.5–4  $\mu$ m, 0–6-septate, medium brown, smooth, wall  $\leq$  0.75  $\mu$ m, penicillate apex formed by a terminal conidiogenous cell giving rise to a single set of ramoconidia. *Conidiogenous cells* terminal, integrated, subcylindrical, straight, 8–12  $\times$  1.5–2(–2.5)  $\mu$ m, pale brown, thin-walled, smooth, apex obtusely rounded to somewhat clavate; loci terminal, occasionally subterminal or lateral, unthickened or almost so to slightly thickened and darkened, not refractive, 1–1.5  $\mu$ m wide. *Conidia* in branched chains; ramoconidia subcylindrical, with 1–3 terminal loci, olivaceous-brown, smooth; secondary conidia ellipsoidal, with one side frequently straight and the other convex, straight to slightly curved, (8–)10–12(–20)  $\times$  2(–3)  $\mu$ m, subhyaline to olivaceous-brown, smooth, thin-walled; hila unthickened or almost so to somewhat thickened and darkened, not refractive, 1  $\mu$ m wide.

*Cultural characteristics:* Colonies on PDA erumpent, spreading, with abundant, dense to woolly aerial mycelium, and uneven, feathery margins; surface pale olivaceous grey, reverse iron-grey. Colonies reaching 25 mm diam after 1 mo at 25  $^{\circ}$ C in the dark; colonies fertile.

*Specimen examined:* **Russia**, Yaroslavl Region, Rybinsk Reservoir, mouth of Sutka River, isolated from leaf of *Carex* sp. (*Cyperaceae*), from stagnant water, S. Ozerskaya, **holotype** BKMf-2534, culture ex-type CBS 114484.

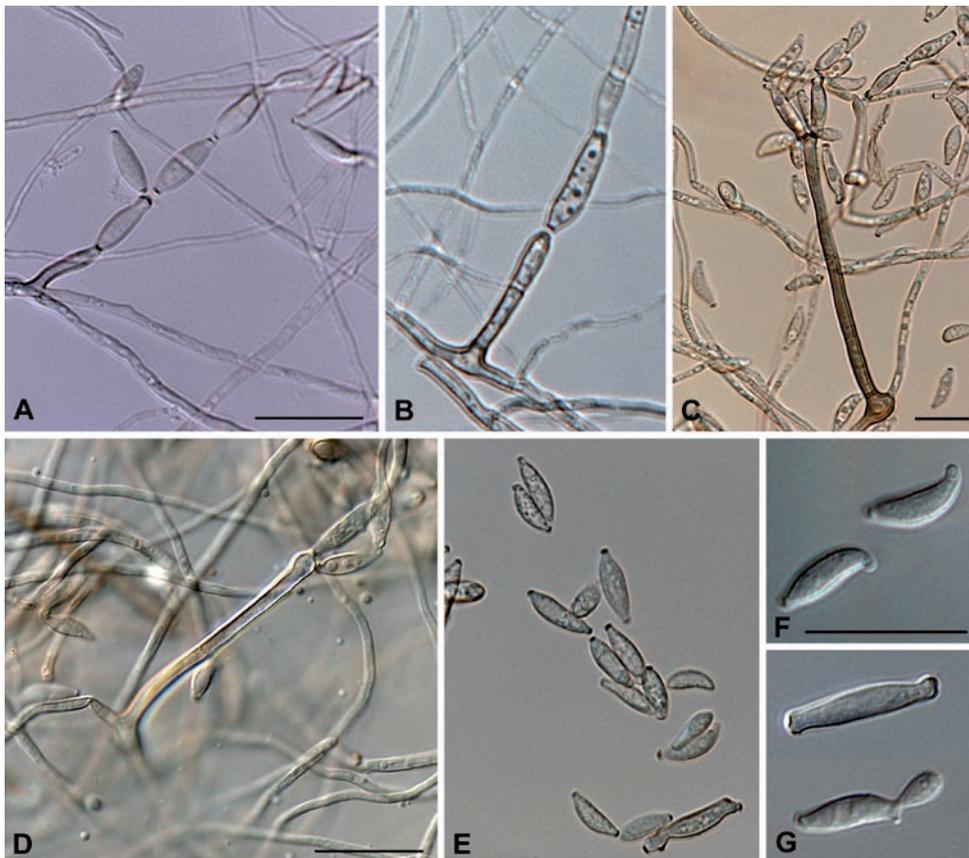


Fig. 14. *Penidiella strumelloidea* (type material). A–B. Micronematous conidiophores. C–D. Macronematous conidiophores. E–G. Conidia. Scale bars = 10  $\mu$ m.

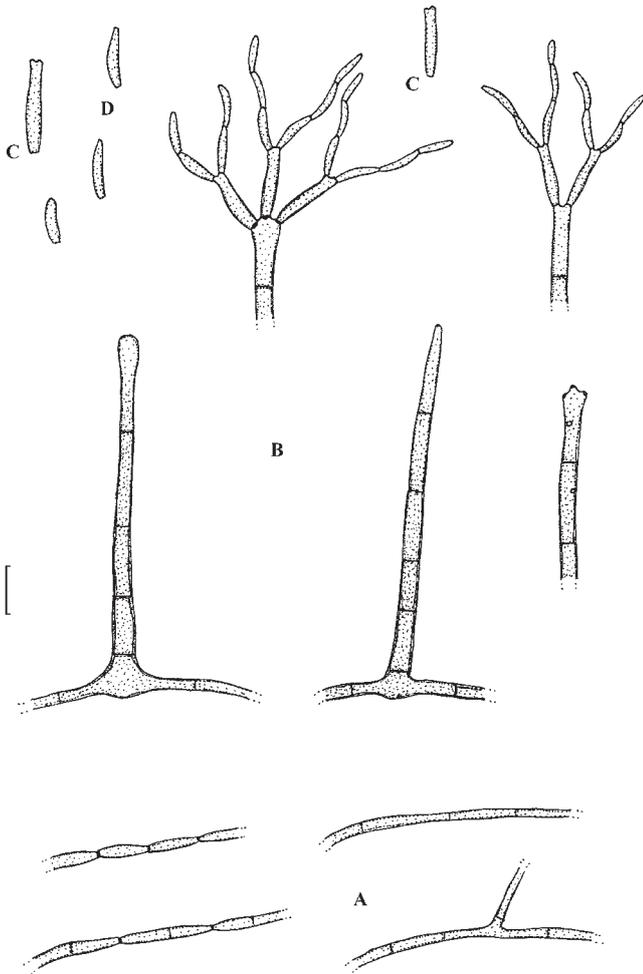


Fig. 15. *Penidiella strumelloidea* (type material). A. Hyphae. B. Conidiophores. C. Ramoconidia. D. Secondary conidia. Scale bars = 10 µm. U. Braun del.

**Notes:** *Penidiella strumelloidea* resembles other species of *Penidiella* by having penicillate conidiophores with a conidiogenous apparatus giving rise to branched conidial chains. It differs, however, from all other species of this genus in having a rather simple penicillate apex composed of a single terminal conidiogenous cell giving rise to one set of ramoconidia which form frequently somewhat curved conidia. It is also phylogenetically distinct from the other taxa of *Penidiella* (see Fig. 1 in Crous *et al.* 2007a – this volume).

***Penidiella venezuelensis*** Crous & U. Braun, **sp. nov.** MycoBank MB504515. Figs 16–17.

**Etymology:** Named after the geographic location of its type strain, Venezuela.

Differt a *P. columbiana* conidiophoris beavioribus et angustioribus, ad 120 × 3–4 µm, subnodulosis, apice plus minusve laxe penicillatis et conidiis brevioribus, (4–)5–7(–8) µm longis.

**Mycelium** consisting of branched, septate, smooth to faintly rough-walled, thin-walled, subhyaline, pale olivaceous to medium brown, (1.5–)2–3 µm wide hyphae. **Conidiophores** solitary, erect, macronematous, subcylindrical, straight to flexuous to once geniculate, up to 120 µm long, 3–4 µm wide, 1–12-septate, pale to medium olivaceous-brown or brown, thin-walled (up to about 1 µm), terminally penicillate, branched portion composed of true branchlets and/or a single set or several sets of ramoconidia, branchlets up to 50 µm long; occasionally with a few additional micronematous

conidiophores, about 10–15 × 2–3 µm. **Conidiogenous cells** terminal and intercalary, unbranched, subcylindrical, 5–12 × 3–4 µm, medium brown, smooth or almost so to finely verruculose, apex of conidiogenous cells frequently swollen, up to 6 µm diam, with 1–3(–4) flat-tipped, non to slightly thickened, non to slightly darkened-refractive loci, 1–1.5 µm wide, frequently appearing subdentate, up to 1.5 µm long, intercalary conidiogenous cells also slightly swollen at the conidiogenous portion just below the upper septum, which render the conidiophores subnodulose to nodulose, swellings round about the conidiophore axis or unilateral. **Conidia** ellipsoid-ovoid, subcylindrical, pale to medium olivaceous-brown or brown, finely verruculose, wall ≤ 0.5 µm wide, guttulate or not, occurring in branched chains. **Ramoconidia** 0–1(–3)-septate, 5–15(–22) × 3–4(–5) µm, with 1–3 subdentate apical hila; secondary conidia 0(–1)-septate, ellipsoid, obovoid to irregular, (4–)5–7(–8) × (2–)2.5–3(–4) µm; hila non to slightly thickened, non to slightly darkened-refractive, (0.5–)1(–1.5) µm wide.

**Cultural characteristics:** Colonies on OA erumpent, spreading, with dense, compact aerial mycelium, and even, smooth margins; olivaceous-grey (surface), margins iron-grey. Colonies reaching 22 mm diam after 1 mo at 25 °C in the dark.

**Specimen examined:** Venezuela, isolated from man with *tinea nigra*, Jan. 1975, D. Borelli, **holotype** CBS H-19934, culture ex-type CBS 106.75.

**Notes:** The type culture of *Penidiella venezuelensis* was originally determined as *Stenella araguata* from which it is, however, quite distinct by having smooth mycelium, long penicillate conidiophores with subdentate conidiogenous loci, smaller conidia, and agreeing with the concept of the genus *Penidiella*. It is phylogenetically distinct from the other taxa of *Penidiella* (see Fig. 1 in Crous *et al.* 2007a – this volume).

***Pseudotaeniolina*** J.L. Crane & Schokn., *Mycologia* 78: 88. 1986.  
? = *Friedmanniomyces* Onofri, *Nova Hedwigia* 68: 176. 1999.

**Type species:** *Pseudotaeniolina convolvuli* (Esfand.) J.L. Crane & Schokn., *Mycologia* 78: 88. 1986.

**Description:** Crane & Schoknecht (1986, figs 3–19).

**Notes:** No cultures or sequence data are available of the type species, and *Pseudotaeniolina globosa* De Leo, Urzi & de Hoog was placed in *Pseudotaeniolina* based on its morphology and ecology. The genus *Friedmanniomyces* is presently known from two species (Selbmann *et al.* 2005). Morphologically *Friedmanniomyces* is similar to *Pseudotaeniolina*, but fresh material of *Pseudotaeniolina convolvuli* needs to be recollected before this can be clarified.

***Readeriella*** Syd. & P. Syd., *Ann. Mycol.* 6: 484. 1908.

= *Kirramyces* J. Walker, B. Sutton & Pascoe, *Mycol. Res.* 96: 919. 1992.

= *Colletogloeopsis* Crous & M.J. Wingf., *Canad. J. Bot.* 75: 668. 1997.

**Synanamorphs:** ***Cibiessia*** Crous, *Fungal Diversity* 26: 151. 2007; also pseudocercospora-like, see Crous (1998).

**Type species:** *Readeriella mirabilis* Syd. & P. Syd., *Ann. Mycol.* 6: 484. 1908.

**Description:** Crous *et al.* (2004b; figs 36–38).

**Notes:** Several coelomycete genera are presently available to accommodate anamorphs of *Capnodiales* that reside in *Teratosphaeriaceae*, for which *Readeriella* is the oldest name. Other genera such as *Phaeophleospora* Rangel, *Sonderhenia* H.J. Swart & J. Walker and *Lecanosticta* Syd. belong to *Mycosphaerellaceae*.

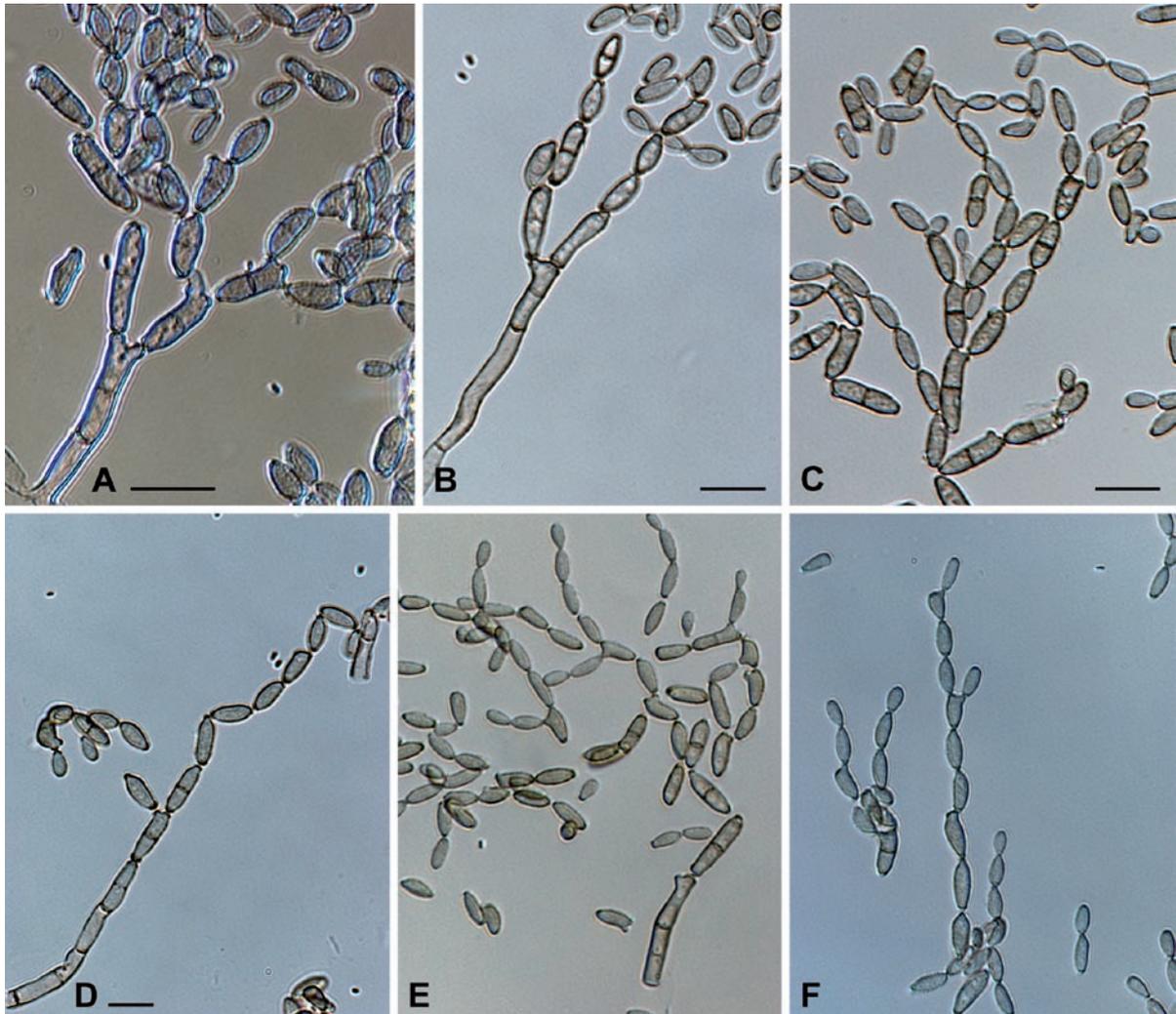
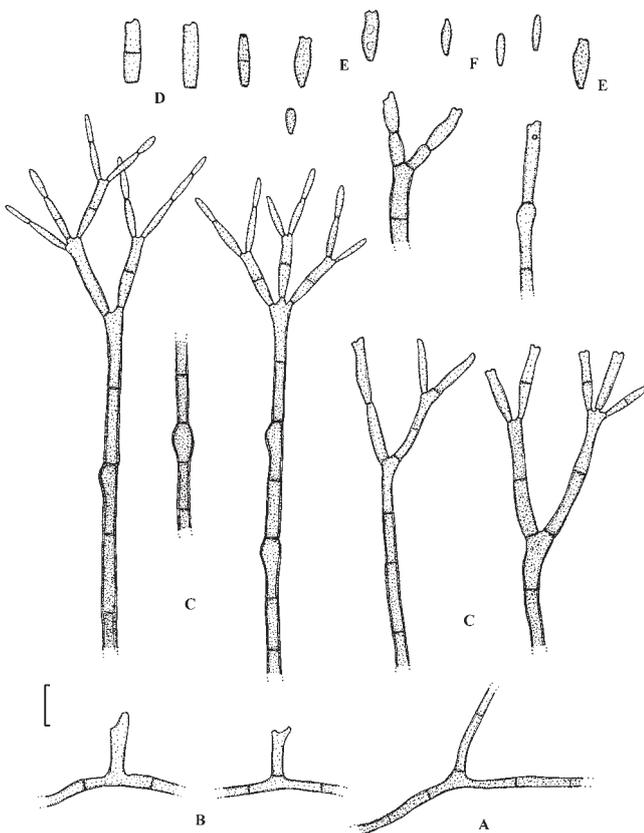


Fig. 16. *Penidiella venezuelensis* (type material). A. Microconidiophore. B. Apical part of macroconidiophore. C–F. Chains of conidia. Scale bars = 10  $\mu$ m.



*Readeriella* is polyphyletic within *Teratosphaeriaceae*. The recognition and circumscription (synonymy) of this genus follows the principles for anamorph genera within *Capnodiales* as outlined in the introduction to this volume. The only unifying character is conidial pigmentation, and the mode of conidiogenesis. Conidiogenous cells range from mono- to polyphialides with periclinal thickening, to phialides with percurrent proliferation, as observed in the type species, *R. mirabilis* (Fig. 18). Within the form genus conidia vary from aseptate to multiseptate, smooth to rough, and have a range of synanamorphs. *Readeriella mirabilis* has a synanamorph with cylindrical, aseptate conidia, while other species of *Readeriella* again have *Cibiessia* synanamorphs (scytalidium-like, with chains of dry, disarticulating conidia), suggesting the conidial morphology to be quite plastic. A re-examination of *R. readeriellophora* Crous & Mansilla revealed pycnidia to form a central cushion on which the conidiogenous cells are arranged (Fig. 18). This unique feature is commonly known in genera such as *Coniella* Höhn. and *Pilidiella* Petr. & Syd. (*Diaporthales*) (Van Niekirk *et al.* 2004), and has never been observed among anamorphs of the *Capnodiales*. Another species of *Readeriella*, namely "*Phaeophleospora*" *toledana* Crous & Bills, again forms paraphyses interspersed among conidiogenous cells, a rare feature in this group of fungi, while several species

Fig. 17. *Penidiella venezuelensis* (type material). A. Hypha. B. Micronematous conidiophores. C. Macronematous conidiophores. D–E. Ramoconidia. F. Secondary conidia. Scale bar = 10  $\mu$ m. U. Braun *del.*

have conidiomata ranging from acervuli to pycnidia (Cortinas *et al.* 2006). Phylogenetically this coelomycete morphology, with its characteristic conidiogenesis, has evolved several times in *Teratosphaeriaceae*.

***Readeriella blakelyi*** (Crous & Summerell) Crous & U. Braun, **comb. nov.** MycoBank MB504516.

*Basionym:* *Colletogloeopsis blakelyi* Crous & Summerell, Fungal Diversity 23: 342. 2006.

***Readeriella brunneotagens*** Crous & Summerell, **sp. nov.** MycoBank MB504517. Fig. 19.

*Etymology:* Named after the diffuse brown pigment visible in agar when cultivated on MEA.

*Readeriellae gauchensis* similis, sed coloniis viridi-atris et pigmento brunneo in agaro diffundente distinguenda.

*Leaf spots* amphigenous, irregular specks up to 3 mm diam, medium brown with a thin, raised, concolorous border. *Conidiomata* amphigenous, substomatal, exuding conidia in black masses; conidiomata pycnidial *in vivo* and *in vitro*, globose, brown to black, up to 120 µm diam; wall consisting of 3–4 cell layers of brown cells of *textura angularis*. *Conidiogenous cells* brown, verruculose, aseptate, doliform to ampulliform, or reduced to inconspicuous loci on hyphae (*in vitro*), proliferating percurrently near the apex, 5–7 × 3–5 µm; sympodial proliferation also observed in culture. *Conidia* brown, smooth to finely verruculose, ellipsoidal to subcylindrical, apex obtuse to subobtuse, tapering to a subtruncate or truncate base (1–1.5 µm wide) with inconspicuous, minute marginal frill, (5–)6–7(–8) × 2–3(–3.5) µm *in vitro*, becoming 1-septate; in older cultures becoming swollen, and up to 2-septate, 15 µm long and 5 µm wide.

*Cultural characteristics:* Colonies on MEA reaching 20 mm diam after 2 mo at 25 °C; colonies erumpent, aerial mycelium sparse to absent, margins smooth but irregularly lobate; surface irregularly folded, greenish black, with profuse sporulation, visible as oozing black conidial masses; a diffuse dark-brown pigment is also produced, resulting in inoculated MEA plates appearing dark-brown.

*Specimen examined:* **Australia**, Queensland, Cairns, Eureka Creek, 48 km from Mareeba, S 17° 11' 13.2", E 145° 02' 27.4", 468 m, on leaves of *Eucalyptus tereticornis*, 26 Aug. 2006, P.W. Crous, CBS-H 19838 **holotype**, culture ex-type CPC 13303 = CBS 120747.

*Notes:* Conidial dimensions of *R. brunneotagens* closely match those of *Readeriella gauchensis* (M.-N. Cortinas, Crous & M.J. Wingf.) Crous (Cortinas *et al.* 2006). The two species can be distinguished in culture, however, in that colonies of *R. brunneotagens* are greenish black in colour, sporulate profusely, and exude a diffuse, brown pigment into the agar, whereas colonies of *R. gauchensis* are more greenish olivaceous, and exude a yellow pigment into the agar (Cortinas *et al.* 2006).

***Readeriella considenianae*** (Crous & Summerell) Crous & U. Braun, **comb. nov.** MycoBank MB504518.

*Basionym:* *Colletogloeopsis considenianae* Crous & Summerell, Fungal Diversity 23: 343. 2006.

***Readeriella destructans*** (M.J. Wingf. & Crous) Crous & U. Braun, **comb. nov.** MycoBank MB504519.

*Basionym:* *Kirramyces destructans* M.J. Wingf. & Crous, S. African J. Bot. 62: 325. 1996.

≡ *Phaeophleospora destructans* (M.J. Wingf. & Crous) Crous, F.A. Ferreira & B. Sutton, S. African J. Bot. 63: 113. 1997.

***Readeriella dimorpha*** (Crous & Carnegie) Crous & U. Braun, **comb. nov.** MycoBank MB504520.

*Basionym:* *Colletogloeopsis dimorpha* Crous & Carnegie, Fungal Diversity 23: 345. 2006.

***Readeriella gauchensis*** (M.-N. Cortinas, Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504521.

*Basionym:* *Colletogloeopsis gauchensis* M.-N. Cortinas, Crous & M.J. Wingf., Stud. Mycol. 55: 143. 2006.

***Readeriella pulcherrima*** (Gadgil & M. Dick) Crous & U. Braun, **comb. nov.** MycoBank MB504522.

*Basionym:* *Septoria pulcherrima* Gadgil & M. Dick, New Zealand J. Bot. 21: 49. 1983.

≡ *Stagonospora pulcherrima* (Gadgil & M. Dick) H.J. Swart, Trans. Brit. Mycol. Soc. 90: 285. 1988.

= *Cercospora eucalypti* Cooke & Massee, *Grevillea* 18: 7. 1889.

≡ *Kirramyces eucalypti* (Cooke & Massee) J. Walker, B. Sutton & Pascoe, Mycol. Res. 96: 920. 1992.

≡ *Phaeophleospora eucalypti* (Cooke & Massee) Crous, F.A. Ferreira & B. Sutton, S. African J. Bot. 63: 113. 1997.

*Notes:* The epithet “*eucalypti*” is preoccupied by *Readeriella eucalypti* (Gonz. Frag.) Crous (Summerell *et al.*, 2006), and thus the synonym “*pulcherrima*” becomes the next available name for this species.

*Readeriella readeriellophora*, see *Teratosphaeria readeriellophora*. Fig. 18.

***Readeriella stellenboschiana*** (Crous) Crous & U. Braun, **comb. nov.** MycoBank MB504523.

*Basionym:* *Colletogloeopsis stellenboschiana* Crous, Stud. Mycol. 55: 110. 2006.

***Readeriella zuluensis*** (M.J. Wingf., Crous & T.A. Cout.) Crous & U. Braun, **comb. nov.** MycoBank MB504524.

*Basionym:* *Coniothyrium zuluense* M.J. Wingf., Crous & T.A. Cout., Mycopathologia 136: 142. 1997.

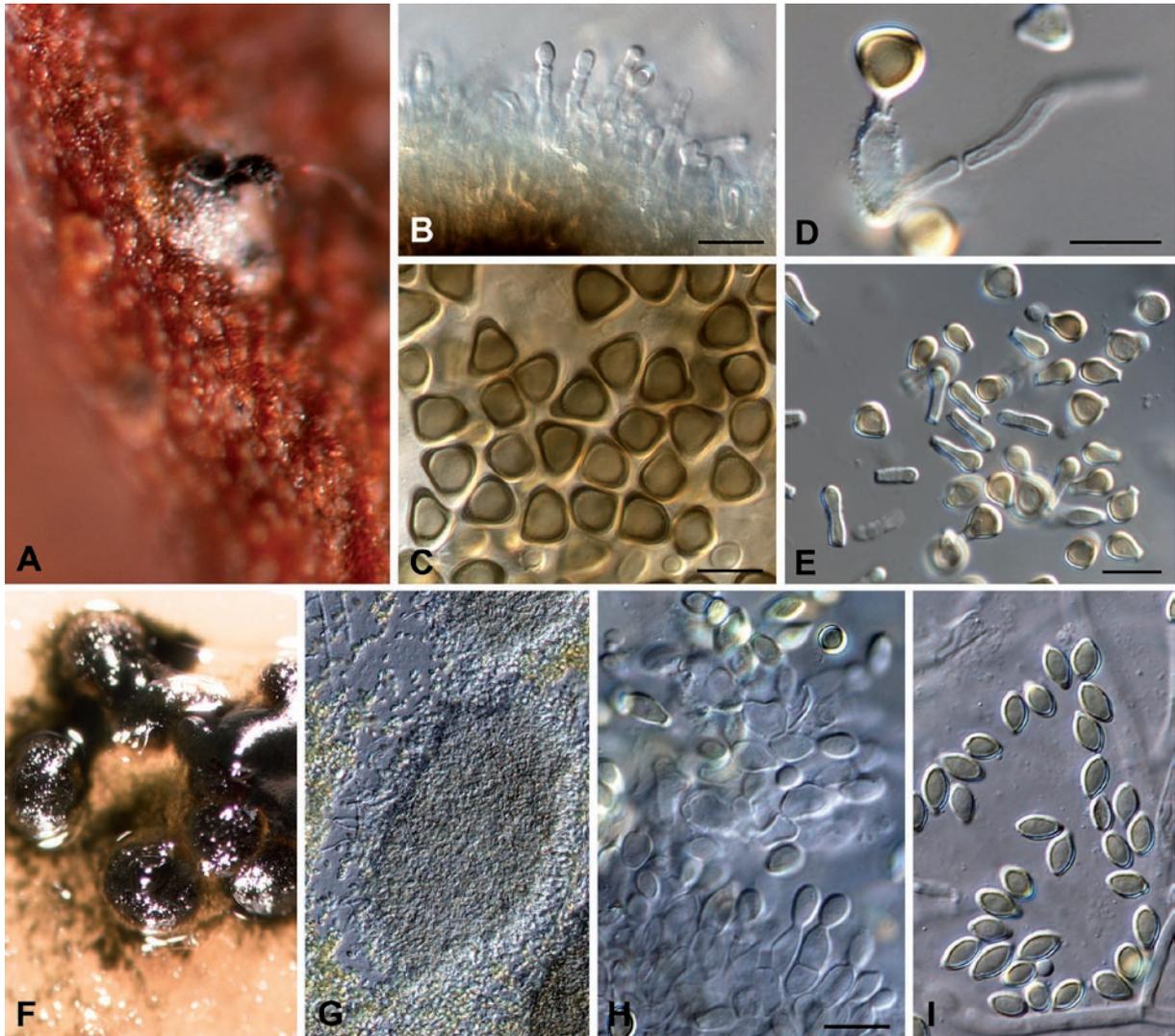
≡ *Colletogloeopsis zuluensis* (M.J. Wingf., Crous & T.A. Cout.) M.-N. Cortinas, M.J. Wingf. & Crous (*zuluense*), Mycol. Res. 110: 235. 2006.

***Staninwardia*** B. Sutton, Trans. Br. Mycol. Soc. 57: 540. 1971.

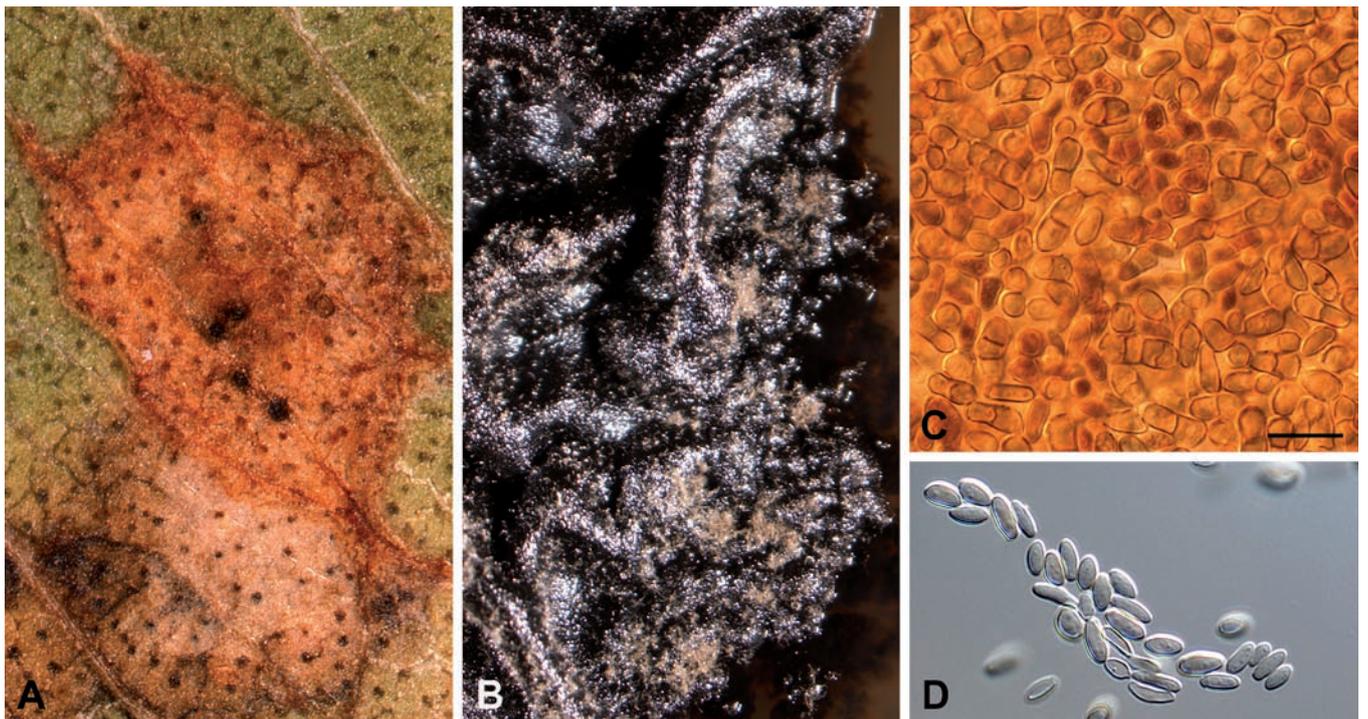
*Type species:* *Staninwardia breviuscula* B. Sutton, Trans. Br. Mycol. Soc. 57: 540. 1971.

*Description:* Sutton (1971; fig. 1).

*Notes:* The genus *Staninwardia* presently contains two species, namely *S. breviuscula* and *Staninwardia suttonii* Crous & Summerell (Summerell *et al.* 2006), though its placement in *Capnodiales* was less well resolved. The genus forms acervuli on brown leaf spots, with brown, catenulate conidia covered in a mucilaginous sheath.



**Fig. 18.** A–E. *Readeriella mirabilis*. A. Conidium with conidial cirrus. B. Conidiogenous cells with percurrent proliferation. C. Macroconidia. D. Slightly pigmented, verruculose conidiogenous cell. E. Macro- and microconidia. F–I. *Readeriella readeriellophora* (type material). F. Colony on OA. G. Central stomatal tissue giving rise to conidiophores. H. Conidiogenous cells. I. Conidia. Scale bars = 10 µm.



**Fig. 19.** *Readeriella brunneotingens* (type material). A. Leaf spot. B. Colony on MEA. C–D. Conidia. Scale bar = 10 µm.

## Schizothyriaceae clade

**Schizothyrium** Desm., Ann. Sci. Nat., Bot., sér. 3: 11. 1849.

*Type species: Schizothyrium acerinum* Desm., Ann. Sci. Nat., Bot., sér. 3: 11. 1849.

*Description:* Batzer *et al.* (2007; figs 3–7).

*Notes:* Species of *Schizothyrium* (*Schizothyriaceae*) have *Zygothiala* E.W. Mason anamorphs, and were recently shown to be allied to *Mycosphaerellaceae* (Batzer *et al.* 2007). Although species of *Schizothyrium* have thyrothecia, they cluster among genera with pseudothecial ascomata, questioning the value of this character at the family level. Based on its bitunicate asci and 1-septate ascospores, the teleomorph is comparable to others in the *Capnodiales*.

## Mycosphaerellaceae clade

### Mycosphaerella subclade

**Mycosphaerella** Johanson, Öfvers. Förh. Kongl. Svenska Vetensk.-Akad. 41(9): 163. 1884.

*Type species: Mycosphaerella punctiformis* (Pers. : Fr.) Starbäck, Bih. Kongl. Svenska Vetensk.-Akad. Handl. 15(3, 2): 9. 1889.

*Anamorph: Ramularia endophylla* Verkley & U. Braun, Mycol. Res. 108: 1276. 2004.

*Description:* Verkley *et al.* (2004; figs 3–16).

*Notes:* The genus *Mycosphaerella* has in the past been linked to 23 anamorph genera (Crous *et al.* 2000), while additional genera have been linked via DNA-based studies, bringing the total to at least 30 genera (Crous & Braun 2003, Crous *et al.* 2007b). However, based on ITS and SSU DNA phylogenetic studies and a reassessment of morphological characters and conidiogenesis, several anamorph genera have recently been reduced to synonymy (Crous & Braun 2003, Crous *et al.* 2006a). Furthermore, the DNA sequence data generated to date clearly illustrate that the anamorph genera in *Mycosphaerella* are polyphyletic, residing in several clades within *Mycosphaerella*. If future collections not known from culture or DNA sequences are to be described in form genera, we recommend that the concepts as explained in Crous & Braun (2003) be used until such stage as they can be placed in *Mycosphaerella*, pending a modification of Art. 59 of the International Code of Botanical Nomenclature. The genus *Mycosphaerella* and its anamorphs represent a future topical issue of the *Studies in Mycology*, and will thus be treated separately.

### Dissoconium subclade

**Dissoconium** de Hoog, Oorschot & Hijwegen, Proc. K. Ned. Akad. Wet., Ser. C, Biol. Med. Sci. 86(2): 198. 1983.

*Type species: Dissoconium aciculare* de Hoog, Oorschot & Hijwegen, Proc. K. Ned. Akad. Wet., Ser. C, Biol. Med. Sci. 86(2): 198. 1983.

? = *Uwebraunia* Crous & M.J. Wingf., Mycologia 88: 446. 1996.

*Teleomorph: Mycosphaerella*-like.

*Description:* de Hoog *et al.* (1983), Crous (1998), Crous *et al.* (2004b; figs 3–10).

*Notes:* The genus *Dissoconium* presently encompasses six species (Crous *et al.* 2007b), of which two, *M. lateralis* Crous & M.J. Wingf. (*D. dekkeri* de Hoog & Hijwegen), and *M. communis* Crous & Mansilla (*D. commune* Crous & Mansilla) are also known from their *Mycosphaerella*-like teleomorphs. No teleomorph genus will be introduced for this clade, however, until more sexual species have been collected to help clarify the morphological features of this genus. A further complication lies in the fact that yet other species, morphologically distinct from *Dissoconium*, also cluster in this clade (Crous, unpubl. data).

### “Passalora” zambiae subclade

**“Passalora” zambiae** Crous & T.A. Cout., Stud. Mycol. 50: 209. 2004.

*Description:* Crous *et al.* (2004b; figs 32–33).

*Notes:* This fungus was placed in the form genus “*Passalora*” based on its smooth mycelium, giving rise to conidiophores forming branched chains of brown conidia with thickened, darkened, refractive hila. Although derived from single ascospores, the teleomorph material was lost, and thus it needs to be recollected before the relevance of its phylogenetic position can be fully understood.

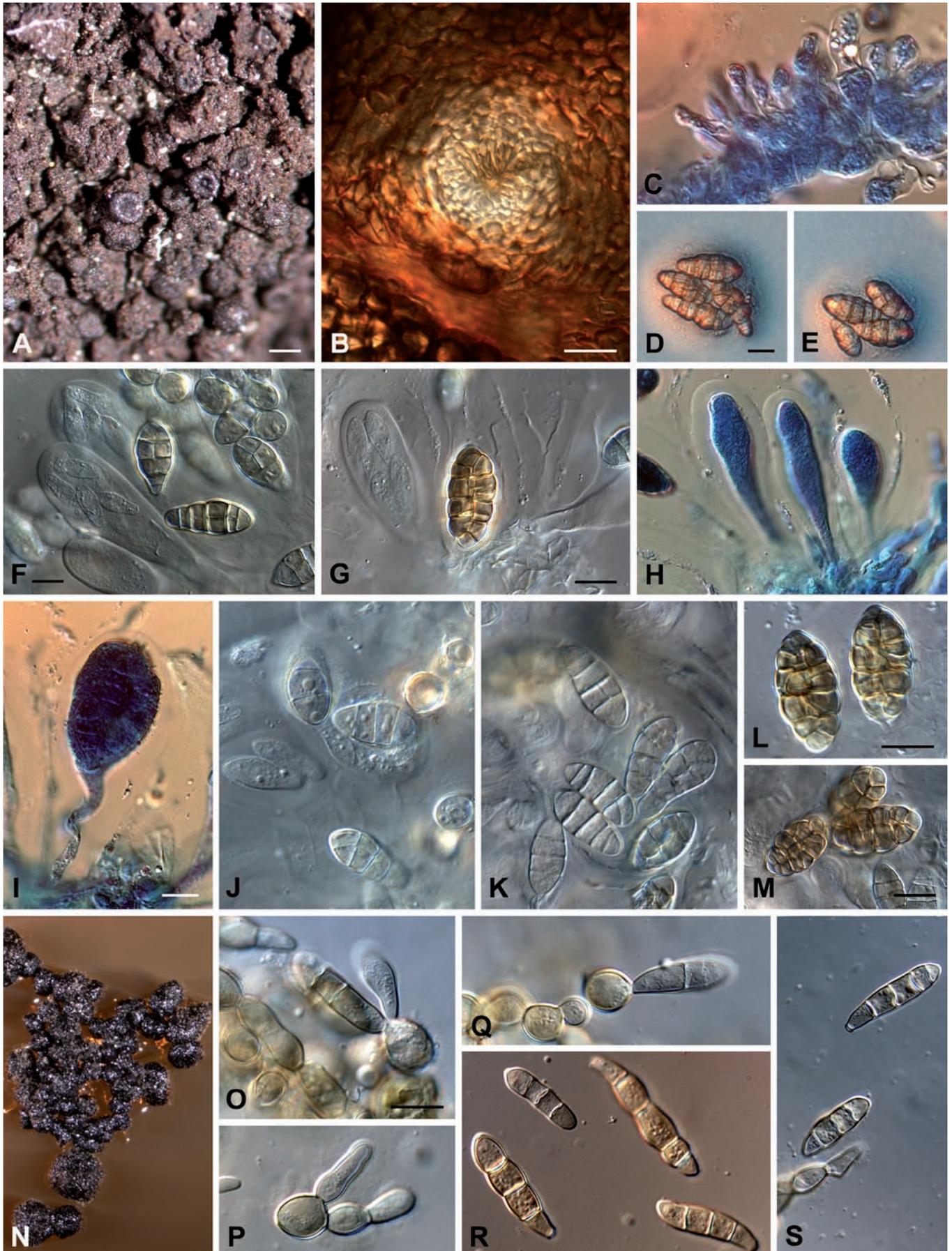
## Additional teleomorph genera considered

**Coccodinium** A. Massal., Atti Inst. Veneto Sci. Lett. Arti, Série 2, 5: 336. 1860. (Fig. 20).

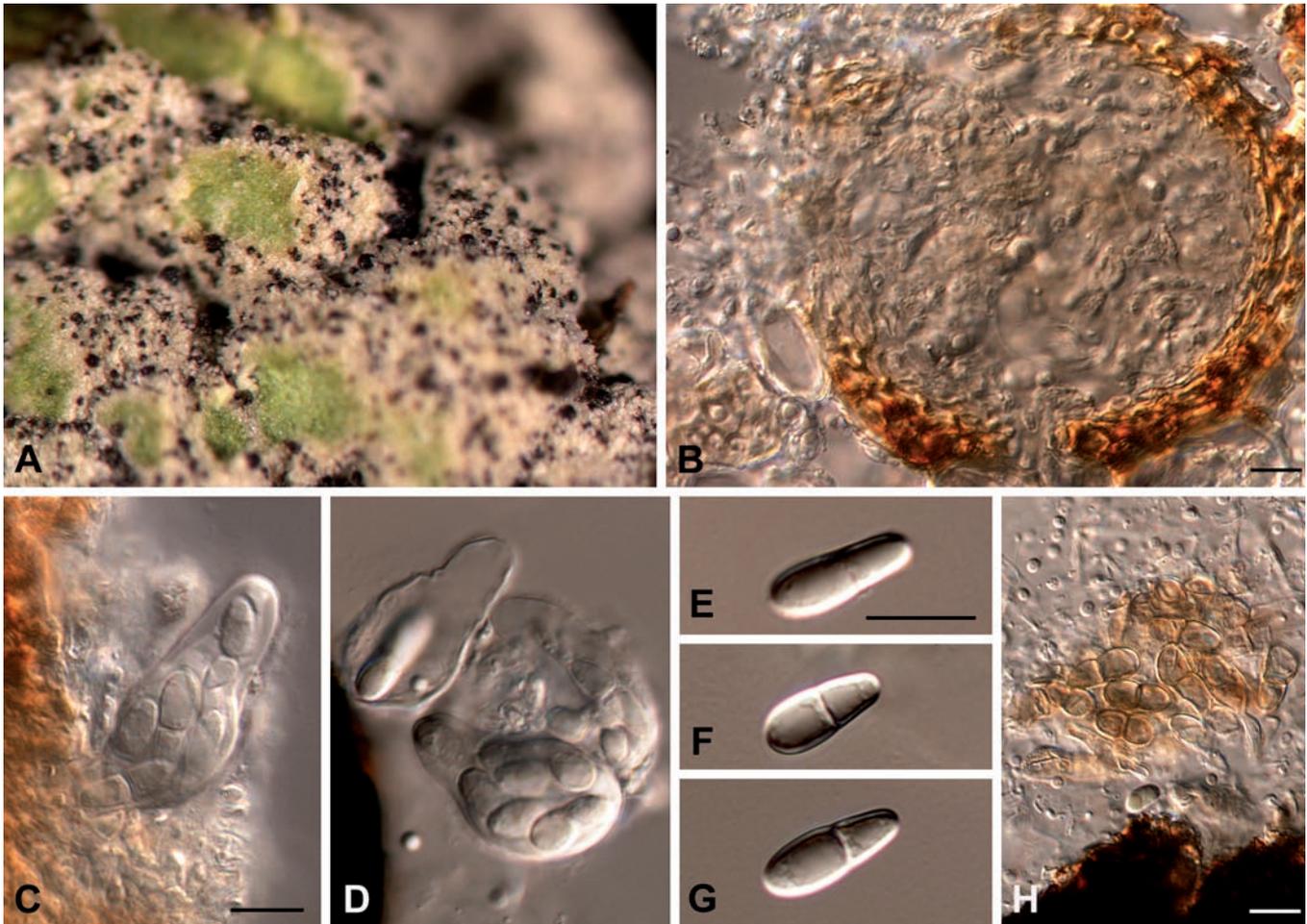
*Type species: Coccodinium bartschii* A. Massal., Atti Inst. Veneto Sci. Lett. Arti, Série 2, 5: 337. 1860.

*Description:* Eriksson (1981, figs 34–35).

*Notes:* The genus *Coccodinium* (*Coccodiniaceae*) is characterised by having ascomata that are sessile on a subiculum, or somewhat immersed, semiglobose, collapsed when dry, brownish, uniloculate, with a centrum that stains blue in IKI (iodine potassium iodide). *Asci* are bitunicate, stalked, 8-spored, saccate, and have a thick, undifferentiated endotunica. *Periphyses* and *periphysoids* are well-developed and numerous. *Ascospores* are elongate, fusiform, ellipsoidal or clavate, transversely septate or muriform, hyaline or brownish (Eriksson 1981), and lack a mucous sheath. Based on a SSU sequence (GenBank accession U77668) derived from a strain identified as *C. bartschii* (Winka *et al.* 1998), *Coccodinium* appears to be allied to the taxa treated here in *Teratosphaeria*. Freshly collected cultures are relatively slow growing, and on MEA they form erumpent round, black colonies with sparse hyphal growth. On the surface of these colonies hyphal strands, consisting of brown, globose cells, give rise to conidia. Older cells (up to 15 µm diam) become fertile, giving rise to 1–3 conidia via inconspicuous phialidic loci. Conidia are fusoid-ellipsoidal to clavate, 3–5-septate, becoming constricted at the transverse septa, apex obtuse, base subtruncate, guttulate, smooth, widest in the upper third of the conidium, 15–40 × 4–7 µm. Phylogenetically *Coccodinium* is thus allied to the *Chaetothyriales* (Fig. 1), and not the *Teratosphaeriaceae*.



**Fig. 20.** *Coccodinium bartschii*. A. Ascomata on host. B. Ostiolar area. C. Periphysoids. D–E. Ascospores shot onto agar. F–I. Asci with thick ectotunica. J–K. Young ascospores. L–M. Mature ascospores. N. Colony on MEA. O–Q. Conidiogenous cells giving rise to conidia. R–S. Conidia. Scale bars: A, N = 250, B, D, F–G, I, L–M, O = 10  $\mu$ m.



**Fig. 21.** *Stigmatidium schaeferi*. A. Lichenicolous habit on *Dacampia hookeri*. B. Vertical section through an ascoma. C–D. Asci. E–G. Ascospores. H. Older, brown ascospores. Scale bars = 10 µm.

***Stigmatidium*** Trevis., Consp. Verruc.: 17. 1860. (Fig. 21).

*Type species:* *Stigmatidium schaeferi* (A. Massal.) Trevis., Consp. Verruc.: 17. 1860.

*Description:* Roux & Triebel (1994, figs 47–50).

*Notes:* The type species of the genus is lichenicolous, characterised by semi-immersed, black, globose ascomata with ostiolar periphyses and periphysoids. *Asci* are 8-spored, fasciculate, bituncate, (endotunica not giving a special reaction in Congo red or toluidine blue). *Ascospores* are fusoid-ellipsoidal, medianly 1-septate, guttulate, thin-walled, lacking a sheath. Presently no culture is available, and thus the placement of *Stigmatidium* remains unresolved.

## DISCUSSION

From the LSU sequence data presented here, it is clear that *Mycosphaerella* is not monophyletic as previously suggested (Crous *et al.* 2001, Goodwin *et al.* 2001). The first step to circumscribe natural genera within this complex was taken by Braun *et al.* (2003), who separated *Cladosporium* anamorphs from this complex, and erected *Davidiella* (*Davidiellaceae*; Schoch *et al.* 2006) to accommodate their teleomorphs. The present study reinstates the genus *Teratosphaeria* for a clade of largely extremotolerant fungi (Selbmann *et al.* 2005) and foliar pathogens of *Myrtaceae* and *Proteaceae* (Crous *et al.* 2004a, b, 2006b, 2007b), and further

separates generic subclades within the *Mycosphaerellaceae*, while Batzer *et al.* (2007) again revealed *Schizothyrium* Desm. (*Schizothyriaceae*) to cluster within the *Mycosphaerellaceae*. Our results, however, provide support for recognition of *Schizothyrium* as a distinct genus, although *Schizothyriaceae* was less well supported as being separate from *Mycosphaerellaceae* (*Capnodiales*).

Although pleomorphism represents a rather unstudied phenomenon in this group of fungi, it has been observed in several species. Within the *Teratosphaeria* clade, Crous *et al.* (2007b) recently demonstrated teleomorphs to have *Readeriella* and *Cibiessia* synanamorphs, while the black yeast genera that belong to this clade, commonly have more than one anamorph state in culture. The present study also revealed *Readeriella mirabilis* to have two conidial types in culture, and to be highly plastic regarding its mode of conidiogenesis, and *Readeriella* to be the oldest generic name available for a large group of leaf-spotting coelomycetes in the *Teratosphaeriaceae* (*Capnodiales*).

Although not commonly documented, there are ample examples of synanamorphs in *Capnodiales*. Within *Mycosphaerella*, Beilharz *et al.* (2004) described *Passalora perplexa* Beilharz, Pascoe, M.J. Wingf. & Crous as a species with a coelomycete and yeast synanamorph, while Crous & Corlett (1998) described *Mycosphaerella stigmata-platani* F.A. Wolf to have a *Cercostigmata* U. Braun and *Xenostigmata* Crous synanamorph, and recent collections also revealed the presence of a similar species that has typical “*Stigmata*” (distoseptate conidia) and *Pseudocercospora* (euseptate conidia) synanamorphs (Crous, unpubl. data), and Crous (1998) reported *Readeriella epicoccoides* (coelomycete) to

have a *Cercostigmina* (hyphomycete) synanamorph in culture.

Although the *Mycosphaerella* complex encompasses thousands of names, it may appear strange that it is only now that more clarity is obtained regarding the phylogenetic relationships among taxa in this group. This is partly due to the fact that these organisms are cultivated with difficulty, and also that the first paper to address the taxonomy of this complex based on DNA sequence data was only relatively recently published (Stewart *et al.* 1999). In the latter study, the genus *Paracercospora* Deighton (scars minutely thickened along the rim), was shown to be synonymous with the older genus *Pseudocercospora*. Similarly, Crous *et al.* (2001) showed that *Cercostigmina* (rough, irregular percurrent proliferations) was also synonymous with *Pseudocercospora*. This led Crous & Braun (2003) to conclude that conidiomatal type, conidial catenulation, septation and proliferation of conidiogenous cells were of less importance in separating species at the generic level. *Mycovellosiella* Rangel and *Phaeoramularia* Munt.-Cvetk. were subsequently reduced to synonymy with the older name, *Passalora* Fr., and characters identified as significant at the generic level were pigmentation (*Cercospora* vs. *Passalora*), scar structure (*Passalora* vs. *Pseudocercospora*), and verruculose superficial hyphae (*Stenella* vs. *Passalora*). Due to the unavailability of cultures, no decision was made regarding *Stenella* (verrucose conidia and mycelium), *Stigmia* (distoseptate conidia), and several other, less well-known genera such as *Asperisporium* Maubl., *Denticularia* Deighton, *Distocercospora* N. Pons & B. Sutton, *Prathigada* Subram., *Ramulispora*, *Pseudocercosporidium* Deighton, *Stenellopsis* B. Huguenin and *Verrucisporota* D.E. Shaw & Alcorn. In a recent study, however, Crous *et al.* (2006a) were able to show that *Phaeoisariopsis* (synnemata, conidia with slightly thickened hila) and *Stigmia* (distoseptate conidia) were also synonyms of *Pseudocercospora*.

The present study shows that most anamorph genera are polyphyletic within *Teratosphaeria*, and paraphyletic within *Capnodiales*. In some cases, generic concepts of anamorphs based on morphology and conidium ontogeny conform well with phylogenetic relationships, though this is not true in all cases due to convergence. Nevertheless, anamorphs still convey valuable morphological information that is contained in the anamorph name, and naming anamorphs continue to provide a practical system to identify the various asexual taxa encountered.

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## Eyespot of cereals revisited: ITS phylogeny reveals new species relationships

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### Abstract

Four species so far classified in *Pseudocercospora* or *Ramulispora* (hyphomycetes) are associated with eyespot disease symptoms of cereals. Two of these have been linked to teleomorphs that were described in *Tapesia*. Sequence data derived from the Internal Transcribed Spacer region (ITS1, 5.8S and ITS2) of the rDNA operon showed, however, that the eyespot fungi associated with *Tapesia* are not congeneric with *Ramulispora sorghi*, the type of *Ramulispora*. The genus name *Tapesia* is now rejected in favour of the conserved name *Mollisia*, which appears to comprise heterogeneous fungi. *Tapesia yallundae* is not closely related to the type of *Mollisia*, *M. cinerea*, but clusters separately, being more closely allied to species with *Cadophora* anamorphs. A new holomorph genus, *Oculimacula*, is therefore proposed for teleomorphs of the eyespot fungi, while the anamorphs are accommodated in *Helgardia* gen. nov.

### Introduction

Eyespot disease of cereals is widespread throughout the temperate regions of the world, and causes a damaging stem-base infection of these hosts (Fitt et al., 1990). Severe eyespot lesions girdle the stem and soften the stem-base, resulting in lodging and heavy crop losses (Scott and Hollins, 1974). Four cercosporoid species are known to be associated with eyespot disease of cereals (Nirenberg, 1981; Robbertse et al., 1995), while a sexual state is known for two of these species (Robbertse et al., 1995). The cercosporoid species associated with eyespot disease are rather unusual in resembling leaf spot pathogens of *Pseudocercospora* Deighton.

The eyespot fungus was originally described as *Cercospora herpotrichoides* Fron (Fron, 1912). Deighton (1973) established the new genus *Pseudocercospora* for anamorphs of *Mycosphaerella* Johanson that were *Cercospora*-like, but had unthickened and inconspicuous conidial

scars. He included *C. herpotrichoides* in this genus. Nirenberg (1981) found that the best-known eyespot fungus on wheat, *Pseudocercospora herpotrichoides*, includes two varieties, *P. herpotrichoides* (Fron) Deighton var. *herpotrichoides* and var. *acuformis* Nirenberg. These varieties were initially thought to correlate with two pathotypes, respectively known as the wheat-type (W-type) and the rye-type (R-type) (Priestley et al., 1992), though an examination of more strains found this to not always be the case (Lucas et al., 2000). In her treatment of this complex, Nirenberg (1981) followed Deighton (1973), and chose *Pseudocercospora* in which to place *C. herpotrichoides* together with the new variety, as well as two new species which she described from eyespot lesions on cereals in Germany, namely *P. anguioides* Nirenberg and *P. aestiva* Nirenberg.

Nirenberg's treatment received wide recognition and was the first to highlight the fact that several taxa are involved in this disease complex. Von Arx

(1983), however, recognized that the eyespot fungi are unrelated to the *Mycosphaerella* anamorphs included in *Pseudocercospora*. He observed them to have a mode of conidiogenesis similar to that of *Ramulispora sorghi* (Ellis & Everh.) Olive & Lefebvre, the type of *Ramulispora* Miura. He also found that conidia in all these species developed lateral branches. Robbertse et al. (1995) later demonstrated that the lateral conidial branches were, in most cases, the result of microcyclic conidiation, which is not uncommon among the cercosporoid taxa (Fernandez et al., 1991).

Von Arx (1983) expanded the genus *Ramulispora* to include those species that are indeed *Pseudocercospora*-like, with or without lateral branches in the conidia that are formed in slimy masses, and parasitize the culm base of gramineous hosts. He transferred *P. herpotrichoides* to *Ramulispora* and indicated that the other species treated by Nirenberg (1981) also had to be allocated in this genus. This recommendation was followed by Boerema et al. (1992), in their treatment of the two varieties of *R. herpotrichoides*. In a later revision of this species complex, Robbertse et al. (1995) found that the two varieties shared a very low percentage RAPD similarity, exhibited differences in spore and colony morphology, infection pathway, fungicide sensitivity, virulence to specific hosts (Scott and Hollins, 1980) and distinct mating populations (Daniels et al., 1991; Dyer et al., 1994; Robbertse et al., 1994). These taxa were therefore recognized as separate species of *Ramulispora* (Robbertse et al., 1995), a genus known to represent pathogens of gramineous plants (Von Arx, 1983; Braun, 1995).

The discovery that the teleomorphs of the eyespot pathogens were actually discomycetes belonging to the genus *Tapesia* (Pers.) Fuckel (Wallwork and Spooner, 1988; Boerema et al., 1992) seemed to support the position taken by Von Arx (1983), namely to remove these pathogens from the *Mycosphaerella* anamorphs in *Pseudocercospora*. *Tapesia* resides well outside *Mycosphaerella* (Stewart et al., 1999) in the *Helotiales*. But *Tapesia* is now recognized to be congeneric with species of the younger but better-known genus *Mollisia* (Fr.) P. Karst. (Dennis, 1968; Baral, 1985), and the name was therefore rejected in favour of the conserved name *Mollisia* (Hawksworth and David, 1989). Species of *Tapesia* thus require transfer to the recognized generic name *Mollisia*.

*Ramulispora* is typified by *R. sorghi*, a pathogen that causes prominent leaf spots on sorghum called sooty

stripe, due to the abundant production of microsclerotia on the leaf surface (Olive et al., 1946; Braun, 1995). The latter pathogen was recently encountered on sorghum in the KwaZulu-Natal Province of South Africa, where it was associated with a severe outbreak of sooty leaf stripe (Mchau et al., 1996). In an attempt to clarify the taxonomic position of *R. sorghi*, as well as the eyespot pathogens of cereals, the present study was undertaken to infer a phylogeny for these fungi in comparison with other members representing their respective anamorph (*Ramulispora*) and teleomorph (*Tapesia*) genera. This was achieved by sequencing the Internal Transcribed Spacer region (ITS1, 5.8S and ITS2) of the rDNA operon, and comparing sequence data from the eyespot and *Ramulispora* isolates with those of known *Mycosphaerella* species (Crous et al., 2001).

## Materials and methods

### *Isolates and DNA amplification*

Isolates studied were obtained from the culture collections of the Centraalbureau voor Schimmelcultures (CBS), and the Department of Plant Pathology at the University of Stellenbosch (STE-U) (Table 1). Single-conidium subcultures were grown on malt extract agar (Biolab, Midrand, Johannesburg) (MEA) plates for 7 days. The isolation protocol of Crous et al. (2000) was used to isolate genomic DNA from fungal mycelia grown on MEA plates. The primers ITS1 (5' TTT CCG TAG GTG AAC CTG C3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC') (White et al., 1990) were used to amplify part of the nuclear rRNA operon using polymerase chain reaction (PCR). The amplified region included the 3' end of the 18S (small subunit) rRNA gene, the first ITS (ITS1), the 5.8S rRNA gene, the second ITS (ITS2) region and the 5' end of the 26S (large subunit) of the rRNA gene. The reaction mixture contained 5 µl of diluted sample, 1 × buffer, 8 mM MgCl<sub>2</sub>, 500 µM of each of the dNTPs, 2.5 U (Bioline) Taq polymerase and 10 pM of each primer and made up to a total volume of 25 µl with sterile water. The cycling conditions comprised denaturing at 96 °C for 5 min, followed by 30 cycles of denaturation at 96 °C (30 s), annealing 55 °C (30 s) and elongation at 72 °C (90 s). A final elongation step at 72 °C for 7 min was included. PCR products were separated by electrophoresis at 75 V for 1 h in a 0.8% (w/v) agarose gel in 0.5 × TAE buffer (0.4 M Tris, 0.05 M NaAc and 0.01 M EDTA, pH 7.85) and visualized under UV light

Table 1. Strains sequenced in the present study

Teleomorph	Anamorph	Accession no.	Collector	Substrate	Origin	GenBank no. (ITS)
<i>M. cinerea</i>	Unknown	STE-U 5092 = CBS 412.81	O. Petrini	<i>Juniperus communis</i>	Switzerland	AY259135
<i>M. dextrinospora</i>	Unknown	STE-U 5093 = CBS 401.78	R.P. Korf	Decaying wood	Spain	AY259134
<i>M. fusca</i>	<i>T. fusca</i>	CBS 234.71	B. Aebi	<i>Fagus sylvatica</i>	Switzerland	AY259138
<i>M. fusca</i>	<i>T. fusca</i>	CBS 486.48	Unknown	<i>Azalea</i> sp.	Netherlands	AY259137
<i>M. melaleuca</i>	Unknown	STE-U 5094 = CBS 89.84	H. Butin	<i>Picea abies</i> needle	Germany	AY259136
<i>Mycosphaerella capsellae</i>	<i>P. capsellae</i>	CBS 112032, 112033	R. Evans	<i>Pisum sativum</i>	UK	AY259139, AY259140
Unknown	<i>R. sorghi</i>	STE-U 905 = CBS 110578	D. Nowell	<i>Sorghum bicolor</i>	South Africa	AY259131
Unknown	<i>R. sorghi</i>	STE-U 906 = CBS 110579	D. Nowell	<i>Sorghum bicolor</i>	South Africa	AY259132
Unknown	<i>R. sorghi</i>	STE-U 908 = CBS 110580	D. Nowell	<i>Sorghum bicolor</i>	South Africa	AY259133

using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK) following ethidium bromide staining.

Polymerase chain reaction products were purified using a NucleoSpin Extract 2 in 1 Purification Kit (Macherey-Nagel GmbH, Germany). The cycle sequencing reaction with 20–40 ng of purified PCR products and 10 pmol primer in a total volume of 10 µl was carried out with an ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA, USA) containing AmpliTaq DNA Polymerase. The reaction was set up as denaturing at 94 °C for 5 min, followed by 25 cycles of 96 °C for 10 s, 55 °C for 10 s and 60 °C for 4 min, with a final incubation of 30 s at 60 °C. The resulting fragments were analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut).

#### Phylogenetic analysis

The nucleotide sequences of the rDNA gene generated in this study were added to the outgroup, *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (AF027741) and other sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>) and TreeBASE (<http://www.treebase.org/>), which were assembled using Sequence Alignment Editor v2.0 (Rambaut, 2002). The sequences were aligned using CLUSTAL W software (Thompson et al., 1994). Adjustments for improvement were made by eye where necessary. Phylogenetic analyses were undertaken using PAUP Version 4.0b10 (Swofford, 2000). Alignment gaps were treated as missing characters and all characters were unordered and of equal weight. Heuristic searches were conducted using 1000 replicates of random addition sequences

and tree bisection and reconstruction (TBR) as the branch-swapping algorithm to find maximum parsimony trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees was evaluated by 1000 bootstrap replications (Hillis and Bull, 1993). Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC) were also calculated. Resulting trees were printed with TreeView Version 1.6.6 (Page, 1996) and decay indices were calculated with AutoDecay Version 4.0.2 (Eriksson, 1998).

## Results

#### Phylogenetic analysis

Approximately 520–560 bases were determined for each isolate, of which approximately 450–490 bases per sequence (spanning ITS1, 5.8S rRNA gene, ITS2 and the first part of the small subunit gene) were added to the alignment. The manually adjusted alignments of the nucleotide sequences contained 601 characters including alignment gaps (data not shown). Of the aligned nucleotide sites for the data set, 245 characters were parsimony-informative, 61 variable characters were parsimony-uninformative and 295 were constant. Sequences were deposited in GenBank (Table 1), and the alignment in TreeBASE (SN 1392).

Aligned sequences of 39 isolates and an outgroup were subjected to maximum parsimony analysis using the heuristic search option with 1000 random taxon-additions in PAUP (Swofford, 2000). The 14th most parsimonious tree obtained from the heuristic search was evaluated with 1000 bootstrap replications. The

three *R. sorghi* isolates (STE-U 905, 906 and 908) grouped in a strongly supported clade (100%), sharing 55% support with a subclade containing *P. capsellae* (Ellis & Everh.) Deighton (*M. capsellae* A.J. Inman &

Sivan.) within *Mycosphaerella* (Figure 1). Species of *Mollisia* and *Tapesia* grouped in a large clade (100% bootstrap support), consisting of three well-defined subclades outside of the *Mycosphaerellaceae*

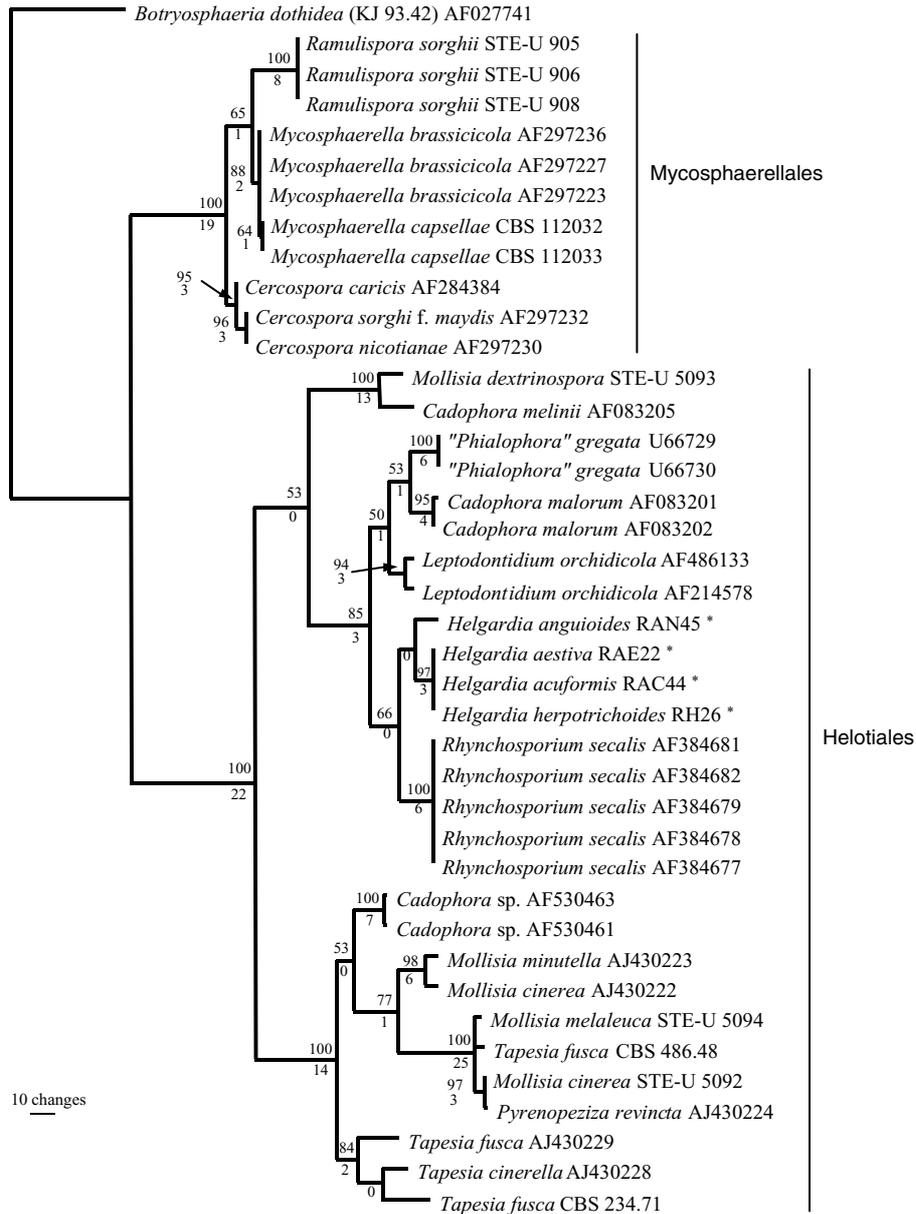


Figure 1. One of 14 most parsimonious trees (length = 606 steps, CI = 0.738, RI = 0.919, RC = 0.678) obtained from a heuristic search with 1000 random taxon-additions using a 601 bp alignment of ITS1, the 5.8S rRNA gene and ITS2. Bootstrap support values from 1000 replicates are shown above and decay values below the nodes. *B. dothidea* was used as outgroup (\*Sequences from TreeBASE matrix M691).

(*Mycosphaerellales*), comprising species of *Mollisia*, *Tapesia* and *Pyrenopeziza* Fuckel of the *Dermateaceae* (*Helotiales*). *Mollisia dextrinospora* Korf and *Cadophora melinii* Nannf. clustered apart from the main clade. *M. cinerea* (Batsch) P. Karst. and *M. melaleuca* (Fr.) Sacc. grouped in a clade (100% bootstrap support) together with *M. minutella* (Sacc.) Rehm, *Pyrenopeziza revincta* (P. Karst.) Gremmen, *Tapesia fusca*, *T. cinerella* Rehm and *Cadophora* sp. The eyespot 'Ramulispora' spp. clustered in a clade containing *Phialophora* Medlar (or rather *Cadophora* Lagerb. & Melin *sensu* Gams, 2000), *M. dextrinospora* Korf (STE-U 5093), *Leptodontidium* de Hoog, and *Rhynchosporium* Heinsen ex A.B. Frank isolates (97% bootstrap support). Within this clade, the four species of 'Ramulispora' together with *Rhynchosporium secalis* (Oudem.) Davis formed a subclade with 88% bootstrap support.

#### Taxonomy

The four species associated with cereal eyespot are obviously not congeneric with *R. sorghi*. For the teleomorphs of these cereal pathogens, the genus *Tapesia* is not available being a rejected name in favour of the conserved name *Mollisia* (Hawksworth and David, 1989), with which it is considered as being synonymous. Furthermore, *Mollisia* also appears to be morphologically and ecologically heterogeneous, and is linked to several different anamorph genera.

Species of *Mollisia* in a broad sense, including the eyespot pathogens, grouped in a large clade containing two well-defined subclades. The first subclade includes the type of *Mollisia*, *M. cinerea* (CBS 412.81, STE-U 5092), with a phialidic anamorph suggestive of a moderately branched *Cystodendron* Bubák, and *Pyrenopeziza revincta*. Species of *Pyrenopeziza* have in the past been linked to *Cystodendron/Cadophora*-like anamorphs (Hütter, 1958). *T. fusca* (Pers.) Fuckel, the type of *Tapesia*, has also been linked to a *Cystodendron* anamorph (Aebi, 1972), and is thus distinct from the eyespot pathogens. Isolates identified as *T. fusca*, clustered with *M. cinerea*, apart from the eyespot pathogens.

Species of the second subclade have *Cadophora* (incl. several taxa presently still in *Phialophora*), *Leptodontidium* and *Rhynchosporium* anamorphs. The *Ramulispora*-like anamorphs of the eyespot pathogens of cereals are quite distinct from all these anamorphs

of the *Dermateaceae*, though phylogenetically appear closely related to *Rhynchosporium* (Figure 1). *Ramulispora*, as typified by *R. sorghi*, is a member of the *Mycosphaerellaceae*. Therefore, it cannot be congeneric with a fungus having a Helotialean teleomorph (viz. the eyespot complex). The latter fungi do therefore not belong in *Ramulispora*, but require a new anamorph genus. *Mollisia*, as typified by *M. cinerea*, occurs in a separate cluster to the eyespot fungi, and has a different anamorph. Likewise, *Tapesia*, typified by *T. fusca*, has a different anamorph, and clusters with *Mollisia*, separate from the eyespot fungi. A new teleomorph genus thus needs to be described for the eyespot fungi.

#### *Oculimacula Crous & W. Gams, gen. nov.*

Apothecia sessilia, gregaria, 0.5–2.5 mm diam., circularia vel lobata, subiculo hypharum plus minusve brunnearum persistentium insidentia, texto superficiali hypharum pallide brunnearum, angustarum substrato affixa. Discus levis, griseus, marginem versus pallide griseus, maturus emarginatus, applanatus ad convexus. Receptaculum pallide brunneum ad griseo-brunneum, crateriforme. Asci 8-spori, unitunicati, clavati vel subcylindrici vel fusoidi, breviter stipitati, poro apicali iodi ope caerulescente. Ascospores biseriatae ad multiseriatae, hyalinae, leves, unicellulares, fusoidae vel subcylindricae-fusoidae vel clavatae, utrinque rotundatae, plerumque rectae. Paraphyses filiformes, sursum obtusatae, ascis longitudine similes. Excipulum medullare ex hyphis multiseptatis, hyalinis compositum, excipulum ectale e cellulis tenuitunicatis, fuscis, angularibus, marginem versus magis elongatis, constans.

*Anamorphe*: Helgardia Crous & W. Gams.

*Type*: AUSTRALIA. Yallunda Flat, on wheat stubble, 18 Nov. 1986, H. Wallwork and B. Spooner, K (holotype), ADW (isotype), of *Oculimacula yallundae* (Wallwork & Spooner) Crous & W. Gams.

*Etymology*: *Oculimacula* = Latin for eyespot, named after the characteristic lesions induced on stems of cereals.

*Apothecia* sessile, gregarious, 0.5–2.5 mm diam., circular to lobate, situated on a subiculum consisting of white to dark brown persistent hyphae, attached to the substrate via a superficial mat of pale brown, thin

hyphae. *Disk* smooth, grey with a pale grey margin, becoming emarginate and flattened to convex at maturity. *Receptacle* pale brown to grey-brown, cup-shaped. *Asci* 8-spored, unitunicate, clavate to subcylindrical or fusoid, with a short stalk, and an apical pore staining blue in Melzer's reagent. *Ascospores* bi- to multiseriate, hyaline, smooth, aseptate, fusoid to subcylindrical-fusoid or clavate with rounded ends, mostly straight. *Paraphyses* filiform with obtuse ends, similar in length to the asci. *Medullary excipulum* consisting of multi-septate, hyaline hyphae. *Ectal excipulum* consisting of thin-walled, dark brown, angular cells, becoming more elongated towards the margin. Anamorph *Helgardia* Crous & W. Gams.

*Helgardia* Crous & W. Gams, *gen. nov.*

Conidiophora fasciculata vel solitaria in hyphis superficialibus, vel e stromate pallide brunneo oriunda, subcylindrica vel geniculato-sinuosa, raro ramosa, hyalina ad pallide olivacea, levia, seu tantum e cellulis conidiogenis constantia seu uno vel duobus septis divisa, paulo distincta; cellulae conidiogenae integratae, ad apicem dense sympodialiter elongascentes; loci conidiogeni haud inspissati, inconspicui nec fuscescentes. Conidia solitaria, hyalina, levia, in acervis mucidis aggregata, acicularia-filiformia, recta vel curvata, uni- vel multiseptata, saepe conidia secundaria statim proferentia.

Type: FRANCE, holotype of *Helgardia herpotrichoides* (could not be traced in herb. PC); SOUTH AFRICA. Western Cape Province, Moorreesburg, on wheat stubble, 1991, F. Bester, CBS 110665 (Dried culture in herb. CBS designated here as *neotype*) of *Helgardia* (isolate genetically identical and sexually compatible with European isolates).

Etymology: *Helgardia*, named after the German mycologist and phytopathologist, Dr. Helgard I. Nirenberg, who first recognized the distinctiveness of these anamorphs on cereals.

*Conidiophores* fasciculate or solitary on the superficial mycelium, or arising from pale brown stromata, subcylindrical to geniculate-sinuuous, rarely branching, hyaline to pale olivaceous, smooth, consisting of conidiogenous cells only, or slightly differentiated with up to 2 septa, conidiogenous cells integrated, proliferating sympodially at the apex, with inconspicuous, dense geniculations; loci unthickened,

inconspicuous, not darkened. Conidia solitary, hyaline, smooth, arranged in slimy packets, acicular-filiform, straight to curved, one- to multiseptate, forming smaller, secondary conidia via microcyclic conidiation.

*Oculimacula yallundae* (Wallwork & Spooner)

Crous & W. Gams, *comb. nov.* Figures 2–6

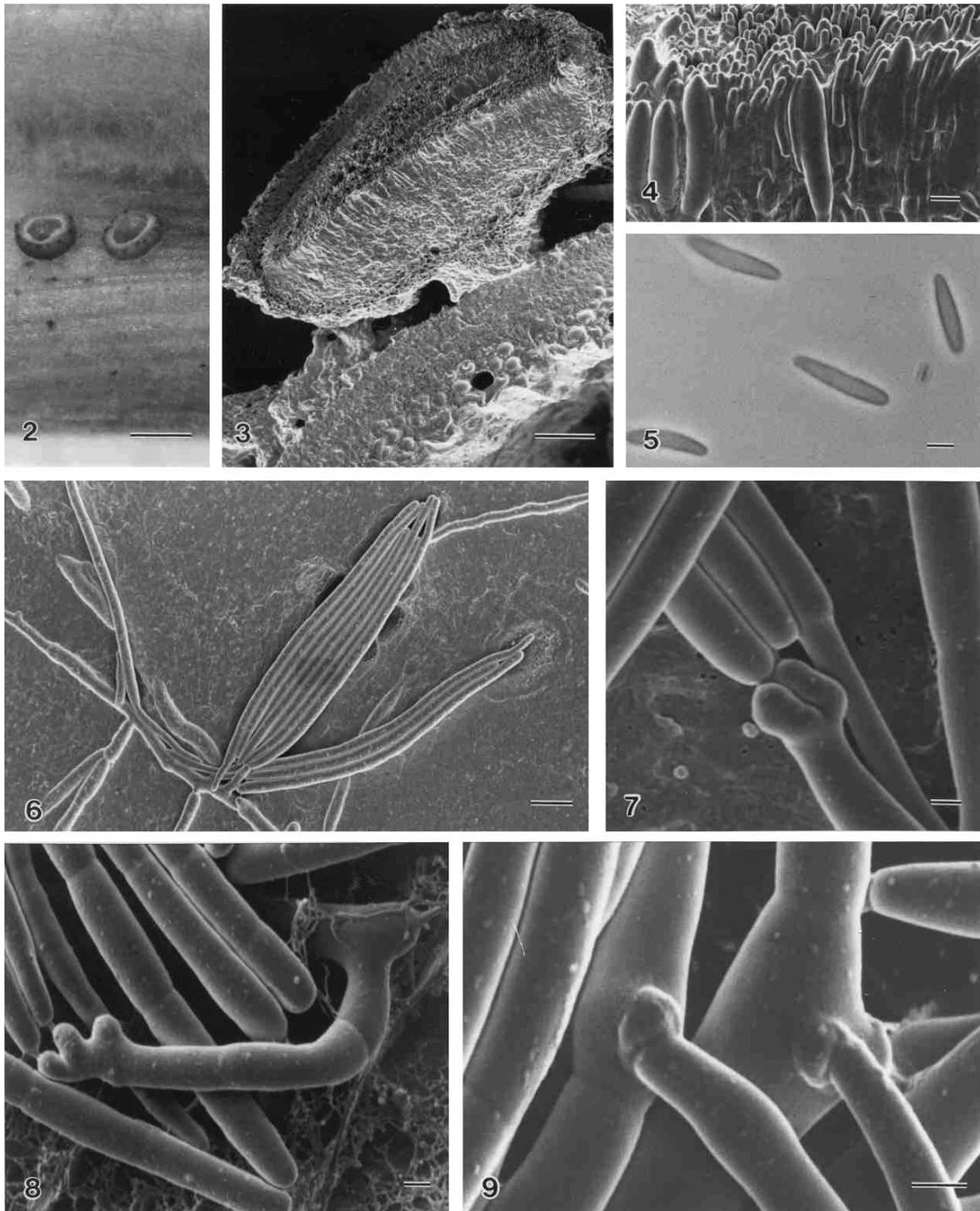
- ≡ *Tapesia yallundae* Wallwork & Spooner, Trans. Brit. Mycol. Soc. 71: 703 (1988). Anamorph: *Helgardia herpotrichoides* (Fron) Crous & W. Gams, *comb. nov.*
- ≡ *Cercospora herpotrichoides* Fron, Ann. Sci. Agron. Franç. Étrangère, Sér. 4, 1: 11 (1912).
- ≡ *Pseudocercospora herpotrichoides* (Fron) Deighton, Mycol. Pap. 133: 46 (1973).
- ≡ *Ramulispora herpotrichoides* (Fron) Arx, Proc. K. Ned. Akad. Wet. C 86(1): 36 (1983).

*Oculimacula aciformis* (Boerema, R. Pieters & Hamers) Crous & W. Gams, *comb. nov.* Figure 7

- ≡ *Tapesia yallundae* Wallwork & Spooner var. *aciformis* Boerema, R. Pieters & Hamers, Neth. J. Pl. Pathol. 98(Suppl 1): 22 (1992).
- ≡ *Tapesia aciformis* (Boerema, R. Pieters & Hamers) Crous, S. Afr. J. Bot. 61: 46 (1995). Anamorph: *Helgardia aciformis* (Nirenberg) Crous & W. Gams, *comb. nov.*
- ≡ *Pseudocercospora herpotrichoides* var. *aciformis* Nirenberg, Z. Pflanzenkr. Pflanzensch. 88: 244 (1981).
- ≡ *Ramulispora herpotrichoides* var. *aciformis* (Nirenberg) Boerema, R. Pieters & Hamers, Neth. J. Pl. Pathol. 98(Suppl 1): 22 (1992) (combination also made by U. Braun, Nova Hedwigia 56: 423, 1993).
- ≡ *Ramulispora aciformis* (Nirenberg) Crous, S. Afr. J. Bot. 61: 46 (1995).

*Helgardia anguioides* (Nirenberg) Crous & W. Gams, *comb. nov.* Figure 8

- ≡ *Pseudocercospora anguioides* Nirenberg, Z. Pflanzenkr. Pflanzensch. 88: 244 (1981).
- ≡ *Ramulispora herpotrichoides* var. *anguioides* (Nirenberg) U. Braun, Nova Hedwigia 56: 423 (1993).



Figures 2–9. Apothecia of *Oculimacula*, with *Helgardia* anamorphs. (2) Apothecia of *O. yallundae* on wheat stubble. (3) Vertical section through an apothecium of *O. yallundae*. (4) Section through an apothecium of *O. yallundae*, showing ascus layer. (5) Ascospores of *O. yallundae*. (6) Conidia and conidiogenous cells of *H. herpotrichoides*. (7) Conidial hila and conidiogenous cell of *H. acufomis*. (8) Conidial hila and conidiogenous cell of *H. anguioides*. (9) Conidia of *H. aestiva* giving rise to secondary conidia via microcyclic conidiation. Bars = 2 mm, 100, 5, 2, 10  $\mu\text{m}$  in (a)–(e), and 1  $\mu\text{m}$  in (f)–(h).

*Helgardia aestiva* (Nirenberg) Crous & W. Gams, *comb. nov.* Figure 9

- ≡ *Pseudocercospora aestiva* Nirenberg, Z. Pflanzenkr. Pflanzensch. 88: 246 (1981).
- ≡ *Ramulispora aestiva* (Nirenberg) E.L. Stewart & Crous, Mycol. Res. 103: 1497 (1999).

## Discussion

A recent reclassification of the eyespot pathogens in *Ramulispora* seemed to correct the inadequacy of their placement in *Pseudocercospora*, which comprises anamorphs of *Mycosphaerella*. The present study has revealed that these assumptions about the phylogenetic position and affinity of the genus *Ramulispora* were incorrect, as was the placement of the sexual state of the eyespot fungi in the genus *Tapesia*. To address this issue, a new teleomorph genus, *Oculimacula*, with its associated anamorph genus *Helgardia*, are proposed. Although it can be argued that a teleomorph genus alone would suffice for these organisms, two related species, namely *H. anguoides* and *H. aestiva*, have not yet been linked to teleomorphs, and thus they require anamorph names for the present. Our data suggest, however, that their teleomorphs, if found, would reside in *Oculimacula*.

The genus *Mollisia* is known to have anamorphs that reside in the *Phialophora* complex, particularly *Cadophora* (Gams, 2000). As shown in the present study, and reported elsewhere (Webster et al., 1993; Nauta and Spooner, 2000), *Mollisia* is heterogeneous. The eyespot taxa reside in one clade together with some species of *Mollisia* that have *Cadophora* or *Cystodendron* or other anamorphs such as *Leptodontidium* and *Rhynchosporium*. The type species of *Mollisia*, *M. cinerea*, has an inconspicuously phialidic, unnamed anamorph, which is distinct from *Cadophora*. The molecular divergence also suggests that *Mollisia* species with *Cadophora* anamorphs will require a new teleomorph genus, while *Tapesia* might possibly be available for species with *Cystodendron* anamorphs (Aebi, 1972). The eyespot pathogens are sufficiently distinct ecologically and in their anamorphs from these two groups to warrant the introduction of a new holomorph. However, the ascumata offer relatively few criteria for this distinction.

The presence or absence of a subiculum has in the past been regarded as significant to separate

genera such as *Tapesia* from *Mollisia* (Boudier, 1885; Saccardo, 1889; Rehm, 1891). In later years, less weight was placed in this feature, which appeared insignificant at the generic level (Dennis, 1968; Aebi, 1972; Baral, 1985; 1994), and hence Aebi (1972) reduced *Mollisia* (1871) to synonymy under *Tapesia* (1870). The genus *Mollisia* encompasses more than 100 species, and is better known than *Tapesia* (20 spp.) (Hawksworth and David, 1989). Therefore, Hawksworth and David (1989) proposed conservation of *Mollisia* over *Tapesia*, a proposal that was accepted by the Committee for Fungi and Lichens (Gams, 1992), and the conservation is now listed in the Code. *Mollisia*, however, consists of several different groups that can be distinguished primarily on the basis of their anamorph associations.

Deighton (1973) introduced the genus *Pseudocercospora* to accommodate taxa with unthickened, not darkened or refractive conidial hila that were formerly placed in *Cercospora* Sacc. He did not, however, consider the morphological similarity of *Pseudocercospora* with *Ramulispora*, and hence placed *C. herpotrichoides* in *Pseudocercospora*. Braun (1995) stated that if *R. sorghi*, the type of *Ramulispora*, had a teleomorph other than *Tapesia*, a new anamorph genus would have to be introduced to accommodate *R. herpotrichoides* and related taxa. We have shown here that *R. sorghi* (and hence *Ramulispora*) represents an anamorph of *Mycosphaerella*, as does *Pseudocercospora*. *Ramulispora* is distinct from *Helgardia* in that *R. sorghi* induces characteristically sooty leaf spots, which is due to the abundant sclerotia that form on the leaf surface. The latter are, however, not produced in culture. Colonies of *R. sorghi* grow more slowly than those of *Helgardia*. They are compact, grey to black, and sporulate by forming masses of pink, slimy conidia. Slimy conidial masses are known to also occur in *Pseudocercospora* and *Helgardia*.

A further issue not addressed in the present paper concerns the distinction between and priority of the genera *Pseudocercospora* (1973) and *Ramulispora* (1920). Although Deighton (1973) did not compare these two genera when he introduced *Pseudocercospora*, Von Arx (1983) chose to retain *Ramulispora* for taxa occurring on gramineous hosts. Morphologically, these two genera are similar, and also cluster closely together (Figure 1). With *Ramulispora* being the older name, the International Code for Botanical Nomenclature determines that all names in *Pseudocercospora* actually would have to

be transferred to *Ramulispora*. To reach a final conclusion, however, more species of *Pseudocercospora* and *Ramulispora* need to be compared in a larger morphological and molecular study. If these two genera were indeed shown to be synonymous, it is evident that the name *Pseudocercospora* would deserve conservation over the lesser-known *Ramulispora*. A further 13 species of *Ramulispora* are known ([www.speciesfungorum.org](http://www.speciesfungorum.org)), but without cultures and molecular analyses, their correct phylogenetic affinities remain unclear. *Pseudocercospora* has recently been monographed (Braun, 1995). It contains more than 100 species that are well known to plant pathologists and mycologists, and the genus should thus be retained. The erection of new generic names for the eyespot pathogens of cereals was necessary, however, as neither *Pseudocercospora* nor *Ramulispora* is available for the anamorphs, nor are *Tapesia* or *Mollisia* for the teleomorphs.

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# Species of *Cercospora* associated with grey leaf spot of maize

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**Abstract:** Grey leaf spot is a serious yield-reducing disease of maize (*Zea mays*) in many parts of the world where this crop is cultivated. The causal organism associated with the disease is *Cercospora zeae-maydis*. Two potential sibling species have been recognized as Groups I and II. The DNA sequences for the internal transcribed spacers (ITS1 & ITS2), the 5.8S rRNA gene, elongation factor 1- $\alpha$ , histone H3, actin and calmodulin gene regions suggest that Groups I and II are two distinct species. Furthermore, *Cercospora zeae-maydis* (Group I) can be distinguished from *C. zeina* sp. nov. (Group II) by its faster growth rate on artificial media, the ability to produce cercosporin, longer conidiophores, and broadly fusiform conidia. A PCR-based test that distinguishes the two species was developed using species-specific primers designed from the histone H3 gene.

**Taxonomic novelties:** *Cercospora zeina* Crous & U. Braun sp. nov.

**Key words:** Ascomycetes, *Cercospora zeae-maydis*, *Cercospora zeina*, grey leaf spot, maize, *Mycosphaerella*, systematics.

## INTRODUCTION

Grey leaf spot of maize is a serious foliar disease of *Zea mays* in many countries where it is cultivated, especially in the eastern U.S.A. and Africa (Ward *et al.* 1999, Crous & Braun 2003). Since it was recognized as a “disease on the move” by Latterell & Rossi (1983), grey leaf spot has become increasingly important and is currently seen as one of the most serious yield-limiting diseases of maize (Nutter & Jenco 1992, Ward & Nowell 1998). The causal agent of grey leaf spot is generally regarded as *Cercospora zeae-maydis* Tehon & E.Y. Daniels, though *C. sorghi* Ellis & Everh. has also been reported from maize (Crous & Braun 2003). Chupp (1954) referred to a *C. sorghi* var. *maydis* Ellis & Everh., which is morphologically similar to *C. sorghi*, but suspected to represent a distinct species due to its lack of pathogenicity to sorghum. In recent years, it has become accepted that more than one species of *Cercospora* is associated with grey leaf spot of maize, namely *C. zeae-maydis* Group I, which is dominant in the U.S.A. and occurs elsewhere in the world, and *C. zeae-maydis* Group II, which is genetically and phenotypically distinct and occurs in the U.S.A., Africa and possibly elsewhere (Wang *et al.* 1998, Dunkle & Levy 2000, Goodwin *et al.* 2001).

The aim of the current study was to characterise the *Cercospora* species associated with grey leaf spot symptoms occurring on maize in South Africa. To achieve this goal isolates were subjected to DNA sequence analysis of several loci, namely the internal transcribed spacers (ITS1 & ITS2), the 5.8S rRNA gene, the elongation factor 1- $\alpha$ , histone 3, actin and calmodulin gene regions. Furthermore, South African isolates were morphologically compared to those

isolates from the U.S.A., and the type specimen of *C. zeae-maydis*.

## MATERIALS AND METHODS

### Isolates

Single-conidial isolates were obtained from symptomatic maize leaves, and cultured as explained in Crous (1998). Cultural characteristics and morphology of *Cercospora* isolates (Table 1) were determined on plates containing 2% malt extract agar (MEA) (20 g/L), 2% potato-dextrose agar (PDA), oatmeal agar (OA), and carnation leaf agar (CLA) [1% water agar (10 g/L) with autoclaved carnation leaves placed onto the medium] (Gams *et al.* 1998). Plates were incubated at 25 °C under continuous near-UV light, to promote sporulation.

### DNA phylogeny

Isolates of *C. zeae-maydis*, *C. beticola*, *C. apii*, and an unidentified *Cercospora* sp. (Table 1) were used for phylogenetic analysis. The protocol of Lee & Taylor (1990) was used to isolate genomic DNA from fungal mycelium of monoconidial cultures grown on MEA in Petri dishes. The primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify part (ITS) of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. To obtain additional sequence information, four other loci were also sequenced. Part of the elongation factor 1- $\alpha$  gene (EF) was amplified with primers EF1-728F and EF1-986R, part of the actin gene (ACT) with primers ACT-512F and ACT-783R,

**Table 1.** *Cercospora* isolates used for sequence analysis.

Species	Accession number <sup>1</sup>	Host	Country	Collector	GenBank numbers <sup>2</sup> (ITS, EF ACT, CAL, HIS)	
<i>Cercospora apii</i>	CBS 114418; CPC 10924	<i>Apium graveolens</i>	Italy	Meutri	AY840517, AY840484, AY840448, AY840415, AY840382	
	CBS 116455; CPC 11556*	<i>A. graveolens</i>	Germany	K. Schrameyer	AY840519, AY840486, AY840450, AY840417, AY840384	
	CBS 116504; CPC 11579	<i>A. graveolens</i>	Germany	K. Schrameyer	AY840520, AY840487, AY840451, AY840418, AY840385	
	CBS 119.25; CPC 5086	<i>A. graveolens</i>	—	L. J. Klotz	AY840512, AY840479, AY840443, AY840410, AY840377	
	CBS 121.31; CPC 5073	<i>Beta vulgaris</i>	Austria	—	AY840513, AY840480, AY840444, AY840411, AY840378	
	CBS 127.31; CPC 5119	<i>B. vulgaris</i>	Hungary	—	AY840514, AY840481, AY840445, AY840412, AY840379	
<i>Cercospora beticola</i>	CBS 116456; CPC 11557*	<i>B. vulgaris</i>	Italy	V. Rossi	AY840527, AY840494, AY840458, AY840425, AY840392	
	CBS 116501; CPC 11576	<i>B. vulgaris</i>	Iran	A. A. Ravanlou	AY840528, AY840495, AY840459, AY840426, AY840393	
	CBS 116502; CPC 11577	<i>B. vulgaris</i>	Germany	S. Mittler	AY840529, AY840496, AY840460, AY840427, AY840394	
	CBS 116.47; CPC 5074	<i>B. vulgaris</i>	Netherlands	G. E. Bunschoten	AY752135, AY752168, AY752196, AY752227, AY752258	
	CBS 124.31; CPC 5070	<i>B. vulgaris</i>	Romania	—	AY840523, AY840490, AY840454, AY840421, AY840388	
	CBS 126.31; CPC 5064	<i>B. vulgaris</i>	Germany	—	AY840525, AY840492, AY840456, AY840423, AY840390	
	CPC 5125	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY752137, AY752170, AY752198, AY752229, AY752260	
	CPC 5128	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY752138, AY752171, AY752199, AY752230, AY752261	
	CPC 10168	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY840533, AY840500, AY840464, AY840431, AY840398	
<i>Cercospora canescens</i>	ATCC 32779	<i>Vigna radiata</i>	Taiwan	—	AY266164, —, —, —, —	
<i>Cercospora sorghi</i>	—	<i>Sorghum bicolor</i>	U.S.A., Texas	—	AF291707, —, —, —, —	
<i>Cercospora sorghi</i> var. <i>maydis</i>	—	<i>Zea mays</i>	U.S.A., North Carolina	—	AF297233, —, —, —, —	
—	—	<i>Z. mays</i>	Kenya	—	AF297232, —, —, —, —	
<i>Cercospora</i> sp.	CPC 12062	<i>Z. mays</i>	South Africa, KwaZulu-Natal	P. Caldwell	DQ185071, DQ185083, DQ185095, DQ185107, DQ185119	
<i>Cercospora zeae-maydis</i>	—	<i>Z. mays</i>	U.S.A., Indiana	—	AF291709, —, —, —, —	
	CBS 117755 = A358	<i>Z. mays</i>	U.S.A., Indiana	B. Fleener	DQ185072, DQ185084, DQ185096, DQ185108, DQ185120	
	CBS 117756 = A359	<i>Z. mays</i>	U.S.A., Delaware	B. Fleener	DQ185073, DQ185085, DQ185097, DQ185109, DQ185121	
	CBS 117757* = A360	<i>Z. mays</i>	U.S.A., Wisconsin	B. Fleener	DQ185074, DQ185086, DQ185098, DQ185110, DQ185122	
	CBS 117758 = A361	<i>Z. mays</i>	U.S.A., Iowa	B. Fleener	DQ185075, DQ185087, DQ185099, DQ185111, DQ185123	
	CBS 117759 = A362	<i>Z. mays</i>	U.S.A., Tennessee	B. Fleener	DQ185076, DQ185088, DQ185100, DQ185112, DQ185124	
	CBS 117760 = A363	<i>Z. mays</i>	U.S.A., Pennsylvania	B. Fleener	DQ185077, DQ185089, DQ185101, DQ185113, DQ185125	
	CBS 117761 = A364	<i>Z. mays</i>	U.S.A., Indiana	B. Fleener	DQ185078, DQ185090, DQ185102, DQ185114, DQ185126	
	CBS 117762 = A365	<i>Z. mays</i>	U.S.A., Missouri	B. Fleener	DQ185079, DQ185091, DQ185103, DQ185115, DQ185127	
	CBS 117763 = A367	<i>Z. mays</i>	U.S.A., Iowa	B. Fleener	DQ185080, DQ185092, DQ185104, DQ185116, DQ185128	
	<i>Cercospora zeina</i>	CBS 118820 = CPC 11995*	<i>Z. mays</i>	South Africa, KwaZulu-Natal	P. Caldwell	DQ185081, DQ185093, DQ185105, DQ185117, DQ185129
		CPC 11998	<i>Z. mays</i>	South Africa, KwaZulu-Natal	P. Caldwell	DQ185082, DQ185094, DQ185106, DQ185118, DQ185130
		—	<i>Z. mays</i>	U.S.A., North Carolina	—	AF291710, —, —, —, —

<sup>1</sup>CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.<sup>2</sup>ITS: internal transcribed spacer region, EF: partial elongation factor 1-alpha gene, ACT: partial actin gene, CAL: partial calmodulin gene, HIS: partial histone H3 gene.

\*Ex-type cultures.

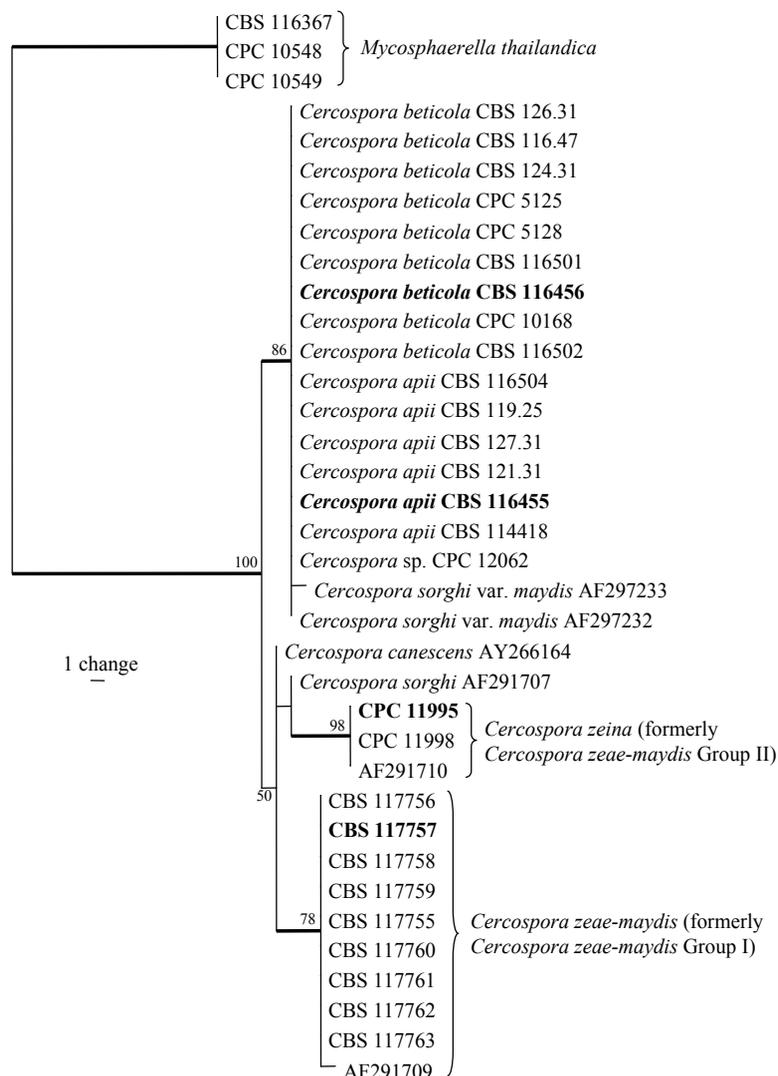


and part of the calmodulin gene (CAL) with primers CAL-228F and CAL-737R (Carbone & Kohn 1999). Part of the histone H3 gene (HIS) was amplified with primers CylH3F and CylH3R (Crous *et al.* 2004a). Sequencing was done with the same PCR primers. The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Crous *et al.* (2004b). The new sequences were added to a subset of the alignment (TreeBASE matrix M2038) of Crous *et al.* (2004b) and additional sequences were obtained from GenBank. Sequence data were deposited in GenBank and alignments in TreeBASE (S1509, M2712).

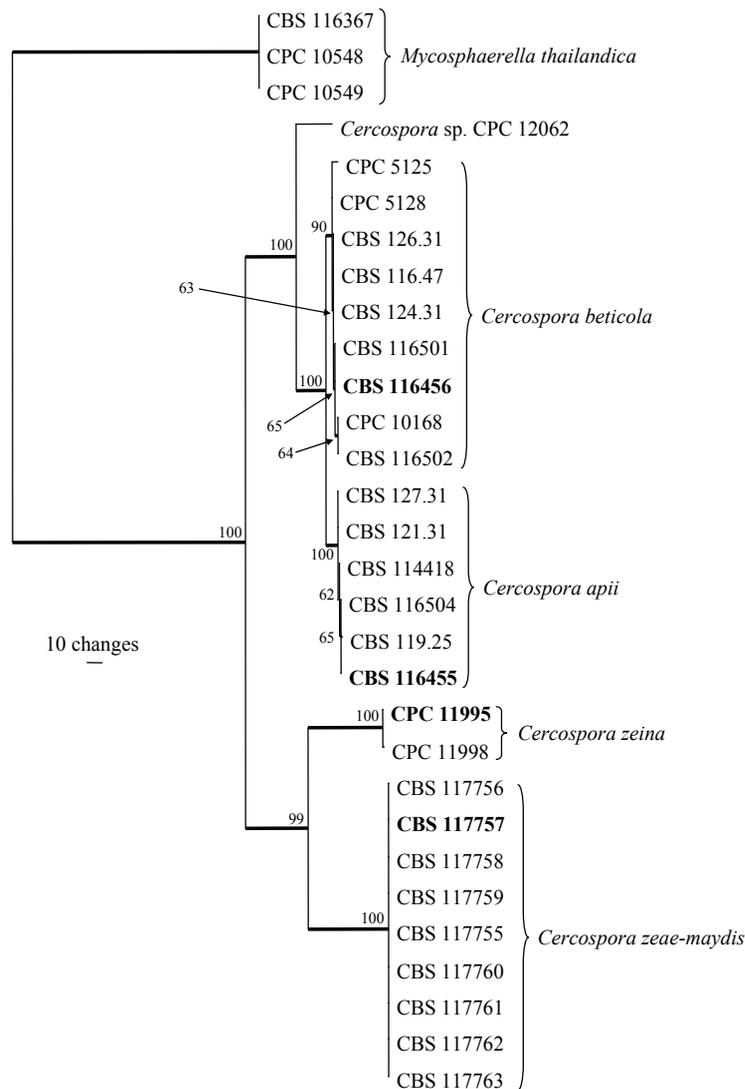
#### Development of a species-specific diagnostic test

The histone H3 gene was found to be most effective in separating the three species described in the present study. Therefore, this area was targeted for the development of a species-specific diagnostic test. Primers CylH3F and CylH3R were used as external primers and their amplification product functions as a positive control. Three species-specific primers were designed for *C. zea-maydis*, *C. zeina* sp. nov. and

an undescribed *Cercospora* species, respectively: CzeaeHIST (5'-TCGACTCGTCTTTCACTTG-3'), CzeinaHIST (5'-TCGAGTGGCCCTCACCGT-3') and CmaizeHIST (5'-TCGAGTCACTTCGACTTCC-3'); all of them species-specific. These internal, species-specific primers, together with the external primers, were used in separate PCR reactions in a total volume of 12.5 µl, containing 1 µl of diluted genomic DNA, 1× PCR buffer, 2 mM MgCl<sub>2</sub>, 48 µM of each of the dNTPs, 0.7 pmol CylH3F, 3 pmol of CylH3R, 4 pmol of the specific internal primer and 0.7 units (Bioline) *Taq* polymerase. The amplification reactions were done on a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Connecticut). The initial denaturation step was done at 94 °C for 5 min, followed by 15 cycles of denaturation at 94 °C (20 s), annealing at 58 °C (30 s) and elongation at 72 °C (40 s) as well as 25 cycles of denaturation at 94 °C (20 s), annealing at 55 °C (30 s) and elongation at 72 °C (40 s). A final elongation step at 72 °C (5 min) was included to ensure that full length products are obtained. The PCR products were separated on a 1 % agarose gel and visualized under UV-light after ethidium bromide staining.



**Fig. 1.** One of six most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows a single change, and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches, and ex-type strains are shown in bold print. The tree was rooted to three *Mycosphaerella thailandica* strains.



**Fig. 2.** One of two most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined ITS, elongation factor 1- $\alpha$ , actin, calmodulin and histone H3 sequence alignment. The scale bar shows 10 changes, and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches, and type strains are shown in bold print. The tree was rooted to three *Mycosphaerella thailandica* strains.

### Taxonomy

Morphological examinations were made from cultures sporulating on CLA, as well as on host material. Structures were mounted in lactic acid, and 30 measurements at  $\times 1000$  magnification were made of each structure. The 95 % confidence levels were determined and the extremes of spore measurements given in parentheses. Colony colours were noted after 3 wk growth on MEA, PDA and OA at 25 °C in the dark, using the colour charts of Rayner (1970). All cultures studied are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. Type specimens were deposited at the National Collection of Fungi in Pretoria (PREM), South Africa (Table 1).

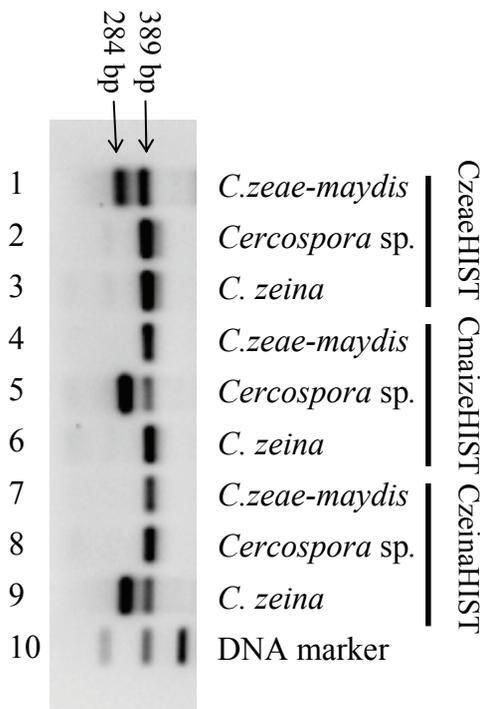
## RESULTS

### DNA phylogeny

Approximately 500, 310, 230, 320 and 400 bases were determined for ITS, EF, ACT, CAL, and HIS loci, respectively, of the isolates listed in Table 1. Because

sequences for the last four loci were not available for other isolates, a separate tree that included more isolates was generated using only ITS sequences (Fig. 1). A partition homogeneity test showed that all loci could be combined ( $p = 0.747$ ) into a single analysis (Fig. 2).

The ITS data matrix contained 36 taxa (including the three outgroup isolates) and 487 characters including alignment gaps. Of these characters, 40 were parsimony-informative, one was variable and parsimony-uninformative, and 446 are constant. Neighbour-joining analysis using three substitution models (uncorrected "p", Jukes-Cantor and HKY85) on the sequence data yielded trees with similar topology and bootstrap values. Parsimony analysis of the alignment yielded six most parsimonious trees (TL = 44 steps; CI = 0.955; RI = 0.986; RC = 0.942), one of which is shown in Fig. 1. Three distinct clades were obtained. The first clade (86 % bootstrap support) contained *C. apii* and *C. beticola* together with two isolates of *C. sorgh* var. *maydis* and an undescribed *Cercospora* sp. (CPC 12062) from *Zea mays* in South Africa. The second clade (98 % bootstrap support) contained three



**Fig. 3.** Identification of *C. zaeae-maydis*, an unidentified *Cercospora* sp. and *C. zeina* using the species-specific primers. Lane 10 contains the DNA marker. The 389 bp fragment, which acts as the positive control, is present in all PCR amplifications (lanes 1–9). The species-specific fragment (284 bp) is observed when the amplification reaction contains *C. zaeae-maydis* DNA and primer CzeaeHIST (lane 1, strain CBS 117757), *Cercospora* sp. DNA and primer CmaizeHIST (lane 5, strain CPC 12062) or *C. zeina* DNA and primer CzeinaHIST (lane 9, strain CPC 11995).

isolates of the new species (*C. zeina*, formerly *C. zaeae-maydis* Group II). The isolates of *C. sorghi* var. *sorghii* and *C. canescens* had ITS sequences similar to those of *C. zaeae-maydis* Group II (= *C. zeina*), but there was no bootstrap support for this branch. The third clade (78 % bootstrap support) contained isolates of *C. zaeae-maydis* (formerly *C. zaeae-maydis* Group I). The neighbour-joining and parsimony analyses provided trees with similar topologies (data not shown).

The combined data matrix contained 30 taxa (including the three outgroup taxa) and 1643 characters including alignment gaps. Of these characters, 406 were parsimony-informative, 10 were variable and parsimony-uninformative, and 1227 were constant. Parsimony analysis of the alignment yielded two most parsimonious trees (TL = 519 steps; CI = 0.948; RI = 0.986; RC = 0.935), one of which is shown in Fig. 2. Three distinct clades were obtained, the first (100 % bootstrap support) containing clades with *C. beticola* (90 % bootstrap support) and *C. apii* (100 % bootstrap support) with *Cercospora* sp. CPC 12062 as a sister taxon (100 % bootstrap support). Similar to the ITS tree, the *C. zeina* and *C. zaeae-maydis* isolates formed distinct and well-supported clades (each with a bootstrap support value of 100 %). Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values to that obtained using parsimony (data not shown).

### Development of a species-specific diagnostic test

Easy and rapid identification of *C. zaeae-maydis*, *C. zeina* and the new *Cercospora* sp. is possible using three multiplex PCR amplifications. A 389 bp fragment, which serves as the positive control, is present for all three species, while the second 284 bp fragment is only observed for the *Cercospora* species recognised by the specific internal primer (Fig. 3). Primers CzeaeHIST, CzeinaHIST, and CmaizeHIST are therefore specific for *C. zaeae-maydis*, *C. zeina* and the *Cercospora* sp., respectively, and can be used for their identification and detection.

### Taxonomy

***Cercospora zaeae-maydis*** Tehon & E.Y. Daniels, Mycologia 17: 248. 1925. Fig. 4.

**Leaf spots** oblong, forming extended streaks or irregular, greyish to brownish spots, shape and size variable, often with a narrow brown border line or margin. **Caespituli** amphigenous, mostly hypophyllous, punctiform to subeffuse, brown. **Mycelium** internal. **Stromata** lacking or small, with a few swollen substomatal brown cells. **Conidiophores** in small to moderately large fascicles (3–14), emerging through the stomata, usually divergent, erect, straight, subcylindrical to flexuous, distinctly geniculate–sinuous, unbranched, 40–180 × 4–8 μm, obscurely (0–)1–8-septate, uniformly pale olivaceous to medium brown, thin-walled, smooth; conidiogenous cells integrated, terminal, occasionally intercalary, 10–40 μm long, conidiogenous loci conspicuously thickened and darkened, 2–3 μm wide. **Conidia** solitary, broadly obclavate–subcylindrical, 30–100 × 4–9 μm, 1–10-septate, hyaline, thin-walled, smooth, apex obtuse, base obconically truncate, hila somewhat thickened and darkened, 2–3 μm wide (based on type specimen).

**Specimens examined:** U.S.A., Illinois, Alexander Co., McClure, on *Zea mays*, 29 Aug. 1924, P.A. Young (ILLS 4276) **holotype**, BPI 442569 **isotype**; Indiana, Princeton, 2003, B. Fleener, YA-03 = A358 = CBS 117755; Delaware, 1997, B. Fleener, DE-97 = A359 = CBS 117756; Wisconsin, Janesville, 2002, B. Fleener, **epitype designated here**, CBS H-17774, JV-WI-02 = A360 = CBS 117757, culture ex-type; Iowa, Johnston, 2004, B. Fleener, JH-IA-04 = A361 = CBS 117758; Tennessee, Union City, 1999, B. Fleener, UC-TN-99 = A362 = CBS 117759; Pennsylvania, New Holland, 1999, B. Fleener, NH-PA-99 = A363 = CBS 117760; Indiana, Princeton, 1999, B. Fleener, PR-IN-99 = A364 = CBS 117761; Missouri, Dexter, 2000, B. Fleener, DEXTER-MO-00 = A365 = CBS 117762; Iowa, Reinbeck, 1999, B. Fleener, RENBECK-IA-99 = A367 = CBS 117763.

**Cultural characteristics:** Colonies on PDA reaching 15–25 mm diam after 3 wk, and forming ample spermatogonia; colonies on MEA erumpent, with sparse aerial mycelium; margins smooth, but irregular; surface olivaceous-grey with irregular patches of white or smoke-grey; reverse iron-grey; colonies fertile. On OA colonies spreading with moderate aerial mycelium; margins smooth but irregular; surface red with patches of white and pale olivaceous-grey; fertile.

**Substrate:** *Zea mays*.

**Distribution:** Azerbaijan, Brazil, Cameroon, Canada, China, Colombia, Congo, Costa Rica, Ecuador,



**Fig. 4.** *Cercospora zeae-maydis*. A. Conidiophore with darkened, refractive conidiogenous locus. B. Germinating conidium. C–E. Conidia in vitro. Bars = 10  $\mu$ m.

Ethiopia, Georgia, Guatemala, Kenya, Malawi, Mexico, Mozambique, Nigeria, Panama, Peru, South Africa, Swaziland, Tanzania, Trinidad and Tobago, Uganda, USA (CO, DE, IA, IL, KS, KY, MD, MN, NC, OH, PA, SC, TN, VA, WI, WV), Venezuela, Zambia, Zimbabwe (Crous & Braun 2003).

***Cercospora zeina*** Crous & U. Braun, **sp. nov.**  
 MycoBank MB500863. Fig. 5.

*Cercospora zeae-maydis* affinis, a qua imprimis differt conidiophoris brevioribus (ad 100  $\mu$ m longis), conidiis late fusiformibus, coloniis in cultura crescentibus tardioribus, sine pigmento rubro.

**Leaf spots** amphigenous, confined by leaf veins, 2–3 mm wide, variable in length from 5–40 mm; lesions becoming confluent, pale grey to pale brown; borders indistinct, chlorotic in younger leaf spots. **Caespituli** fasciculate, amphigenous, punctiform to subeffuse, grey to brown on leaves, up to 120  $\mu$ m high and wide. **Mycelium** internal, consisting of pale brown, septate, branched, smooth hyphae, 3–4  $\mu$ m wide. **Stromata** lacking or small, a few swollen substomatal cells, brown, up to 30  $\mu$ m diam. **Conidiophores** aggregated (3–20) in loose to semi-dense fascicles arising from the upper cells of an inconspicuous brown stroma, emerging through stomata, usually divergent, erect, straight, subcylindrical to flexuous, distinctly geniculate-sinuuous, unbranched or branched above, 40–100  $\times$  5–7  $\mu$ m, 1–5-septate, uniformly pale olivaceous to medium brown, thin-walled, smooth; conidiogenous cells integrated, terminal, 40–60  $\times$  5–6  $\mu$ m, with several conidiogenous loci that are conspicuously thickened, darkened and refractive, 2–3  $\mu$ m wide. **Conidia** solitary, broadly fusiform, (40–)60–75(–100)  $\times$  (6–)7–8(–9)  $\mu$ m, (1–)3–5(–10)-septate, hyaline, thin-walled, smooth, apex subobtuse, base subtruncate, hila somewhat thickened, darkened and refractive, 2–3  $\mu$ m wide (based on type specimen).

**Specimen examined:** South Africa, KwaZulu-Natal, Pietermaritzburg, on *Zea mays*, 2005, P. Caldwell, CBS H-17775 **holotype**, CBS 118820 = CPC 11995, culture ex-type.

**Cultural characteristics:** Colonies on PDA reaching 10–15 mm diam after 3 wk, and forming spermatogonia; colonies on MEA erumpent, with sparse aerial mycelium; margins smooth, but irregular; surface olivaceous-grey with irregular patches of white or iron-grey; reverse iron-grey; colonies fertile. On OA colonies are spreading with moderate whitish aerial mycelium; margins smooth but irregular, olivaceous-grey; fertile.

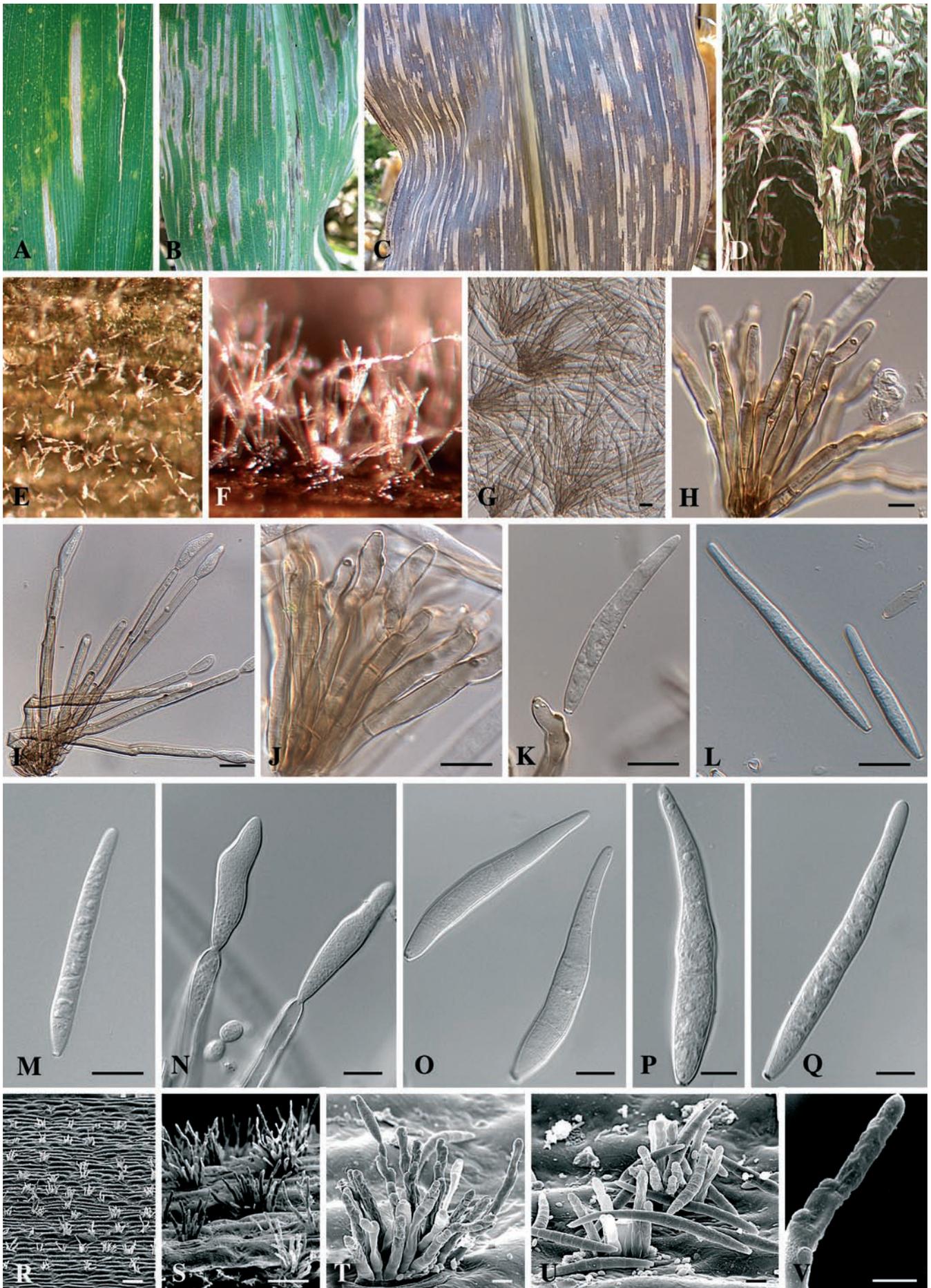
**Substrate:** *Zea mays*.

**Distribution:** South Africa, Uganda, U.S.A. (NC, NY, OH, VA), Zambia, Zimbabwe (Wang *et al.* 1998, Dunkle & Levy 2000).

**Notes:** *Cercospora zeae-maydis* has conidia of similar dimensions to those of *C. zeina*. However, *C. zeina* can be distinguished by having shorter conidiophores (up to 100  $\mu$ m) and more broadly fusiform conidia, versus longer conidiophores (up to 180  $\mu$ m) and broadly obclavate–subcylindrical conidia of *C. zeae-maydis*. Colonies of *C. zeina* grow more slowly in culture and lack the red pigment associated with cercosporin production, typical of *C. zeae-maydis* (Goodwin *et al.* 2001).

## DISCUSSION

In a recent review of grey leaf spot of maize, Ward *et al.* (1999) discussed the complexities and importance of this disease in the U.S.A., as well as in Africa. Several papers have commented on the disease being associated with two or more species (Wang *et al.* 1998, Dunkle & Levy 2000, Goodwin *et al.* 2001). A review



**Fig. 5.** *Cercospora zeina*. A–C. Close-up of grey leaf spot lesions on maize. D. Heavily infected plant. E–G. Conidiophores fascicles on leaf surface. H–J. Conidiophores. K, N. Conidiogenous cells giving rise to conidia. L–M, O–Q. Conidia. R–U. Scanning electron micrographs of conidiophores and conidia. V. Conidiogenous cell showing thickened loci. Scale bars: G–Q = 10  $\mu$ m, R = 100  $\mu$ m, S = 50  $\mu$ m, T–U = 8  $\mu$ m, V = 5  $\mu$ m.

of the literature suggests that there are two possible species complexes associated with grey leaf spot, namely the *C. sorghi* complex (*C. sorghi* and *C. sorghi* var. *maydis*), and the *C. zea-maydis* complex (Groups I and II).

The description of *C. zeina* has now resolved some of this taxonomic uncertainty, by demonstrating that Group II is, in fact, a distinct species (*C. zeina*) and that Group I, to which the name *C. zea-maydis* applies, apparently does not occur in South Africa. Further collections from other African countries, as well as other locations in South Africa would be required, however, to determine if *C. zea-maydis* is truly absent from the continent.

Grey leaf spot disease was first recorded from South Africa in 1988 (Ward *et al.* 1997). The possible source of inoculum was later postulated by Ward *et al.* (1999) to have been from infested maize residues imported from the U.S.A. However, as argued by Dunkle & Levy (2000), if this was indeed the case, such inoculum would have more likely contained *C. zea-maydis*, which dominates over *C. zeina* throughout most of the maize-producing areas of the eastern and midwestern U.S.A. Given the distribution of *C. zeina* throughout Africa and the fact that there is more genetic diversity of the pathogen in Africa than in the U.S.A. (Dunkle & Levy 2000), it was thought to be more likely that *C. zeina* was introduced to the U.S.A. from Africa, than *vice versa*. Dunkle & Levy (2000) also considered a third possibility, namely that *C. zeina* was introduced to Africa and the U.S.A. on another host, as maize is not native to Africa. However, the most likely hypothesis may be that *C. zeina* is indeed native to Africa, but that it has jumped from another indigenous host (such as sorghum) onto maize. It is interesting to note that the ITS sequence of the *C. zeina* isolates was more similar to that of an isolate of *C. sorghii* var. *sorghii* than to that of the presumably American species *C. zea-maydis*. Although they are morphologically distinct, further comparisons between *C. zeina* and *C. sorghi* are needed.

Although species of *Mycosphaerella* and their anamorphs are generally assumed to be host-specific (Corlett 1991, Crous & Braun 2003), some species have been observed to also have the ability to colonise hosts other than those on which they are assumed to be primary pathogens. This was recently observed for the greasy leaf-spot pathogen of *Citrus*, *Mycosphaerella citri* Whiteside, which was isolated from other hosts such as *Acacia* and *Musa* (Crous *et al.* 2004b). This finding subsequently led to the formulation of the pogo stick hypothesis (Crous & Groenewald 2005), where species of *Mycosphaerella* can jump to another host as a secondary colonizer, where they sporulate on lesions of the primary *Mycosphaerella* pathogen, producing enough inoculum to enable them to continue the search for their real host.

A further interesting finding was the isolation of a single, fast-growing isolate from grey leaf spot lesions caused by *C. zeina*. Although it was originally suspected that this isolate may represent *C. zea-maydis* (fast growing and forming a red pigment in agar), this has proven to not be the case. Morphologically this isolate

(CPC 12062) appeared more similar to isolates in the *Cercospora apii* complex (*C. apii* and *C. beticola*). Although only a few of the species in this complex are known from culture, CPC 12062 proved distinct based on DNA sequence data when compared to the more than 100 sequences currently available in our unpublished database. This isolate may represent an unrelated pathogen from another host that has “jumped” onto maize (Crous & Groenewald 2005). By using the PCR-based method described here as a diagnostic tool, it is relatively easy to identify the three *Cercospora* species on maize that are treated in this study.

Both *C. zea-maydis* and *C. zeina* have the ability to form ample spermatogonia on host tissue as well as in culture. Although there has been an earlier report of a possible *Mycosphaerella* teleomorph (Latterell & Rossi 1977), this has remained unconfirmed. Wang *et al.* (1998) were unable to find evidence of the MAT-2 mating type idiomorph in isolates of *Cercospora zea-maydis*, and our current mating studies with isolates of *C. zea-maydis* and *C. zeina* have also given negative results. Further population-level studies are thus needed to determine the level of variation present in populations, and whether sexual reproduction occurs within populations of these two fungi. Published results do not support the existence of cryptic sex, however, as Wang *et al.* (1998) reported the variation to be rather low in populations of both species.

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## Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*

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**Abstract:** Species of *Eucalyptus*, mostly native to Australia, are widely planted as exotics in the tropics and Southern Hemisphere. These plantations represent an important source of fuel-wood, structural timber and pulp. *Eucalyptus* plantations are, however, vulnerable to infection by pathogens, including *Mycosphaerella* spp. and their anamorphs, which have caused substantial damage, in many parts of the world. More than 30 species of *Mycosphaerella*, and close to 30 anamorph species for which the *Mycosphaerella* state remains unknown, are associated with leaf and shoot disease on *Eucalyptus* spp., worldwide. Although several studies using DNA sequence data have been applied to resolve the phylogenetic relationships between *Mycosphaerella* spp. on *Eucalyptus*, the number of species treated has been incomplete. In the present study, isolates of 44 *Mycosphaerella* species or their anamorphs associated with lesions on *Eucalyptus* leaves were compared based on DNA sequence data for the internal transcribed spacer region (ITS1 & ITS2) and the 5.8S gene. In addition, DNA sequence data from the elongation factor 1- $\alpha$  and the  $\beta$ -tubulin gene regions were used to resolve species in the *M. nubilosa* species complex. As a result of these comparisons, 11 new species are described. *Mycosphaerella juvenis* is reduced to synonymy with *M. nubilosa* and an epitype specimen and ex-epitype culture are designated for the latter. *Mycosphaerella nubilosa* is recorded as a serious agent of *Mycosphaerella* leaf blotch on *E. globulus* in Spain. This is also the first definitive record of this pathogen occurring on *Eucalyptus* in Europe.

**Taxonomic novelties:** *Mycosphaerella madeirae* Crous & Denman sp. nov., *M. toledana* Crous & G. Bills sp. nov. (anamorph *Phaeophleospora toledana* Crous & G. Bills sp. nov.), *M. readeriellophora* Crous & J.P. Mansilla sp. nov. (anamorph *Readeriella readeriellophora* Crous & J.P. Mansilla sp. nov.), *M. communis* Crous & J.P. Mansilla sp. nov. (anamorph *Dissoconium commune* Crous & J.P. Mansilla sp. nov.), *M. ohnowa* Crous & M.J. Wingf. sp. nov., *Passalora zambiae* Crous & T. Coutinho sp. nov., *Pseudocercospora pseudoeucalyptorum* Crous sp. nov., *Readeriella novaezealandiae* Crous sp. nov.

**Key words:** Ascomycetes, *Dissoconium*, DNA sequence comparisons, *Mycosphaerella*, *Passalora*, *Phaeophleospora*, *Pseudocercospora*, *Readeriella*, systematics.

## INTRODUCTION

Species of *Eucalyptus* L'Hérit., primarily native to Australia, are widely planted as exotics in the tropics, Mediterranean region and Southern Hemisphere. These plantations that cover more than 8 million hectares, sustain major industries producing timber products and pulp. They also represent important sources of income and fuel wood for resource-poor farmers. *Eucalyptus* spp. planted as exotics are well-known for their exceptional growth, probably due to the separation of these trees from their natural enemies (Wingfield 2001). However, diseases have had a serious negative impact on plantations in some parts of the world, and this is a situation that appears to be worsening. *Mycosphaerella* leaf blotch (MLB) was one of the first diseases to seriously damage plantations of *Eucalyptus* outside their native range (Crous 1998). For example, early plantations of *Eucalyptus*

*globulus* Labill. in South Africa were devastated by MLB, and the disease resulted in the abandonment of this species for plantation development (Purnell & Lundquist 1986).

Several species of *Mycosphaerella* Johanson, such as *M. cryptica* (Cooke) Hansf. and *M. nubilosa* (Cooke) Hansf., cause severe defoliation and leaf blotch symptoms, particularly of *E. globulus* and *E. nitens* Maiden in Australia, South Africa, and elsewhere (Carnegie *et al.* 1994, Crous & Wingfield 1996, Dungey *et al.* 1997). In New Zealand, *M. cryptica* is documented to have caused an epidemic in over 1000 ha of *E. delegatensis* R.T. Bak. (Cheah 1977). More recently, an asexual state of *Mycosphaerella*, *Phaeophleospora destructans* (M.J. Wingf. & Crous) Crous, F.A. Ferreira & B. Sutton, has begun to cause devastating leaf and shoot blight of *E. grandis* W. Hill ex Maiden, *E. camaldulensis* Dehnh. and hybrids of these and other species in South-East Asia (Wingfield *et al.*

1996). These fungi are clearly amongst the most important and most threatening pathogens of *Eucalyptus* spp., and they are likely to become increasingly important in the future.

*Mycosphaerella* is one of the largest genera of *Ascomycetes*, for which more than 2000 species names have been proposed (Corlett 1991). It also has several thousand anamorph species that lack known teleomorphs (Crous & Braun 2003). In a recent taxonomic treatment, Crous (1998) included 55 species that were known from *Eucalyptus*, although subsequent studies have shown that many more species are present on this host (Carnegie & Keane 1998, Braun & Dick 2002, Maxwell *et al.* 2003, Hunter *et al.* 2004).

Species identification in *Mycosphaerella* is extremely difficult. This is particularly because 4–5 different species frequently inhabit the same lesion, and these often also overlap in morphological characteristics. Ascospore germination patterns, characteristics of the fungi in culture and anamorph morphology, have made it possible to distinguish some of these taxa (Crous 1998). The more recent incorporation of DNA sequence data has allowed for more accurate species delimitation and has elucidated phylogenetic relationships in these fungi (Crous *et al.* 2000, 2001a, b). DNA sequence comparisons have, however, also shown that there can be several phylogenetic species encompassed in what have been perceived to represent well-defined morphological taxa (Crous *et al.* 2000, 2001a, b).

The aim of this study was to compare the largest possible number of *Mycosphaerella* species from *Eucalyptus*, based on DNA, cultural characteristics and morphology. In this way we wished to test the reliability of the morphological species defined by Crous (1998). All isolates used in the study were compared based on the sequences of their internal transcribed spacer region (ITS-1 & ITS-2) and the 5.8S gene. Furthermore, isolates of *M. juvenis* Crous & M.J. Wingf., a species that is morphologically similar to the important pathogen, *M. nubilosa*, were compared using sequences for the elongation factor 1- $\alpha$  and the  $\beta$ -tubulin gene regions.

## MATERIALS AND METHODS

### Isolates

Leaves showing symptoms of MLB or leaf and shoot blight associated with *Mycosphaerella* spp. and their anamorphs, were chosen for isolations. Excised lesions were placed in water for approximately 2 h, after which they were placed on double-sided tape and fastened to the insides of Petri dish lids, suspended over 2 % malt extract agar (MEA) (2 g/L) (Biolab, Midrand, South Africa). Germination patterns of ascospores were examined after 24 h, and single-ascospore and conidial cultures established as ex-

plained by Crous (1998). Colonies were sub-cultured onto carnation leaf agar (CLA) [1 % water agar (1 g/L) (Biolab) with autoclaved carnation leaves placed onto the medium] and incubated at 25 °C under continuous near-ultraviolet light, to promote sporulation. To resolve the ascospore germination patterns of *M. juvenis* and *M. nubilosa*, original material, slides and cultures used by Crous (1998) were re-examined. Fresh material was also studied from South Africa (Hunter *et al.* 2004), as well as Australia, New Zealand and Spain.

### DNA phylogeny

The protocol of Lee & Taylor (1990) was used to isolate genomic DNA from fungal mycelium, grown on MEA in Petri dishes. The primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify part of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. The PCR reaction mixture consisted of 0.75 units Biotaq (Bioline, London, U.K.), 1 $\times$  PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2  $\mu$ M of each dNTP, 5 pmol of each primer, approximately 10 to 30 ng of fungal genomic DNA and was made up to a total volume of 25  $\mu$ L with sterile water. Reactions were performed on a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA) and the cycling conditions consisted of denaturation for 5 min at 96 °C, followed by 30 cycles at 96 °C (30 s), 55 °C (30 s), 72 °C (90 s) and a final 7 min extension step at 72 °C to complete the reaction.

For isolates of *M. juvenis* and *M. nubilosa* part of the elongation factor 1- $\alpha$  (EF-1 $\alpha$ ) gene was amplified with primers EF1-728F and EF1-986R (Carbone & Kohn 1999) and part of the  $\beta$ -tubulin gene was amplified with primers T1 (O'Donnell & Cigelnik 1997) and Bt-2b (Glass & Donaldson 1995). PCR conditions for EF-1 $\alpha$  and  $\beta$ -tubulin genes were the same as those for ITS, except for the MgCl<sub>2</sub> concentration, which was increased to 2.0 mM for  $\beta$ -tubulin. PCR products were separated by electrophoresis at 80 V for 1 h in a 0.8 % (w/v) agarose gel in 0.5 $\times$  TAE running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, U.K.) following ethidium bromide staining. The amplification products were purified according to the manufacturer's instructions using a commercial kit (GFX PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech Europe GmbH, Germany). Sequencing reactions were carried out using the PCR primers in ABI PRISM Big Dye Terminator Cycle v 3.0 Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's recommendations. The reactions were analysed on an ABI Prism 3100 Genetic Analyser (Applied Biosystems).

**Table 1.** *Mycosphaerella* isolates included in this study for sequence analysis and morphological comparison.

Teleomorph	Anamorph	Accession no. <sup>1</sup>	Substrate	Country	Collector	GenBank accession <sup>3</sup>				
						ITS	EF	TUB		
Unknown	"Coniothyrium ovatum"	CBS 110906; CPC 40	<i>E. cladocalyx</i>	South Africa	P.W. Crous	AY725513				
		CBS 111149; CPC 23	<i>E. cladocalyx</i>	South Africa	P.W. Crous	AY725514				
		CBS 113621; CPC 42	<i>E. cladocalyx</i>	South Africa	P.W. Crous	AY725515				
		CBS 116427; CPC 10941	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	AY725516				
		CPC 18	<i>E. cladocalyx</i>	South Africa	P.W. Crous	AY725517				
Unknown	"Coniothyrium" sp.	CBS 116428; CPC 10886	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	AY725518				
Unknown	<i>Dissoconium aciculare</i>	CBS 201.89	<i>Brassica</i> sp.	Netherlands	T. Hijwegen	AY725519				
		CBS 204.89	<i>Astragalus</i> sp.	Germany	T. Hijwegen	AY725520				
		CBS 116429; CPC 10805	<i>Amorpha fruticosa</i>	Korea	H.D. Shin	AY725521				
Unknown	<i>Passalora zambiae</i>	CBS 112970 <sup>2</sup> ; CPC 1228	<i>E. globulus</i>	Zambia	T. Coutinho	AY725522				
		CBS 112971 <sup>2</sup> ; CPC 1227	<i>E. globulus</i>	Zambia	T. Coutinho	AY725523				
Unknown	<i>Pseudocercospora "eucalyptorum"</i>	CBS 116291; CPC 10503	<i>Eucalyptus</i> sp.	China	A. Aptroot	AY725525				
Unknown	<i>Ps. pseudoeucalyptorum</i>	CPC 10390 <sup>2</sup> ; CBS 114242	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725526				
		CPC 10500; CBS 114243	<i>E. nitens</i>	New Zealand	W. Gams	AY725527				
		CBS 116371; CPC 10507	<i>E. nitens</i>	New Zealand	W. Gams	AY725528				
Unknown	<i>Readeriella mirabilis</i>	CBS 116293; CPC 10506	<i>E. fastigata</i>	New Zealand	W. Gams	AY725529				
	<i>M. ambiphylla</i>	<i>Phaeophleospora</i> sp.	CBS 110499 <sup>2</sup>	<i>E. globules</i>	Australia	A. Maxwell	AY725530			
		CPC 4577	<i>Eucalyptus</i> sp.	Australia	A. Maxwell	AY725524				
<i>M. aurantia</i>	Unknown	CBS 110500 <sup>2</sup>	<i>E. globulus</i>	Australia	A. Maxwell	AY725531				
<i>M. colombiensis</i>	<i>Ps. colombiensis</i>	CBS 110967 <sup>2</sup> ; CPC 1104	<i>E. urophylla</i>	Colombia	M.J. Wingfield	AY725532				
		CBS 110968 <sup>2</sup> ; CPC 1105	<i>E. urophylla</i>	Colombia	M.J. Wingfield	AY725533				
		CBS 110969 <sup>2</sup> ; CPC 1106	<i>E. urophylla</i>	Colombia	M.J. Wingfield	AY725534				
<i>M. communis</i>	<i>Dissoconium commune</i>	CBS 110747; CPC 831	<i>E. nitens</i>	South Africa	P.W. Crous	AY725535				
		CBS 110809; CPC 830	<i>E. nitens</i>	South Africa	P.W. Crous	AY725536				
		CBS 110976; CPC 849	<i>E. cladocalyx</i>	South Africa	P.W. Crous	AY725537				
		CBS 111270; CPC 1190	<i>E. nitens</i>	South Africa	M.J. Wingfield	AY725538				
		CBS 112889; CPC 3359	<i>Protea magnifica</i>	Australia	P.W. Crous	AY725539				
		CBS 112890; CPC 1189	<i>E. nitens</i>	South Africa	M.J. Wingfield	AY725540				
		CPC 10440 <sup>2</sup> ; CBS 114238	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725541				
		CPC 10492; CBS 114239	<i>E. globulus</i>	New Zealand	W. Gams	AY725542				
		CBS 116284; CPC 10510	<i>E. globulus</i>	New Zealand	W. Gams	AY725543				
		CBS 116286; CPC 10515	<i>E. globulus</i>	New Zealand	W. Gams	AY725544				
		<i>M. ellipsoidea</i>	<i>Uwebraunia ellipsoidea</i>	CBS 110843 <sup>2</sup> ; CPC 850	<i>E. cladocalyx</i>	South Africa	P.W. Crous	AY725545		
		<i>M. intermedia</i>	Unknown	CPC 10902; CBS 114356	<i>E. saligna</i>	New Zealand	M. Dick	AY725546		
				CPC 10922; CBS 114415	<i>E. saligna</i>	New Zealand	M. Dick	AY725547		
<i>M. juvenis</i>	<i>Uwebraunia juvenis</i>	CPC 933 <sup>2</sup> ; CBS 115669	<i>E. nitens</i>	South Africa	M.J. Wingfield	AY725548	AY725582	AY725597		
		CBS 116292; CPC 934 <sup>2</sup>	<i>E. nitens</i>	South Africa	M.J. Wingfield	AY725549	AY725583	AY725598		
<i>M. lateralis</i>	<i>Uwebraunia dekkeri</i>	CBS 111169; CPC 1232	<i>E. globulus</i>	Zambia	T. Coutinho	AY725550				
		CBS 111272; CPC 1188	<i>E. nitens</i>	South Africa	M.J. Wingfield	AY725551				
		CBS 111282; CPC 1233	<i>E. globulus</i>	Zambia	T. Coutinho	AY725552				

<i>M. madeirae</i>	Unknown	CBS 112895; CPC 3745	<i>Eucalyptus</i> sp.	Madeira	S. Denman	AY725553		
<i>M. marksii</i>	Unknown	CBS 116290; CPC 10873	<i>E. botryoides</i>	New Zealand	M. Dick	AY725554		
		CBS 116285; CPC 10876	<i>E. botryoides</i>	New Zealand	M. Dick	AY725555		
		CBS 116288; CPC 10892	<i>E. botryoides</i>	New Zealand	M. Dick	AY725556		
		CBS 116287; CPC 10359	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725557		
<i>M. mexicana</i>	Unknown	CBS 110502	<i>E. globulus</i>	Australia	A. Maxwell	AY725558		
<i>M. molleriana</i>	<i>Colletogloeum molleriana</i>	CBS 116368; CPC 10391	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725559		
		CBS 116369; CPC 10394	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725560		
		CBS 116370; CPC 10397	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725561		
		CBS 111968; CPC 1079	<i>E. globulus</i>	Kenya	M.J. Wingfield	AY725562	AY725584	AY725599
		CBS 111969; CPC 1078	<i>E. globulus</i>	Kenya	M.J. Wingfield	AY725563	AY725585	AY725600
		CBS 112972; CPC 1007	<i>E. nitens</i>	South Africa	M.J. Wingfield	AY725564	AY725586	AY725601
		CBS 113064; CPC 4665	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725565	AY725587	AY725602
		CPC 10360; CBS 114241	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725566	AY725588	AY725603
		CPC 1099	<i>Eucalyptus</i> sp.	Tanzania	M.J. Wingfield	AY725567	AY725589	AY725604
		CPC 3722	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725568	AY725590	AY725605
		CBS 111445; CPC 4659	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725569	AY725591	AY725606
		CPC 4661	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725570	AY725592	AY725607
CPC 4663	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725571	AY725593	AY725608		
CBS 116005 <sup>2</sup> ; CPC 937	<i>E. globulus</i>	Australia	A. Carnegie	AY725572	AY725594	AY725609		
<i>M. "nubilosa"</i>	Unknown	CBS 116283; CPC 10495	<i>E. globulus</i>	New Zealand	W. Gams	AY725573	AY725595	AY725610
		CPC 10497; CBS 114419	<i>E. globulus</i>	New Zealand	W. Gams	AY725574	AY725596	AY725611
<i>M. ohnowa</i>	Unknown	CBS 110949 <sup>2</sup> ; CPC 1006	<i>E. grandis</i>	South Africa	M.J. Wingfield	AY725575		
<i>M. parva</i>	Unknown	CBS 116289; CPC 10935	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	AY725576		
<i>M. readeriellophora</i>	<i>R. readeriellophora</i>	CPC 10375 <sup>2</sup> ; CBS 114240	<i>E. globulus</i>	Spain	J.P.M. Vazquez	AY725577		
<i>Mycosphaerella</i> sp.	<i>R. novaezelandiae</i>	CPC 10895 <sup>2</sup> ; CBS 114357	<i>E. botryoides</i>	New Zealand	M. Dick	AY725578		
<i>M. suberosa</i>	Unknown	CPC 515 <sup>2</sup>	<i>E. dunnii</i>	Brazil	M.J. Wingfield	AY725579		
<i>M. toledana</i>	<i>Ph. toledana</i>	CBS 113313 <sup>2</sup>	<i>Eucalyptus</i> sp.	Spain	P.W. Crous	AY725580		
		CPC 10840; CBS 115513	<i>Eucalyptus</i> sp.	Spain	P.W. Crous	AY725581		

<sup>1</sup>CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS. <sup>2</sup>Ex-type cultures. <sup>3</sup>GenBank accession numbers for sequence data.



The ITS nucleotide sequences generated in this study were added to other sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov>) and the alignment was assembled using Sequence Alignment Editor v 2.0a11 (Rambaut 2002) with manual adjustments for visual improvement where necessary.

Phylogenetic analyses of sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2000). Phylogenetic analysis of the complete ITS alignment consisted of neighbour-joining analysis with the uncorrected ("p"), the Jukes-Cantor and the Kimura 2-parameter substitution model in PAUP. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. When they were encountered, ties were broken randomly.

For parsimony analysis of *M. juvenis* and *M. nubilosa* isolates, alignment gaps were treated as both a fifth character state and as missing and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxon additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Measures calculated for parsimony included tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC, respectively). The robustness of the resulting phylogenetic trees was evaluated by 1000 bootstrap replications (Hillis & Bull 1993) and the trees were printed with TreeView v. 1.6.6 (Page 1996). A partition homogeneity test (Farris *et al.* 1994) was conducted in PAUP to consider the feasibility of combining the various sequence data sets used for the *M. juvenis* and *M. nubilosa* isolates. Sequence data were deposited in GenBank (Table 1) and the alignments in TreeBASE (accession number S1157).

## Taxonomy

Wherever possible, thirty measurements ( $\times 1000$  magnification) were made of structures mounted in lactic acid, and the extremes of spore measurements given in parentheses. Colony colours (surface and reverse) were assessed after 1 mo on MEA at 25 °C in the dark, using the colour charts of Rayner (1970). All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands (Table 1).

## RESULTS

### DNA Phylogeny

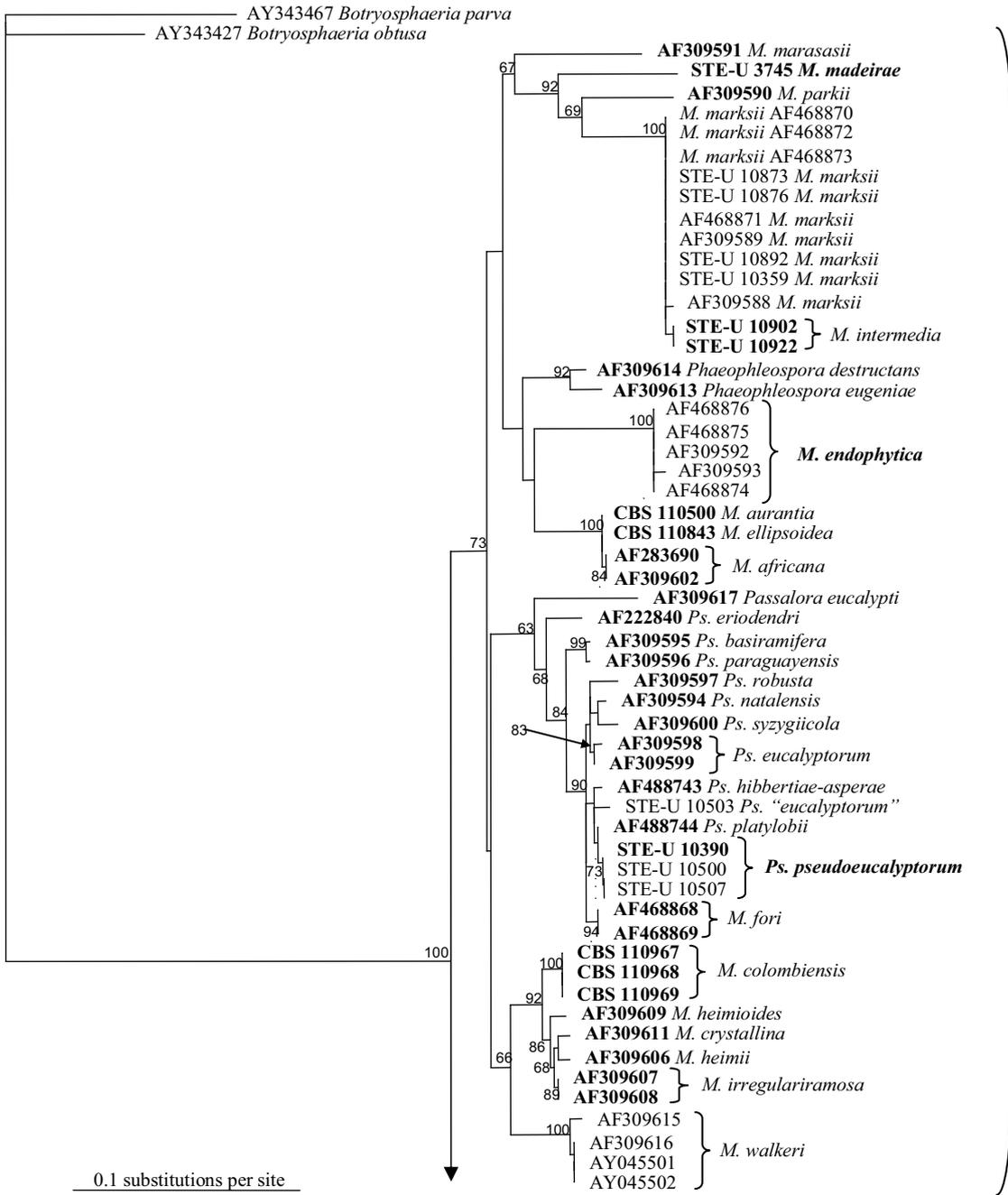
For the ITS region, approximately 500 to 560 bases were determined for all isolates (Table 1). The manually adjusted alignment of the ITS nucleotide sequences contained 134 taxa (including the two out-

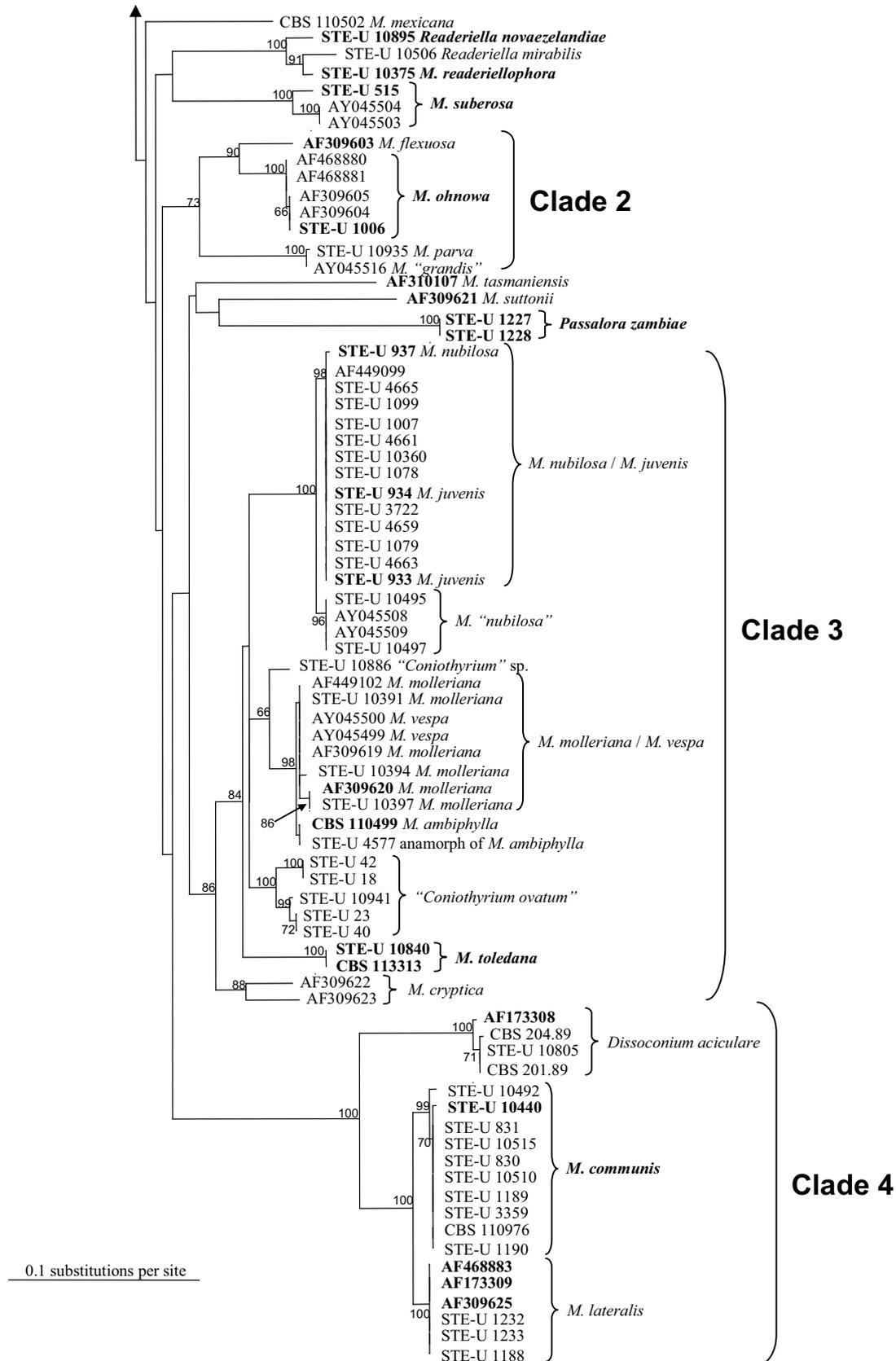
groups) and 572 characters including alignment gaps (TreeBASE accession number S1157). Neighbour-joining analysis using the three substitution models, yielded trees with similar topology and bootstrap values. The topology of the trees generated with the Jukes-Cantor and Kimura-2-parameter models were identical, whereas the uncorrected "p" model yielded a tree that differed from the other two models mainly in the higher hierarchy (data not shown). The distance tree obtained using the Kimura 2-parameter substitution model is shown in Fig. 1.

Four well-supported major clades, each containing several sub-clades, were delimited in the tree (Fig. 1). The major clade (clade 1) (73 % bootstrap support), contained a sub-clade (100 % bootstrap support) with isolates of *M. marksii* Carnegie & Keane and *M. intermedia* M. Dick & K. Dobbie. Clade 1 also included two *Phaeophleospora* Rangel species (92 % bootstrap support), a sub-clade of five isolates of *M. endophytica* Crous & H. Smith (100 % bootstrap support) and a sub-clade (100 % bootstrap support) containing *M. ellipsoidea* Crous & M.J. Wingf., *M. aurantia* A. Maxwell and two *M. africana* Crous & M.J. Wingf. isolates. Clade 1 also included a sub-clade with *Pseudocercospora* Speg. species as well as *Mycovellosiella eucalypti* Crous & Alfenas and two isolates of *M. fori* G.C. Hunter, Crous & M.J. Wingf. (63 % bootstrap support). Another sub-clade (66 % bootstrap support) included *M. colombiensis* Crous & M.J. Wingf., *M. irregulariramosa* Crous & M.J. Wingf. and *M. walkeri* R.F. Park & Keane, as well as single isolates of *M. heimioides* Crous & M.J. Wingf., *M. crystallina* Crous & M.J. Wingf. and *M. heimii* Crous.

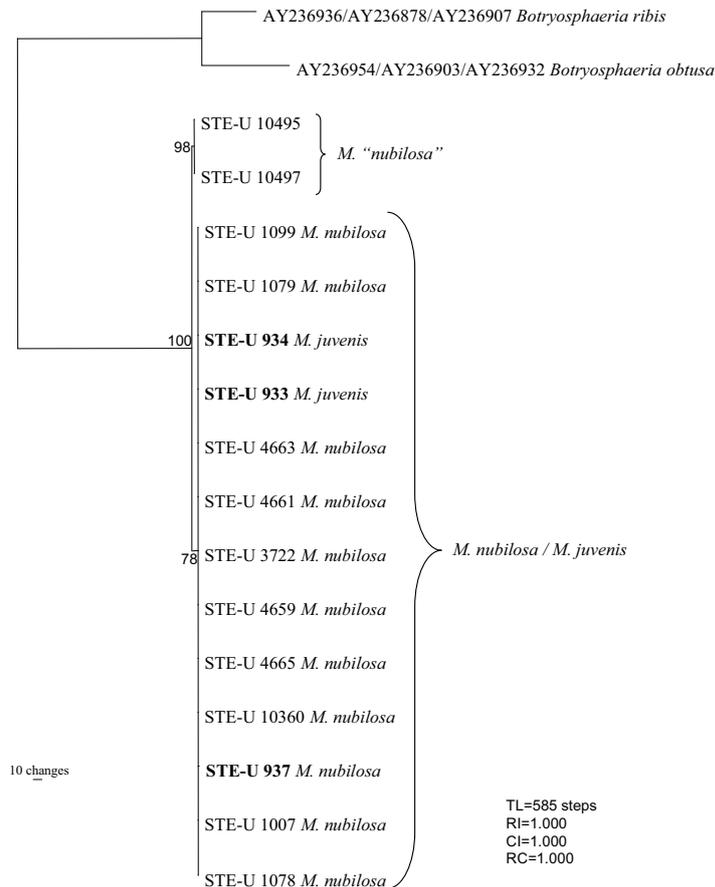
The second major clade (clade 2) in the phylogenetic tree (Fig. 1) (73 % bootstrap support) contained isolates and sub-clades that are basal to each other. Single isolates that did not form clear groupings with significant bootstrap support were those of *M. mexicana* Crous, *M. tasmaniensis* Crous & M.J. Wingf. and *M. suttonii* Crous & M.J. Wingf. Isolates of *Readeriella* Syd. and *M. readeriellophora* sp. nov. clustered together (100 % bootstrap support), as did those of *M. suberosa* Crous, F.A. Ferreira, Alfenas & M.J. Wingf. (100 % bootstrap support) and *Passalora zambiae* sp. nov. (100 % bootstrap support). However, these isolates did not form well-supported associations with other isolates in the tree. Clade 2 (Fig. 1) contained *M. flexuosa* Crous & M.J. Wingf. and sequences of *M. ohnowa*. A sequence of *M. parva* R.F. Park & Keane and *M. "grandis"* Carnegie & Keane grouped with a 100 % bootstrap support in this clade.

The third major clade (clade 3) (86 % bootstrap support) in the phylogenetic tree contained a well-supported sub-clade grouping *M. nubilosa* (Cooke) Hansf. and *M. juvenis* Crous & M.J. Wingf. isolates (98 % bootstrap support) that clustered together (100 % bootstrap support) with four other isolates that had tentatively been assigned to *M. "nubilosa"* (96 % bootstrap support).





**Fig. 1.** Neighbour-joining tree obtained from a distance analysis using the Kimura-2-parameter substitution model on ITS sequence data. The scale bar shows 0.1 substitutions per site and bootstrap replicate values from 1000 replicates are shown at the nodes (only values higher than 64 %). Ex-type strains are shown in bold print. The tree was rooted to two *Botryosphaeria* species.



**Fig. 2.** Single most parsimonious tree obtained from a heuristic search with 100 random taxon additions of a combined ITS, elongation factor 1- $\alpha$  and  $\beta$ -tubulin sequence alignment. The scale bar shows 10 changes and bootstrap replicate values from 1000 replicates are shown at the nodes. The tree was rooted to two *Botryosphaeria* species.

Clade 3 also included a well-supported sub-clade (98 % bootstrap support) containing *M. vespa* Carnegie & Keane and *M. molleriana* (Thüm.) Lindau isolates as well as isolates of *M. ambiphylla* A. Maxwell and its *Phaeophleospora* anamorph. This sub-clade also contained five isolates tentatively assigned to “*Coniothyrium ovatum*” H.J. Swart (100 % bootstrap support), a single isolate of a “*Coniothyrium*” sp., two isolates of *M. toledana* sp. nov. (100 % bootstrap support) and two isolates of *M. cryptica* (88 % bootstrap support).

Clade 4 (100 % bootstrap support) consisted of *Mycosphaerella* isolates with *Dissoconium* de Hoog, Oorschot & Hijwegen anamorphs and included four isolates of *Dissoconium aciculare* de Hoog, Oorschot & Hijwegen and two separate sub-clades, one with a bootstrap support value of 99 % containing *M. commune* sp. nov. isolates, and the other with a 100 % bootstrap support containing *M. lateralis* Crous & M.J. Wingf. isolates.

Approximately 500 bases of the  $\beta$ -tubulin gene and 300 bases of the EF-1 $\alpha$  were determined for isolates of *M. juvenis* and *M. nubilosa* and these were added to the alignment (TreeBASE accession number S1157). The manually adjusted alignment of the combined ITS, EF-1 $\alpha$  and  $\beta$ -tubulin nucleotide sequences contained seventeen isolates (including the two outgroups) and 1184 characters (489, 268 and

427 bases, respectively) including alignment gaps. Of the aligned nucleotide sites for the data set, 348 characters were parsimony-informative, 163 variable characters were parsimony-uninformative and 673 were constant. The results of the pairwise and combined partition homogeneity tests did not reject the null hypothesis of congruence ( $P = 1.000$  for all tests) and indicated that the ITS,  $\beta$ -tubulin and EF-1 $\alpha$  data sets could be combined. A single most parsimonious tree (Fig. 2) was obtained for the combined data and in this tree the two New Zealand isolates (bootstrap support value of 98 % for the group) grouped separately from the rest of the isolates, which formed a strongly supported clade (bootstrap = 78 %).

### Taxonomy

Results from the phylogenetic analysis have revealed five new species of *Mycosphaerella*, three of which have undescribed anamorphs, and a further three species that are known only from their anamorph states. Furthermore, these data also revealed that two species occurring on *Eucalyptus* should be reduced to synonymy. These species are described below.

*Mycosphaerella communis* Crous & J.P. Mansilla, **sp. nov.** MycoBank MB500050. Figs 3–10. *Anamorph*: *Dissoconium commune* Crous & J.P. Mansilla, **sp. nov.**

*Etymology*: Referring to the common occurrence of this species.

*Mycosphaerellae nubilosa* similis, sed coloniis avellaneis distinguenda.

*Leaf spots* amphigenous, sub-circular to circular, 4–12 mm diam, medium brown, surrounded by a thin, raised, concolorous border. *Ascomata* pseudothecial, hypophyllous, single, black, immersed becoming erumpent, globose, up to 120 µm diam; apical ostiole 10–15 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* paraphysate, fasciculate, bitunicate, sessile, obovoid to broadly ellipsoid, straight or slightly incurved, 8-spored, 35–50 × 10–14 µm. *Ascospores* 2–3-seriate, overlapping, hyaline, guttulate, thick-walled, straight to slightly curved, obovoid with subobtuse ends, medianly or unequally 1-septate, widest in middle of apical cell, or close to the apex of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (12–)13–15(–17) × (3.5–)4–4.5 µm *in vivo*.

*Dissoconium commune* Crous & J.P. Mansilla, **sp. nov.** MycoBank MB500051.

*Dissoconio dekkeri* simile, sed coloniis avellaneis distinguendum.

*Mycelium* internal and external, consisting of smooth, branched, septate, pale brown to olivaceous, 1.5–3 µm wide hyphae. *Conidiophores* arising from mycelium, single, 0–1-septate, smooth, medium brown, base subulate, upper part subcylindrical, simple or branched, 15–30 × 4–6 µm. *Conidiogenous cells* smooth, pale brown, subcylindrical, tapering to a truncate apex with 1–2 loci, straight to curved, 15–20 × 3–4 µm. *Conidia* terminal, pale olivaceous, smooth, obclavate with obtuse apex and obconical-truncate base, 0–1-septate, constricted at the septum, straight or curved, 20–30 × 4–5 µm (avg. 25 × 4.5 µm); hila inconspicuous. *Secondary conidia* developing from loci at the same level as the primary conidia, hyaline to pale olivaceous, aseptate, pyriform with a truncate base, 4–5 × 3–4 µm; hila inconspicuous.

*Holotypes*: **Spain**, Pontevedra, Lourizán, Areiro, on leaves of *E. globulus*, Dec. 2002, J.P. Mansilla, herb. CBS 9900, **holotype** of *M. communis* and *D. commune*; culture ex-type CBS 114238 = CPC 10440.

*Ascospore germination on MEA after 24 h*: Type F. Ascospores not darkening on MEA, and germinating

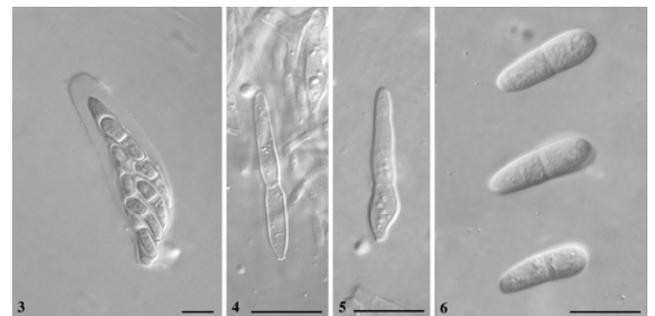
from both ends, with germ tubes parallel to the long axis of the spore, and distorting prominently upon germination, becoming 7–9 µm diam.

*Cultures*: Colonies irregular, erumpent, uneven, folded, aerial mycelium moderate to sparse, 19”i, hazel (surface), 27”m, olivaceous-black (reverse). Colonies reaching 20–35 mm diam on MEA after 1 mo at 25 °C in the dark; readily producing conidiophores of *D. commune* in culture after 14 d.

*Hosts*: *E. globulus*, *Protea* sp.

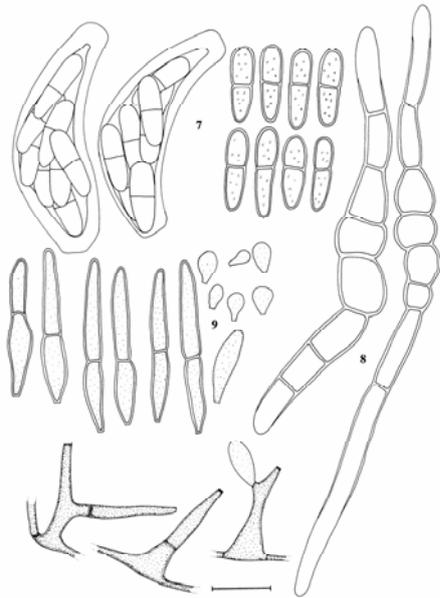
*Distribution*: Australia, New Zealand, South Africa, Spain.

*Notes*: *Mycosphaerella communis* is relatively common, and appears to have a wide host range beyond *Eucalyptus*, as well as a wide geographic distribution. In the past, isolates representing this species were erroneously treated as either *M. lateralis* or *M. juvenis* (= *M. nubilosa*). Although *M. lateralis* has a similar *Dissoconium* anamorph, its ascospores are fusoid–ellipsoidal, and are thus distinct from the obovoid ascospores of *M. communis*. Ascospore morphology of *M. communis* is similar to that of *M. nubilosa*, *M. ohnowa* and *M. readeriellophora*. In culture, colonies of *M. communis* are hazel in colour, while those of these other, morphologically similar species are pale olivaceous-grey (*M. nubilosa*), greenish black (*M. ohnowa*) or olivaceous (*M. readeriellophora*).



**Figs 3–6.** *Mycosphaerella communis* and its anamorph *Dissoconium commune* (CBS 114238). 3. Ascus. 4, 5. Conidia. 6. Ascospores. Scale bars = 10 µm.

*Additional specimens and cultures examined*: **Australia**, NSW, Mount Tomah Botanic Gardens, on leaves of *Protea magnifica*, Aug. 1999, P.W. Crous & B. Summerell, CPC 3359 = CBS 112889. **New Zealand**, on leaves of *E. globulus*, Feb. 2003, W. Gams, CPC 10510, 10515, 10492 = CBS 114239. **South Africa**, Western Cape province, Grabouw, on leaves of *E. nitens*, Nov. 1994, P.W. Crous (PREM 51914, cultures CPC 830–832, 831 = CBS 110747; KwaZulu-Natal, Seven Oaks Plantation, on leaves of *E. nitens*, 12 Jul. 1995, M.J. Wingfield (CPC 1188–1190 = CBS 111272, 112890, 111270).



**Figs 7–10.** *Mycosphaerella communis* and its anamorph *Dissoconium commune* (CBS 114238). 7. Asci and ascospores. 8. Germinating ascospores. 9. Primary and secondary conidia. 10. Conidiophores. Scale bar = 10  $\mu\text{m}$ .

***Mycosphaerella madeirae* Crous & Denman, sp. nov.** MycoBank MB500052. Figs 11–13.

*Anamorph:* *Pseudocercospora* sp. (unconfirmed).

*Etymology:* Named after the location from which it was collected.

*Mycosphaerellae heimioide* similis, sed ascosporis germinantibus ad septum non constrictis distinguenda.

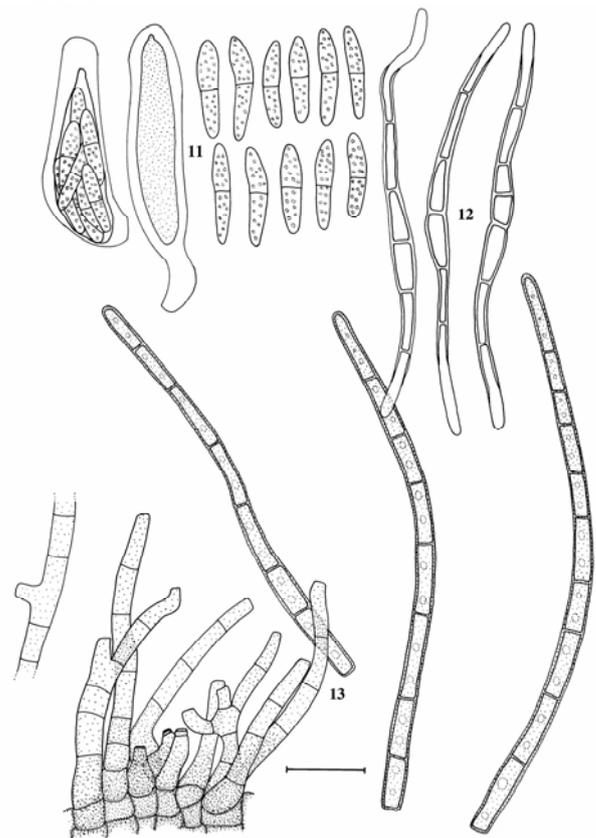
*Leaf spots* amphigenous, subcircular, 2–15 mm diam, medium brown, surrounded by a slightly raised, red-purple border. *Ascomata* pseudothecial, predominantly epiphyllous, single, black, immersed, becoming erumpent, globose, up to 120  $\mu\text{m}$  diam; apical ostiole 10–15  $\mu\text{m}$  diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, sessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 30–50  $\times$  8–12  $\mu\text{m}$ . *Ascospores* 3- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid–ellipsoid with subobtuse ends, apex frequently acutely rounded, medianly 1-septate, widest in the middle of the apical cell, not constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (9–)10–13(–15)  $\times$  2.5–3(–3.5)  $\mu\text{m}$  *in vivo*.

*Mycelium* internal and external, consisting of smooth, branched, septate, pale to medium brown, 3–6  $\mu\text{m}$  wide hyphae; external mycelium extensive on abaxial leaf surface. *Conidiomata* fasciculate, hypophyllous, medium brown, up to 90  $\mu\text{m}$  wide and 150  $\mu\text{m}$  high. *Conidiophores* arising from superficial mycelium, or aggregated in loose fascicles arising from the upper cells of a brown stroma up to 80  $\mu\text{m}$

wide and 90  $\mu\text{m}$  high; conidiophores pale to medium brown, smooth, unbranched or branched, 1–5-septate, subcylindrical, straight to variously curved, 15–45  $\times$  2.5–4  $\mu\text{m}$ . *Conidiogenous cells* terminal or lateral, unbranched, subcylindrical, pale brown, smooth, proliferating sympodially, or 1–4 times percurrently near apex, 7–15  $\times$  2.5–3  $\mu\text{m}$ ; conidial scars inconspicuous. *Conidia* solitary, pale brown, smooth, subcylindrical, but tapering from a subtruncate base towards a subobtuse apex, 3–6- or multiseptate, 35–85  $\times$  2.5–4  $\mu\text{m}$ ; hila inconspicuous.

*Specimen examined:* **Madeira**, Party Farm, on leaves of *E. globulus*, Apr. 2000, S. Denman, herb. CBS 9898 **holotype**, cultures ex-type CPC 3745 = CBS 112895, CPC 3747 = CBS 112301.

*Ascospore germination on MEA after 24 h:* Type C. Ascospores not darkening on MEA, and germinating from both ends, with germ tubes parallel to the long axis of the spore, and with no or slight constriction at the ascospore septum, with ascospores becoming 3–4  $\mu\text{m}$  diam.



**Figs 11–13.** *Mycosphaerella madeirae* and its presumed *Pseudocercospora* anamorph (holotype). 11. Asci and ascospores. 12. Germinating ascospores. 13. Conidia and conidiogenous cells. Scale bar = 10  $\mu\text{m}$ .

*Cultures:* Colonies olivaceous-grey (21''''1) on the surface, iron-grey (23''''k) in reverse; erumpent, folded, with sparse aerial mycelium, and a smooth, catenulate margin. Colonies 20–30 mm diam on MEA after 1 mo at 25  $^{\circ}\text{C}$  in the dark; teleomorph but no conidia formed in culture.

Host: *E. globulus*.

Distribution: Madeira.

Notes: *Mycosphaerella madeirae* is most similar to *M. heimioides* Crous & M.J. Wingf. (Crous 1998), but can be distinguished by its ascospore germination pattern as well as on its cultural characteristics. A *Pseudocercospora* occurred in close proximity to *M. madeirae*, but the connection between these states could not be established in culture and remains unconfirmed. The *Pseudocercospora* species resembled *P. robusta* in conidium shape, but was distinct in having paler conidia. As no cultures could be obtained of the *Pseudocercospora* species to facilitate a more detailed comparison, this fungus will not be treated further here.

***Mycosphaerella nubilosa*** (Cooke) Hansf., Proc. Linn. Soc. N.S.W. 81: 36. 1965. Figs 14–19.

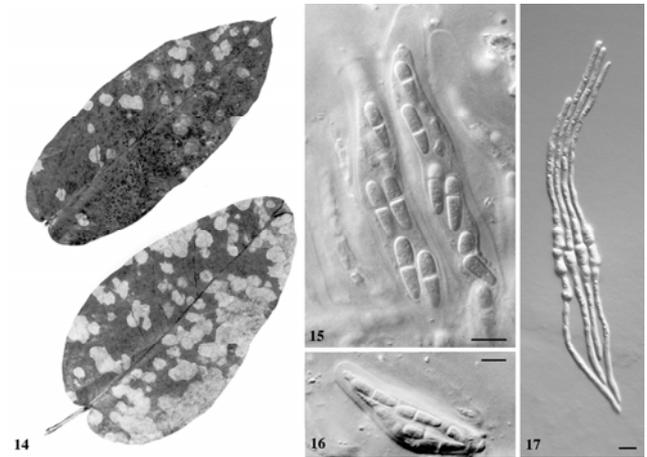
≡ *Sphaerella nubilosa* Cooke, Grevillea 19: 61. 1892.

= *Mycosphaerella juvenis* Crous & M.J. Wingf., Mycologia 88: 453. 1996.

Anamorph: *Uwebraunia juvenis* Crous & M.J. Wingf., Mycologia 88: 453. 1996.

*Leaf spots* amphigenous, varying from pin spots or flecks to small, round or irregular spots, frequently circular to irregular, up to 15 mm diam, becoming confluent to form larger blotches up to 3 cm diam on older leaves, pale brown, surrounded by a raised dark brown border, and a thin red-purple diffuse margin. *Ascomata* pseudothecial, amphigenous, predominantly hypophyllous, single, black, immersed, becoming erumpent, globose, up to 150 µm diam; apical ostiole 10–15 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, obovoid to ellipsoid, straight or incurved, 8-spored, 30–50(–68) × 9–14(–18) µm. *Ascospores* bi- to triseriate, overlapping, hyaline, non-guttulate, thin-walled, but the septum appearing thicker than the side walls, straight to slightly curved, obovoid with obtuse ends, medianly or unequally 1-septate, not or slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)13–14(–16) × (2.5–)3–4(–4.5) µm *in vivo*; apical cell (4–)5–6 µm, basal cell (4–)7–9 µm long.

*Types*: **Australia**, Victoria, Melbourne, on leaves of *Eucalyptus* sp., Martin 584 (K), **holotype**. **Australia**, Victoria, Briagalong, on leaves of *E. globulus*, 16 Sep. 1994, A. Carnegie, herb. CBS 9902 **epitype designated here**, ex-epitype culture CPC 937 = CBS 116005.



**Figs 14–17.** *Mycosphaerella nubilosa* (CBS 113064). 14. Leaf symptoms on upper and lower leaf surfaces of *Eucalyptus globulus* leaves. 15, 16. Asci. 17. Germinating ascospores. Scale bars = 10 µm.

*Ascospore germination on MEA after 24 h*: Type F, not type C as reported in Crous (1998). Ascospores not darkening on MEA, germinating from both ends, with germ tubes parallel to long axis of spore, with a gross distortion of the original spore; ascospores becoming 6–8 µm diam.

*Cultures*: Margins irregular but not feathery; surface folded; aerial mycelium moderate to sparse, more whitish in the centre, becoming pale olivaceous-grey, 23''''b, towards margins (surface), olivaceous-grey, 23''''i (reverse). Colonies 10–30 mm diam on MEA after 1 mo at 25 °C in the dark; conidiophores of *U. juvenis* rarely formed in culture.

*Hosts*: *E. bridgesiana*, *E. cytellocarpa*, *E. globulus*, *E. nitens* and *E. quadrangulata* (Crous 1998). Records on *E. grandis* and *E. botryoides* are doubtful.

*Distribution*: Australia, Kenya, New Zealand, South Africa, Spain, Tanzania, Zambia.

*Notes*: Confusion regarding the ascospore germination pattern for *M. nubilosa* (Park & Keane 1982, Crous & Wingfield 1996, Crous 1998), and the presence or absence of an anamorph, led Crous & Wingfield (1996) to describe *M. juvenis* as a distinct species, and also led Crous (1998) to conclude that *M. juvenis* was the major pathogen causing MLB on *E. globulus* and *E. nitens* in South Africa. Hunter *et al.* (2004) have, however, recently shown that *M. nubilosa* is the major pathogen on *E. nitens* in South Africa. Results of the present study show that *M. juvenis* should be treated as a synonym of this species.

Original slides of germinating ascospores in MEA were re-examined in this study, along with fresh collections obtained from Australia, South Africa, New Zealand and Spain. Germinating ascospores of *M. nubilosa* were seen to have the same germination pattern as that described for *M. juvenis* (type F), with

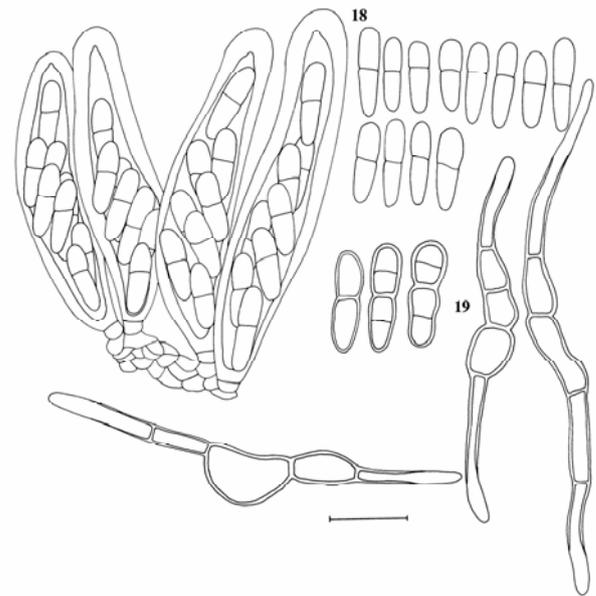
massive distortion within 24 h after germination. A re-examination of the original slide with germinating ascospores described by Crous (1998), received from A. Carnegie, showed that only 3 of the spores present had germinated. Hence the process had been terminated before 24 h had passed, and the germination pattern was described as type C. The same is presumably true for the illustrations provided by Park & Keane (1982). Fresh material studied from several plantations in South Africa, Spain, as well as a few randomly collected specimens from Australia and New Zealand, have shown that spores germinate, then become constricted (type C), and after 24 h become distorted (type F), similar to those observed for *M. juvenis* (Crous 1998).

**Additional specimens and cultures examined:** **Australia**, On leaves of *E. globulus*, Sep. 2000, M.J. Wingfield (CMW 6210 = CBS 114706, 6211 = CBS 114707). **Kenya**, on leaves of *E. globulus*, May 1995, T. Coutinho (PREM 54972, cultures CPC 1078 = CBS 111969, CPC 1079 = CBS 111968). **New Zealand**, on leaves of *E. globulus*, Feb. 2003, W. Gams (CPC 10495, 10497 = CBS 114419). **Spain**, Reboreda, on leaves of *E. globulus*, Dec. 2001, J.P. Mansilla (cultures CPC 4666, 4665 = CBS 113064); Lago, on leaves of *E. globulus*, Dec. 2001, J.P. Mansilla (cultures CPC 4659, 4660 = CBS 114445); Pontearreas, on leaves of *E. globulus*, Dec. 2001, J.P. Mansilla (cultures CPC 4661, 4662 = CBS 114513); Castrove, on leaves of *E. globulus*, Dec. 2001, J.P. Mansilla (cultures CPC 4663, 4664 = CBS 114244); Pontevedra, Lourizán, Areiro, on leaves of *E. globulus*, 2003, J.P. Mansilla (CPC 10360 = CBS 114241); on leaves of *E. globulus*, 2001, J.P. Mansilla, CPC 3722. **South Africa**, KwaZulu-Natal, Pietermaritzburg, on leaves of *E. nitens*, Jan. 1995, M.J. Wingfield (PREM 51910 teleomorph, PREM 51915 anamorph, cultures CPC 932–934, ex-type of *M. juvenis* and *U. juvenis*); KwaZulu-Natal, Pietermaritzburg, Bulwer, on leaves of *E. nitens*, June 2000, G.C. Hunter (CMW 9000); Mpumalanga, Witbank, on leaves of *E. grandis*, Mar. 1995, M.J. Wingfield (PREM 51913, cultures CPC 1007 = CBS 112972); KwaZulu-Natal, Pietermaritzburg, Bulwer, on leaves of *E. nitens*, May 2000, G.C. Hunter (CMW 9002); KwaZulu-Natal, Pietermaritzburg, Bulwer, on leaves of *E. nitens*, May 2000, G.C. Hunter (CMW 9003 = CBS 114708). **Tanzania**, on leaves of *Eucalyptus* sp., May 1995, T. Coutinho (PREM 54971, culture CPC 1099).

***Mycosphaerella ohnowa* Crous & M.J. Wingf., sp. nov.** MycoBank MB500053. Figs 20–23.

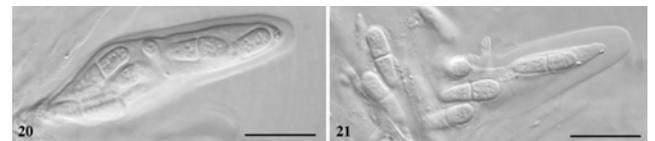
**Etymology:** Exclamation upon finding this morphologically nondescript, but genetically and culturally distinct taxon.

*Mycosphaerellae nubilosae* similis, sed coloniis mucidis viridi-atris distinguenda.



**Figs 18, 19.** *Mycosphaerella nubilosa* (CBS 113064). 18. Asci and ascospores. 19. Germinating ascospores. Scale bar = 10  $\mu$ m.

**Leaf spots** amphigenous, irregular to subcircular, 2–10 mm diam, medium brown, with a raised border which is red-brown on the adaxial surface, and medium brown on the abaxial surface. **Ascomata** pseudothecial, amphigenous, single, black, immersed, becoming erumpent, globose, up to 100  $\mu$ m diam; apical ostiole 5–10  $\mu$ m diam; wall of 2–3 layers of medium brown *textura angularis*. **Asci** paraphysate, fasciculate, bitunicate, sessile, obovoid to broadly ellipsoid, straight or slightly incurved, 8-spored, 40–60  $\times$  8–11  $\mu$ m. **Ascospores** 2–3-seriate, overlapping, hyaline, guttulate, thick-walled, straight, obovoid with obtuse ends, medianly to unequally 1-septate, widest near the apex of the apical cell, not constricted at the septum, tapering towards both ends, but more prominently towards lower end, (10–)12–14(–15)  $\times$  (3–)3–4  $\mu$ m *in vivo*.



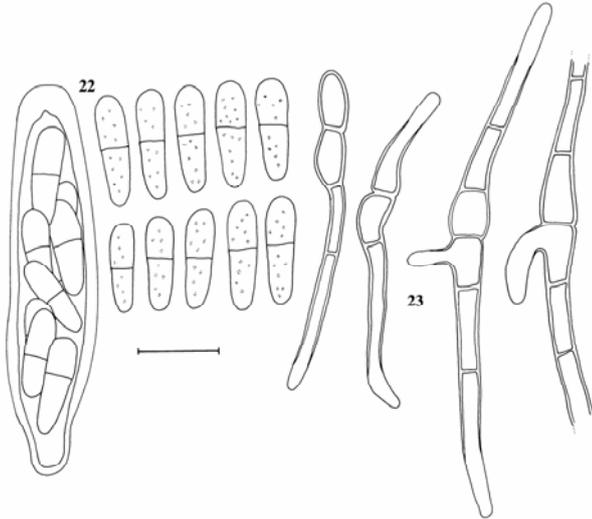
**Figs 20, 21.** *Mycosphaerella ohnowa* (PREM 51912). 20. Ascus. 21. Broken ascus with ascospores. Scale bar = 10  $\mu$ m.

**Holotype:** **South Africa**, Mpumalanga, Hazy View, on leaves of *E. grandis*, 27 Mar. 1995, M.J. Wingfield, PREM 51912 **holotype**; cultures ex-type CPC 1004 = CBS 112896, CPC 1005 = CBS 112973, CPC 1006 = CBS 110949.

**Ascospore germination on MEA after 24 h:** Type C. Ascospores not darkening on MEA, germinating predominantly from one end, but also from both ends, with germ tubes parallel to the long axis of the

spore, and with a constriction at the ascospore septum; ascospores becoming 3.5–5 µm diam.

**Cultures:** Colonies smooth, with extensive aerial mycelium that collapses with age, giving a flat, slimy surface, 33''''k, greenish black (surface and reverse); margins smooth. Colonies reaching 40–50 mm diam on MEA after 1 mo at 25 °C in the dark; cultures remaining sterile on a variety of media.



**Figs 22, 23.** *Mycosphaerella ohnowa* (PREM 51912). 22. Ascus with ascospores. 23 Germinating ascospores. Scale bar = 10 µm.

**Hosts:** *E. grandis*, *E. smithii*.

**Distribution:** South Africa.

**Notes:** *Mycosphaerella ohnowa* is morphologically similar to *M. nubilosa*, and is also associated with similar leaf spots, and hypophyllous fruiting. It can be distinguished, from the latter species by its colonies that become slimy, greenish black, whereas those of *M. nubilosa* are pale olivaceous-grey.

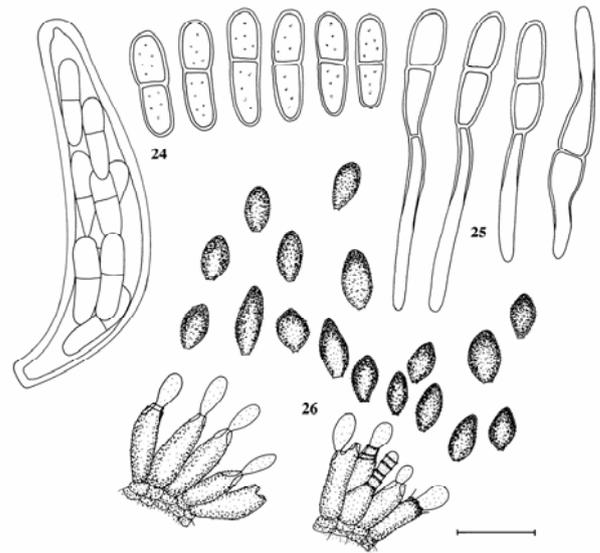
**Additional specimens and cultures examined:** **South Africa**, Eastern Cape Province, Umtata, on leaves of *E. grandis*, 2001, G.C. Hunter (CMW 9101 = CBS 113289); KwaZulu-Natal, Richmond, on leaves of *E. smithii*, Jun. 2000, G.C. Hunter (CMW 9102 = CBS 113290); KwaZulu-Natal, Richmond, on leaves of *E. smithii*, Jun. 2000, G.C. Hunter (CMW 9103 = CBS 113291).

***Mycosphaerella readeriellophora*** Crous & J.P. Mansilla, **sp. nov.** MycoBank MB500054. Figs 24–26.

**Anamorph:** *Readeriella readeriellophora* Crous & J.P. Mansilla, **sp. nov.**

**Etymology:** Named after the anamorph genus *Readeriella*.

*Mycosphaerellae nubilosae* similis, sed coloniis olivaceis distinguenda.



**Figs 24–26.** *Mycosphaerella readeriellophora* and its anamorph *Readeriella readeriellophora* (CBS 114240). 24. Ascus and ascospores. 25. Germinating ascospores. 26. Conidiogenous cells and conidia. Scale bar = 10 µm.

**Leaf spots** amphigenous, subcircular, 4–6 mm diam, grey to medium brown, with a raised, red-brown border. **Ascomata** pseudothecial, amphigenous, single, black, immersed becoming erumpent, globose, up to 90 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown *textura angularis*. **Asci** aparaphysate, fasciculate, bitunicate, sessile, obovoid to broadly ellipsoid, straight or slightly incurved, 8-spored, 35–60 × 8–11 µm. **Ascospores** 2–3-seriate, overlapping, hyaline, guttulate, thick-walled, straight, obovoid with obtuse ends, unequally 1-septate, widest in the middle of the apical cell, not to slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (11–)13–14(–16) × (3.5–)4–4.5 µm *in vivo*.

***Readeriella readeriellophora*** Crous & J.P. Mansilla, **sp. nov.** MycoBank MB500055.

*Readeriellae mirabili* similis, sed conidiis minoribus, (5–)6–7(–9) × (3–)4(–4.5) µm, distinguenda.

**Mycelium** internal, consisting of branched, septate, medium brown, smooth, 2.5–3.5 µm wide hyphae. **Conidiomata in vitro** pycnidial, globose to subglobose, up to 130 µm diam; wall of 2–4 layers of dark brown *textura angularis*. **Conidiogenous cells** discrete, doliiiform to subcylindrical, hyaline, smooth, monophialidic, rarely polyphialidic, with prominent periclinal thickening, but also percurrent, becoming pale yellow-brown, finely verruculose, later verruculose, green-brown, with irregular, percurrent proliferation and flared collarettes, 8–15 × 3–4 µm. **Conidia** holoblastic, solitary, ellipsoidal to limoniform, tapering towards a bluntly rounded, subobtuse, thickened apex, base subtruncate, initially hyaline, becoming yellow- to green-brown, and finally dark

brown, aseptate, finely verruculose, (5–)6–7(–9) × (3–)4(–4.5) µm; inconspicuous marginal frill present.

**Holotypes:** **Spain**, Pontevedra, Lourizán, Areiro, on leaves of *E. globulus*, 2003, J.P. Mansilla, herb. CBS 9901, **holotype** of *M. readeriellophora* and *R. readeriellophora*; culture ex-type for both morphs CBS 114240 = CPC 10375.

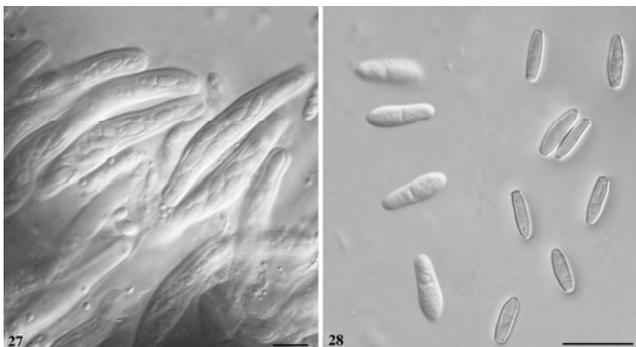
**Ascospore germination on MEA after 24 h:** Type C. Ascospores not darkening on MEA, germinating predominantly from one end, but also from both ends, with germ tubes parallel to the long axis of the spore, and with a constriction at the ascospore septum; ascospores becoming 4–5 µm diam.

**Cultures:** Colonies with extensive, pale brown aerial mycelium, surface becoming slimy, with green-brown masses of exuding conidia becoming visible in older cultures; colonies 21''k, olivaceous (surface), 27'''m, olivaceous-black (reverse); reaching 50 mm diam on MEA after 1 mo at 25 °C in the dark; readily forming conidiomata of *R. readeriellophora* in culture.

**Host:** *E. globulus*.

**Distribution:** Spain.

**Notes:** *Mycosphaerella readeriellophora* is morphologically similar to *M. nubilosa*, and is also associated with similar leaf spots, and hypophyllous fruiting on *E. globulus*. It can be distinguished from the latter species by its colonies that are olivaceous, producing a *Readeriella* anamorph, while those of *M. nubilosa* are pale olivaceous-grey, sterile, or produce an *Uwebraunia* anamorph. Furthermore, ascospores of *M. readeriellophora* do not distort on MEA (type C), while those of *M. nubilosa* and *M. ohnowa* do (type F).



**Figs 27, 28.** *Mycosphaerella toledana* and its anamorph *Phaeophleospora toledana* (CBS 113313). 27. Asci. 28. Ascospores (left) and conidia (right). Scale bars = 10 µm.

***Mycosphaerella toledana* Crous & G. Bills, sp. nov.** MycoBank MB500056. Figs 27–31.

**Anamorph:** *Phaeophleospora toledana* Crous & G. Bills, sp. nov.

**Etymology:** Named after the location from which it was collected.

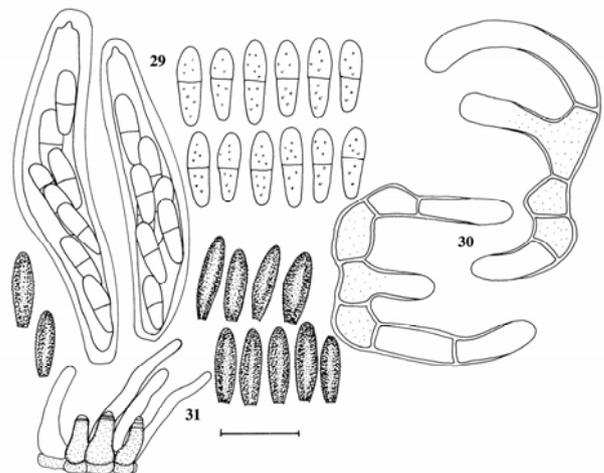
*Mycosphaerellae ellipsoideae* similis, sed ascosporis fusciscentibus multis hyphis germinantibus distinguenda.

**Leaf spots** amphigenous, irregular to subcircular or angular, frequently confined by leaf veins, 3–6 mm diam, medium brown, with or without a red-purple border, spots aggregating with age, forming irregular blotches. **Ascomata** pseudothecial, amphigenous, single, black, immersed becoming erumpent, globose, up to 80 µm diam; apical ostiole 10–20 µm diam; wall of 2–3 layers of medium brown *textura angularis*. **Asci** aparaphysate, fasciculate, bitunicate, subsessile, narrowly ellipsoid, straight or slightly incurved, 8-spored, 35–50 × 8–10 µm. **Ascospores** 2–3-seriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid–ellipsoid with subobtuse ends, medianly 1-septate, widest in the middle of the apical cell, not constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (7–)8–10(–11) × 3(–3.5) µm.

***Phaeophleospora toledana* Crous & G. Bills, sp. nov.** MycoBank MB500057.

*Colletogloeopsi nubilosae* (Ganap. & Corbin) Crous & M.J. Wingf. similis sed pycnidiis clausis et conidiis minoribus, (8–)10–12(–14) × (2.5–)3–3.5(–4) µm, distinguenda.

**Mycelium** internal, consisting of smooth, branched, septate, medium brown, 3–4 µm wide hyphae. **Conidiomata** amphigenous, pycnidial, substomatal; wall consisting of 3–4 layers of *textura angularis*. **Conidiophores** reduced to conidiogenous cells.



**Figs 29–31.** *Mycosphaerella toledana* and its anamorph *Phaeophleospora toledana* (CBS 113313). 29. Asci and ascospores. 30. Germinating ascospores. 31. Conidiogenous cells, paraphyses and conidia. Scale bar = 10 µm.

*Conidiogenous cells* ampulliform to subcylindrical, pale brown, smooth to finely verruculose, proliferating 1–3 times percurrently near apex,  $6\text{--}10 \times 3\text{--}4 \mu\text{m}$ ; occurring intermixed between hyaline, smooth, subcylindrical, aseptate paraphyses,  $10\text{--}20 \times 2\text{--}2.5 \mu\text{m}$ . *Conidia* fusoid with acutely rounded apices and truncate bases, medium brown, verruculose, aseptate,  $(8\text{--})10\text{--}12\text{--}(14) \times (2.5\text{--})3\text{--}3.5\text{--}(4) \mu\text{m}$ ; base with minute marginal frill.

*Holotypes*: **Spain**, Toledo, on leaves of *Eucalyptus* sp., May 2003, P.W. Crous & G. Bills, herb. CBS 9896, **holotype** of *M. toledana* and *P. toledana*; culture ex-type of both morphs CBS 113313.

*Ascospore germination on MEA after 24 h*: Type E. Ascospores becoming pale brown on MEA, germinating from both ends, with multiple germ tubes growing irregular to the long axis of spore, with prominent distortion; ascospores becoming  $5\text{--}6 \mu\text{m}$  diam.

*Cultures*: Colonies smooth, irregular, with moderate aerial mycelium, grey in the centre, white towards the margin, 21''''b, grey-olivaceous (surface), 15''''i, greyish sepia (reverse). Colonies reaching 55 mm diam on MEA after 1 mo at 25 °C in the dark; cultures sterile.

*Host*: *Eucalyptus* sp.

*Distribution*: Spain.

*Notes*: The only other *Mycosphaerella* species known from *Eucalyptus* that has a type E germination pattern is *M. suberosa* (Crous 1998). *Mycosphaerella toledana* can easily be distinguished from *M. suberosa* by its smaller, fusoid–ellipsoid ascospores, and a *Phaeophleospora* anamorph.

*Additional specimen examined*: **Spain**, Ribera del Alberche, on leaves of *Eucalyptus* sp., May 2003, P.W. Crous & G. Bills, herb. CBS: 9897, culture CPC 10840 = CBS 115513.

***Passalora zambiae* Crous & T. Coutinho, sp. nov.** MycoBank MB500058. Figs 32, 33.

*Etymology*: Named after the country from which it was collected.

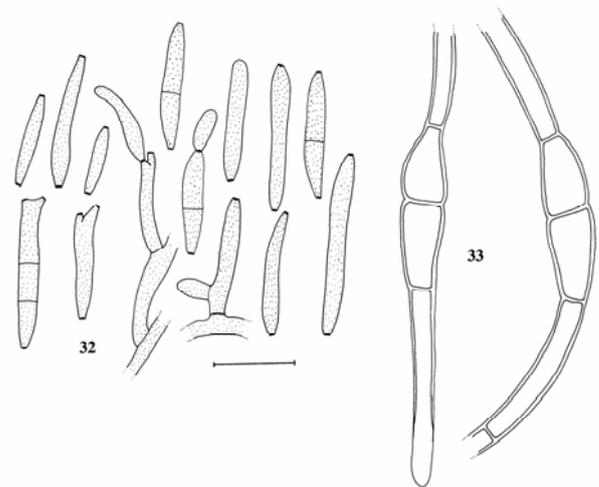
*Passalorae morrisii* similis, sed conidiis minoribus, 0(–2)-septatis, anguste ellipsoideis,  $10\text{--}20 \times 2\text{--}3 \mu\text{m}$ , distinguenda.

*Leaf spots* amphigenous, subcircular, 3–10 mm diam, medium brown, surrounded by a raised, brown border. *Mycelium* consisting of smooth to rough, irregularly branched, septate, brown, 2–7  $\mu\text{m}$  wide hyphae; frequently with hyphal swellings that develop into thick-walled, dark brown chlamydospore-like struc-

tures, up to 15  $\mu\text{m}$  diam. *Conidiophores* arising from the mycelium, medium brown, smooth, branched or unbranched, 0–2-septate, subcylindrical, straight to variously curved,  $10\text{--}30 \times 2\text{--}4 \mu\text{m}$ . *Conidiogenous cells* terminal and intercalary, subcylindrical, tapering to truncate apices, pale to medium brown, smooth, proliferating sympodially,  $10\text{--}30 \times 2\text{--}4 \mu\text{m}$ ; conidial scars conspicuous, darkened, refractive. *Conidia* catenulate, chains simple or branched, medium brown, smooth, narrowly ellipsoidal, tapering to subtruncate, with flattened ends, straight or slightly curved, 0(–2)-septate,  $10\text{--}20 \times 2\text{--}3 \mu\text{m}$  *in vitro*.

*Holotype*: **Zambia**, on leaves of *E. globulus*, 21 Aug. 1995, T. Coutinho, herb. CBS 9895 **holotype**; cultures ex-type CPC 1227 = CBS 112971, CPC 1228 = CBS 112970).

*Ascospore germination on MEA after 24 h*: Type I. Ascospores not darkening on MEA, germinating from both ends, with germ tubes parallel to the long axis of the spore, and with a constriction at the ascospore septum; ascospores becoming 4–5  $\mu\text{m}$  diam. Lateral branches also commonly observed 24–48 h after germination.



**Figs 32, 33.** *Passalora zambiae* (CBS 112971). 32. Conidia and conidiogenous cells. 33. Germinating ascospores. Scale bar = 10  $\mu\text{m}$ .

*Cultures*: Colonies irregular with smooth margins, and sparse aerial mycelium, 21''''i, olivaceous-grey (surface), 27''''m, olivaceous-black (reverse). Colonies reaching 30 mm diam on MEA after 1 mo at 25 °C in the dark; colonies initially producing conidia of *P. zambiae*, but becoming sterile upon transfer.

*Host*: *E. globulus*.

*Distribution*: Zambia.

*Notes*: This species is phylogenetically distant from other *Mycosphaerella* spp. known from *Eucalyptus*. Only a slide preparation with asci and ascospores is available for the teleomorph of this fungus, and this

is insufficient on which to base a description of this state. However, it is clear that the fungus resembles other species in the *M. nubilosa* complex that occur on *E. globulus*. The anamorph has been observed only in culture. The cultures described here were derived from germinating ascospores.

***Pseudocercospora pseudoecalyptorum* Crous, sp. nov.** MycoBank MB500059. Figs 34, 35.

**Etymology:** Morphologically similar to *P. eucalyptorum*.

*Pseudocercosporae eucalyptorum* similis, sed conidiomatus brunneis et conidiis medio-brunneis distinguenda.

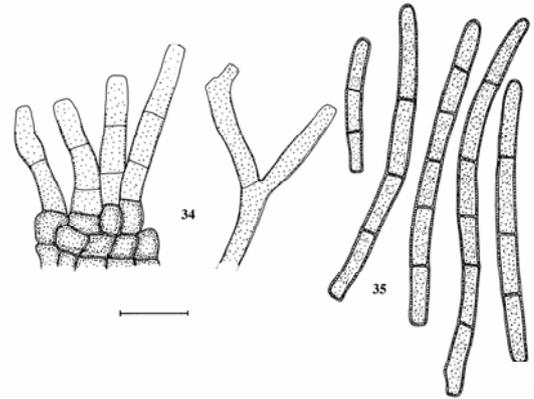
**Leaf spots** amphigenous, subcircular to angular, 3–10 mm diam, pale to medium brown, surrounded by a raised, brown border. **Conidiomata** amphigenous, brown (not grey as in *P. eucalyptorum*); stromata lacking to well developed, brown, 10–100 µm diam. **Mycelium** internal and external, consisting of smooth, branched, septate, medium brown, 2.5–4 µm wide hyphae; external mycelium extensive on the abaxial leaf surface. **Conidiophores** in small, loose or dense fascicles arising from the upper cells of a brown stroma, or from superficial hyphae; conidiophores medium brown, smooth, branched or unbranched, 0–2-septate, subcylindrical, straight to geniculate-sinuuous, 10–50 × 2.5–5 µm. **Conidiogenous cells** terminal, subcylindrical, tapering to truncate or bluntly rounded apices, medium brown, smooth, proliferating sympodially, 10–30 × 2.5–4 µm; conidial scars inconspicuous. **Conidia** solitary, pale brown, smooth, cylindrical, bases truncate, apices bluntly rounded, thick-walled with irregular swellings, straight or curved, 3–7-septate, (25–)59–70(–90) × 2.5–3(–4) µm *in vivo*, 30–65 × 2.5–3 µm, 3–6-septate *in vitro*; hila inconspicuous.

**Holotype:** Spain, Pontevedra, Lourizán, Areeiro, on leaves of *E. globulus*, 2003, J.P. Mansilla, herb. CBS 9893 **holotype**; culture ex-type CPC 10390 = CBS 114242.

**Cultures:** Colonies folded, with irregular, smooth margins; aerial mycelium sparse to moderate, 21''''i, olivaceous-grey (surface), 27''''m, olivaceous-black (reverse). Colonies reaching 25 mm diam on MEA after 1 mo at 25 °C in the dark; cultures fertile.

**Host:** *E. globulus*.

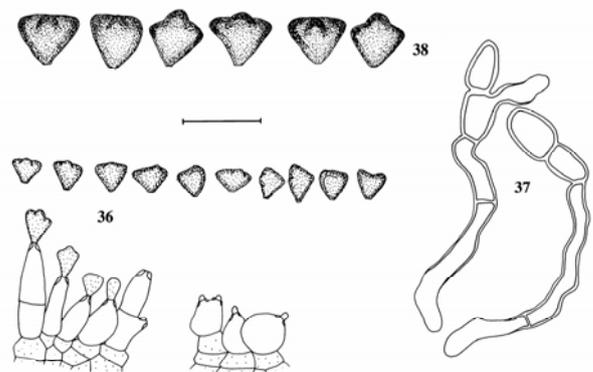
**Distribution:** ?China, Spain, New Zealand.



**Figs 34, 35.** *Pseudocercospora pseudoecalyptorum* (CBS 114242). 34. Conidiophores. 35. Conidia. Scale bar = 10 µm.

**Notes:** *Pseudocercospora pseudoecalyptorum* differs from *P. eucalyptorum* in having brown, not grey conidiomata, and conidia that are medium brown and not olivaceous in colour. The dimensions of the conidia in these two species overlap. A further collection obtained from China (CBS 10503), also belongs to this complex, but appears to represent a distinct species. The Chinese collection was from a *Eucalyptus* sp. with amphigenous, medium brown, angular to irregular leaf spots, 2–6 mm diam, frequently delimited by leaf veins. This particular collection had abundant superficial mycelium, and conidia that were paler in colour than *P. pseudoecalyptorum*, but similar in length, (25–)50–70(–75) × 2.5–3 µm, thus resembling those of *P. eucalyptorum*. Because the Chinese material needed to be incubated for sporulation to occur, the morphological features of the fungus under natural conditions are not known. For this reason, we have chosen not to describe this species until additional material can be obtained.

**Additional specimens and cultures examined:** China, Yunnan Province, Lunan Co., 5 km NE of Lunan, Chuxiong town, garden of Zixishan Hotel, 1700 m alt., on leaf litter of *Eucalyptus* sp., 27 Oct. 2002, A. Aptroot, Herb. CBS 9894, culture CBS 10503. New Zealand, on leaves of *E. nitens*, Feb. 2003, W. Gams, CPC 10500 = CBS 114243, CPC 10507.



**Figs 36–38.** *Readeriella* species. 36. Conidia and conidiogenous cells of *R. novaezealandiae*. 37. Germinating ascospores of the *Mycosphaerella* teleomorph of *R. novaezealandiae* (CBS 114357). 38. Conidia of *R. mirabilis* (CPC 10506). Scale bar = 10 µm.



*Readeriella novaezelandiae* Crous, sp. nov.  
MycoBank MB500060. Figs 36, 37.

*Teleomorph*: *Mycosphaerella* sp.

*Etymology*: Named after the country where this fungus was collected.

*Readeriellae mirabilis* similis, sed conidiis minoribus, 3–5 µm longis et latis, distinguenda.

Occurring on leaf spots associated with *M. marksii*, which is dominant and assumed to be the primary pathogen. *Leaf spots* irregular to subcircular, medium brown to red-brown, margins raised, 2–15 mm diam. Intact pseudothecia not observed, but epiphyllous remnants intermixed with those of *M. marksii*. *Mycelium* internal, consisting of branched, septate, medium brown, smooth, 3–4 µm wide hyphae. *Conidiomata in vitro* pycnidial, aggregated, globose to subglobose, up to 400 µm diam; wall of 4–6 layers of medium brown *textura angularis*. *Conidiophores* hyaline, smooth, subcylindrical to doliiform or reduced to conidiogenous cells and ovoid, 0–1-septate, 10–25 × 2–4 µm. *Conidiogenous cells* doliiform to subcylindrical or ovoid, hyaline, smooth, mono- or polyphialidic, with prominent periclinal thickening, 5–15 × 2–4 µm. *Conidia* holoblastic, solitary, aseptate, pale to medium brown, finely verruculose, base subtruncate, apex flattened with three lateral, obtuse projections, deltoid, whole conidia 3–5 µm long and wide.

*Holotype*: New Zealand, North Island, Kerikeri, on leaves of *E. botryoides*, 17 Oct. 2003, M.A. Dick, herb. CBS 9892 **holotype**, cultures ex-type CBS 114357 = CPC 10895).

*Ascospore germination on MEA after 24 h*: Type D. Ascospores not darkening on MEA, germinating predominantly from both ends, but with an irregular germination pattern; germ tubes varying in width, appearing irregular, and with a prominent constriction at the ascospore septum; ascospores 3.5–5 µm diam upon germination.

*Cultures*: Colonies with moderate, grey, fluffy aerial mycelium, which is interspersed with slimy black dots representing aggregated, black pycnidia exuding brown, slimy conidial masses; surface pale mouse-grey (15°"d), with feathery margins, reverse olivaceous (19°"k); reaching 50 mm diam on MEA after 1 mo at 25 °C in the dark; cultures fertile.

*Host*: *E. botryoides*.

*Distribution*: New Zealand.

*Notes*: *Readeriella novaezelandiae* is morphologically similar to *R. mirabilis* (Fig. 38), but it can be distinguished by its smaller conidia, 3–5 µm long and

wide, in contrast to the larger conidia of *R. mirabilis*, 7–9.5 µm long, and 7–9 µm wide. Cultures of *R. novaezelandiae* were obtained from single ascospores. However, these were few in number, and no mature pseudothecia were found on the leaves, precluding a description of the *Mycosphaerella* teleomorph. Further collections are required to fully elucidate the morphology of this species.

## DISCUSSION

This study has included the largest number of isolates of *Mycosphaerella* spp. from *Eucalyptus* that has ever been considered based on DNA sequence comparisons. Comparisons using ITS sequence data for this large collection, followed by those including three gene regions for isolates in the *M. nubilosa* species complex, have shown the presence of many new species of *Mycosphaerella*. All of these species can be identified based on a combination of morphological and cultural characteristics, but unequivocal identifications demand DNA sequence data. A similar situation is arising with many other fungi, such as for example *Fusarium* spp. (O'Donnell *et al.* 2000) where large numbers of cryptic species are emerging from DNA sequence comparisons.

It might seem surprising that there should be in excess of 60 species of *Mycosphaerella* on *Eucalyptus*. However, this needs to be viewed against the background that there are more than 700 species of *Eucalyptus* (Potts & Pederick 2000). Many plant species are infected by more than one species of *Mycosphaerella* (Crous & Mourichon 2002, Crous & Braun 2003, Taylor *et al.* 2003), and we might expect that many more species of *Mycosphaerella* will be found on *Eucalyptus* in the future. This is clearly a group of fungi that has undergone extensive radiation, presumably associated with the substantial variation in the host genus.

The description of *Pseudocercospora eucalyptorum* Crous *et al.* (1989) resolved considerable confusion regarding species of coelomycetes that were initially described as *Cercospora eucalypti* Cooke & Massee and *C. epicoccoides* Cooke & Massee (Chupp 1954). These fungi were later placed in *Kirramyces* J. Walker, B. Sutton & Pascoe (Walker *et al.* 1992), and subsequently transferred to *Phaeophleospora* Rangel (Crous *et al.* 1997). An assemblage of different morphological species, however, remained aggregated under the epithet *Pseudocercospora eucalyptorum*. This confusing situation was later addressed by Crous (1998), who also provided a key to the various species of *Pseudocercospora* Speg. occurring on *Eucalyptus*. The species occurring in New Zealand were treated by Braun & Dick (2002), which led to the description of several new taxa, of which, *P. pseudobasitruncata* U. Braun & M. Dick, appears to be synonymous with *P. sublata* Z.Q. Yuan, de Little & C. Mohammed (Yuan *et al.* 2000),

described at approximately the same time from Australia.

Based on its morphology, *P. eucalyptorum* is accepted to have a wide geographic distribution, occurring on many different species of *Eucalyptus* (Crous 1998, Braun & Dick 2002). The present study is the first to consider this species based on DNA sequence data. These comparisons (Fig. 1), show clearly that *P. eucalyptorum* represents a species complex, and that further collections are required to fully recognise these cryptic species.

Recent collections from *Eucalyptus* leaves in South Africa have revealed a *Mycosphaerella* species that resembles *M. africana* Crous & M.J. Wingf. in morphology (CPC 10935). However, this fungus differs from *M. africana* in having ascospores that germinate at an angle from one end of the ascospore (Type N), thus closely fitting the pattern of *M. parva* R.F. Park & Keane. The ITS sequences for this isolate is identical to an unpublished sequence deposited in GenBank for *M. grandis* Carnegie & Keane (AY145516), which Crous (1998) considered a synonym of *M. parva*. Maxwell *et al.* (2003) report that *M. parva* is widespread in Australia, but this is the first record of the species from South Africa.

Park *et al.* (2000) regarded *Readeriella mirabilis* Syd. & P. Syd. as a common saprobe or secondary colonist of leaf spots caused by other primary pathogens such as *M. cryptica* and *Tracylla aristata* (Cooke) Tassi. Furthermore, *R. mirabilis* has been recorded from a range of eucalypt species in Australia, New Zealand, the U.K. and Brazil (Sutton 1980). The fact that *Readeriella* Syd. & P. Syd. (typified by *R. mirabilis*) belongs to *Mycosphaerella*, is surprising. An isolate of *R. mirabilis* obtained from New Zealand (CPC 10506), was phylogenetically closely related to a new species of *Mycosphaerella* from Spain, *M. readeriellophora*, which also has a *Readeriella* anamorph. Furthermore, ascospores of a *Mycosphaerella* sp. obtained from New Zealand produced the new species, *Readeriella novaezealandiae* in culture. The genus *Readeriella* now includes three species, which all occur on *Eucalyptus*.

One of the major agents of MLB disease of *Eucalyptus* is *M. nubilosa* (Carnegie & Ades 2002). *Mycosphaerella nubilosa* has few definitive morphological characters, and also resembles several other species occurring on *Eucalyptus*. For this reason, its taxonomy has been confused and controversial. The first modern treatment of *M. nubilosa*, including ascospore germination studies, was provided by Park & Keane (1982). Later, Crous *et al.* (1991) argued that *M. nubilosa* should be treated as a synonym of *M. molleriana*, but after obtaining fresh collections, Crous & Wingfield (1996) showed that the two species were distinct. This distinction was given strong support using some of the first DNA sequence comparisons for *Mycosphaerella* species (Crous *et al.* 2001a). In South Africa, this species has been a serious impediment to the propagation of *E. globulus*

and certain provenances of *E. nitens* (Crous 1998). The causal agent of the disease has been ascribed to either *M. molleriana* (Thüm.) Lindau (Doidge 1950, Crous *et al.* 1991), or *M. nubilosa* (Lundquist & Purnell 1987). In a later study using morphological characteristics, Crous & Wingfield (1996) described a morphologically similar species, *M. juvenis*. Subsequently Crous (1998) regarded this fungus as the major causal agent of leaf blotch on *E. nitens* in South Africa. In a later DNA-based comparison, Crous *et al.* (2001a) identified some isolates as *M. juvenis* based on morphology (CBS 112973); they were shown to be phylogenetically distant from *M. nubilosa* and *M. molleriana*. However, the ex-type strain of *M. juvenis* was not included in that analysis.

Hunter *et al.* (2004) sampled several *E. nitens* plantations in the KwaZulu-Natal province of South Africa. Although *M. juvenis* was present (determined based on morphology, and sequence similarity to strains presumed to be *M. juvenis* *vide* Crous *et al.* 2001a), the dominant pathogen on *E. nitens* in South Africa was found to be *M. nubilosa*. Ex-type cultures of *M. juvenis* that were subjected to DNA sequence analysis in the present study, were shown to be identical to *M. nubilosa*. Furthermore, germinating ascospores of *M. nubilosa* were shown to initially have some constriction at the median septum (type C), but to distort prominently after 24 h (type F). Because the exact time that germination of ascospores was terminated by Park & Keane (1982) and Crous (1998) did not correspond, confusion resulted over the exact germination pattern of *M. nubilosa*, and what was later to be described as *M. juvenis*. This distinction between strains was further supported by the fact that some strains of *M. juvenis* produced an *Uwebraunia* anamorph (Crous & Wingfield 1996), while those of *M. nubilosa* did not.

Several *Mycosphaerella* collections from Africa (South Africa, Kenya, Tanzania, Zambia), were originally identified based on morphology, as representing *M. juvenis*. Cultures, however, were reported by Crous (1998) to be variable in colour, and in some, the *Uwebraunia* anamorph formed readily (brown colonies), whereas it formed with difficulty in the olivaceous-black to grey-olivaceous colonies. DNA sequence comparisons in this study showed that these isolates included several distinct species. These isolates are associated with similar symptoms on *Eucalyptus* leaves, hypophyllous fruiting, and have similar asci, ascospores, and ascospore germination patterns (type F). Surprisingly, the majority of these isolates, including the ex-type cultures of *M. juvenis*, were shown to represent *M. nubilosa*. This suggests that *M. nubilosa* has *U. juvenis* as its anamorph. This anamorph is rarely observed in culture, and upon sub-culturing, strains lose their ability to produce conidia. The fungus with brown colonies that readily formed the anamorph (Crous 1998), was shown to represent a new species that is described here as *M. communis*. This fungus appears to have a



wide host range, including *Protea* spp. in Australia (Crous *et al.* 2000). The cultures originally sequenced and regarded as *M. juvenis* (Crous *et al.* 2001a), represent a third species in this complex, namely *M. ohnowa*.

*Mycosphaerella nubilosa* is a serious pathogen of *E. globulus* and *E. nitens*. The presence of this pathogen has recently been confirmed from South Africa (Hunter *et al.* 2004), and in the present study; it has also been identified from several populations collected during 2001 and 2002 on *E. globulus* in four regions in Spain, namely Lago, Pontearreas, Castrove and Reboredo. This is the first definitive record of *M. nubilosa* on eucalypts in Spain, and probably Europe. Although it is not known how long this pathogen has been present in Spain, it is likely to present a serious threat to *E. globulus* on the continent.

Although all *Mycosphaerella* spp. presently known from *Eucalyptus* that are available in culture were included in the current study, analysis of sequence data resulted in only one species being reduced to synonymy. This clearly emphasises the fact that there is more, rather than less variation amongst the *Mycosphaerella* spp., which have been described on *Eucalyptus*. It is highly probable that additional collections from *Eucalyptus* will reveal new species. Furthermore, once additional genes are sequenced, other cryptic species will be revealed within presently accepted morphological species. At present, nearly all of these species, other than those in the clade with *Dissoconium* anamorphs, appear to be specific to *Eucalyptus*. It will be interesting to note whether this will remain true, as additional species of *Mycosphaerella* spp. from other hosts are also currently being subjected to DNA sequence comparisons.

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## Cryptic speciation and host specificity among *Mycosphaerella* spp. occurring on Australian *Acacia* species grown as exotics in the tropics

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**Abstract:** Species of *Mycosphaerella* and their anamorphs represent serious pathogens of two phyllodenous species of *Acacia*, *A. mangium* and *A. crassicarpa*. In recent years, these fungi have been collected during surveys in South America and South-East Asia, where these trees are widely planted as exotics. In this study, the *Mycosphaerella* spp. and their anamorphs were identified based on morphological and cultural characteristics. Identifications were confirmed using comparisons of DNA sequences for the internal transcribed spacers (ITS1 & ITS2), the 5.8S rRNA gene, elongation factor 1- $\alpha$ , histone 3, actin and calmodulin gene regions. The data revealed six new taxa, of which three are named in this study, along with their anamorphs. *Cercospora acaciae-mangii*, which is morphologically part of the *C. apii sensu lato* species complex, is distinguished based on its distinct phylogeny. *Mycosphaerella acaciigena*, collected in Venezuela, is distinguished from *M. konae* and *M. heimii*, and described as new. *Mycosphaerella thailandica*, a new species occurring on *Acacia* and *Musa*, is shown to be a sibling species to *M. colombiense*, a foliar pathogen of *Eucalyptus*. *Mycosphaerella citri*, an important leaf and fruit pathogen of *Citrus* (*Rutaceae*), is shown to also occur on *Musa* (*Musaceae*) and *Acacia* (*Leguminosae*).

**Taxonomic novelties:** *Cercospora acaciae-mangii* Crous, Pongpanich & M.J. Wingf. sp. nov., *Mycosphaerella acaciigena* Crous & M.J. Wingf. sp. nov. (anamorph *Pseudocercospora acaciigena* Crous & M.J. Wingf. sp. nov.), *Mycosphaerella thailandica* Crous, Himaman & M.J. Wingf. sp. nov. (anamorph *Pseudocercospora thailandica* Crous, Himaman & M.J. Wingf. sp. nov.).

**Key words:** *Acacia*, *Ascomycetes*, *Cercospora*, *Mycosphaerella*, *Pseudocercospora*, *Stenella*, systematics.

### INTRODUCTION

Plantations of exotic tree species in the tropics and Southern Hemisphere sustain important industries producing solid wood products and pulp. In many situations, they provide an alternative to logging of native forest trees and they contribute substantially to the economies of many developing countries. The most extensively planted trees in these plantations are species of *Pinus* L., *Eucalyptus* L'Herit. and *Acacia* L. Australian *Acacia* species have been planted as exotics in the tropics and Southern Hemisphere for many years. Until relatively recently, however, these have been less extensively planted than *Pinus* or *Eucalyptus* spp. In areas with temperate climates, *Acacia* spp. with pinnate leaves such as *Acacia mearnsii* De Wild. and *A. dealbata* Link are planted, although on a limited scale. More recently, phyllodenous *Acacia* spp. such as *Acacia mangium* Willd., *A. crassicarpa* A. Cunn. ex Benth. and *A. auriculiformis* A. Cunn. ex Benth. have been planted extensively in plantations in the tropics (Old *et al.* 2000).

The success of exotic plantation forestry can, to some extent, be attributed to the separation of trees from their natural enemies (Wingfield *et al.* 2001). In terms of *Acacia* spp., virtually nothing is known regarding the diseases that affect these trees, particularly where they are planted as exotics. A preliminary synthesis of the diseases of phyllodenous *Acacia* spp. was made by Old *et al.* (2000), and from this study it was clear that many pathogens were poorly defined and required rigorous taxonomic study.

Leaf and shoot pathogens belonging to the genus *Mycosphaerella* Johanson, have had a very distinct impact on plantations in the tropics and Southern Hemisphere. The pine pathogen *Dothistroma septosporum* (Dorog.) M. Morelet (teleomorph *M. pini* E. Rostrup) that has devastated plantings of *P. radiata* D. Don in many Southern Hemisphere countries is one example (Stone *et al.* 2003). Likewise, species of *Mycosphaerella* have had a very marked impact on *Eucalyptus* species planted in this area. For example, *Mycosphaerella* leaf blight resulted in the abandonment of *E. globulus* Labill. as a plantation species in South Africa (Purnell & Lundquist 1986), and this and

other species in the genus continue to seriously threaten *Eucalyptus* plantings (Crous 1998).

Species of *Mycosphaerella* and its anamorphs have been recorded on phyllodinous *Acacia* spp. grown in the tropics (Old *et al.* 1996). These fungi have tentatively been recognised as members of two anamorph genera of *Mycosphaerella*, namely *Cercospora* Fresen. and *Pseudocercospora* Speg. (Old *et al.* 1996, Cannon *et al.* 1997). However, no intensive taxonomic studies have been conducted on these fungi, and the names used are tentative. Although the disease is known to occur widely on species of *Acacia* (Fig. 1), the correct identity of the causal organisms remains unresolved. This again has negative implications for disease management and quarantine programmes, which are aimed at restricting the movement of pathogens between countries.

This present study results from a collection of *Mycosphaerella* species and their anamorphs on two phyllodinous species of *Acacia*, *A. mangium* and *A. crassicaarpa*, which are widely planted as exotics in the tropics and the Southern Hemisphere. These fungi have been collected in surveys in South America and South-East Asia during the course of the past four years. Their identification will hopefully contribute to a better understanding of their biology and the diseases that they cause. Identification of species included both morphological and cultural characteristics. More importantly for this group of fungi, however, identifications were also confirmed using comparisons of DNA sequences for the internal transcribed spacer (ITS1 & ITS2) and the 5.8S regions of the ribosomal RNA operon, as well as the elongation factor 1- $\alpha$ , histone, actin and calmodulin gene regions.



Fig. 1A–D. Typical *Mycosphaerella* leaf blotch symptoms on *Acacia mangium* leaves collected in Thailand.



## MATERIALS AND METHODS

### Isolates

Symptomatic leaves with leaf spots or blight were chosen for isolations. Excised lesions were placed in distilled water for approximately 2 h, after which they were placed on double-sided tape and fastened to the insides of Petri dish lids, suspended over 2 % malt extract agar (MEA) (Biolab, Midrand, South Africa). Germinating ascospores were examined after 24 h, and single-ascospore and conidial cultures established as explained by Crous (1998). Colonies were sub-cultured onto oatmeal agar (OA) (Gams *et al.* 1998) and incubated at 25 °C under continuous near-ultraviolet light, to promote sporulation.

### DNA phylogeny

Genomic DNA was isolated from fungal mycelium grown on malt extract agar plates following the protocol of Lee & Taylor (1990). The primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify part (ITS) of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. Part of the elongation factor 1- $\alpha$  gene (EF) was amplified with primers EF1-728F and EF1-986R, part of the actin gene (ACT) with primers ACT-512F and ACT-783R and part of the calmodulin gene (CAL) with primers CAL-228F and CAL-737R (Carbone & Kohn 1999). Part of the histone H3 gene (HIS) was amplified with primers H3-1a and H3-1b (Glass & Donaldson 1995). PCR conditions and protocols, as well as alignment of the subsequent data and DNA phylogeny were treated and generated as explained in Crous *et al.* (2004b) elsewhere in this volume. Sequence data were deposited in GenBank (Table 1) and the alignments in TreeBASE (study accession number S1178). Uniquely fixed characters were identified by manual comparison of the aligned *Cercospora* sequences and unique character positions were calculated using the sequences of *C. apii* CPC 5087 and *C. acaciae-mangii* CPC 10526 as references. The nucleotides shown in the description represent the *C. acaciae-mangii* allele.

### Taxonomy

Fungal structures were mounted in lactic acid. The extremes of spore measurements (30 observations) are given in parentheses. Colony colours (surface and reverse) were rated after 1–2 mo on OA at 25 °C in the dark, using the colour charts of Rayner (1970). All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands (Table 1).

## RESULTS

### DNA Phylogeny

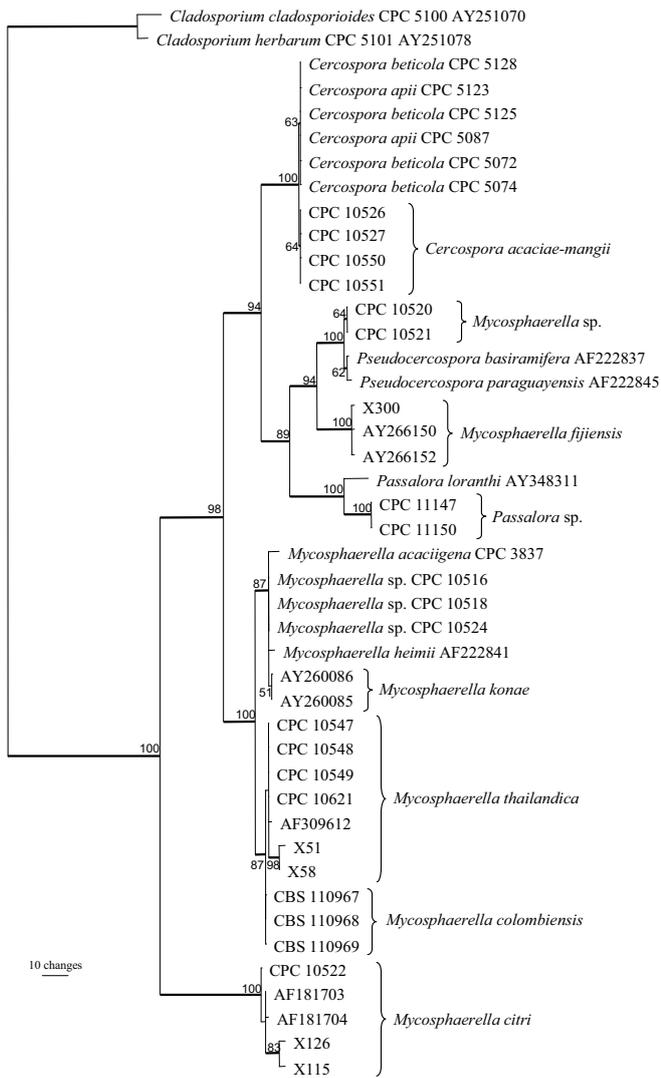
For each of the five loci sequenced, approximately 500, 320, 230, 320 and 395 bases were determined for ITS, EF, ACT, CAL, and HIS, respectively. A partition homogeneity test using the sequence data showed that only some loci could be combined ( $p > 0.05$ ) in a phylogenetic analysis and these were ITS / ACT ( $p = 0.131$ ), ACT / CAL ( $p = 0.698$ ), ACT / HIS ( $p = 0.186$ ), CAL / HIS ( $p = 0.430$ ) and ACT / CAL / HIS ( $p = 0.145$ ). Therefore, the ITS dataset, which contains additional sequences obtained from GenBank and for which sequence data for the other loci were not available, and the EF dataset were analysed separately and the ACT, CAL and HIS datasets were combined into a single analysis.

The manually adjusted alignment of the ITS sequences contains 44 taxa (including the two out-groups) and 521 characters including alignment gaps (TreeBASE study accession number S1178). Of these characters, 195 are parsimony-informative, 11 are variable and parsimony-uninformative, and 315 are constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values. Parsimony analysis of the alignment yielded 96 most parsimonious trees (TL = 350 steps; CI = 0.794; RI = 0.944; RC = 0.750), one of which is shown in Fig. 2. The neighbour-joining and parsimony analyses supported the same main clades (data not shown). Several well-supported clades are seen in the tree, the first of which (100 % bootstrap support) contains sequences of *Cercospora apii* and *C. beticola* (63 % bootstrap support) and four isolates of *C. acaciae-mangii* (64 % bootstrap support). Two *Mycosphaerella* species (CPC 10520 and 10521) cluster with *Pseudocercospora basiramifera* and *Ps. paraguayensis* (100 % bootstrap support). Three sequences of *M. fijiensis* (100 % bootstrap support) form a sister clade to the *Pseudocercospora* isolates (94 % bootstrap support). The sequences of the two *Passalora* sp. isolates (100 % bootstrap support) cluster with *Passalora loranthi* with a bootstrap support value of 100 %. Another well-supported clade (87 % bootstrap support) contains three *Mycosphaerella* species as well as *M. mangium*, *M. heimii* and *M. konaie*.

**Table 1.** Isolates of *Mycosphaerella* spp. and their anamorphs included for sequence analysis.

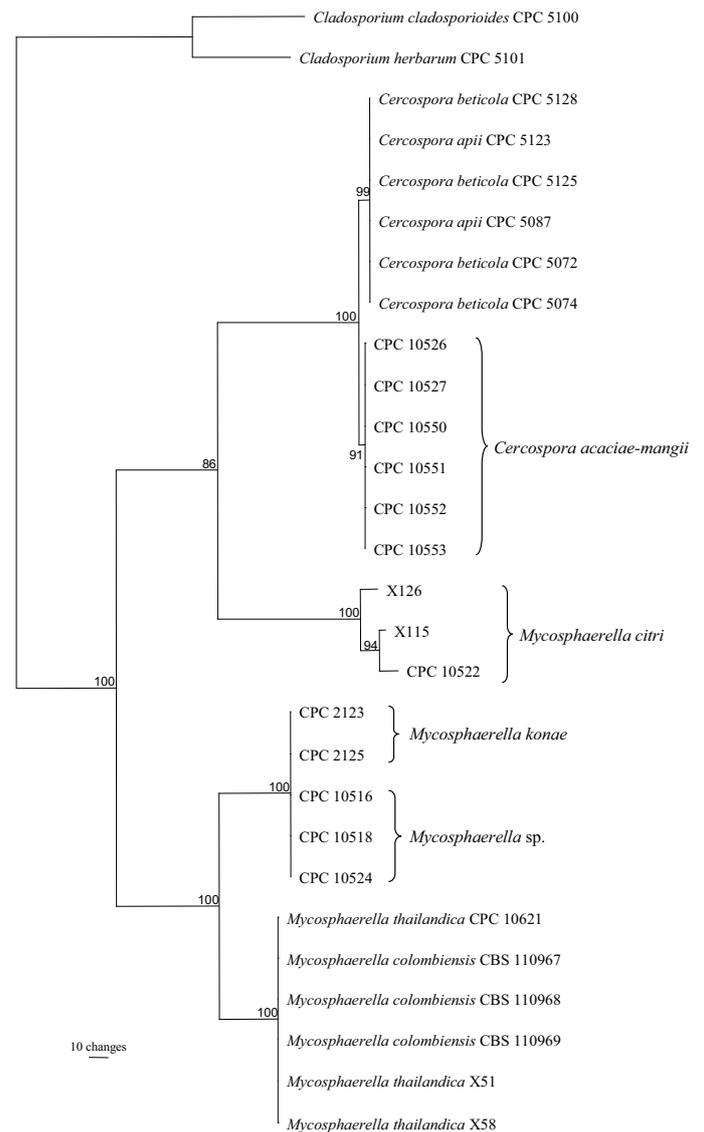
Species	Accession number <sup>1</sup>	Host	Country	Collector	GenBank numbers (ITS, EF 1- $\alpha$ , ACT, CAL, HIS)
<i>Cladosporium cladosporioides</i>	ATCC 66669 / CPC 5100	Creosote-treated southern pine pole	U.S.A.	–	AY251070, AY752164, AY752192, AY752223, AY752254
<i>Cladosporium herbarum</i>	ATCC 66670 / CPC 5101	CCA-treated Douglas-fir pole	U.S.A.	–	AY251078, AY752165, AY752193, AY752224, AY752255
<i>Cercospora apii</i>	CBS 536.71 / CPC 5087	<i>Apium graveolens</i>	Romania	–	AY752133, AY752166, AY752194, AY752225, AY752256
	CPC 5123	<i>A. graveolens</i>	New Zealand	C.F. Hill	AY752134, AY752167, AY752195, AY752226, AY752257
<i>Cercospora beticola</i>	CBS 116.47 / CPC 5074	<i>Beta vulgaris</i>	Netherlands	–	AY752135, AY752168, AY752196, AY752227, AY752258
	CBS 122.31 / CPC 5072	<i>Beta vulgaris</i>	Germany	–	AY752136, AY752169, AY752197, AY752228, AY752259
	CPC 5125	<i>Beta vulgaris</i>	New Zealand	C.F. Hill	AY752137, AY752170, AY752198, AY752229, AY752260
	CPC 5128	<i>Beta vulgaris</i>	New Zealand	C.F. Hill	AY752138, AY752171, AY752199, AY752230, AY752261
<i>Cercospora acaciae-mangii</i>	CPC 10550	<i>Acacia mangium</i>	Thailand	–	AY752139, AY752172, AY752200, AY752231, AY752262
	CPC 10551	<i>A. mangium</i>	Thailand	–	AY752140, AY752173, AY752201, AY752232, AY752263
	CPC 10552	<i>A. mangium</i>	Thailand	–	–, AY752174, AY752202, AY752233, AY752264
	CPC 10553	<i>A. mangium</i>	Thailand	–	–, AY752175, AY752203, AY752234, AY752265
	CPC 10526	<i>A. mangium</i>	Thailand	M.J. Wingfield	AY752141, AY752176, AY752204, AY752235, AY752266
	CPC 10527	<i>A. mangium</i>	Thailand	M.J. Wingfield	AY752142, AY752177, AY752205, AY752236, AY752267
<i>Mycosphaerella acaciigena</i>	CPC 3837	<i>Acacia</i> sp.	Venezuela	M.J. Wingfield	AY752143 (ITS only)
<i>Mycosphaerella citri</i>	X126	<i>Citrus</i> sp.	Florida	–	AY752144, AY752178, AY752206, AY752237, AY752268
	CPC 10522	<i>A. mangium</i>	Thailand	M.J. Wingfield	AY752145, AY752179, AY752207, AY752238, AY752269
	X115 / rCRB2 / CBS 116426	<i>Musa</i> sp.	Florida	J. Cavaletto	AY752146, AY752180, AY752208, AY752239, AY752270
<i>Mycosphaerella colombiensis</i>	CBS 110967 / CPC 1104	<i>Eucalyptus urophylla</i>	Colombia	M.J. Wingfield	AY752147, AY752181, AY752209, AY752240, AY752271
	CBS 110968 / CPC 1105	<i>E. urophylla</i>	Colombia	M.J. Wingfield	AY752148, AY752182, AY752210, AY752241, AY752272
	CBS 110969 / CPC 1106	<i>E. urophylla</i>	Colombia	M.J. Wingfield	AY752149, AY752183, AY752211, AY752242, AY752273
<i>Mycosphaerella fijiensis</i>	X300	<i>Musa</i> sp.	Tonga	F. Sumich	AY752150 (ITS only)
<i>Mycosphaerella konae</i>	CPC 2123	<i>Leucadendron</i> sp.	Hawaii	P.W. Crous	AY260086, AY752184, AY752212, AY752243, AY752274
	CPC 2125	<i>Leucadendron</i> sp.	Hawaii	P.W. Crous	AY260085, AY752185, AY752213, AY752244, AY752275
<i>Mycosphaerella</i> sp.	CPC 10516	<i>A. mangium</i>	Thailand	M.J. Wingfield	AY752151, AY752186, AY752214, AY752245, AY752276
	CPC 10518	<i>A. mangium</i>	Thailand	M.J. Wingfield	AY752152, AY752187, AY752215, AY752246, AY752277
	CPC 10520	<i>Acacia aulacocarpa</i>	Thailand	M.J. Wingfield	AY752153 (ITS only)
	CPC 10521	<i>A. aulacocarpa</i>	Thailand	M.J. Wingfield	AY752154 (ITS only)
	CPC 10524	<i>A. mangium</i>	Thailand	M.J. Wingfield	AY752155, AY752188, AY752216, AY752247, AY752278
<i>Mycosphaerella thailandica</i>	CPC 10547	<i>A. mangium</i>	Thailand	–	AY752156, –, AY752217, AY752248, AY752279
	CPC 10548	<i>A. mangium</i>	Thailand	–	AY752157, –, AY752218, AY752249, AY752280
	CPC 10549	<i>A. mangium</i>	Thailand	–	AY752158, –, AY752219, AY752250, AY752281
	CPC 10621	<i>A. mangium</i>	Thailand	–	AY752159, AY752189, AY752220, AY752251, AY752282
	X51	<i>Musa</i> sp.	Windward Isles	E. Reid	AY752160, AY752190, AY752221, AY752252, AY752283
	X58	<i>Musa</i> sp.	Windward Isles	E. Reid	AY752161, AY752191, AY752222, AY752253, AY752284
<i>Passalora</i> sp.	CPC 11147	<i>Acacia crassiparva</i>	Indonesia	M.J. Wingfield	AY752162 (ITS only)
	CPC 11150	<i>A. crassiparva</i>	Indonesia	M.J. Wingfield	AY752163 (ITS only)

<sup>1</sup>CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; C.P.C.: Culture collection of Pedro Crous, housed at CBS; ATCC: American Type Culture Collection, Virginia, U.S.A.; <sup>2</sup>Ex-type cultures. <sup>3</sup>ITS: internal transcribed spacer region, EF 1- $\alpha$ : elongation factor 1-alpha, ACT: actin, CAL: calmodulin, HIS: histone 3-a.



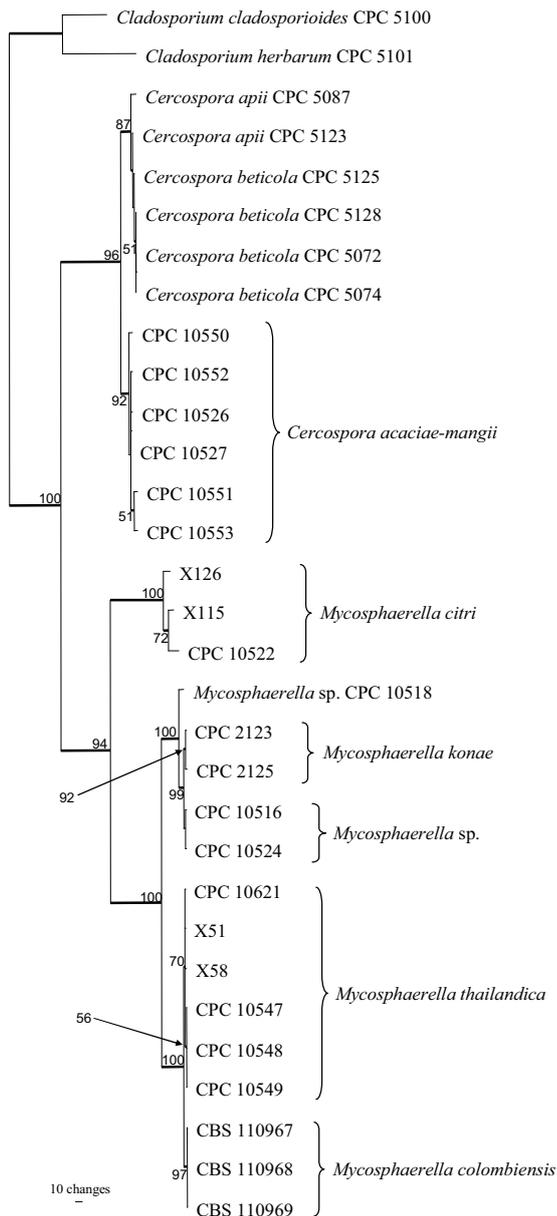
**Fig. 2.** One of 96 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows 10 changes; bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches. The tree was rooted to two *Cladosporium* species.

The sequences of *M. thailandica* and *M. colombiensis* all cluster in the same clade (87 % bootstrap support), with only isolates X51 and X58 forming a distinct group (98 % bootstrap support). Five sequences of *M. citri*, two of which were obtained from GenBank, also formed a well-supported (100 % bootstrap support) clade.



**Fig. 3.** Single most parsimonious tree obtained from a heuristic search with 100 random taxon additions of the EF 1- $\alpha$  sequence alignment. The scale bar shows 10 changes; bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to two *Cladosporium* species.

The manually adjusted EF sequence alignment (TreeBASE study accession number S1178) contains 28 taxa (including the two outgroups) and 300 characters including alignment gaps; of these characters 233 are parsimony-informative, 24 are variable and parsimony-uninformative, and 43 are constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology (data not shown). Between the neighbour-joining and parsimony analyses, the trees supported the same main clades (data not shown). Parsimony analysis of the alignment yielded a single most parsimonious tree (TL = 611 steps; CI = 0.876; RI = 0.966; RC = 0.846), which is shown in Fig. 3.



**Fig. 4.** One of 18 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined ACT, CAL and HIS sequence alignment. The scale bar shows 10 changes and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches. The tree was rooted to two *Cladosporium* species

Several well-supported clades are seen in the tree, one of which (100 % bootstrap support) contains sequences of *C. apii* and *C. beticola* (99 %) and isolates of *C. acaciae-mangii* (91 %). The *M. citri* clade (100 %) contains three isolates, two of which are more closely related, grouping with a bootstrap support value of 94 %. The three *Mycosphaerella* spp. form a well-supported clade (100 %) together with the *M. konae* isolates. Three sequences of each of *M. thailandica* and *M. colombiensis* all cluster in the same clade (100 %). The EF dataset failed to separate *M. konae* and the *Mycosphaerella* sp., as well as *M. thailandica* and *M. colombiensis*.

For ACT, CAL and HIS, respectively 209, 312 and 388 bases (including alignment gaps) were included in the manually adjusted alignment consisting of all three loci for 44 taxa (including the two outgroups). The combined data set (TreeBASE study accession number S1178) used for phylogenetic analysis contains a total of 909 characters, of which 306 are parsimony-informative, 54 were variable and parsimony-uninformative, and 549 were constant. The topology of the trees generated with neighbour-joining analysis using the three substitution models were identical (data not shown). Parsimony analysis of the combined data yielded 18 most parsimonious trees, one of which is shown in Fig. 4. Between the neighbour-joining and parsimony analyses, the trees differed only in the placement of the *M. citri* clade (data not shown). Distance analysis grouped the *M. citri* clade with the *Cercospora* clade (bootstrap support value of approximately 70 % irrespective of which substitution model is used), whereas it groups (94 %) with the clades containing the other *Mycosphaerella* species when a parsimony analysis is performed. As with the ITS and EF trees, a clear separation is found between the clade containing *C. apii*/*C. beticola* isolates (87 %) and *C. acaciae-mangii* (92 %). The *M. citri* clade (100 %) contains three isolates, two of which once again are more closely related, and is supported by a lower bootstrap support value of 72 %. The clade containing the three *Mycosphaerella* sp. and two *M. konae* isolates is also well-supported (100 %), with *M. konae* clustering with a bootstrap support of 92 % and the isolate CPC 10518 sitting outside of the cluster (99 %) formed by the rest of the isolates in this clade. Another well-supported clade (70 %) in this tree contains the *M. thailandica* (70 %) and *M. colombiensis* (97 %) isolates.

## Taxonomy

***Cercospora acaciae-mangii*** Crous, Pongpanich & M.J. Wingf., **sp. nov.** MycoBank MB500118.  
*Teleomorph:* *Mycosphaerella* sp. Fig. 5.

*Etymology:* Named after its host *Acacia mangium*.

Maculae amphigenae, medio-brunneae, inter marginem et costam, margine atro-brunneo, leviter elevato cinctae. Stromata nulla vel bene evoluta, brunnea, ad 30 µm diam. Conidiophora medio-brunnea, levia, longa, fasciculata (3–20), recta vel apice geniculato-sinuoso. Cellulae conidiogenae integratae, terminales vel intercalares, ad 100 µm longae, sympodiales; cicatrices conidiales incrassatae, fuscatae, refractivae, ad 3 µm latae. Conidia solitaria, hyalina, levia, aciculares, 50–350 × 3.5–5 µm, pluriseptata, basi (in hilo) incassata, fuscata, refractiva. *Cercosporae apii* similis, sed hospite *Acacia* et nonnullis nucleotideis differens: elongation factor 1-alpha (EF) in positionibus 42 (T), 47 (C), 144 (C), 198 (G), 217 (A), 224 (A), 235 (A), 245 (C), 257 (G); actinum (ACT) in positionibus 70 (T), 172 (A), 175 (A); calmodulinum (CAL) in positionibus 37

(C), 81 (A), 109 (C), 114 (C), 117 (A), 148 (A), 149 (T), 189 (T), 270 (G), 279 (T); histonum H3 (HIS) in positionibus 112 (A), 114 (T), nucleotide delecto inter positiones 122 et 123, 135 (G), 148 (C), 151 (T), 381 (C).

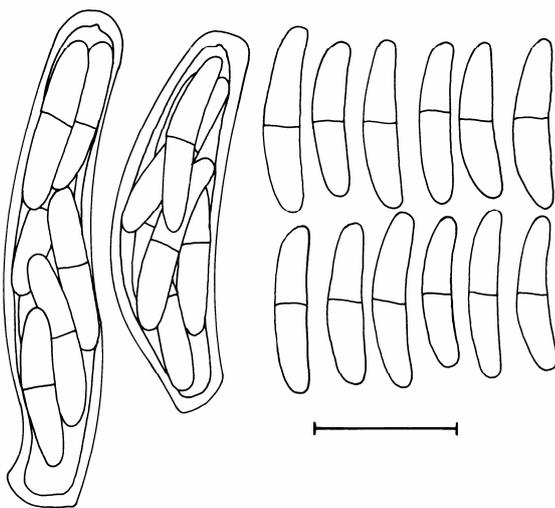
*Leaf spots* amphigenous, covering up to half of the leaf lamina from the margin to the mid rib; infections intermixed with that of *M. thailandica*; lesions medium brown, surrounded by a raised, dark brown border. *Stromata* lacking to well developed, brown, up to 30 µm diam, giving rise to conidiophores. *Conidiophores* medium brown, smooth, long, flexuous, in fascicles that vary in number from 3–20, straight, or with upper part geniculate-sinuous. *Conidiogenous cells* integrated, terminal or intercalary, up to 100 µm long, proliferating sympodially, loci thickened, darkened, refractive, up to 3 µm wide. *Conidia* solitary, hyaline, smooth, acicular, 50–350 × 3.5–5 µm, multi-septate, with a thickened, darkened, refractive scar. Morphologically indistinguishable from *C. apii* s. l. (Crous & Braun 2003).

*Holotype*: **Thailand**, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, **holotype** herb. CBS 9874; culture ex-type CBS 116365 = CPC 10526.

*Host*: *Acacia mangium*.

*Cultures*: Colonies irregular, fast growing, covering the dish after 1 mo; aerial mycelium fluffy to woolly, surface white to pale olivaceous-grey (21''''d), with patches of grey-olivaceous (21''''b) sporulation; reverse iron-grey (25''''k).

*Distribution*: Thailand.



**Fig. 5.** Asci and ascospores of a *Mycosphaerella* sp. commonly found associated with fascicles of *Cercospora acaciae-mangii*. Scale bar = 10 µm.

*Notes*: When leaf tissues were treated for ascospore discharge, several ascospores of a *Mycosphaerella* sp.

were obtained that gave rise to a *Cercospora* anamorph. Upon germination, however, these ascospores could not with certainty be traced back to the *Mycosphaerella* state, as they were only harvested after 48 h, and had hence started to distort. The formal naming of the *Mycosphaerella* teleomorph thus awaits further collections of fresh material. A probable candidate which occurred on the lesions from which the cultures were derived has the following morphology: *Ascomata* pseudothecial, amphigenous, erumpent, black, aggregated in moderately dense clusters, globose, up to 90 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* fasciculate, bitunicate, sessile, obovoid to narrowly ellipsoid or subcylindrical, straight or slightly incurved, 8-spored, 30–40 × 7–9 µm. *Ascospores* tri- to multiseriate, overlapping, hyaline, non-guttulate, thin-walled, curved, fusoid-ellipsoidal with obtuse ends, medianly 1-septate, widest at the median, unstricted septum, tapering towards both ends, (10–)12–13(–15) × (2–)2.5–3 µm *in vivo*.

The *Cercospora* anamorph closely matched others within the *C. apii* s. l. complex (Crous & Braun 2003), but could be separated phylogenetically, and is thus described as *C. acaciae-mangii*.

*Additional specimens and cultures examined*: **Thailand**, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, herb. CBS 9876, CPC 10550, 10526–10528 (single-ascospore isolates), CPC 10551–10553 (single-conidial isolates of *C. acaciae-mangii*).

*Mycosphaerella acaciigena* Crous & M.J. Wingf., **sp. nov.** MycoBank MB500119. Figs 6–9.

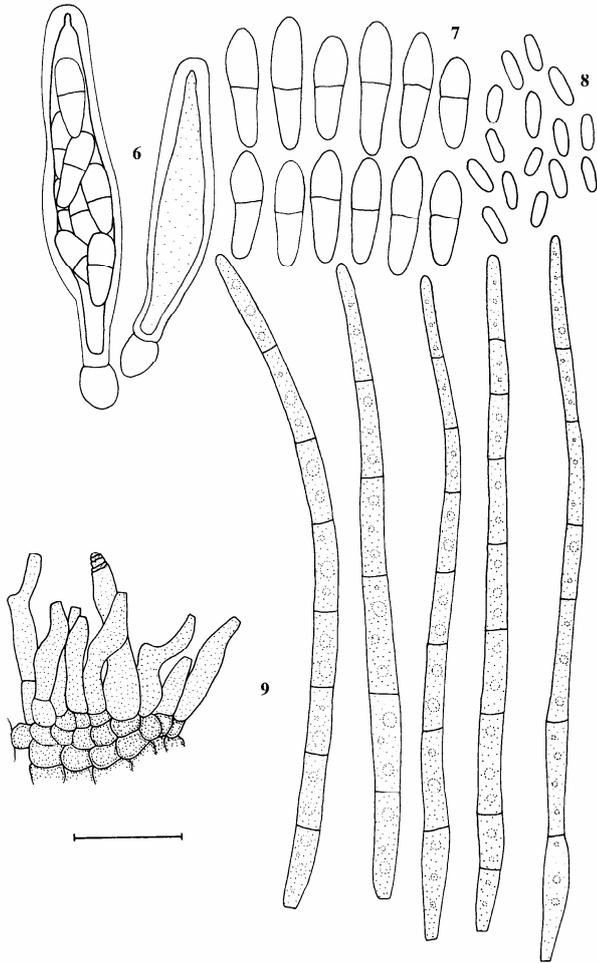
*Anamorph*: *Pseudocercospora acaciigena* Crous & M.J. Wingf., **sp. nov.**

*Etymology*: Named after the host genus, *Acacia*.

*Mycosphaerella heimii* similis sed ascosporis ad septum modice constrictis differens.

*Leaf spots* amphigenous, elongated along the length of the leaf, not confined to the margins, variable in width, up to 2 cm diam, medium brown, surrounded by a raised, dark brown border. *Ascomata* pseudothecial, amphigenous, erumpent, black, aggregated in clusters of up to 100, forming black spots up to 1 mm diam on the lesions; ascomata globose, up to 80 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* fasciculate, bitunicate, sessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 25–40 × 8–11 µm. *Ascospores* tri- to multiseriate, overlapping, hyaline, non-guttulate, thin-walled, straight, fusoid-

ellipsoidal with obtuse ends, medianly 1-septate, widest in the middle of the apical cell, slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)9–10(–11) × (2.5–)3 µm *in vivo*. *Spermogonia* intermixed with and similar to the ascomata in general morphology. *Spermatia* rod-shaped, hyaline, 3–6 × 1 µm *in vivo*.



**Figs 6–9.** *Mycosphaerella acaciigena* and its *Pseudocercospora* anamorph. 6. Asci. 7. Ascospores. 8. Spermatia. 9. Conidiophores and conidia. Scale bar = 10 µm.

***Pseudocercospora acaciigena*** Crous & M.J. Wingf., **sp. nov.** MycoBank MB500120.

Differt a *P. thailandica* conidiis longioribus, ad 15-septatis; a *P. acaciae-confusae*, *P. hyaloconidiophora* et *P. acaciae* conidiis obclavatis, pallide brunneis, 2–2.5(–3) µm latis.

*Conidiomata* amphigenous, pale brown, up to 80 µm diam; stromata well developed, brown, up to 60 µm wide and 30 µm high. *Mycelium* predominantly internal, consisting of smooth, branched, septate, pale brown, 3–4 µm wide hyphae. *Conidiophores* aggregated in dense fascicles arising from the upper cells of the stroma; conidiophores pale brown, smooth, unbranched or branched, 0–3-septate, subcylindrical, straight to geniculate-sinuuous, 15–30 × 3–5 µm.

*Conidiogenous cells* terminal, pale brown, smooth, subcylindrical, tapering to flat tipped apical loci, proliferating sympodially, or several times percurrently, 15–20 × 3–4 µm; conidial scars inconspicuous. *Conidia* solitary, pale brown, smooth, guttulate, narrowly obclavate, apex subobtuse, base long obconically subtruncate, straight to curved, 3–15-septate, (40–)50–75(–80) × 2–2.5(–3) µm *in vivo*; hila inconspicuous.

**Holotype:** Venezuela, Acarigua, on leaves of *A. mangium*, May 2000, M.J. Wingfield, herb. CBS 9873, **holotype** of *M. acaciigena* and *P. acaciigena*; cultures ex-type CBS 115432, 112515, 112516 = CPC 3836–3838.

**Cultures:** Colonies on OA with thin yellow-brown line of pigment diffusing into the agar; margin thin, smooth, slimy, white (1–2 mm wide); surface pale olivaceous-grey (21''''d), with sparse aerial mycelium. On MEA margin smooth, regular, aerial mycelium sparse; surface colour variable, predominantly pale olivaceous-grey (23''''d), with patches of smoke-grey (19''''d) and olivaceous-grey (21''''i); reverse olivaceous-grey (21''''i).

**Host:** *Acacia mangium* (Leguminosae).

**Distribution:** Venezuela.

**Notes:** The dense black clusters of raised ascomata on both sides of the leaf lamina is a very characteristic feature of this species. The holotype specimen of *M. acaciigena* is also colonized by a species of *Cercospora*. The latter appears to be distinct from the *C. apii* s. l. complex, as conidia tend to have more rounded bases, and be more subcylindrical in shape and shorter than the typical conidia of *C. apii*, which have more truncate bases, and are longer and acicular in shape. A few conidia of a *Stenella* sp. were also found to be present, though fructification was sparse. As no cultures of the latter two fungi were obtained, they are not treated further and await additional collections.

***Mycosphaerella citri*** Whiteside, *Phytopathology* 62: 263. 1972. Fig. 10.

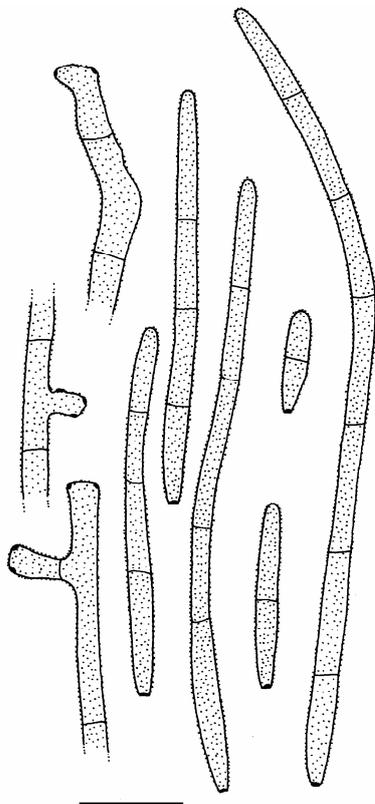
**Anamorph:** *Stenella citri-grisea* (F.E. Fisher) Sivan., In: Sivanesan, Bitunicate ascomycetes and their anamorphs: 226. 1984.

≡ *Cercospora citri-grisea* F.E. Fisher, *Phytopathology* 51: 300. 1961.

*Leaf spots* amphigenous, covering up to half of the leaf lamina from the margin to the mid rib; infections intermixed with that of *M. thailandica* and *C. acaciae-mangii*; lesions medium brown, surrounded by a raised, dark brown border. *Mycelium* consisting of verruculose, branched, septate, red-brown to medium

brown hyphae, 2–3 µm wide. *Conidiophores* arising singly from superficial mycelium, red-brown to medium brown, verruculose, subcylindrical to irregular, 1–3-septate, straight to variously curved, 5–20 × 2.5–4 µm. *Conidiogenous cells* terminal, verruculose, medium brown, unbranched, tapering to rounded apices with flat, thickened, darkened, refractive loci, proliferating sympodially, 5–10 × 2.5–4 µm. *Conidia* solitary, medium brown to red-brown, verruculose, narrowly obclavate, apex subobtuse, base long obconically subtruncate, straight to curved, (0–)3–5(–10)-septate, (10–)35–65(–120) × (2–)2.5(–3) µm *in vivo* (description based on *Acacia* isolate CPC 10522 = CBS 116366).

*Cultures*: Colonies with smooth, regular margins, moderately fast growing, covering the dish after 2 mo; aerial mycelium moderate, surface olivaceous-grey (21 °C), reverse greenish black (33 °C); cultures fertile.



**Fig. 10.** Conidiophores and conidia of *Stenella citri-grisea* formed *in vitro* from isolate CBS 116366. Scale bar = 10 µm.

*Hosts*: *Acacia mangium*, *Musa* sp., and species of *Aeglopsis* Swingle, *Citrus*, *Fortunella* Swingle, *Murraya* L., *Poncirus* Rafin. (Rutaceae) (Pretorius *et al.* 2003).

*Distribution*: Thailand (*Acacia*), on Rutaceae in Brazil, Costa Rica, Cuba, Dominican Republic, El Salvador, Gabon, Haiti, Hong Kong, Japan, Puerto Rico, Surinam, Taiwan, Thailand, USA (FL, HI, TX), Venezuela, Virgin Islands (Pretorius *et al.* 2003).

*Notes*: In culture, conidia of CBS 116366 closely resembled the morphology of isolates described from *Citrus* (Fisher 1961, Sivanesan 1984).

*Culture examined*: **Thailand**, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, CBS 116366 = CPC 10522 (single-ascospore isolate).

*Mycosphaerella thailandica* Crous, Himaman & M.J. Wingf., **sp. nov.** MycoBank MB500121. Figs 11–15.

*Anamorph*: *Pseudocercospora thailandica* Crous, Himaman & M.J. Wingf., **sp. nov.**

*Etymology*: Named after its country of origin, Thailand.

*Mycosphaerellae colombiensi* similis, sed ascosporis ad septum modice constrictis differens; ascosporae modo C germinantes.

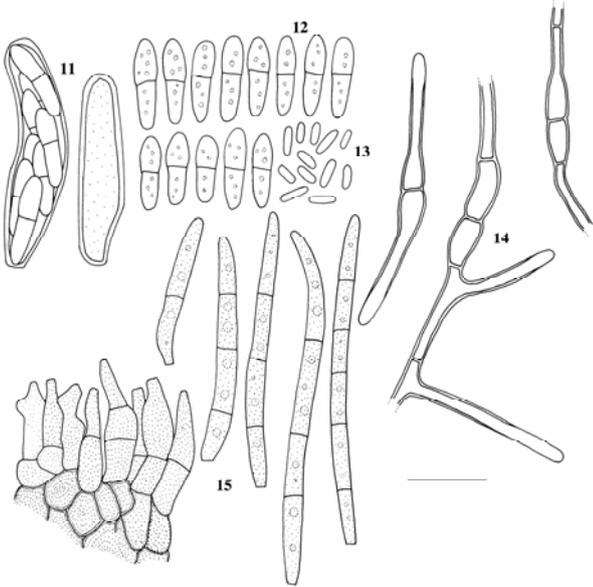
*Leaf spots* amphigenous, irregular blotches covering large parts of the leaf lamina; associated symptoms include tip blight, or lesions all along the margin of the leaf, frequently extending to the middle of the leaf lamina; lesions medium brown, surrounded by a raised, dark brown border. *Ascomata* pseudothecial, amphigenous, subepidermal, becoming erumpent, black, globose, up to 80 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* fasciculate, bitunicate, sessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 30–40 × 6–8 µm. *Ascospores* tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, medianly 1-septate, widest in middle of the apical cell, slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (9–)10–11(–12) × (2–)2.5–3 µm *in vivo*. Spermogonia intermixed with and similar to the ascomata in general morphology. Spermata rod-shaped, hyaline, 3–5 × 1 µm *in vivo*.

*Pseudocercospora thailandica* Crous, Himaman & M.J. Wingf., **sp. nov.** MycoBank MB500122.

Differt a *P. acaciigena* conidiis brevioribus, ad 6-septatis; a *P. acaciae-confusae*, *P. hyaloconidiophora* et *P. acaciae* conidiis obclavatis-subcylindratis, pallide brunneis, 2–2.5(–3) µm latis.

*Conidiomata* amphigenous, pale brown, up to 60 µm diam; stromata well developed, brown, up to 25 µm wide and 30 µm high. *Mycelium* predominantly internal, consisting of smooth, branched, septate, medium brown, 3–4 µm wide hyphae. *Conidiophores* aggregated in dense fascicles arising from the upper cells of the stroma; conidiophores pale brown, smooth, un-

branched, 0–2-septate, subcylindrical, straight to variously curved,  $10\text{--}20 \times 5\text{--}6 \mu\text{m}$ . *Conidiogenous cells* terminal, pale brown, smooth, subcylindrical, tapering to flat tipped apical loci, proliferating sympodially,  $10\text{--}15 \times 3\text{--}5 \mu\text{m}$ ; conidial scars inconspicuous. *Conidia* solitary, pale brown, smooth, guttulate, narrowly obclavate to subcylindrical, apex subobtuse, base long obconically subtruncate, straight to curved, 3–6-septate,  $(25\text{--})30\text{--}45(\text{--}60) \times 2\text{--}2.5(\text{--}3) \mu\text{m}$  *in vivo*; hila inconspicuous.



**Figs 11–15.** *Mycosphaerella thailandica* and its *Pseudocercospora* anamorph. 11. Asci. 12. Ascospores. 13. Spermatia. 14. Germinating ascospores on MEA. 15. Conidiophores and conidia. Scale bar =  $10 \mu\text{m}$ .

**Holotype:** Thailand, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, herb. CBS 9875, **holotype** of both *M. thailandica* and *P. thailandica*, cultures ex-type CBS 116367 = CPC 10547–10549.

**Ascospore germination on MEA after 24 h:** Germinating with germ tubes parallel to the long axis of the ascospore, constricted at the original septum, ascospores becoming  $2.5\text{--}3 \mu\text{m}$  wide, developing several lateral branches.

**Cultures:** Colonies slightly erumpent, having smooth, regular margins, fast growing, covering the dish after 60 d; aerial mycelium fluffy, surface grey-olivaceous (21 ""b), reverse olivaceous-black (25 ""k); cultures sterile.

**Host:** *Acacia mangium*.

**Distribution:** Thailand.

**Notes:** Morphologically *M. acaciigena* is similar to *M. thailandica*, except that the *Pseudocercospora* conidia of *M. acaciigena* tend to be longer, and ascomata of *M. acaciigena* are arranged in dense, superficial clusters, which differ from what was observed on the

type of *M. thailandica*. However, additional specimens studied from Thailand (herb. CBS 9879, Mar. 2003) also tend to have ascomata arranged in clusters, though not as pronounced as observed for *M. acaciigena*. This could indicate that the clustering is a result of the host tissue, or that *M. acaciigena* also occurs in Thailand. Further collections and cultures would be required, however, to resolve this issue.

*Mycosphaerella thailandica* is morphologically similar to *M. colombiensis* Crous & M.J. Wingf., which is a pathogen of *Eucalyptus* (Crous 1998). Although the latter two species can be distinguished based on ascospore morphology and germination patterns.

**Additional specimens and cultures of unidentified spp. examined:** Thailand, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, May 2002, W. Himaman, herb. CBS 9878; Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, Mar. 2003, K. Pongpanich, herb. CBS 9879; Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 2003, K. Pongpanich, ascospore cultures CPC 10516–10525, 10621–10625.

**Cultures of unidentified *Mycosphaerella* spp. examined:** Thailand, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, CPC 10516, 10518, 10524 (single-ascospore isolates of *Mycosphaerella* sp. in the *M. konae* clade); CPC 10520, 10521 (single-ascospore isolates of *Mycosphaerella* sp. in the *M. basiramifera* clade).

## DISCUSSION

Results of this study have clearly emphasised the paucity of knowledge regarding the taxonomy of leaf pathogens of *Acacia* spp. that are of considerable economic importance to the forestry industry. In the review of diseases of *Acacia* spp. grown in plantations in the tropics, Old *et al.* (2000) noted that two species, tentatively identified as species of *Cercospora* and *Pseudocercospora*, occur on *A. mangium*, *A. auriculiformis* and *A. crassicarpa*. In this study we have described three species of *Mycosphaerella*, and one that is currently known only from its anamorph. We have also identified at least three other, as yet undescribed species from these trees in various tropical countries. Several of these fungi are peripherally similar to each other and this probably explains why they have not previously been recognised.

In their revision of the genus *Cercospora*, Crous & Braun (2003) regarded 281 names to be synonymous with the older *C. apii*, and treated these as part of the *C. apii* s. l. species complex. Currently there are no *Mycosphaerella* teleomorphs known within this complex. The collection of a *Mycosphaerella* sp. that gave rise to a *Cercospora* anamorph matching the description of *C. apii* s. l. in the present study is thus an exciting development. Isolates were obtained from



single conidia, as well as single ascospores. Comparison of DNA sequence data for several genes (Figs 2–4) showed that these ascospore and conidial isolates cluster closely together within the *C. apii* clade, but that they represent a distinct lineage. We have described these as morphologically similar to *C. apii*, but representing a phylogenetically distinct species, named *C. acaciae-mangii*. These isolates will add a valuable indication of the variation that can be expected within the *C. apii s. l.* species complex. They will also promote our understanding of the species limits and genetic entities within this complex.

The *Pseudocercospora* anamorphs of *M. acaciigena* and *M. thailandica* are morphologically very similar, differing chiefly in conidial size and septation, and are quite distinct from *P. acaciae-confusae* (Sawada) Goh & W.H. Hsieh, which has pale yellowish brown, cylindrical conidia, and causes irregularly angular spots 0.5–2 mm diam (Hsieh & Goh 1990). *Pseudocercospora hyaloconidiophora* Goh & W.H. Hsieh is distinguished by having hyaline conidiophores and conidia (Hsieh & Goh 1990). Furthermore, *P. acaciae* Kamal & R.P. Singh is distinguished by its very long (up to 270 µm), thick-walled, smooth conidiophores, and obclavate conidia that are much wider than observed in the present collections (21.5–70 × 7–11 µm) (Kamal & Singh 1980).

*Mycosphaerella acaciigena*, which was collected in Venezuela, is morphologically similar, but phylogenetically distinct from the *M. heimii* Crous/*M. kona* Crous, Joanne E. Taylor & M.E. Palm species complex (Crous 1998, Crous *et al.* 2004a). Several other isolates obtained from Thailand (CPC 10516, 10518, 10524), could, however, represent one of the latter species, and this will be resolved once fertile collections have been obtained for morphological comparison. Isolates CPC 10520 and CPC 10521 appear to represent another, undescribed species closely related to *P. basiramifera* Crous/*P. paraguayensis* (Kobayashi) Crous (Fig. 2). The *Passalora* sp. (CPC 11147, 11150) from *A. crassicaarpa* which clusters with *Cercospora loranthi* McAlpine (= *Passalora fide* V. Beilharz, in press), is clearly distinguishable based on morphological and phylogenetic differences. This species is treated elsewhere in this volume (Beilharz *et al.* 2004).

*Mycosphaerella thailandica* is morphologically very similar to *M. colombiensis*, which is a leaf pathogen of *Eucalyptus* in Colombia (Crous 1998). Morphologically, the two species can be distinguished by the constricted ascospores of *M. thailandica*, while those of *M. colombiensis* are not constricted. In the ITS dataset (Fig. 2), these species cluster together. However, in both the EF-1 $\alpha$ , and combined actin, calmodulin & histone datasets (Figs 3, 4), it is clear that *M. thailandica* is a cryptic species closely related to, but distinct from *M. colombiensis*.

*Mycosphaerella citri* is an important foliar and fruit pathogen of *Citrus*, causing premature leaf drop, as well as reduced tree vigour, yield and fruit size (Mondal *et al.* 2003). In a recent phylogenetic study of the genus *Cercospora*, Goodwin *et al.* (2001) included one isolate from a *Musa* sp. (rCRB2 = CBS 116426), which, although identified as *M. fijiensis* M. Morelet, clustered with an isolate of *M. citri*. They subsequently concluded that the isolate was either misidentified or contaminated. The same isolate was obtained from Dr S.B. Goodwin for inclusion in the present study. We can now confirm that this isolate represents *M. citri*, and not *M. fijiensis*. Furthermore, an ex-ascospore isolate obtained from leaves of *Acacia mangium* in Thailand in the present study, also represented *M. citri*. As far as we are aware, this is the first record confirmed based on DNA sequence data, of a serious *Mycosphaerella* pathogen having alternative hosts. Species of *Acacia*, *Citrus*, and *Musa* are all native to parts of South-East Asia, and this might explain the host-sharing observed here. The fact that these trees are also widely planted as exotics in tropical and sub-tropical parts of the world, and that the important pathogen *M. citri* could infect three unrelated hosts, is cause for considerable concern. An examination of the various gene trees generated in the current study support the view of Pretorius *et al.* (2003) that *M. citri* is more variable than previously believed. Furthermore, our results show that speciation is occurring in *M. citri*. Although the isolates occurring on *Musa* and *Acacia* appear to fall within the morphological variation accepted for *M. citri*, this appears to be changing. We expect that in the future, this species will evolve into separate, cryptic species or lineages depending on its host.

Host sharing was also found in the *M. colombiensis/thailandica* complex, where *M. thailandica*, occurs on *Acacia* and *Musa*. However, in this case, lineages are more distinct than those in the *M. citri* complex, and the fungus on *Acacia* and *Musa* could thus be named as *M. thailandica*. In the *Cercospora apii s. l.* complex, *C. acaciae-mangii* represents an additional example of a morphologically similar species, which can be separated based on its host and phylogeny. Ironically, in all three examples where host sharing has been observed, isolates were obtained from ascospores, again suggesting that the presence of the teleomorph enhances speciation. Other taxa in the *C. apii s. l.* complex lack teleomorphs, and still cluster together in clades emerging from comparisons of the various gene regions sequenced, despite their different hosts.

An intriguing question relating to the fungi described in this study is where they might have originated. The host trees are native to tropical parts of Australia and Papua New Guinea, and it is logical to assume that the fungi have been introduced into plantation areas from one or more of these native tree

populations. Alternatively, and as illustrated, they could have jumped from completely unrelated hosts. The two undescribed cercosporoid fungi reported by Old *et al.* (2000) were both found in Northern Australia (Old *et al.* 1996, Cannon *et al.* 1997), and match the description of the fungi described here. The remaining species might have evolved together with the *Acacia* spp. on which they occur. However, there is growing evidence to show that pathogens of *Eucalyptus* have adapted from native plants to infect these important plantation trees (Wingfield *et al.* 2001).

There are many native species of *Acacia* and trees of related genera in areas where Australian *Acacia* spp. are being propagated commercially. It seems likely that both fungi occurring on *Acacia* spp. in their native environment, and others that have more recently adapted to infect these trees as exotics will be encountered. The latter group of new pathogens could seriously threaten the trees in their native environment, if they were to be transferred back to these areas.

This situation would be similar to that found with Eucalyptus rust caused by *Puccinia psidii* G. Winter, which is native in Latin America on various *Myrtaceae*, and has adapted to infect *Eucalyptus* in that area (Coutinho *et al.* 1998). This rust fungus is presently considered to be one of the most serious threats to *Eucalyptus* in areas such as Australia where there are no rust pathogens of these trees.

*Mycosphaerella* spp. and their anamorphs include some of the most important leaf and shoot pathogens of forest plantation trees, fruit trees and shrubs (Old *et al.* 2000, Park *et al.* 2000, Stone *et al.* 2003, Crous *et al.* 2004a, b). In the case of *Eucalyptus*, plantations in the tropics and the Southern Hemisphere have been seriously damaged by these fungi (Crous 1998). We might thus expect the same situation for *Acacia* spp. in the future. It is thus imperative that these fungi are correctly characterised and named. Management strategies to reduce the impact of the diseases associated with these fungi will rest strongly on a clear understanding of the relative importance of the various species. Likewise, quarantine measures aimed at excluding these fungi from new areas will depend on our ability to identify them.

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# Re-evaluating the taxonomic status of *Phaeoisariopsis griseola*, the causal agent of angular leaf spot of bean

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**Abstract:** Angular leaf spot of *Phaseolus vulgaris* is a serious disease caused by *Phaeoisariopsis griseola*, in which two major gene pools occur, namely Andean and Middle-American. Sequence analysis of the SSU region of nrDNA revealed the genus *Phaeoisariopsis* to be indistinguishable from other hyphomycete anamorph genera associated with *Mycosphaerella*, namely *Pseudocercospora* and *Stigmina*. A new combination is therefore proposed in the genus *Pseudocercospora*, a name to be conserved over *Phaeoisariopsis* and *Stigmina*. Further comparisons by means of morphology, cultural characteristics, and DNA sequence analysis of the ITS, calmodulin, and actin gene regions delineated two groups within *P. griseola*, which are recognised as two formae, namely f. *griseola* and f. *mesoamericana*.

**Taxonomic novelties:** *Pseudocercospora griseola* (Sacc.) Crous & U. Braun comb. nov., *P. griseola* f. *mesoamericana* Crous & U. Braun f. nov.

**Key words:** Ascomycetes, DNA sequence comparisons, *Mycosphaerella*, *Phaeoisariopsis*, *Phaseolus vulgaris*, *Pseudocercospora*, systematics.

## INTRODUCTION

Angular leaf spot (ALS) of beans (*Phaseolus vulgaris*) is caused by *Phaeoisariopsis griseola* (Sacc.) Ferraris. The disease is of major importance in tropical and subtropical areas, causing yield losses of up to 80 % (Schwartz *et al.* 1981, Saettler 1991, Liebenberg & Pretorius 1997). The disease affects pods and foliage, and is particularly destructive in warm, humid areas (Saettler 1991). Pod symptoms consist of circular to elliptical red-brown lesions, while leaf lesions start as small, brown or grey spots that become angular and necrotic, being confined by leaf veins. Leaf spots eventually coalesce, causing premature defoliation (Correa-Victoria *et al.* 1989, Saettler 1991). Furthermore, the disease also affects the quality and marketability of seed across bean-producing areas of the world (Pastor-Corrales *et al.* 1998).

In the Great Lakes Region of Africa, losses attributed to ALS have been estimated to be around 374 800 t (Wortmann *et al.* 1998). Disease control is best achieved via the selection of resistant varieties. Breeding for resistance against ALS is complicated, as the pathogen is highly variable with regard to pathogenicity, which means that durable resistance is difficult to achieve (Pastor-Corrales *et al.* 1998). High levels of pathogenic and genetic variation have been reported in *P. griseola* by various authors (Guzmán *et al.* 1995, Boshoff *et al.* 1996, Busogoro *et al.* 1999, Mahuku *et al.* 2002, Wagara *et al.* 2004).

There are indications of at least two main, morphologically distinguishable domestication events for the common bean, which in turn gave rise to two main gene pools, namely large-seeded beans of Andean origin, and small to medium-sized beans of Middle-American origin (Brown *et al.* 1982, Gepts &

Bliss 1985, 1986, Gepts *et al.* 1986, Koenig & Gepts 1989, Sprecher & Isleib 1989, Koenig *et al.* 1990, Singh *et al.* 1991a, b, Miklas & Kelly 1992, Skroch *et al.* 1992, Chacón *et al.* 2005).

Several fungal pathogens of *P. vulgaris*, in particular *Phaeoisariopsis griseola*, causal organism of ALS, *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara, the causal organism of anthracnose, and *Uromyces appendiculatus* (Pers. : Pers.) Unger var. *appendiculatus*, the causal organism of bean rust, have undergone parallel micro-evolution with the host. Although there is considerable variation within gene pools, differences are particularly evident when the reactions of isolates to differential lines of known Andean and Middle-American origin are compared. Isolates originating from the Andes are virulent only on large-seeded lines, whereas those originating from countries such as Central America, Mexico, Bolivia and Brazil are generally virulent on lines from both groups (Steadman 1995, Liebenberg 1996, Pastor-Corrales 1996, Chacón *et al.* 1997, Araya & Steadman 1998, Sandlin *et al.* 1999, Araya *et al.* 2004). Using isozyme analysis, Correa-Victoria (1987) could distinguish two groups in 55 *P. griseola* isolates from Africa, the U.S.A. and Latin America. All 26 isolates from Africa clustered in one group, whereas Latin American isolates clustered in both groups. However, recently the presence of both groups was reported from Africa (Liebenberg 1996, Wagara *et al.* 2004), which was also supported by data derived from isozyme analysis (Boshoff *et al.* 1996). Guzmán *et al.* (1995) used RAPD analysis to divide 62 *P. griseola* isolates from Brazil, Wisconsin (U.S.A.) and Malawi into two broad groups. Isolates in the Andean group, collected predominantly from Andean bean host genotypes, were more pathogenic on Andean genotypes, whereas those from the second group,

originating predominantly from Middle-American bean genotypes, were more pathogenic on Middle-American bean genotypes. The 11 Brazilian isolates fell in the second group, whereas 39 of the 42 Malawian isolates belonged to the Andean group. This grouping reflects the preference for small-seeded beans in Brazil, and large-seeded beans in Malawi. A third, more virulent group reported in Africa (CIAT 1996, Liebenberg 1996) appears to be a variation of the Andean group (Mahuku *et al.* 2002).

Buruchara (1983) observed differences in conidial size and amount of septation between isolates. However, he concluded that, due to the extent of variation within groups, these characteristics could not be used for grouping isolates. Several authors have attempted to associate lesion size with pathogenicity differences. Verma & Sharma (1984) observed two types of lesions in the field that differed in size, but found no significant differences in the number and size of lesions caused by the two groups of isolates, or in their radial growth in culture. Lesion size can vary considerably, but Correa-Victoria (1987) found no significant correlation between disease severity and lesion size, and no correlation between spore production and lesion size, but reported it to be highly dependent on the host cultivar (Correa-Victoria 1987). Lesion size may be affected by the interaction between host gene pool and pathogen origin (Liebenberg *et al.* 1996). These phenomena gave rise to questions as to the extent of differences between the Andean and Middle-American groups.

Ferraris (1909) erected the genus *Phaeoisariopsis* Ferraris for four *Isariopsis*-like species, including *Isariopsis griseola* Sacc. (Saccardo 1878), the type species, characterised by having synnematos conidiophore fascicles and pigmented conidiophores and conidia. In subsequent years several diverse elements were included in the genus (Ellis 1971, 1976, von Arx 1983). Chupp (1954) described a bean pathogen in his monograph under *Cercospora columnaris* Ellis & Everh., but cited the older name *Phaeoisariopsis griseola* as synonym. In his notes he stressed to favour the retention of *Phaeoisariopsis*. Deighton (1990) reassessed the genus, and considered the synnematos arrangement of conidiophores to be unsuitable as sole character for generic differentiation. Subsequently he confined *Phaeoisariopsis* to a few species similar to *P. griseola*, having non-geniculate conidiogenous cells with flattened, but conspicuous scars. Deighton placed species with conspicuously geniculate conidiogenous cells and thickened, darkened scars in *Passalora* Fr., whereas taxa with quite inconspicuous conidiogenous loci were reallocated to *Pseudocercospora* Speg. Von Arx (1983) and Braun (1992, 1995a, b) preferred to maintain *Phaeoisariopsis*, based on synnematos conidiomata, but confined it to species with conspicuous (slightly thickened, not darkened) conidiogenous loci.

The primary aim of the present study was to resolve the generic status of *Phaeoisariopsis* within *Mycosphaerella* Johanson, for which a subset of isolates were subjected to DNA sequence analysis of the SSU region. A further aim was to compare isolates of the Andean and Middle-American groups to address

the question if they represent two groups or species. For this purpose isolates were compared by means of morphology, cultural characteristics, and DNA sequence analysis of their internal transcribed spacer region (ITS-1, ITS-2 and 5.8S), calmodulin, and actin regions.

## MATERIALS AND METHODS

### Isolates

*Phaseolus* leaves exhibiting ALS symptoms, collected in Africa and South America, were studied (Table 1). Single-conidial cultures were established on 2 % malt extract agar (MEA) (Biolab, Midrand, South Africa) as outlined by Crous (1998). Colonies were subcultured onto 2 % potato-dextrose agar (PDA; Gams *et al.* 1998) and incubated at 25 °C under continuous near-ultraviolet light to promote sporulation.

### DNA phylogeny

Genomic DNA was isolated from fungal mycelium grown on MEA in Petri dishes and the ITS, actin (ACT) and calmodulin (CAL) regions were amplified and sequenced using the protocols and primers as described by Crous *et al.* (2004). The 5' end of the 18S rRNA gene (SSU) was amplified and sequenced as described by Braun *et al.* (2003).

The nucleotide sequences generated in this study were added to other sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov>) and the alignment was assembled using Sequence Alignment Editor v. 2.0a11 (Rambaut 2002) with manual improvement of the alignment where necessary. Sequence data were analysed as explained in Braun *et al.* (2003) using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2002) with both neighbour-joining and parsimony algorithms. Neighbour-joining analyses were conducted with the uncorrected ("p"), the Kimura 2-parameter and the HKY85 substitution models in PAUP. When they were encountered, ties were broken randomly. For parsimony analysis, alignment gaps were treated as new character states and all characters were unordered and of equal weight. Heuristic searches were performed with 10 random taxon additions. A partition homogeneity test (Farris *et al.* 1994) was conducted in PAUP to consider the feasibility of combining the ITS, actin and calmodulin data sets. Sequence data were deposited in GenBank (Table 1) and the alignments in TreeBASE (S1507, M2709-10).

### Determination of virulence phenotypes

The monoconidial isolates studied (Table 1) have previously been subjected to virulence phenotype characterisation on ALS differential lines from both the large- and small-seeded gene pools, as published previously (Liebenberg 1996, Mahuku *et al.* 2002).

### Morphology and cultural characteristics

Wherever possible, thirty measurements ( $\times$  1000 magnification) were made of structures mounted in

lactic acid, and the extremes of spore measurements given in parentheses. Colony colours (surface and reverse) were assessed after 14 d on PDA at 25 °C in the dark, using the colour charts of Rayner (1970). Cardinal temperatures for growth (from 9–33 °C, in 3° intervals) were determined on PDA plates as explained in Crous (1998). All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands (Table 1).

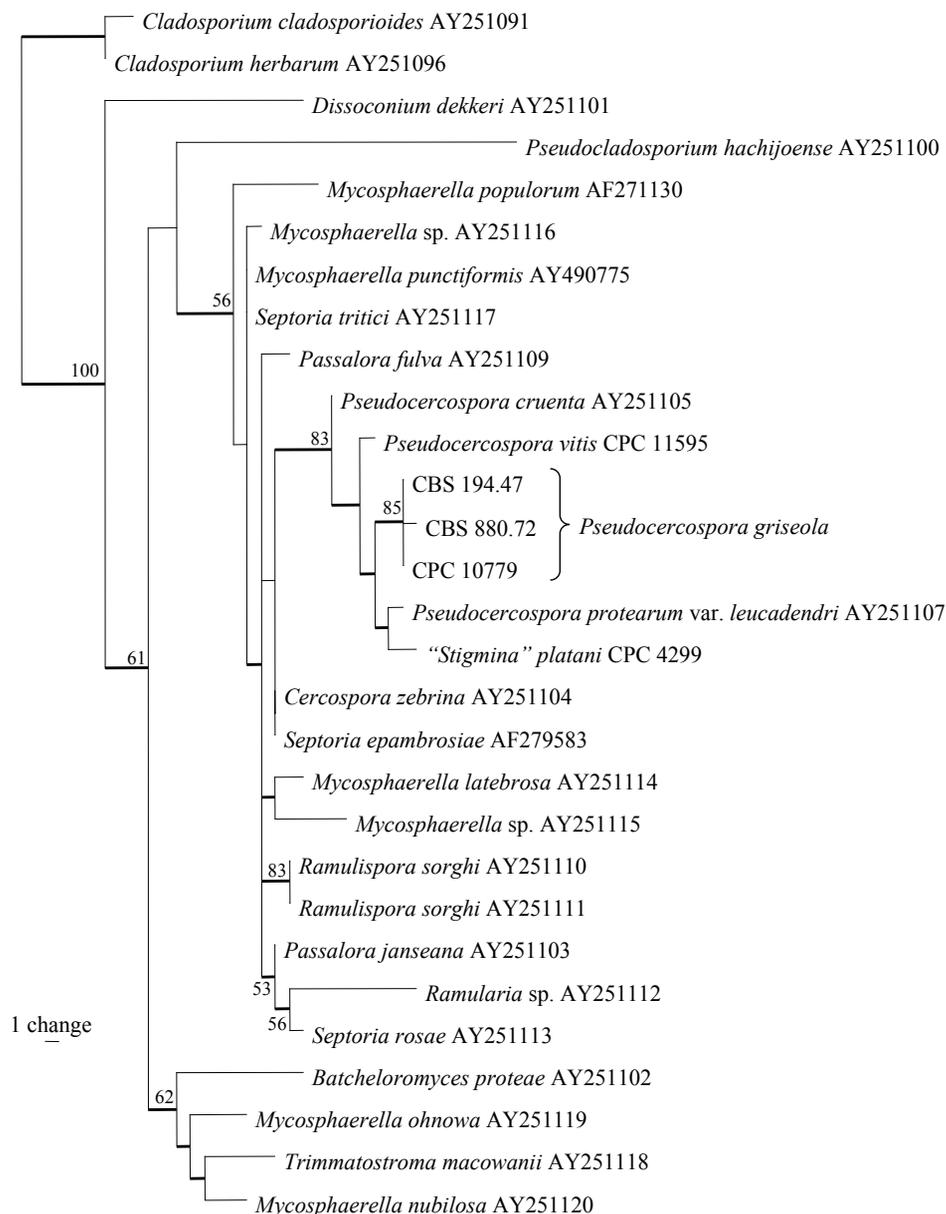
## RESULTS

### DNA phylogeny

The manually adjusted SSU sequence alignment contains 29 isolates (including the two outgroups) and 1029 characters including alignment gaps; of

these characters 38 are parsimony-informative, 57 are variable and parsimony-uninformative, and 934 are constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with identical topologies (data not shown). The same overall topology was also obtained with the parsimony analysis, which yielded 13 most parsimonious trees (TL = 135 steps; CI = 0.807; RI = 0.809; RC = 0.653), one of which is shown in Fig. 1. In this tree, species of *Pseudocercospora* and *Stigmata* form a well-defined clade (bootstrap support value of 83 %) within *Mycosphaerella*.

The ITS region was sequenced to provide better resolution of the order of the species within the *Pseudocercospora* clade. The manually adjusted ITS sequence alignment contains 45 isolates (including the two outgroups) and 499 characters including alignment gaps; of these characters 168 are parsimony-informative, 25 are variable and parsimony-uninformative, and 306



**Fig. 1.** One of 15 most parsimonious trees obtained from a heuristic search with 10 random taxon additions of the 18S rRNA gene sequence alignment. The scale bar shows a single change and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and the tree was rooted to two *Cladosporium* species.

**Table 1.** Isolates used for sequence analysis.

Species	Accession number <sup>1</sup>	Host	Virulence type	Origin	Collector	GenBank numbers <sup>2</sup> (ITS, CAL, SSU, ACT)
<i>Cladosporium herbarum</i>	CBS 572.78	<i>Polyporus radiatus</i>	—	Russia	—	DQ289799, DQ289831, —, DQ289866
<i>Davidiella tassiana</i>	CPC 11600	<i>Delphinium barbeyi</i>	—	U.S.A.	A. Ramalay	DQ289800, DQ289832, —, DQ289867
<i>Pseudocercospora griseola</i> f. <i>griseola</i>	CBS 194.47; ATCC 22393	<i>Phaseolus vulgaris</i>	—	Portugal	—	DQ289801, DQ289833, DQ289861, DQ289868
	CBS 880.72	<i>Phaseolus vulgaris</i>	—	Netherlands	H. A. v. Kesteren	DQ289802, DQ289834, DQ289862, DQ289869
	CPC 5592; Pg97MZ41	<i>Phaseolus vulgaris</i>	Andes	Zambia	R. Buruchara	DQ289803, DQ289835, —, DQ289870
	CPC 5594; Pg97LB48	<i>Phaseolus vulgaris</i>	Andes	South Africa	M.M. Liebenberg	DQ289804, DQ289836, —, DQ289871
	CPC 10457; Pg97MZ64	<i>Phaseolus vulgaris</i>	Andes	Zambia	R. Buruchara	DQ289805, DQ289837, —, DQ289872
	CPC 10458; Pg96CE7	<i>Phaseolus vulgaris</i>	Andes	South Africa	M.M. Liebenberg	DQ289806, DQ289838, —, DQ289873
	CPC 10459; Pg97CE78	<i>Phaseolus vulgaris</i>	Andes	South Africa	M.M. Liebenberg	DQ289807, DQ289839, —, DQ289874
	CPC 10460; Pg97AT101	<i>Phaseolus vulgaris</i>	Andes	Tanzania	F.S. Ngulu; C. Mushi	DQ289808, DQ289840, —, DQ289875
	CPC 10464; Pg97CE105	<i>Phaseolus vulgaris</i>	Andes	—	—	DQ289809, DQ289841, —, DQ289876
	CPC 10465; Pg97CE106	<i>Phaseolus vulgaris</i>	Andes	—	—	DQ289810, DQ289842, —, DQ289877
	CPC 10467; Pg97MZ42	<i>Phaseolus vulgaris</i>	Andes	Zambia	R. Buruchara	DQ289811, DQ289843, —, DQ289878
	CPC 10468; Pg97AT95	<i>Phaseolus vulgaris</i>	Andes	Tanzania	F.S. Ngulu; C. Mushi	DQ289812, DQ289844, —, DQ289879
	CPC 10469; Pg97KZ44	<i>Phaseolus vulgaris</i>	Andes	Zambia	R. Buruchara	DQ289813, DQ289845, —, DQ289880
	CPC 10477; Pg97CE23	<i>Phaseolus vulgaris</i>	Andes	South Africa	M.M. Liebenberg	DQ289814, DQ289846, —, DQ289881
	CPC 10480; Pg96VI90	<i>Phaseolus vulgaris</i>	Andes	South Africa	M.M. Liebenberg	DQ289815, DQ289847, —, DQ289882
	CPC 10481; Pg95GT5	<i>Phaseolus vulgaris</i>	Andes	South Africa	A.J. Liebenberg	DQ289816, DQ289848, —, DQ289883
	CPC 10484; Pg95CE7	<i>Phaseolus vulgaris</i>	Andes	South Africa	M.M. Liebenberg	DQ289817, DQ289849, —, DQ289884
	CPC 10779	<i>Phaseolus vulgaris</i>	—	Korea	H.D. Shin	DQ289818, DQ289850, DQ289863, DQ289885
	CPC 12238; Pg350	<i>Phaseolus vulgaris</i>	Andes	Colombia	G. Mahuku	DQ289819, DQ289851, —, DQ289886
	CPC 12239; Pg3	<i>Phaseolus vulgaris</i>	Andes	Colombia	G. Mahuku	DQ289820, DQ289852, —, DQ289887
CPC 12240; Pg266	<i>Phaseolus vulgaris</i>	Andes	Colombia	G. Mahuku	DQ289821, DQ289853, —, DQ289888	
<i>Ps. griseola</i> f. <i>mesoamericana</i>	CPC 5596; Pg99GT4	<i>Phaseolus vulgaris</i>	Middel-Amerikaans	South Africa	A.J. Liebenberg	DQ289822, DQ289854, —, DQ289889
	CPC 5597; Pg97TM109	<i>Phaseolus vulgaris</i>	Middel-Amerikaans	Malawi	A.J. Liebenberg	DQ289823, DQ289855, —, DQ289890
	CPC 10463; Pg96GT35	<i>Phaseolus vulgaris</i>	Middel-Amerikaans	South Africa	M.M. Liebenberg	DQ289824, DQ289856, —, DQ289891
	CPC 10474; Pg96GT32	<i>Phaseolus vulgaris</i>	Middel-Amerikaans	South Africa	M.M. Liebenberg	DQ289825, DQ289857, —, DQ289892
	CPC 10479; Pg99CE5	<i>Phaseolus vulgaris</i>	Middel-Amerikaans	South Africa	M.M. Liebenberg	DQ289826, DQ289858, —, DQ289893
	CPC 12241; Pg8	<i>Phaseolus vulgaris</i>	Middel-Amerikaans	Honduras	G. Mahuku	DQ289827, DQ289859, —, DQ289894
CPC 12242; Pg32	<i>Phaseolus vulgaris</i>	Middel-Amerikaans	Colombia	G. Mahuku	DQ289828, DQ289860, —, DQ289895	
<i>Pseudocercospora vitis</i>	CPC 11595	<i>Vitis vinifera</i>	—	Korea	H.D. Shin	DQ289829, —, DQ289864, —
	CPC 11660	<i>Vitis flexuosa</i>	—	Korea	H.D. Shin	DQ289830, —, —, —
" <i>Stigmina</i> " <i>platani</i>	CBS 110755; CPC 4299; IMI 136770	<i>Platanus orientalis</i>	—	India	—	AY260090, —, DQ289865, —

<sup>1</sup>ATCC: American Type Culture Collection, Virginia, U.S.A.; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, U.K.

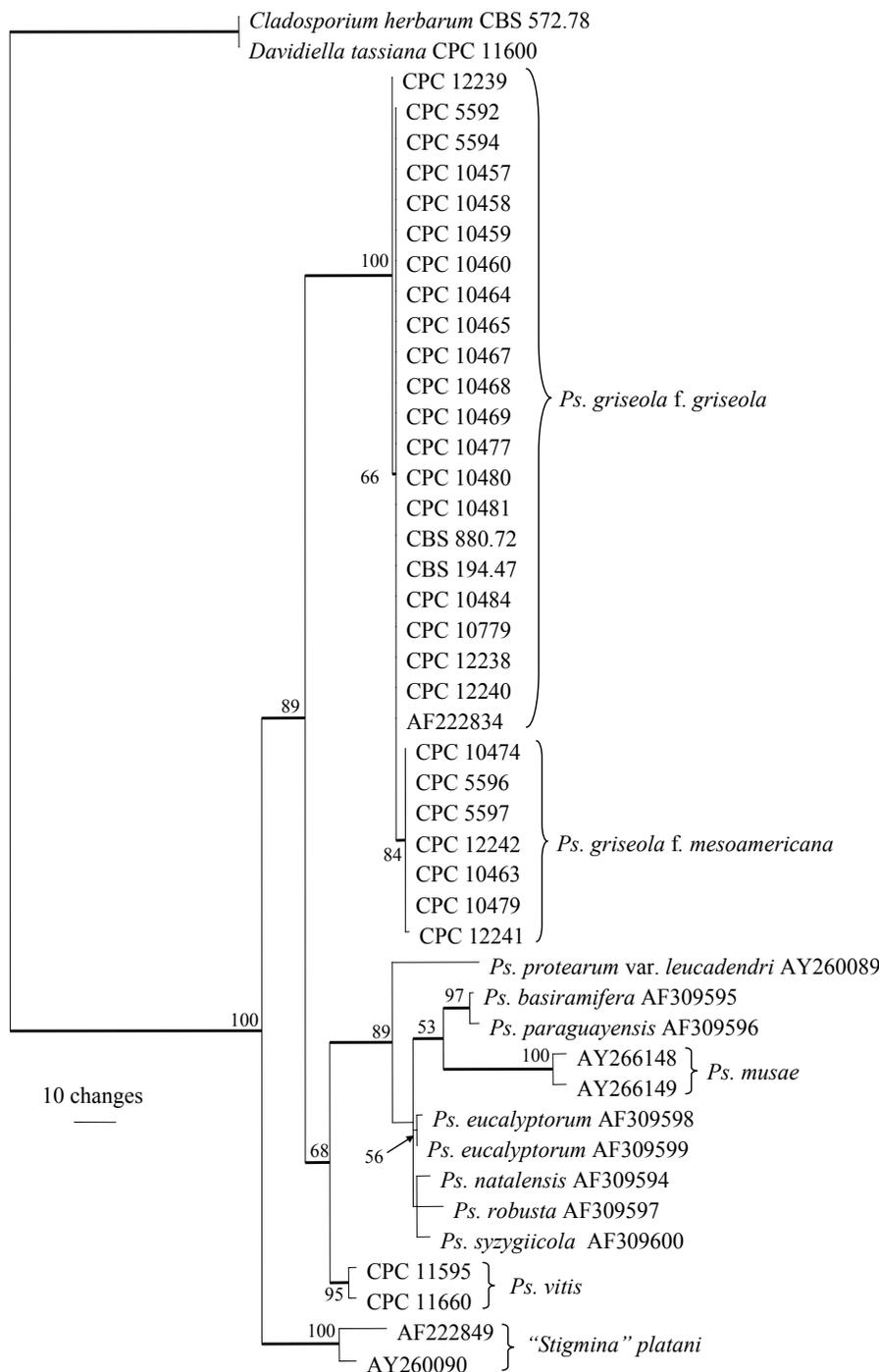
<sup>2</sup>ITS: internal transcribed spacer region, CAL: partial calmodulin gene, SSU: partial 18S rRNA gene, ACT: partial actin gene. All DQ numbers refer to newly generated sequences.



are constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with identical topologies (data not shown). Only the order and grouping of the deeper nodes differed between the neighbour-joining and parsimony analyses (data not shown). Parsimony analysis yielded 13 most parsimonious trees (TL = 293 steps; CI = 0.816; RI = 0.918; RC = 0.749), one of which is shown in Fig. 2. In this tree, isolates of *Ps. griseola* are grouped together with a bootstrap support value of 100 %, with the Middle-American isolates (*Ps. griseola* f. *mesoamericana*) grouping together with a bootstrap support value of 84 %. Also in the tree are other *Pseudocercospora* species

(89 % bootstrap support), two strains of *Ps. vitis* (type species of *Pseudocercospora*, 95 % bootstrap support) and a basal well-defined clade (bootstrap support value of 100 %) of two GenBank sequences of *Stigmina platani*.

To determine whether *Ps. griseola* isolates from Middle-American and Andean origin can be distinguished phylogenetically, the ACT (235 characters) and CAL (316 characters) sequences were combined with the ITS sequences. The partition homogeneity test showed that the three loci were combinable into a single analysis ( $P = 0.6550$ ). The manually adjusted combined alignment consists of 1050 bases (including alignment gaps) and



**Fig. 2.** One of 13 most parsimonious trees obtained from a heuristic search with 10 random taxon additions of the ITS sequence alignment. The scale bar shows 10 changes and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and the tree was rooted to *Cladosporium herbarum* and *Davidiella tassiana*.

30 isolates (including the two outgroups). Of the 1050 characters, 288 are parsimony-informative, 42 were variable and parsimony-uninformative, and 720 were constant. The topologies of the trees obtained from the neighbour-joining analyses were identical to each other and also to that obtained from the parsimony analysis (data not shown). Parsimony analysis of the combined data yielded three most parsimonious trees (TL = 353 steps; CI = 0.994; RI = 0.994; RC = 0.988), one of which is shown in Fig. 3. The tree shows two distinct clades, namely *Ps. griseola* f. *griseola* and what we call here the *Ps. griseola* f. *mesoamericana* clade. Bootstrapping using parsimony results in support values of 53 % and 71 % for each clade, respectively. These values increase to 62 % and 98 %, respectively, if neighbour-joining with the HKY85 substitution model is used for bootstrapping. The *Ps. griseola* f. *griseola* clade is further split into two groups (62 / 95 % and

52 / 71 % bootstrap support, respectively), which is the result of three characters that changed in the CAL sequence of isolates CPC 12238 and CPC 12239 (99.04 % sequence similarity to the other *Ps. griseola* f. *griseola* isolates).

### Taxonomy

***Pseudocercospora griseola*** (Sacc.) Crous & U. Braun, **comb. nov.** MycoBank MB500855. Fig. 4.

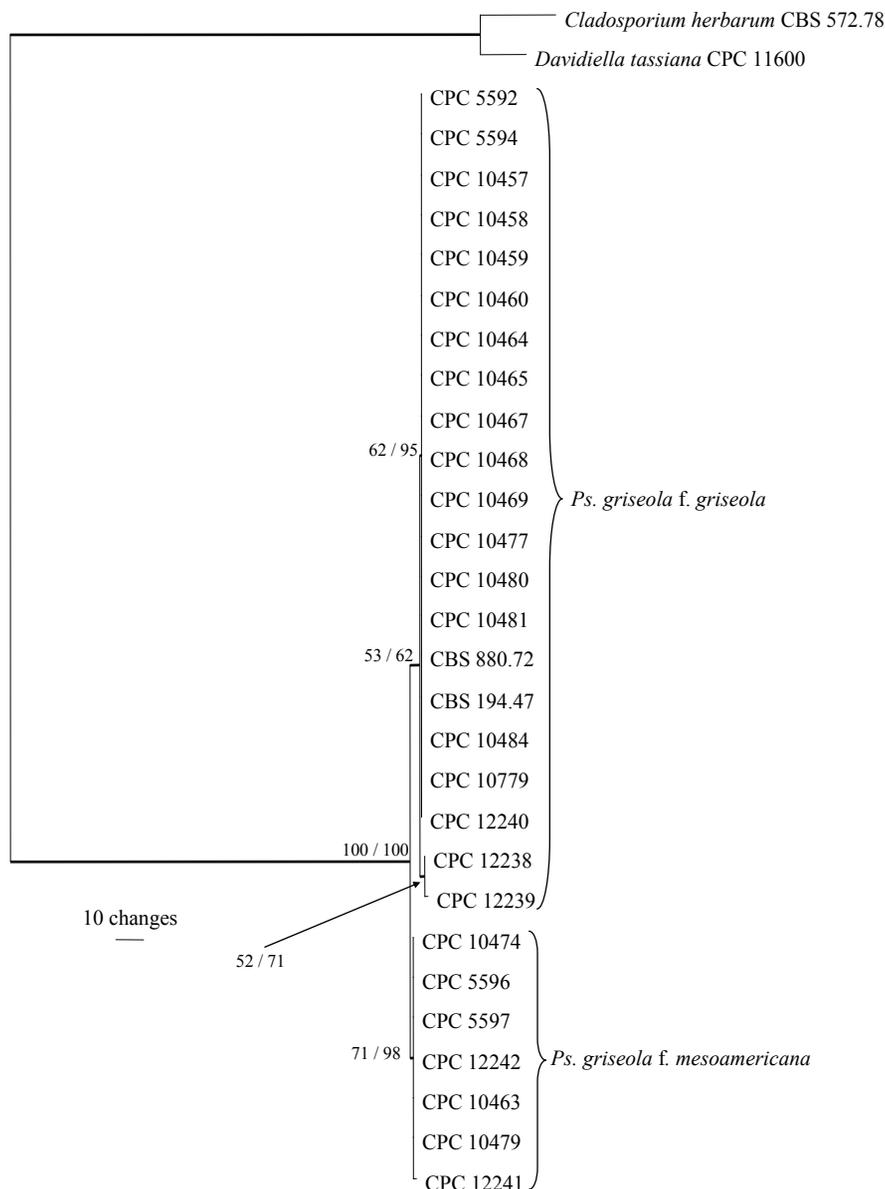
Basionum: *Isariopsis griseola* Sacc., *Michelia* 1: 273. 1878.

≡ *Phaeoisariopsis griseola* (Sacc.) Ferraris, *Ann. Mycol.* 7: 273. 1909.

≡ *Lindaumyces griseolus* (Sacc.) Gonz. *Frag. (as "g riseola")*, *Mem. R. Acad. Ci. Exact. Madrid, Ser. 2*, 6: 339. 1927.

≡ *Cercospora griseola* (Sacc.) Ragunath. & K. Ramakr., *J. Madras Univ.* 35–36: 11. (1965–1966) 1968.

= *Cylindrosporium phaseoli* (*Cylindrospora*) Rabenh., *Klotzschii Herbarium vivum mycologicum, Editio nova, Series Prima, Centuria 4, No. 327, Dresden 1856, nom. nud.*, also *Bot. Zeitung* 15(6): 94. 1857, *nom. nud.* and *Flora* 15(9): 134. 1857, *nom. nud.*



**Fig. 3.** One of three most parsimonious trees obtained from a heuristic search with 10 random taxon additions of a combined ITS, actin and calmodulin sequence alignment. The scale bar shows 10 changes and bootstrap support values from 1000 replicates are shown at the nodes (values from parsimony before the slash and neighbour-joining with the HKY85 substitution model after the slash). Thickened lines indicate branches found in the strict consensus parsimony tree and the tree was rooted to *Cladosporium herbarum* and *Davidiella tassiana*.



- = *Graphium laxum* Ellis, Bull. Torrey Bot. Club 8: 64. 1881.
- ≡ *Isariopsis laxa* (Ellis) Sacc., Syll. Fung. 4: 631. 1886.
- ≡ *Phaeoisariopsis laxa* (Ellis) S.C. Jong & E.F. Morris, Mycopathol. Mycol. Appl. 34: 269. 1968.
- = *Cercospora solimani* Speg. (*solimani*), Anales Soc. Ci. Argent. 16: 167. 1883.
- = *Cercospora columnaris* Ellis & Everh. (as "*columnare*"), Proc. Acad. Nat. Sci. Philadelphia 46: 380. 1894.
- ≡ *Pseudocercospora columnaris* (Ellis & Everh.) J.M. Yen, in Yen & Lim, Gard. Bull., Singapore 33: 172. 1980.
- = *Arthrobotryum puttemansii* Henn., Hedwigia 41: 309. 1902.
- = *Cercospora stuhlmannii* Henn., Bot. Jahrb. Syst. 33: 40. 1904.

**Syntypes:** on *Phaseolus vulgaris*, Italy, Selva, Aug. 1877, Saccardo, Mycotheca Veneta 1247 (e.g., B, HAL, PAD).

**Formae novae:**

***Pseudocercospora griseola*** (Sacc.) Crous & U. Braun, f. *griseola*

**Specimen examined:** Tanzania, on *Phaseolus vulgaris*, F.S. Ngulu & C. Mushi, CBS H-19683, **epitype designated here**, CBS 119906 = CPC 10468. culture ex-epitype. The epithet "*griseola*" was based on European material, and from our analysis, it appears that European material is representative of *P. griseola* f. *griseola*.

***Pseudocercospora griseola*** (Sacc.) Crous & U. Braun, f. ***mesoamericana*** Crous & U. Braun f. **nov.** MycoBank MB500856.

Differt a f. *griseola* variatione virulentiae majore, culturis crescentibus ad  $\geq 30$  °C.

Morphologically similar to *P. griseola* f. *griseola*, but distinct by having a broader range of virulence on different bean types, and being able to grow at or above 30 °C, which is not the case for f. *griseola*.

**Specimen examined:** South Africa, on *Phaseolus vulgaris*, M.M. Liebenberg, CBS H-19684, **holotype**, culture ex-type CBS 119113 = CPC 10463.

**Descriptions (selection):** Gonzáles Fragoso (1927: 339), Chupp (1954: 295, as *Cercospora columnaris*), Ellis (1971: 269), Shin & Kim (2001: 151–153).

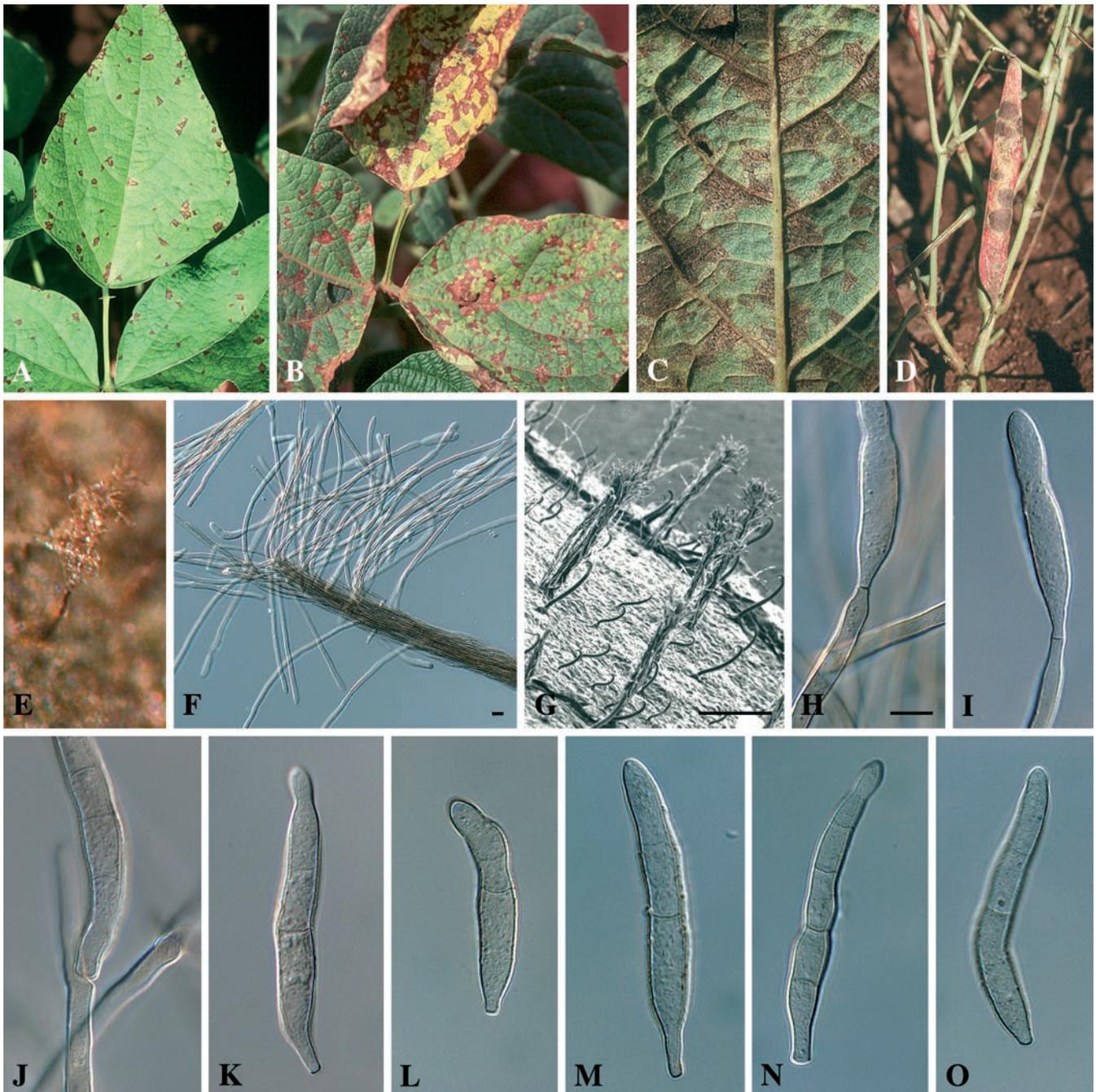
**Illustrations (selection):** Saccardo, Fungi italici, Pl. 838, Padova 1881; Briosi & Cavara, Funghi parassiti delle piante coltivate od utili, Fasc. I, No. 17, figs 1–2, Pavia 1888; Gonzáles Fragoso (1927: 340, fig. 79); Ellis (1971: 269, fig. 183); Deighton (1990: 1098, figs 2–3); Shin & Kim (2001: 153, fig. 65).

**Description in vivo:** On leaves, petioles, stems and pods; *leaf spots* amphigenous, angular–irregular, rarely subcircular–elliptical, mostly vein-limited, 1–8 mm wide, finally sometimes confluent, forming larger patches, brown, ranging from pale olivaceous, olivaceous-brown, yellowish brown, greyish brown to dark brown, on pods often reddish brown and more regular, subcircular–elliptical, margin indefinite, only delimited by veins, or surrounded by a narrow, dark brown border or marginal line. *Caespituli* on petioles, pods, stems and leaves, amphigenous, mostly hypophyllous, usually scattered, occasionally aggregated, conspicuous, punctiform, dark brown to blackish grey. *Mycelium* internal. *Stromata* almost lacking to well-developed,

subglobose, depressed to lacrimoid, up to 70  $\mu$ m diam, brown. *Conidiophores* numerous, up to approx. 40, in dense fascicles, often forming synnematosus conidiomata, erumpent, 100–500  $\times$  20–70  $\mu$ m, rarely longer, olivaceous-brown, composed of a more or less firm stipe of closely appressed conidiophores and a terminal, loose capitulum, i.e. conidiophores splaying out at the end of the conidiomata, free ends usually up to 100  $\mu$ m long, individual conidiophores filiform, appressed threads 2–5  $\mu$ m wide, up to 7  $\mu$ m wide towards the apex, pluriseptate, subhyaline to olivaceous-brown, thin-walled, occasionally becoming rough-walled with age. *Conidiogenous cells* integrated, terminal, 20–100  $\mu$ m long, subcylindrical to subclavate, usually not or only barely geniculate, but moderately geniculate in some collections; conidiogenous loci terminal and lateral, quite inconspicuous to subconspicuous, i.e. unthickened or almost so, but slightly darkened-refractive, in surface view visible as minute circles, 1.5–2.5  $\mu$ m diam, usually flat, non-protruding. *Conidia* solitary, obclavate-cylindrical, broadly subfusiform, short conidia sometimes ellipsoid-ovoid to short cylindrical, straight to curved, 20–75(–85)  $\times$  4–9  $\mu$ m, (0–)1–5(–6)-septate, usually not constricted at the septa, rarely with slight constrictions, subhyaline to pale olivaceous or olivaceous-brown, thin-walled, smooth, sometimes rough-walled, with obtuse apex, and obconically truncate to rounded base, 1.5–2.5(–3)  $\mu$ m wide, hila unthickened or almost so, at most somewhat refractive.

**Cultural characteristics:** Forma *griseola*; on OA colonies flat to slightly erumpent, spreading with moderate aerial mycelium; margins smooth, regular, surface with patches of olivaceous-grey and smoke-grey to dirty-white; on PDA erumpent with moderate aerial mycelium, surface pale olivaceous-grey to olivaceous-grey in the central part; margin iron-grey, and also iron-grey in reverse. Cardinal temperature requirements for growth: minimum 6 > °C, optimum = 24 °C, maximum < 30 °C. Forma *mesoamericana*; on OA flat to slightly erumpent, spreading, with moderate aerial mycelium; margins irregular, feathery to smooth, even; surface with the central part dirty-white to pale or darker olivaceous-grey, outer region iron-grey; on PDA spreading, erumpent, with moderate aerial mycelium; surface olivaceous-grey in the central part; outer region and reverse iron-grey, margins feathery, irregular. Cardinal temperature requirements for growth: minimum 6 > °C, optimum 24 °C, maximum > 30 °C.

**Herbarium specimens examined:** On *Lablab niger*, Japan, Tokyo, Toyoda, Itino-machi, Minamitama-gun, 9 Aug. 1962, S. Takamoto (IMI 96372). On *Phaseolus vulgaris*, Italy, Selva, Aug. 1877, Sacc., Mycoth. Ven. 1247 (HAL), type of *Isariopsis griseola*; Italy, Pavia, Casatisma e Albaredo Arnaboldi, 1888, Briosi & Cavara, Funghi parass. 17 (HAL); Russia, Czernigov, Borzova, Aug. 1914, G. Nevodovsky, Petr. Mycoth. gen. 249 (B); South Korea, Chunchon, 7 Oct. 2003, H.D. Shin (HAL). On *Phaseolus* sp., Brazil, São Paulo, Botanical Garden, 26 Dec. 1901, Puttemans, No. 413 (B), type of *Arthrobotryum puttemansii*; Italy, Bugellae et Vercellis, Cesati, Rabenh., Herb. mycol., Ed. 2, No. 327 (HAL), type of *Cylindrosporium phaseoli*; USA, N.J., Newfield, 27 Sep. 1894, J.B. Ellis (NY), type of *Cercospora columnaris*. Unidentified host (*Phaseolus* sp.), Paraguay, Caá-guazú, Jan. 1882, B. Balansa, No. 3492 (LSP 918), type of *Cercospora solimani*.



**Fig. 4.** *Pseudocercospora griseola*. A–C. Leaf disease symptoms. D. Lesions on bean pod. E–G. Fasciculate conidiophores. H–J. Conidiogenous cells giving rise to conidia. K–O. Conidia. Scale bars: F = 8  $\mu$ m, G = 200  $\mu$ m, H = 10  $\mu$ m.

**Hosts and distribution:** *Lablab niger*, ?*L. purpureus*, ?*Lathyrus odoratus*, ?*Macroptilium atropurpureum*, *Phaseolus acutifolius*, *P. aureus*, *P. coccineus*, *P. lunatus*, *P. pubescens*, *P. vulgaris*, *Vigna angularis*, *V. mungo*, *V. radiata*, *V. sinensis*, *V. unguiculata* (*Leguminosae*), worldwide, including Angola, Argentina, Armenia, Australia, Austria, Bhutan, Brazil, Bulgaria, Burundi, Cameroon, Canada, China, Colombia, Congo, Costa Rica, Croatia, Cuba, Dominican Republ., Ecuador, El Salvador, Ethiopia, Fiji, France, Georgia, Germany, Ghana, Great Britain, Greece, Guatemala, Haiti, Hungary, Jamaica, Japan, India, Indonesia, Iran, Ireland, Israel, Italy, Ivory Coast, Jamaica, Japan, Kenya, Korea, Laos, Latvia, Malawi, Madagascar, Malaysia, Mauritius, Mexico, Mozambique, Nepal, Netherlands, Netherlands Antilles, New Caledonia, New Zealand,

Nicaragua, Nigeria, Norfolk Island, Panama, Papua New Guinea, Paraguay, Peru, Philippines, Poland, Portugal, Puerto Rico, Réunion, Romania, Russia, Rwanda, Saint Helena, Senegal, Sierra Leone, Singapore, Slovenia, Solomon Islands, Somalia, South Africa, Spain, Sudan, Suriname, Swaziland, Switzerland, Taiwan, Tanzania, Thailand, Trinidad and Tobago, Turkey, Uganda, Ukraine, U.S.A. (CT, DE, Eastern states, FL, HI, IN, MA, MD, ME, MI, MS, NC, NH, NJ, NY, OK, PA, SC, TX, VA, WI), Vanuatu, Venezuela, Virgin Islands, Yugoslavia, Zambia, Zimbabwe (Crous & Braun 2003).

**Notes:** As a consequence of molecular sequence analyses (Figs 1–3), and re-examination and reassessments of the synnematous conidiomata and scar and hilum structures (Fig. 4, see Discussion),

*Phaeoisariopsis griseola* proved to be congeneric with *Pseudocercospora*. The proposed assignment of this species to *Pseudocercospora* presupposes acceptance of a formal proposal to conserve the latter genus against the older names *Phaeoisariopsis* and *Stigmina* (Braun & Crous 2006). All other taxa formerly placed in *Phaeoisariopsis* have already been treated and reallocated elsewhere (Crous & Braun 2003).

*Cylindrosporium phaseoli* Rabenh. is the oldest name coined for this species, which appeared first on the printed label of 'Rabenh., Herb. mycol. 327, 1856'. This name was repeated in Fürnrohr (1857), Schlechtendal (1857) and Saccardo (1884), but in all cases without any description (*nom. nud.*). González Frago (1927: 339) was the first author who correctly cited this name as synonym of *Phaeoisariopsis griseola*, which we confirm after having re-examined Rabenhorst's original material.

Deighton (1990) reduced *Cercospora solimanii* Speg. to synonymy with *Ph. griseola*, but without any comments and references. Braun (2000) examined type material of this species and confirmed Deighton's (1990) synonymy.

Although there are two clear entities associated with the angular leaf spot disease of bean on pathological or molecular grounds, we were unable to find enough morphological, cultural or phylogenetic support to separate these as two species. Because isolates can readily be classed as either one or the other type based on their host reaction on differential cultivars, we have chosen to designate them as *formae* of the same species.

## DISCUSSION

A primary aim of the present study was to determine the species status of the Andean and Middle-American groups of the angular leaf spot pathogen of beans. Because we have been unable to obtain good morphological differences between the two groups (other than cardinal temperatures for growth), nor clear phylogenetic support for the separation based on various gene loci, we have chosen to recognise these two operational units as *formae* of the same species, namely f. *griseola* and f. *mesoamericana*.

Two basic characters have in the past been used for the discrimination of *Phaeoisariopsis* and *Pseudocercospora*, namely the structure of the conidiomata and the type of conidiogenous loci and conidial hila. In molecular studies, the conidiomatal structures were shown to be unreliable at the genus level for anamorphs of *Mycosphaerella*. This is aptly illustrated by the examples of *Septoria* Sacc. (pycnidia) and *Phloeospora* Wallr. (acervuli) (Verkley *et al.* 2004), *Colletogloeopsis* Crous & M.J. Wingf. (acervuli) and *Phaeophloeospora*-like species with aseptate conidia and pycnidia (Cortinas *et al.* 2005), *Ramularia* Unger (normal fascicles) and *Phacellium* Bonord. (synnemata) (Crous *et al.*, unpubl. data), which are all irregularly scattered among the cladogrames. The coelomycete

genus *Septoria* (pycnidia) always clusters basal to *Cercospora* Fresen. (fasciculate hyphomycete) (Crous *et al.* 2000, 2001). The presence of synnemata is thus insufficient to separate *Phaeoisariopsis* from *Pseudocercospora* (Crous *et al.* 2001, Crous & Braun 2003). Furthermore, *Pseudocercospora* already includes some synnematosus species [e.g. the type species, *P. vitis* (Lév.) Speg.]. Several species originally placed in *Phaeoisariopsis*, but with inconspicuous conidial scars, have already been reallocated in *Pseudocercospora* (Deighton 1990). There are also some other genera of hyphomycetes with synnematosus as well as non-synnematosus species, e.g., *Spiropes* Cif. (Ellis 1971).

The structure of the conidiogenous loci and conidial hila represent another important character used for the distinction of *Phaeoisariopsis* and *Pseudocercospora*. Prior to the introduction of the scar structure as basic feature in the taxonomy of cercosporoid genera (Deighton 1967, 1973, 1974, 1976), *Phaeoisariopsis* was mainly or even solely based on the synnematosus arrangement of the conidiophores, combined with pigmented conidia formed singly. Therefore, it was hardly surprising that Sawada (1922) transferred *Septonema vitis* Lév., the type species of *Pseudocercospora*, to *Phaeoisariopsis*, and thus reduced *Pseudocercospora* to synonymy with *Phaeoisariopsis*. The heterogeneity of *Phaeoisariopsis* is also reflected by the exclusion of all species, except for the type species, *I. griseola*, originally placed in this genus by Ferraris (1909): *Isariopsis grayiana* Ellis (= *Fusicladium grayianum* (Ellis) Deighton & M.B. Ellis), *I. mexicana* Ellis & Everh. (= *Exosporium mexicanum* (Ellis & Everh.) M.B. Ellis) and *I. pilosa* Earle (= *Morrisographium persicae* (Schwein.) Deighton) (see Deighton 1990). Von Arx (1983), Deighton (1990) and Braun (1992, 1995, 1998) considered the conidiogenous loci and conidial hila in *Phaeoisariopsis* to be conspicuous or at least subconspicuous, i.e., barely to slightly thickened and darkened. However, Yen (Yen & Lim 1980) already placed the ALS pathogen in *Pseudocercospora* (conidiogenous loci inconspicuous), although the wrong combination [*Pseudocercospora columnaris* (Ellis & Everh.) J.M. Yen] was introduced, and the correct basionym, *Isariopsis griseola*, cited as synonym. The inclusion of *Phaeoisariopsis griseola* in *Pseudocercospora* (Sawada 1922) thus reduces *Pseudocercospora* to synonymy with *Phaeoisariopsis*. We have re-examined the scars and hila in *Ph. griseola* in detail, based on a wide range of samples *in vivo* and *in vitro*, including type material of *Isariopsis griseola*, *Cylindrosporium phaseoli*, *Cercospora columnaris* and *C. solimanii*. The conidiogenous cells are usually not or barely geniculate, the conidiogenous cells are terminal to lateral, non-protruding, quite inconspicuous to subconspicuous, i.e. unthickened or almost so, but slightly darkened-refractive. There are collections with completely inconspicuous conidiogenous loci, e.g. the types of *Isariopsis griseola* and *Cercospora solimanii*. In other samples, the loci range from being quite inconspicuous to subconspicuous. The African collection illustrated by Deighton (1990) is an example of subconspicuous loci. However, as demonstrated earlier by molecular examinations, taxa

with subconspicuous loci and hila (unthickened or almost so, but slightly darkened-refractive or only the ultimate rim slightly thickened and darkened) clustered together with *Pseudocercospora* species, so that further segregate-genera like *Paracercospora* Deighton and *Pseudophaeoramularia* U. Braun had to be reduced to synonymy with *Pseudocercospora* (Crous et al. 2000, 2001; Crous & Braun 2003).

Based on the molecular data presented here, the type species of *Pseudocercospora* (*P. vitis*) clusters with the type of *Phaeoisariopsis* (*P. griseola*), and the type of *Stigmina* Sacc. [*S. platani* (Fuckel) Sacc.]. The close affinity of these three genera underlines earlier suspicions of mycologists that criteria such as 1) slightly thickened conidial hila and scars, 2) synnematos to fasciculate to sporodochial conidiomata, 3) transverse to muriformly septate conidia, 4) euseptate to distoseptate conidia, 5) smooth percurrent proliferations and sympodial proliferation, versus irregular, rough percurrent proliferations on conidiogenous cells, are an insufficient basis to separate anamorph genera in *Mycosphaerella*.

Given the fact that these three genera represent anamorph forms of *Mycosphaerella*, and that they phylogenetically reside in the same clade, the next predicament arises as to what name should be applied: *Pseudocercospora* (1910; 1171 names), *Phaeoisariopsis* (1909, 65 names), or *Stigmina* (1880, 161 names). Although *Stigmina* is the oldest name, *Pseudocercospora* is the most commonly used, and many species of *Stigmina* in fact represent other fungi. *Phaeoisariopsis*, which also is older than *Pseudocercospora*, has been reduced to its type species, with most other species being placed in either *Passalora* or *Pseudocercospora*. *Stigmina* predates *Phaeoisariopsis*. If the Code of Botanical Nomenclature were to be strictly applied, all species in this complex should be transferred to *Stigmina*. As the latter is a poorly resolved, still heterogeneous genus, we choose to avoid this upheaval, and support conservation of the commonly used and accepted generic name, *Pseudocercospora* (Braun & Crous 2006). The latter genus should be used for the whole complex of hyphomycetes formerly placed in *Phaeoisariopsis* and some of *Stigmina*. A formal conservation proposal to this extent has been prepared for Taxon (Braun & Crous 2006).

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## Metulocladosporiella gen. nov. for the causal organism of Cladosporium speckle disease of banana

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### ABSTRACT

*Cladosporium musae*, a widespread leaf-spotting hyphomycete on *Musa* spp., is genetically and morphologically distinct from *Cladosporium* s. str. (*Davidiella* anamorphs, *Mycosphaerellaceae*, *Dothideales*). DNA sequence data derived from the ITS and LSU gene regions of *C. musae* isolates show that this species is part of a large group of hyphomycetes in the *Chaetothyriales* with dematiaceous blastoconidia in acropetal chains. *Cladosporium adianticola*, a foliicolous hyphomycete known from leaf litter in Cuba is also a member of this clade and is closely related to *C. musae*. A comparison with other genera in the *Cladosporium* complex revealed that *C. musae* belongs to a lineage for which no generic name is currently available, and for which the genus *Metulocladosporiella* gen. nov. is proposed. Two species of *Metulocladosporiella* are currently known, namely *M. musae*, which is widely distributed, and *M. musicola* sp. nov., which is currently known from Africa.

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### Introduction

The name *Cladosporium musae* was introduced by Mason (*in Martyn 1945*) for a leaf-spotting hyphomycete causing *Cladosporium* speckle disease of banana. This disease occurs in most countries where banana is cultivated (*Jones 2000*). Although the disease is generally regarded as insignificant, it can be serious depending on the cultivar and location. Symptoms initially appear as pale green flecks that elongate into brown streaks of about 2 cm or longer. Leaf specks frequently turn orange in colour, with sparse grey-green blotching becoming evident on the adaxial surface of older leaves. Lesions eventually become dark brown, coalesce, and occupy large areas of the photosynthetic leaf surface (*SurrIDGE et al. 2003*).

*C. musae* was described in *Cladosporium* because of its pigmented conidiophores and conidia that are formed in

acropetal chains. *Cladosporium* s. lat. is heterogeneous, composed of many kinds of superficially similar, but unrelated dematiaceous hyphomycetes with acroblastic conidial formation. A total of 772 names have thus far been assigned to this genus (*Dugan et al. 2004*). *Roquebert (1981)* and *David (1997)* examined the conidiogenesis and structure of the conidiogenous loci of *Cladosporium* species in detail and demonstrated that *Cladosporium* s. str. is well-characterised by having a unique 'coronate' scar type (scars more or less protuberant, with a central dome surrounded by a raised periclinal rim). *Braun et al. (2003)* published a phylogenetic study of cladosporioid hyphomycetes (i.e. *Cladosporium* s. lat.), based on sequences of the ITS (ITS-1, 5.8 S, ITS-2) and 18 S rRNA genes. This study supported *David's (1997)* narrow circumscription of *Cladosporium* s. str. *Braun et al. (2003)* also proposed the new genus *Davidiella* for teleomorphs of *Cladosporium* s. str.

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Numerous other groups of cladosporioid hyphomycetes (*Cladosporium* s. lat.) have already been excluded on the basis of morphological reassessments and molecular data. For example, human pathogenic 'Cladosporium' species belonging to the *Herpotrichiellaceae* are presently placed in *Cladophialophora* (Masclaux et al. 1995; Untereiner 1997; de Hoog et al. 2000) and cladosporioid *Venturia* anamorphs are accommodated in *Fusicladium* (Schubert et al. 2003). Other species, originally placed in *Cladosporium*, proved to be *Mycosphaerella* anamorphs belonging in *Passalora*, *Pseudocercospora* and *Stenella* (Crous & Braun 2003; Schubert & Braun 2005). On account of morphological, molecular and ecological features, Seifert et al. (2004) recently separated *Cladosporium staurophorum* from *Cladosporium* s. str. and introduced the new genus *Devriesia* to accommodate a group of five heat-resistant species that also appeared *Cladosporium*-like in their general morphology.

In this study, morphological characters and DNA sequence data of the ITS and 28 S nrDNA were used to taxonomically and phylogenetically characterise *C. musae*. Preliminary morphological examinations suggested that the conidiogenesis and structure of the conidiogenous loci differ from *Cladosporium* s. str.

## Materials and methods

### Isolates

Isolates used in this study were retrieved from the Centraal-bureau voor Schimmelcultures (CBS; Utrecht), and CABI Bioscience (IMI; Egham). Freshly isolated strains included in this study were obtained from symptomatic *Musa* leaves collected in South Africa and Mozambique (Table 1).

Leaves were incubated in a moist chamber for 3 d and observed under a dissecting microscope. Conidia were removed from *Cladosporium*-like conidiophores with the help of a sterile glass needle, and streaked out on 2% malt extract agar (MEA) (Sigma-Aldrich Chemie, Zwijndrecht) containing streptomycin and penicillin (Gams et al. 1998).

### DNA isolation, amplification and sequencing

General methods used for DNA isolation, amplification, and sequencing, as well as for phylogenetic analyses are those used by Halleen et al. (2004). Amplification of the rDNA was performed using the primers V9G/LR5 (de Hoog & Gerrits van den Ende 1998; Vilgalys & Hester 1990) or ITS1/ITS4 (White et al. 1990). The amplicons were sequenced with the BigDye terminator cycle (Applied Biosystems, Foster City, CA) or DYEnamicET dye terminator (Amersham Biosciences, Freiburg) sequencing kits and analysed on an ABI Prism 3700 (Applied Biosystems) by using the standard conditions recommended by the vendor. The PCR primers were used as sequence primers for both genes. To ensure a good-quality sequences across the length of the LSU sequence, primers LR0R (Rehner & Samuels 1994) and LR16 (Moncalvo et al. 1993) were used as additional, internal sequence primers. Newly generated sequences were compared with published sequences of a broad range of taxa downloaded from GenBank. The selection of the sequences partly followed results obtained by

BLAST-searches, in which sequences similar to those of *C. musae* strains were retrieved. Obtained LSU trees were rooted using a sequence of *Peziza nathrophila* as outgroup, and a sequence of *Mycosphaerella punctiformis* was used as outgroup for the ITS tree. A gap caused by the longer ITS2 region of the *Metulocladosporiella musae* sequences in the ITS alignment was coded as a single indel (characters 585–617 of the alignment). Tree topologies were obtained from the aligned sequences by the maximum parsimony and neighbour-joining criteria as implemented in PAUP 4.0b10 (Swofford 2003). For parsimony analyses, heuristic searches with 100 random taxon additions were performed using parsimony-informative, unordered, and equally weighted characters. Gaps were treated as both new character states and missing characters and a maximum of 1000 trees was allowed. For neighbour-joining analyses, the uncorrected "p", Kimura 2-parameter and F84 substitution models were tested and ties were broken randomly if encountered. Branch robustness in the analyses was tested by 1000 bootstrap replicates. Newly generated sequences and the alignments were deposited in GenBank (DQ008125–DQ008163) and TreeBASE (SN2290), respectively (Table 1).

### Morphology

Isolates were inoculated onto potato–dextrose agar (PDA), synthetic nutrient-poor agar (SNA), and oatmeal agar (OA) (Gams et al. 1998), and incubated under continuous near-ultraviolet light at 25 °C for 6 d. Microscopic observations were made from colonies cultivated on SNA, and preparations mounted in lactic acid. Conidial branching patterns were studied by placing squares of transparent adhesive tape (1 cm<sup>2</sup>) on conidiophores at colony margins, and mounting these between two drops of clear lactic acid under a glass coverslip. Cultural characteristics were determined from colonies cultivated on PDA and OA using the colour charts of Rayner (1970).

## Results

### Phylogeny

Neighbour-joining analyses on the LSU and ITS datasets resulted in the same tree topology irrespective of the substitution model tested (data not shown). Some rearrangements of the deep nodes were observed when the most parsimonious trees were compared with the trees obtained from the neighbour-joining analyses (data not shown). Parsimony analysis of the datasets with gaps coded as missing data or as new states did not alter the consensus tree topologies obtained.

Approximately 975 nucleotides were sequenced for the LSU gene for the isolates studied (Table 1). The manually adjusted alignment contained 60 taxa (including the outgroup) and 611 characters including alignment gaps. Of the 611 characters used in the phylogenetic analysis, 245 were parsimony-informative, 79 were variable and parsimony-uninformative and 287 were constant. Twenty equally most parsimonious trees, one of which is shown in Fig 1, were obtained from the parsimony analysis. Two classes are represented in this

**Table 1 – Isolates subjected to DNA analysis and morphological examination**

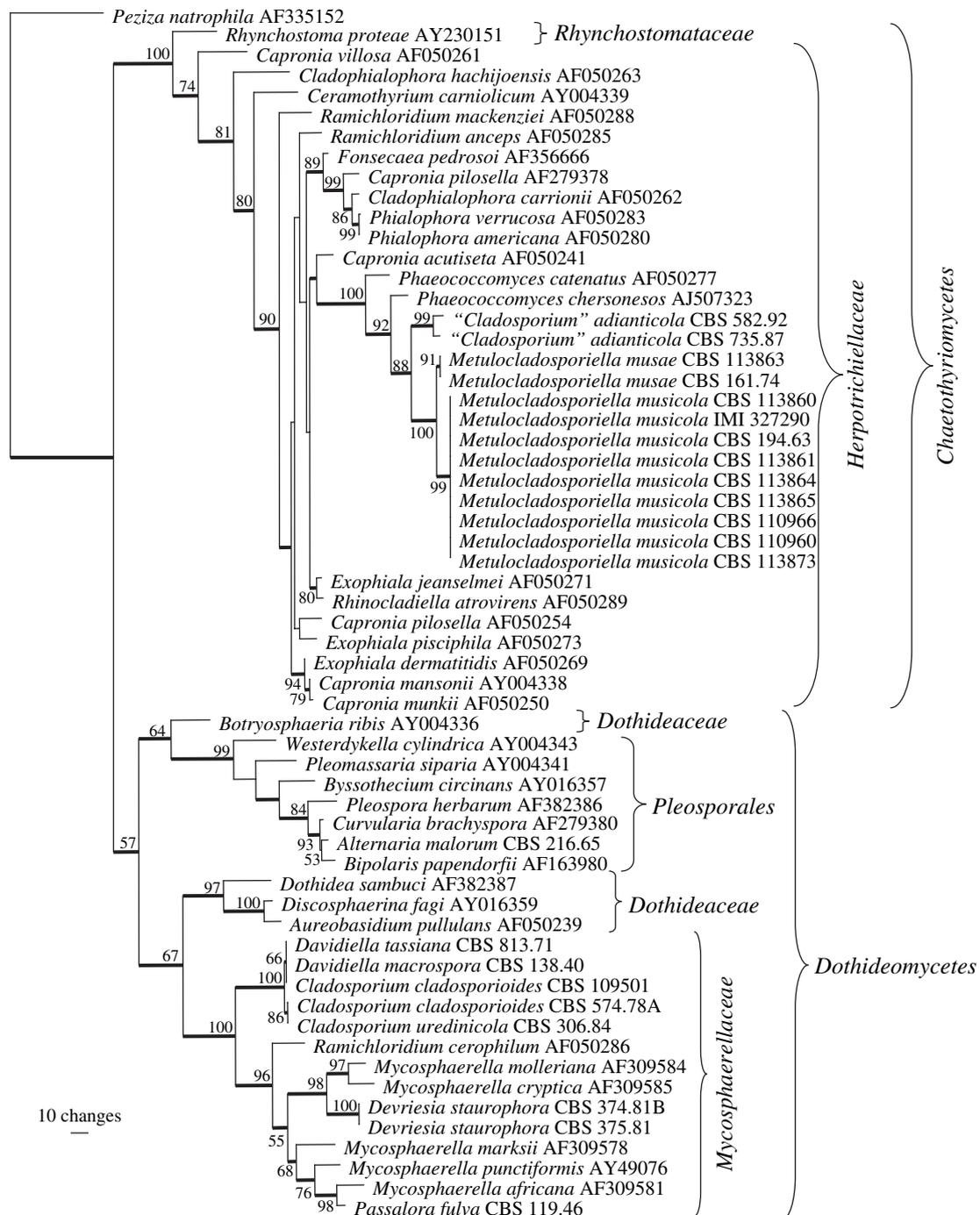
Species	Accession No. <sup>b</sup>	Source	Origin	GenBank accession no. (ITS, LSU)
<i>Alternaria malorum</i>	CBS 216.65, NRRL A-13702	<i>Triticum aestivum</i>	USA	—, DQ008142
' <i>Cladosporium</i> ' <i>adianticola</i>	CBS 582.92 CBS 735.87 <sup>a</sup>	<i>Adiantum tenerum</i> <i>Adiantum</i> sp.	Cuba Cuba	—, DQ008143 DQ008125, DQ008144
<i>C. cladosporioides</i>	CBS 574.78A CBS 109501	Mycophilic Deep mycosis of human patient	USSR Turkey	—, DQ008145 —, DQ008146
<i>C. uredinicola</i>	CBS 306.84	<i>Puccinia allii</i>	UK	—, DQ008147
<i>Davidiella macrospora</i>	CBS 138.40	<i>Iris</i> sp.	The Netherlands	—, DQ008148
<i>D. tassiana</i>	CBS 813.71	<i>Polygonatum odoratum</i>	Czech Republic	—, DQ008149
<i>Devriesia staurophora</i>	CBS 374.81B CBS 375.81	Páramo soil Páramo soil	Colombia Colombia	—, DQ008150 —, DQ008151
<i>Metulocladosporiella musicola</i>	CBS 194.63, ATCC 36952 CBS 110959, CPC 4628 CBS 110960, CPC 4629 <sup>a</sup> CBS 110963, CPC 4632 CBS 110964, CPC 4633 CBS 110966, CPC 4635 CBS 110965, CPC 4634 CBS 113860, IMI 380629 CBS 113861, IMI 295939 CBS 113862, IMI 380793 CBS 113864, IMI 374551 CBS 113865, IMI 380626 CBS 113873 IMI 327290	<i>Musa</i> sp. <i>Musa</i> sp. <i>Musa</i> sp. <i>Musa</i> sp. <i>Musa</i> sp. <i>Musa</i> sp. <i>Musa</i> sp. <i>M. sapientum</i> <i>M. sapientum</i> <i>M. sapientum</i> <i>Musa</i> sp. <i>M. sapientum</i> <i>M. sapientum</i> <i>M. paradisiaca</i>	France South Africa South Africa South Africa South Africa South Africa South Africa Uganda Zimbabwe Kenya Uganda Uganda Mozambique Uganda	DQ008126, DQ008152 — DQ008127, DQ008153 DQ008128, — — —, DQ008154 DQ008129, — DQ008130, DQ008155 DQ008131, DQ008156 DQ008132, — DQ008133, DQ008157 DQ008134, DQ008158 DQ008135, DQ008159 DQ008136, DQ008160
<i>M. musae</i>	CBS 161.74, ATCC 36973 <sup>a</sup> CBS 113863, IMI 380798	<i>Musa</i> sp. <i>M. sapientum</i>	Honduras Cameroon	DQ008137, DQ008161 DQ008138, DQ008162
<i>Passalora fulva</i>	CBS 119.46, CPC 3688	<i>Lycopersicon esculentum</i>	The Netherlands	AY251069, DQ008163
' <i>Pseudocladosporium</i> ' sp.	CBS 115142, FRR 5582, CPC 11044 CBS 115143, FRR 5599, CPC 11047 CBS 115144, FRR 3318, CPC 11048	Fruit-based drink Bottled spring water Apple juice drink	Australia Australia Australia	DQ008139, — DQ008140, — DQ008141, —

a Ex-type strain.  
b CBS, Centraalbureau voor Schimmelcultures, (Utrecht); C.P.C., culture collection of Pedro Crous (at CBS); ATCC, American Type Culture Collection (Manassas); F.R.R., CSIRO Division of Food Science & Technology (Sydney); IMI, CABI Bioscience UK Centre (Egham); NRRL, ARS Culture Collection, Northern Regional Research Laboratory, USA, (Peoria).

tree, namely the *Chaetothyriomycetes* (100 % bootstrap support) and the *Dothideomycetes* (57 % bootstrap support). In the *Chaetothyriomycetes*, a representative of the *Rhynchostomataceae* and several taxa from the *Herpotrichiellaceae* are present. In the *Herpotrichiellaceae*, a clade supported by a bootstrap support value of 92 % contains two sequences of two species of *Phaeococcomyces* and three well-supported clades containing strains of *Cladosporium adianticola* (99 % bootstrap support), *Metulocladosporiella musicola* (91 % bootstrap support) and *Metulocladosporiella musae* (99 % bootstrap support). The two

*Metulocladosporiella* species are joined with a bootstrap support value of 100 %. Sequences of '*Ramichloridium*' *mackenziei* and '*Ramichloridium*' *anceps* are found in the *Herpotrichiellaceae* clade. Representatives of the *Pleosporales*, *Dothideaceae* and *Mycosphaerellaceae* are present in the *Dothideomycetes* clade. A sequence of *Ramichloridium cerophilum* clustered with *Mycosphaerella* species in the *Mycosphaerellaceae* clade.

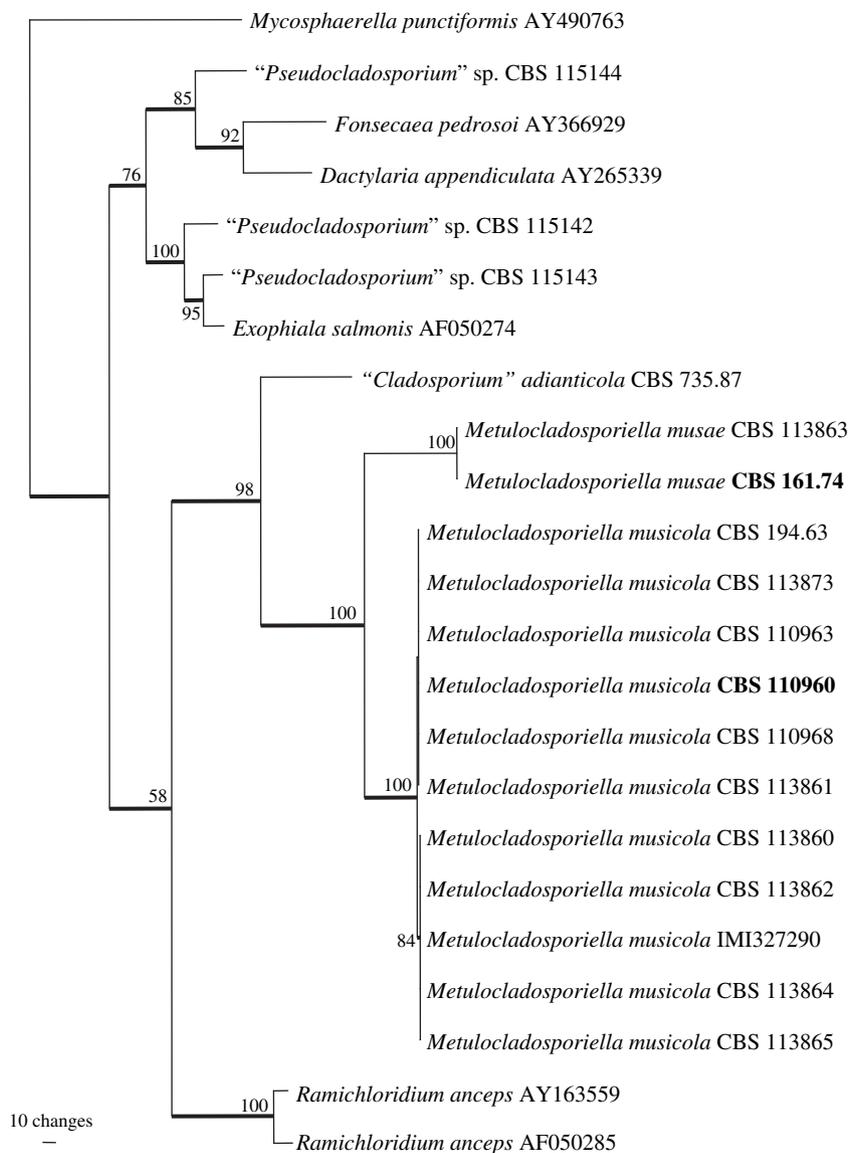
Approximately 600 bases were determined for the ITS region for the isolates studied (Table 1). The manually adjusted alignments contained 23 taxa (including the outgroup) and



**Fig 1** – One of 20 equally most parsimonious trees obtained from large subunit sequence data (TL = 1247 steps, CI = 0.460, RI = 0.820, RC = 0.377). The scale bar indicates a 10 changes and the numbers at the nodes represent bootstrap support values based on 1000 resamplings. Branches that appear in the strict consensus tree are indicated by thickened lines. The GenBank sequence of *Peziza natrophila* (AF335152) was included as outgroup.

612 characters including alignment gaps. Of the 612 characters used in the phylogenetic analysis, 296 were parsimony-informative, 83 were variable and parsimony-uninformative and 233 were constant. Two equally most parsimonious trees, one of which is shown in Fig 2, were obtained from the parsimony analysis. As with the LSU tree, strains of '*Cladosporium*' *adianticola*, *Metulocladosporiella musicola* (100 % bootstrap

support) and *Metulocladosporiella musae* (100 % bootstrap support) cluster together with a bootstrap support value of 100 %. Two sequences of '*Ramichloridium*' *anceps* obtained from GenBank formed a distant, highly supported sister clade (bootstrap support = 100 %) to the clade containing the *Metulocladosporiella* species and *C. adianticola*. The *Ramichloridium*–*Metulocladosporiella*–*C. adianticola* clade is weakly supported (58 %).



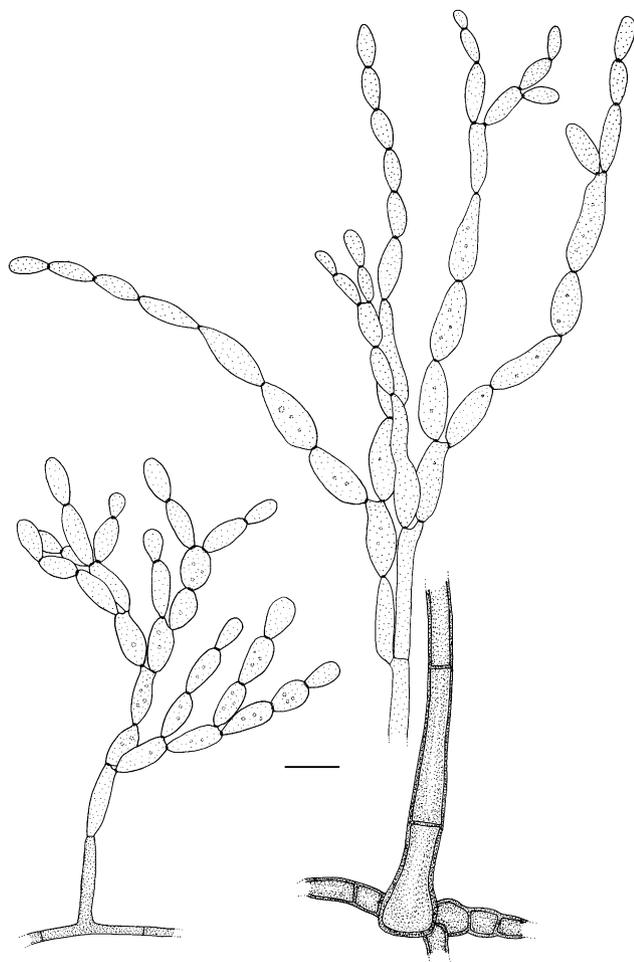
**Fig 2 – One of two equally most parsimonious trees obtained from ITS sequence data (TL = 1008 steps, CI = 0.718, RI = 0.819, RC = 0.588). The scale bar indicates 10 changes and the numbers at the nodes represent bootstrap support values based on 1000 resamplings. Branches that appear in the strict consensus tree are indicated by thickened lines. The GenBank sequence of *Mycosphaerella punctiformis* (AY490763) was included as outgroup. Ex-type strains are in bold.**

## Taxonomy

Conidia in *Cladosporium musae* are formed holoblastically in acropetal, often branched chains. This pattern is similar as in *Cladosporium* s. str. The examination of cultures and herbarium specimens of *C. musae* revealed clear differences in the conidiogenesis and structure of the conidiogenous loci and conidial hila in comparison with species of *Cladosporium* s. str., typified by *C. herbarum*. A septum separating the maturing conidia is formed, which is cleft in the middle. The structure of the walls of the conidiogenous loci and the conidial hila is uniform and remains unchanged. The conidiogenous loci are subdentate, apically truncate, unthickened to slightly so, and somewhat darkened-refractive. A convex central dome

surrounded by a raised periclinal rim, as in *Cladosporium* s. str., is not formed. On account of the quite distinct conidiogenous loci and conidial hila, supported by molecular analyses of DNA sequences (see below), *C. musae* has to be excluded from *Cladosporium* s. str. Based on its peculiar features, *C. musae* belongs to a group of hyphomycetes that have been classified by Kiffer and Morelet (1999) as 'Acroblastosporae', i.e. hyphomycetes with holoblastic conidiogenesis and conidia formed in acropetal, often branched chains. Most genera in this group are phaeoacroblastic, i.e. they are pigmented, and they are morphologically, ecologically and, as far as known, genetically clearly distinct (Fig 3).

A comparison with phaeoblastosporic hyphomycetous genera (see below) revealed that *C. musae* does not fit into the concepts of any of the genera concerned. The present



**Fig 3 – *Metulocladosporiella musae* (CBS 161.74, ex-epitype). Micro- and macronematous conidiophores and conidia. Bar = 10  $\mu$ m.**

fungus is well-characterised and distinguished by frequently branched, metuloid, pigmented conidiophores with paler tips. The ultimate branchlets are composed of conidiogenous cells and ramoconidia, giving rise to pale, mostly subhyaline conidia. Therefore, the new genus is proposed below for *C. musae* and another newly described species. Fig 4.

***Metulocladosporiella*** Crous, Schroers, Groenewald, U. Braun & K. Schubert, **gen. nov.**

Mycobank MB500224.

Hyphae ramosae, septatae, hyalinae, subhyalinae vel pallide olivaceae, tenuitunicatae. Conidiophora solitaria vel laxae aggregata, erecta, subcylindrica, septata, brunnea, levi; ramuli terminales ex cellulis conidiogenis et ramoconidiis compositi; cellulae conidiogenae integratae, terminales, polyblasticae; cicatrices conidiales subconspicuae vel conspicuae. Conidia et ramoconidia catenata vel rami-catenata, ellipsoidea, ovoidea, subcylindrica vel fusiformia septata, subhyalina vel pallide olivacea, hila non-incrassata, leviter fuscata-refractiva, secessio schizolytica.

**Typus:** *Metulocladosporiella musae* (E.W. Mason) Crous, Schroers, Groenewald, U. Braun & K. Schubert 2006.

**Hyphomycetes. Acroblastosporae.** On living leaves. Mycelium internal and external, hyphae branched, septate, hyaline, subhyaline to pale olivaceous, thin-walled. Stromata lacking. Conidiophores macronematous, mononematous (occasionally

with some intermixed micronematous conidiophores), solitary or in loose groups, arising from hyphae, erect, composed of a long, subcylindrical, simple stipe and a branched terminal part; stipe septate, pigmented, smooth or almost so, usually swollen at the very base; branched part loose to dense, metuloid, composed of short to long branchlets and ramoconidia, tips paler than the stipes, subhyaline to very pale olivaceous; conidiogenous cells integrated, terminal, occasionally intercalary, polyblastic, sympodial, conidiogenous loci (conidial scars) subconspicuous to conspicuous, subdenticulate, truncate, unthickened to slightly thickened, and somewhat darkened-refractive. Conidia and ramoconidia in simple and branched chains, ellipsoid, ovoid, subcylindrical, fusiform, 0–1-septate, subhyaline to very pale olivaceous, thin-walled, smooth, hila truncate, unthickened to slightly thickened and slightly darkened-refractive, secession schizolytic.

***Metulocladosporiella musae*** (E.W. Mason) Crous, Schroers, Groenewald, U. Braun & K. Schubert, **comb. nov.**

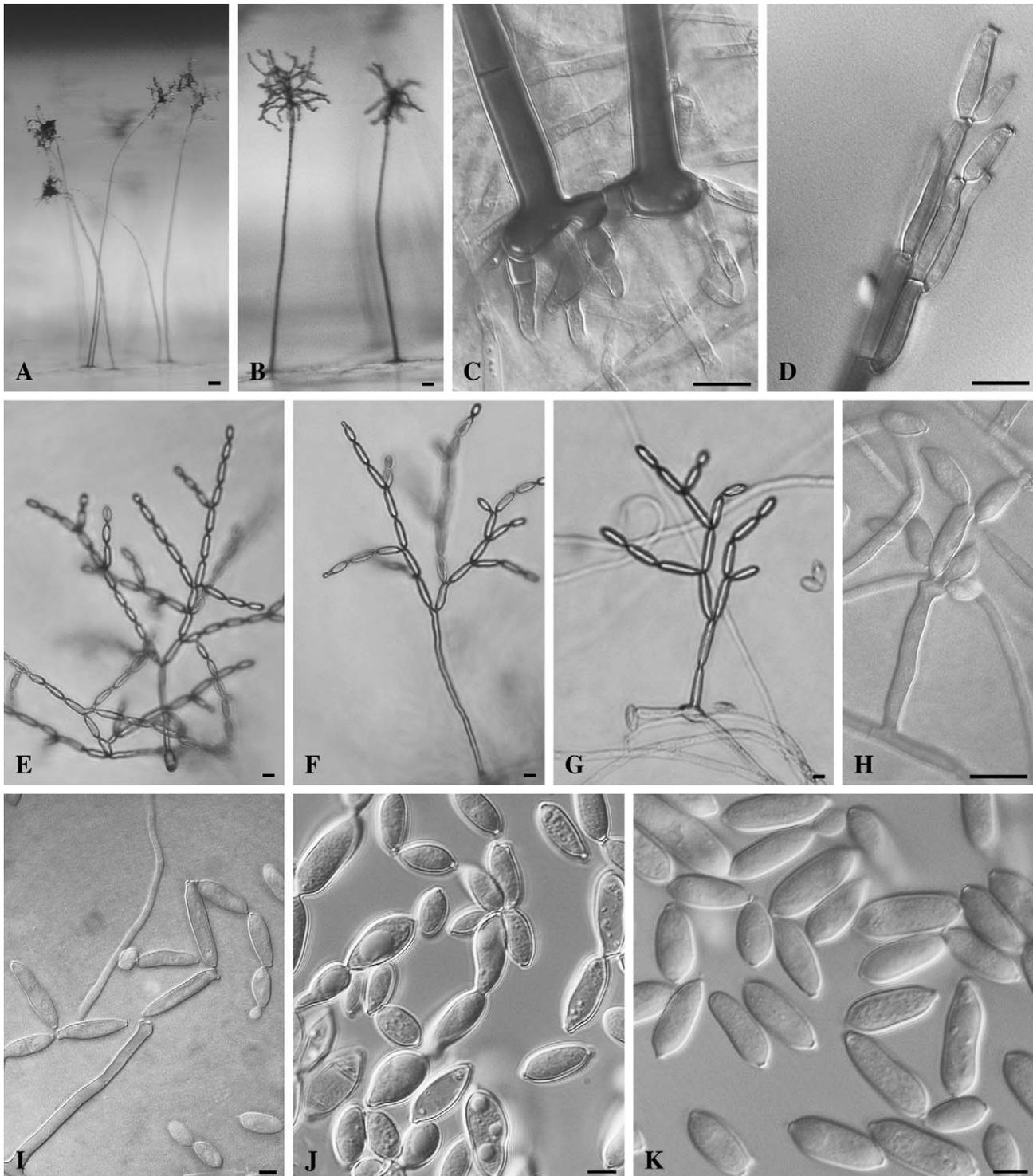
Mycobank MB500185

**Basionym:** *Cladosporium musae* E.W. Mason, in Martyn, *Mycol. Pap.* 13: 2 (1945).

**Synonym:** *Periconiella sapientumicola* Siboe, *African J. Mycol. Biotechnol.* 2: 4 (1994); non *Periconiella musae* M.B. Ellis 1967.

(Figs 3–4)

Leaf spots amphigenous, at first visible as pale greenish flecks, ellipsoid to oblong, forming streaks up to 2 cm or even longer, pale to blackish brown, occasionally somewhat zonate, with age turning orange in colour, later often dark brown, finally often confluent, forming large patches, in severe infections entire leaves occasionally becoming necrotic, often with dark, sunken, water-soaked lesions along the midrib, 10–20 mm wide. Mycelium internal and external, superficial; external hyphae branched, 1–3(–4)  $\mu$ m wide, septate, occasionally slightly constricted at the septa, with small swellings, hyaline, subhyaline to very pale olivaceous, thin-walled, smooth, hyphae occasionally aggregated, forming ropes; sometimes with some intermixed micronematous conidiophores, erect from the vegetative mycelium, intercalary, straight to flexuous, unbranched, subhyaline, usually with simple terminal conidial chains. Macronematous conidiophores arising from superficial hyphae, erect, solitary to loosely aggregated, 45–500(–600)  $\mu$ m long, composed of a subcylindrical stipe, 3–8  $\mu$ m wide, 2–12-septate, swollen or lobed at the base, 10–17  $\mu$ m diam, with short rhizoid hyphae growing from the base, medium to dark brown in the lower half, paler towards the apex, tips pale olivaceous or even subhyaline, thick-walled below, thin-walled towards the apex, smooth; apex persistently branched, branched part composed of usually fairly compact, closely arranged subcylindrical branchlets; primary branches aseptate, 15–30  $\times$  3.5–5  $\mu$ m, giving rise to 1–2 secondary branches, or to conidiogenous cells; secondary branches 0(–1)-septate, 30–50  $\times$  3–4.5  $\mu$ m, giving rise to 1(–3) conidiogenous cells; conidiogenous cells subcylindrical, 10–45  $\times$  3–4  $\mu$ m, terminal or occasionally intercalary, sympodial, polyblastic, conidiogenous loci subconspicuous to conspicuous, subdenticulate, somewhat protuberant, truncate, wall unthickened, but somewhat darkened-refractive, 1–2  $\mu$ m wide. Conidia in simple and branched acropetal chains, ellipsoid-ovoid, fusiform, subcylindrical, (6–)8–11(–16)  $\times$  (3–)4(–5)  $\mu$ m [ramoconidia (10–)15–19(–25)  $\times$  (3.5–)5(–6)  $\mu$ m]], 0(–1)-septate,



**Fig 4 – A–K – *Metulocladosporiella musae* (CBS 161.74, ex-epitype). Figs A–B – Macronematous conidiophores. Fig C – Lobed to swollen bases of conidiophores. Fig D – Conidiogenous apparatus. Figs E–I – Micronematous conidiophores. Figs J–K – Conidia. Bars: (A) = 16  $\mu\text{m}$ , (B) = 20  $\mu\text{m}$ , (C–D), (H) = 10  $\mu\text{m}$ , (E–G) = 8  $\mu\text{m}$ , (I–K) = 4  $\mu\text{m}$ .**

hyaline, subhyaline to very pale olivaceous, thin-walled, smooth, with 1–3 hila, truncate, 1–2  $\mu\text{m}$  wide (up to 3  $\mu\text{m}$  wide at the base of ramoconidia), unthickened or almost so, but somewhat darkened-refractive, secession schizolytic.

**Cultures:** Colonies 37–50 mm diam on PDA after 14 d under  $\text{NUV}$  at 25 °C. Colonies on PDA and OA spreading, with smooth, regular

margins and sparse aerial mycelium; surface on PDA pale mouse-grey to mouse-grey due to profuse sporulation; margins of submerged mycelium, mouse-grey; reverse on PDA greenish black.

**Host range and distribution:** On *Musa* spp., incl. *M. ×paradisica* (incl. var. *sapientum*) and *M. schweinfurthii* (syn. *Ensete gillesii*); Africa (Burundi, Cameroon, Côte d'Ivoire,

Democratic Republic of Congo, Egypt, Ethiopia, Ghana, Guinea, Kenya, Mozambique, Rwanda, Sierra Leone, South Africa, Sudan, Togo, Uganda, Zimbabwe), Asia (Bangladesh, Hong Kong, Indonesia, Malaysia, Nepal, Sabah, Sri Lanka, Thailand, Vietnam), Australasia and Oceania (Solomon Islands, W. Samoa), Central America (Mexico), Latin America, Caribbean (Cuba, Ecuador, Honduras, Jamaica) (Jones 2000).

Literature: Ellis (1971), Stover (1972), David (1988), Siboe (1994), Jones (1994, 2000), Ho et al. (1999), Surridge et al. (2003).

Specimens examined: All on *Musa* sp.: Jamaica: 7 Sept. 1942, E. B. Martyn [slide ex type coll.] (IMI 7521)-*lectotypus hic designatus*. Honduras: R.H. Stover (CBS herb. 14788-*epitypus hic designatus*; culture ex-epitype CBS 161.74 = ATCC 36973).-Mexico: 12 Feb. 1983, J. M. van Valkenburgh (BPI 427272).

Notes: Mason (in Martyn 1945) described long conidiophores, 60–500 × 3.5–6 µm, and aseptate conidia, 6–22 × 2.5–4 µm. In the type material from IMI (slide only), the conidiophores are much shorter, 45–150 × 3–6 µm, but the conidia agree well with the original description [5–16 × 3–5 µm, ramo-conidia 11–17(–22) µm long].

*Cladosporium pannosum* (Cooke 1883) is an additional *Cladosporium* species described from banana leaves. Type material of this species has been re-examined (USA: South Carolina, on *Musa* sp., Ravenel, K 121564) and proved to be a true species of *Cladosporium* s. str. It was introduced in connection with the ascomycete *Chaetophoma musae*. However, this species is undoubtedly not hyperparasitic but probably saprobic on the *Musa* leaves (Heuchert et al. 2005).

Mason (in Martyn 1945) cited three collections, viz. from Jamaica (on *Musa* sp., Sept. 1942, E.B. Martyn), from Sierra Leone (on *Musa schweinfurthii*) and Ghana. The collection from Jamaica was marked as type material. Herbarium material of this collection could not be traced and is probably not preserved, but a slide based on the type collection has been found at IMI. This sample has thus been selected as lectotype. Morphologically this material closely resembles a culture obtained from Honduras, which is selected as ex-epitype strain, with a dried down specimen as epitype.

***Metulocladosporiella musicola*** Crous, Schroers & Groenewald, sp. nov.

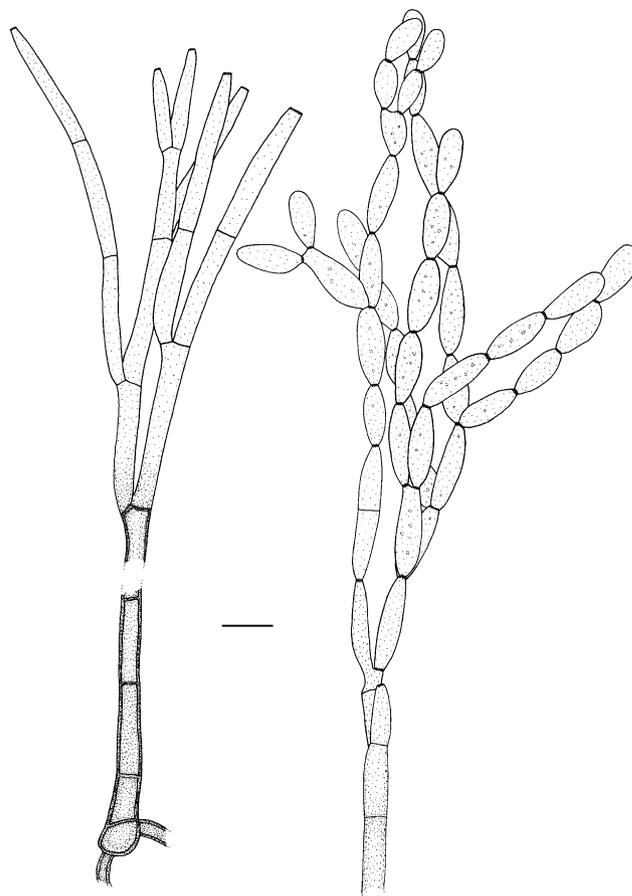
Mycobank MB500186

(Figs 5–6)

Differt a *M. musae* conidiophoris ad apicem valde ramosis, conidiis (9–)11–13(–16) µm longis, locis conidiogenis latioribus, (1–)2(–4) µm, saepe distinctioribus.

*Typus*: South Africa: Northern Province: Levubu, on *Musa acuminata* subgr. “Cavendish ‘Grand Nain’”, Mar. 2000, A. Viljoen (CBS herb. 14787-*holotypus*; culture ex-type CBS 110960 = CPC 4629).

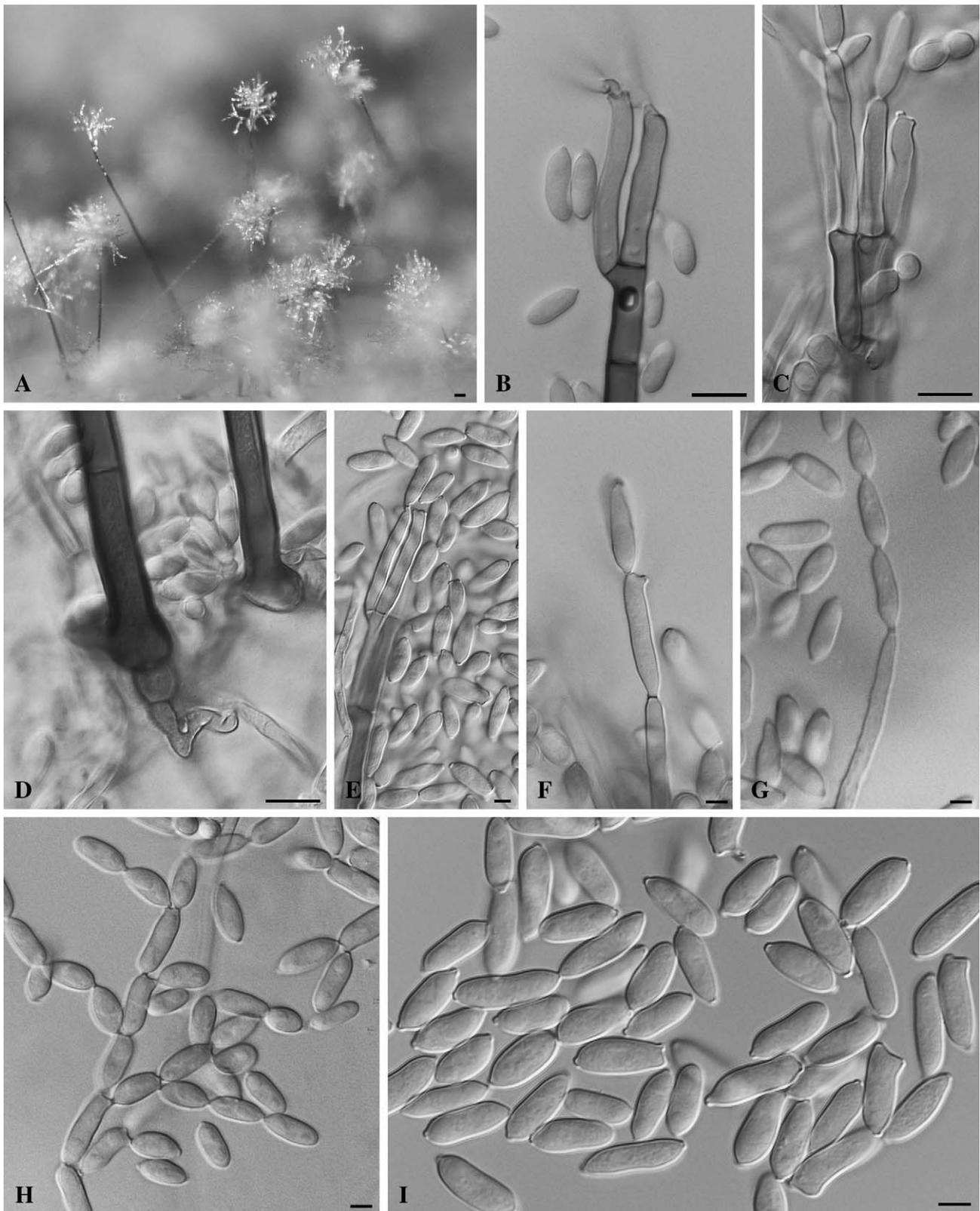
Leaf spots similar to those of *M. musae*. Mycelium internal and external, superficial; hyphae branched, 1–3(–4) µm wide, septate, occasionally slightly constricted at the septa, with small swellings, hyaline, subhyaline to very pale olivaceous, thin-walled, smooth, hyphae occasionally aggregated, forming ropes; sometimes with some intermixed micronematous conidiophores (but less common than in *M. musae*), erect from the vegetative mycelium, intercalary, straight to flexuous, unbranched, subhyaline, usually with simple terminal conidial chains. Macronematous conidiophores arising from superficial hyphae, erect, solitary to loosely aggregated, 80–600(–700) µm long, composed of a subcylindrical stipe, 3–8 µm wide, 2–18 septate, swollen or lobed at the base, 10–15 µm diam, with short rhizoid hyphae growing from the base,



**Fig 5 – *Metulocladosporiella musicola* (CBS 113865, ex-holotype). Conidiophores and conidia. Bar = 10 µm.**

medium to dark brown in the lower half, paler towards the apex, tips pale olivaceous or even subhyaline, thick-walled below, thin-walled towards the apex, smooth; apex persistently branched, branched part composed of usually fairly compact, closely arranged subcylindrical branchlets; primary branches 0(–2)-septate, 15–85 × 3.5–6 µm, giving rise to 1–3 secondary branches, or to conidiogenous cells; secondary branches 0(–1)-septate, 20–40 × 3–4 µm, giving rise to (1–)2–3 conidiogenous cells; conidiogenous cells subcylindrical, 20–30 × 3–5 µm, terminal or occasionally intercalary, sympodial, polyblastic, conidiogenous loci subconspicuous to conspicuous, subdenticulate, somewhat protuberant, truncate, wall unthickened to somewhat so, darkened-refractive, 1–2 µm wide. Conidia in simple and branched acropetal chains, ellipsoid-ovoid, fusiform, subcylindrical, (9–)11–13(–16) × (3.5–)4(–5) µm [ramoconidia (12–)15–20(–25) × (3.5–)5(–6) µm], 0(–1)-septate, hyaline, subhyaline to very pale olivaceous, thin-walled, smooth, with 1–3 hila, truncate, (1–)2 µm diam (up to 4 µm diam at the base of ramoconidia), unthickened or almost so, and somewhat darkened-refractive (more prominent than in *M. musae*), secession schizolytic.

*Cultures*: Colonies 20–30 mm diam on PDA after 14 d under NUV at 25 °C. Colonies on PDA and OA spreading, with smooth, regular margins and sparse aerial mycelium; centres of colonies darker than margins due to grey-white aerial mycelium;



**Fig 6 - *Metulocladosporiella musicola* (CBS 113865, ex-holotype). Fig A - Macronematous conidiophores. Figs B-C, E - Conidiogenous apparatus. Fig D - Lobed bases of conidiophores. Figs F-G - Micronematous conidiophores. Figs H-I - Conidia. Bars: (A) = 20  $\mu$ m, (B-D) = 10  $\mu$ m, (E-I) = 4  $\mu$ m.**



surface on PDA pale mouse-grey to dirty white–grey (centre); margins leaden-black to olivaceous–grey.

**Host range and distribution:** on *Musa* spp.; Africa (Kenya, Mozambique, South Africa, Uganda, Zimbabwe; one record, CBS 194.63 = ATCC 36952, is incorrectly cited in the literature as a distribution record for France, but in fact represents the French territories outside Europe, the exact location being unknown).

**Notes:** *Metulocladosporiella musicola* is morphologically distinguishable from *M. musae* in having: (1) conidiophores that are more frequently branched in their apical region (more secondary branches and conidiogenous cells); (2) longer conidia, (9–)11–13(16) vs (6–)8–11(–16)  $\mu\text{m}$ ; and (3) wider loci, (1–)2(–4) vs 1–2(–3)  $\mu\text{m}$ . *M. musae* is also more prone to form micronematous conidiophores in culture than *M. musicola*, and has conidial scars which are barely thickened, and only somewhat refractive, while those of *M. musicola* are more prominently visible.

### Comparison of *Metulocladosporiella* and other genera

The following genera are easily distinguishable from *Metulocladosporiella* by having little differentiated, micronematous or semi-micronematous to semi-macronematous conidiophores: *Bispora* (saprobic), *Cladophialophora* (human pathogenic), *Devriesia* (heat-resistant soil fungi), *Dimorphospora* (saprobic on submerged leaves), *Pseudocladosporium* (saprobic), *Torula* (mostly saprobic), *Xylohypha*, *Xylohyphopsis* (saprobic, human pathogenic) (Ellis 1971, 1976; Carmichael et al. 1980; Braun 1998; Kiffer & Morelet 1999; Partridge et al. 2000; Seifert et al. 2004).

*Polyscytalum* (Ellis 1971) and *Websteromyces* (Partridge et al. 2000) are two comparable genera that also have branched conidiophores. They differ, however, in having semi-micronematous to semi-macronematous conidiophores. Furthermore, the lignicolous genus *Websteromyces* is easily distinguishable by inconspicuous conidiogenous loci. *Polyscytalum* species are leaf litter fungi, differing in having little differentiated, rather inconspicuous conidiogenous loci (Ellis 1971).

*Periconia* species are characterised by basipetal conidial maturation; *Haplobasidium* and *Haplographium* possess conidiogenous cells arranged in terminal penicilli. Species of *Cladosporium*, *Passalora* emend. (incl. *Mycovellosiella*, *Phaeoramularia*), and *Stenella* (all anamorphs of either *Davidiella* or *Mycosphaerella*, *Mycosphaerellaceae*) as well as species of the hyperparasitic genus *Cladosporiella* are easily distinguishable by having conspicuously thickened and darkened conidiogenous loci (Kiffer & Morelet 1999; Partridge & Morgan-Jones 2003). Species of the genus *Fusicladium* are anamorphs of the *Venturiaceae*. The conidiophores are usually unbranched and the conidia are more or less concolorous with the conidiophores (Schubert et al. 2003). *Fusicladosporium* (2003) was introduced for *Fusicladium* species with catenate conidia, although two older generic names were available for this taxon, viz., *Hormocladium* and *Ramalia*. Based on a re-assessment of the conidial formation, conidiogenesis and molecular data, *Fusicladosporium* has recently been reduced to synonymy with *Fusicladium* (Beck et al. 2005).

The conidiophores and conidial chains in *Anungitea*, *Castaneda*, *Hormiactella*, *Lobatopedis*, *Pleurotheciopsis* and

*Parapleurotheciopsis* are unbranched (Ellis 1971, 1976; Carmichael et al. 1980; Kiffer & Morelet 1999; Partridge et al. 2001c). The conidiogenous cells in *Anungitea* are raduliform. The conidiophores in *Castaneda* are inflated at the very base, as in *Metulocladosporiella*, but they proliferate percurrently and the conidiogenous cells are verruculose and non-cicatrised. The conidia in *Parapleurotheciopsis* are pale, subhyaline as in *Metulocladosporiella*, but the conidiophores arise from lobed basal cells. Species of *Pleurotheciopsis* are also close to *Metulocladosporiella* as the conidiophores may arise from a swollen basal cell and the conidiogenous cells and conidia are hyaline or subhyaline. However, the latter genus is easily separable by its unbranched, percurrently proliferating conidiophores and conidia formed in simple chains. Several other genera are characterised by conidiophores arising from enlarged basal cells, e.g. *Beltrania*, *Beltraniopsis*, *Beltraniella*, *Hemibeltrania* and *Pseudobeltrania*, but these genera belong to the ‘Sympodulosporae’ (sensu Kiffer & Morelet 1999, i.e., conidia formed singly). Within the ‘Sympodulosporae’ they form a group of genera with more or less rhombic, biconic to turbinate conidia (‘Rhombospores’ sensu Kiffer & Morelet 1999; obovoid in *Hemibeltrania*). *Cordana*, *Parapyricularia* and *Sterigmatobotrys* are additional genera in which the conidiophores arise from inflated bases, but they form solitary conidia.

Species of *Septonema* have inconspicuous conidiogenous loci, pigmented conidia and they are ecologically distinct, and those of *Heteroconium* possess monoblastic, determinate to percurrent conidiogenous cells (Ellis 1971, 1976).

Siboe (1994) assigned ‘*Cladosporium*’ *musae* to *Periconiella*. However, this treatment is not tenable as *Periconiella* species are characterised by having conspicuously thickened and darkened conidiogenous loci and conidial hila. The conidia are usually formed singly.

*Haplotrichum* (syn. *Acladium*, *Alysidium*; Partridge et al. 2001a), *Sorocybe resiniae* (Partridge & Morgan-Jones 2002), *Parahaplotrichum* (Partridge et al. 2001b), *Phaeoblastophora* and *Subramaniomyces* are some morphologically comparable genera with branched conidiophores. *Haplotrichum*, comprising wood-inhabiting hyphomycetes, differs in having quite distinct, denticulate conidiogenous cells and pigmented unicellular conidia. *Parahaplotrichum* species are also lignicolous, denticulate, amerosporous and pigmented throughout. *Sorocybe resiniae* is morphologically very close to *Metulocladosporiella*, but ecologically, genetically and also morphologically distinguishable. *S. resiniae* occurs on resinous wood, does not cluster within the *Chaetothyriales* (Braun et al. 2003), and differs morphologically from *Metulocladosporiella* in having unthickened, non-pigmented conidiogenous loci and conidial hila as well as pigmented conidia. The wood-inhabiting *Phaeoblastophora* species have often inflated conidiogenous cells with inconspicuous conidiogenous loci and pigmented, amerosporous conidia with relatively broad, truncate, unthickened, non-pigmented hila. *Subramaniomyces* species are saprobic and possess conidiophores with lobed bases as well as aseptate conidia (amerospores).

*Cladosporium adianticola*, a foliicolous fungus described from Cuba (Castañeda 1987), clustered close to *Metulocladosporiella*. Type material of *C. adianticola* has been examined (Cuba: Prov. Matanzas: San Miguel de los Baños, on *Adiantum* sp., 23 Jan. 1987, R.F. Castañeda, INIFAT C87/44-holotype; permanent slide at HAL; culture ex-type CBS 735.87). In some

basic features, such as the branched conidiophores, paler conidiogenous cells and subhyaline conidia, this species resembles *Metulocladosporiella*, but, *C. adianticola* is distinguished from the latter species by having loosely branched, non-metuloid conidiophores and strongly dimorphic conidia [ramo-conidia narrowly subclavate, subcylindrical, filiform, 15–25 × 1.5 µm, 0(–1)-septate; conidia broadly ellipsoid-ovoid, subglobose, 7–18 × 4–10 µm, 0–1-septate, subhyaline to very pale olivaceous]. Furthermore, conspicuous basal swellings of the conidiophores are lacking, and the conidiogenous loci and conidial hila are rather inconspicuous, unthickened, neither darkened nor refractive. *C. adianticola* must be excluded from *Cladosporium* s. str., but a final conclusion about its generic affinity is not yet possible, and awaits the recollection of fertile cultures. It seems to be close to *Metulocladosporiella*, but it is not yet clear if it is congeneric.

### Key to *Metulocladosporiella* and morphologically similar genera (bearing branched acropetal chains of dematiaceous blastoconidia)

- 1 Conidiophores micronematous to semi-macronematous, little-differentiated .....2  
 Conidiophores macronematous .....3
- 2(1) Conidiophores little branched, with short lateral branches; conidia broadly ellipsoid-ovoid to somewhat clavate, 4–5 µm wide, verruculose, with broadly rounded ends, with inconspicuous hila; on dead wood .....**Websteromyces**  
 Conidiophores often branched, branches short to long; conidia narrowly cylindrical, 1–3 µm wide, smooth, ends attenuated to a more or less pointed hilum; on leaf and stem litter or parasitic on *Solanum tuberosum* tubers .....**Polyscytalum**
- 3(1) Conidiophores composed of a long stipe and a complex, mostly dense head of branches; conidia aseptate to septate .....4  
 Conidiophores without branched head, irregularly branched, sometimes deeply cleft; conidia consistently aseptate .....5
- 4(3) Branched head of the conidiophores loose to dense, but not typically metuloid; conidiogenous loci conspicuous, thickened and darkened, non-denticulate .....**Periconiella**  
 Branched head of the conidiophores dense, often metuloid; conidiogenous loci more or less inconspicuous, unthickened or slightly thickened, slightly darkened-refractive, subdenticulate .....**Metulocladosporiella**
- 5(3) Conidiophores arising from an inflated, more or less lobed base; saprobic, mostly on leaf-litter .....**Subramaniomyces**  
 Without inflated, lobed base; wood-inhabiting .....6

- 6(5) Colonies effuse, dark, blackish; conidiophores simple or occasionally branched; conidiogenous cells often inflated, ampulliform, doliiform or clavate, non-denticulate; conidia at least partly subglobose, dark brown when mature .....**Phaeoblastophora**  
 Conidiogenous cells not inflated or, if somewhat inflated, conidiogenous cells distinctly denticulate .....7
- 7(6) Conidiogenous cells distinctly denticulate; conidia broad, about 7–13 µm wide .....**Haplotrichum**  
 Conidiogenous cells non-denticulate or at most subdenticulate; conidia narrower, 3–6 µm wide .....8
- 8(7) Colonies effuse, dense, resupinate, hypochnoid, powdery, chocolate brown; conidiophores mononematous, densely caespitose; conidiogenous cells terminal and intercalary; conidia subhyaline to very pale yellowish .....**Parahaplotrichum**  
 Colonies effuse, dense, but felted, black, brittle and appearing carbonaceous when dry; conidiophores solitary, mononematous and arranged in synnemata; conidiogenous cells terminal and pleurogenous; conidia pale brown to brown .....**Sorocybe**

### Discussion

*Metulocladosporiella* is an additional segregate of *Cladosporium* s. lat., which demonstrates that a combination of morphological re-examination, molecular analyses and ecological data are useful approaches to find and define more natural anamorph genera reflecting monophyletic fungal groups. The new genus belongs to a large assemblage of dematiaceous hyphomycetes with holoblastic conidia formed in acropetal, often branched chains (*sensu* Kiffer & Morelet 1999). However, it differs from morphologically allied genera in having frequently branched, pigmented conidiophores with much paler tips and paler, often subhyaline conidia. The conidiogenous loci are subconspicuous to conspicuous, i.e. unthickened or almost so, but somewhat darkened-refractive. The phylogenetic analyses showed that *Metulocladosporiella* belongs to the *Chaetothyriales*. The conidiogenous loci and conidial hila in *Cladophialophora* (anamorphs of *Capronia*, *Herpotrichiellaceae*, *Chaetothyriales*) resemble those of *Metulocladosporiella*, but the conidiophores are unbranched, micronematous to semimacronematous, the conidia are concolorous with the conidiophores, and the species of this genus are human pathogenic.

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## Foliicolous *Mycosphaerella* spp. and their anamorphs on *Corymbia* and *Eucalyptus*

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The genus *Eucalyptus* is host to numerous species of *Mycosphaerella*, several of which are only known as anamorphs, and for which no *Mycosphaerella* state is known. In this study new *Mycosphaerella* teleomorph states are described for *Nothostrasseria dendritica* and *Trimmatostroma excentrica*. Two new hyphomycete genera are introduced. Of these, *Cibiessia* gen. nov., with three new species accommodates an arthroconidial synanamorph of *Readeriella*. *Phaeothecoidea* gen. nov. is described for species with brown, thick-walled endoconidia. Four additional new species of *Mycosphaerella* are introduced with several new anamorph species described in *Dissoconium*, *Phaeophleospora*, *Pseudocercospora*, *Ramularia* and *Stenella*. Furthermore, an epitype is designated for *Mycosphaerella molleriana*. This study also presents new *Eucalyptus* host and distribution records including *M. mexicana* from Hawaii, *M. ohnowa* from Australia, *M. acaciigena* from Australia and Venezuela, *M. heimii* from Venezuela and Thailand, *M. kona* from Venezuela, and *M. thailandica* from Thailand.

**Key words:** *Cibiessia*, *Dissoconium*, DNA sequence comparisons, *Mycosphaerella*, *Phaeotheca*, *Phaeophleospora*, *Pseudocercospora*, *Ramularia*, *Septoria*, *Stenella*, systematics.

### Introduction

The genus *Mycosphaerella* includes more than 3000 species names (Aptroot, 2006), and several thousand anamorphs that lack known teleomorph

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connections (Crous and Braun, 2003). The *Mycosphaerellaceae* (*Capnodiales*) (Schoch *et al.*, 2006) includes species that are plant pathogens, saprobes, endophytes (saprobitic or plant-pathogenic), or those that have mutualistic (in lichen) associations (Crous *et al.*, 2000, 2001; Verkley *et al.*, 2004).

More than 100 species of *Mycosphaerella* and associated anamorphs have been described from *Corymbia* and *Eucalyptus* (Cortinas *et al.*, 2006; Crous *et al.*, 2004b, 2006d, g; Hunter *et al.*, 2006a, b; Andjic *et al.*, 2007; Carnegie *et al.*, 2007). The fact that these host genera are so extraordinarily species-rich in *Mycosphaerella* spp. might not be surprising, as they include more than 700 species (Brooker and Kleinig, 1994), many of which are known to harbour a wide range of diverse fungal species (Adams *et al.*, 2005; Crous *et al.*, 2006f, b, e; de Beer *et al.*, 2006; Gryzenhout *et al.*, 2006). Although the genera *Corymbia* and *Eucalyptus* are indigenous to Australia, many species also occur in other parts of the world (chiefly *Eucalyptus*), where they are planted as exotics to provide fibre for timber and paper pulp industries.

Several species of *Mycosphaerella* have been associated with *Mycosphaerella* Leaf Disease (MLD) of eucalypts, causing severe leaf spot, defoliation and shoot die-back (Crous, 1998; Crous *et al.*, 2004b, 2006g; Hunter *et al.*, 2006a, b; Burgess *et al.*, 2007). Many species, however, cause minor leaf spots, rarely resulting in severe disease (Crous *et al.*, 2004b, 2006g, Burgess *et al.*, 2007). Although little is known regarding the host-specificity of *Mycosphaerella* species, the majority are thought to be highly host-specific. Several recent studies have reported species of *Mycosphaerella* that are known pathogens of other hosts to be associated with leaf spots of *Eucalyptus*, where they occurred with other *Mycosphaerella* spp. (Crous *et al.*, 2004c; Burgess *et al.*, 2007). The co-occurrence of *Mycosphaerellaceae* on a single leaf spot appears to be a common phenomenon on diverse plant hosts (Crous, 1998; Crous and Groenewald, 2005; Crous *et al.*, 2006a), and it might have led to incorrect assumptions regarding host range and pathogenicity. Crous and Groenewald (2005) also drew attention to the fact that in some cases these species could be major pathogens of hosts other than *Myrtaceae*. The “pogo stick hypothesis” was proposed to explain this unusual behavioural pattern, where propagules of a presumed host-specific species show some restricted ability to colonize dead tissue of a non-host, possibly to produce propagules to facilitate onwards dispersal. In *Mycosphaerella* this behavioural pattern has been observed for teleomorph as well as anamorph states (Crous and Groenewald, unpubl.).

This study is part of a series of investigations, in which *Mycosphaerella* spp. occurring on eucalypts are characterised. The primary aim was to use comparisons of DNA sequence data to clarify obscure anamorph-teleomorph

connections, and also to recognise new species. These are compared with taxa known in culture and from sequence data, contributing to a global database of *Mycosphaerella* names, cultures and sequences ([www.MycBank.org](http://www.MycBank.org)).

## Materials and methods

### *Isolates*

*Mycosphaerella* leaf spots were excised, soaked in water for approximately 2 hours, after which they were placed in the bottom of Petri dish lids, with the top half of the dish containing 2% malt extract agar (MEA; Oxoid). Ascospore germination patterns were examined after 24 h, and single-ascospore and conidial cultures established as described by Crous (1998). Colonies were sub-cultured onto 2% potato-dextrose agar (PDA; Difco) and oatmeal agar (OA; Gams *et al.*, 2007), and incubated at 25°C under continuous near-ultraviolet light to promote sporulation.

### *DNA isolation, amplification and phylogeny*

Fungal colonies were established on MEA plates, and genomic DNA was isolated following the protocol of Lee and Taylor (1990). The primers V9G (Hoog and Gerrits van den Ende 1998) and ITS4 (White *et al.* 1990) were used to amplify part (ITS) of the nuclear rDNA operon spanning the 3' end of the 18S rDNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S rDNA gene, the second ITS region and the 5' end of the 28S rDNA gene (LSU). The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Crous *et al.* (2004c). To ensure optimal alignment and to simplify the presentation of the trees, the sequence alignment was split into two, whilst keeping phylogenetic lineages together. Sequence data were deposited in GenBank (Table 1) and alignments in TreeBASE (accession number SN3229).

### *Taxonomy*

Fungal structures were mounted in lactic acid and examined under a light microscope. Wherever possible, 30 measurements ( $\times 1000$  magnification) were made of structures, with the extremes of spore measurements given in parentheses. Colony colours (surface and reverse) were assessed weekly on PDA, MEA or OA at 25°C in the dark, using the colour charts of Rayner (1970). All cultures obtained in this study are maintained in the culture

collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands (Table 1). Nomenclatural novelties are listed and descriptions have been deposited in MycoBank <[www.Mycobank.org](http://www.Mycobank.org)>.

## Results and Discussion

### *DNA phylogeny*

Two alignments of DNA sequences were subjected to phylogenetic analyses. The resulting neighbour-joining trees were congruent for the separate alignments when the substitution models were changed from uncorrected “p” to the Kimura 2-parameter model and to the HKY85 model as implemented in PAUP. The obtained equally most parsimonious trees mainly differed in the order of taxa at the terminal nodes. The first alignment consisted of 70 taxa including the two outgroups and 512 characters (including alignment gaps) were included in the analyses. Of these characters, 249 were parsimony-informative, 21 were variable and parsimony-uninformative, and 242 were constant. Parsimony analysis with gaps treated as new states yielded 480 equally most parsimonious trees (TL = 897 steps; CI = 0.586; RI = 0.915; RC = 0.536), one of which is shown in Fig. 1. Although the same lineages were found for the neighbour-joining analyses, the order of the lineages at the deeper nodes differed (data not shown). For example, the clade containing *M. fimbriata* and *Colletogloeopsis* spp. is swapped with the clade including *M. mexicana* and the *Readeriella* spp. when compared to the figure. The second alignment consisted of 57 taxa including the two outgroups and 494 characters (including alignment gaps) were included in the analyses. Of these characters, 185 were parsimony-informative, 44 were variable and parsimony-uninformative, and 265 were constant. Parsimony analysis with gaps treated as new state yielded 390 equally most parsimonious trees (TL = 637 steps; CI = 0.597; RI = 0.833; RC = 0.497), one of which is shown in Fig. 2. Similar to the results obtained for the first alignment, the same lineages were found but their order differed in the backbone of the tree. All new species were well-supported, except for the *Septoria* sp. and *Dissoconium eucalypti*. The phylogenetic placement suggested by the sequences is discussed in the descriptive notes below each of the treated species.

### *Taxonomy*

Several anamorph and teleomorph specimens collected in the present study were morphologically and phylogenetically distinct from those presently known. These fungi are described as new taxa as follows:

**Table 1.** Isolates of *Mycosphaerella* spp. and its anamorphs included for sequence analysis and morphological comparison.

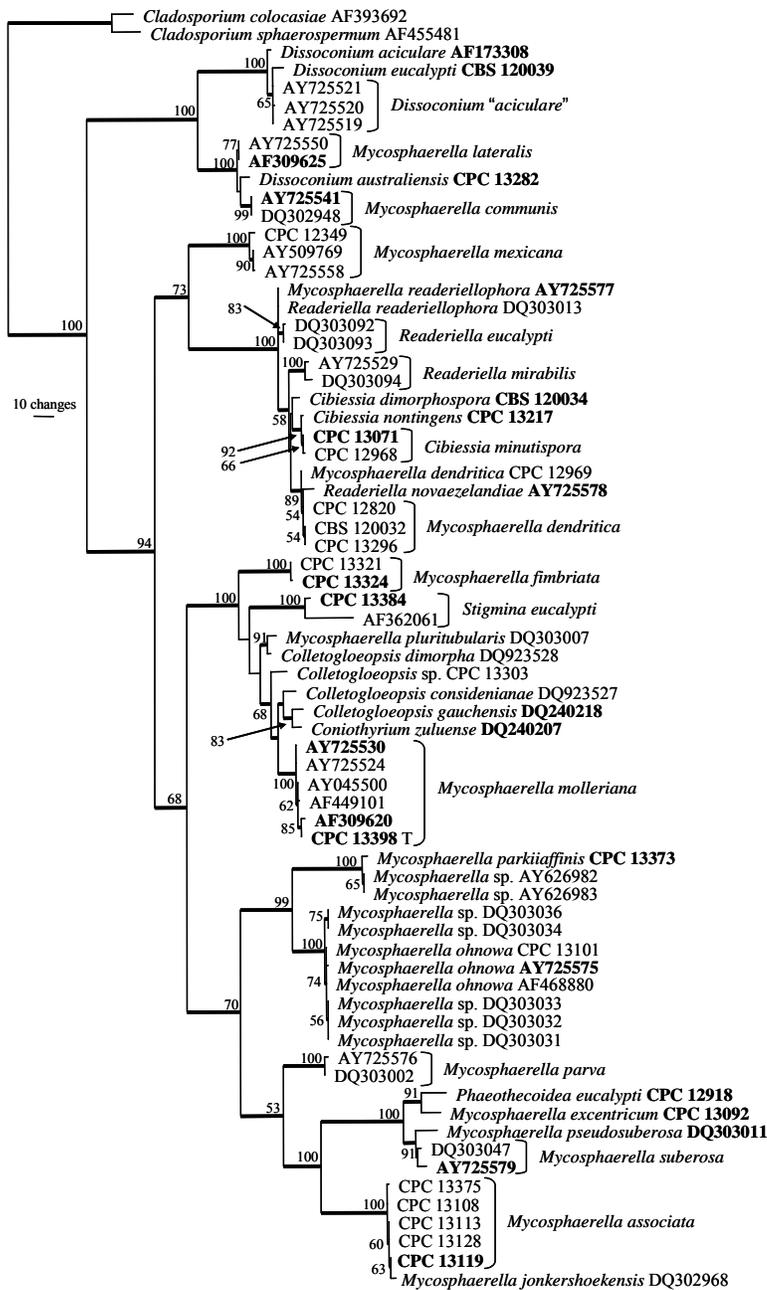
Teleomorph	Anamorph / Synanamorph	Strain no. <sup>1</sup>	Substrate	Country	Collector	GenBank number
<i>Mycosphaerella acaciigena</i>		CPC 13290 = CBS 120740	<i>Eucalyptus</i> sp.	Australia	B. Summerell	EF394822
		CPC 13350	<i>Eucalyptus camaldulensis</i> × <i>E. urophylla</i>	Venezuela	M.J. Wingfield	EF394823
<i>Mycosphaerella associata</i>		CPC 13108 = CBS 120732	<i>Eucalyptus dunnii</i>	Australia	A.J. Carnegie	EF394824
		CPC 13113	<i>Eucalyptus dunnii</i>	Australia	A.J. Carnegie	EF394825
		*CPC 13119 = CBS 120730	<i>Corymbia henryii</i>	Australia	A.J. Carnegie	EF394826
		CPC 13128 = CBS 120731	<i>Corymbia variegata</i>	Australia	A.J. Carnegie	EF394827
		CPC 13375	<i>Eucalyptus tereticornis</i>	Australia	B. Summerell	EF394828
<i>Mycosphaerella dendritica</i>	<i>Nothostrasseria dendritica</i>	*CPC 12709 = CBS 120032	<i>Eucalyptus deanei</i>	Australia	B.A. Summerell	EF394829
		CPC 12820 = CBS 120733	<i>Eucalyptus nitens</i>	Australia	A.J. Carnegie	EF394830
		CPC 12969	<i>Eucalyptus deanei</i>	Australia	B.A. Summerell	EF394831
		CPC 13296 = CBS 120734	<i>Eucalyptus globulus</i>	Australia	C. Mohammed	EF394832
<i>Mycosphaerella elongata</i>		*CPC 13378 = CBS 120735	<i>Eucalyptus camaldulensis</i> × <i>E. urophylla</i>	Venezuela	M.J. Wingfield	EF394833
<i>Mycosphaerella excentrica</i>	<i>Trimmatostroma excentricum</i>	*CPC 13092 = CBS 121102	<i>Eucalyptus agglomerata</i>	Australia	G. Price	EF394834
<i>Mycosphaerella fimbriata</i>		CPC 13321 = CBS 120893	<i>Corymbia</i> sp.	Australia	P.W. Crous	EF394835
		*CPC 13324 = CBS 120736	<i>Corymbia</i> sp.	Australia	P.W. Crous	EF394836
<i>Mycosphaerella heimii</i>		CPC 13276 = CBS 120741	<i>Eucalyptus platyphylla</i>	Australia	P.W. Crous	EF394837
		CPC 13356 = CBS 120743	<i>Eucalyptus urophylla</i>	Venezuela	M.J. Wingfield	EF394838
		CPC 13359	<i>Eucalyptus urophylla</i>	Venezuela	M.J. Wingfield	EF394839
		CPC 13371	<i>Eucalyptus urophylla</i>	Venezuela	M.J. Wingfield	EF394840
		CPC 13474 = CBS 120742	<i>Eucalyptus camaldulensis</i>	Thailand	W. Himaman	EF394841
<i>Mycosphaerella konae</i>	<i>Pseudocercospora</i> sp.	CPC 13469 = CBS 120748	<i>Eucalyptus camaldulensis</i>	Thailand	W. Himaman	EF394842
<i>Mycosphaerella mexicana</i>		CPC 12349 = CBS 120744	<i>Eucalyptus</i> sp.	Hawaii	W. Gams	EF394843
<i>Mycosphaerella molleriana</i>	<i>Colletogloeopsis molleriana</i>	*CPC 13398 = CBS 120746	<i>Eucalyptus</i> sp.	Portugal	P.W. Crous & A.J.L. Phillips	EF394844
<i>Mycosphaerella ohnowa</i>		CPC 13101 = CBS 120745	<i>Eucalyptus dunnii</i>	Australia	A.J. Carnegie	EF394845
<i>Mycosphaerella parkii</i>		*CPC 13373 = CBS 120737	<i>Eucalyptus urophylla</i>	Venezuela	M.J. Wingfield	EF394846
<i>Mycosphaerella</i> sp.	<i>Cibiessia nontingens</i> / <i>Readeriella</i> sp.	*CPC 13217 = CBS 120725	<i>Eucalyptus tereticornis</i>	Australia	B. Summerell	EF394847

**Table 1.** Isolates of *Mycosphaerella* spp. and its anamorphs included for sequence analysis and morphological comparison.

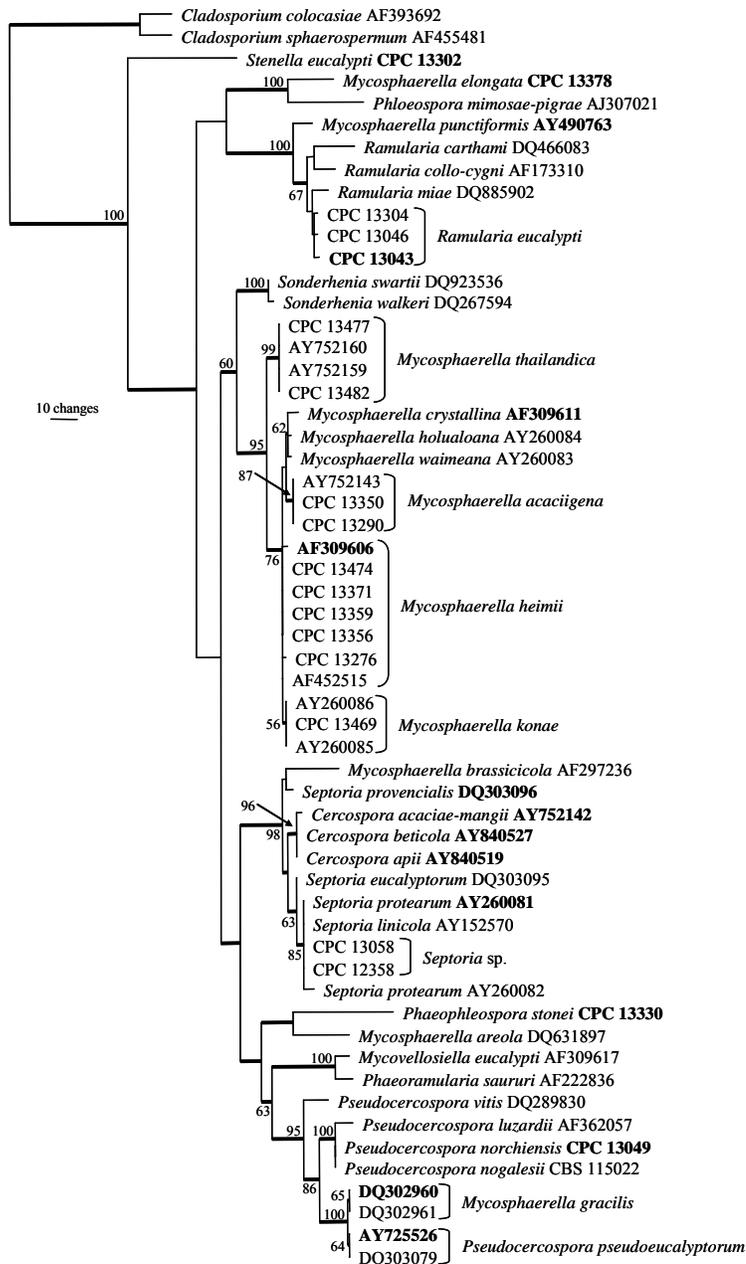
Teleomorph	Anamorph / Synanamorph	Strain no. <sup>1</sup>	Substrate	Country	Collector	GenBank number
<i>Mycosphaerella thailandica</i>	<i>Pseudocercospora thailandica</i>	CPC 13478 = CBS 120723	<i>Eucalyptus camaldulensis</i>	Thailand	W. Himaman	EF394848
		CPC 13482	<i>Eucalyptus camaldulensis</i>	Thailand	W. Himaman	EF394849
	<i>Cibiessia dimorphospora</i> / <i>Readeriella</i> sp.	*CPC 12636 = CBS 120034	<i>Eucalyptus nitens</i>	Australia	C. Mohammed	EF394850
	<i>Cibiessia minutispora</i>	CPC 12968 = CBS 120749	Leaf litter of <i>Cussonia</i> sp.	South Africa	P.W. Crous	EF394851
		*CPC 13071 = CBS 120894	<i>Corymbia henryii</i>	Australia	A.J. Carnegie	EF394852
	<i>Colletogloeopsis</i> sp.	CPC 13303	<i>Eucalyptus tereticornis</i>	Australia	P.W. Crous	EF394853
	<i>Dissoconium australiensis</i>	*CPC 13282 = CBS 120729	<i>Eucalyptus platyphylla</i>	Australia	P.W. Crous	EF394854
	<i>Dissoconium eucalypti</i>	*CPC 13004 = CBS 120039	<i>Eucalyptus tereticornis</i>	Australia	A. Carnegie	EF394855
	<i>Phaeophleospora stonei</i>	*CPC 13330 = CBS 120830	<i>Eucalyptus</i> sp.	Australia	P.W. Crous	EF394856
	<i>Phaeothecoidea eucalypti</i>	*CPC 12918 = CBS120831	<i>Eucalyptus botryooides</i>	Australia	B. Summerell	EF394857
	<i>Pseudocercospora nogalesii</i>	CBS 115022	<i>Chamaecytisus proliferus</i>	New Zealand	C.F. Hill	EF394858
	<i>Pseudocercospora norchiensis</i>	*CPC 13049 = CBS 120738	<i>Eucalyptus</i> sp.	Italy	W. Gams	EF394859
	<i>Ramularia eucalypti</i>	*CPC 13043 = CBS 120726	<i>Eucalyptus grandiflora</i>	Italy	W. Gams	EF394860
		CPC 13046 = CBS 120727	<i>Eucalyptus grandiflora</i>	Italy	W. Gams	EF394861
		CPC 13304 = CBS 120728	<i>Eucalyptus tereticornis</i>	Australia	P.W. Crous	EF394862
	<i>Septoria</i> sp.	CPC 12358	<i>Eucalyptus</i> sp.	Italy	W. Gams	EF394863
		CPC 13058 = CBS 120739	<i>Eucalyptus</i> sp.	Italy	W. Gams	EF394864
	<i>Stenella eucalypti</i>	*CPC 13302 = CBS 121101	<i>Eucalyptus tereticornis</i>	Australia	P.W. Crous	EF394865
	<i>Stigmia eucalypti</i>	*CPC 13384 = CBS 121100	<i>Corymbia variegata</i>	Australia	G. Price	EF394866

<sup>1</sup>CBS: Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.

\*Denotes ex-type cultures.



**Fig. 1.** One of 480 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows ten changes, and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches, and ex-type strains are shown in bold print. The tree was rooted to two *Cladosporium* species.



**Fig. 2.** One of 390 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows 10 changes, and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches, and type strains are shown in bold print. The tree was rooted to two *Cladosporium* species.

***Cibiessia* Crous, gen. nov.**

MycoBank: 501091

*Etymology*: Named for the Centraalbureau voor Schimmelcultures (“CBS”), where the fungus was first discovered by students during a mycological training course. Its unique conidiogenesis captured the imagination of several mycologists who gathered to examine it, and hence the suggestion arose that its name should reflect the unique concentration of mycologically interested persons at CBS.

Genus hyphomycetium ad *Mycosphaerellaceas pertinens*. Hyphae pallide brunneae, leves, 3–5  $\mu\text{m}$  latae, in conidia dilute brunnea, cylindrica, 0–3-septata, utrinque subtruncata disarticulatae, synanamorphe *Readeriella*.

Hyphomycetous, *Mycosphaerellaceae*. *Hyphae* pale brown, smooth, 3–5  $\mu\text{m}$  wide, disarticulating to form pale brown, cylindrical, 0–3-septate conidia with subtruncate ends. A *Readeriella* synanamorph also formed in culture.

*Type species*: *Cibiessia dimorphospora* Crous & C. Mohammed, sp. nov.

***Cibiessia dimorphospora* Crous & C. Mohammed, sp. nov.**

(Fig. 3)

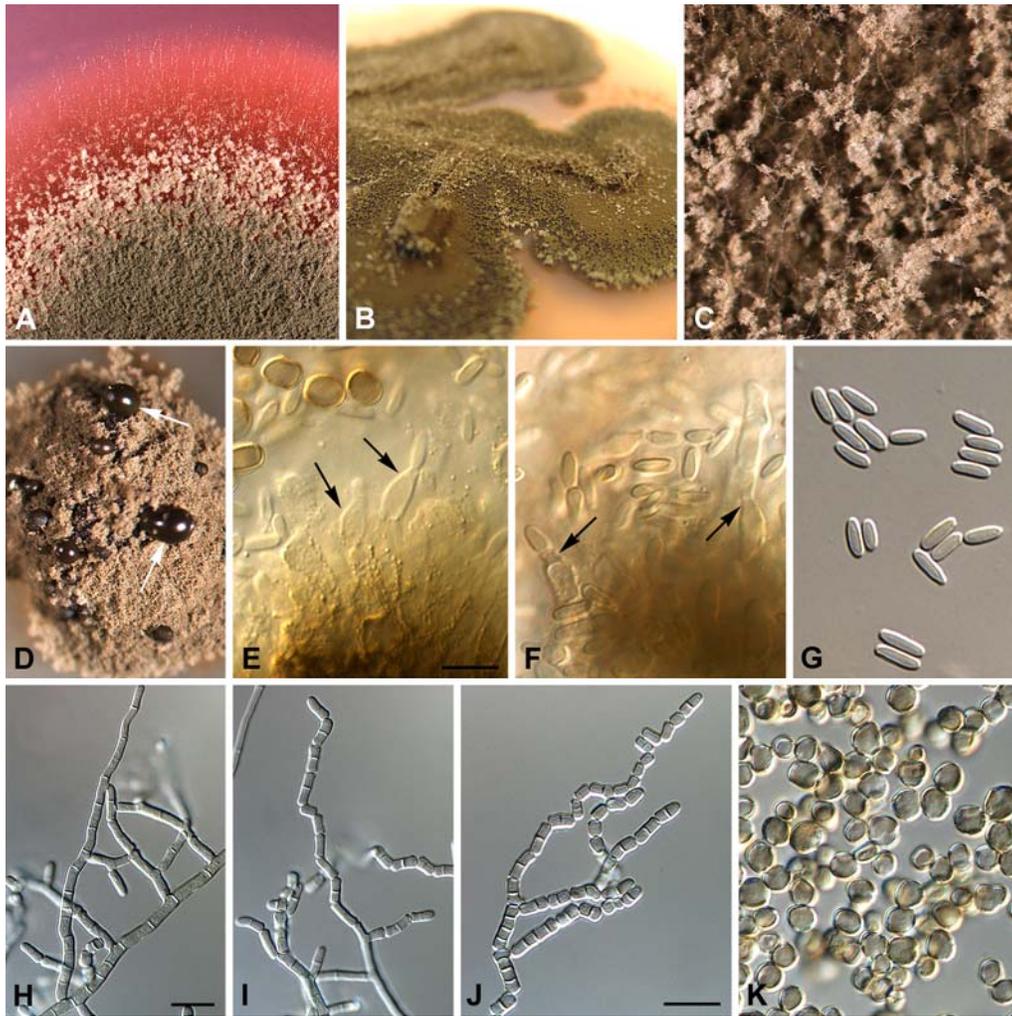
MycoBank: 501092.

*Synanamorph*: *Readeriella* sp.

*Etymology*: Name refers to the two asexual states (anamorphs) with different conidial types.

*Arthroconidia* dilute brunnea, cylindrica, utrinque subtruncata, 5–9  $\times$  2–3  $\mu\text{m}$ , 1(–3)-septata, synanamorphe *Readeriellae* in vitro formata, conidia ellipsoidea vel subcylindrica, dilute brunnea, 4.5–7  $\times$  2–2.5  $\mu\text{m}$ .

*Arthroconidia* occurring on brown lesions associated with a *Pseudocercospora* sp. *Hyphae* pale brown, smooth, 3–5  $\mu\text{m}$  wide, disarticulating at septa to form short, pale brown, cylindrical conidia with obtusely rounded to subtruncate ends; aseptate conidia 5–7  $\times$  2–3  $\mu\text{m}$ , 1(–3)-septate conidia 5–9  $\times$  2–3  $\mu\text{m}$ ; conidia developing further, becoming medium brown, predominantly aseptate, verruculose, ellipsoidal to subglobose or globose, 5–7  $\mu\text{m}$  diam, with dehiscence scars clearly visible on conidial body; inner layer of the dehiscence scar extends past the outer layer. *Readeriella* synanamorph: Only observed in culture, and absent in young and older colonies, with *Cibiessia* state dominant. *Conidiomata* oozing a dark brown conidial mass; conidiomata pycnidial, subglobose, unilocular; wall consisting of 3–4 layers of brown *textura angularis*. *Conidiophores* 0–1-septate, subcylindrical to ampulliform, hyaline to pale brown, smooth, 5–8  $\times$  3–4  $\mu\text{m}$ , mono- or polyphialide with visible periclinal thickening, or phialide proliferating percurrently near apex; frequently intermingled with cylindrical paraphyses that can extend 5–10  $\mu\text{m}$  above the conidiophores. *Conidia* narrowly ellipsoid to subcylindrical with rounded ends, pale brown, smooth to finely verruculose, 4.5–7  $\times$  2–2.5  $\mu\text{m}$ .



**Fig. 3.** *Cibiessia dimorphospora* (CBS H-19762). **A–C.** Colonies on PDA. **D.** Slimy conidial mass of *Readeriella* state (arrows). **E, F.** Conidiogenous cells of *Readeriella* state (arrows). **G.** Conidia of *Readeriella* state. **H–K.** Conidia of *Cibiessia* *in vitro*. Scale bars = 10  $\mu$ m.

*Cultural characteristics:* Colonies on PDA slow growing, reaching 30 mm diam after 2 months at 25°C. Surface appearing grey-olivaceous to green-olivaceous due to aerial mycelium and profuse sporulation; margins regular, smooth to slightly feathery; reverse greenish black; young colonies producing a red soluble pigment, but this is inconspicuous in older colonies.

*Specimen examined:* **Australia**, Tasmania, on *Eucalyptus nitens* leaves, Oct 2005, C. Mohammed, **holotype** CBS-H 19762, cultures ex-type CPC 12636 = CBS 120034, CPC 12637–12638.

*Notes:* Although there are several genera available for species with chains of disarticulating conidia (arthroconidia), none are represented in the *Mycosphaerellaceae*, and none have ever been linked to *Readeriella*. As the *Readeriella* synanamorph of *C. dimorphospora* rarely occurs in culture, and was not observed on the host, a new genus has been proposed to accommodate the novel arthroconidial anamorph. Species of *Cibiessia* are present with high bootstrap support (100%) in the *Readeriella* clade.

***Cibiessia minutispora*** Crous & Carnegie, **sp. nov.** (Fig. 4)  
MycoBank 501258.

*Etymology:* Name refers to the conidia that are smaller than those of the other species presently known.

*Cibiessiae dimorphosporae* similis, vel conidia  $4\text{--}6 \times 2\text{--}3 \mu\text{m}$ .

*Hyphae* pale brown, smooth,  $2\text{--}3 \mu\text{m}$  wide, disarticulating at septa to form short, pale brown, cylindrical conidia with obtusely rounded to subtruncate ends; aseptate conidia  $4\text{--}6 \times 2\text{--}3 \mu\text{m}$ , 1(–2)-septate conidia  $6\text{--}10 \times 2\text{--}3 \mu\text{m}$ ; conidia developing further, becoming medium brown, predominantly aseptate, verruculose, ellipsoidal to subglobose or globose, with dehiscence scars clearly visible on conidial body. *Readeriella* synanamorph not seen.

*Cultural characteristics:* Colonies flat with even margins, spreading with moderate to prominent aerial mycelium, reaching 25 mm diam after 1 month on PDA; colonies on OA iron-grey, becoming olivaceous-grey on surface due to aerial mycelium; having prominent, diffuse red pigment in agar when cultivated on PDA, colony surface and reverse iron-grey.

*Specimen examined:* **Australia**, New South Wales, South Grafton, Grafton City Council Landfill Plantation,  $152^{\circ} 54' 38''$  E,  $29^{\circ} 46' 21''$  S, on leaves of *Corymbia henryii*, 16 Feb. 2006, A.J. Carnegie, **holotype** CBS-H 19839, **isotype** DAR 78030, cultures ex-type CPC 13071 = CBS 120894, CPC 13072–13073. **South Africa**, Western Cape Province, Betties Bay, Harold Porter Botanical Garden, leaf litter of *Cussonia* sp., Jan. 2006, P.W. Crous, CPC 12968 = CBS 120749 (single ascospore isolate).

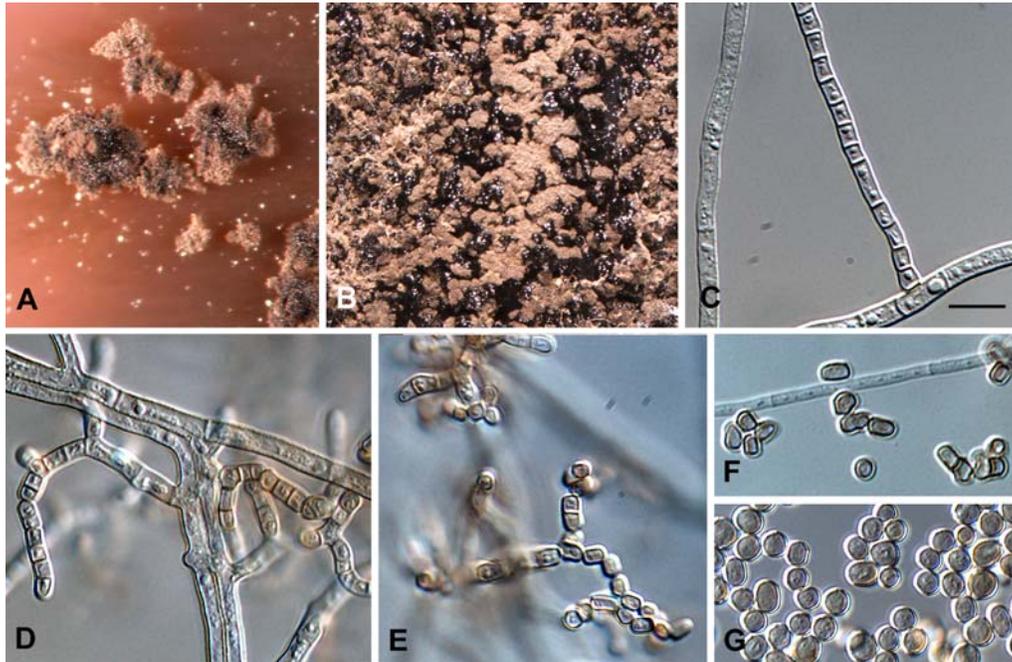
*Notes:* *Cibiessia minutispora* is similar to *C. dimorphospora* in producing a prominent red pigment in agar, but is distinct due to the absence of a *Readeriella* synanamorph, and in the fact that it has much smaller conidia. This species is known from two collections, and seems to not be host specific. The South African collection arose from an actively discharged ascospore [using the technique as explained in Summerell *et al.* (2006), with spores shot upwards onto clean plates], while the Australian isolates occurred with several *Mycosphaerella* spp. on leaves of *E. henryii*.

*Cibiessia nontingens* Crous & Summerell, **sp. nov.**

(Fig. 5)

Mycobank 501259.

*Synanamorph: Readeriella* sp.



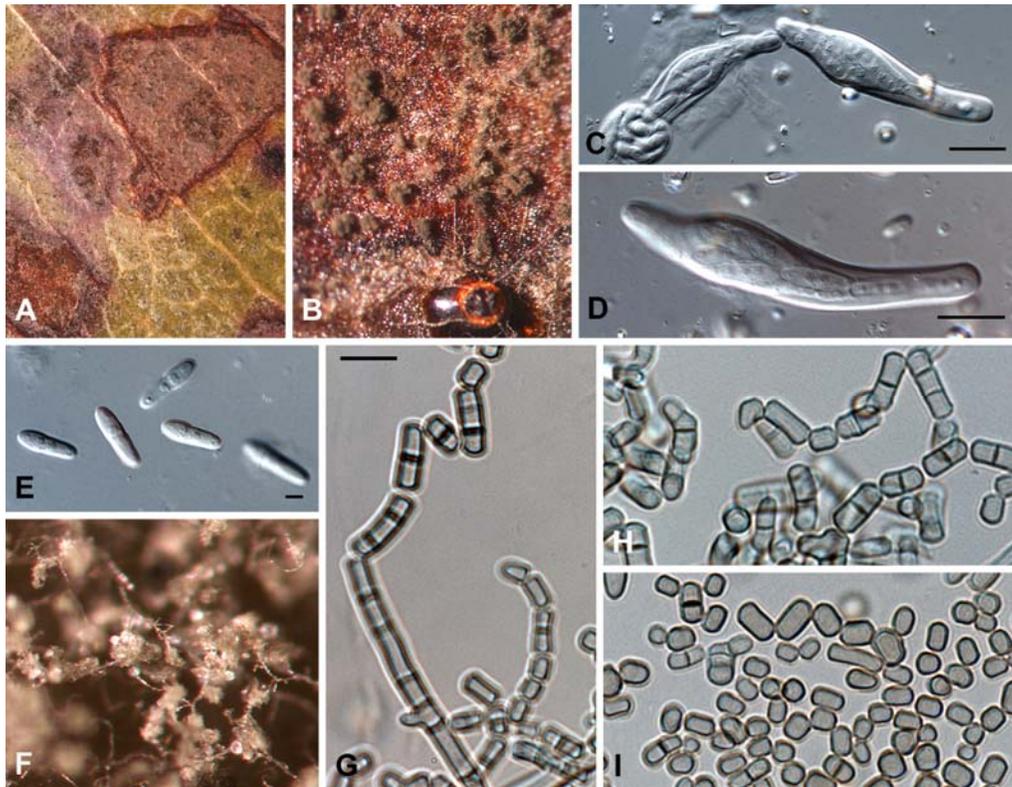
**Fig. 4.** *Cibiessia minutispora* (CBS H-19839). **A, B.** Colonies *in vitro* on PDA. **C–G.** Conidia and conidiogenous cells *in vitro*. Scale bar = 10  $\mu$ m.

*Teleomorph: Mycosphaerella* sp.

*Etymology:* Lacking a red pigment in culture, that is present in other species of the genus presently known.

*Cibiessiae dimorphosporae* similis, sed synanamorphe *Readeriellae* et pigmento rubro diffundente carens.

*Leaf spots* amphigenous, irregular to subcircular; spots variable from small specks (1 mm diam) to larger spots (7 mm diam), or coalescing to form larger blotches, medium brown, with a raised border and thin, red-purple margin. *Ascomata* pseudothecial, amphigenous, but predominantly epiphyllous, black, subepidermal, globose, up to 90  $\mu$ m wide; apical ostiole 5–10  $\mu$ m wide; wall consisting of 2–3 layers of medium brown textura angularis. *Asci* aparaphysate, fasciculate, bitunicate, sessile, obovoid to narrowly ellipsoid to subcylindrical, straight to slightly curved, 8-spored, 35–45  $\times$  8–11  $\mu$ m. *Ascospores* tri- to multi-seriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest just above the septum, medianly 1-septate, constricted at the septum, tapering towards both ends, but



**Fig. 5.** *Cibiessia nontingens* (CBS H-19840). **A.** Leaf lesion. **B.** Colonies *in vivo*. **C, D.** Asci. **E.** Asci. **F–I.** Conidia *in vitro*. Scale bars = 10  $\mu$ m.

more prominently towards the lower end, (9–)10–11  $\times$  2–3(–3.5)  $\mu$ m; several ascospores showed remnants of a mucus sheath; no single ascospore cultures were obtained to confirm the anamorph link, though the anamorph formed on top of these ascomata, and the synanamorph among these ascomata. *Hyphae* pale brown, smooth, 3–5  $\mu$ m wide, disarticulating at septa to form short, pale brown, cylindrical conidia with obtusely rounded to subtruncate ends; aseptate conidia 4–10  $\times$  3–5  $\mu$ m, 1(–3)-septate conidia 7–12  $\times$  3–5  $\mu$ m; conidia developing further, becoming medium brown, predominantly aseptate, verruculose, ellipsoidal to subglobose or globose, with dehiscence scars clearly visible on conidial body; inner layer of the dehiscence scar extends past the outer layer. *Readeriella* synanamorph: Not observed in culture. *Conidiomata* intermingled among ascomata of a *Mycosphaerella* sp.; oozing a dark brown conidial mass; conidiomata pycnidial, subglobose, unilocular; wall consisting of 3–4 layers of brown *textura angularis*. *Conidiophores* 0–1-septate, subcylindrical to ampulliform, hyaline to pale brown, smooth, 5–7  $\times$  3–4  $\mu$ m,

mono- or polyphialidic. *Conidia* narrowly ellipsoid to subcylindrical with rounded ends, pale brown, smooth,  $4\text{--}6 \times 2\text{--}3 \mu\text{m}$ .

*Cultural characteristics*: Colonies flat, spreading, with moderate aerial mycelium and even margins, reaching 40 mm diam after 1 month on OA at 25°C, 50 mm diam on PDA. Colonies on OA olivaceous-grey, on PDA iron-grey to greenish black, with numerous mucus droplets on colony surface; colonies greenish black in reverse.

*Specimen examined*: **Australia**, New South Wales, McWilliam Drive, Douglas Park 34 11 0 S 150 43 0 E, on leaves of *Eucalyptus tereticornis*. Open woodland (Cumberland Plains Woodland) of *E. molucanna* and *E. tereticornis* on shale derived clay, Jul. 2006, B. Summerell, **holotype** CBS-H 19840, cultures ex-type CPC 13217 = CBS 120725, CPC 13218–13219.

*Notes*: Characteristic differences between *C. nontingens* and *C. dimorphospora* are the absence of the *Readeriella* synanamorph in culture, as well as the diffuse red pigment, which are prominent features in the latter species.

### Key to species of *Cibiessia*

1. Red pigment produced in colonies on PDA ..... 2
1. Red pigment absent in colonies on PDA, aseptate conidia up to 10  $\mu\text{m}$  long and 5  $\mu\text{m}$  wide ...  
..... *C. nontingens*
2. *Readeriella* state produced in culture; aseptate *Cibiessia* conidia up to 7  $\mu\text{m}$  long and 2.5  $\mu\text{m}$  wide ..... *C. dimorphospora*
2. *Readeriella* state not produced in culture; aseptate *Cibiessia* conidia up to 6  $\mu\text{m}$  long and 3  $\mu\text{m}$  wide ..... *C. minutispora*

***Dissoconium australiensis* Crous & Summerell, sp. nov.** (Fig. 6)  
MycoBank 501260.

*Etymology*: Named for Australia, the country of origin.

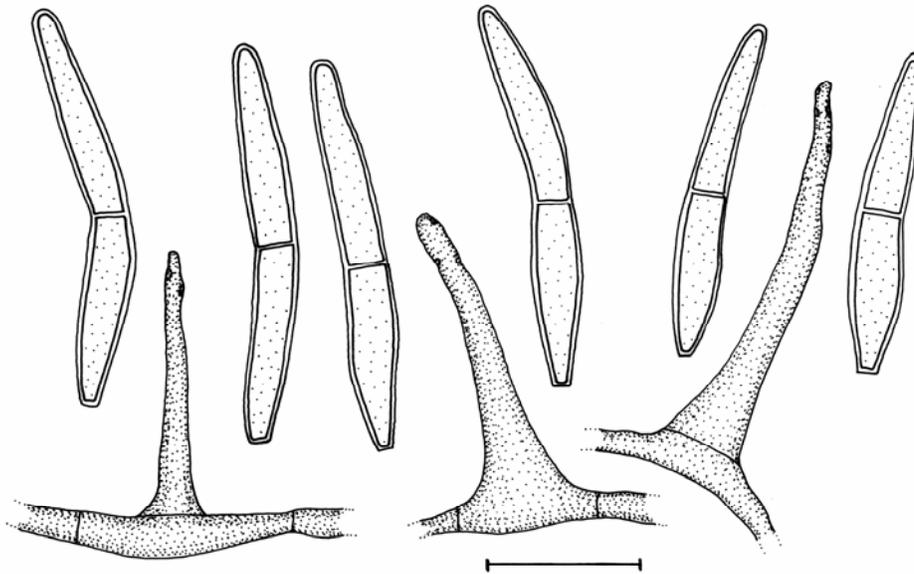
*Dissoconio communi* simile, sed conidiis minoribus,  $(20\text{--})23\text{--}25(\text{--}27) \times (3\text{--})4(\text{--}5) \mu\text{m}$ , distinguendum.

*Mycelium* internal and external, consisting of branched, septate, smooth, hyaline to pale brown hyphae, 2–3  $\mu\text{m}$  wide. *Conidiophores* separate, arising from hyphae, subcylindrical, subulate or lageniform, tapering to a bluntly rounded or truncate apex, straight to curved, smooth, medium brown, aseptate,  $20\text{--}27 \times 4\text{--}5 \mu\text{m}$ ; loci terminal and lateral, indistinct. *Conidia*  $(20\text{--})23\text{--}25(\text{--}27) \times (3\text{--})4(\text{--}5) \mu\text{m}$ , solitary, pale olivaceous-brown, smooth, ellipsoid to obclavate, 1-septate, apex obtuse, base obconic-truncate, hilum unthickened, 1–1.5  $\mu\text{m}$  wide. *Secondary conidia* not observed on MEA or on SNA.

*Cultural characteristics*: Colonies on MEA reaching 30 mm diam after 1 month at 25°C; erumpent with sparse aerial mycelium, hazel to isabelline, with feathery margins; umber in reverse.

*Specimen examined:* **Australia**, Queensland, Cairns, nr Kuranda, S 16° 56' 23.3", E 145° 32' 34.6", on leaves of *Eucalyptus platyphylla*, 26 Aug. 2006, P.W. Crous, **holotype** CBS-H 19837, culture ex-type CPC 13282 = CBS 120729.

*Notes:* Morphologically and phylogenetically *D. australiensis* is similar to *D. commune* and *D. dekkeri* (= *M. lateralis*). Conidia of *D. australiensis* (20–23–25(–27) × (3–)4(–5) μm are on average smaller than those of *D. commune* (20–30 × 4–5 μm, av. 25 × 4.5 μm), and somewhat larger than the common range of *D. dekkeri* (15–)17–21(–35) × (2–)3.5–4(–4.5) μm (Crous, 1998). Although the present strain failed to produce microconidia on various culture media, this cannot be seen as a species character until more strains have been collected and studied.



**Fig. 6.** *Dissoconium australiensis* (CBS H-19837). Conidia and conidiogenous cells *in vitro*. Scale bar = 10 μm.

***Dissoconium eucalypti* Crous & Carnegie, sp. nov.**

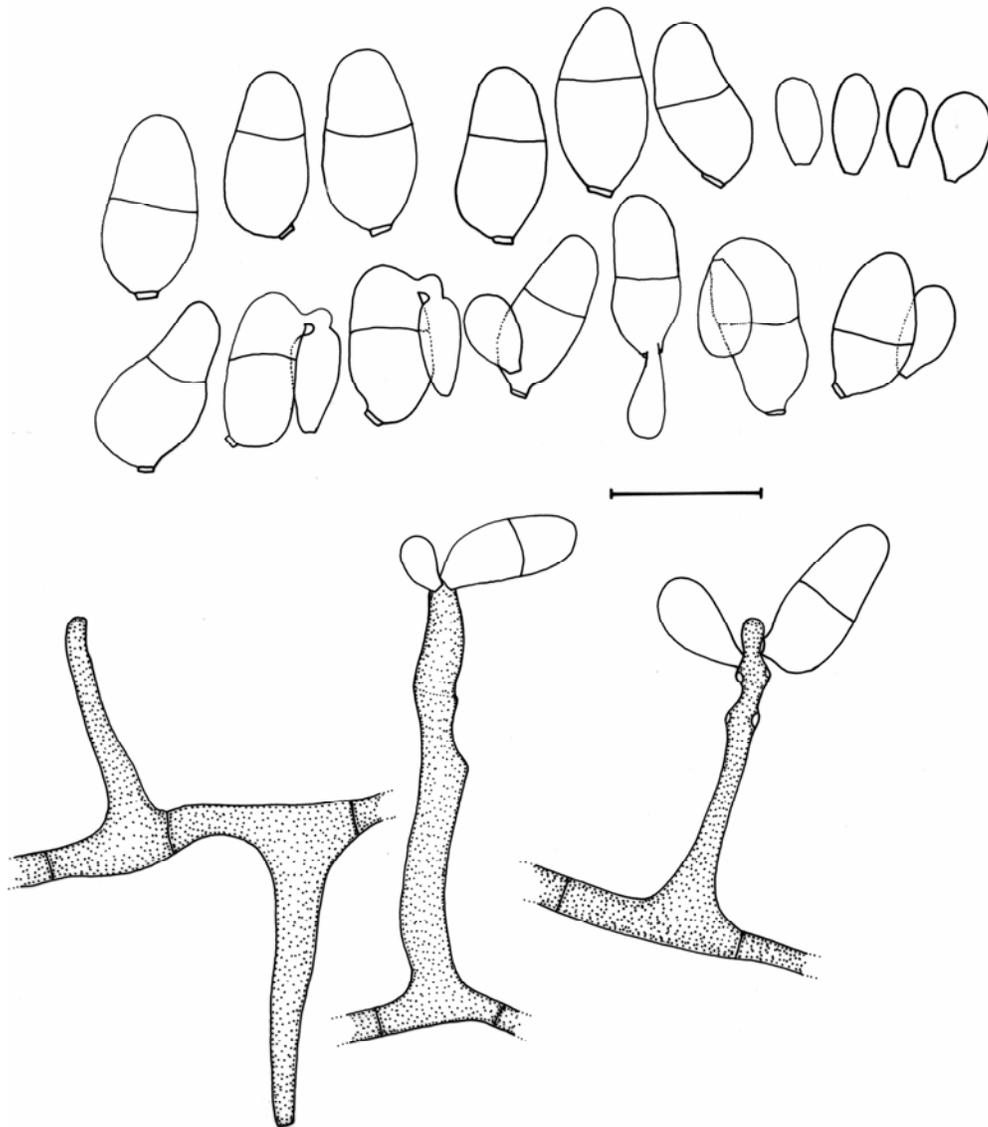
(Fig. 7)

MycoBank 501103.

*Etymology:* Named after its host plant, *Eucalyptus*.

*Dissoconia aciculari* simile, sed conidiis primariis minoribus, (8–)10–12(–14) × (4.5–)5–6 μm, secundariis majoribus, 4–7 × 2.5–3 μm, differens.

*Mycelium* internal and external, consisting of branched, septate, smooth, hyaline to pale brown hyphae, 2–3 μm wide. *Conidiophores* separate, arising from hyphae, subcylindrical, subulate or lageniform, tapering to a bluntly rounded or truncate apex, straight to once geniculate, smooth, medium brown, aseptate, 10–30 × 4–8 μm; loci terminal and lateral, visible as slightly



**Fig. 7.** *Dissoconium eucalypti* (CBS H-19770). Conidia and conidiogenous cells *in vitro*. Scale bar = 10  $\mu$ m.

thickened, darkened scars, 0.5–1  $\mu$ m wide. *Conidia* (8–)10–12(–14)  $\times$  (4.5–)5–6  $\mu$ m, solitary, pale olivaceous-brown, smooth, ellipsoid to obclavate, 1-septate, apex obtuse, base obconic-truncate, hilum thickened, somewhat darkened, 1–1.5  $\mu$ m wide. *Secondary conidia* developing adjacent to primary

conidia, pale olivaceous to subhyaline, aseptate, pyriform, with a truncate base,  $4\text{--}7 \times 2.5\text{--}3 \mu\text{m}$ ; anastomosing with primary conidia after active discharge.

*Cultural characteristics*: Colonies on MEA reaching 15 mm diam after 3 weeks at 25°C; erumpent with sparse aerial mycelium, buff to olivaceous-buff, with feathery margins; cinnamon in reverse.

*Specimen examined*: **Australia**, New South Wales, Morpeth Park, Plantation, Bonalbo, 152° 36' 47" E, 28° 46' 3", on leaves of *Eucalyptus tereticornis*, 8 Feb 2006, A. Carnegie, **holotype** CBS-H 19770, cultures ex-type CPC 13004 = CBS 120039, CPC 13005–13006.

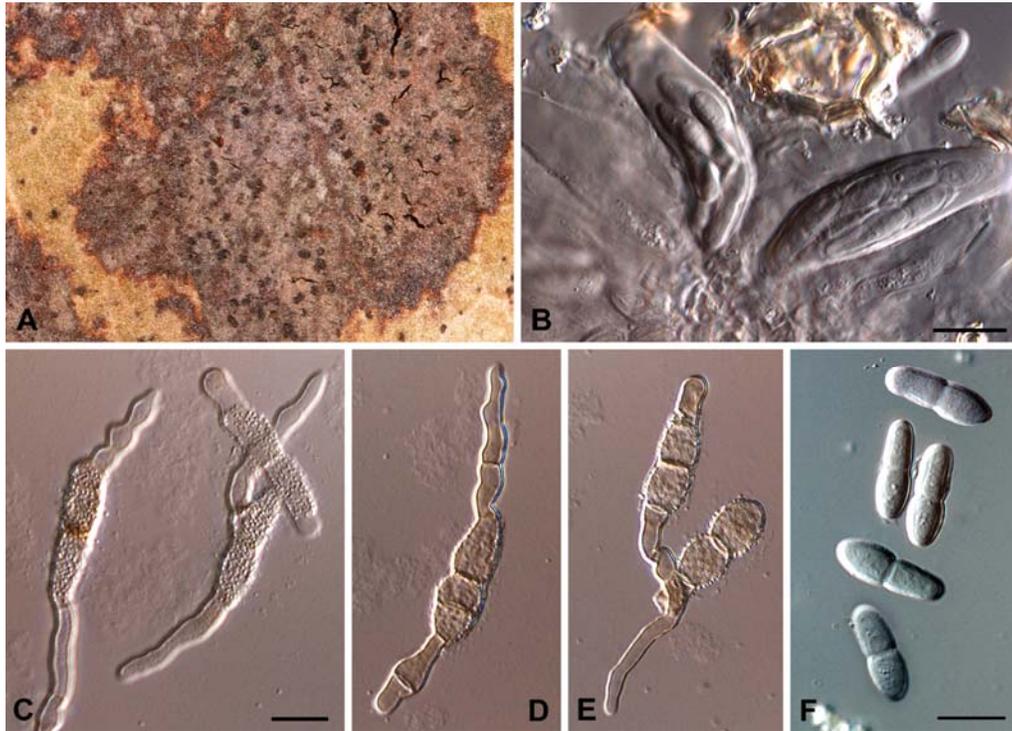
*Notes*: Although several species of *Dissoconium* have been described from *Eucalyptus* (Crous *et al.*, 2004b), *D. eucalypti* is distinct in having smaller primary and larger secondary conidia than those species known to date. Phylogenetically it clusters close to the ex-type strain of *D. aciculare*, which has larger primary ( $12\text{--}25 \times 3.5\text{--}6 \mu\text{m}$ ), and secondary ( $7.5\text{--}12 \times 3.5\text{--}6 \mu\text{m}$ ) conidia (De Hoog *et al.*, 1983). However, *D. eucalypti* differs with 5 nucleotides in the ITS1 region when compared to strains identified as *D. aciculare*.

***Mycosphaerella associata* Crous & Carnegie, sp. nov.** (Fig. 8)  
MycoBank 501261.

*Etymology*: Name refers to its co-occurrence with other species of *Mycosphaerella* on the same leaf spots.

Ascospores fusioideae-ellipsoideae,  $(12\text{--})13\text{--}16(\text{--}17) \times (3.5\text{--})4\text{--}5(\text{--}6) \mu\text{m}$ .

*Leaf spots* amphigenous, irregular to subcircular, 4–6 mm diam, medium brown, with a thin, raised, dark brown border on the adaxial surface; dark brown with patches of grey due to the lifting cuticle on the abaxial surface, displaying numerous small cracks within the lesion tissue. *Ascomata* pseudothecial, amphigenous, but predominantly hypophyllous, black, subepidermal to erumpent, globose, up to 120  $\mu\text{m}$  wide; apical ostiole 10–15  $\mu\text{m}$  wide; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* paraphysate, but with remains of hamathecium visible, fasciculate, bitunicate, sessile, obovoid to broadly ellipsoidal, straight to slightly curved, 8-spored,  $30\text{--}38 \times 9\text{--}12 \mu\text{m}$ . *Ascospores* tri- to multi-seriate, overlapping, hyaline, guttulate, thick-walled, straight, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, medianly 1-septate, constricted at the septum, tapering towards both ends, but more prominently towards the lower end,  $(12\text{--})13\text{--}16(\text{--}17) \times (3.5\text{--})4\text{--}5(\text{--}6) \mu\text{m}$ ; ascospores with persistent mucus sheath. Ascospores germinate from polar ends, with germ tubes parallel to the long axis of the spore; spore distorting and becoming prominently constricted at the septum, verruculose and brown; germ tubes pale brown, not straight and even, but irregularly crenate, 5–8  $\mu\text{m}$  wide, at times developing 1–2 additional spore septa and additional germ tubes (germination Type H *sensu* Crous, 1998).



**Fig. 8.** *Mycosphaerella associata* (CBS H-19833). **A.** Leaf spot. **B.** Asci. **C–E.** Germinating ascospores. **F.** Ascospores. Scale bars = 10 µm.

*Cultural characteristics:* Colonies erumpent with moderate aerial mycelium; margins catenulate, smooth; surface uneven on OA, olivaceous-grey with patches of pale olivaceous-grey to iron-grey, reaching 15 mm diam after 1 month at 25°C (on OA and PDA); on PDA olivaceous-grey with patches of pale olivaceous-grey to grey-olivaceous.

*Specimens examined:* **Australia**, New South Wales, South Grafton, Grafton City Council Landfill Plantation, 152° 54' 38" E, 29° 46' 21" S, on leaves of *Corymbia henryii*, 16 Feb. 2006, A.J. Carnegie, **holotype** CBS-H 19833, **isotype** DAR 78031, cultures ex-type CPC 13119 = CBS 120730, CPC 13120 (occurring with *Lembosina* sp.); New South Wales, Bungawalbin, Robertson Plantation, 153° 15' 39" E, 29° 5' 34" S, on leaves of *Corymbia variegata*, 23 Jan. 2005, A.J. Carnegie, DAR 78032, cultures CPC 13128 = CBS 120731, CPC 13129–13130 (occurring with *Lembosina* sp.); New South Wales, Bungawalbin, Robertson Plantation, 153° 15' 39" E, 29° 5' 34" S, on leaves of *Eucalyptus dunnii*, 14 Feb. 2006, A.J. Carnegie, cultures CPC 13108 = CBS 120732, CPC 13109–13110, 13113–13114 (occurring with *M. suberosa*).

*Notes:* *Mycosphaerella associata* frequently colonizes lesions of other ascomycetes, but it also occurs singly. Although several species have overlapping ascospore dimensions that overlap with those of *M. associata* (Crous, 1998), none share its rather unique mode of ascospore germination.

*Mycosphaerella jonkershoekensis* (GenBank DQ302968) clusters with 100% bootstrap support in the *M. associata* clade, but differs with one nucleotide in both the ITS1 and ITS2 region from this species.

***Mycosphaerella dendritica*** Crous & Summerell, **sp. nov.** (Fig. 9)  
MycoBank 501102.

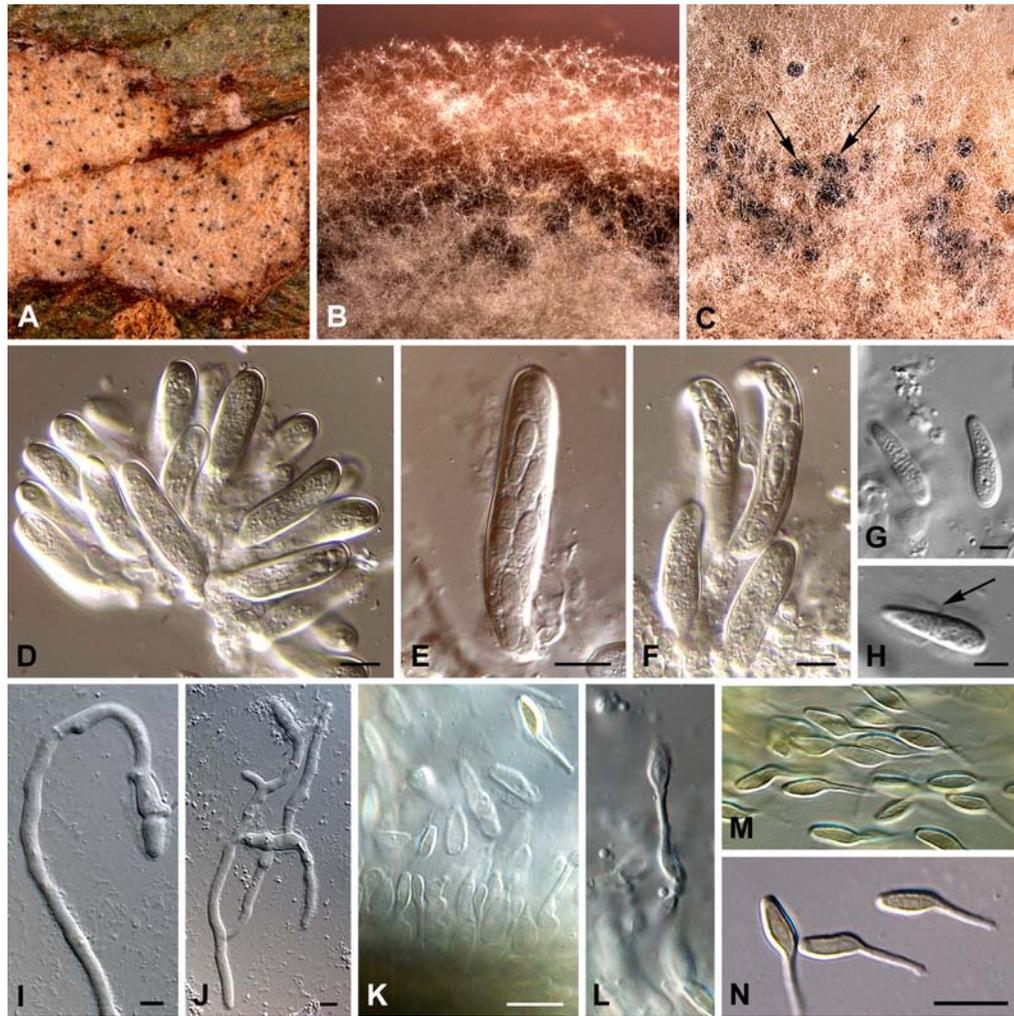
*Anamorph: Nothotrasseria dendritica* (Hansf.) Nag Raj, Can. J. Bot. 61: 25. 1983.

(Basionym) *Spilomyces dentriticus* Hansf., Proc. Linn. Soc. N. S. W. 81: 32. 1956.

Ascosporae fusoidae-ellipsoideae, (11–)12–13(–15) × 3–3.5(–4.5) µm, anamorphe *Nothotrasseria dendritica* formata in vitro.

*Leaf spots* amphigenous, irregular to subcircular, 2–8 mm diam, pale to gray-brown, with raised borders and thin, dark brown margins. *Ascomata* pseudothecial, amphigenous, black, subepidermal, globose, up to 150 µm wide; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, sessile, broadly ellipsoid, straight to slightly curved, 8-spored, 25–50 × 9–11 µm. *Ascospores* bi- to triseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest just above the septum, medianly 1-septate, not to slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (11–)12–13(–15) × 3–3.5(–4.5) µm; encased in a mucus sheath. Ascospores germinate irregularly, but mostly from polar ends, with germ tubes parallel to the long axis, but also with secondary germ tubes forming on the spore, at right angles to the long axis of the spore (Type D or I, *sensu* Crous 1998); spore distorting, becoming constricted, but remaining hyaline, 3.5–4.5 µm diam. *Conidiomata* black, globose, pycnidial, scattered, immersed in leaf tissue, but immersed to almost superficial on agar, up to 250 µm diam. *Conidiophores* ampulliform to lageniform, hyaline, smooth, 0–1-septate, mono- to polyphialidic, rarely proliferating percurrently, rarely branched, with loci terminal but also lateral, 5–10 × 2.5–4 µm. *Conidia* consisting of an ellipsoid body with obtuse apex, tapering to a tubular basal appendage; body medium brown, verruculose, 6–12 × 3–4 µm; tubular appendage separated from the conidium body by a septum, unbranched, hyaline, smooth, 4–15 × 1–1.5 µm.

*Cultural characteristics:* Colonies on PDA reaching 35 mm diam after 5 weeks at 25°C; colonies erumpent, with moderate, woolly aerial mycelium, pale olivaceous-grey to olivaceous-grey, margins smooth, regular; reverse iron-grey with zones of olivaceous-grey; colonies produce a faint, diffuse, pink pigment in agar. Colonies form numerous erumpent, black, globose, dark brown to black conidiomata on PDA and MEA.



**Fig. 9.** *Mycosphaerella dendritica* and its anamorph, *Nothostrasseria dendritica* (CBS H-19772). **A.** Leaf spot. **B, C.** Colonies on MEA (arrows indicate conidiomata). **D–F.** Asci. **G, H.** Ascospores (arrow indicates sheath). **I, J.** Germinating ascospores. **K, L.** Conidiogenous cells. **M, N.** Conidia. Scale bars: E, F, K, N = 10, G–J = 3.5  $\mu$ m.

*Specimens examined:* **Australia**, New South Wales, Wollemi National Park, on leaves of *Eucalyptus deanei*, Feb 2006, B.A. Summerell, **holotype** CBS-H 19772, cultures ex-type CPC 12709 = CBS 120032, CPC 12710–12711; New South Wales, Laurel Hill, Bago State Forest, research trial, on leaves of *E. nitens*, 22 Dec. 2005, A.J. Carnegie, CPC 12820 = CBS 120733; Tasmania, on leaves of *E. globulus*, 31 Aug. 2006, C. Mohammed, CPC 13296 = CBS 120734, CPC 13297–13298.

*Notes:* As far as we could establish, this is the first record of *Nothostrasseria dendritica* grown in pure culture. This is also the first record of

its teleomorph, which is a species of *Mycosphaerella*, described here as *M. dendritica*. Phylogenetically *Nothostrasseria* clusters with species of *Readeriella*, but is different from *R. novaezealandiae* at three nucleotide positions in the ITS1 region and one in the ITS2 region. Although species of *Readeriella* have brown conidia that have up to three obtuse, apical projections, they lack basal appendages, and are thus tentatively retained as separate genera. The conidiogenesis of both genera is, however, similar, with conidia forming on mono- or polyphialides, which can also proliferate percurrently.

***Mycosphaerella elongata*** Crous & M.J. Wingf., **sp. nov.** (Fig. 10)  
MycoBank 501262.

*Etymology*: Named after its characteristic long ascospores.

Ascospores fusoid-ellipsoideae, (18–)20–25 × (4–)4.5(–5) µm.

*Leaf spots* amphigenous, irregular to subcircular, 3–13 mm diam, medium brown, with a thin, raised, dark brown to red-brown border. *Ascomata* pseudothecial, amphigenous, but predominantly epiphyllous, dark brown, subepidermal to somewhat erumpent, globose, up to 150 µm wide; apical ostiole up to 30 µm wide; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* paraphysate, fasciculate, bitunicate, sessile, obovoid to broadly ellipsoidal, straight to slightly curved, 8-spored, 45–60 × 11–15 µm. *Ascospores* tri- to multi-seriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, 1-septate, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (18–)20–25 × (4–)4.5(–5) µm; basal cell frequently 1–4 µm longer than apical cell. Ascospores germinate from both ends, with germ tubes parallel to the long axis of the spore; spore not darkening, nor distorting, becoming up to 5 µm wide (germination Type C *sensu* Crous, 1998).

*Cultural characteristics*: Colonies on MEA erumpent, convex, radially striated; margins smooth, even; surface cinnamon with patches of pale vinaceous aerial mycelium in centre; reverse brown-vinaceous; reaching 11 mm diam after 2 months at 25°C.

*Specimen examined*: **Venezuela**, El Piñal Lotes farm near Acarigua, on leaves of *Eucalyptus camaldulensis* × *urophylla*, Oct. 2006, M.J. Wingfield, **holotype** CBS-H 19824, cultures ex-type CPC 13378 = CBS 120735, CPC 13379–13380.

*Notes*: *Mycosphaerella elongata* has characteristically long ascospores (up to 25 µm long), somewhat reminiscent of *M. longibasalis* (22–30 × 3.5–5 µm; Crous, 1998), but shorter. It also has different lesions, with those of *M. longibasalis* being pale brown in colour.



**Fig. 10.** *Mycosphaerella elongata* (CBS H-19824). **A.** Leaf spot. **B–D.** Asci and ascospores. **E–G.** Germinating ascospores. Scale bar = 10  $\mu$ m.

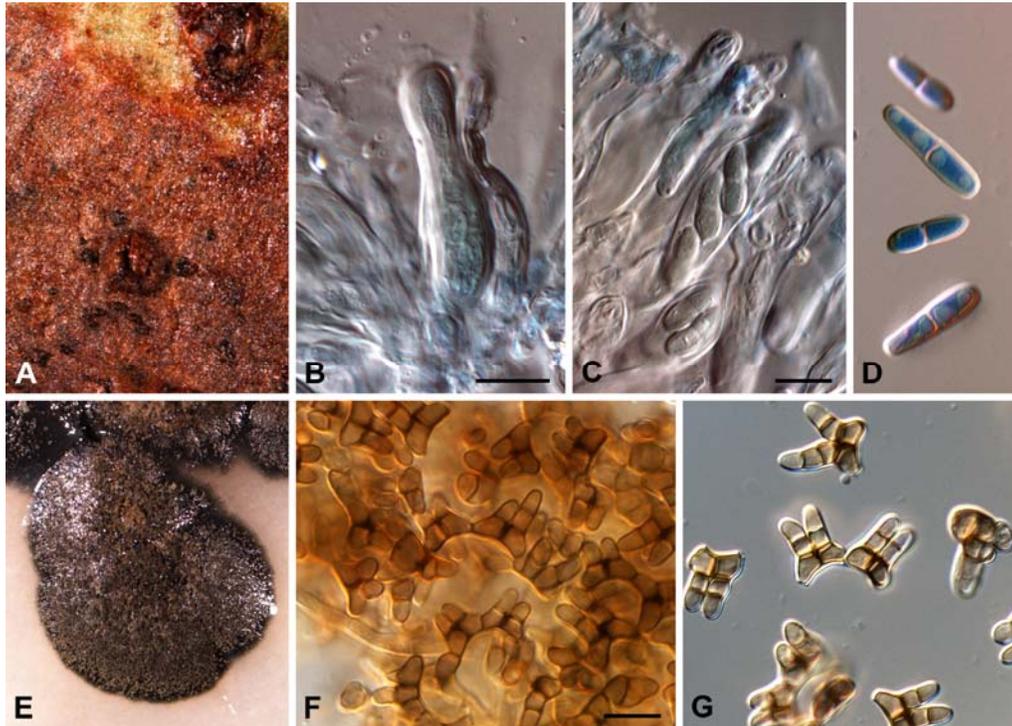
***Mycosphaerella excentrica*** Crous & Carnegie, **sp. nov.**  
 MycoBank 501263.

(Fig. 11)

*Anamorph:* *Trimmatostroma excentricum* B. Sutton & Ganap., N.Z. J. Bot. 16: 529. 1978.

Ascospores fusoidae-ellipsoideae, (10–)15–18(–23)  $\times$  (3–)4  $\mu$ m, anamorphe *Trimmatostroma excentrica* formata in vitro.

*Leaf spots* amphigenous, irregular, corky, medium to dark brown, raised, with an irregular margin and thin, red-brown border, 2–12 mm diam. *Ascomata* amphigenous, separate, dark brown, subepidermal, becoming superficial, globose, up to 160  $\mu$ m wide; apical ostiole up to 20  $\mu$ m wide, but frequently opening by means of irregular rupture; wall of 2–3 layers of dark brown, thick-walled *textura angularis*. *Asci* fasciculate, bitunicate, aparaphysate (through remains of the hamathecium observed in some ascomata), 8-spored, obovoid to broadly ellipsoidal, straight to slightly incurved, 40–50  $\times$  8–10  $\mu$ m. *Ascospores*



**Fig. 11.** *Mycosphaerella excentrica* and its anamorph *Trimmatostroma excentricum* (CBS H-19829). **A.** Leaf spot. **B, C.** Asci. **D.** Ascospores. **E.** Colony on OA. **F, G.** Conidia and conidiogenous cells *in vitro*. Scale bars = 10  $\mu$ m.

tri to multiseriate, fusoid-ellipsoidal with obtuse ends, hyaline, smooth, but pale brown and verruculose in old asci, becoming 3-septate, not constricted at median septum, thick-walled, guttulate, widest in the middle of the apical cell, with persistent mucous sheath, (10–)15–18(–23)  $\times$  (3–)4  $\mu$ m. *Conidia in vitro* formed in basipetal chains, smooth, medium brown, 4-celled, consisting of two basal cells with truncate lateral sides (adhesion scars present when catenulate), each giving rise to a secondary globose apical cell, that can extend and develop two additional septa in some cases; primary cells 9–11  $\times$  3–4  $\mu$ m, secondary cells 2.5–4.5  $\mu$ m wide, 4–6  $\mu$ m long, but with additional septa these arms can become up to 15  $\mu$ m long (excluding the basal cell); septa separating the primary and secondary cells are dark-brown and thick-walled.

*Cultural characteristics:* Colonies on OA erumpent, black, powdery, uneven with catenulate margins; aerial mycelium absent, reaching 10 mm diam after 2 months on OA at 25°C; fertile forming anamorph.

*Specimen examined:* **Australia**, New South Wales, Mackenzie Creek Road, Kempsey, Byrne Plantation, 152° 27' 47" E, 30° 53' 15" S, on leaf spots of *E. agglomerata*, 13 Apr. 2005,

G. Price, **holotype** CBS-H 19829, **isotype** DAR 78033, culture ex-type CPC 13092 = CBS 121102.

*Notes:* No teleomorph has previously been linked to *T. excentricum*, and this is the first record of this species grown in pure culture. Although the anamorph has a different conidial morphology to those of other species of *Trimmatostroma*, it clusters with other members of the genus based on its DNA phylogeny.

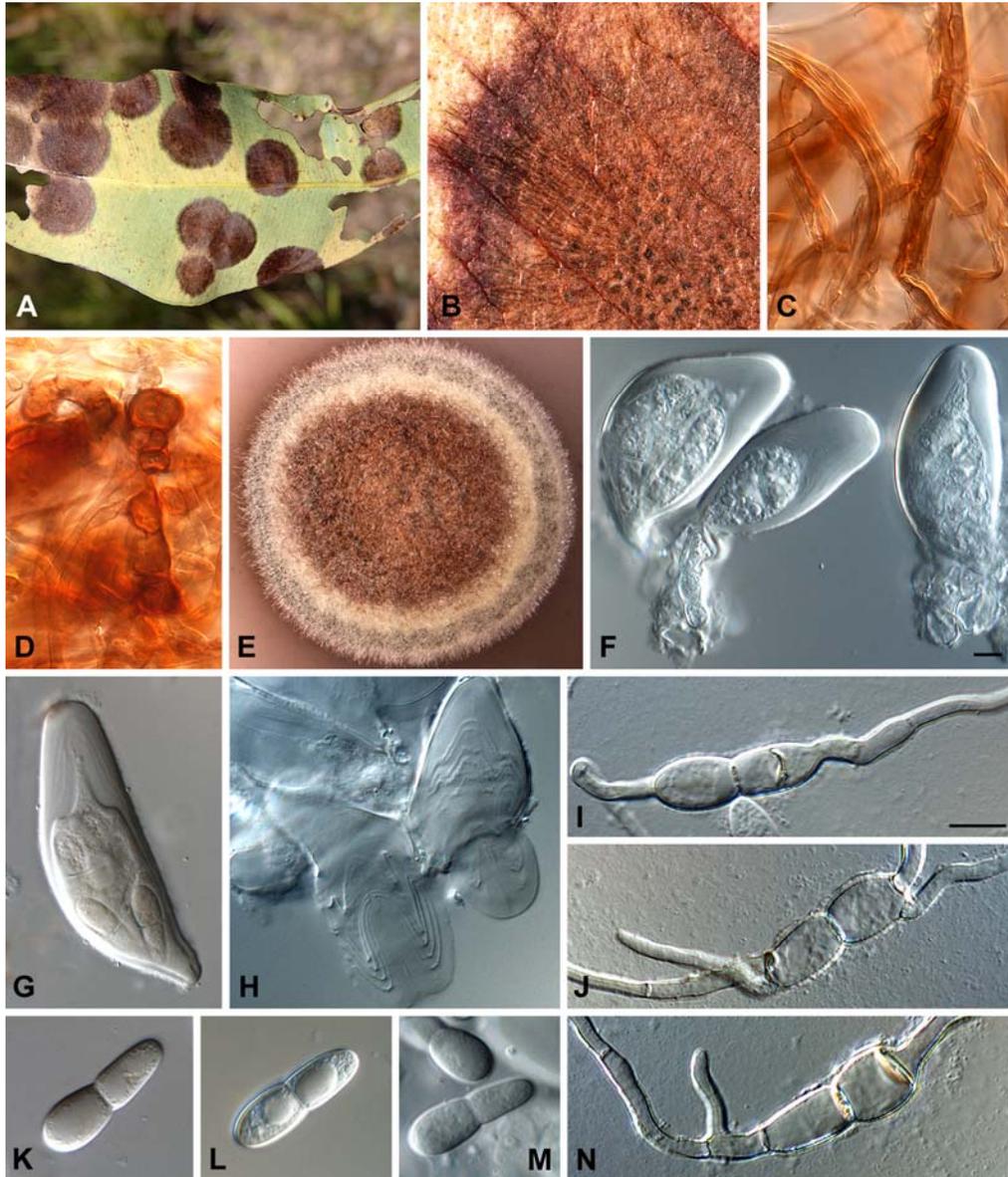
***Mycosphaerella fimbriata*** Crous & Summerell, **sp. nov.** (Fig. 12)  
Mycobank 501264.

*Etymology:* Named after its characteristic leaf spots with radiating hyphal strands.

Ascospores obovoidea, (18–)22–17(–30) × (6–)7(–8) μm.

*Leaf spots* amphigenous, irregular to circular, 5–15 mm diam, medium to dark brown, with radiating superficial mycelium, spreading from ascomata that are predominantly in the middle of the lesion; hyphae red-brown, 5–8 μm wide, thick-walled, verruculose, aggregating in hyphal strands (also *in vitro*), with chlamydospore-like cells, up to 15 μm diam, aggregating in clusters; forming spermatogonia in the outer region of the lesion (also formed *in vitro*). *Ascomata* pseudothecial, amphigenous, black, subepidermal, but becoming erumpent, globose, up to 120 μm wide; apical ostiole 15–20 μm wide; wall consisting of 6–8 layers of medium brown *textura angularis*. *Asci* paraphysate, fasciculate, bitunicate, sessile, obovoid to broadly ellipsoidal, straight to slightly curved, 8-spored, with the endotunica having 3–5 well differentiated layers, visible when mounted in clear lactic acid, 30–90 × 17–22 μm. *Ascospores* multi-seriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, obovoid, with obtuse ends, widest near the apex of the apical cell, mostly medianly 1-septate, constricted at the septum; larger ascospores tend to be unequally 1-septate, with the upper cell being up to 13 μm long, and the bottom cell up to 17 μm long, tapering towards both ends, but more prominently towards the lower end, (18–)22–17(–30) × (6–)7(–8) μm; ascospores frequently with a persistent mucous sheath. Ascospores germinate from both ends, but not necessarily polar, with 2–4 germ tubes more or less parallel to the long axis of the spore (or germ tubes 3–4 irregular); original spore becoming transversely septate, constricted, with mucus sheath prominently visible; spore becoming up to 10 μm wide, darkening and becoming verruculose (germination Type I *sensu* Crous, 1998).

*Cultural characteristics:* Colonies on MEA slow growing, reaching 5 mm diam after 2 months; colonies erumpent, with moderate aerial mycelium and uneven, feathery margins; surface olivaceous-grey, at times fawn in centre due to superficial mycelium; reverse dark-brick. On OA erumpent, spreading with



**Fig. 12.** *Mycosphaerella fimbriata* (CBS H-19828). **A, B.** Leaf spots with radiating superficial hyphae. **C.** Hyphal strands. **D.** Chlamyospore-like structures. **E.** Colony on OA. **F–H.** Asci, with layered endotunica. **I, J, N.** Germinating ascospores. **K–M.** Ascospores. Scale bars: F = 7, I = 10  $\mu$ m.

even, smooth margins; surface dark-brick in centre, outer zone olivaceous-grey, forming a diffuse, dark-vinaceous pigment in the agar, reaching 10 mm diam

after 2 months at 25°C; colonies forming numerous spermatogonia when inoculated onto OA.

*Specimen examined:* **Australia**, Queensland, Cairns, S 16° 56' 23.3", E 145° 32' 34.6", on leaves of *Corymbia* sp., 26 Aug. 2006, P.W. Crous, **holotype** CBS-H 19828, cultures ex-type CPC 13324 = CBS 120736, CPC 13325–13326; Cairns, Mareeba Wetlands, Peninsula Development Road, S 16° 47' 11.3", E 145° 21' 3.2", 380 m, on leaves of *Corymbia* sp., 27 Aug. 2006, P.W. Crous, CBS-H 19827, cultures CPC 13321 = CBS 120893, CPC 13322–13323.

*Notes:* *Mycosphaerella fimbriata* is unique among the species known on *Eucalyptus* in having distinct brown leaf spots covered by strands of red-brown, radiating hyphae, and having a multi-layered ascal endotunica. Phylogenetically it is related to *Stigmina eucalypti* and *Colletogloeopsis* spp.

***Mycosphaerella parkiiaffinis* Crous & M.J. Wingf., sp. nov.** (Fig. 13)  
Mycobank 501265.

*Etymology:* Name refers to its morphology which is similar to that of *Mycosphaerella parkii*.

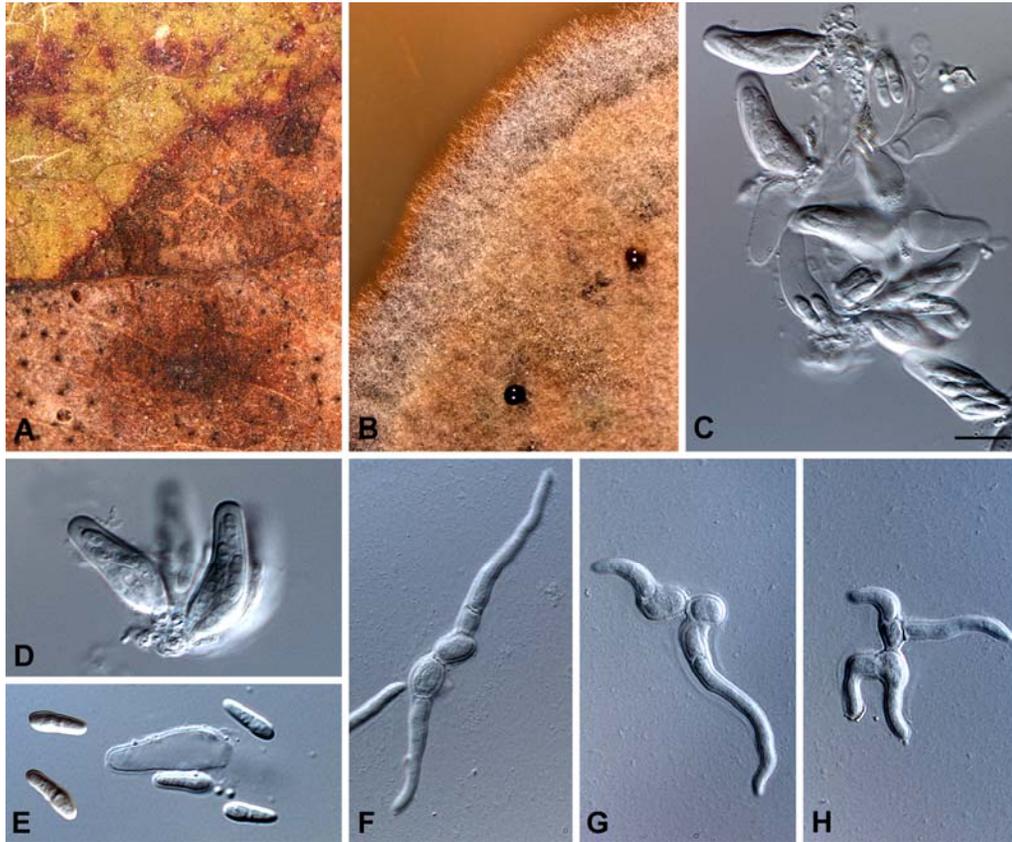
Ascospores fusoid-ellipsoid, (8–)9–10 × 3(–3.5) µm.

*Leaf spots* amphigenous, irregular to subcircular, 6–30 mm diam, pale to medium brown, with a thin, raised, dark brown border, and a red-purple margin. *Ascomata* pseudothecial, amphigenous, dark brown, subepidermal to somewhat erumpent, globose, up to 80 µm wide; apical ostiole 10–15 µm wide; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* paraphysate, fasciculate, bitunicate, subsessile, obovoid to ellipsoidal, straight to slightly curved, 8-spored, 18–30 × 7–10 µm. *Ascospores* tri- to multi-seriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, medianly 1-septate, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)9–10 × 3(–3.5) µm. Ascospores germinate from both ends, with germ tubes parallel or irregular to the long axis of the spore, with 2–4 germ tubes developing; spore not darkening, but distorting, becoming up to 6 µm wide (germination Type D *sensu* Crous, 1998).

*Cultural characteristics:* Colonies on MEA reaching 18 mm diam after 2 months at 25°C; colonies erumpent, spreading, with moderate aerial mycelium, and smooth, but somewhat feathery margins; surface olivaceous-grey in the centre, pale olivaceous-grey in outer region; reverse olivaceous-black.

*Specimen examined:* **Venezuela**, near Acarigua, on leaves of *Eucalyptus urophylla*, Oct. 2006, M.J. Wingfield, **holotype** CBS-H 19823, cultures ex-type CPC 13373 = CBS 120737, CPC 13374.

*Notes:* In comparison to other *Mycosphaerella* spp., *M. parkiiaffinis* has small, nondescript spores, and an irregular ascospore germination pattern (Type D), similar to species in the *M. parkii* complex. It is distinct in lacking a



**Fig. 13.** *Mycosphaerella parkiiaffinis* (CBS H-19823). **A.** Leaf spot. **B.** Colony on MEA. **C, D.** Asci. **E.** Ascospores. **F–H.** Germinating ascospores. Scale bar = 10  $\mu$ m.

*Stenella* anamorph, and having smaller ascospores than those of *M. parkii*, which are up to 15  $\mu$ m long (Crous, 1998; Crous *et al.*, 2006g). Phylogenetically, it is most closely related to *M. ohnowa*.

***Phaeophleospora stonei* Crous, sp. nov.**

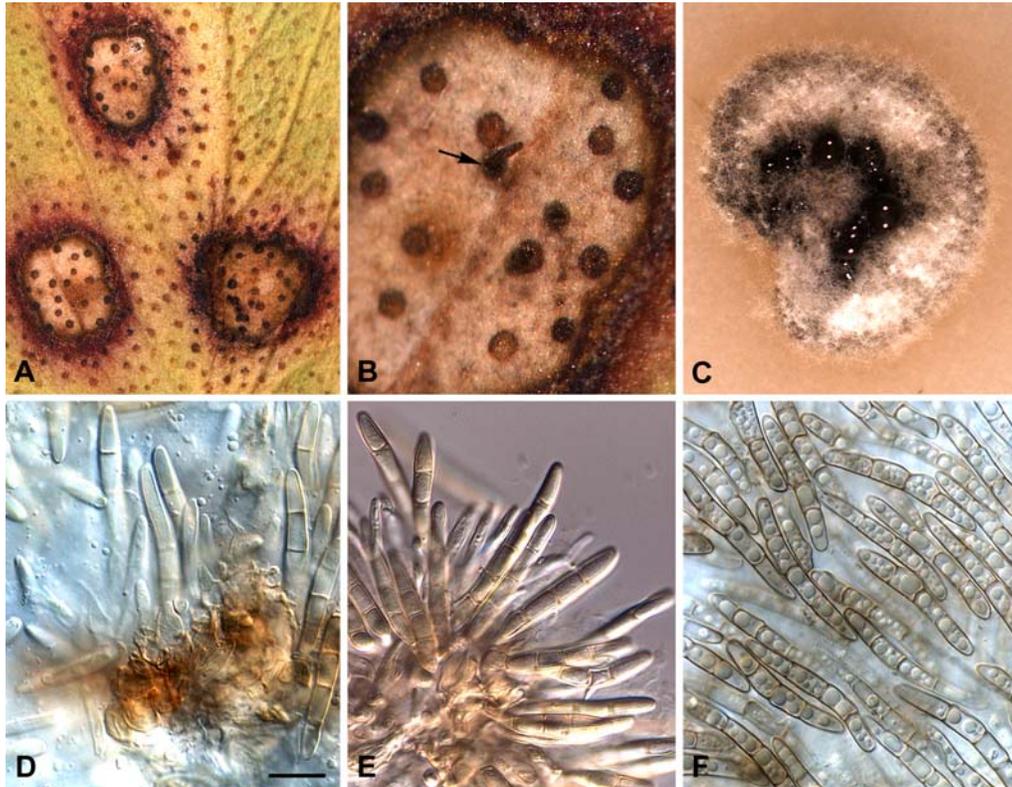
(Fig. 14)

Mycobank 501266.

*Etymology:* Named for Dr. Jeff Stone, who collected this fungus with P.W.C. along the river bank in Kuranda before the IMC8 congress.

*Phaeophleosporae lilianae* similis, sed conidiis minoribus, (25–)30–33(–35)  $\times$  (3.5)4(–5)  $\mu$ m, distinguenda.

*Leaf spots* amphigenous, circular to subcircular, pale brown with a raised, dark brown border, and thin, red-purple margin, 1–4 mm diam. *Conidiomata* amphigenous, subepidermal with a central ostiole, from where conidia exude in a brown cirrus; scattered, globose, dark brown, up to 200  $\mu$ m diam; wall of 3–4 layers of dark brown *textura angularis*. *Conidiogenous cells* pale brown,



**Fig. 14.** *Phaeophleospora stonei* (CBS H-19835). **A, B.** Leaf spots (arrow indicates conidial cirrus). **C.** Colony on OA. **D, E.** Conidiogenous cells and conidia. **F.** Conidia. Scale bar = 10  $\mu\text{m}$ .

smooth, ampulliform to doliiform,  $3\text{--}7 \times 3\text{--}5 \mu\text{m}$ , proliferating percurrently near apex. *Conidia* subcylindrical to narrowly obclavate, widest at basal septum, tapering to a subtruncate, flattened hilum with minute marginal frill, and tapering in the apical cell to an obtuse apex; cellular content granular to not so in vivo, conidia 3(–6)-euseptate (septa appear thicker in Shear’s than in clear lactic acid, but never distoseptate); conidia guttulate and darker brown *in vitro*, but similar in dimensions,  $(25\text{--})30\text{--}33(35) \times (3.5)4(5) \mu\text{m}$ .

*Cultural characteristics:* Colonies slow-growing, reaching 7 mm diam on OA after 2 months at 25°C; erumpent, with moderate aerial mycelium and uneven, but smooth margins, pale mouse-grey to olivaceous-grey.

*Specimen examined:* **Australia**, Queensland, Cairns, Kuranda, Karoomba River Walk, S 16° 49’ 08.8”, E 145° 38’ 24.7”, on leaves of *Eucalyptus* sp., 19 Aug. 2006, P.W. Crous & J. Stone, **holotype** CBS-H 19835, culture ex-type CPC 13330 = CBS 120830, CPC13331–13332.

*Notes:* Swart and Walker (1988) erected the genus *Sonderhenia* to separate taxa with distoseptate conidia from those with transversely euseptate

conidia. Walker *et al.* (1992) placed several similar taxa with eu-septate conidia in a new genus, *Kirramyces*. Crous *et al.* (1997) treated *Kirramyces* as synonym of *Phaeophleospora*. The type species of *Kirramyces* (*K. epicoccoides*) clusters apart from that of *Phaeophleospora* (*P. eugeniae*) within the *Mycosphaerellaceae*. The fact that *P. stonei* does not cluster with the type of *Phaeophleospora* nor *Kirramyces*, suggests that *Phaeophleospora* is polyphyletic, as are most anamorph genera in the *Mycosphaerellaceae*. The phylogenetic analysis places it closest to *M. areola* and *Pseudocercospora* spp.

***Phaeothecoidea* Crous, gen. nov.**

MycoBank 501267

*Etymology*: Its characteristic endoconidia resemble *Phaeotheca*, but the structures are always dark and thick-walled.

*Phaeothecae* similis, sed structuris omnino fuscis et crassitunicatis.

Hyphomycetous, *Mycosphaerellaceae*. *Hyphae* pale to medium brown, verruculose, 4–6 µm wide, end cells dividing into several endoconidia. *Endoconidia* pale to medium brown, verruculose, thick-walled, ellipsoid to obovoid, obclavate or irregularly triangular, 4–10 × 4–5 µm, becoming 1(–2) septate, medium to dark brown, verruculose to verrucose, 10–15 × 5–7 µm, giving rise to additional endoconidia.

*Type species*: *Phaeothecoidea eucalypti* Crous & Summerell., sp. nov.

***Phaeothecoidea eucalypti* Crous & Summerell, sp. nov.**

(Fig. 15)

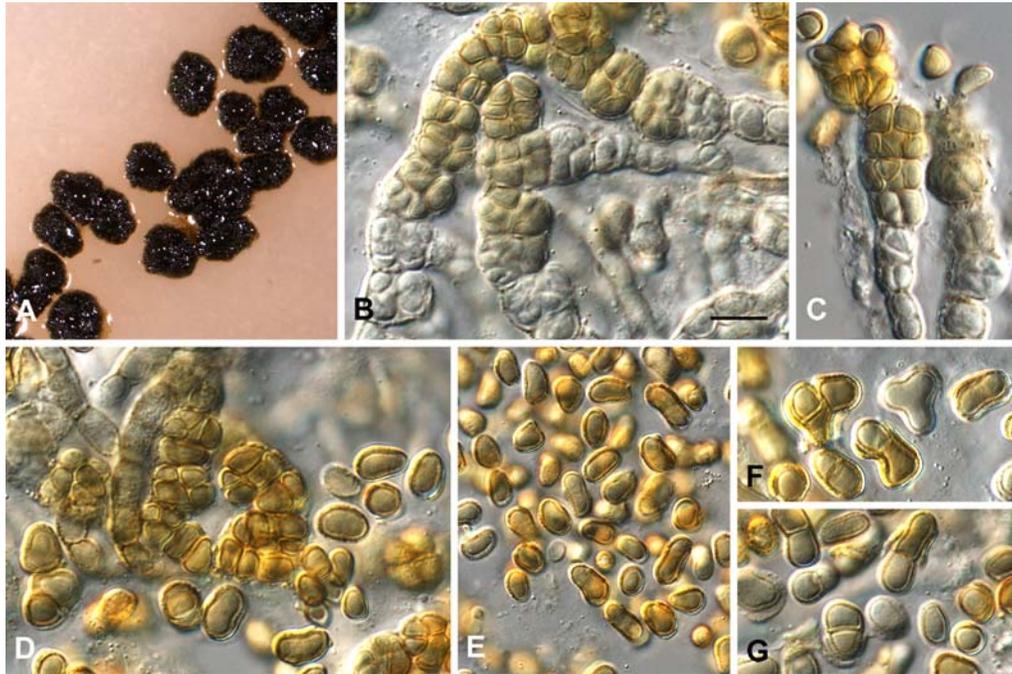
MycoBank 501268.

*Etymology*: Named after its host genus, *Eucalyptus*.

Conidia matura brunnea, verruculosa, crassitunicata, ellipsoidea vel irregulariter triangularia, 4–10 × 4–5 µm.

*Hyphae in vitro* creeping, subhyaline, verruculose, branched, septate, 4–6 µm wide, becoming swollen, up to 15 µm wide, verruculose, medium brown; end cells dividing into several endoconidia, which are released upon rupture of the cell wall. *Endoconidia* pale to medium brown, verruculose, thick-walled, ellipsoid to obovoid, obclavate or irregularly triangular, 4–10 × 4–5 µm after liberation; swelling, becoming 1(–2) septate, medium to dark brown, verruculose to verrucose, 10–15 × 5–7 µm; conidia giving rise to 1–2(–4) additional endoconidia, with outer wall of primary conidium visible as prominent collarete around endoconidia during rupture, and on outer wall of primary conidium after conidial release.

*Cultural characteristics*: Colonies on OA and PDA black, slimy, shiny, irregular, elevated with a catenulate margin, lacking aerial mycelium, but having slimy droplets on the surface; growing 5 mm diam in 3 weeks on OA, 1 cm on PDA.



**Fig. 15.** *Phaeothecoidea eucalypti* (CBS H-19836). **A.** Colonies on OA. **B, C.** Hyphal ends with endoconidia. **D–F.** Conidia. Scale bar = 10  $\mu$ m.

*Specimen examined:* **Australia**, New South Wales, Clareville Beach Reserve, on leaves of *Eucalyptus botryoides*, Feb. 2006, B. Summerell, **holotype** CBS-H 19836, culture ex-type CPC 12918 = CBS 120831.

*Notes:* The genus *Melanothecoidea* is reminiscent of the genera *Hyphospora* (teleomorph: *Cumminutispora*) and *Phaeotheca*, which both have endoconidia, and are placed in the Dothideomycetes. However, neither of these genera cluster within *Mycosphaerella*, and they are also morphologically distinct by tending to have more thin-walled conidia, that become pigmented with age (Zalar *et al.*, 1998). *Melanothecoidea eucalypti* clusters among species of *Trimmatostroma* within *Mycosphaerella*, but is distinct in that members of *Trimmatostroma* generally have dry, disarticulating conidia, while colonies of *Melanothecoidea* are wet and slimy, and have endoconidia. The phylogenetic analysis places it closest to *M. excentrica* and *M. suberosa*.

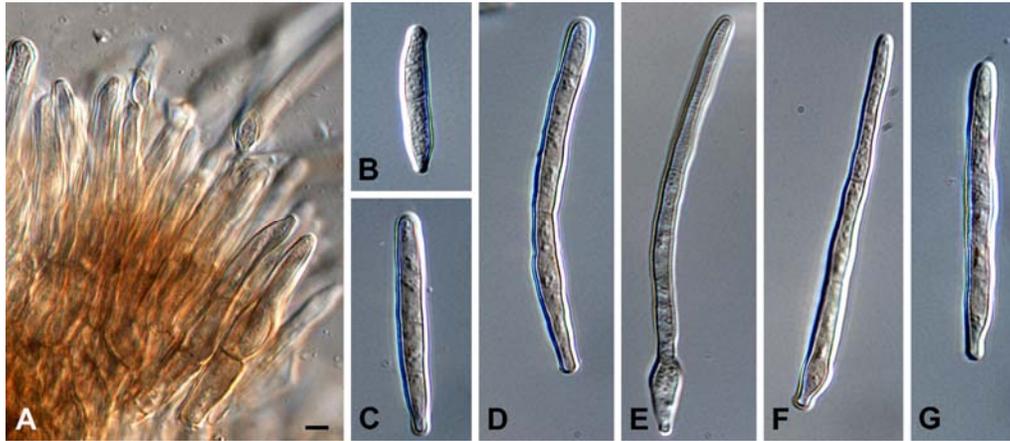
***Pseudocercospora norchiensis* Crous, sp. nov.**

(Fig. 16)

Mycobank 501269.

*Etymology:* Named after the type locality in Italy, Norchia, Prov. Viterbo.

Conidia obclavata, (0–)5–9(–12)-septata, (50–)80–120(–140)  $\times$  (5–)6(–7)  $\mu$ m.



**Fig. 16.** *Pseudocercospora norchiensis* (CBS H-19841). A. Conidiophores. B–F. Conidia. Scale bar = 10  $\mu$ m.

*Leaf spots* amphigenous, irregular to subcircular, 2–6 mm diam, medium brown with a raised border, and a thin red-purple margin. Mycelium internal, smooth, consisting of branched, septate, smooth, pale brown hyphae, 3–4  $\mu$ m wide; superficial mycelium developing once incubated in moist chambers. *Caespituli* fasciculate, epiphyllous, pale brown on leaves, up to 160  $\mu$ m wide and 150  $\mu$ m high. *Conidiophores* aggregated in highly dense fascicles arising from the upper cells of a brown stroma up to 160  $\mu$ m wide and 90  $\mu$ m high; conidiophores medium brown, smooth, 3–5-septate, subcylindrical, straight to variously curved, unbranched, 40–70  $\times$  4–7  $\mu$ m. *Conidiogenous cells* terminal, unbranched, medium brown, smooth, tapering to flat-tipped apical loci, proliferating sympodially, rarely percurrently near apex, 12–45  $\times$  4–6  $\mu$ m. *Conidia* solitary, medium brown, smooth, prominently guttulate, obclavate, apex subobtuse, base short obconically truncate, straight to slightly curved, (0–) 5–9(–12)-septate, (50–)80–120(–140)  $\times$  (5–)6(–7)  $\mu$ m; hila inconspicuous, 2–3  $\mu$ m wide.

*Cultural characteristics:* Colonies on MEA erumpent, raised, convex, with moderate aerial mycelium and feathery, uneven margins; on MEA surface pale olivaceous-grey, with patches of smoke-grey; outer margin olivaceous-grey to iron-grey; reverse iron-grey, reaching 20 mm diam after 2 months at 25°C.

*Specimen examined:* **Italy**, Viterbo, Norchia, on leaves of *Eucalyptus* sp., Apr. 2005, W. Gams, **holotype** CBS-H 19841, cultures ex-type CPC 13049–13051 = CBS 120738, CPC 13050–13051.

*Notes:* The ITS sequence of *P. norchiensis* is identical to that of *P. nogalesii*, which was described from *Chamaecytisus* in New Zealand (Braun *et al.*, 2003). It can be distinguished morphologically, however, by having

extremely dense caespituli, lacking superficial mycelium, having conidia that are more obclavate in shape, and also being larger and wider than those of *P. nogalessii* (20–70 × 2.5–5 µm; Braun *et al.*, 2003). Based on the key of Braun and Dick (2002), as well as recently described species (Crous *et al.*, 2004b; Hunter *et al.*, 2006a), *P. norchiensis* is morphologically distinct from the taxa presently known from *Eucalyptus*.

***Ramularia eucalypti* Crous, sp. nov.**

(Fig. 17)

MycoBank 501270.

*Etymology*: Named after its host plant genus, *Eucalyptus*.

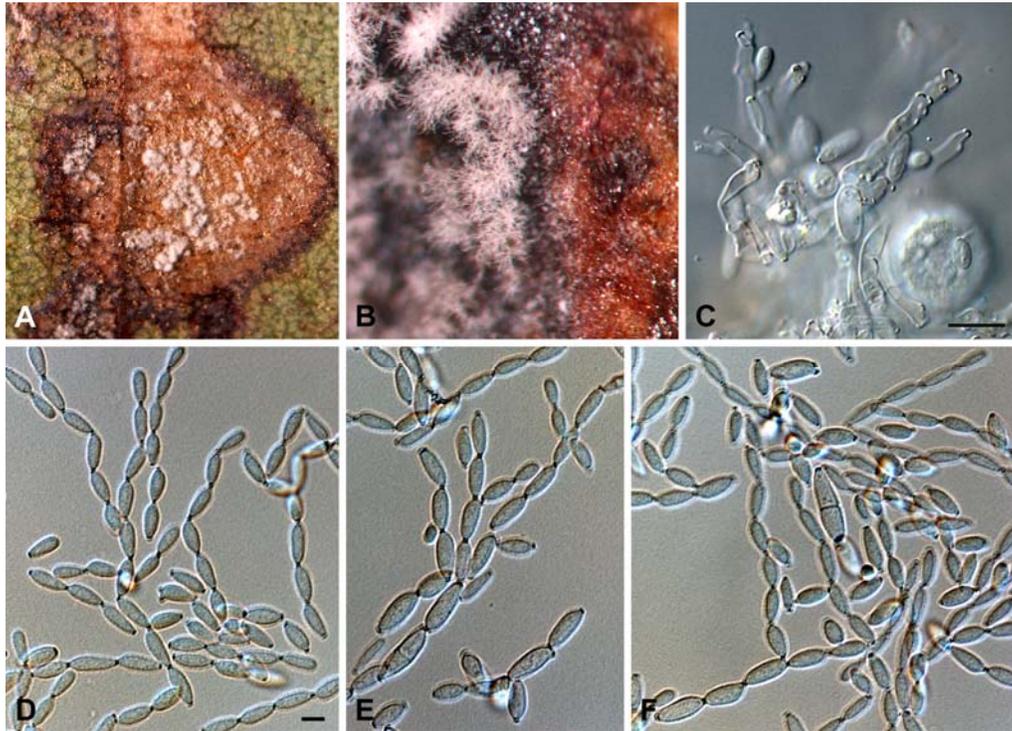
Conidia catenulata, levia, hyalina, subcylindrica vel fusoido-ellipsoidea, 0–1-septata, (10–)12–15(–18) × (2.5–)3(–4) µm.

*Leaf spots* amphigenous, irregular, subcircular or angular, confined by leaf veins, medium brown with a thin, red-brown border, specks 1–2 mm diam, or larger spots and blotches up to 4 cm diam. *Mycelium* internal and external, hyaline, smooth, consisting of branched, septate, hyphae, 3–4 µm wide. *Caespituli* fasciculate, amphigenous, hyaline, up to 80 µm wide and 50 µm high, situated on a poorly developed substomatal stroma, up to 40 µm wide. *Conidiophores* arising in dense fascicles from a subhyaline stroma (rarely separate on superficial mycelium), smooth, hyaline, 1–7-septate, subcylindrical, straight to geniculate-sinuuous, unbranched or branched below, 10–60 × 3–4 µm. *Conidiogenous cells* terminal or lateral, integrated, hyaline, smooth, tapering to flat-tipped apical loci, 10–20 × 2.5–3.5 µm; scars darkened, refractive, thickened, 1–1.5 µm wide. *Conidia* catenulate in branched chains, smooth, hyaline; ramiconidia subcylindrical to fusoid-ellipsoidal, 0–1-septate, (10–)12–15(–18) × (2.5–)3(–4) µm; secondary conidia fusoid-ellipsoidal, occurring in branched chains of up to 15 µm long, (5–)6–7(–8) × 3(–3.5) µm; hila darkened, thickened, refractive, up to 1 µm wide.

*Cultural characteristics*: Colonies on MEA spreading, erumpent, convex with uneven, convoluted surface, radially striated, with sparse to moderate aerial mycelium and submerged, uneven, feathery margins; surface dirty white, reverse brown-vinaceous in centre, becoming fawn in middle zone, and brown-vinaceous in outer region; reaching 20 mm after 2 months at 25°C.

*Specimens examined*: **Italy**, Norchia, on living leaves of *Eucalyptus grandiflora*, Apr. 2006, W. Gams, **holotype** CBS-H 19832, culture ex-type CPC 13043 = CBS 120726, CPC 13044–13045; Viterbo, on living leaves of *E. grandiflora*, Apr. 2006, W. Gams, CPC 13046 = CBS 120727, CPC 13047–13048. **Australia**, Queensland, Cairns, Kuranda, Karoomba River Walk, S 16° 49' 08.8", E 145° 38' 24.7", on leaves of *Eucalyptus* sp., 19 Aug. 2006, P.W. Crous & J. Stone, CPC 13304 = CBS 120728.

*Notes*: Presently this is the only true member of *Ramularia* known from *Eucalyptus*, as *Ramularia pitereka* and aggregate species are now accommodated in the genus *Quambalaria* (*Quambalariaceae*) (De Beer *et al.*,



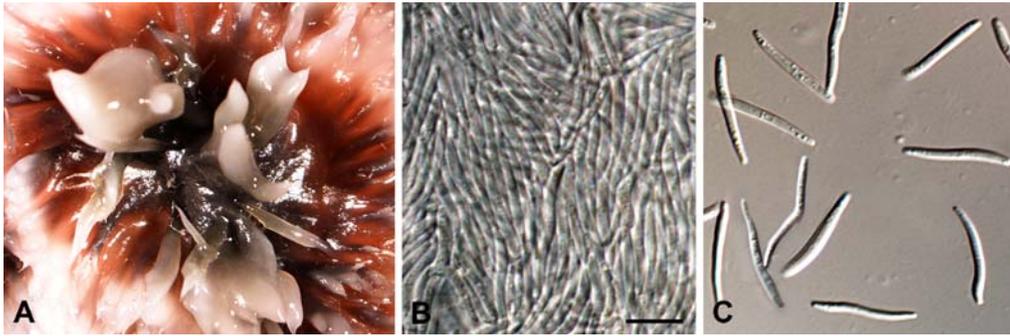
**Fig. 17.** *Ramularia eucalypti* (CBS H-19832). **A, B.** Leaf spots. **C.** Conidiophores. **D–F.** Conidia in chains. Scale bars: C = 10, D = 6  $\mu$ m.

2006). *Ramularia eucalypti* was collected from several locations in Italy, where it was associated with severe leaf spotting symptoms of mature *Eucalyptus* trees. It is interesting that the disease has not previously been reported from Australia, where eucalypts are native. Based on the species of *Ramularia* known from culture, *R. eucalypti* appears to be new, though further collections from other hosts will have to address the potential host specificity of this species. Currently *Ramularia* is accepted as being a host-specific genus of phytopathogenic fungi (Braun, 1998), though some exceptions are likely to emerge.

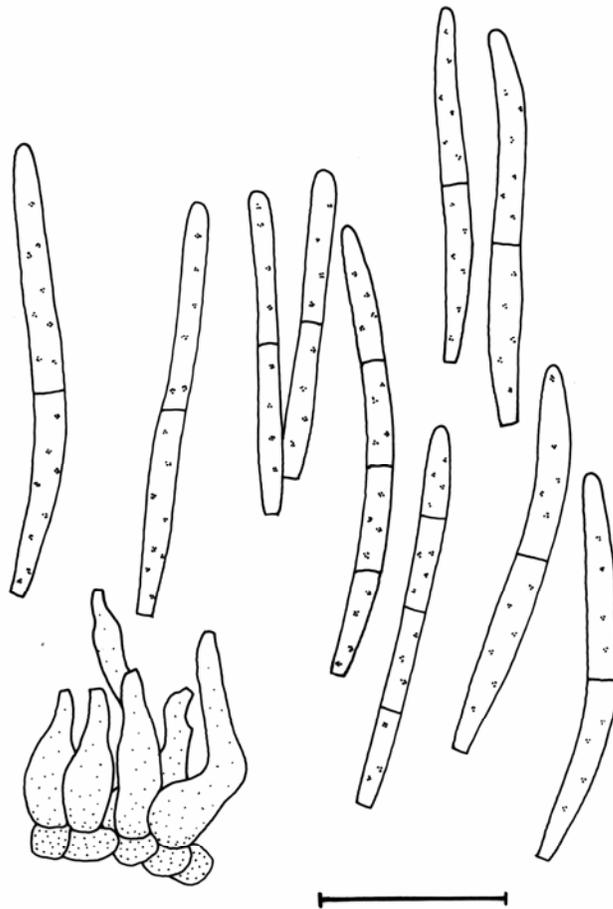
***Septoria* sp.**

(Figs 18, 19)

*Leaf spots* absent, conidiomata associated with leaf litter. *Mycelium* internal, consisting of smooth, branched, septate, pale brown, 1.5–2  $\mu$ m wide hyphae. *Conidiomata* pycnidial, immersed, brown, globose in OA, up to 100  $\mu$ m diam; wall consisting of 3–4 cell layers of *textura angularis*. *Conidiogenous cells* lining the inner layer of the conidioma, densely aggregated, ampulliform to subcylindrical, straight to curved, unbranched,



**Fig. 18.** *Septoria* sp. (CBS H-19831). **A.** Colony on OA. **B, C.** Conidia. Scale bar = 10  $\mu$ m.



**Fig. 19.** Conidia and conidiogenous cells of *Septoria* sp. (CBS H-19831). Scale bar = 10  $\mu$ m.

hyaline, smooth, proliferating sympodially near the apex,  $6\text{--}12 \times 3\text{--}8 \mu\text{m}$ . *Conidia* solitary, hyaline, smooth, finely guttulate or not, subcylindrical to narrowly obclavate, with subobtuse apex, and long subtruncate base, straight to curved, 1(–3)-septate,  $(9\text{--})17\text{--}20(\text{--}24) \times 1.5(\text{--}2) \mu\text{m}$ ; hila inconspicuous,  $0.5\text{--}1 \mu\text{m}$  diam.

*Cultural characteristics*: Colonies erumpent, spreading, with even, lobate margins; on OA with moderate, dirty pink to white aerial mycelium, umber in outer region, which lacks aerial mycelium; reaching 35 mm diam after 1 month at 25°C; on PDA erumpent, central part with dense tufts of dirty white aerial mycelium, outer zone chestnut; reverse chestnut; reaching 25 mm diam after 1 month at 25°C.

*Specimen examined*: **Italy**, Viterbo, Norchia, on leaves of *Eucalyptus* sp., Apr 2005, W. Gams, CBS-H 19831, cultures CPC 13058 = CBS 120739, CPC 13059–13060.

*Notes*: Based on their ITS DNA sequence data, these isolates are similar to those of *S. protearum* Viljoen & Crous, known from *Protea* leaf spots in South Africa (Crous *et al.*, 2004a). However, the conidia are somewhat narrower than those of *S. protearum*. Additional genes will therefore have to be sequenced to fully resolve the status of the *Eucalyptus* isolates.

***Stenella eucalypti* Crous & Summerell, sp. nov.**

(Fig. 20)

Mycobank 501271.

*Etymology*: Named after its host genus, *Eucalyptus*.

*Stenellae pseudoparkii* similis, sed conidiis et conidiophoris longioribus distinguenda.

*Leaf spots* amphigenous, irregular to angular specks, 1–3 mm diam, pale brown with dark brown, with raised, dark brown spots inside lesions, presumably due to insect damage; borders raised, margins absent to red-purple, but the latter may be due to co-colonization of a *Pseudocercospora* sp. *Mycelium* internal and external, consisting of branched, septate, medium brown, finely verruculose hyphae, 3–4  $\mu\text{m}$  wide; terminal hyphal ends characteristically ending in clusters of globose, multi-celled chlamyospore-like structures, frequently surrounded by a mucus sheath; clusters 10–30  $\mu\text{m}$  diam. *Conidiophores* arising singly from superficial mycelium, dark brown, finely verruculose, multi-septate, subcylindrical, straight to geniculate-sinuuous, mostly unbranched, or branched below,  $50\text{--}200 \times 5\text{--}8 \mu\text{m}$ . *Conidiogenous cells* terminal, mostly unbranched, medium brown, smooth to finely verruculose, tapering to flat-tipped apical loci, proliferating sympodially,  $10\text{--}15 \times 4\text{--}5 \mu\text{m}$ ; scars thickened, darkened, refractive. *Conidia* solitary, pale brown, finely verruculose, guttulate, subcylindrical to narrowly obclavate, apex subobtuse, base long obconically subtruncate to obconically subtruncate, straight to slightly curved, (0–)1–3(–5)-septate,  $(10\text{--})20\text{--}35(\text{--}60) \times (2\text{--})3\text{--}4(\text{--}6) \mu\text{m}$ ; hila thickened, darkened, refractive,  $1.5\text{--}2 \mu\text{m}$  wide.



**Fig. 20.** *Stenella eucalypti* (CBS H-19830). **A.** Leaf spot. **B.** Colony on MEA. **C–E.** Conidiophores. **F, G.** Chlamydospore-like structures. **H.** Conidia. Scale bar = 10 µm.

*Cultural characteristics:* Colonies on MEA reaching 15 mm diam after 2 months at 25°C; erumpent, with moderate aerial mycelium and smooth, uneven margins; surface mouse-grey to olivaceous-grey; reverse greenish-black.

*Specimen examined:* **Australia**, Queensland, Cairns, Eureka Creek, 48 km from Mareeba, S 17° 11' 13.2", E 145° 02' 27.4", 468 m, on leaves of *Eucalyptus tereticornis*, 26 Aug. 2006, P.W. Crous, **holotype** CBS-H 19830, CPC 13302 = CBS 121101.

*Notes:* Several species of *Stenella* are known from *Eucalyptus* (Crous, 1998; Crous *et al.*, 2006g). *Stenella eucalypti* has conidia that are 10–60 × 2–6 µm, 0–5-septate, showing some overlap with those of *S. pseudoparkii* (20–50 × 2.5–3 µm, 1–5-septate) and *S. xenoparkii* (12–50 × 3–5 µm, 1–2-septate), but is distinct in having somewhat longer and wider conidia, and very long conidiophores. The phylogenetic analysis could not confidently place this species; the parsimony analysis places it basal, whereas with neighbour-joining

it clustered with *Phaeophleospora stonei*. A Blast search with the ITS sequence reveals the highest similarity with species of *Cercospora* and *Septoria*.

### New and interesting records

***Mycosphaerella acaciigena*** Crous & M.J. Wingfield, Stud. Mycol. 50: 463. 2004.

*Specimens examined:* **Australia**, New South Wales, on leaves of *Eucalyptus* sp. Aug. 2006, B. Summerell, CPC 13290 = CBS 120740, CPC 13291–13292. **Venezuela**, El Piñal Lotes farm near Acarigua, on leaves of *E. camaldulensis* × *E. urophylla*, Aug. 2006, M.J. Wingfield, CPC 13350–13352.

*Notes:* *Mycosphaerella acaciigena* was recently described from leaf spots on *Acacia mangium* leaves collected in Venezuela (Crous *et al.*, 2004c). Although this is the first report of this fungus from *Eucalyptus*, and also the first report from Australia, several species of *Mycosphaerella* are now known to move between *Eucalyptus* and *Acacia* hosts (Crous and Groenewald, 2005).

***Mycosphaerella heimii*** Crous, S. African For. J. 172: 2. 1995.

*Specimens examined:* **Australia**, Queensland, Cairns, close to Kuranda, Kennedy Highway, S 16° 52' 4.5", E 145° 35' 54.5", on leaves of *Eucalyptus platyphylla*, 26 Aug. 2006, P.W. Crous, CPC 13276 = CBS 120741, CPC 13277–13278. **Thailand**, Thatakiab District, Chachoengsao Province, on leaves of *E. camaldulensis*, 12 Oct. 2006, W. Himaman, CPC 13474 = CBS 120742, CPC 13475–13476. **Venezuela**, on leaves of *E. urophylla*, Aug. 2006, M.J. Wingfield, CPC 13371–13374, 13359–13361, 13356 = CBS 120743, CPC 13357–13358.

*Notes:* Since *M. heimii* was originally described from *Eucalyptus* leaves collected in Madagascar, it has been reported on this host from several countries (Crous, 1998), including a recent report from Australia (Whyte *et al.*, 2005), which is confirmed by the present collection.

***Mycosphaerella konae*** Crous, Joanne E. Taylor & M.E. Palm, Mycotaxon 78: 459. 2001.

*Anamorph:* *Pseudocercospora* sp.

*Specimen examined:* **Thailand**, Thatakiab District, Chachoengsao Province, on leaves of *E. camaldulensis*, 12 Oct. 2006, W. Himaman, CPC 13469 = CBS 120748, CPC 13470.

*Notes:* *Mycosphaerella konae* is known to be a pathogen of *Banksia* and *Leucospermum* spp. cultivated in Hawaii (Crous *et al.*, 2004a). This is the first report of this fungus on *Eucalyptus* in Thailand. The present collection closely matches the type with regards to ascospore dimensions and germination patterns, and similar cultural characteristics.

***Mycosphaerella mexicana*** Crous, Mycol. Mem. 21: 81. 1998.

*Specimen examined:* **Hawaii**, Waimea, ascomata occurring on older lesions of *Aulographina eucalypti*, on *Eucalyptus* leaves, Aug. 2005, W. Gams, CPC 12349 = CBS 120744, CPC 12350–12351.

*Notes:* *Mycosphaerella mexicana* was originally described from eucalypt leaves collected in Mexico (Crous, 1998), and has subsequently been recorded from Australia (Maxwell *et al.*, 2003). This is, however, the first report of this fungus from Hawaii. Although there are a few base pair differences compared to the sequences derived from the Australian cultures, sparse material made it difficult to compare morphologically to *M. mexicana*.

***Mycosphaerella molleriana*** (Thüm.) Lindau in Engler & Prantl., *Natürlichen Pflanzenf.* 1: 424. 1897. (Fig. 21)

(Basionym) *Sphaerella molleriana* Thüm., *Revista Inst. Sci. Lit. Coimbra* 28: 31. 1881.

*Anamorph:* *Colletogloeopsis molleriana* Crous & M.J. Wingf., *Can. J. Bot.* 75: 670. 1997

*Specimens examined:* **Portugal**, Lusitania, leaves of *E. globulus*, Jul. 1879, Fr. Moller, K (**holotype** of teleomorph); Abrantes, leaves of *E. globulus*, Jul. 1995, S. McCrae, PREM 54395 (**holotype** of anamorph), cultures ex type CPC 1214 = CBS 111164, CPC 1215 = CBS 111165; Lisbon, N 40° 00' 39", W 8° 36' 2.3", 77 m, on leaves of *Eucalyptus* sp., 13 Oct. 2006, P.W. Crous & A.J.L. Phillips, CBS-H 19826, **epitype of teleomorph designated here**, cultures ex-epitype CPC 13398 = CBS 120746, CPC 13399–13400.

*Notes:* Crous and Wingfield (1997) described the anamorph of *M. molleriana*, and this culture has since been used as representative of the species. The present collection contains numerous ascomata, and is morphologically and genetically similar to the anamorph strain, while the morphology matches that observed on the holotype of the teleomorph. This fresh collection, which has ample fruiting of both states, can thus be used to epitypify the holomorph.

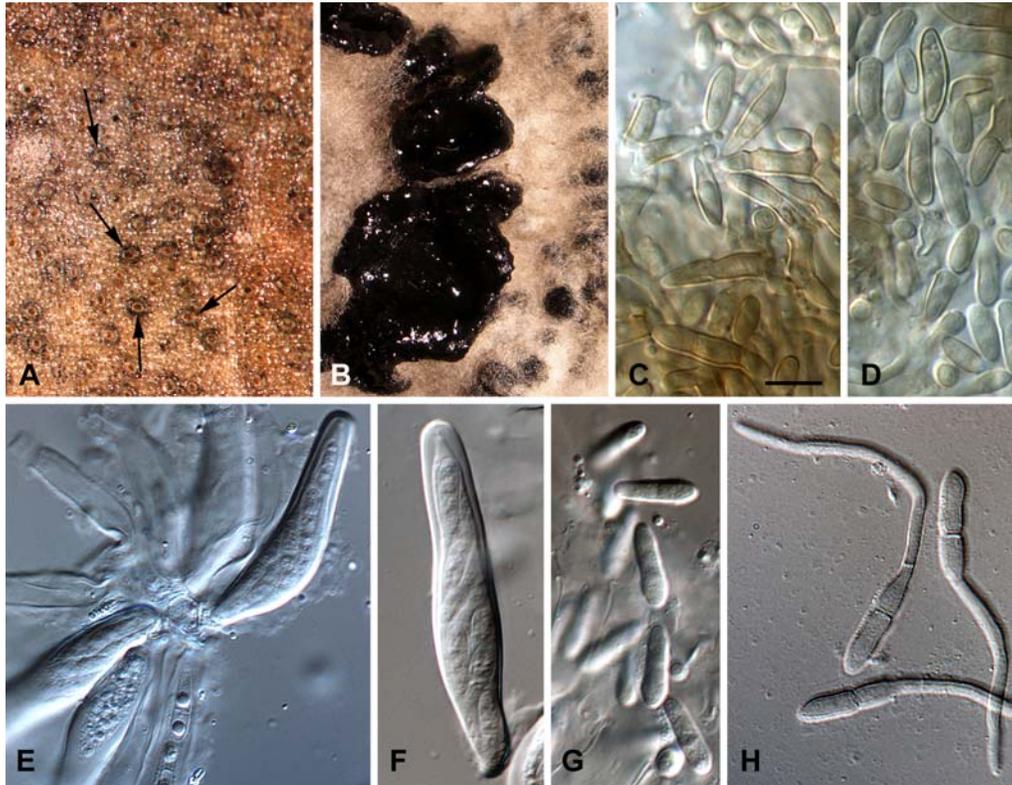
***Mycosphaerella ohnowa*** Crous & M.J. Wingf., *Stud. Mycol.* 50: 206. 2004.

*Specimen examined.* **Australia**, New South Wales, Dilkoon, Hourne Plantation, 153° 1' 47" E, 29° 29' 26" S, on leaves of *Eucalyptus dunnii*, 12 Feb. 2006, A.J. Carnegie, CPC 13101 = CBS 120745, CPC 13102–13103.

*Notes:* *Mycosphaerella ohnowa* is presently known to occur on *E. grandis* leaves in South Africa (Crous *et al.*, 2004b), and this is the first record from Australia. The present collection agrees well with that of the type strain in cultural characteristics (colour, growth rate and slimy aerial hyphal tufts) and morphology.

***Mycosphaerella thailandica*** Crous, Himaman & M.J. Wingf., *Stud. Mycol.* 50: 465. 2004.

*Anamorph:* *Pseudocercospora thailandica* Crous, Himaman & M.J. Wingf., *Stud. Mycol.* 50: 465. 2004.



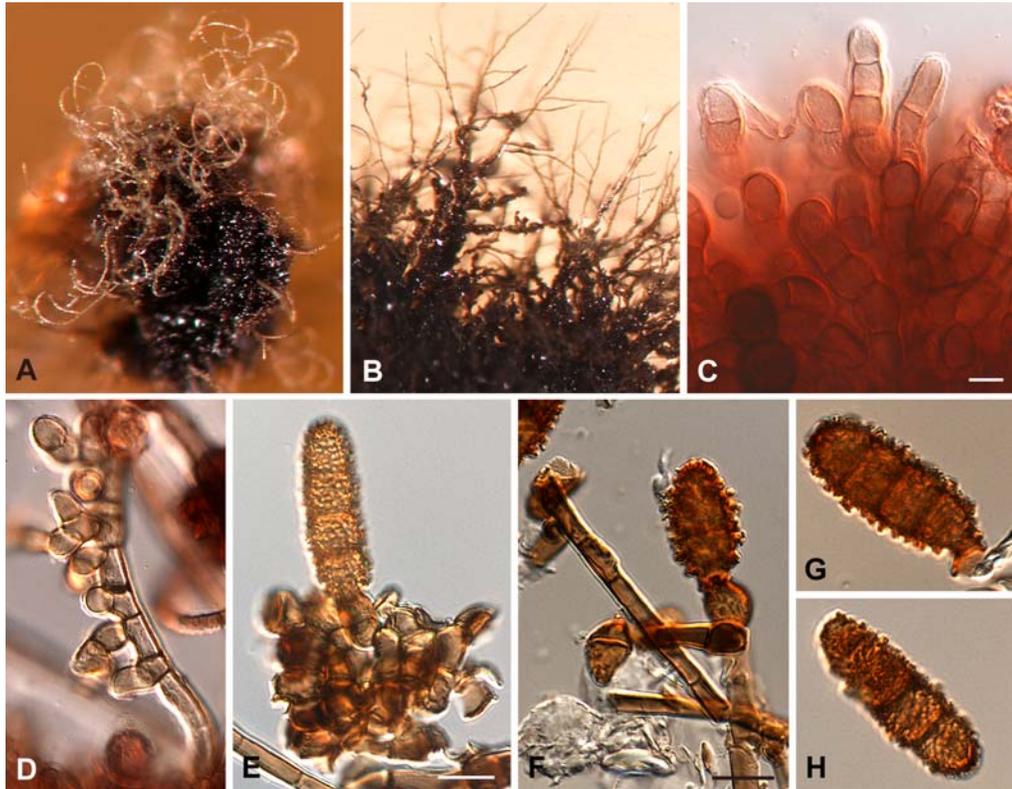
**Fig. 21.** *Mycosphaerella molleriana* and its anamorph *Colletogloeopsis molleriana* (CBS H-19826). **A.** Leaf spot (arrows indicated subepidermal ascomata). **B.** Sporulation on MEA. **C, D.** Conidia and conidiogenous cells. **E, F.** Asci. **G.** Ascospores. **H.** Germinating ascospores. Scale bar = 10  $\mu$ m.

*Specimen examined:* **Thailand**, Thatakiab District, Chachoengsao Province, on leaves of *E. camaldulensis*, 12 Oct. 2006, W. Himaman, CPC 13477, 13481–13482, 13478 = CBS 120723.

*Notes:* *Mycosphaerella thailandica* is associated with leaf spots of *Acacia mangium* in Thailand (Crous *et al.*, 2004c). This is the first report of this fungus on *Eucalyptus* in Thailand. The present collection closely matches the type with regards to ascospore dimensions, germination patterns, and cultural characteristics. Cultures remained sterile, and did not produce the anamorph.

***Stigmina eucalypti*** Alcorn, Trans. Brit. Mycol. Soc. 60: 151. 1973. (Fig. 22)

*Specimens examined:* **Australia**, Queensland, Brisbane, on leaves of *Eucalyptus tessellaris*, 19 Nov. 1969, A. Skoien, **holotype** IMI 161747; **Australia**, New South Wales, Jackadgery, Singh Plantation (adjacent Inglebar State Forest), 152° 32' 13" E, 29° 34' 24" S, on leaves of *Corymbia variegata*, 11 Mar. 2006, G. Price, CBS-H 19834, cultures CPC 13384 = CBS 121100, CPC 13385–13386.



**Fig. 22.** *Stigmina eucalypti* (CBS H-19834). **A, B.** Colonies on MEA. **C–F.** Conidiogenous cells. **G, H.** Conidia. Scale bars = 10 µm.

*Notes:* A recent study by Crous *et al.* (2006c) confirmed *Stigmina* to be synonymous with *Pseudocercospora* and *Phaeoisariopsis*. *Stigmina eucalypti*, however, clusters apart from *Stigmina s.str.* (typified by *S. platani*). Because the generic affinity of *S. eucalypti* is uncertain, this species is tentatively retained in *Stigmina* until more molecular data become available.

### Acknowledgements

We thank Drs W. Gams, A.J.L. Phillips, S. Mohali, I. Smith and M.J. Wingfield (MJW), who provided specimens without which this study would not have been possible. MJW is also thanked for comments on a draft of the script. Several technicians assisted with this project, namely A. van Iperen (cultures), M. Vermaas (photo plates), and M. Starink (DNA sequencing). Dr R.C. Summerbell is thanked for his comments on the morphology of the fungi named in the genus *Cibiessia*.

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## Host specificity and speciation of *Mycosphaerella* and *Teratosphaeria* species associated with leaf spots of Proteaceae

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### Key words

ITS  
*Leucadendron*  
*Leucospermum*  
*Mycosphaerella*  
*Protea*  
*Teratosphaeria*

**Abstract** Species of *Mycosphaerella* and *Teratosphaeria* represent important foliicolous pathogens of Proteaceae. Presently approximately 40 members of these genera (incl. anamorphs) have been recorded from Proteaceae, though the majority are not known from culture, and have never been subjected to DNA sequence analysis. During the course of this study, epitypes were designated for several important species, namely *Batcheloromyces leucadendri*, *B. proteae*, *Catenulostroma macowanii*, *Mycosphaerella marksii*, *Teratosphaeria bellula*, *T. jonkershoekensis*, *T. parva*, and *T. proteae-arboreae*. Several species were also newly described, namely *Batcheloromyces sedgfieldii*, *Catenulostroma wingfieldii*, *Dissoconium proteae*, *Teratosphaeria persoonii*, *T. knoxdavesii*, and *T. marasasii*. Although accepted as being highly host specific, some species were shown to have wider host ranges, such as *M. communis* (*Eucalyptus*, *Protea*), *M. konae* (*Leucospermum*, *Eucalyptus*), *M. marksii* (*Eucalyptus*, *Leucadendron*), *T. associata* (*Eucalyptus*, *Protea*), and *T. parva* (*Eucalyptus*, *Protea*), which in most cases were found to co-occur with other species of *Mycosphaerella* or *Teratosphaeria* on Proteaceae. Furthermore, earlier records of *T. jonkershoekensis* on Proteaceae in Australia were shown to be representative of two recently described species, *T. associata* and *T. maxii*. A phenomenon of underdeveloped, or micro-ascospores was also newly observed in asci of *T. maculiformis* and *T. proteae-arboreae*. The exact purpose of asci with two distinct types of ascospores remains to be clarified, as both types were observed to germinate on agar.

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### INTRODUCTION

Several genera of South African Proteaceae, especially *Protea*, *Leucospermum* and *Leucadendron* are routinely cultivated for the local and export cut-flower industry. Due to popular demand, these flowers are also now being cultivated in various countries around the world (Crous et al. 2004a). In spite of the popularity of these crops, fungal pathogens still represent a serious impediment to their cultivation. Several groups of fungal pathogens of Proteaceae have in recent years been characterised morphologically as well as phylogenetically, such as the Botryosphaeriaceae stem cankers (Denman et al. 1999, 2000, 2003, Crous et al. 2006b), Armillaria and Cyliandrocladium root rot (Schoch et al. 1999, Crous 2002, Coetzee et al. 2003), Elsinoë scab disease (Swart et al. 2001) and Phomopsis cankers (Mostert et al. 2001a, b). However, this is generally not true for the pathogens associated with leaf diseases, as most have not been studied in culture.

Species of *Mycosphaerella* and *Teratosphaeria* are widespread on Proteaceae, and cause leaf spots and blights on numerous plant hosts in this family (Swart et al. 1998, Crous & Palm 1999, Crous et al. 2000a, Taylor & Crous 2000). In the compendium of Proteaceae diseases, Crous et al. (2004a) listed 13 species of *Mycosphaerella* (incl. *Teratosphaeria*), and 18 associated anamorph species, while Crous & Groenewald (2006a, b)

recently described a further two *Teratosphaeria* spp. from *Protea*. Although several studies have focused on the distribution of *Mycosphaerella* spp. of Proteaceae in native and exotic habitats (Crous et al. 2000b, 2004a, Taylor & Crous 2000, Taylor et al. 2001a, b), their phylogenetic relationships have remained largely unresolved (Taylor et al. 2003).

The genus *Mycosphaerella* includes more than 3 000 names (Aptroot 2006), which together with names in associated anamorph genera probably represent close to 10 000 names (Crous et al. 2000a, 2001, 2004a, b, 2006a–c, 2007a–c, Crous & Braun 2003, Arzanlou et al. 2007). Although previous phylogenetic studies based on the ITS rDNA region have suggested *Mycosphaerella* to be monophyletic (Crous et al. 2000a, 2001, Goodwin et al. 2001), recent studies employing LSU sequence data have refuted this (Hunter et al. 2006), and split off several genera such as *Davidiella* (Davidiellaceae, Braun et al. 2003, Schoch et al. 2006, Crous et al. 2007b, Schubert et al. 2007), *Schizothyrium* (Schizothyriaceae, Batzer et al. 2008), and *Teratosphaeria* (Teratosphaeriaceae, Crous et al. 2007a). Although Crous et al. (2004a) listed eight species of *Mycosphaerella* from Proteaceae in South Africa, Crous et al. (2007a) have recently placed several of these in *Teratosphaeria*.

The genus *Teratosphaeria* is separated from *Mycosphaerella* s.str. based on several characters such as the presence of superficial stromatic tissue, ascospores that darken in their asci, remnants of the hamathecial tissue, ascospores that are frequently covered by a mucoid sheath, asci with a multi-layered endotunica, and the presence of ostiolar periphyses (Crous et al. 2007a). Presently 12 anamorph genera have been linked to *Teratosphaeria* (see Crous et al. 2007a for key), with the majority

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**Table 1** Details of isolates included for morphological and / or molecular examination in this study. The GenBank accession numbers of isolates for which ITS sequences were generated for the first time are printed in **bold face**.

Teleomorph	Anamorph	Accession number <sup>1</sup>	Host	Country	Collector	GenBank Accession number
<i>Mycosphaerella buckinghamiae</i>		CBS 111996; CPC 3006*	<i>Buckinghamia</i> sp.	Australia	P.W. Crous & B. Summerell	<b>EU707855</b>
		CBS 112175; CPC 3360	<i>Buckinghamia</i> sp.	–	–	<b>EU707856</b>
<i>M. communis</i>	<i>Dissoconium commune</i>	CBS 112889; CPC 3359	<i>Protea magnifica</i>	Australia	P.W. Crous	AY725539
		CBS 114238; CPC 10440*	<i>Eucalyptus globulus</i>	Spain	J.P. Mansilla	AY725541
<i>M. holualoana</i>		CBS 110698; CPC 2126*	<i>Leucospermum</i> sp.	USA: Hawaii	P.W. Crous & M.E. Palm	AY260087
<i>M. konae</i>	<i>Pseudocercospora</i> sp.	CBS 111261; CPC 2123*	<i>Leucadendron</i> sp.	USA: Hawaii	P.W. Crous & M.E. Palm	AY260086
		CBS 111028; CPC 2125	<i>Leucadendron</i> sp.	USA: Hawaii	P.W. Crous & M.E. Palm	AY260085
		CBS 120748; CPC 13469	<i>E. camaldulensis</i>	Thailand	W. Himaman	EF394842
<i>M. marksii</i>		CBS 110942; CPC 982*	<i>E. botryoides</i>	Australia	A.J. Carnegie	AF309589
		CBS 110974; CPC 984	<i>E. botryoides</i>	Australia	A.J. Carnegie	–
		CBS 115501; CPC 5358	<i>Leucadendron tinctum</i>	Madeira Islands	S. Denman	DQ302979
<i>M. stromatosa</i>	<i>Pseudocercospora stromatosa</i>	CBS 101953; CPC 1731*	<i>Protea</i> sp.	South Africa	S. Denman	EU167598
<i>M. waimeana</i>	<i>Stenella</i> sp.	CBS 110697; CPC 2179*	<i>Leucospermum</i> sp.	USA: Hawaii	P.W. Crous & M.E. Palm	AY260083
<i>Teratosphaeria alistairii</i>	<i>Batcheloromyces</i> sp.	CBS 120035; CPC 12730*	<i>P. repens</i>	South Africa	P.W. Crous & A. Smith	DQ885901
<i>T. associata</i>		CBS 112224; CPC 3116	<i>P. lepidocarpodendron</i>	Australia	P.W. Crous & B. Summerell	DQ302968
		CBS 112627; CPC 3115	<i>P. lepidocarpodendron</i>	Australia	P.W. Crous & B. Summerell	<b>EU707857</b>
		CBS 114165; CPC 3117	<i>P. lepidocarpodendron</i>	Australia	P.W. Crous & B. Summerell	<b>EU707858</b>
		CBS 120730; CPC 13119*	<i>Corymbia henryii</i>	Australia	A.J. Carnegie	EF394826
		CBS 120731; CPC 13128	<i>C. variegata</i>	Australia	A.J. Carnegie	EF394827
		CBS 120732; CPC 13108	<i>E. dunnii</i>	Australia	A.J. Carnegie	EF394824
<i>T. bellula</i>		CBS 111699; CPC 1816	<i>Leucospermum</i> sp.	South Africa	J.E. Taylor	<b>EU707859</b>
		CBS 111700; CPC 1821	<i>P. eximia</i>	South Africa	J.E. Taylor	EU019301
		CBS 114145; CPC 2795	<i>Leucadendron</i> sp.	South Africa	L. Swart	<b>EU707860</b>
		CPC 14908	<i>Protea</i> sp.	South Africa	P.W. Crous	<b>EU707861</b>
<i>T. fibrillosa</i>		CBS 121707; CPC 13960*	<i>Protea</i> sp.	South Africa	P.W. Crous & L. Mostert	<b>EU707862</b>
		CPC 1876	<i>P. nitida</i>	South Africa	J.E. Taylor	AY260094
		CPC 13969	<i>Protea</i> sp.	South Africa	P.W. Crous	<b>EU707863</b>
<i>T. jonkershoekensis</i>		CBS 122897; CPC 13984*	<i>Protea</i> sp.	South Africa	P.W. Crous & L. Mostert	<b>EU707864</b>
<i>T. knoxdavesii</i>		CBS 122898; CPC 14960*	<i>Protea</i> sp.	South Africa	P.W. Crous & M. Crous	<b>EU707865</b>
		CPC 14905	<i>Protea</i> sp.	South Africa	P.W. Crous & M. Crous	<b>EU707866</b>
<i>T. maculiformis</i>		No culture available	<i>Protea</i> sp.	South Africa	P.W. Crous & K.L. Crous	<b>EU707867</b>
<i>T. marasasii</i>		CBS 122899; CPC 14889*	<i>Protea</i> sp.	South Africa	P.W. Crous & M. Crous	<b>EU707868</b>
<i>T. maxii</i>		CBS 112231; CPC 3321	<i>Protea</i> sp.	Australia	P.W. Crous & B. Summerell	<b>EU707869</b>
		CBS 112232; CPC 3323	<i>Protea</i> sp.	Australia	P.W. Crous & B. Summerell	<b>EU707870</b>
		CBS 112496; CPC 3322	<i>Protea</i> sp.	Australia	P.W. Crous & B. Summerell	<b>EU707871</b>
		CBS 120137; CPC 12805*	<i>P. repens</i>	South Africa	M. Crous & P.W. Crous	DQ885899
		CPC 12943	<i>P. repens</i>	South Africa	P.W. Crous	DQ885898
<i>T. microspora</i>	<i>Catenulostroma microsporium</i>	CBS 101951; CPC 1960*	<i>P. cynaroides</i>	South Africa	S. Denman & J.E. Taylor	<b>EU707872</b>
		CBS 110890; CPC 1832	<i>P. cynaroides</i>	South Africa	L. Swart	AY260097
		CBS 111031; CPC 1848	<i>P. cynaroides</i>	South Africa	J.E. Taylor	AY260098
		CBS 111697; CPC 1597	<i>P. cynaroides</i>	South Africa	P.W. Crous	<b>EU707873</b>
<i>T. parva</i>		CBS 114761; CPC 1217	<i>P. repens</i>	South Africa	P.W. Crous	<b>EU707874</b>
		CBS 122892; CPC 12421*	<i>E. globulus</i>	Australia	I. Smith	<b>EU707875</b>
		CBS 122893; CPC 14898	<i>P. repens</i>	South Africa	L. Mostert	<b>EU707876</b>

	CBS 122894; CPC 13896	<i>P. nitida</i>	South Africa	P.W. Crous & L. Mostert	<b>EU707877</b>
	CPC 2120	<i>P. repens</i>	South Africa	G. Matthews	AY260091
	CPC 12418	<i>E. globulus</i>	Australia	I. Smith	<b>EU707878</b>
	CPC 12419	<i>E. globulus</i>	Australia	I. Smith	<b>EU707879</b>
<i>T. persoonii</i>	CBS 122895; CPC 13972*	<i>Protea</i> sp.	South Africa	P.W. Crous & L. Mostert	<b>EU707880</b>
	CBS 122896; CPC 14846; STE-U 6389	<i>Euchaetis meridionalis</i>	South Africa	A.R. Wood	<b>EU707881</b>
<i>T. proteae-arboreae</i>	CPC 12952*	<i>P. nitida</i>	South Africa	M.K. Crous & P.W. Crous	<b>EU707882</b>
	CPC 12954*	<i>P. nitida</i>	South Africa	M.K. Crous & P.W. Crous	<b>EU707883</b>
	CPC 14963	<i>Protea</i> sp.	South Africa	P.W. Crous & M. Crous	<b>EU707884</b>
<i>Teratosphaeria</i> sp.	CPC 13917	<i>P. nitida</i>	South Africa	P.W. Crous & L. Mostert	<b>EU707885</b>
<i>Teratosphaeria</i> sp.	CPC 13963	<i>P. nitida</i>	South Africa	P.W. Crous & L. Mostert	<b>EU707886</b>
<i>Teratosphaeria</i> sp.	CPC 13981	<i>P. repens</i>	Portugal	M.F. Moura	<b>EU707887</b>
<i>Teratosphaeria</i> sp.	CPC 14957	<i>Protea</i> sp.	South Africa	P.W. Crous & M. Crous	<b>EU707888</b>
	<i>Batcheloromyces leucadendri</i>	<i>Leucadendron</i> sp.	South Africa	L. Swart	AY260100
	CBS 110892; CPC 1837	<i>Leucadendron laureolum</i>	South Africa	L. Swart	AY260101
	CBS 111577; CPC 1838*	<i>Leucadendron</i> sp.	South Africa	L. Swart	<b>EU707889</b>
	CBS 111937; CPC 2822	<i>Leucadendron</i> sp.	South Africa	L. Swart	<b>EU707890</b>
	CBS 114024; CPC 2794	<i>Leucadendron</i> sp.	South Africa	L. Swart	<b>EU707891</b>
	CBS 114144; CPC 2823	<i>Leucadendron</i> sp.	South Africa	L. Swart	<b>EU707892</b>
	CBS 114146; CPC 2820	<i>Leucadendron</i> sp.	South Africa	L. Swart	EU552103
	CBS 119344; CMW 20456; PREM 58041	<i>Leucadendron salignum</i>	South Africa	S. Lee	–
	CPC 1839	<i>Leucadendron</i> sp.	South Africa	L. Swart	–
	CPC 1840	<i>Leucadendron gandogerii</i>	South Africa	L. Swart	–
<i>B. proteae</i>	CBS 110696; CPC 1518*	<i>P. cynaroides</i>	South Africa	L. Swart	AY260099
	CPC 1833	<i>P. cynaroides</i>	South Africa	L. Swart	–
	CPC 1834	<i>P. repens</i>	South Africa	L. Swart	–
	CPC 1835	<i>Protea</i> sp.	South Africa	L. Swart	–
	CPC 1836	<i>P. neriifolia</i>	South Africa	L. Swart	–
<i>B. sedgfieldii</i>	CBS 112119; CPC 3026*	<i>P. repens</i>	South Africa	J.E. Taylor	<b>EU707893</b>
<i>Catenulostroma elginense</i>	CBS 111030; CPC 1958	<i>P. grandiceps</i>	South Africa	J.E. Taylor & S. Denman	AY260093
<i>C. macowanii</i>	CBS 110756; CPC 1872	<i>P. nitida</i>	South Africa	J.E. Taylor	AY260095
	CBS 111029; CPC 1488	<i>P. nitida</i>	South Africa	P.W. Crous	AY260096
	CBS 122901; CPC 13899*	<i>P. nitida</i>	South Africa	P.W. Crous & L. Mostert	<b>EU707894</b>
	CPC 13966	<i>P. nitida</i>	South Africa	P.W. Crous & L. Mostert	<b>EU707895</b>
<i>C. wingfieldii</i>	CBS 112163; CPC 2944*	<i>P. nitida</i>	South Africa	J.E. Taylor	<b>EU707896</b>
<i>Dissoconium proteae</i>	CBS 122900; CPC 13853*	<i>Protea</i> sp.	Canary Islands: Tenerife	P.W. Crous	<b>EU707897</b>
<i>Phaeothecoidea proteae</i>	CBS 114129; CPC 2831*	<i>P. repens</i>	South Africa	S. Denman	<b>EU707898</b>
<i>Pseudocercospora protearum</i> var. <i>leucadendri</i>	CPC 1869	<i>Leucadendron</i> sp.	South Africa	P.W. Crous & S. Denman	AY260089
<i>Ramularia proteae</i>	CBS 112161; CPC 3075*	<i>P. longifolia</i>	Australia	A. Macfadyen	<b>EU707899</b>
<i>Readeriella guyanensis</i>	CBS 117550; MUCL 46082	Leaf litter	French Guiana	–	<b>EU707900</b>
<i>Septoria protearum</i>	CBS 778.97; ATCC 201159; CPC 1470; IMI 375230 CPC 5212	<i>P. cynaroides</i> <i>Protea</i> sp.	South Africa Canary Islands: Tenerife	L. Viljoen S. Denman	AY260081 AY260082

<sup>1</sup> ATCC: American Type Culture Collection, Virginia, USA; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW: Culture collection of Mike Wingfield, housed at FABI, Pretoria, South Africa; CPC: Culture collection of Pedro Crous, housed at CBS; IMI: International Mycological Institute, CAB International, Egham, Basingstoke, UK; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; PREM: National Collection of Fungi, Pretoria, South Africa; STE-U: Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa.

\* Ex-type cultures.

being quite distinct from those found in *Mycosphaerella* s. str. Although the genus *Teratosphaeria* was initially established for species occurring on Proteaceae, the genus remains poorly understood, as many of the taxa are not known from culture, and their phylogenetic position remains uncertain. The aim of the present study was thus to recollect these taxa from Proteaceae, and designate epitype specimens for many of the older names, thereby enabling us to clarify their phylogeny.

**MATERIALS AND METHODS**

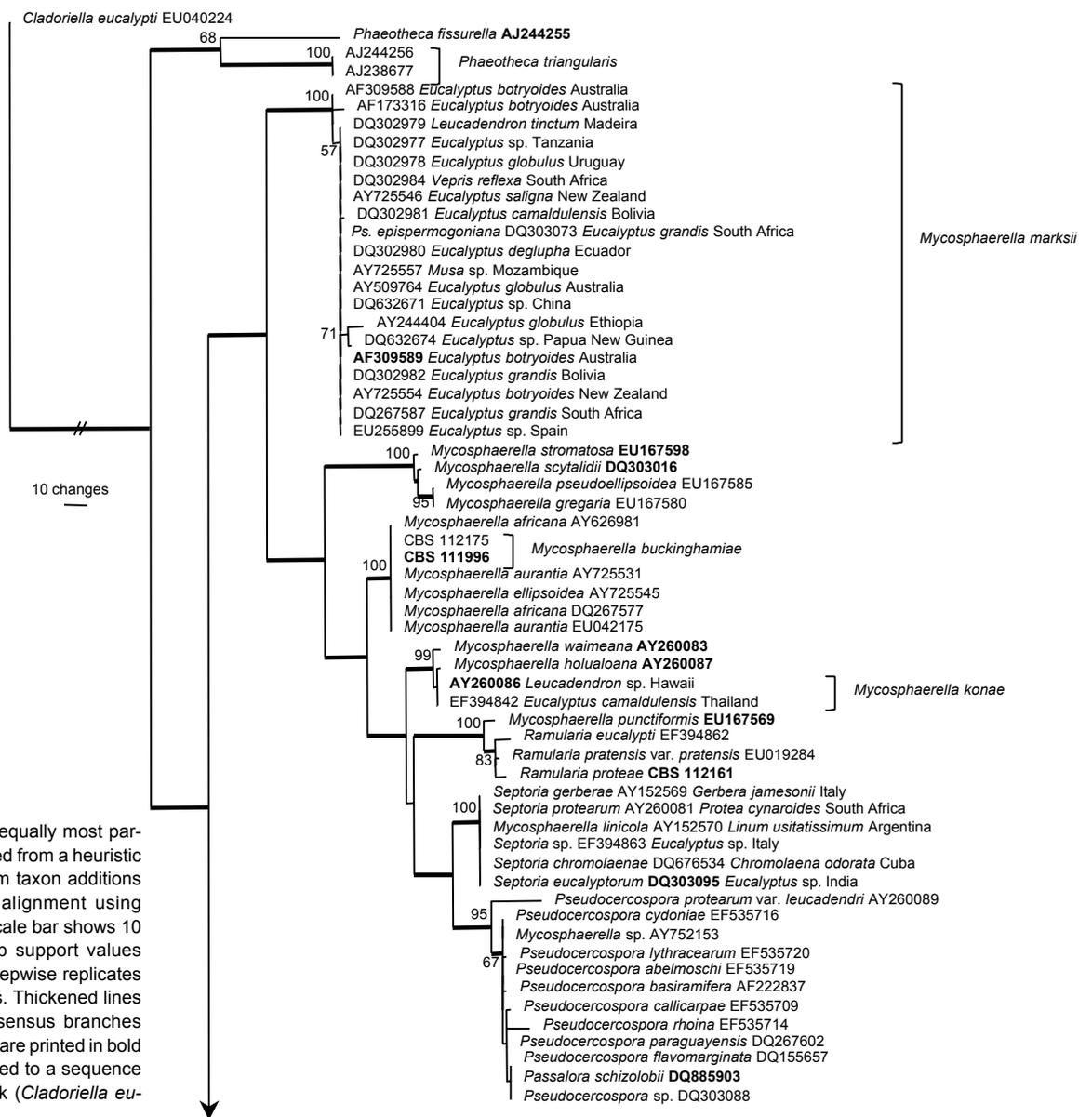
**Isolates**

Proteaceae leaves bearing ascomata, or with leaf spots were chosen for study. Excised lesions were soaked in water for approximately 2 h, after which they were placed in the bottom of of

Petri dish lids, with the top half of the dish containing 2 % malt extract agar (MEA; Oxoid, Hampshire, England). Ascospore germination patterns were examined after 24 h, and single ascospore and conidial cultures established as described by Crous (1998). Colonies were sub-cultured onto 2 % potato-dextrose agar (PDA), synthetic nutrient-poor agar (SNA), MEA, and oatmeal agar (OA) (Gams et al. 2007), and incubated under continuous near-ultraviolet light at 25 °C to promote sporulation. All cultures obtained in this study are maintained in the culture collection of the CBS (Table 1). Nomenclatural novelties, descriptions and trace files of the ITS DNA barcodes were deposited in MycoBank (www.MycoBank.org).

**DNA phylogeny**

Fungal colonies were established on agar plates, and genomic DNA was isolated following the CTAB-based protocol described



**Fig. 1** One of 10 000 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment using PAUP v. 4.0b10. The scale bar shows 10 changes and bootstrap support values from 10 000 000 fast stepwise replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and ex-type sequences are printed in bold face. The tree was rooted to a sequence obtained from GenBank (*Cladoriella eucalypti* EU040224).

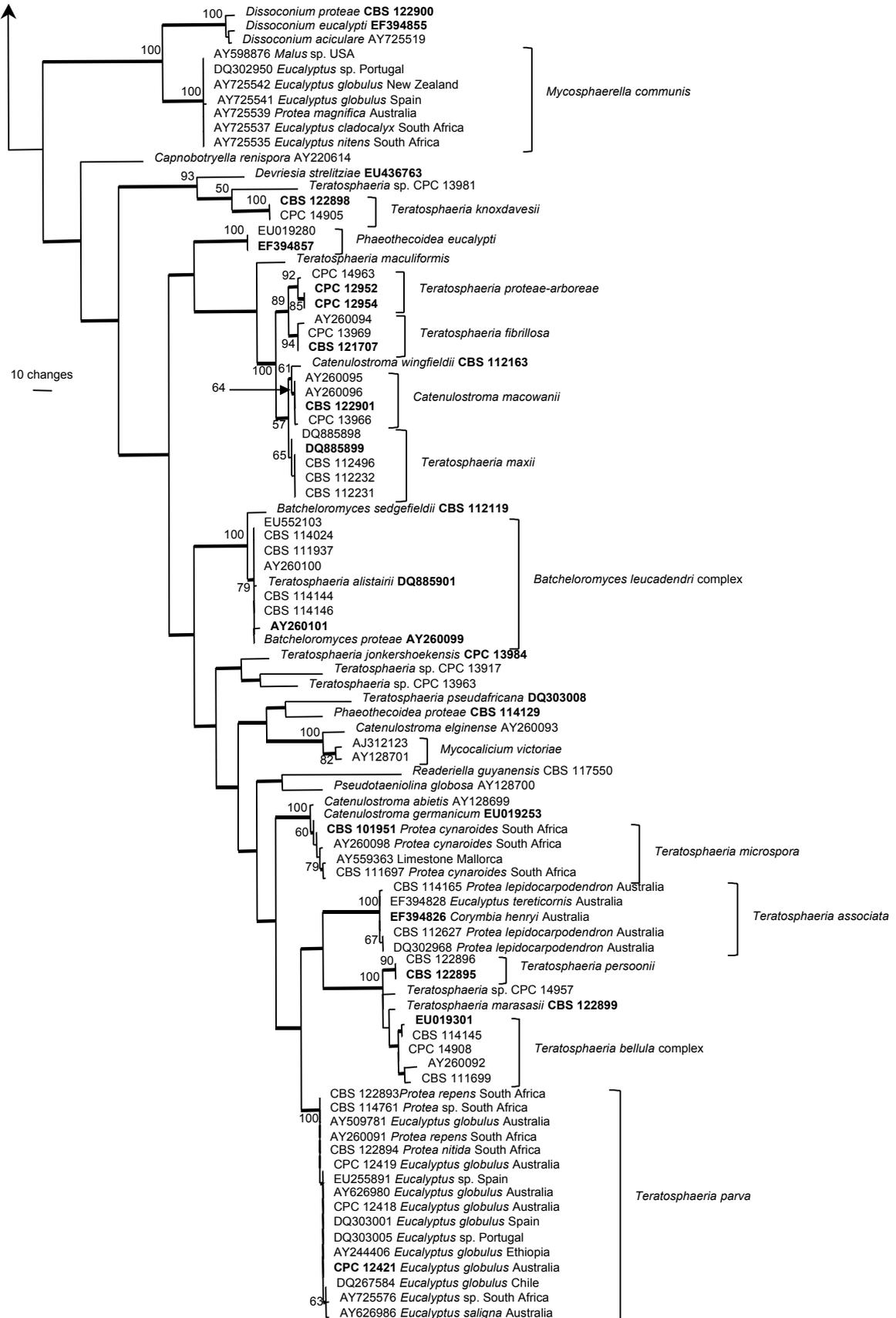
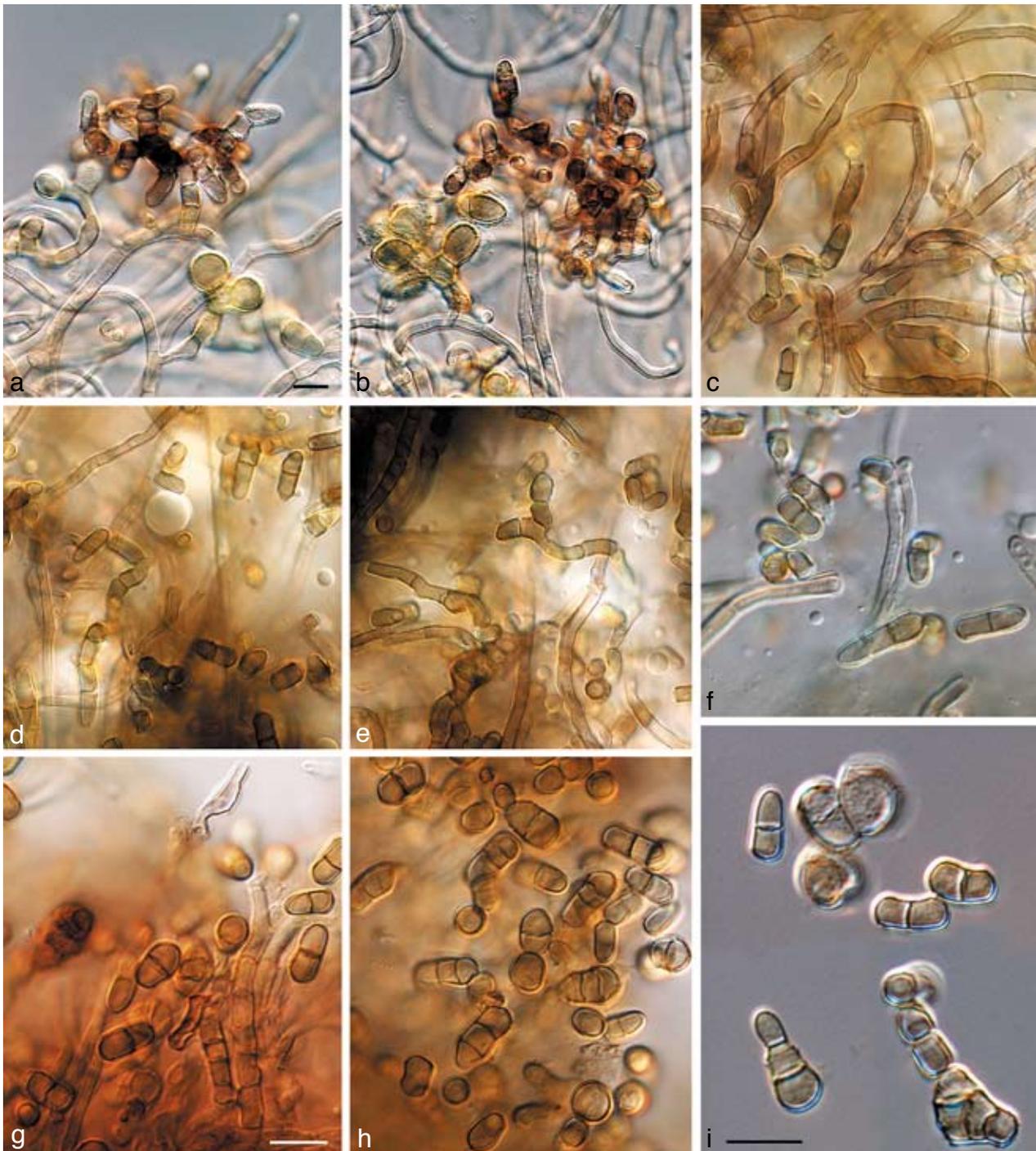


Fig. 1 (cont.)



**Fig. 2** *Batcheloromyces leucadendri* and *B. proteae*. a–f. *Batcheloromyces leucadendri* (CBS 110892). a, b. Sporodochia formed in culture; c–e. chains of conidia formed in aerial mycelium; f. conidia. — g–i. *Batcheloromyces proteae* (CBS 110696). g, h. conidia formed in aerial mycelium; i. catenulate conidia (note percurrent proliferation on solitary conidiogenous cell). — Scale bars = 10 µm.

in Gams et al. (2007). The primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990) were used to amplify part (ITS) of the nuclear rDNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. The primer ITS4 (White et al. 1990)

was used in combination with primer V9G for sequencing to ensure good quality overlapping sequences were obtained. The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Crous et al. (2006b). The ITS1, ITS2 and 5.8S rRNA gene were sequenced only for those isolates for which these data were not available.

Gaps longer than 10 bases were coded as single events for the phylogenetic analyses; the remaining gaps were treated as missing data. Sequence data were deposited in GenBank (Table 1) and the alignment and trees in TreeBASE (www.treebase.org).

### Taxonomy

Wherever possible, 30 measurements ( $\times 1\ 000$  magnification) were made of structures mounted in lactic acid, with the extremes of spore measurements given in parentheses. Colony colours (surface and reverse) were assessed after 2–8 wk on MEA, OA and PDA at 25 °C in the dark, using the colour charts of Rayner (1970).

## RESULTS

### DNA phylogeny

Amplicons of approximately 1 700 bases were obtained for the isolates listed in Table 1. The ITS sequences were used to obtain additional sequences from GenBank, which were added to the alignment. The manually adjusted ITS alignment contained 151 sequences (including the outgroup sequence) and 973 characters including alignment gaps (available in TreeBASE). Of the 540 characters used in the phylogenetic analysis, 279 were parsimony-informative, 55 were variable and parsimony-uninformative, and 206 were constant. Neighbour-joining analyses using three substitution models on the sequence alignment yielded trees with identical topologies to one another, except for the position of *Capnobotryella renispora*, which differed when the uncorrected 'p' substitution model was compared with the Kimura 2-parameter and HKY85 models (it is placed basal to *Teratosphaeria* and *Mycosphaerella* in the latter two models). The neighbour-joining trees support the same clades as obtained from the parsimony analysis, but with a different arrangement at the deep nodes, for example the position of the *Dissoconium* and *Phaeotheca* clades. Because of the large number of different strain associations, for example in the *Mycosphaerella marksii*, *Pseudocercospora* and *Teratosphaeria parva* clades (as evident from the strict consensus branches shown in Fig. 1), only the first 10 000 equally most parsimonious trees (TL = 1725 steps; CI = 0.386; RI = 0.870; RC = 0.336) were saved, one of which is shown in Fig. 1. The phylogenetic results obtained are discussed where applicable in the descriptive notes below.

### Taxonomy

Although numerous species of *Mycosphaerella*, *Teratosphaeria* and associated anamorphs have been described from Proteaceae (Crous & Wingfield 1993, Crous & Braun 1994, 1996, Swart et al. 1998, Crous & Palm 1999, Crous et al. 2000b, Taylor & Crous 2000, Taylor et al. 2001a–c, 2003, Sivanesan & Shivas 2002), only those known from culture are discussed below.

***Batcheloromyces leucadendri*** P.S. van Wyk, Marasas & Knox-Dav., S. African J. Bot. 51: 344. 1985 — Fig. 2

Descriptions — Taylor et al. (1999), Crous et al. (2004a).

Cultural characteristics — Colonies on MEA iron-grey, reverse olivaceous-black, smooth with irregular margins; aerial mycelium sparse and grey or lacking; moderately slow growing, up to 40 mm in diam after 2 mo.

*Specimens examined.* SOUTH AFRICA, Western Cape Province, Betty's Bay, living leaves of *Leucadendron gandogeri*, 27 Mar. 1984, P.S. van Wyk, holotype PREM 47423; Cape Town, Kirstenbosch Botanical Gardens, on a living leaf of *Leucadendron coniferum*, 1996, L. Swart, F54, PREM 55954, CPC 1840; Porterville, Osdam Farm, on a living leaf of *Leucadendron* sp., 15 Jan. 1998, J.E. Taylor, JT84, PREM 55940; Stellenbosch, Helderberg Nature Reserve, on a living leaf of *Leucadendron* sp., 19 Jan. 1998, J.E. Taylor, JT107, PREM 55941; Stellenbosch, J.S. Marais Nature Reserve, on a living leaf of *Leucadendron* sp., 1996, L. Swart, F46, CPC 1837 = CBS 110892; Stellenbosch, Protea Heights farm, on a living leaf of *Leucadendron laureolum*, 1996, L. Swart, epitype designated here PREM 55949, cultures ex-epitype, CPC 1838 = CBS 111577; *ibid.*, *Leucadendron* cultivar 'Pisa', F49, PREM 55950; Stellenbosch, J.S. Marais Park, on a living leaf of *Leucadendron salicifolium*, 1996, L. Swart, F50, PREM 55951; *ibid.*, *Leucadendron* sp., F51, PREM 55952, CPC 1839; *ibid.*, *Leucadendron elimense*, F52, PREM 55953; Western Cape Province, *Leucadendron* sp., 27 May 1999, L. Swart, CPC 2820 = CBS 114146; *ibid.*, CPC 2823 = CBS 114144; *ibid.*, CPC 2794 = CBS 114024; *ibid.*, CPC 2822 = CBS 111937; Jonkershoek Nature Reserve, 6 June 2000, leaf litter of *Leucadendron salignum*, S. Marincowitz, S.L.130, PREM 58041, cultures CBS 119344 = CMW 20456.

***Batcheloromyces proteae*** Marasas, P.S. van Wyk, & Knox-Dav., J. S. African Bot. 41: 43. 1975 — Fig. 2

= *Stigmia proteae* (Marasas, P.S. van Wyk, & Knox-Dav.) B. Sutton & Pascoe, Mycol. Res. 92: 214. 1989.

Descriptions — Taylor et al. (1999), Crous et al. (2004a).

Cultural characteristics — Colonies olivaceous, the same in reverse, smooth with irregular margins, sectored; aerial mycelium lacking; slow-growing, approximately 10–18 mm diam after 2 mo.

*Specimens examined.* SOUTH AFRICA, Western Cape Province, Stellenbosch, on living leaves of *Protea cynaroides*, 15 Aug. 1973, P.S. van Wyk, holotype PREM 44850; Betty's Bay, Harold Porter Botanical Garden, on a living leaf of *P. magnifica*, 1997, L. Swart, F44, PREM 55947; *ibid.*, *P. nerifolia*, F45, PREM 55948, CPC 1836; Stellenbosch, J.S. Marais Park, on a living leaf of *P. cynaroides*, 28 Feb. 1998, J.E. Taylor, JT119, PREM 55942; *ibid.*, 1996, L. Swart, F42, PREM 55946; *ibid.*, 26 Aug. 1996, L. Swart, F25, PREM 55943, CPC 1833; *ibid.*, *P. repens*, F40, PREM 55944, CPC 1834; *ibid.*, *Protea* sp., F41, PREM 55945, CPC 1835; Stellenbosch, Devon Valley, on a living leaf of *P. cynaroides*, 30 Aug. 1996, L. Swart, epitype designated here CBS H-20087, culture ex-epitype CPC 1518 = CBS 110696.

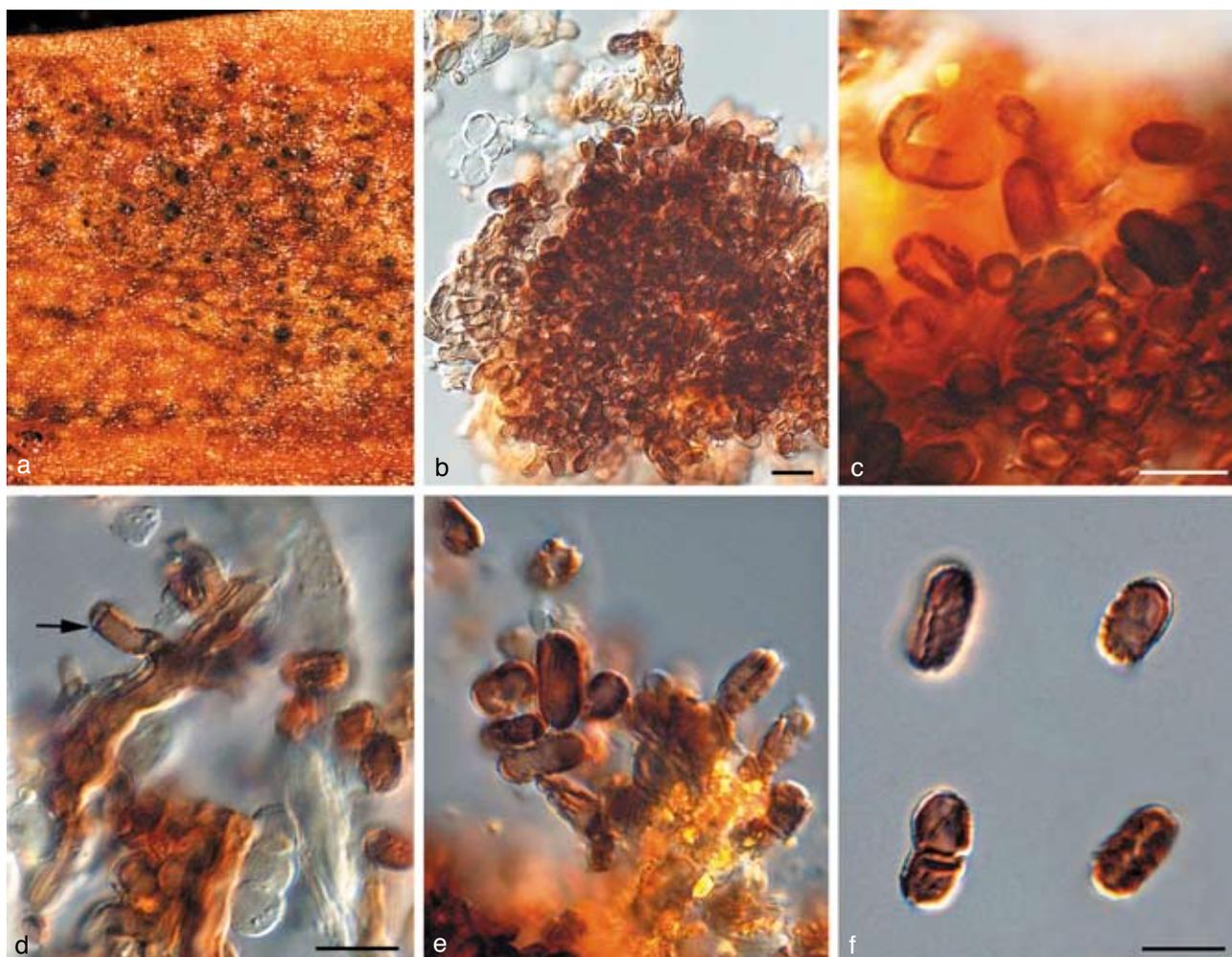
Notes — Although morphologically distinct, *B. proteae* could not be distinguished phylogenetically from *B. leucadendri* in the present study (Fig. 1).

***Batcheloromyces sedgefieldii*** Crous, *sp. nov.* — MycoBank MB506591; Fig. 3

*Batcheloromyces proteae* similis, sed in agar (MEA) coloniis tarde crescentibus, usque ad 5 mm diam post 30 dies 25 °C.

*Etymology.* Named after Sedgefield, a town in the Southern Cape of South Africa, from where this fungus was collected.

*Leaf spots* containing black sporodochial plates, not extending through leaf lamina, on both sides of leaf surface, irregular, non-necrotic, becoming somewhat erumpent, up to 7 mm diam.



**Fig. 3** *Batcheloromyces sedgefieldii* in vivo (CBS H-20088). a. Sporodochia on leaf; b. sporodochium; c–e. conidiogenous cells giving rise to conidia; f. verruculose conidia. — Scale bars = 10 µm.

*Conidiomata* sporodochial, composed of a single layer of radiating, septate, branched, dark brown, thick-walled hyphae which are formed from a stroma within the substomatal cavity, forming pulvinate plates, 100–120 µm diam; hyphae radiating from sporodochial plates and adhering closely to the host surface or growing into the stomata. *Conidiophores* erect or ascending, short lateral branches on the superficial hyphae, simple, brown, effuse, but occurring mainly towards the centre of the sporodochial plates, above the stomata, terminating to produce conidiogenous cells. *Conidiogenous cells* mainly subcylindrical, but occasionally doliiform, proliferating percurrently resulting in up to three irregular, ragged annellides, 5–10 × 3–5 µm. *Conidia* arising singly from blown out ends of the conidiogenous cells, solitary, unicellular, but often remaining in fragile chains of 2 conidia, ellipsoidal, oblong, with thick, verrucose walls, and a basal marginal frill, (5–)6.5–7(–10) × (3.5–)4–5 µm.

**Cultural characteristics** — Colonies on MEA olivaceous, with smooth margins, lacking aerial mycelium; extremely slow-growing, up to 5 mm diam after 2 mo.

**Specimen examined.** SOUTH AFRICA, Western Cape Province, Southern Cape, Sedgefield, on leaves of *Protea repens*, 10 Aug. 1999, J.E. Taylor, holotype CBS H-20088, culture ex-type CPC 3026 = CBS 112119.

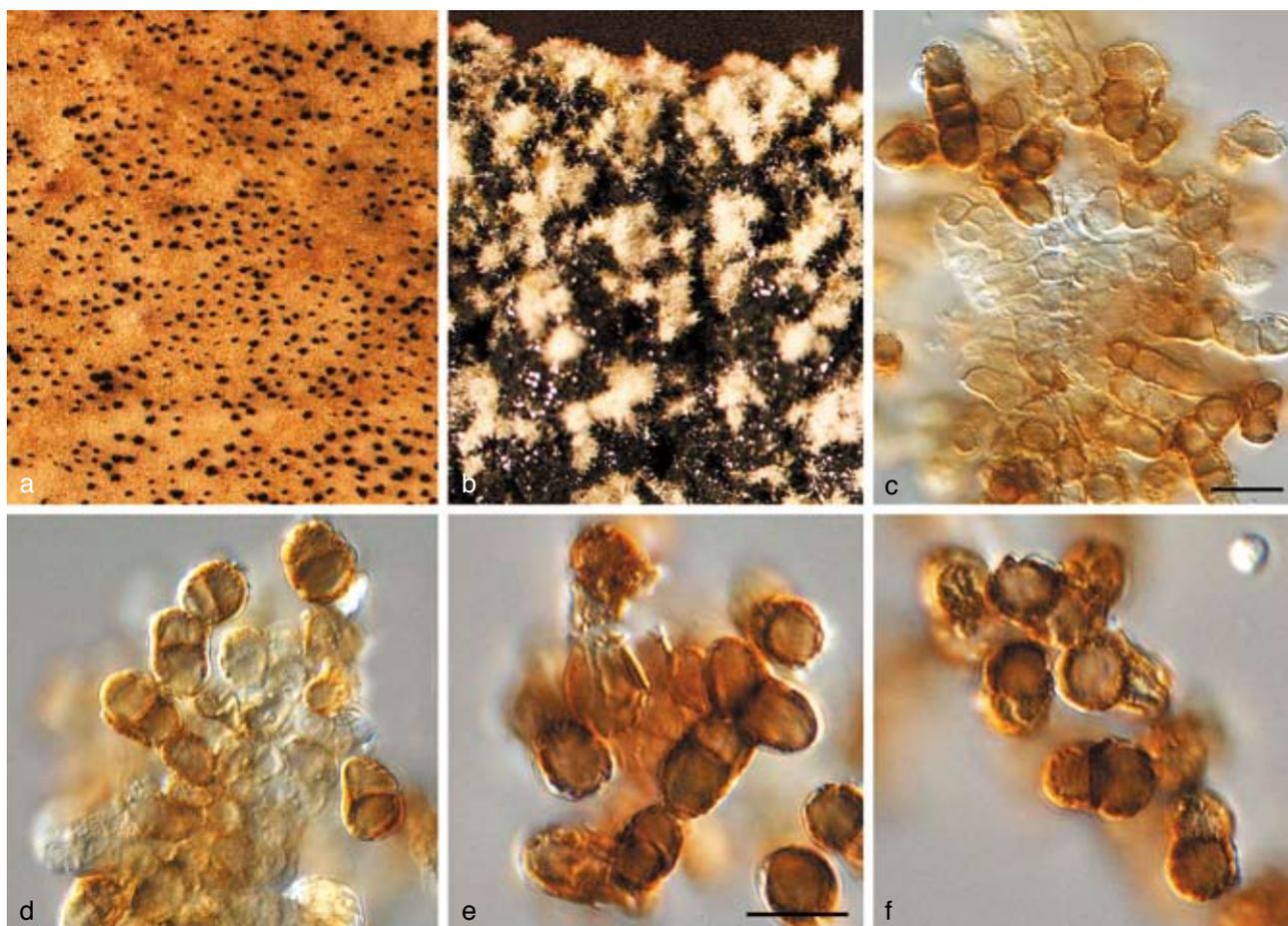
**Notes** — Although phylogenetically distinct, *B. sedgefieldii* is morphologically similar to *B. proteae*. The two species can be distinguished in culture, however, as colonies of *B. proteae* are relatively fast growing compared to those of *B. sedgefieldii*, which only reach 5 mm diam after 2 mo.

***Catenulostroma elginense*** (Joanne E. Taylor & Crous) Crous & U. Braun, Stud. Mycol. 58: 16. 2007

**Basionym.** *Trimmatostroma elginense* Joanne E. Taylor & Crous, Mycol. Res. 104: 633. 2000.

**Descriptions** — Taylor & Crous (2000), Crous et al. (2004a).

**Specimen examined.** SOUTH AFRICA, Western Cape Province, Elgin, Molteno Brothers Farm, on a living leaf of *Protea grandiceps* Tratt., 21 July 1998, J.E. Taylor & S. Denman, holotype PREM 56208, culture ex-type CPC 1958 = CBS 111030.



**Fig. 4** *Catenulostroma macowanii* (CBS 122901). a. Sporodochia on leaf; b. sporulation on MEA; c, d. conidiogenous cells giving rise to conidia; e, f. conidia. — Scale bars = 10 µm.

***Catenulostroma macowanii*** (Sacc.) Crous & U. Braun, Stud. Mycol. 58: 17. 2007 — Fig. 4

*Basionym.* *Coniothecium macowanii* Sacc., Syll. Fung. 4: 512. 1886. nom. nov., based on *Coniothecium punctiforme* G. Winter, Hedwigia 24: 33. 1885, non *C. punctiforme* Corda, Icon. Fungorum (Corda) 1: 2. 1837.

≡ *Trimmatostroma macowanii* (Sacc.) M.B. Ellis, *More Dematiaceous Hyphomycetes*: 29. 1976.

Descriptions — Taylor & Crous (1998f), Crous et al. (2004a).

Cultural characteristics — Colonies on MEA erumpent, spreading, margins irregular, smooth, surface with sparse pale olivaceous-grey aerial mycelium, interrupted by black conidial masses bursting through the layer of aerial mycelium; reaching 4 mm diam after 2 wk.

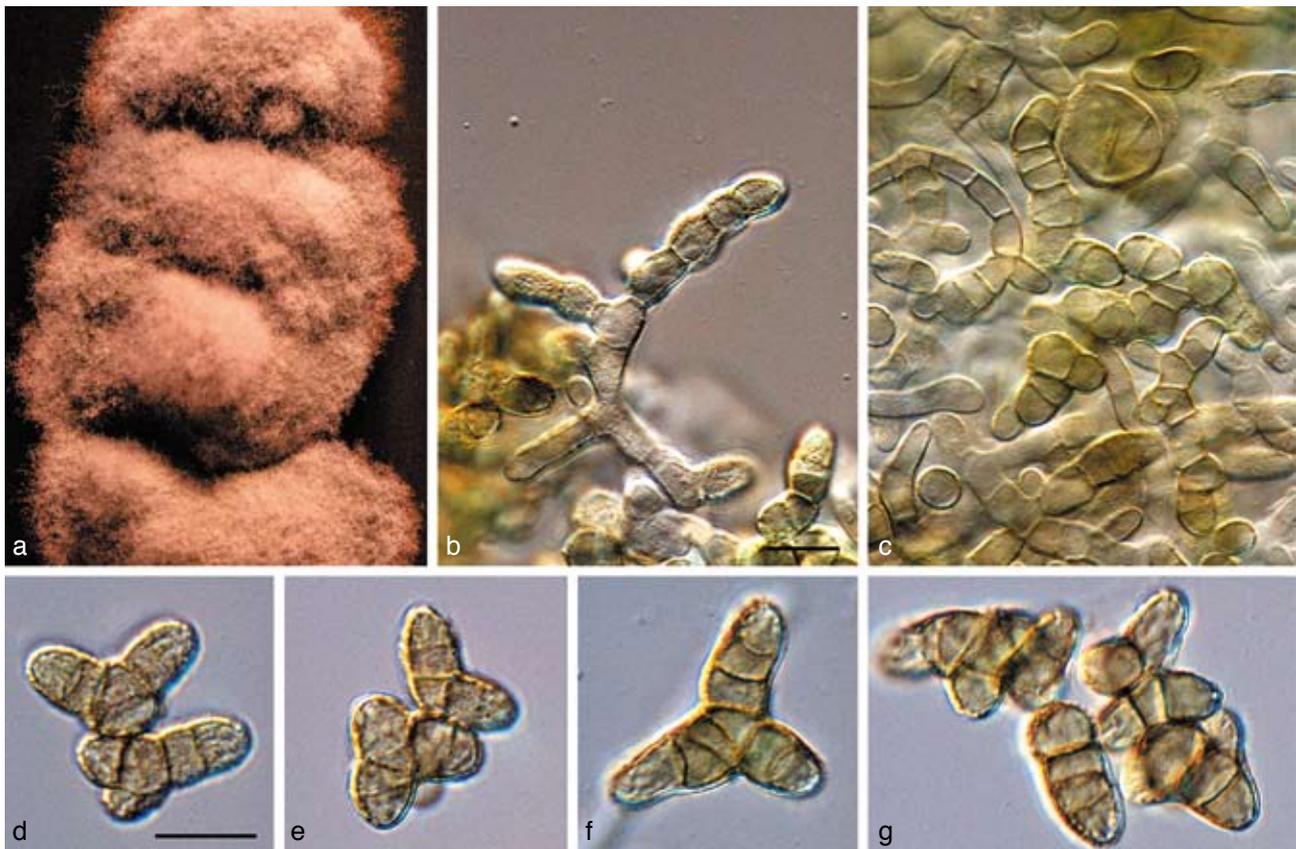
*Specimens examined.* SOUTH AFRICA, Western Cape Province, Table Mountain, on living leaves of *Protea grandiflora*, *P. MacOwan*, holotype JE (of *Coniothecium punctiforme* G. Winter); Cederberge, on living leaves of *P. nitida*, 10 May 1998, *J.E. Taylor*, CPC 1872 = CBS 110756; Hermanus, on a living leaf of *P. nitida*, 13 Aug. 1996, *P.W. Crous*, CPC 1488 = CBS 111029; Jonkershoek, S33°59'11.2" E18°57'14.7", on living leaves of *P. nitida*, 1 Apr. 2007, *P.W. Crous & L. Mostert*, epitype designated here CBS H-20089, cultures ex-epitype CPC 13899, 13901, 13900 = CBS 122901; ibid, CPC 13966–13968.

***Catenulostroma wingfieldii*** Crous, *sp. nov.* — MycoBank MB506592; Fig. 5

*Catenulostromatis macowanii* simile, sed conidiis longioribus, (13–)20–26 (–35) × (5–)6–8 (–12) µm.

*Etymology.* Named in honour of Prof. M.J. Wingfield, who has made a significant contribution to our knowledge of the fungi occurring on Proteaceae.

*Conidiomata* sporodochial, pulvinate, punctiform, brown-black, 40–80 µm diam. *Conidiophores* emerging as fascicles through the stomata, hyaline to pale brown, smooth or verruculose, cylindrical. *Conidiogenous cells* holoblastic, basipetal, forming an unconnected chain of conidia delimited by a septum followed by diffuse wall-building below the previous conidium to form the next conidium which is delimited retrogressively, conidia separating by schizolytic secession. *Conidia* formed in simple and branched chains, highly variable in shape, ellipsoidal to subcylindrical, Y-shaped, curved or straight, with rounded apices, and truncate bases, frequently with a prominent marginal frill, transversely 1- to multi-septate, at times with oblique septa, pale to medium-brown, verrucose, (13–)20–26 (–35) × (5–)6–8 (–12) µm.



**Fig. 5** *Catenulostroma wingfieldii* (CBS 112163). a. Colony growing on MEA; b, c. conidia forming on aerial mycelium; d–g. conidia. — Scale bars = 10 µm.

**Cultural characteristics** — Colonies on MEA erumpent, with smooth, regular margins, and sparse aerial mycelium, pale olivaceous-grey; colonies reaching 7 mm diam after 2 wk on MEA; on OA erumpent with smooth to feathery margins, sparse to moderate aerial mycelium, pale olivaceous-grey to olivaceous-grey; colonies reaching 7 mm diam after 2 wk at 25 °C.

**Specimen examined.** SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Gardens, on living leaves of *Protea nitida*, 15 Aug. 1999, J.E. Taylor, holotype CBS H-20090, cultures ex-type CPC 2944 = CBS 112163, CPC 2945–2946.

**Notes** — *Catenulostroma wingfieldii* is similar to *C. macowanii*, but differs in that colonies are pale olivaceous-grey on MEA, while those of *C. macowanii* are iron-grey to black, and lack aerial mycelium. Furthermore, conidia of *C. wingfieldii* are somewhat larger, being (13–)20–26(–35) × (5–)6–8(–12) µm, while those of *C. macowanii* are (10–)15–17(–23) × (6–)6.5–7(–9) µm.

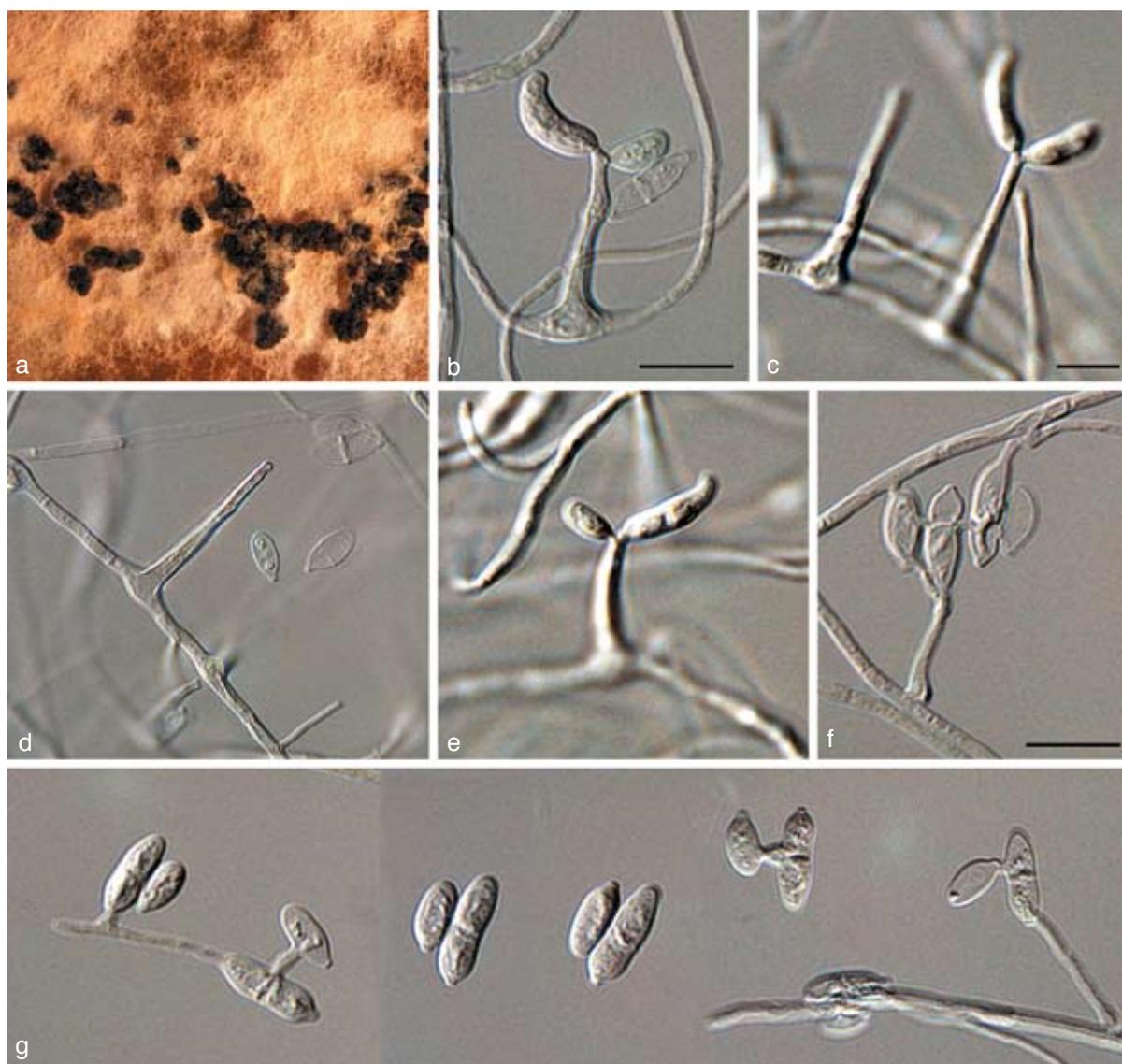
***Dissoconium proteae* Crous, sp. nov.** — MycoBank MB506593; Fig. 6

*Dissoconio aciculari* simile, sed conidiis primariis minoribus, (9–)10–11(–12) × (3–)3.5(–4) µm, conidiis secundariis quoque minoribus, 7–8(–10) × (3–)3.5(–4) µm.

**Etymology.** Named after the host on which it occurs, *Protea*.

**Mycelium** internal and external, consisting of branched, septate, smooth, hyaline to pale brown hyphae, 1.5–2 µm wide. **Coniophores** separate, arising from hyphae, subcylindrical, subulate or lageniform, tapering to a bluntly rounded or truncate apex, straight to gently curved, smooth, hyaline, becoming medium brown with age, aseptate, 10–30 × 3–5 µm; loci terminal and lateral, visible as slightly thickened, darkened scars, 0.5 µm wide. **Conidia** (9–)10–11(–12) × (3–)3.5(–4) µm, solitary, straight to somewhat curved, hyaline to pale olivaceous, smooth, ellipsoid, not to slightly constricted at median septum, apex obtuse, base obconic-truncate, tapering pronounced at somewhat protruding hilum, unthickened, not darkened, 1 µm wide. **Secondary conidia** developing adjacent to primary conidia, hyaline to subhyaline, aseptate, ellipsoid, tapering prominently towards a protruding, truncate base, 7–8(–10) × (3–)3.5(–4) µm; anastomosing with primary conidia after active discharge (in some cases the secondary conidia were observed to germinate, which has never been observed in species with smaller, pyriform secondary conidia).

**Cultural characteristics** — Colonies on OA spreading, with sparse aerial mycelium, and irregular margins; surface sienna, with patches of white and cinnamon; forming clusters of black sclerotia (remaining infertile) on OA, MEA and PDA; reaching 10 mm diam after 1 mo on OA.



**Fig. 6** *Dissoconium proteae* (CBS 122900). a. Sclerotia forming on MEA; b–f. solitary conidiophores giving rise to primary and secondary conidia; g. anastomosing primary and secondary conidia. — Scale bars = 10 µm.

*Specimen examined.* CANARY ISLANDS, Tenerife, on leaves of *Protea* sp., 1 Mar. 2007, P.W. Crous, holotype CBS H-20091, culture ex-type CPC 13853 = CBS 122900.

**Notes** — Of the *Dissoconium* species known to date (Crous et al. 2004b, Zhang et al. 2007, Arzanlou et al. 2008), *D. proteae* is most similar to *D. eucalypti* (primary conidia, 8–14 × 4.5–6 µm, and secondary conidia, 4–7 × 2.5–3 µm), and *D. aciculare* (primary conidia, 12–25 × 3.5–6 µm, and secondary conidia, 7.5–12 × 3.5–6 µm), but has smaller and narrower primary and secondary conidia (9–12 × 3–4 µm, and 7–10 × 3–4 µm, respectively), than both.

***Mycosphaerella buckinghamiae*** Crous & Summerell, Australas. Pl. Pathol. 29: 272. 2000

**Description** — Crous et al. (2000b).

*Specimen examined.* AUSTRALIA, New South Wales, Mangrove Mountain, on leaves of *Buckinghamia* sp., Aug. 1999, P.W. Crous & B. Summerell, holotype DAR 74865, cultures ex-type CPC 3006 = CBS 111996.

**Notes** — Although the ITS DNA sequence of *M. buckinghamiae* is identical to that of *M. africana* (ascospores 7–11 × 2–3 µm, darkening and distorting upon germination), *M. buckinghamiae* has larger ascospores (9–13 × 2.5–3.5 µm), which do not darken at germination, and colonies that contain rose and off-white sectors, which are lacking in *M. africana*, which again has black colonies, forming a brown pigment in MEA (Crous 1998, Crous et al. 2000b).



**Fig. 7** *Phaeothecoidea proteae* (CBS 114129). a. Colony on OA; b–e. hyphae with endoconidia visible; f–i. released endoconidia become brown and verruculose. — Scale bars = 10  $\mu$ m.

***Mycosphaerella communis*** Crous & Mansilla, Stud. Mycol. 50: 203. 2004

*Anamorph.* *Dissoconium commune* Crous & Mansilla, Stud. Mycol. 50: 203. 2004.

Description — Crous et al. (2004b).

*Specimens examined.* SPAIN, Pontevedra, Lourizán, Areeiro, on leaves of *Eucalyptus globulus*, Dec. 2002, *J.P. Mansilla*, holotype of *M. communis* and *D. commune* CBS H-9900, culture ex-type CBS 114238 = CPC 10440. — AUSTRALIA, New South Wales, Mount Tomah Botanic Garden, on leaves of *Protea magnifica*, 2002, *P.W. Crous*, CPC 3359 = CBS 112889.

***Mycosphaerella holualoana*** Crous, Joanne E. Taylor & M.E. Palm, Mycotaxon 78: 458. 2001

Descriptions — Taylor et al. (2001b), Crous et al. (2004a).

*Specimen examined.* USA, Hawaii, Kona district, Holualoa, on a living leaf of *Leucospermum* sp., 17 Nov. 1998, *P.W. Crous & M.E. Palm*, holotype PREM 56926, culture ex-type CPC 2126 = CBS 110698.

***Mycosphaerella konae*** Crous, Joanne E. Taylor & M.E. Palm, Mycotaxon 78: 459. 2001

*Anamorph.* *Pseudocercospora* sp.

Descriptions — Taylor et al. (2001b), Crous et al. (2004a).

*Specimens examined.* USA, Hawaii, Kona district, Holualoa, on a living leaf on *Leucadendron* 'Safari Sunset', 17 Nov. 1998, *P.W. Crous & M.E. Palm*, holotype PREM 56921, cultures ex-type CPC 2123 = CBS 111261, CPC 2125 = CBS 111028. — THAILAND, Thatakiab District, Chachoengsao Province, on leaves of *Eucalyptus camaldulensis*, 12 Oct. 2006, *W. Hima-man*, CPC 13469 = CBS 120748, CPC 13470.

***Mycosphaerella marksii*** Carnegie & Keane, Mycol. Res. 98: 414. 1994

Description — Carnegie & Keane (1994), Crous (1998).

*Specimens examined.* AUSTRALIA, Victoria, Nowa Nowa, on leaves of *Eucalyptus botryoides*, 11 Nov. 1990, *A.J. Carnegie*, holotype IMI 353731; Tostaree, on leaves of *E. botryoides*, Oct. 1994, *A.J. Carnegie*, epitype designated here PREM 51932, cultures ex-epitype CPC 982 = CBS 110942, CPC 984 = CBS 110974. — MADEIRA ISLANDS, Florialis Estate, on leaves of *Leucadendron tinctum*, 1 Apr. 2000, *S. Denman*, CPC 5358 = CBS 115501.

***Mycosphaerella stromatosa*** Joanne E. Taylor & Crous, Mycol. Res. 104: 625. 2000

*Anamorph.* *Pseudocercospora stromatosa* Joanne E. Taylor & Crous, Mycol. Res. 104: 625. 2000.

Description — Taylor & Crous (2000), Crous et al. (2004a).

*Specimen examined.* SOUTH AFRICA, Kwazulu-Natal, Drakensberg, Dragon's Peak, on a living leaf of *Protea* sp., Jan. 1998, *S. Denman*, holotype PREM 56204, culture ex-type CPC 1731 = CBS 101953.

***Mycosphaerella waimeana*** Crous, Joanne E. Taylor & M.E. Palm, Mycotaxon 78: 463. 2001

*Anamorph.* *Stenella* sp.

Descriptions — Taylor et al. (2001b), Crous et al. (2004a).

*Specimen examined.* USA, Hawaii, Kona district, Waimea, on a living leaf of *Leucospermum* hybrid 24, 17 Nov. 1998, *P.W. Crous & M.E. Palm*, holotype PREM 56950, culture ex-type CPC 2179 = CBS 110697.

***Phaeothecoidea proteae*** Crous, *sp. nov.* — MycoBank MB506594; Fig. 7

*Phaeothecoidea eucalypti* similis, sed conidiis majoribus, (6–)8–10(–13) × (4–)5–6(–11) µm.

*Etymology.* Named after the host from which it was collected, *Protea*.

*Hyphae in vitro* creeping, brown, verruculose, branched, septate, 3–5 µm wide, becoming swollen, up to 15 µm wide, verruculose, dark brown, or forming a mucoid capsule filled with endoconidia which are former hyphal cells that turn brown and thick-walled; end cells dividing into several endoconidia, which are released upon rupture of the cell wall. *Endoconidia* medium to dark brown, verruculose to verrucose to warty, thick-walled, ellipsoid to obovoid or obclavate, (6–)8–10(–13) × (4–)5–6(–11) µm; after liberation swelling, becoming transversely 1-septate, or with several oblique septa, again forming endoconidia, becoming warty with age, with the outer layer peeling off once endoconidia are released.

Cultural characteristics — Colonies on MEA slimy, erumpent, lacking aerial mycelium, irregular, folded, with smooth, regular margin; surface iron-grey, reverse fuscous-black; colonies reaching 7 mm diam after 2 wk; on OA lacking aerial mycelium, erumpent with smooth margins, black, reaching 6 mm diam after 2 wk; fertile.

*Specimen examined.* SOUTH AFRICA, Western Cape Province, Stellenbosch, Eisenburg Farm, on leaves of *Protea repens*, 23 July 1999, *S. Denman*, holotype CBS H-20092, cultures ex-type CPC 2828–2830, 2831 = CBS 114129.

Notes — The present species clusters close to the type of the genus *Phaeothecoidea*, *P. eucalypti* (Crous et al. 2007d), and as it shares the feature of brown, verruculose endoconidia, we chose to describe it as a new species of this genus. It is interesting to note that it was originally isolated as a coelomycete, and based on its yeast-like growth in culture, identified as *Coniothyrium leucospermi* (Swart et al. 1998, Taylor & Crous 2001), which has subsequently been allocated to a new genus, *Coniozyma* (Marincowitz et al. 2008). Unfortunately, the original herbarium specimen could not be located, and thus only the cultural synanamorph can be described here.

***Pseudocercospora protearum*** var. *leucadendri* (Cooke) U. Braun & Crous, Mycol. Progr. 1: 22. 2002

*Basionym.* *Cercospora protearum* Cooke var. *leucadendri* Cooke, Grevillea 12: 39. 1883.

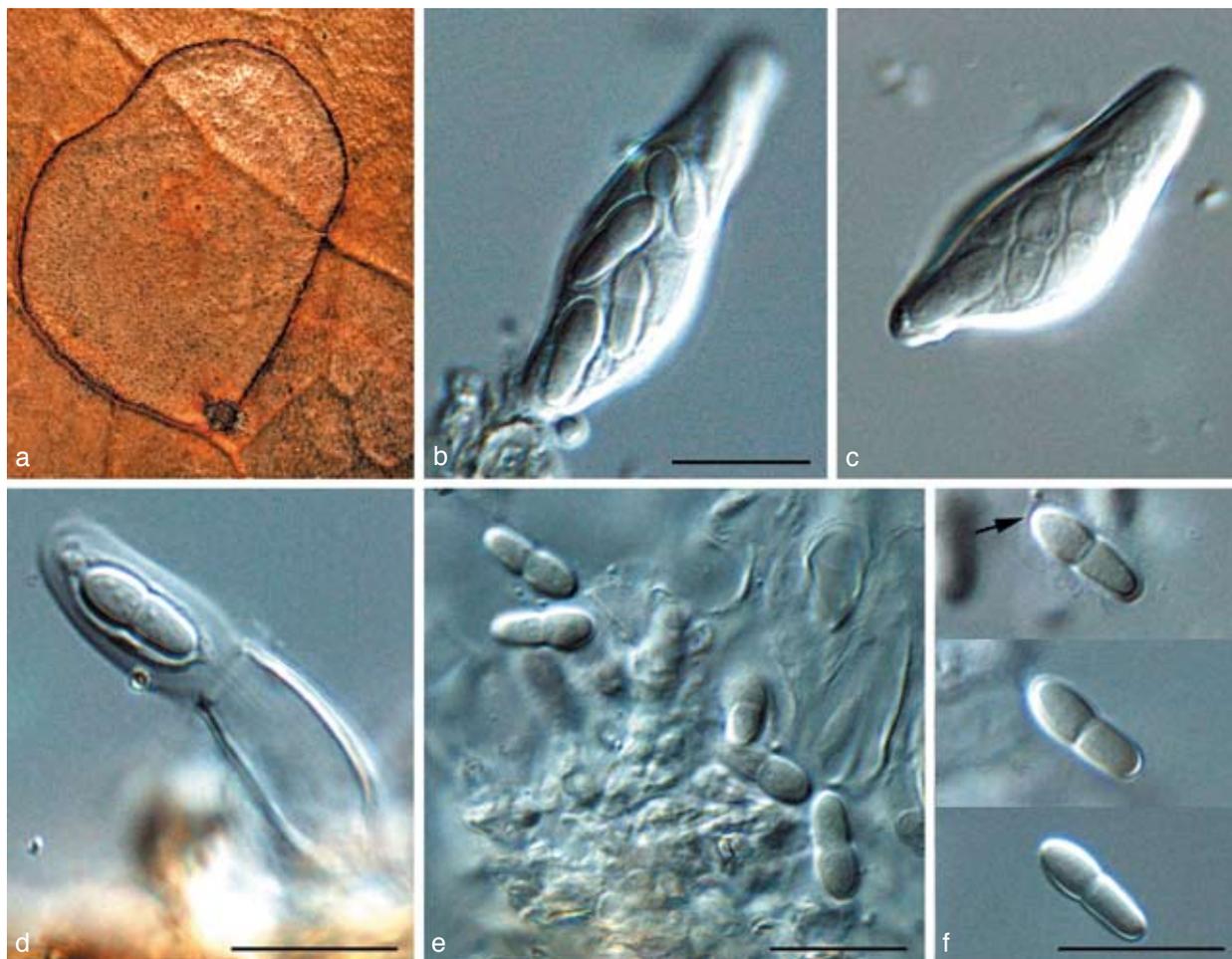
≡ *Stigmia protearum* var. *leucadendri* (Cooke) M.B. Ellis, Mycol. Pap. 131: 7. 1972.

≡ *Cercostigmia protearum* var. *leucadendri* (Cooke) U. Braun & Crous, Sydowia 46: 206. 1994.

= *Passalora protearum* Kalchbr. & Cooke, Grevillea 19: 6. 1890.

Description — Crous et al. (2004a).

*Specimens examined.* SOUTH AFRICA, Cape Province, Cape of Good Hope, Table Mountain, *Leucadendron argenteum*, MacOwan, No 1457, holotype K; Stellenbosch, Devon Valley, Protea Heights, on leaves of *Leucadendron* sp., 3 Apr. 1998, *P.W. Crous & S. Denman*, culture CPC 1869.



**Fig. 8** *Teratosphaeria bellula* (CBS 111699). a. Leaf spot on *Protea eximia*; b, c. asci; d. ascus with jack-in-the-box release of inner sack, showing mucilaginous sheath around ascospore; e, f. ascospores (sheath indicated by arrow). — Scale bars = 10 µm.

***Ramularia proteae*** Crous & Summerell, Australas. Pl. Pathol. 29: 277. 2000

Description — Crous et al. (2000b).

*Specimen examined.* AUSTRALIA, Tasmania, Royal Tasmanian Botanical Gardens, Hobart, on leaves of *Protea longifolia*, Aug. 1999, A. Macfadyen, holotype DAR 74883, culture ex-type CPC 3075 = CBS 112161.

***Septoria protearum*** Viljoen & Crous, S. Afr. J. Bot. 64: 144. 1998

Description — Crous et al. (2004a).

*Specimens examined.* SOUTH AFRICA, Gauteng Province, Pretoria, leaves of *Protea cynaroides*, Sept. 1996, L. Viljoen, holotype PREM 55353, culture ex-type CPC 1470 = IMI 375230 = ATCC 201159 = CBS 778.97. — CANARY ISLANDS, Tenerife, leaves of *Protea* sp., 1 Apr. 2000, S. Denman, CPC 5212.

***Teratosphaeria alistairii*** (Crous) Crous & U. Braun, Stud. Mycol. 58: 9. 2007

*Basionym.* *Mycosphaerella alistairii* Crous, in Crous & Groenewald, Fungal Planet, No. 4. 2006.

*Anamorph.* *Batcheloromyces* sp.

Description — Crous & Groenewald (2006a).

*Specimen examined.* SOUTH AFRICA, Western Cape Province, Hermanus, Rotary Road, close to the Vodacom tower, on leaves of *Protea repens*, 31 Dec. 2005, P.W. Crous & A. Smith, holotype CBS H-19765, cultures ex-type CPC 12730 = CBS 120035, CPC 12731–12732.

*Notes.* — *Teratosphaeria alistairii* resembles *T. jonkershoekensis* in symptomatology on the host, but is distinct in having smaller ascospores, (9–)10–12(–13) × (2.5–)3–4 µm, and a *Batcheloromyces* anamorph (Crous & Groenewald 2006a).

***Teratosphaeria associata*** (Crous & Carnegie) Crous & U. Braun, Stud. Mycol. 58: 9. 2007

*Basionym.* *Mycosphaerella associata* Crous & Carnegie, Fung. Diversity 26: 159. 2007.

Description — Crous et al. (2007d).

*Specimens examined.* AUSTRALIA, New South Wales, South Grafton, Grafton City Council Landfill Plantation, E152°54'38", S29°46'21", on leaves of *Corymbia henryii*, 16 Feb. 2006, A.J. Carnegie, holotype CBS-H 19833, isotype DAR 78031, cultures ex-type CPC 13119 = CBS 120730, CPC 13120 (occurring with *Lembosina* sp.); NSW, Bungawalbin, Robertson Plantation,



**Fig. 9** *Teratosphaeria fibrillosa* in vivo (CBS H-19913). a. Ascomata on leaf surface, linked by stromatic tissue; b, c. germinating ascospores; d–f. asci with darkening ascospores; g–i. ascospores. — Scale bars = 10  $\mu$ m.

E153°15'39", S29°5'34", on leaves of *Corymbia variegata*, 23 Jan. 2005, A.J. Carnegie, DAR 78032, cultures CPC 13128 = CBS 120731, CPC 13129–13130 (occurring with *Lembosina* sp.); NSW, Bungawalbin, Robertson Plantation, E153°15'39", S29°5'34", on leaves of *Eucalyptus dunnii*, 14 Feb. 2006, A.J. Carnegie, cultures CPC 13108 = CBS 120732, CPC 13109–13110, 13113–13114 (occurring with *M. suberosa*); NSW, Mount Tomah Botanic Gardens, on leaves of *Protea lepidocarpodendron*, Aug. 1999, P.W. Crous & B. Summerell, JT 993, DAR 74867, cultures CPC 3115 = CBS 112627, CPC 3116 = CBS 112224, CPC 3117 = CBS 114165.

Notes — *Teratosphaeria associata* was recently described from *Eucalyptus* in Australia (Crous et al. 2007d), where it occurred in association with several other species (hence the name, *associata*). Crous et al. (2000b) reported the presence of *T. jonkershoekensis* from *Protea* spp. in Australia, where this species was observed to be an important primary pathogen (suggesting *Eucalyptus* is not a primary host of *T. associata*). Based on the results obtained here, it appears that the *Protea* isolates were incorrectly identified, and belong to the recently named *T. associata*. Furthermore, the recent description of *T. alistairii* and *T. maxii* from this host, suggest that there could be several more species that resemble *T. jonkershoekensis* in morphology and symptomatology, but which could represent distinct species.

***Teratosphaeria bellula*** (Crous & M.J. Wingf.) Crous & U. Braun, Stud. Mycol. 58: 10. 2007 — Fig. 8

*Basionym.* *Mycosphaerella bellula* Crous & M.J. Wingf., Mycotaxon 46: 20. 1993.

Descriptions — Crous & Wingfield (1993), Taylor & Crous (1998b), Crous et al. (2004a).

*Specimens examined.* SOUTH AFRICA, Western Cape Province, Stellenbosch, Stellenbosch Mountain, living leaves of *Protea repens*, 30 Aug. 1991, P.W. Crous, holotype PREM 51028; Western Cape Province, Stellenbosch, Protea Heights, Devon Valley, on *Leucospermum* sp., 6 Mar. 1998, J.E. Taylor, CBS H-20093, CPC 1815, 1816 = CBS 111699; Western Cape Province, Stellenbosch, J.S. Marais Botanical Garden, on leaves of *Protea eximia*, Apr. 1998, J.E. Taylor, epitype designated here CBS H-20094, culture ex-epitype CPC 1821 = CBS 111700; Western Cape Province, *Leucadendron* sp., 27 May 1999, L. Swart, CPC 2795 = CBS 114145; Western Cape Province, Kirstenbosch Botanical Gardens, on leaves of *Protea* sp., 1 Jan. 2008, P.W. Crous, CPC 14908.

Notes — *Teratosphaeria bellula* is characterised by having ascospores that are strongly constricted at the median septum, with small guttules, and surrounded by a prominent sheath when mounted in water. Attempts to recollect *T. bellula* for

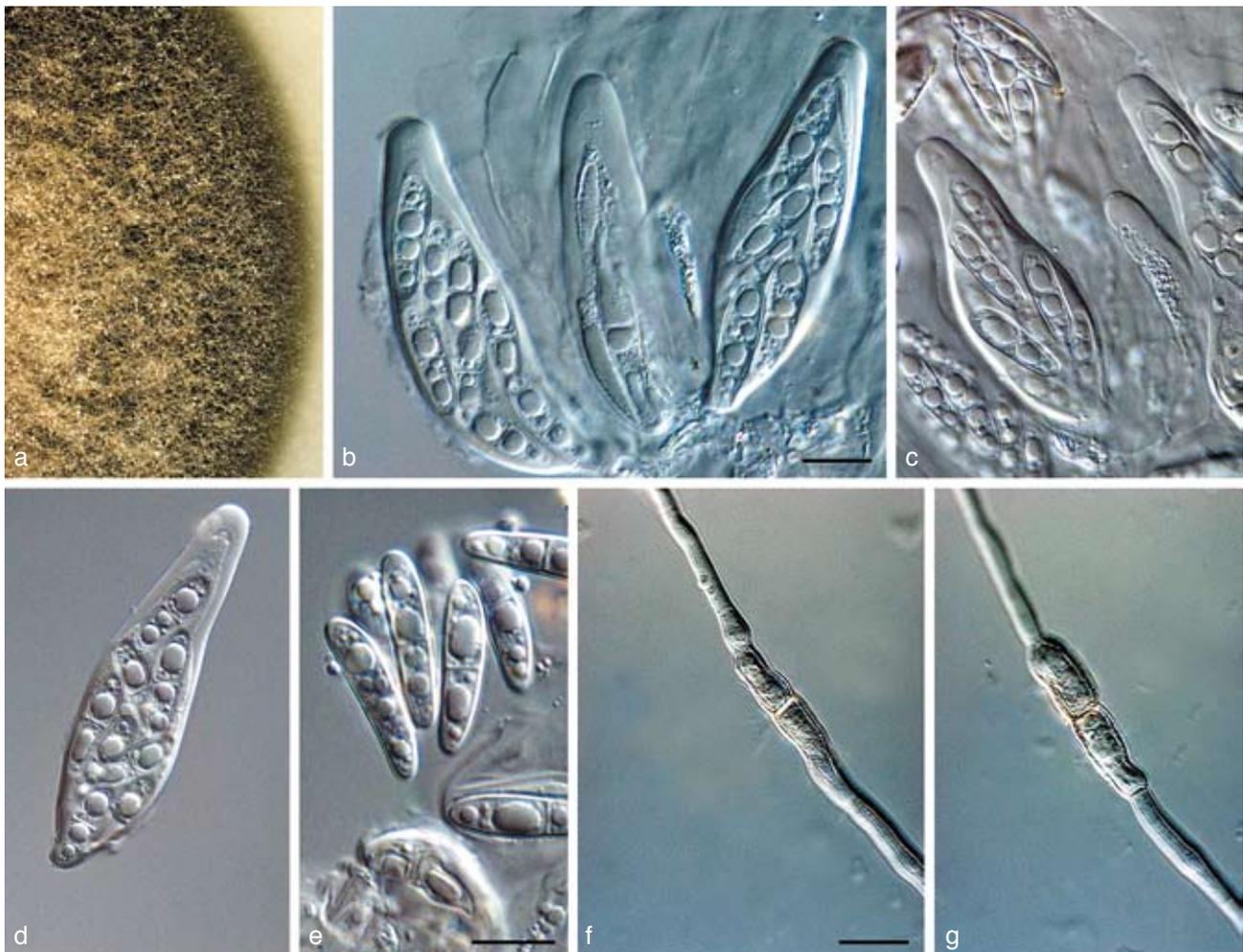


Fig. 10 *Teratosphaeria jonkershoekensis* (CBS 122897). a. Colony on OA; b–d. asci; e. ascospores; f, g. germinating ascospores. — Scale bar = 10 µm.

the purpose of epitypification have revealed it to be a species complex. As the morphology of the strains is quite similar, more isolates from other hosts in the Proteaceae need to be collected to clarify if strains from *Leucadendron* and *Leucospermum* represent *T. bellula* s.str.

***Teratosphaeria fibrillosa*** Syd. & P. Syd., Ann. Mycol. 10: 40. 1912 — Fig. 9

≡ *Mycosphaerella fibrillosa* (Syd. & P. Syd.) Joanne E. Taylor & Crous, Mycol. Res. 107: 657. 2003.

Descriptions — Taylor & Crous (1998d), Crous et al. (2004a, 2007a).

*Specimens examined.* SOUTH AFRICA, Western Cape Province, Bains Kloof near Wellington, on living leaves of *Protea grandiflora*, 26 Feb. 1911, E.M. Doidge, holotype PREM; Stellenbosch, Jonkershoek valley, S33°59'44.7", E18°58'50.6", on leaves of *Protea* sp., 1 Apr. 2007, P.W. Crous & L. Mostert, epitype CBS H-19913, culture ex-epitype CBS 121707 = CPC 13960; Cederberg, on leaves of *P. nitida*, J.E. Taylor, CPC 1876.

Notes — *Teratosphaeria fibrillosa* is the type species of *Teratosphaeria* (Teratosphaeriaceae), and is characterised by having subepidermal ascomata linked by means of stromatic tissue, apical periphyses, asci with a multi-layered endotunica, ascospores that have a sheath and turn brown and verruculose while still in their asci (Crous et al. 2007a).

***Teratosphaeria jonkershoekensis*** (P.S. van Wyk, Marasas & Knox-Dav.) Crous & U. Braun, Stud. Mycol. 58: 10. 2007 — Fig. 10

*Basionym.* *Mycosphaerella jonkershoekensis* P.S. van Wyk, Marasas & Knox-Dav., J. S. African Bot. 41: 234. 1975.

Descriptions — Van Wyk et al. (1975), Taylor & Crous (1998a), Crous et al. (2004a).

Cultural characteristics — Colonies on MEA erumpent with feathery margins and radiating superficial ridges; aerial mycelium sparse, fuscous-black (surface and reverse); on OA with smooth, regular margins and moderate pale olivaceous-grey aerial mycelium; outer margin olivaceous-black; reaching 30 mm diam after 1 mo on OA at 25 °C; sterile.

*Specimens examined.* SOUTH AFRICA, Western Cape Province, Jonkershoek, on living leaves of *Protea repens*, 9 Sept. 1971, P.S. van Wyk, holotype PREM 44830; Jonkershoek, S33°59'4.2" E18°57'16.1", on living leaves of *Protea* sp., 1 Apr. 2007, P.W. Crous & L. Mostert, epitype designated here CBS H-20095, culture ex-epitype CBS 122897 = CPC 13984 (occurring on leaf spots in association with *T. persoonii*).

Notes — No anamorph has thus far been observed for this species. Germ tubes grow parallel to the long axis of the spore, but after 48 h several germ tubes have been produced and germination is irregular. Germinating ascospores become brown, verruculose and constricted at the septum. Optimal ascospore germination occurs at 15 °C, and it is hypothesised that this low temperature requirement is necessary for successful germination and infection of leaf tissue (Swart et al. 1998, Taylor & Crous 1998a). Isolates reported from Australia as representative of *T. jonkershoekensis* (Crous et al. 2000b), are in fact representative of two morphologically similar species

that were recently described in the complex, namely *T. associata* and *T. maxii* (Fig. 1).

***Teratosphaeria knoxdavesii*** Crous, sp. nov. — MycoBank MB506595; Fig. 11

*Teratosphaeriae bellulae* similis, sed ascosporis cum tubis germinalibus parallelis ad axem longum sporae.

*Etymology.* Named in honour of Prof. P.S. Knox-Davies, who dedicated a large part of his career to studying fungal pathogens of Proteaceae.

*Leaf spots* amphigenous, irregular to subcircular, 5–12 mm diam, medium brown with a raised border, and thin, red-purple margin. *Ascomata* amphigenous, black, immersed, substomatal, up to 100 µm diam; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* paraphysate, fasciculate, bitunicate, sessile, obovoid to broadly ellipsoid, straight to slightly curved, 8-spored, 35–45 × 8–12 µm. *Ascospores* tri- to multi-seriate, overlapping, hyaline, non-guttulate, thick-walled, straight, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, prominently constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8.5–)10–11(–12) × (3–)3.5(–4) µm; germinating ascospores on MEA become brown and verruculose, germinating from both polar ends, with germ tubes parallel to the long axis of the spore, constricted at septum, but not distorting, 3.5–4 µm wide.

Cultural characteristics — Colonies on MEA erumpent, spreading, folded, with moderate, pale olivaceous-grey aerial mycelium, and smooth, catenulate, olivaceous-grey margins; reverse olivaceous-grey; colonies reaching 10 mm diam on MEA after 1 mo; on OA erumpent, spreading, grey-olivaceous, with moderate aerial mycelium and even margins; sterile.

*Specimens examined.* SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Protea* sp., 6 Jan. 2008, P.W. Crous & M. Crous, holotype CBS H-200104, cultures ex-type CBS 122898 = CPC 14960, 14961, 14962 (occurring on leaf spots in association with *Coleroa senniana*); Kirstenbosch Botanical Garden, on living leaves of *Protea* sp., 6 Jan. 2008, P.W. Crous & M. Crous, CPC 14905–14907.

Notes — Although several small-spored species of *Teratosphaeria* are known from Proteaceae, *T. knoxdavesii* is distinct in having a very characteristic ascospore germination pattern, germinating from both polar ends, with spores becoming constricted, brown, and verruculose, germ tubes growing parallel to the long axis of the spore.

***Teratosphaeria maculiformis*** (G. Winter) Joanne E. Taylor & Crous, IMI Descriptions of Fungi and Bacteria No. 1346. 1998 — Fig. 12

*Basionym.* *Didymella maculiformis* G. Winter, Hedwigia 23: 169. 1884.

≡ *Oligostroma maculiformis* (G. Winter) Doidge, Bothalia 1: 31. 1921.

= *Oligostroma proteae* Syd., Ann. Mycol. 12: 265. 1914.

≡ *Mycosphaerella proteae* (Syd.) Arx, Beitr. Kryptogamenfl. Schweiz 11(2): 357. 1962.

= *Euryachora maculiformis* Nel, Annals of the University of Stellenbosch 20 Ser. A (2): 11. 1942.

Descriptions — Taylor & Crous (1998c), Crous et al. (2004a).

Cultural characteristics — Colonies black and erumpent, devoid of aerial mycelium, slow-growing; 3 mm diam after 6 mo



**Fig. 11** *Teratosphaeria knoxdavesii* (CBS 122898). a. Leaf spot on *Protea* sp.; b, c. asci; d. germinating ascospores; e, f. close-up of ascospores in asci. — Scale bars = 10 µm.

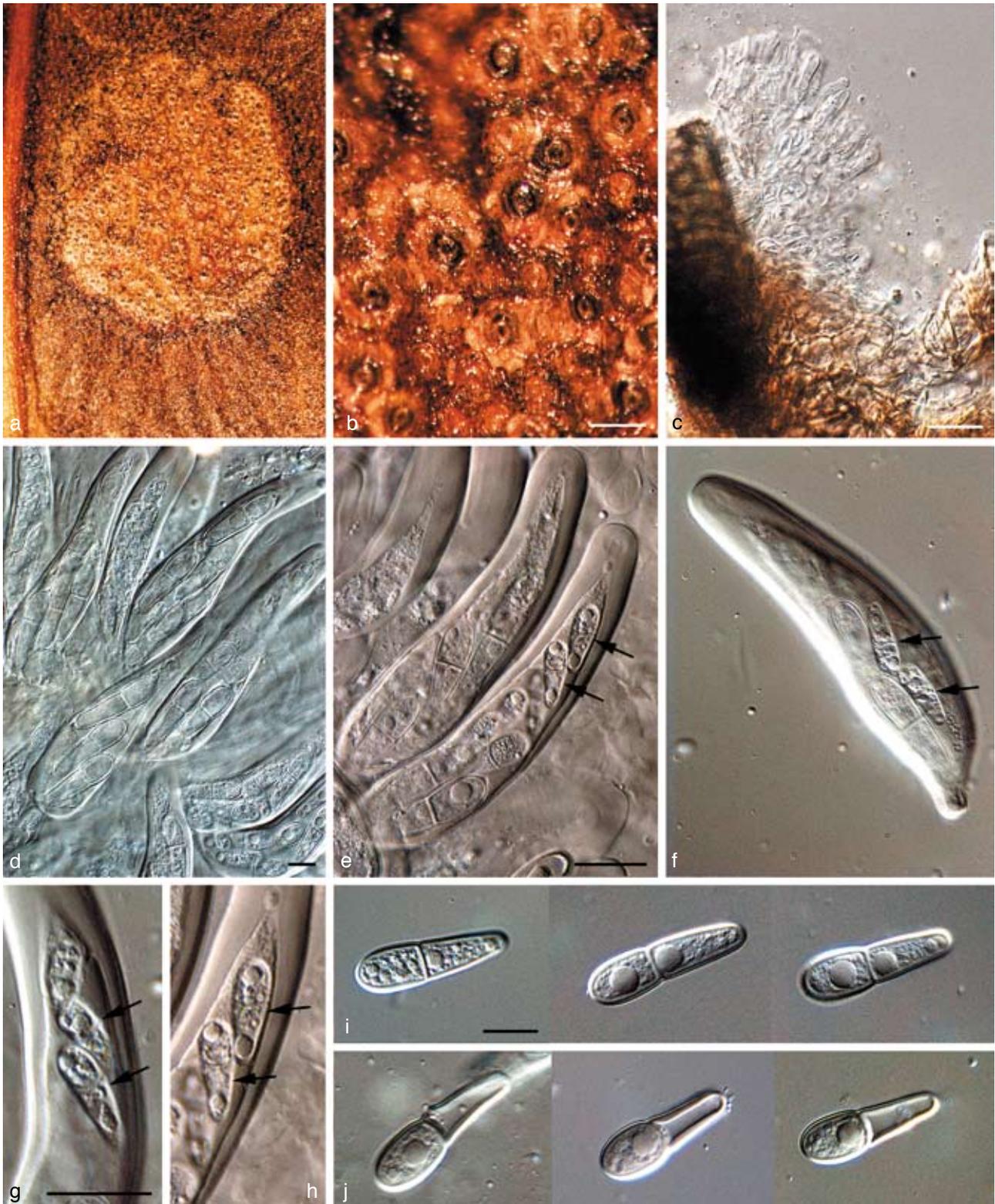
at 25 °C on PDA; colonies did not survive preservation, and numerous subsequent attempts have been unsuccessful in cultivating it again. Germinating ascospores died on OA, MEA and PCA. Ascospores become brown at germination on PDA, and produce germ tubes parallel to the long axis of the ascospore, but the spore wall does not become verruculose.

*Specimens examined.* SOUTH AFRICA, Kentani, on *Protea flanaganii*, 17 July 1912, Pegler, 5163 deposited in PREM (type of *Oligostroma proteae*); Cape Town, *Protea grandiflora*, June 1884, MacOwan (Winter, Fungi Eur. Extraeur. Exs. 3056, type of *Didymella maculiformis*); Western Cape Province, Knysna, S34°1'49.9", E23°1'6.0", on leaves of *Protea* sp., 4 Jan. 2008, P.W. Crous & K.L. Crous, CBS H-20096 (used to harvest ascospores for DNA isolation).

**Notes** — Ascospores were observed to disarticulate at the septum, with each cell frequently becoming 1-septate and germinating (on host tissue) (Crous et al. 2004a). Although the spores are thick-walled, they also seem to get punctured quite easily, in which case cytoplasm leaks out of the damaged

ascospore cell, and the spore sheds this cell by disarticulating relatively easily at the septum. In some cases, this separation was observed even in the absence of ascospore cell damage. This interesting mechanism has not been observed in this group of fungi before, and may be linked to the relatively large ascospore size of *T. maculiformis*. Ascospores often adhere to leaf hairs, with the resulting hyphae growing superficially until they infect the host. Upon germination ascospores become brown and produce germ tubes parallel to the long axis of the ascospore, but the spore wall does not become verruculose. A further interesting phenomenon observed in *T. maculiformis* is the fact that asci frequently have one or two microascospores that appear completely underdeveloped, and much smaller than the 'normal' ascospores. As far as we could establish, these microascospores are viable, and germinate along with the normal, larger ascospores.

Ascospores on the leaf surface are frequently hyperparasitised by a species of *Cladosporium*. A similar *Cladosporium* species



**Fig. 12** *Teratosphaeria maculiformis* in vivo (CBS H-20096). a. Leaf spot on *Protea* sp.; b. close-up of substomatal ascomata; c. periphysoids; d–h. asci with ascospores (microascospores arrowed); i. mature ascospores; j. disarticulating ascospores. — Scale bars = 10 µm.



**Fig. 13** *Teratosphaeria marasasii* (CBS 122899). a. Leaf spot on *Protea* sp.; b, c. germinating ascospores; d. fasciculate asci viewed from above; e. asci with darkening ascospores; f, g. asci; h. ascospores. — Scale bars = 10 µm.

(a member of the *C. cladosporioides* species complex) was also observed to grow on exuding ascospore masses of *T. proteae-arboreae*. Due to the extremely slow growth of *T. maculiformis*, this species could not be deposited in the CBS culture collection. Ascospores germinate on PDA (though they fail to do so upon MEA, OA or SNA), but although germ tubes elongate and branch, they never form mycelium, and stay recognisable as single germinating ascospores, even 6–8 mo after they started germinating. At a certain point (3–5 mo after the onset of germination), all growth ceases, though the spore and the hyphae do not dissolve, but still appear viable, though they enter a dormant phase. The DNA sequence provided in this study was obtained from harvesting a mass of discharged, germinated ascospores (4 mo after ascospore discharge), and extracting their DNA using a commercial DNA isolation kit (E.Z.N.A. Forensic DNA Isolation Kit, Omega Bio-Tek).

***Teratosphaeria marasasii* Crous, sp. nov.** — MycoBank MB506596; Fig. 13

*Teratosphaeriae jonkershoekensis* similis, sed maculis minoribus et ascosporis brevioribus, (15–)16–18(–22) × (3.5–)4(–5) µm.

**Etymology.** Named in honour of Prof. W.F.O. Marasas, who was instrumental in naming *T. jonkershoekensis*, which this species closely resembles in morphology and symptomatology.

**Leaf spots** amphigenous, circular, 2–3 mm diam, pale brown to grey-brown, with a thin, raised, dark brown border. **Ascomata** amphigenous, black, immersed, substomatal, up to 120 µm diam; wall consisting of 2–3 layers of medium brown *textura angularis*. **Asci** paraphysate, fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoid, straight to slightly curved, 8-spored, 35–50 × 11–15 µm; with a well-developed ocular chamber, and multi-layered endotunica. **Ascospores** tri- to multi-seriate, overlapping, hyaline, guttulate, thick-walled, straight, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, not to slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (15–)16–18(–22) × (3.5–)4(–5) µm, becoming brown and verruculose in the asci; germinating ascospores on MEA become brown and verruculose, distorting, with 2–4 germ tubes, growing irregular to the long axis, 6–10 µm wide.

**Cultural characteristics** — Colonies after 1 mo on MEA erumpent, spreading, with sparse aerial mycelium, and smooth, entire margins; surface olivaceous-grey in middle, iron-grey in outer region; reverse greenish black, 10–15 mm diam; on OA with moderate aerial mycelium, and smooth, regular margins, olivaceous-grey; sterile.

**Specimen examined.** SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Protea* sp., 6 Jan. 2008, P.W. Crous & M. Crous, holotype CBS H-20105, cultures ex-type CBS 122899 = CPC 14889, 14890, 14891 (occurring on leaf spots in association with *Coleroa senniana*).

**Notes** — *Teratosphaeria marasasii* closely resembles *T. jonkershoekensis* in morphology (ascospores 15–23 × 4–6 µm), and symptomatology. It is distinct, however, by producing smaller leaf spots and having shorter ascospores.

***Teratosphaeria maxii* (Crous) Crous & U. Braun, Stud. Mycol. 58: 10. 2007**

**Basionym.** *Mycosphaerella maxii* Crous, in Crous & Groenewald, Fungal Planet No. 6. 2006.

**Description** — Crous & Groenewald (2006b).

**Specimens examined.** AUSTRALIA, New South Wales, Mount Tomah Botanic Gardens, on leaves of *Protea* sp., Aug. 1999, P.W. Crous & B. Summerell, DAR 74870, CBS H-20097, cultures CPC 3321 = CBS 112231, CPC 3322 = CBS 112496, CPC 3323 = CBS 112232. — SOUTH AFRICA, Western Cape Province, Bettie's Bay, Harold Porter Botanical Garden, on leaves of *Protea repens*, 4 Jan. 2006, M. Crous & P.W. Crous, holotype CBS H-19774, cultures ex-type CPC 12805 = CBS 120137, CPC 12806–12807; Hermanus, Rotary road on top of mountain, on leaves of *P. repens*, 31 Dec. 2005, P.W. Crous, CPC 12943–12945.

**Notes** — *Teratosphaeria maxii* is associated with leaf spot symptoms reminiscent of those of *T. alistairii*, *T. bellula* and *T. jonkershoekensis*. It is distinct from *T. alistairii* and *T. bellula* in its larger ascospores, (15–)17–19(–22) × 4–5(–6) µm, which closely resemble those of *T. jonkershoekensis* in size. However, ascospores of *T. maxii* do not darken during germination, and colonies have a peculiar, thick-walled, budding aerial mycelium, which eventually form clumps of orange crystals, which has never been observed in *T. jonkershoekensis* (Crous & Groenewald 2006b). It is interesting to note, however, that several strains reported as '*Mycosphaerella jonkershoekensis*' from Australia (Crous et al. 2000b), are in fact *T. maxii*.

***Teratosphaeria microspora* Joanne E. Taylor & Crous, Mycol. Res. 104: 631. 2000**

= *Mycosphaerella microspora* (Joanne E. Taylor & Crous) Joanne E. Taylor & Crous, Mycol. Res. 107: 657. 2003.

**Anamorph.** *Catenulostroma microsporium* (Joanne E. Taylor & Crous) Crous & U. Braun, Stud. Mycol. 58: 10. 2007.

**Basionym.** *Trimmatostroma microsporium* Joanne E. Taylor & Crous, Mycol. Res. 104: 631. 2000.

**Descriptions** — Taylor & Crous (2000), Crous et al. (2004a).

**Specimens examined.** SOUTH AFRICA, Western Cape Province, Somerset West, Hilly Lands Farm, on a living leaf of a *Protea cynaroides*, 21 July 1998, S. Denman & J.E. Taylor, PREM 56207a, holotype of teleomorph, culture ex-type CPC 1960 = CBS 101951; PREM 56207b, holotype of anamorph; Stellenbosch, J.S. Marais Nature Reserve, on a living leaf of *P. cynaroides*, July 1998, L. Swart, CPC 1832 = CBS 110890; Somerset West, on a living leaf of *P. cynaroides*, July 1998, J.E. Taylor, CPC 1848 = CBS 111031; Stellenbosch, J.S. Marais Nature Reserve, on leaves of *P. cynaroides*, 30 Aug. 1996, P.W. Crous, CPC 1597 = CBS 111697.

**Notes** — *Teratosphaeria microspora* is presently the only known teleomorph connection for species of *Catenulostroma* (Crous et al. 2007a). *Catenulostroma microsporium* is part of a species complex that resembles *C. abietis*, which is known from needles of various species of Gymnospermae. Ascospores of *T. microspora* germinate on MEA from both cells, and germ tubes grow parallel to the long axis of the spore. There is no constriction at the septum and the spores do not darken or become verruculose upon germination. Isolate CBS 111697 is listed here under *T. microspora*, but has somewhat larger conidia, and probably represents a cryptic species. More collections are required to fully elucidate the variation present in this species complex.



**Fig. 14** *Teratosphaeria parva* (CBS 122892). a. Jack-in-the-box separation of layers in bitunicate ascus; b. ascus; c–e. germinating ascospores; f, g. ascospores. — Scale bars = 10 µm.

***Teratosphaeria parva*** (R.F. Park & Keane) Crous & U. Braun, *Stud. Mycol* 58: 10. 2007 — Fig. 14

*Basionym.* *Mycosphaerella parva* R.F. Park & Keane, *Trans. Brit. Mycol. Soc.* 79: 99. 1982.

= *Mycosphaerella grandis* Carnegie & Keane, *Mycol. Res.* 98: 414. 1994.

**Descriptions** — Carnegie & Keane (1994), Crous (1998).

*Specimens examined.* AUSTRALIA, Victoria, Nowa Nowa, on leaves of *Eucalyptus globulus*, July 1981, R.F. Park, holotype IMI 263258 (published as 263358); Victoria, Otway Ranges, (near Gellibrand), latitude: -38.568412, longitude: 143.539586, elevation: 175 m, on leaves of *E. globulus*, Sept. 2005, I. Smith, epitype designated here CBS H-20098, cultures ex-epitype CPC 12421 = CBS 122892, CPC 12422, 12423. — SOUTH AFRICA, Western Cape Province, Stellenbosch, Stellenbosch Mountain, on leaves of *Protea repens*, 20 Sept. 1995, P.W. Crous, CPC 1217 = CBS 114761; Western Cape Province, on leaves of *P. repens*, 12 Nov. 1998, G. Matthews, CBS H-20099, CPC 2118–2120; Western Cape Province, Botmaskop, on leaves of *P. repens*, Nov. 2007, L. Mostert, CBS H-20101, CPC 14898 = CBS 122893, CPC 14899, 14900; Western Cape Province, Jonkershoek, S33°59'11.2" E18°57'14.7", on living leaves of *P. nitida*, 1 Apr. 2007, P.W. Crous & L. Mostert, CPC 13896 = CBS 122894, CPC 13897, 13898.

***Teratosphaeria persoonii*** Crous & L. Mostert, *sp. nov.* — MycoBank MB506597; Fig. 15

*Teratosphaeriae bellulae* similis, sed ascosporis sine vagina gelatinosa, ascosporis quidem in asco brunnescentibus.

*Etymology.* Named after Christiaan Hendrik Persoon (31 Dec. 1761 – 16 Nov. 1836) who was born in South Africa, but left for Europe at the age of 12, never to return. His most important work was *Synopsis Fungorum*, published in 1801, which formed the basis of modern mycology.

*Leaf spots* amphigenous, subcircular to circular, 2–7 mm diam, pale brown with a raised, pale to medium brown border. *Ascomata* amphigenous, black, immersed, substomatal, up to 120 µm diam; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, obovoid to ellipsoid, straight to slightly curved, 8-spored, 23–40 × 8–11 µm. *Ascospores* tri- to multi-seriate, overlapping, hyaline, with 1–2 large guttules in every cell, thick-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, prominently constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (7–)8–10(–11) × 3–3.5(–4) µm; ascospores commonly observed to turn brown and verruculose in asci. Germinating ascospores on MEA become brown and verruculose, and one to several germ tubes grow at irregular angles to the long axis of the spore.



**Fig. 15** *Teratosphaeria personii* (CBS 122895). a. Colony on OA; b, c. bitunicate asci; d. germinating ascospores; e. ascospores. — Scale bars = 10 µm.

**Cultural characteristics** — Colonies on MEA erumpent with moderate aerial mycelium and smooth, regular margins; surface pale grey-olivaceous to grey-olivaceous; reverse grey-olivaceous, reaching 10 mm diam after 2 wk on MEA; sterile.

**Specimens examined.** SOUTH AFRICA, Western Cape Province, Jonkershoek, S33°59'4.2" E18°57'16.1", on living leaves of *Protea* sp., 1 Apr. 2007, P.W. Crous & L. Mostert, holotype CBS H-20102, cultures ex-type CPC 13972 = CBS 122895, CPC 13973, 13974 (occurring on leaf spots in association with *T. jonkershoekensis*); Western Cape Province, De Hoop Nature Reserve, Bredasdorp, S34°27'33" E20°27'04", on leaves of *Euchaetis meridionalis* (whole leaf turns brown, covered with brown-black mycelium; leaf and stem necrosis often starts where petiole attaches to branch; several fungi appear to be present, incl. species of *Phoma* and *Leptosphaeria*), 29 June 2006, A.R. Wood, CBS H-20103, culture STE-U 6389 = CPC 14846 = CBS 122896.

**Notes** — Although *T. personii* is morphologically very similar to *T. bellula* with regards to ascospore dimensions and symptomatology, it can be distinguished by having ascospores that have large, prominent guttules, are relatively thick-walled, commonly turn brown and verruculose while still in asci, and lack a mucoid sheath when mounted in water. No anamorph

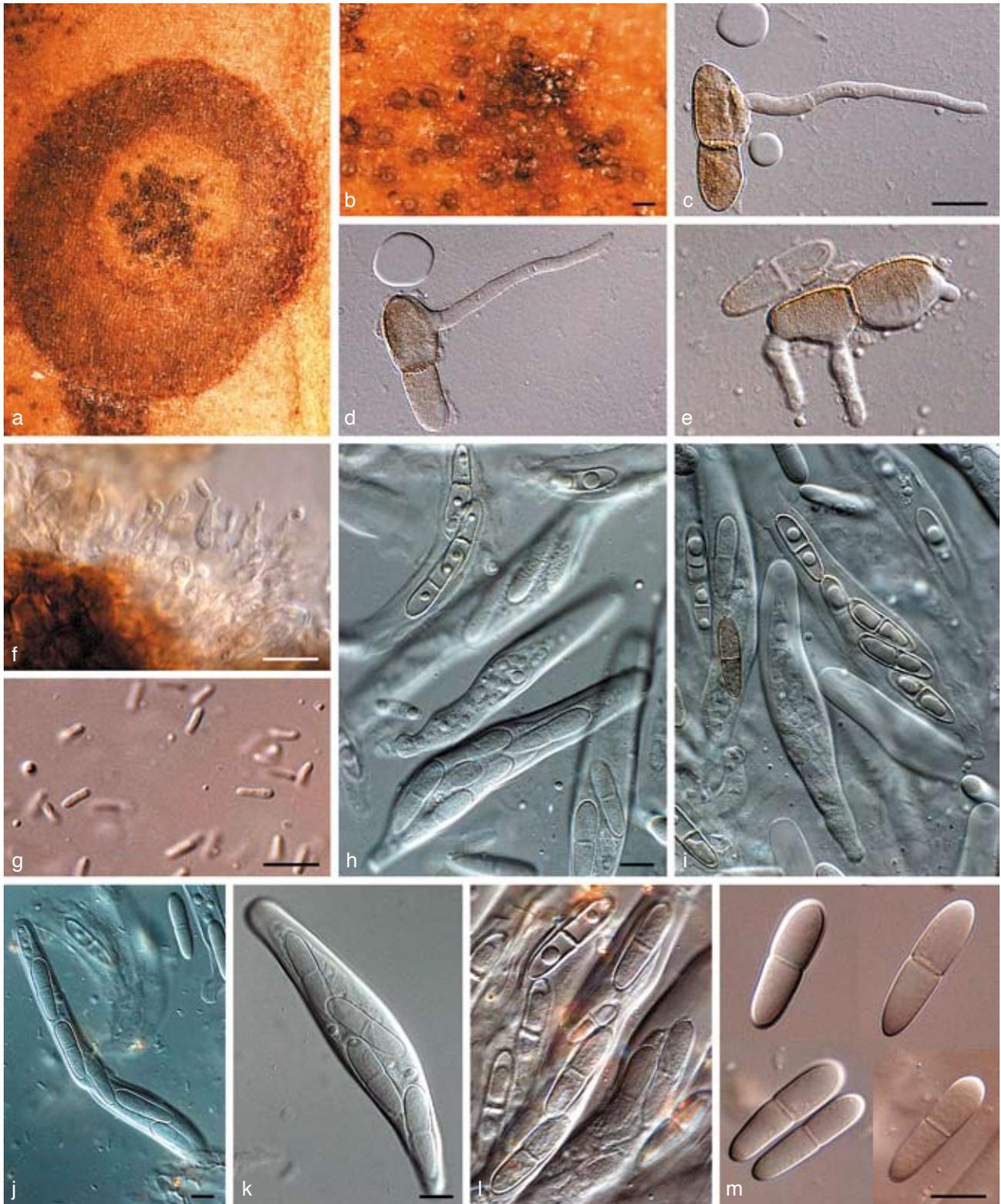
has been observed. *Teratosphaeria personii* also has a wider host range, occurring on *Protea* as well as *Euchaetis*.

***Teratosphaeria proteae-arboreae*** P.S. van Wyk, Marasas & Knox-Dav., J. S. Afr. Bot. 41: 232. 1975 — Fig. 16

≡ *Mycosphaerella proteae-arboreae* (P.S. van Wyk, Marasas & Knox-Dav.) Joanne E. Taylor & Crous, Mycol. Res. 107: 657. 2003.

**Descriptions** — Taylor & Crous (1998e), Crous et al. (2004a).

**Leaf spots** initially indistinct, chlorotic, raised, circular, with catenulate margins, with substomatal ascomata appearing as black spots in lesions; spots not extending through lamina, becoming black in centre, later extending so that the whole spot appears black, 1–3 mm diam. **Ascomata** epiphyllous or hypophyllous, globose, ostiolate, non-papillate, black, singular, gregarious; in section substomatal, subepidermal, globose to slightly pyriform, periphysoids lining the ostiole and the upper ascoma wall, up to 250 µm diam. **Peridium** consisting of three layers of



**Fig. 16** *Teratosphaeria proteae-arboreae* (CPC 12952). a. Leaf spot on *Protea nitida*; b. close-up of substomatal ascomata; c–e. germinating ascospores; f. spermatophores; g. spermatia; h–l. asci with darkening ascospores; m. ascospores. — Scale bars: b = 200  $\mu$ m, all others = 10  $\mu$ m.

compressed, brown *textura angularis*. Asci narrowly ellipsoid to obovoid, tapering abruptly to a small pedicel, narrowing to a rounded apex with a distinct ocular chamber, 2–4 µm diam, mainly straight, fasciculate, bitunicate with fissitunicate dehiscence, with a multi-layered endotunica, 80–120 × 14–20 µm; asci predominantly 8-spored, with two ascospores being underdeveloped. Ascospores bi- to tri-seriate, fusoid-ellipsoidal, straight to slightly curved, 1-septate, prominently guttulate, with septum median or slightly supra-median, constricted, broadest in the middle of the apical cell and tapering to the lower end, hyaline, but becoming brown and verruculose in asci, (17–)25–30(–35) × (5–)6–7(–9) µm. *Spermatogonia* intermixed with ascomata, similar in morphology. *Spermatophores* producing spermatia that are bacilliform, smooth, hyaline with obtuse ends, 4–6 × 1.5 µm. Germinating ascospores on MEA after 24 h with one or several germ tubes, germinating from polar ends, parallel to the long axes, or lateral, from sides of spore; ascospores distorting, brown, verruculose, with outer, brown, verruculose layer frequently becoming separate from inner, hyaline layer.

**Cultural characteristics** — Colonies on MEA erumpent with sparse aerial mycelium and even, catenulate margins; centre hazel, margins isabelline to sepia; reverse fuscous-black; reaching 12 mm diam after 1 mo. On OA erumpent with sparse aerial mycelium and even, catenulate margins, pale olivaceous-grey to olivaceous-grey, reaching 10 mm diam after 1 mo at 25 °C.

**Specimens examined.** SOUTH AFRICA, Western Cape Province, Jonkershoek, Houttuyn, on living leaves of *Protea arborea*, 21 June 1971, P.S. van Wyk, holotype PREM 44801; Western Cape Province, Bettie's Bay, Harold Porter Botanical Garden, on leaves of *P. nitida*, 4 Jan. 2006, M.K. Crous & P.W. Crous, epitype designated here CBS H-20106, cultures ex-epitype CPC 12952–12954; Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Protea* sp., 6 Jan. 2008, P.W. Crous & M. Crous, CBS H-20107, cultures CPC 14963–14965.

**Notes** — *Teratosphaeria proteae-arboreae* is commonly associated with prominent leaf spots on *Protea nitida*. The present collection was obtained from the type location, and could subsequently be designated as epitype to clarify its phylogenetic relationship to other species of *Teratosphaeria*. It is interesting to note that as observed in *T. maculiformis*, asci frequently have one or two underdeveloped microascospores. The reason for the smaller ascospores occurring in asci along with normally developed ascospores is unknown. It is thus tempting to speculate that microascospores (which frequently aggregate at the ascus apex in *T. proteae-arboreae* and *T. maculiformis*) could have a different ecological role, being the first to be discharged. Alternatively, these could simply be weakly developed ascospores that are not viable. Further collections would be required to clarify this aspect.

#### ***Teratosphaeria* sp.**

**Cultural characteristics** — Colonies on MEA erumpent, with sparse aerial mycelium and smooth, catenulate margins; grey-olivaceous in centre, olivaceous-grey at margins and underneath; colonies reaching 7 mm diam after 2 wk at 25 °C.

**Specimen examined.** SOUTH AFRICA, Western Cape Province, Jonkershoek, S33°59'4.2" E18°57'16.1", on living leaves of *Protea nitida*, 1 Apr. 2007, P.W. Crous & L. Mostert, CPC 13917–13919.

**Notes** — This species, which is closely related to *T. proteae-arboreae*, could not be described here due to insufficient material. It was isolated from small, fusoid-ellipsoidal ascospores, 10 × 3.5 µm, that became constricted and distorted upon germination, and germinated from one end only. Based on its DNA phylogeny and ascospore germination pattern, it clearly represents yet another undescribed species of *Teratosphaeria*.

#### ***Teratosphaeria* sp.**

**Cultural characteristics** — Colonies on PDA erumpent, irregular, with smooth, catenulate margins and sparse aerial mycelium, iron-grey (surface), olivaceous-black (reverse); on OA spreading with smooth, regular margins; aerial mycelium sparse, iron-grey (surface); reaching 20 mm diam after 1 mo on OA at 25 °C; sterile.

**Specimen examined.** SOUTH AFRICA, Western Cape Province, Jonkershoek, S33°59'4.2" E18°57'16.1", on living leaves of *Protea nitida*, 1 Apr. 2007, P.W. Crous & L. Mostert, CPC 13963–13965.

**Notes** — This species, which has ascospores that distort and turn brown upon germination, occurred on spots in association with *T. fibrillosa* and *T. proteae-arboreae*. It could not be described, however, due to paucity of material.

#### ***Teratosphaeria* sp.**

**Cultural characteristics** — Colonies on MEA erumpent, folded, with sparse to moderate aerial mycelium and smooth, catenulate to feathery margins; grey-olivaceous in centre, olivaceous-grey at margins and underneath; colonies reaching 12 mm diam after 1 mo at 25 °C.

**Specimen examined.** SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Protea* sp., 6 Jan. 2008, P.W. Crous & M. Crous, CPC 14957–14959.

**Notes** — This species was isolated from ascospores that turned brown and distorted upon germination. It could not be described, however, due to paucity of material.

#### ***Teratosphaeria* sp.**

**Cultural characteristics** — Colonies on PDA erumpent with smooth margins and moderate aerial mycelium; isabelline in middle, becoming olivaceous towards margin; dark mouse-grey underneath. On OA spreading with smooth, catenulate margins and moderate olivaceous-grey aerial mycelium; iron-grey in outer region; colonies reaching 40 mm diam after 1 mo on OA at 25 °C; sterile.

**Specimen examined.** PORTUGAL, on leaves of *Protea repens*, 1 Jan. 2007, M.F. Moura, CPC 13981.

**Notes** — This species was isolated from leaf spots on living leaves of *Protea repens*, but could not be described due to paucity of the material. Based on its cultural characteristics and DNA phylogeny, it appears to be distinct from the *Teratosphaeria* spp. presently known from Proteaceae

## DISCUSSION

In their treatment of the *Mycosphaerella* diseases associated with Proteaceae, Crous et al. (2004a) listed 13 species of *Mycosphaerella* (incl. *Teratosphaeria*) and 18 associated anamorph species. Since the publication of the compendium, several species have been investigated by means of DNA molecular analyses, showing the morphological species concepts used in the past to have been too wide, obscuring the presence of several novel taxa. A good example of this was the report of *M. jonkershoekensis* from Australia based on symptomatology, morphology, and ascospore germination patterns (Crous et al. 2000b), which based on DNA techniques employed here, was revealed to in fact represent two species that were newly described in the *T. jonkershoekensis* complex, namely *T. associata* and *T. maxii* (Crous & Groenewald 2006a, b). A further significant step has been the acknowledgement of Teratosphaeriaceae as being distinct from Mycosphaerellaceae (Crous et al. 2007a), which made it essential to re-evaluate all species occurring on Proteaceae.

The present study also addressed the problem that many of the older names known from Proteaceae have never been studied in culture, and thus were omitted from previous DNA studies. These species had to be recollected, compared to the holotype specimens, and epitype specimens designated, so that ex-epitype cultures could become available for DNA analyses. This was achieved for most, but not all species, namely *Batcheloromyces leucadendri*, *B. proteae*, *Catenulostroma macowanii*, *Mycosphaerella marksii*, *Teratosphaeria bellula*, *T. jonkershoekensis*, *T. parva*, and *T. proteae-arboreae*. Several species are also newly described, namely *Batcheloromyces sedgefieldii*, *Catenulostroma wingfieldii*, *Dissoconium proteae*, *Teratosphaeria knoxdavesii*, *T. marasasii* and *T. persoonii*.

While *Mycosphaerella* and *Teratosphaeria* species are generally accepted to be host specific (Crous & Braun 2003), several species have now been shown to have wider host ranges than was commonly accepted (Burgess et al. 2007, Crous et al. 2004c, 2007d, Crous & Groenewald 2005). These include *M. communis*, which is known to have a wider host range, including *Eucalyptus* (South Africa, Spain, New Zealand), *Musa* (Trinidad) as well as *Protea magnifica* in Australia; *Mycosphaerella konae* (*Leucospermum*, Hawaii), which also occurs on *Eucalyptus* in Thailand (Crous et al. 2007d), *M. marksii* (*Eucalyptus*, Australia, Bolivia, China, Ecuador, Ethiopia, Papua New Guinea, New Zealand, South Africa, Spain, Tanzania, Uruguay), which also occurs on *Leucadendron* on the Madeira Islands, and *Musa* in Mozambique (Arzanlou et al. 2008). *Teratosphaeria associata*, an apparent opportunist on *Eucalyptus* in Australia, appears to be a primary pathogen on *Protea* in the same country (Crous et al. 2000b, 2007d). *Teratosphaeria parva* (on *Eucalyptus* in Australia, Chile, Ethiopia, Portugal, South Africa and Spain), is also found on *Protea* in South Africa. Wider host ranges may also be applicable to *T. microspora* and *Septoria protearum*, though species concepts in these genera are still unresolved.

Based on DNA analysis of single ascospore cultures of *Mycosphaerella* spp. derived from various hosts and substrates, Crous & Groenewald (2005) introduced the pogo stick hypo-

thesis to explain the fact that well-known plant pathogenic species of *Mycosphaerella* (incl. *Teratosphaeria*) are frequently encountered on 'non-hosts', where they appear to colonise leaf spots of other *Mycosphaerella* species that are primary pathogens on these hosts, to enable them to produce a limited amount of progeny to enable onward dispersal. This 'host jumping' phenomenon appears to be much more common in *Mycosphaerella* and *Teratosphaeria* than generally accepted in literature, revealing these necrotrophic species to be able to grow also as saprobes on dead tissue, enabling them to disperse further in an attempt to locate their ideal hosts.

Many species of *Mycosphaerella* and *Teratosphaeria*, which were commonly accepted as host-specific necrotrophic pathogens, thus appear to also exhibit a facultative saprobic behaviour. This indicates that the definitions of 'necrotroph' or 'saprobe' do not clearly define all species of *Mycosphaerella* and *Teratosphaeria*, as some have obviously retained the ability to also grow on dead tissue when they lose the connection to their real host.

The exact mechanism that allows species of *Mycosphaerella* and *Teratosphaeria* to co-colonise the same host tissue, leading to several species co-occurring in the same leaf spot (Crous & Wingfield 1996, Crous 1998), also deserves further study.

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# *Eucalyptus* microfungi known from culture. 1. *Cladoriella* and *Fulvoflamma* genera nova, with notes on some other poorly known taxa

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**Abstract:** A study of microfungi associated with living *Eucalyptus* leaves and leaf litter revealed several novel and interesting taxa. *Cladoriella eucalypti* gen. et sp. nov. is described as a *Cladosporium*-like genus associated with litter collected in South Africa, while *Fulvoflamma eucalypti* gen. et. sp. nov. is newly described from leaf litter collected in Spain. Beta-conidia are newly reported for species of *Pestalotiopsis*, namely *Pestalotiopsis disseminata* in New Zealand, and a *Pestalotiopsis* sp. from Colombia. *Satchmopsis brasiliensis* is reported from litter in Colombia and Indonesia, while *Torrendiella eucalypti* is reported from leaf litter in Indonesia, and shown to have a *Sporothrix*-like anamorph. *Leptospora rubella* is reported from living *Eucalyptus* leaves in Colombia, where it is associated with leaf spots of *Mycosphaerella longibasalis*, while *Macrohilum eucalypti* is reported from leaf spots of *Eucalyptus* in New Zealand.

**Taxonomic novelties:** *Cladoriella eucalypti* Crous gen. et sp. nov., *Fulvoflamma eucalypti* Crous gen. et sp. nov.

**Key words:** *Cladosporium*, *Eucalyptus*, *Leptospora*, *Macrohilum*, microfungi, *Pestalotiopsis*, *Satchmopsis*, systematics.

## INTRODUCTION

The genus *Eucalyptus* (*Myrtaceae*) contains approximately 700 species (Potts & Pederick 2000), most of which are known to host a range of incredibly diverse and interesting microfungi (Crous *et al.* 1989, Sankaran *et al.* 1995). In recent years there have been numerous papers listing and describing the plant-pathogenic fungi occurring on eucalypts in the various countries where these trees are grown as ornamentals, or planted in plantations for timber and paper fibre (Old & Davison 2000, Park *et al.* 2000). As the majority of the plant-pathogenic fungi are known from culture, this has enabled plant pathologists to revise numerous important pathogen complexes such as *Mycosphaerella* leaf blotch (Crous 1998, Crous *et al.* 2000, 2001, 2004a, Hunter *et al.* 2004), *Cylindrocladium* leaf blight (Crous 2002, 2004b), *Cryphonectria* canker (Gryzenhout *et al.* 2004), *Botryosphaeria* canker (Slippers *et al.* 2004a–c), *Coniella* (Van Niekerk *et al.* 2004), *Cytospora* (Adams *et al.* 2005), and *Harknessia* leaf spots (Lee *et al.* 2004), to name but a few. In contrast, however, the saprobic microfungi have largely been neglected, and in spite of checklists and descriptions, very few are in fact known from culture, or are represented in freely accessible culture collections. As such, many of these diverse genera will never be represented in international initiatives like Assembling the Tree of Life (AToL), or the Consortium for the Barcoding of Life (CBOL), and biologists will remain ignorant as to their distribution, host range, importance and various ecological roles.

Because the eucalypt microbial community is so rich and diverse, and appears to harbour numerous undescribed and relatively unstudied fungal species, it was decided to focus on this host substrate to obtain cultures for inclusion in larger projects and international

initiatives such as those cited above. The current paper represents the first in a series aimed at describing eucalypt microfungi from culture, and recollecting and culturing those already known (Sankaran *et al.* 1995), to help elucidate their taxonomy, and resolve their phylogenetic relationships.

## MATERIALS AND METHODS

### Isolates

Leaf litter as well as living, symptomatic leaves were chosen for study. Leaves were incubated in moist chambers (Petri dishes with moist filter paper on the laboratory bench), and inspected daily for microfungi. Hyphomycetes and coelomycetes were cultured on 2 % malt extract agar (MEA) plates (Gams *et al.* 1989) by obtaining single conidial colonies as explained in Crous (2002). Single germinating ascospores were obtained and cultured using the technique as explained in Crous (1998). Colonies were sub-cultured onto fresh MEA, oatmeal agar (OA), cornmeal agar (CMA) and carnation leaf agar (CLA) plates (Gams *et al.* 1989) and incubated at 25 °C under continuous near-ultraviolet light, to promote sporulation.

### DNA amplification and sequence analysis

Genomic DNA was isolated from fungal mycelium grown on malt extract agar plates following the protocol of Lee & Taylor (1990). The primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify part (ITS) of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene (LSU). PCR conditions and protocols were treated and generated

as explained in Crous *et al.* (2004a). Part of the 18S rRNA gene was amplified and sequenced as explained in Braun *et al.* (2003) and part of the 28S rRNA gene as explained in Lee *et al.* (2004). ITS sequences were subjected to a nucleotide-nucleotide BLAST (Altschul *et al.* 1997) of the NCBI sequence database (BLAST-N 2.2.11; <http://www.ncbi.nlm.nih.gov/>). The LSU and / or SSU sequences were also used in cases where ITS sequences did not provide adequate BLAST results.

### Taxonomy

Fungal structures were mounted in lactic acid or in water when stated. The extremes of spore measurements (30 observations) are given in parentheses. Colony colours (surface and reverse) were rated after 7–14 d on MEA and OA at 25 °C in the dark, using the colour charts of Rayner (1970). All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands (Table 1), and type specimens in the mycology herbarium (PREM) at the Biosystematics Division of the Plant Protection Research Institute, Agricultural Research Council of South Africa.

## RESULTS AND DISCUSSION

### Sequence analysis

Sequence data obtained from the amplification products were deposited in GenBank (Table 1). BLAST searches resulted in associations with known fungal species or orders. These results are discussed in the descriptive notes below each of the treated species.

### Taxonomy

***Cladoriella*** Crous, **gen. nov.** MycoBank MB500799.

*Etymology:* Resembling species accommodated in *Cladosporium*.

Genus anamorphosis, hyphomycetium. Devriesiae simile, sed chlamydosporis carens. Hila conidiorum inspissata, fuscata, refringentia, poro centrali minuto praedita.

*Typus:* *Cladoriella eucalypti* Crous, sp. nov.

*External hyphae* coiling on the leaf surface, medium to dark brown, thick-walled, smooth to finely verruculose, branched, septate, with swollen cells giving rise to conidiophores; hyphododium-like structures present, simple, intercalary. *Conidiophores* separate, erect, medium to dark brown, smooth to finely verruculose, thick-walled, subcylindrical, straight, septate. *Conidiogenous cells* terminal or intercalary, monotretic or polytretic, sympodial, with 1–2 conspicuous loci, thickened, darkened, refractive, with a minute central pore, not protruding as in the case of *Cladosporium* s. str. *Conidia* frequently remaining attached in long acropetal chains, simple or branched, narrowly ellipsoidal to cylindrical or fusoid, 0–1-septate, medium brown, thick-walled, finely verruculose, apical conidium with rounded apex, additional conidia with 1–2 truncate, conspicuous hila; thickened, darkened, refractive, with a minute central pore. *Colonies* on MEA

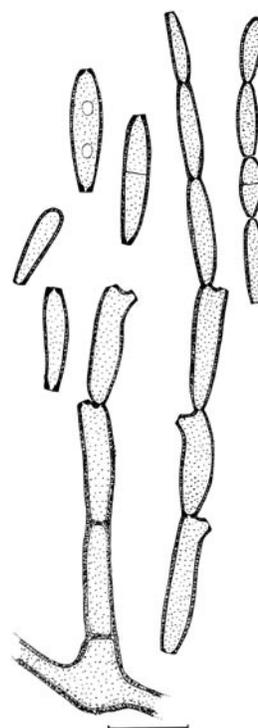
producing abundant amounts of diffusing red pigment. *Chlamydospores* absent.

***Cladoriella eucalypti*** Crous, **sp. nov.** MycoBank MB500800. Figs 1–2.

Devriesiae thermoduranti similis, sed conidiis 0–1-septatis, (11–)13–15(–22) × (2.5–)3–3.5(–4) µm, hilo conspicuo, inspissato, fuscato, refringente, 1.5–2 µm diam, praeditis distinguenda; porus hili centralis 0.5 µm latus; coloniae in agarō multi pigmentum rubrum formantes; chlamydosporae absentes.

*Hyphae* internal and external; external hyphae coiling on the leaf surface, medium to dark brown, thick-walled, smooth to finely verruculose, branched, septate, 2.5–3.5 µm wide, frequently forming a swollen cell which gives rise to a conidiophore; hyphododium-like structures present, simple, intercalary, 2.5–3.5 µm diam. *Conidiophores* separate, erect, medium to dark brown, smooth to finely verruculose, thick-walled, subcylindrical, straight, 1–4-septate, 15–60 × 5–7 µm. *Conidiogenous cells* terminal or intercalary, monotretic or polytretic, sympodial, usually with 1–2 conspicuous loci, 1.5–2 µm wide, thickened, darkened, refractive, with a minute central pore, 0.5–1 µm wide, scar usually within the cell outline, and not protruding as in the case of *Cladosporium* s. str., finely verruculose, medium brown, 10–17 × 4–5 µm. *Conidia* frequently remaining attached in long acropetal chains, simple or branched, narrowly ellipsoidal to cylindrical or fusoid, 0–1-septate, (11–)13–15(–22) × (2.5–)3–3.5(–4) µm, medium brown, thick-walled, finely verruculose, apical conidium with rounded apex, additional conidia with 1–2 truncate, conspicuous hila, 1.5–2 µm wide, thickened, darkened, refractive, with a minute central pore, 0.5 µm wide.

*Cultural characteristics:* Colonies on MEA producing abundant amounts of diffusing red pigment that changes the colour of the medium to red: colonies irregular,



**Fig. 1.** *Cladoriella eucalypti*. Conidiophore and conidia. Scale bar = 10 µm.

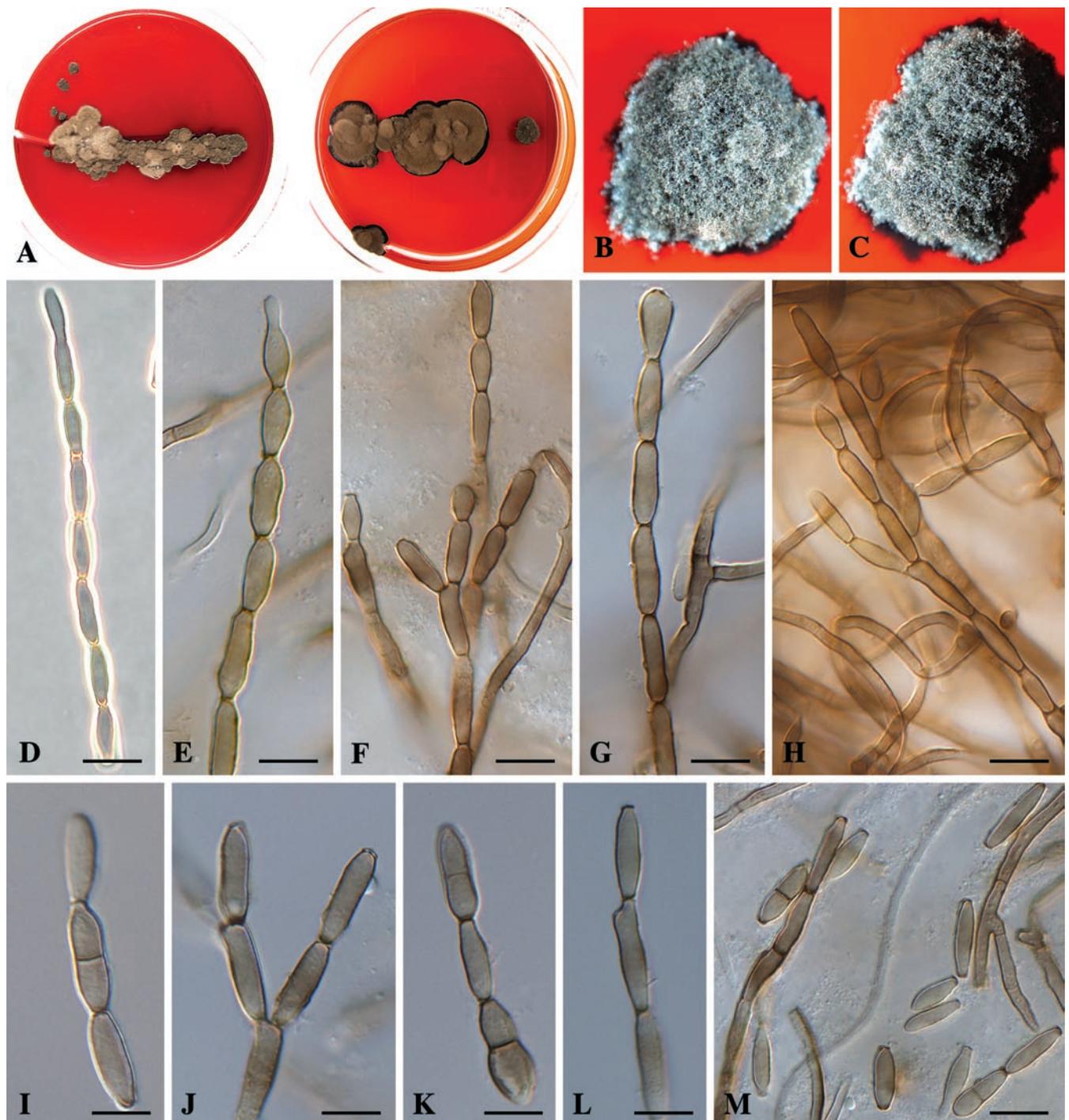
erumpent, with smooth, irregular margins; surface iron-grey; reverse greenish black.

**Substrate and distribution:** *Eucalyptus* sp., South Africa (Western Cape Province).

**Specimen examined:** South Africa, Western Cape Province, Stellenbosch Mountain, on *Eucalyptus* leaf litter, 13 Dec. 2003, P.W. Crous, CBS H-18043, **holotype**, cultures ex-type CPC 10953–10955 = CBS 115898–115890.

**Notes:** The genus *Cladosporium* Link contains 772 names (Dugan *et al.* 2004), many of which represent elements not congeneric with the type species, *C. herbarum* (Pers.: Fr.) Link, which is an anamorph of

*Davidiella* Crous & U. Braun (Braun *et al.* 2003). The recent description of *Devriesia* Seifert & N.L. Nickerson (Seifert *et al.* 2004) for a group of heat-resistant, chlamydospore forming species with slightly thickened conidial scars proves this point. *Cladoriella* resembles *Devriesia* in general morphology, but lacks chlamydospores, forms a distinct red pigment in culture, and clusters apart from the *Cladosporium* complex (*Mycosphaerellaceae*), the *Cladophialophora* Borelli complex (*Herpotrichiellaceae*), or the *Pseudocladosporium* U. Braun complex (*Venturiaceae*). BLAST results of the ITS sequence of this species had an E-value of 1e-90 with ITS sequences of



**Fig. 2.** *Cladoriella eucalypti*. A–B. Colonies on MEA, with diffuse red pigment visible in agar. D–M. Conidiophores and conidia. Scale bars: D, F, H–K, M = 10 µm, E, G, L = 6 µm.

**Table 1.** Isolates used for DNA sequence analysis.

Species	Accession number <sup>1</sup>	Host	Country	Collector	GenBank numbers <sup>2</sup> (ITS, LSU, SSU)
<i>Cladoriella eucalypti</i>	CBS 115898; CPC 10953	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ195778, DQ195790, DQ195801
<i>Fulvoflamma eucalypti</i>	CBS 118549; CPC 11243	<i>Eucalyptus</i> sp.	Spain	M.J. Wingfield	DQ195779, DQ195791, DQ195802
<i>Leptospora rubella</i>	CBS 118550; CPC 11006	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ195780, DQ195792, DQ195803
<i>Macrohilum eucalypti</i>	CBS 118551; CPC 10945	<i>Eucalyptus</i> sp.	New Zealand	J.A. Stalpers	DQ195781, DQ195793, DQ195804
<i>Pestalotiopsis disseminata</i>	CBS 118552; CPC 10950	<i>Eucalyptus botryoides</i>	New Zealand	M.A. Dick	DQ195782, DQ195794, DQ195805
<i>Pestalotiopsis</i> sp.	CBS 118553; CPC 10969	<i>Eucalyptus eurograndis</i>	Colombia	M.J. Wingfield	DQ195783, DQ195795, DQ195806
<i>Satchmopsis brasiliensis</i>	CBS 420.93	<i>Pimenta dioica</i>	Cuba	R.F. Castañeda	DQ195784, DQ195796, DQ195807
	CPC 10972	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ195785, DQ195797, DQ195808
	CBS 118554; CPC 11017	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ195786, DQ195798, DQ195809
<i>Torrendiella eucalypti</i>	CBS 115326; CPC 11049	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ195787, DQ195799, DQ195810
	CBS 115326; CPC 11050	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ195788, DQ195800, DQ195811
	CBS 115326; CPC 11051	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ195789, —, —

<sup>1</sup>CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.

<sup>2</sup>ITS: internal transcribed spacer region, LSU: partial 28S rDNA gene, SSU: partial 18S rDNA gene.

*Claviceps* Tul., *Calonectria* De Not. (*Hypocreales*) and *Gaeumannomyces* Arx & D.L. Olivier (*Sordariomycetes incertae sedis*). A number of similarities with an E-value of 0.0 were obtained from the LSU data: *Hysteropatella clavisporea* (Peck) Seaver (*Hysteriales*), *Candelariella vitellina* (Hoffm.) Müll. Arg. (*Lecanorales*), *Polysporina simplex* (Davies) Vězda (*Lecanorales*), *Botryosphaeria ribis* Grossenb. & Duggar (*Dothideales, incertae sedis*), and others. A number of similarities with an E-value of 0.0 were also obtained from the SSU data: *Sarcinomyces petricola* Wollenz. & de Hoog (*Chaetothyriales*), *Scytalidium dimidiatum* (Penz.) B. Sutton & Dyko, *Botryosphaeria ribis* (*Dothideales incertae sedis*), *Fusicladium convolvulorum* Ondřej (*Pleosporales*) and others.

***Fulvoflamma*** Crous, **gen. nov.** MycoBank MB500801.

**Etymology:** Named after its characteristic conidiomata and spore masses that appear as orange candle flames once plant material is incubated in moist chambers.

Genus anamorphosis coelomyceticum. Satchmopsi similis, sed proliferatione sympodiali cellularum conidiogenarum et setis marginalibus hyalinis tenuitunicatis et conidiis cylindricis distinguenda.

**Typus:** *Fulvoflamma eucalypti* Crous, sp. nov.

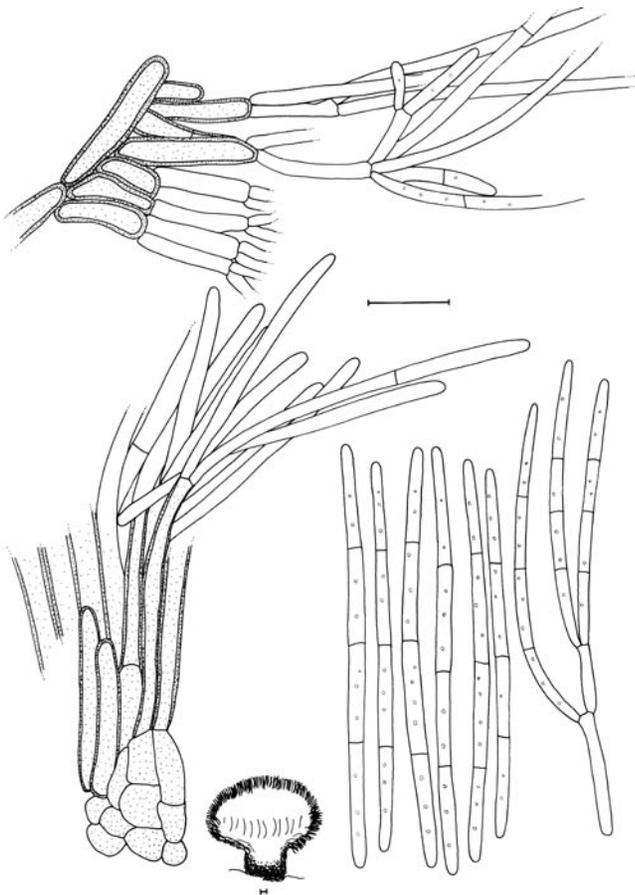
**Mycelium** immersed, consisting of smooth, hyaline, branched, septate hyphae, forming brown stromata that give rise to conidiomata. **Conidiomata** sporodochial, appearing as erect, orange, fusoid structures; basal

region consisting of pale brown *textura angularis* to *textura epidermoidea*, giving rise to thick-walled, pale brown cells of *textura porrecta*, becoming thin-walled, hyaline, and radiating outwards from the narrower, semi-cylindrical sporodochial base, branching sympodially to give rise to hyaline, smooth, thin-walled setae with bluntly rounded ends; inner conidiomatal layer consisting of a mixture of setae and conidiogenous cells. **Conidiogenous cells** hyaline, smooth, subcylindrical, proliferating blastically and sympodially. **Conidia** subcylindrical, straight or slightly curved with obtuse ends, septate, hyaline, smooth, guttulate.

***Fulvoflamma eucalypti*** Crous, **sp. nov.** MycoBank MB500802. Fig. 3.

Conidiomata sporodochialia, erecta, aurantiaca, flammam candelae fingentia. Cellulae conidiogenae hyalinae, leves, subcylindricae, 7–15 × 1.5–2.5 µm, sympodialiter proliferantes. Conidia subcylindrica, recta vel modice curvata, 3-septata, hyalina, levia, (35–)43–55(–60) × 1.5–2 µm.

**Mycelium** immersed, consisting of smooth, hyaline, branched, septate hyphae, 1–1.5 µm wide; aggregating in the epidermis to form a pale to dark brown stroma, up to 50 µm wide, which gives rise to a conidioma. **Conidiomata** sporodochial, appearing as erect, orange, fusoid structures on the leaf surface (like the flame of a candle), up to 100 µm diam and 200 µm high; basal region consisting of pale brown cells of *textura angularis* to *textura epidermoidea*, 3–7 × 2–3 µm, giving rise to thick-walled, pale brown cells of *textura porrecta*, 6–15 × 2–3 µm, becoming thin-walled, hyaline, and radiating outwards from the narrower, semi-



**Fig. 3.** *Fulvoflamma eucalypti*. Conidioma, conidia, conidiogenous cells and setae. Scale bars = 10 µm.

cylindrical sporodochial base, branching sympodially to give rise to hyaline, smooth, thin-walled setae that terminate in bluntly rounded, obtuse ends, and give the conidiomatal margin a feathery appearance; the inner layer of the conidioma gives rise to a mixture of setae and conidiogenous cells. *Conidiogenous cells* hyaline, smooth, subcylindrical, 7–15 × 1.5–2.5 µm, proliferating sympodially, with inconspicuous scars, giving rise to additional conidiogenous cells, or to conidia. *Conidia* subcylindrical, straight or slightly curved, 3-septate, hyaline, smooth, guttulate, widest in the middle, with obtusely rounded ends, (35–)43–55(–60) × 1.5–2 µm.

**Cultural characteristics:** Colonies on MEA spreading, erumpent, folded, with sparse aerial mycelium; surface pale luteous to buff, with diffuse strips of red; reverse luteous. On OA colonies slimy with no aerial mycelium, spreading, appearing to grow more in the agar than on the surface, pale luteous; colonies sporulated when freshly isolated, but became sterile upon first transfer.

**Substrate and distribution:** *Eucalyptus* sp., Spain.

**Specimen examined:** Spain, on *Eucalyptus* leaf litter, Apr. 2004, M.J. Wingfield, CBS H-18045, **holotype**, cultures ex-type CPC 11243 = CBS 118549, CPC 11244–11245.

**Notes:** *Fulvoflamma* is similar to other genera with sporodochial conidiomatal such as *Satchmopsis* B. Sutton & Hodges, *Stevensonula* Petr., *Shawiella* Hansf. and *Zelosatchmopsis* Nag Raj (Sutton 1975, Saikawa *et al.* 1991). It is easily distinguished, however, by its unique

conidiophores, mode of conidiogenesis, presence of marginal, thin-walled setae and its cylindrical conidia. BLAST results of the ITS sequence of this species had an E-value of 5e-130 with the ITS sequence of a foliar endophyte of *Picea glauca*. Similarities with known species include *Potebniamyces pyri* (Berk. & Broome) Dennis (7e-123; *Rhytismatales*), *Phacidiopycnis* sp. (2e-120; *Rhytismatales*) and *Pseudeurotium desertorum* Mouch. (2e-117; *Pseudeurotiaceae*). A number of similarities with an E-value of 0.0 were obtained from the LSU data: *Crinula caliciiformis* Fr. (*Helotiales*), *Leuconeurospora pulcherrima* (G. Winter) Malloch & Cain (*Hypocreales*), *Pseudeurotium zonatum* F.H. Beyma (*Pseudeurotiaceae*), *Aleurodiscus farlowii* Burt (*Stereales*), *Cudoniella clavus* (Alb. & Schwein.) Dennis (*Helotiales*) and others. A number of similarities with an E-value of 0.0 were also obtained from the SSU data: *Phacidium coniferarum* (G.G. Hahn) DiCosmo, Nag Raj & W.B. Kendr. (*Helotiales*), *Bulgaria* spp. (*Helotiales*), *Neofabraea malicorticis* H.S. Jacks. (*Helotiales*) and others.

***Leptospora rubella*** (Pers.: Fr.) Fr., *Herb. mycol.*, ed. 2: no. 532. 1857.

≡ *Sphaeria rubella* Pers., *Syn. meth. fung.* (Göttingen): 63. 1801, sanctioned by Fries, *Syst. Mycol.* 2: 506. 1823.

Ascomata indistinct on host, intermingled with those of *Mycosphaerella longibasalis* Crous & M.J. Wingf. Description based on sporulation obtained on CLA. *Ascomata* dark brown to black, up to 400 µm high and 300 µm wide, flask-shaped with an elongated red-brown neck up to 70 µm long. *Asci* numerous, cylindrical, bitunicate, with a prominent foot cell, 120–160 × 4–6 µm. *Pseudoparaphyses* hyaline, septate, constricted at the septa, 2–3.5 µm wide, not extending beyond the asci. *Ascospores* somewhat spiralled or twisted in the asci, pale brown, subcylindrical, with tapering to subobtusely ends, multiseptate (septa at approx. 10 µm intervals), 130–165 × 1–1.5 µm.

**Cultural characteristics:** Colonies spreading on MEA, slightly erumpent with moderate aerial mycelium and feathery margins; surface on PDA and OA pale mouse grey to mouse grey; reverse chestnut on MEA, iron-grey on OA. Cultures were sterile on MEA, but perithecial initials formed on OA, and fertile perithecia was obtained on CLA.

**Newly observed substrate and distribution:** *Eucalyptus* sp., Colombia.

**Specimen examined:** Colombia, on *Eucalyptus* leaf spots, associated with lesions of *Mycosphaerella longibasalis* Crous & M.J. Wingf., 16 Feb. 2004, M.J. Wingfield, CBS H-18046, culture CPC 11006 = CBS 118550.

**Notes:** Shoemaker (1976) listed numerous hosts for *L. rubella* (as *Ophiobolus rubellus* (Pers.: Fr.) Sacc.), and stated that it is often recognized by the red-purple stain it induces on the host substrate, and the red-brown colour of the apical part of the ascomatal neck. Furthermore, he reported that the fungus is common in Canada, and is suspected to be the teleomorph of *Phoma exigua* Desm. var. *foveata* Foister. BLAST results

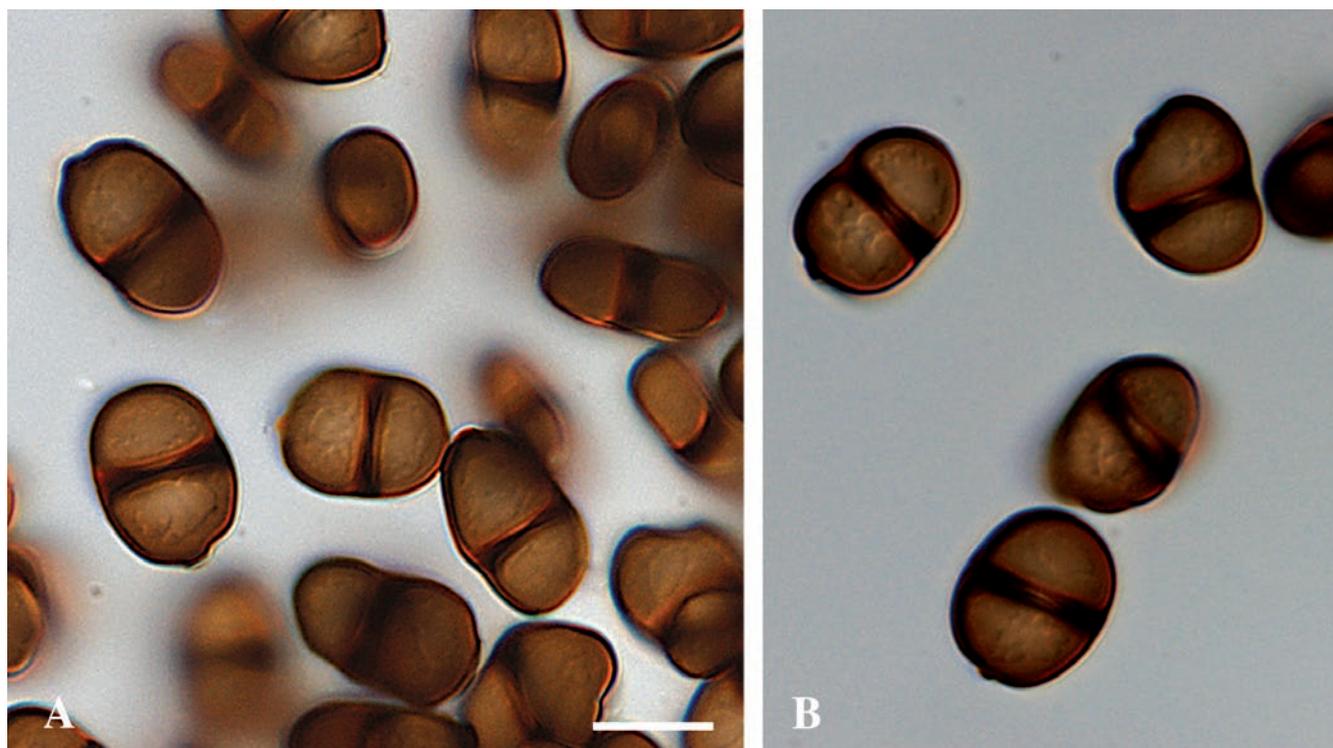


Fig. 4. A–B. Conidia of *Macrohilum eucalypti*. Scale bar = 10  $\mu$ m.

of the ITS sequence of this species had an E-value of 0.0 with an ITS sequence of *Leptospora rubella* on GenBank (AF383951; 99 % similarity). Similarities with *Phaeosphaeria* spp. (*Pleosporales*) ranged from  $9e-175$  to  $4e-109$ . A number of similarities with an E-value of 0.0 were obtained from the LSU data: *Phaeosphaeria avenaria* (G.F. Weber) O.E. Erikss., *Setomelanomma holmii* M. Morelet, *Setosphaeria monoceras* Alcorn (*Pleosporales*) and others. A number of similarities with an E-value of 0.0 were also obtained from the SSU data: *Phaeosphaeria avenaria*, *Paraphaeosphaeria* spp., *Septoria nodorum* (Berk.) Berk., *Ophiobolus fulgidus* (Cooke & Peck) Sacc. (*Pleosporales*) and others.

***Macrohilum eucalypti*** H.J. Swart, Trans. Br. Mycol. Soc. 90: 288. 1988. Fig. 4.

A single conidioma was observed on the host, from which a culture was obtained, and thus the description is based on features *in vitro*. Conidiomata were sparingly formed on MEA, medium brown, globose, up to 400  $\mu$ m diam. Conidiogenous cells pale brown, cylindrical, proliferating percurrently near the apex,  $10-15 \times 3-5 \mu$ m. Conidia medium to dark brown, ovoid, smooth, guttulate, developing a single supramedian septum, thick-walled, frequently constricted at the septum, apex obtuse, base truncate with a visible scar,  $2-3 \mu$ m wide,  $(15-17-19(-20) \times (8-10-12(-13) \mu$ m.

**Cultural characteristics:** Colonies flat on MEA, spreading, with moderate aerial mycelium and submerged, smooth margins. Surface pale luteous on MEA, cream to pale white on OA; reverse with patches of luteous to umber on MEA, pale luteous on OA; fertile on MEA.

**Substrate and distribution:** *Eucalyptus* sp., New

Zealand; also known from *Eucalyptus* spp. in Australia (Sankaran *et al.* 1995).

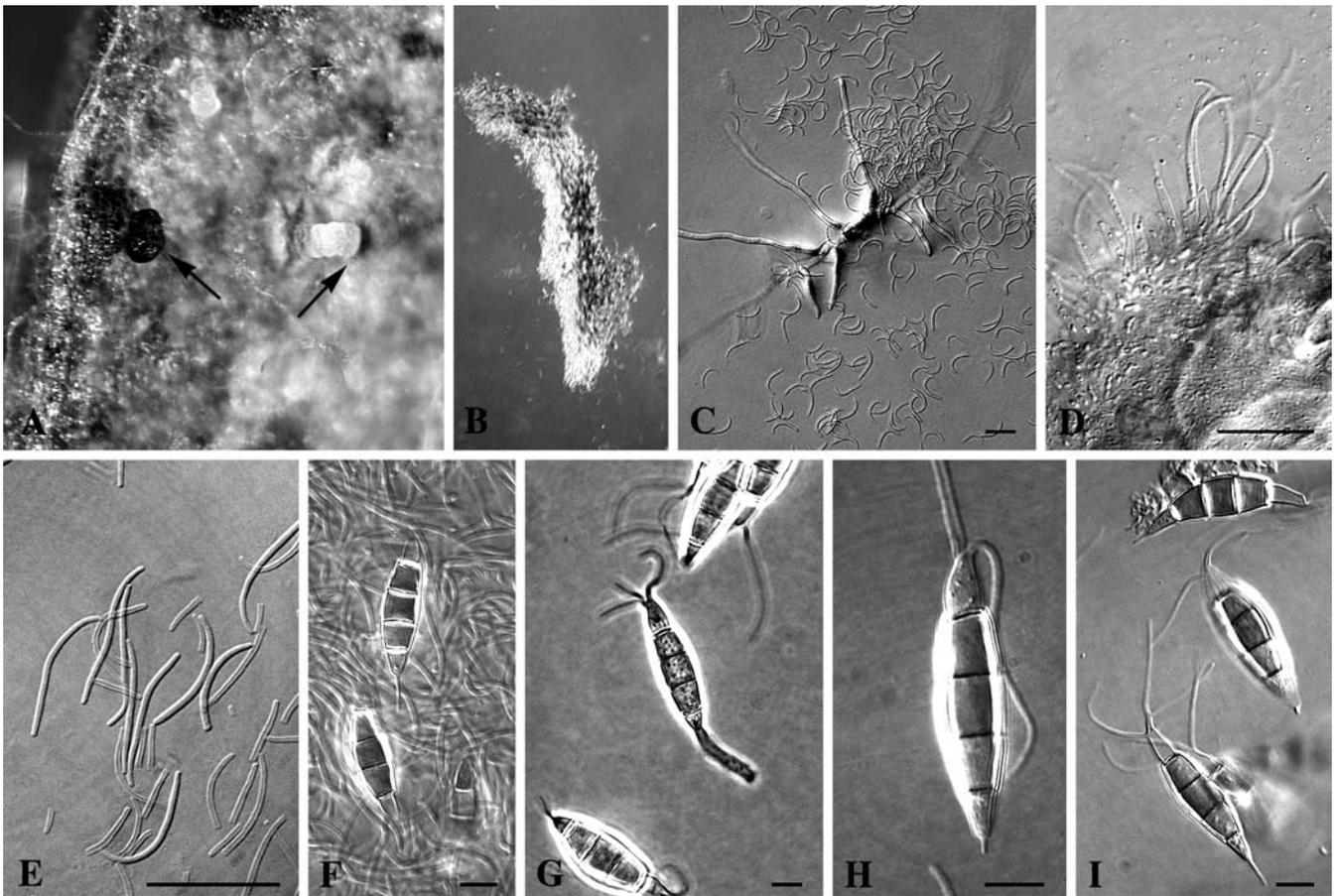
**Specimen examined:** New Zealand, on *Eucalyptus* sp., 2004, J.A. Stalpers, CPC 10945 = CBS 118551.

**Notes:** As far as we could establish, *M. eucalypti* has not previously been known from culture (Swart 1988). BLASTn results of the ITS sequence of this species had an E-value of  $9e-98$  with an ITS sequence of *Valsa sordida* Nitschke (*Diaporthales*). Similarities with known species include *Phomopsis* spp. (1e-96), *Diaporthe helianthi* Munt.-Cvetk., Mihajč. & M. Petrov (1e-96; *Diaporthales*) and *Monilinia* sp. (6e-96; *Helotiales*). A number of similarities with an E-value of 0.0 were obtained from the LSU data: *Diaporthe* spp. (*Diaporthales*), *Cryphonectria* spp. (*Diaporthales*), *Harknessia* spp. (*Diaporthales*) and others. A number of similarities with an E-value of 0.0 were also obtained from the SSU data: *Leucostoma persoonii* (Nitschke) Höhn. (*Diaporthales*), *Cryphonectria* spp., *Prosopidicola mexicana* Crous & C.L. Lennox, *Endothia gyrosa* (Schwein.) Fr. (*Diaporthales, incertae sedis*) and others.

***Pestalotiopsis disseminata*** (Thüm.) Steyaert, Bulletin Jard. Bot. I Etat Bruxelles 19: 319. 1949. Fig. 5.

$\equiv$  *Pestalotia disseminata* Thüm., Inst. Rev. Sci. Coimbra 18: 501. 1880.

**Conidiomata** developing from 10–14 d (none after 7 d) mainly on the surface of the colony. **Conidia** broadly fusoid to fusoid-clavate, straight or somewhat curved, 5-celled, upper cell conical to cylindrical, hyaline, fairly thin-walled, apical setulae central, (2–)3(–4), rather stout, up to 1.2  $\mu$ m wide, 11–20  $\mu$ m long, with a blunt tip, three intermediate cells concolorous or the upper



**Fig. 5.** *Pestalotiopsis disseminata*. A. Conidiomata with exuding alpha- (black) and beta- (cream) conidial masses (arrowed). B. Conidial cirrus containing back (alpha-) and hyaline (beta-) conidia. C. Germinating alpha-conidium, among infertile beta-conidia on MEA plate. D. Conidiogenous cells giving rise to beta-conidia. E. Beta-conidia. F–I. Alpha-conidia. Scale bars: C–E = 10 µm, F–I = 7 µm.

two intermediate cells slightly darker, dull olivaceous-brown to vinaceous-brown, contents guttulate, walls smooth, slightly constricted at the septa when mounted in water, and thickened up to 1 µm especially in the upper two intermediate cells and in the septa, basal cell hyaline, thin-walled, tapering into a filiform pedicel (2–)2.5–4.5(–5) µm long; conidium body (18–)20–24(–25) × 6.5–7(–8) µm (OA).

**Cultural characteristics:** Colonies on OA reaching 52–54 mm diam in 7 d with an even, glabrous, colourless margin; immersed mycelium colourless, aerial mycelium pure white, fluffy, covering most of the colony surface, and very dense and high in the centre and in concentric zones after 7 d; reverse in the centre buff. Colonies on CMA reaching 52–55 mm diam after 7 d, as on OA, but aerial mycelium less well-developed, and reverse colourless. Colonies on MEA reaching 56 mm diam in 7 d, with an even or slightly undulating colourless margin; immersed mycelium colourless, but surface of the colony completely covered by a high, dense mat of pure white, in the centre yellowish, fluffy aerial mycelium, the margin also covered by a diffuse layer of aerial hyphae; reverse with a faint cinnamon tinge.

**Substrate and distribution:** *Eucalyptus botryoides*, New Zealand (North Island).

**Specimen examined:** New Zealand, North Island, Kerikeri, living leaves of *Eucalyptus botryoides*, 17 Oct. 2003, M.A. Dick, CPC 10950 = CBS 118552, CPC 10951.

**Notes:** BLASTn results of the ITS sequence of this species had an E-value of 0.0 with ITS sequences of *Pestalotiopsis* spp.

***Pestalotiopsis* sp.**

**Conidiomata** developing on agar surface and in the aerial mycelium after 3–5 d (OA, MEA & CMA). **Conidia** narrowly fusoid to fusoid-clavate, straight or somewhat curved, 5-celled, upper cell conical to cylindrical, hyaline, fairly thin-walled, without visible cellular contents, bearing (2–)3(–4) rather stout central apical appendages, 10–19 µm long, up to 1.2 µm wide, with a blunt tip, three intermediate cells concolorous or the upper two intermediate cells slightly darker, dull olivaceous-brown to vinaceous-brown, contents guttulate, walls smooth, thickened up to 1 µm especially in the upper two intermediate cells and in the septa, basal cell hyaline, thin-walled, tapering into a filiform pedicel (3–)4–5(–6) µm long; conidium body (19–)20–24(–27) × (5.2–)5.5–6 µm (OA).

**Cultural characteristics:** Colonies on OA reaching 50–53 mm diam in 7 d with an even to undulating, glabrous, colourless margin; immersed mycelium colourless, aerial mycelium pure white, woolly-cottony, covering most of the colony surface without distinct concentric zonations, almost absent in the marginal zone after 7 d; reverse concolorous, in the centre buff (where sporulation occurs). Colonies on CMA reaching 50 mm diam after 7 d, as on OA, but colony margin undulating

to ruffled, and aerial mycelium less well-developed. Colonies on MEA reaching 49–55 mm diam in 7 d, with an irregularly undulating, colourless, glabrous margin; immersed mycelium colourless, but surface of the colony completely covered by a moderately high, densely woolly mat of pure white, locally faintly sulphur-yellow, aerial mycelium; reverse ochreous to fulvous, brown where conidiomata develop.

**Substrate and distribution:** *Eucalyptus eurograndis?*, Colombia.

**Specimen examined:** Colombia, living leaves of *Eucalyptus eurograndis*, 2004, M.J. Wingfield, CBS H-18044, cultures CPC 10969 = CBS 118553, CPC 10970–10971.

**Notes:** BLASTn results of the ITS sequence of this species had an E-value of 0.0 with ITS sequences of *Pestalotiopsis* spp., including *Pestalotiopsis disseminata* and *Pestalotiopsis uvicola* (Speg.) Bissett (both 99 % similar).

The primary reason for the inclusion of these *Pestalotiopsis* spp. in the present paper is the presence of a synanamorph, which has never before been reported for species of *Pestalotiopsis* in the literature (Nag Raj 1993). According to unpublished notes in the CBS database, this has once before been observed for a culture of a *Pestalotiopsis* sp. in the collection. Conidiomata were observed in host tissue to exude a mixture of black and hyaline spores in a typical cirrhus associated with *Pestalotiopsis* conidiomata. The cirrhus consisted of two conidial types, namely typical *Pestalotiopsis* conidia (alpha), and long, narrow, bent, needle-like cylindrical conidia (beta) resembling the beta conidia observed in species of *Phomopsis*, or the conidia typically associated with *Libertella* anamorphs. Conidia were 25–30 × 1–1.5 µm, widest in the middle, tapering to a subobtuse apex, and a truncate base. Conidia were formed on slightly tapering, hyaline, subcylindrical conidiogenous cells that terminated in an apex with 1–2 loci which gave rise to conidia in a sympodial arrangement. In some cases the conidiogenous cells were situated on 1–3-septate conidiophores that were 10–20 × 2–3 µm.

Beta-conidia were initially observed in the collection obtained from Colombia. Although they occurred in the same conidioma, none could be induced to germinate on MEA (observed over 2 wk), while all alpha conidia germinated within 1–2 d. The second collection which had a mixture of both conidial types was obtained from New Zealand. Again, the beta-conidia could not be induced to germinate, and thus their ecological role as potential conidia, or spermatia, still needs to be resolved. None of the colonies derived from alpha conidia could be induced to form beta conidia on MEA, OA or CLA. In this regard it is interesting to note that, contrary to common opinion, it has only recently been proven that beta-conidia of *Phomopsis* spp. do, in fact, germinate in culture (Sergeeva *et al.* 2003).

***Satchmopsis brasiliensis*** B. Sutton & Hodges, Nova Hedwigia 26: 3. 1975. Fig. 6.

**Conidiomata** cupulate, superficial, up to 180 µm wide and 60

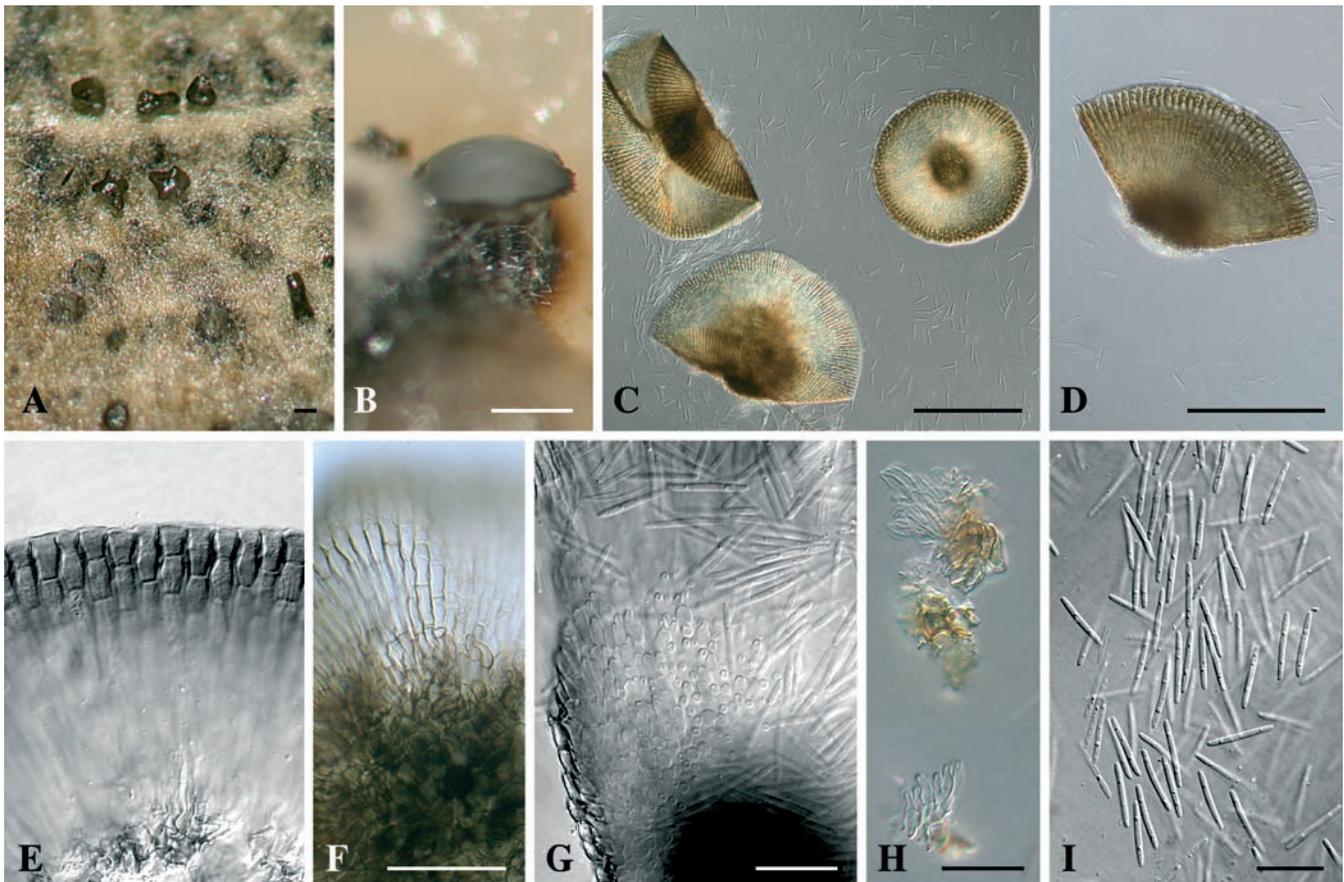
100 µm deep, dark brown, attached centrally to a stroma of dark brown cells that occupy the stomatal chamber; wall consisting of two regions, the lower region having thick-walled dark-brown cells, up to 5 layers thick, the upper region consisting of thin-walled, paler cells, up to 5 layers thick. *Conidiogenous cells* restricted to the lower part of the basal wall, 3–7 × 2–3 µm, doliform to lageniform, phalidic with periclinal thickening, hyaline, with an indistinct collarete. *Conidia* hyaline, aseptate, guttulate, subcylindrical, predominantly straight, with obtuse ends, 11–17 × 1–1.5 µm.

**Cultural characteristics:** Colonies spreading on MEA, flat with sparse aerial mycelium and smooth margins; surface sienna to umber with patches of white, and dark brown conidiomata; reverse umber (centre) to sienna (margins); on OA umber with no aerial mycelium, and dark brown conidiomata.

**Substrate and distribution:** *Eucalyptus* spp., Colombia, Indonesia.

**Specimens examined:** Colombia, on *Eucalyptus* leaf litter, Feb. 2004, M.J. Wingfield, CBS H-18048, cultures CPC 10972–10974. Indonesia, on *Eucalyptus* leaf litter, Mar. 2004, M.J. Wingfield, CBS H-18049, cultures CPC 11017 = CBS 118554, CPC 11018–11019.

**Notes:** The collections from Indonesia and Colombia are morphologically similar. Colonies appear similar on MEA, and conidia of the Indonesian collection (11–17 × 1–1.5 µm) are similar to those of the Colombian collection (12–14 × 1–1.5 µm), and fit within the range given for the species, namely 11.5–15.5 × 1–1.5 µm (Sutton 1975). However, from the sequence data (data not shown) it is clear that there are some base pair differences between these isolates, suggesting that these strains may in fact represent different species. The only obvious morphological difference observed was that conidiomata of the Colombian collection were pale brown, with cells at the margin of the wall being up to 5 µm wide. In contrast, conidiomata from the Indonesian collection were darker brown, with cells at the margins being narrower, namely 3–4 µm wide. Whether these morphological differences can be related to the differences observed in the DNA sequences, can only be resolved once further collections have been obtained. BLASTn results of the ITS sequence of this species has E-values of 5e-167 to 1e-115 with ITS sequences of unidentified leaf litter and mycorrhizal ascomycetes. The closest known species include *Pezicula frangulae* (Pers.) Fuckel (2e-110), *Pezicula ocellata* (Pers.) Seaver (9e-107; *Helotiales*), and *Cryptosporiopsis* spp. (4e-106; *Helotiales*). A number of similarities with an E-value of 0.0 were obtained from the LSU data: *Crinula caliciiformis* Fr. (*Helotiales*), *Leuconeurospora pulcherrima* (G. Winter) Malloch & Cain (*Hypocreales*), *Vibrissea albobusca* G.W. Beaton (*Helotiales*) and others. A number of similarities with an E-value of 0.0 were also obtained from the SSU data: *Phacidium coniferarum* (G.G. Hahn) DiCosmo, Nag Raj & W.B. Kendr., *Bulgaria* spp., *Neofabraea malicorticis* H.S. Jacks. (all *Helotiales*) and others.



**Fig 6.** *Satchmopsis brasiliensis*. A–D. Conidiomata. E–F. Conidiomatal wall. G. Upper view of conidioma, showing aggregated conidiogenous cells. H. Conidiogenous cells. I. Conidia. Scale bars: A–D = 90  $\mu$ m, E–I = 15  $\mu$ m.

***Torrendiella eucalypti*** (Berk.) Spooner, *Bibl. Mycol.* 116: 322. 1987. Figs 7–8.

≡ *Peziza eucalypti* Berk., *Flora Tasman.* 2: 274. 1860.

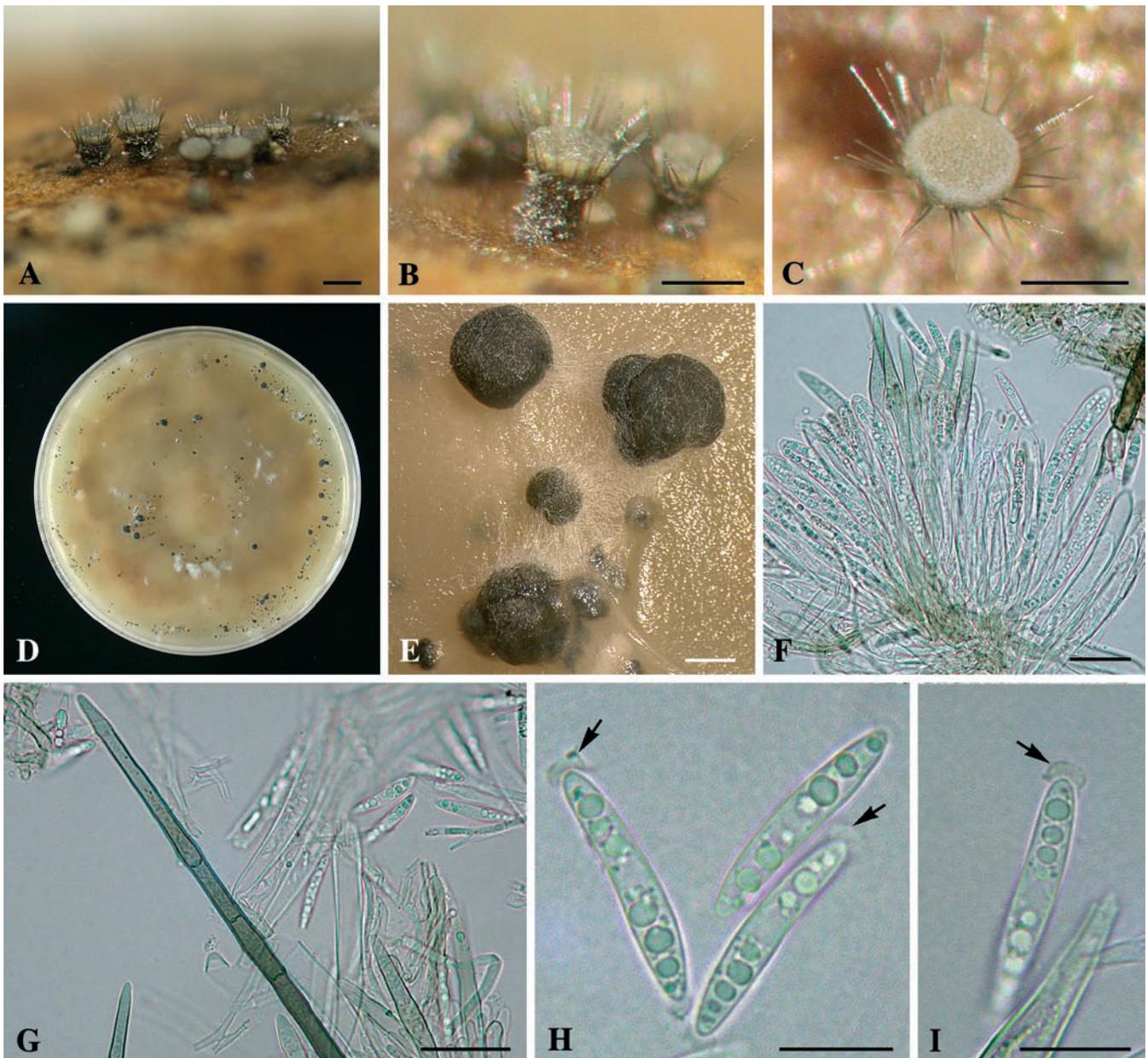
**Apothecia** on host scattered or gregarious in large groups, erumpent, stipitate, arising from a subepidermal stroma visible around the stipe as a dark discoloration. **Disc** plane to convex, greyish brown to olivaceous, smooth, 0.4–1.5 mm diam. **Receptacle** cupulate, concolorous but usually darker than the hymenium, bearing dark brown to reddish brown setae. **Stipe** central, smooth and dark brown, 0.4–1.8 mm high. **Setae** mostly 20–50 per apothecium, (150–)200–250  $\mu$ m long, smooth, with dark brown walls thickened up to 1.5  $\mu$ m, septate, paler at the blunt top, attenuated and bent at the base. **Asci** cylindrical-clavate, apex conical-rounded, the apical apparatus blueing in Melzer's reagent, croziers present, 8-spored, 75–100  $\times$  7–9  $\mu$ m; **ascospores** fusoid, 0-septate, narrowly rounded at both ends, contents guttulate, hyaline, each end provided with a central, everted (umbrella-shaped) mucelaginous appendage, 17–25  $\times$  3–4  $\mu$ m; sometimes producing ellipsoid microspores 3–5.5  $\times$  1.5–2  $\mu$ m directly from apertures at one or both ends. **Paraphyses** simple or branched near the base, obtuse, hyaline, somewhat inflated and up to 3.5  $\mu$ m wide at the top.

**Cultural characteristics:** Colonies on OA reaching a diam of 15–20(–30) mm in 14 d, with an even to slightly ruffled, glabrous and colourless margin; immersed mycelium at first colourless, then very faintly yellowish (primrose) or reddish (apricot), after 10–20 d gradually

developing a mixture of several tinges, pale hazel, ochreous and amber, in the centre sometimes also greyish to olivaceous buff, most of the surface almost glabrous and without aerial mycelium, locally with patches of woolly, pure-white aerial mycelium. Colonies on MEA reaching 33–37 mm diam in 14 d, with a ruffled, glabrous, colourless margin; most of the colony surface covered by a fairly dense, woolly but low mat of pure white aerial mycelium; reverse in centre ochreous to umber, fading to the colourless margin.

**Apothecia** formed on OA after about 10 wk, mostly on the agar surface, most very similar in shape and size to those formed *in planta*, but with less setae; however, large abnormally shaped apothecia are also formed: hymenium convex, protruding from the agar surface as a greyish-black, globular mass with a smooth surface, 1–2.5 mm diam, receptacle reduced, hairs present or absent, lacking a stipe.

**Anamorph in vitro:** *Conidiophores* developing on the surface of globular ascomatal initials after 2–3 wk, smooth-walled, variable, simple, but mostly branched near the base, 15–30  $\times$  2–4(–5)  $\mu$ m thick, hyaline or somewhat yellowish brown, conidiogenesis blastic, sympodial, sometimes seemingly retrogressive, apertures mostly terminal but also immediately below septa (acropleurogenous), scars visible but not thickened or protruding; *conidia* hyaline, ellipsoid, broadly rounded at the top, slightly attenuated into a blunt base, with one or two small guttules, 4–5.2(–6)  $\times$  (1.5–)1.8–2  $\mu$ m.

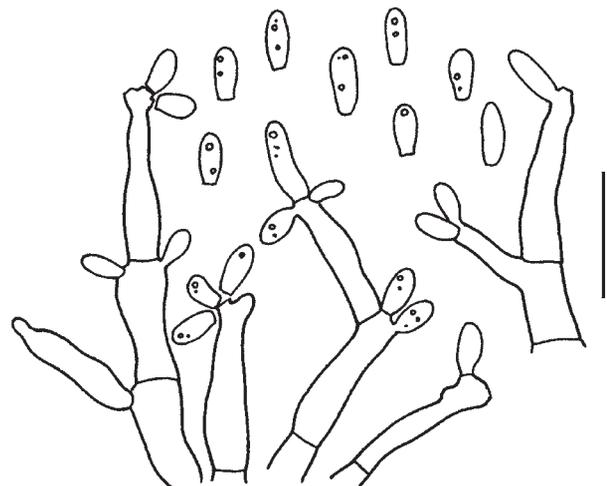


**Fig. 7.** *Torrendiella eucalypti*. A–C. Apothecia on the plant. D–E. in culture on OA. D. 15-wk-old culture with apothecia. E. Abnormally shaped apothecia. F. Asci and ascospores. G. Apothecial hair. H–I. Ascospores with apical appendages (arrows). Scale bars: A–C = 1.5 mm, E = 100 µm, F–G = 25 µm, H–I = 10 µm.

**Substrate and distribution:** *Eucalyptus* sp., Indonesia.

**Specimen examined:** **Indonesia**, on *Eucalyptus* leaf litter, in association with *Coccomyces antillarum* Sherwood, M.J. Wingfield, Mar. 2004, CBS H-18041, single-ascospore isolates, CPC 11049 = CBS 115326, CPC 11050–11051.

**Notes:** The material used in this study generally agrees well with the description given by Spooner (1987). There are, however, some additional observations that were not reported by this author, particularly, the presence of apical appendages on the ascospores, and the production of microspores from liberated ascospores. We observed 3–8 large guttules in ascospores of *T. eucalypti*, while Spooner reported only two or three guttules per spore. After drying, the guttules in our material often merged into larger bodies, and this could explain the difference between our observations and those of Spooner, which were based on herbarium specimens. After drying of our specimen, the



**Fig. 8.** Anamorph of *Torrendiella eucalypti*. Conidiophores and conidia on OA. Scale bar = 10 µm.



appendages of the ascospores were barely visible. The present study is also the first to report on observations in pure culture. The anamorph was only observed in culture, and showed plasticity in conidiogenesis making it very difficult to assign it to a particular anamorph genus. It could be circumscribed as *Sporothrix*-like, although it lacks the denticles characteristic of that anamorph, and it also differs by branched and septate conidiophores. *Sporothrix schenkii* Hektoen & C.F. Perkins, the type species of *Sporothrix* Hektoen & C.F. Perkins, is commonly isolated from *Eucalyptus* wood, but is linked to *Ophiostoma* Syd. & P. Syd. BLASTn results of the ITS sequence of this species had an E-value of 0.0 with ITS sequences of *Torrendiella eucalypti* and *Torrendiella madsenii* (G.W. Beaton & Weste) Spooner (both 94 % similar). Similarities with known species include *Cyathicula coronata* (Bull.) De Not. (2e-135), *Hymenoscyphus fructigenus* (Bull.) Fr. (8e-135) and *Pezizella amenti* (Batsch) Dennis (6e-96; all Helotiales). A number of similarities with an E-value of 0.0 were obtained from the LSU data: *Hymenoscyphus scutula* (Pers.) W. Phillips, *Cudoniella clavus* (Alb. & Schwein.) Dennis (both Helotiales) and others.

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# Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. II.

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**Abstract:** Species of *Eucalyptus* are widely planted as exotics in the tropics and Southern Hemisphere and to some extent in southern Europe, for timber and fibre production. Species of *Mycosphaerella* are commonly associated with leaves and twigs of *Eucalyptus* and can result in defoliation, dieback, and even tree death. In the present study, numerous isolates of *Mycosphaerella* species were collected from leaf litter, living leaves exhibiting leaf spot symptoms or severe *Mycosphaerella* leaf blotch symptoms. Isolates were compared based on DNA sequence data for the internal transcribed spacer region (ITS1 & ITS2) and the 5.8S gene. These data, together with characteristics of the fungal growth on three different media, morphology of the anamorph and teleomorph structures as well as ascospore germination patterns were used to describe 21 new species.

**Taxonomic novelties:** *Colletogloeopsis stellenboschiana* Crous sp. nov., *Mycosphaerella davisoniellae* Crous sp. nov. (anamorph *Davisoniella eucalypti* H.J. Swart), *Mycosphaerella eucalyptorum* Crous & M.J. Wingf. sp. nov. *Mycosphaerella gamsii* Crous sp. nov., *Mycosphaerella perpendicularis* Crous & M.J. Wingf. sp. nov., *Mycosphaerella pluritubularis* Crous & J.P. Mansilla sp. nov., *Mycosphaerella pseudaficana* Crous & T. Coutinho sp. nov., *Mycosphaerella pseudocryptica* Crous sp. nov. (anamorph *Colletogloeopsis* sp.), *Mycosphaerella pseudoendophytica* Crous & G. Hunter sp. nov. (anamorph *Pseudocercospora* sp.), *Mycosphaerella pseudosuberosa* Crous & M.J. Wingf. sp. nov. (anamorph *Trimmatostroma* sp.), *Mycosphaerella quasercospora* Crous & T. Coutinho sp. nov., *Mycosphaerella scytalidii* Crous & M.J. Wingf. sp. nov. (anamorph *Stenella* sp., synanamorph, *Scytalidium*-like.), *Mycosphaerella secundaria* Crous & A.C. Alfenas sp. nov., *Mycosphaerella stramentii* Crous & A.C. Alfenas sp. nov., *Mycosphaerella stramenticola* Crous & A.C. Alfenas sp. nov., *Mycosphaerella sumatrensis* Crous & M.J. Wingf. sp. nov., *Mycosphaerella verrucosiafricana* Crous & M.J. Wingf. sp. nov., *Septoria eucalyptorum* Crous sp. nov., *Septoria provencialis* Crous sp. nov., *Stenella pseudoparkii* Crous & M.J. Wingf. sp. nov. (teleomorph *Mycosphaerella* sp.), *Stenella xenoparkii* Crous & M.J. Wingf., sp. nov. (teleomorph *Mycosphaerella* sp.).

**Key words:** Ascomycetes, *Colletogloeopsis*, *Davisoniella*, DNA sequence comparisons, *Mycosphaerella*, *Pseudocercospora*, *Pseudocercospora*, *Scytalidium*, *Septoria*, *Stenella*, systematics, *Trimmatostroma*.

## INTRODUCTION

*Eucalyptus* spp. are widely planted in the tropics and Southern Hemisphere, providing important sources of structural timber and fibre. Fungal diseases have, however, had a negative impact on their cultivation in many parts of the world (Wingfield *et al.* 2001). *Mycosphaerella* leaf blotch (MLB) was one of the first diseases to seriously damage plantations of *Eucalyptus* outside their native range, leading to the abandonment of some species for plantation development (Lundquist & Purnell 1987).

*Mycosphaerella* leaf blotch has been associated with severe defoliation, shoot die-back, and even tree death. This damage has mostly been attributed to *M. cryptica* (Cooke) Hansf. and *M. nubilosa* (Cooke) Hansf. (Carnegie *et al.* 1994, Crous & Wingfield 1996, Wingfield *et al.* 1996, Cheah 1977, Dungey *et al.* 1997). In recent years, it has become apparent that there are many more species of *Mycosphaerella* Johanson occurring on eucalypts than previously realised. While some of these fungi cause serious disease problems, others cause minor leaf spots, rarely resulting in severe disease (Crous 1998, Crous *et al.* 2004b). Little is known regarding some of these less important species but some could become more important in genetically uniform plantations of susceptible clonal hybrids or where trees are exposed to conditions of stress.

The genus *Mycosphaerella* Johanson includes more

than 2000 species names (Corlett 1991), and several thousand anamorphs that lack known teleomorphs (Crous & Braun 2003). Of these, 55 species from eucalypts were treated by Crous (1998) and several additional species have been described more recently (Carnegie & Keane 1998, Braun & Dick 2002, Maxwell *et al.* 2003, Crous *et al.* 2004b, Hunter *et al.* 2004). Species of *Mycosphaerella* are usually assumed to be host-specific, and presently there are little data available that can be used to refute this supposition. Although some taxa have been found to infect other, secondary hosts (Crous *et al.* 2004c, Groenewald *et al.* 2005), most seem to have narrow host ranges. Interestingly, where species have been reported to have wider host ranges within a plant family, e.g. as reported for *Ramularia* Unger anamorphs by Braun (1998), DNA-based techniques have clearly shown that in most cases these morphologically similar taxa are phylogenetically quite distinct (Crous & Groenewald, unpubl. data). Further confusion could result from species colonising atypical host tissue in an attempt to jump to an ideal host when this becomes available. Crous & Groenewald (2005) have referred to this unusual behavioural pattern as the “pogo stick hypothesis”. In *Mycosphaerella* it has been observed to be true for teleomorph as well as anamorph states. When isolates of these fungi colonising atypical substrates are collected without proving their pathogenicity, incorrect conclusions pertaining to host range could arise.

**Table 1.** *Mycosphaerella* and anamorph isolates included in this study for sequence analysis and morphological comparison.

Teleomorph	Anamorph	Strain no. <sup>1</sup>	Substrate	Country	Collector	ITS GenBank number
<i>Mycosphaerella communis</i>	<i>Dissoconium commune</i>	CPC 11700	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ302948
		CPC 11703	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ302949
		CPC 11792	<i>Eucalyptus</i> sp.	Portugal	A.J.L. Phillips	DQ302950
<i>Mycosphaerella cryptica</i>	<i>Colletogloeopsis nubilosum</i>	CBS 111679; CPC 1576	<i>Eucalyptus nitens</i>	Australia	M.J. Wingfield	DQ302951
<i>Mycosphaerella endophytica</i>	<i>Pseudocercospora endophytica</i>	CBS 111519; CPC 1191	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ302952
		CBS 114662; CPC 1193	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ302953
<i>Mycosphaerella eucalyptorum</i>	—	CBS 118496; CPC 11174	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ302954
<i>Mycosphaerella flexuosa</i>	<i>Stenella</i> sp.	CBS 110743; CPC 673	<i>Eucalyptus globulus</i>	Colombia	M.J. Wingfield	DQ302955
		CBS 111055; CPC 1200	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ302956
		CBS 111163; CPC 1201	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ302957
		CPC 10995	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ302958
<i>Mycosphaerella gamsii</i>	—	CBS 118495; CPC 11138	<i>Eucalyptus</i> sp.	India	W. Gams	DQ302959
<i>Mycosphaerella gracilis</i>	<i>Pseudocercospora gracilis</i>	CBS 111189; CPC 1315	<i>Eucalyptus urophylla</i>	Indonesia	M.J. Wingfield	DQ302960
		CPC 11144	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ302961
		CPC 11181	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ302962
<i>Mycosphaerella heimii</i>	<i>Pseudocercospora heimii</i>	CPC 11441	<i>Eucalyptus</i> sp.	Brazil	A.C. Alfenas	DQ302963
		CPC 11453	<i>Eucalyptus</i> sp.	Brazil	A.C. Alfenas	DQ302964
		CPC 11548	<i>Eucalyptus</i> sp.	Brazil	A.C. Alfenas	DQ302965
		CPC 11716	—	Brazil	A.C. Alfenas	DQ302966
		CPC 11879	<i>Eucalyptus</i> sp.	Portugal	A.J.L. Phillips	DQ302967
<i>Mycosphaerella jonkershoekensis</i>	—	CBS 112224; CPC 3116	<i>Protea lepidocarpodendron</i>	Australia	P.W. Crous	DQ302968
<i>Mycosphaerella lateralis</i>	<i>Dissoconium dekkeri</i>	CPC 11218	<i>Eucalyptus comaldulensis</i>	Bolivia	M.J. Wingfield	DQ302969
		CPC 11293	<i>Eucalyptus tereticornis</i>	Bolivia	M.J. Wingfield	DQ302970
		CPC 11484	<i>Eucalyptus</i> sp.	Spain	P. Mansilla	DQ302971
		CPC 11706	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ302972
		CPC 11729	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ302973
		CPC 11732	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ302974
		CPC 11789	<i>Eucalyptus</i> sp.	Portugal	J.P. Sampaio	DQ302975
<i>Mycosphaerella madeirae</i>	—	CPC 3746	<i>Eucalyptus grandis</i>	Madeira	S. Denman	DQ302976
<i>Mycosphaerella marksii</i>	<i>? Pseudocercospora epispermogoniana</i>	CBS 110981; CPC 1073	<i>Eucalyptus</i> sp.	Tanzania	M.J. Wingfield	DQ302977
		CBS 111670; CPC 1499	<i>Eucalyptus globulus</i>	Uruguay	M.J. Wingfield	DQ302978
		CBS 115501; CPC 5358	<i>Leucadendron tinctum</i>	Madeira	S. Denman	DQ302979
		CBS 116316; CPC 3715	<i>Eucalyptus deglupha</i>	Ecuador	M.J. Wingfield	DQ302980
		CPC 11215	<i>Eucalyptus comaldulensis</i>	Bolivia	M.J. Wingfield	DQ302981

Table 1. (Continued).

Teleomorph	Anamorph	Strain no. <sup>1</sup>	Substrate	Country	Collector	ITS GenBank number
		CPC 11221	<i>Eucalyptus grandis</i>	Bolivia	M.J. Wingfield	DQ302982
		CPC 11222	<i>Eucalyptus grandis</i>	Bolivia	M.J. Wingfield	DQ302983
		CPC 11795	<i>Vepris reflexa</i>	South Africa	P.W. Crous	DQ302984
<i>Mycosphaerella molleriana</i>	<i>Colletogloeopsis molleriana</i>	CPC 11187	<i>Eucalyptus</i> sp.	Spain	M.J. Wingfield	DQ302985
		CPC 11685	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ302986
		CPC 11688	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ302987
		CPC 11709	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ302988
		CPC 11842	<i>Eucalyptus</i> sp.	Portugal	A.J.L. Phillips	DQ302989
		CPC 11845	<i>Eucalyptus</i> sp.	Portugal	A.J.L. Phillips	DQ302990
		CPC 12056	<i>Eucalyptus</i> sp.	Uruguay	M.J. Wingfield	DQ302991
<i>Mycosphaerella nubilosa</i>	? <i>Uwebraunia juvenis</i>	CPC 11246	<i>Eucalyptus globulus</i>	Spain	M.J. Wingfield	DQ302992
		CPC 11249	<i>Eucalyptus globulus</i>	Spain	M.J. Wingfield	DQ302993
		CPC 11487	<i>Eucalyptus</i> sp.	Spain	P. Mansilla	DQ302994
		CPC 11559	<i>Eucalyptus</i> sp.	Spain	P. Mansilla	DQ302995
		CPC 11723	<i>Eucalyptus globulus</i>	Portugal	A.C. Alfenas	DQ302996
		CPC 11761	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ302997
		CPC 11767	<i>Eucalyptus globulus</i>	Portugal	L.P. Phillips	DQ302998
		CPC 11882	<i>Eucalyptus globulus</i>	Portugal	A.J.L. Phillips	DQ302999
		CPC 11885	<i>Eucalyptus</i> sp.	Portugal	A.J.L. Phillips	DQ303000
<i>Mycosphaerella parva</i>	—	CPC 11273	<i>Eucalyptus globulus</i>	Spain	M.J. Wingfield	DQ303001
		CPC 11758	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ303002
		CPC 11759	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ303003
		CPC 11764	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ303004
		CPC 11888	<i>Eucalyptus</i> sp.	Portugal	A.J.L. Phillips	DQ303005
<i>Mycosphaerella perpendicularis</i>	—	CBS 118367; CPC 10983	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ303006
<i>Mycosphaerella pluritubularis</i>	—	CBS 118508; CPC 11697	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ303007
<i>Mycosphaerella pseudoafricana</i>	—	CBS 114782; CPC 1230	<i>Eucalyptus globulus</i>	Zambia	T.A. Coutinho	DQ303008
<i>Mycosphaerella pseudocryptica</i>	<i>Colletogloeopsis</i> sp.	CPC 11264	<i>Eucalyptus</i> sp.	New Zealand	J.A. Stalpers	DQ303009
		CBS 118504; CPC 11267	<i>Eucalyptus</i> sp.	New Zealand	J.A. Stalpers	DQ303010
<i>Mycosphaerella pseudosuberosa</i>	<i>Trimmatostroma</i> sp.	CBS 118911; CPC 12085	<i>Eucalyptus</i> sp.	Uruguay	M.J. Wingfield	DQ303011
<i>Mycosphaerella quasicercospora</i>	—	CBS 111161; CPC 1098	<i>Eucalyptus</i> sp.	Tanzania	M.J. Wingfield	DQ303012
<i>Mycosphaerella readeriellophora</i>	<i>Readeriella readeriellophora</i>	CPC 11711	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ303013
<i>Mycosphaerella scytalidii</i>	—	CBS 516.93; CPC 653	<i>Eucalyptus globulus</i>	Brazil	F.A. Ferreira	DQ303014
		CPC 10988	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ303015

Table 1. (Continued).

Teleomorph	Anamorph	Strain no. <sup>1</sup>	Substrate	Country	Collector	ITS GenBank number
<i>Mycosphaerella secundaria</i>	—	CBS 118493; CPC 10998	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ303016
		CBS 111002; CPC 1112	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303017
		CBS 115608; CPC 504	<i>Eucalyptus grandis</i>	Brazil	A.C. Alfenas	DQ303018
		CPC 10989	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ303019
<i>Mycosphaerella</i> sp.	<i>Stenella pseudoparkii</i>	CBS 118507; CPC 11551	<i>Eucalyptus</i> sp.	Brazil	A.C. Alfenas	DQ303020
		CBS 110988; CPC 1090	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303021
		CBS 110992; CPC 1092	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303022
		CBS 110999; CPC 1087	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303023
		CBS 111000; CPC 1088	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303024
		CBS 111049; CPC 1089	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303025
		<i>Mycosphaerella</i> sp.	<i>Stenella xenoparkii</i>	CBS 111088; CPC 1299	<i>Eucalyptus</i> sp.	Indonesia
CBS 111089; CPC 1301	<i>Eucalyptus</i> sp.			Indonesia	M.J. Wingfield	DQ303027
CBS 111185; CPC 1300	<i>Eucalyptus</i> sp.			Indonesia	M.J. Wingfield	DQ303028
<i>Mycosphaerella</i> sp.	—	CBS 208.94 / CPC 727	<i>Eucalyptus grandis</i>	Indonesia	A.C. Alfenas	DQ303029
<i>Mycosphaerella</i> sp.	—	CBS 209.94 / CPC 728	<i>Eucalyptus grandis</i>	Indonesia	A.C. Alfenas	DQ303030
<i>Mycosphaerella</i> sp.	—	CBS 110678; CPC 652	<i>Eucalyptus globulus</i>	Brazil	F.A. Ferreira	DQ303031
<i>Mycosphaerella</i> sp.	—	CBS 110679; CPC 653	<i>Eucalyptus globulus</i>	Brazil	F.A. Ferreira	DQ303032
<i>Mycosphaerella</i> sp.	—	CBS 110745; CPC 651	<i>Eucalyptus globulus</i>	Brazil	F.A. Ferreira	DQ303033
<i>Mycosphaerella</i> sp.	—	CBS 110987; CPC 1093	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303034
<i>Mycosphaerella</i> sp.	—	CBS 110991; CPC 1091	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303035
<i>Mycosphaerella</i> sp.	—	CBS 111036; CPC 1101	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303036
<i>Mycosphaerella</i> sp.	—	CPC 10986	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ303037
<i>Mycosphaerella</i> sp.	—	CPC 11002	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ303038
<i>Mycosphaerella</i> sp.	—	CPC 11004	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ303039
<i>Mycosphaerella</i> sp.	—	CPC 12200	<i>Eucalyptus</i> sp.	South Africa	Z.A. Pretorius	DQ303040
<i>Mycosphaerella</i> sp.	—	CPC 12147	<i>Acacia mangium</i>	Thailand	W. Himaman	DQ303041
<i>Mycosphaerella stramentii</i>	—	CBS 118909; CPC 11545	<i>Eucalyptus</i> sp.	Brazil	A.C. Alfenas	DQ303042
<i>Mycosphaerella stramenticola</i>	—	CBS 118506; CPC 11438	<i>Eucalyptus</i> sp.	Brazil	A.C. Alfenas	DQ303043
<i>Mycosphaerella suberosa</i>	—	CPC 11032	<i>Eucalyptus</i> sp.	Colombia	M.J. Wingfield	DQ303044
		CPC 11190	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ303045
		CPC 11276	<i>Eucalyptus comaldulensis</i>	Spain	M.J. Wingfield	DQ303046
		CPC 12193	<i>Eucalyptus</i> sp.	—	A.C. Alfenas	DQ303047
<i>Mycosphaerella sumatrensis</i>	—	CBS 118499; CPC 11171	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ303048



Table 1. (Continued).

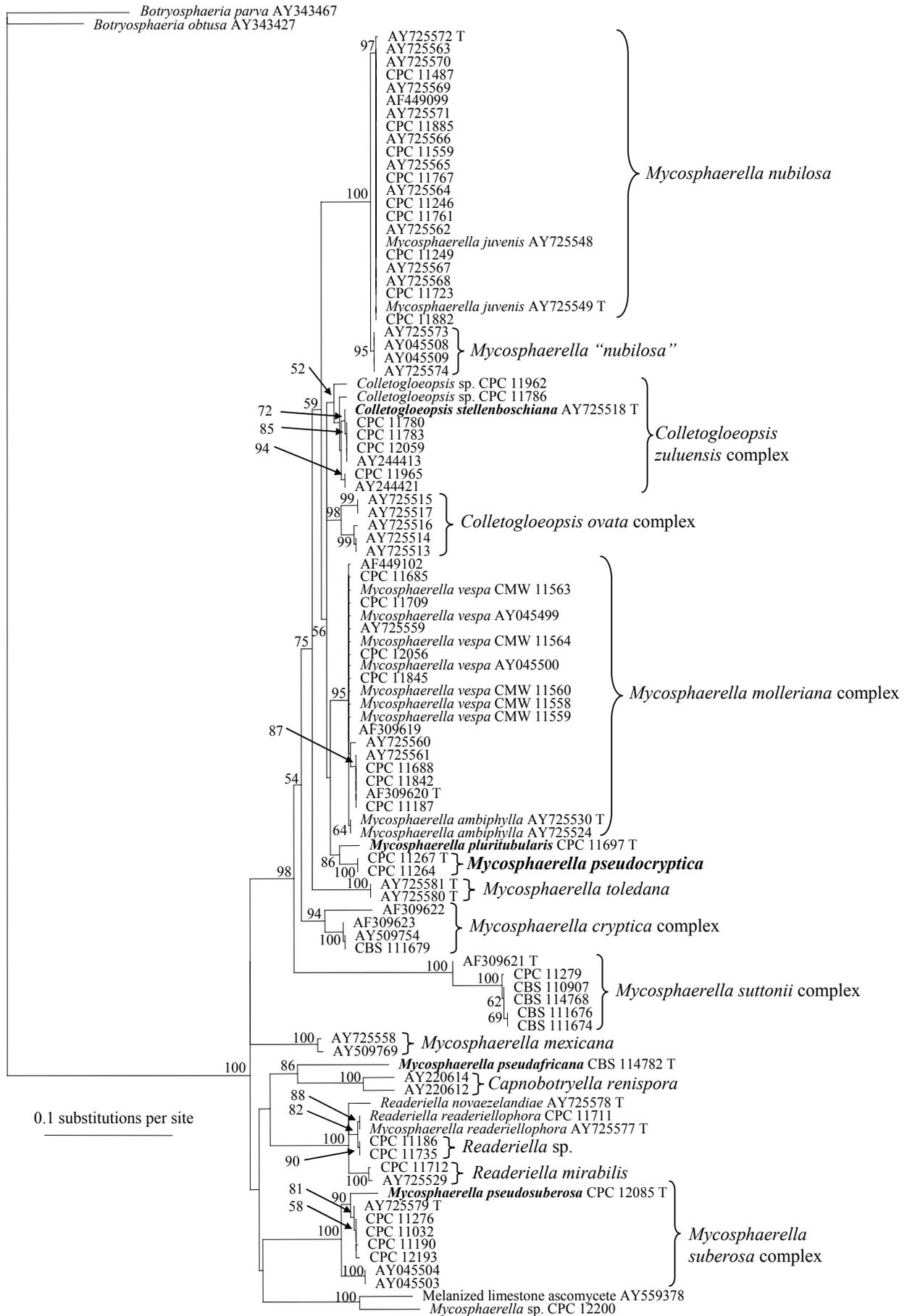
Teleomorph	Anamorph	Strain no. <sup>1</sup>	Substrate	Substrate	Collector	ITS GenBank number
<i>Mycosphaerella suttonii</i>	<i>Kirramyces epicoccoides</i>	CBS 118501; CPC 11175	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ303049
		CBS 118502; CPC 11178	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ303050
		CBS 111676; CPC 1550	<i>Eucalyptus grandis</i>	Australia	M.J. Wingfield	DQ303051
		CBS 114768; CPC 1409	<i>Eucalyptus</i> sp.	Brazil	P.W. Crous	DQ303052
		CBS 110907; CPC 63	<i>Eucalyptus grandis</i>	South Africa	P.W. Crous	DQ303053
		CBS 111674; CPC 1581	<i>Eucalyptus grandis</i>	Australia	M.J. Wingfield	DQ303054
<i>Mycosphaerella verrucosiafricana</i>	—	CPC 11279	<i>Eucalyptus tereticornis</i>	Bolivia	M.J. Wingfield	DQ303055
		CBS 118496; CPC 11167	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ303056
		CBS 118497; CPC 11169	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ303057
<i>Mycosphaerella vespa</i>	<i>Colletogloeopsis</i> sp.	CBS 118498; CPC 11170	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ303058
		CMW 11558	<i>Eucalyptus</i> sp.	Australia	—	DQ303059
		CMW 11559	<i>Eucalyptus</i> sp.	Australia	—	DQ303060
		CMW 11560	<i>Eucalyptus</i> sp.	Australia	—	DQ303061
		CMW 11563	<i>Eucalyptus</i> sp.	Australia	—	DQ303062
		CMW 11564	<i>Eucalyptus</i> sp.	Australia	—	DQ303063
<i>Mycosphaerella walkeri</i>	<i>Sonderhenia eucalypticola</i>	CPC 11252	<i>Eucalyptus globulus</i>	Spain	M.J. Wingfield	DQ303064
—	<i>Colletogloeopsis zuluensis</i>	CPC 11780	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ303065
—	—	CPC 11783	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ303066
—	—	CPC 11962; CMW 17322	<i>Eucalyptus</i> sp.	South Africa	M.J. Wingfield	DQ303067
—	—	CPC 11965; CMW 17326	<i>Eucalyptus</i> sp.	Uruguay	M.J. Wingfield	DQ303068
—	—	CPC 12059	<i>Eucalyptus</i> sp.	Uruguay	M.J. Wingfield	DQ303069
—	<i>Colletogloeopsis</i> sp.	CPC 11786	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ303070
—	<i>Pseudocercospora basitruncata</i>	CBS 114664; CPC 1202	<i>Eucalyptus grandis</i>	Colombia	M.J. Wingfield	DQ303071
—	<i>Pseudocercospora clematidis</i>	CPC 11657	<i>Clematis</i> sp.	U.S.A.	M.A. Palm	DQ303072
—	<i>Pseudocercospora epispermogoniana</i>	CBS 110693; CPC 823	<i>Eucalyptus grandis</i>	South Africa	G. Kemp	DQ303073
—	—	CBS 110694; CPC 824	<i>Eucalyptus grandis</i>	South Africa	G. Kemp	DQ303074
—	—	CBS 110750; CPC 822	<i>Eucalyptus grandis</i>	South Africa	G. Kemp	DQ303075
—	<i>Pseudocercospora fatouae</i>	CPC 11648	<i>Fatoua villosa</i>	Korea	H.D. Shin	DQ303076
—	<i>Pseudocercospora natalensis</i>	CBS 111069; CPC 1263	<i>Eucalyptus nitens</i>	South Africa	T.A. Coutinho	DQ303077
—	<i>Pseudocercospora pseudoecalyptorum</i>	CBS 116359; CPC 3751	<i>Eucalyptus</i> sp.	Madeira	S. Denman	DQ303078
—	—	CPC 10916	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ303079
—	—	CPC 11713	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ303080
—	<i>Pseudocercospora robusta</i>	CBS 111175; CPC 1269	<i>Eucalyptus robur</i>	Malaysia	M.J. Wingfield	DQ303081
—	<i>Pseudocercospora</i> sp.	CBS 111072; CPC 1266	<i>Eucalyptus pellita</i>	Thailand	M.J. Wingfield	DQ303082

Table 1. (Continued).

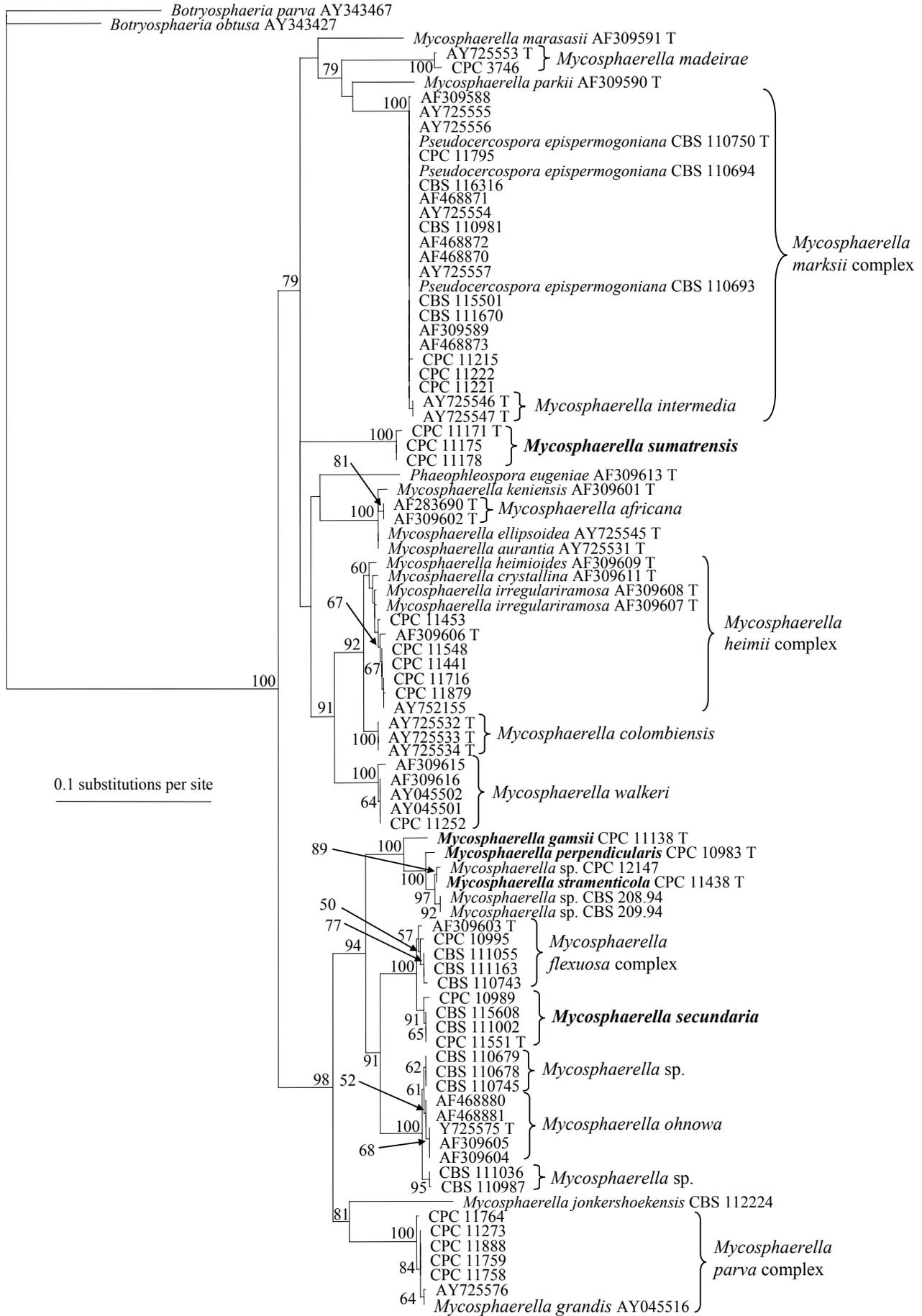
Teleomorph	Anamorph	Strain no. <sup>1</sup>	Substrate	Country	Collector	ITS GenBank number
		CBS 111373; CPC 1493	<i>Eucalyptus globulus</i>	Uruguay	M.J. Wingfield	DQ303083
		CPC 11591	<i>Brachybotrys paridiformis</i>	Korea	H.D. Shin	DQ303084
		CPC 11592	<i>Zelkova serrata</i>	Korea	H.D. Shin	DQ303085
		CPC 11654	<i>Morus bombycis</i>	Korea	H.D. Shin	DQ303086
		CPC 11668	<i>Pilea hamaoi</i>	Korea	H.D. Shin	DQ303087
		CPC 11680	<i>Ampelopsis brevipedunculata</i> var. <i>heterophylla</i>	Korea	H.D. Shin	DQ303088
		CPC 11726	<i>Platanus occidentalis</i>	Korea	H.D. Shin	DQ303089
—	<i>Pseudocercospora subulata</i>	CBS 118489; CPC 10849	<i>Eucalyptus botryoides</i>	New Zealand	M. Dick	DQ303090
—	<i>Pseudocercospora capsellae</i>	CPC 11677	<i>Draba nemorosa</i> var. <i>hebecarpa</i>	Korea	H.D. Shin	DQ303091
—	<i>Readeriella</i> sp.	CPC 11186	<i>Eucalyptus globulus</i>	Spain	M.J. Wingfield	DQ303092
		CPC 11735	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ303093
—	<i>Readeriella mirabilis</i>	CPC 11712	<i>Eucalyptus globulus</i>	Spain	P. Mansilla	DQ303094
—	<i>Septoria eucalyptorum</i>	CBS 118505; CPC 11282	<i>Eucalyptus</i> sp.	India	W. Gams	DQ303095
—	<i>Septoria provencialis</i>	CBS 118910; CPC 12226	<i>Eucalyptus</i> sp.	France	P.W. Crous	DQ303096
—	<i>Stenella</i> sp.	CPC 11671	<i>Lonicera japonica</i>	Korea	H.D. Shin	DQ303097

<sup>1</sup>CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; CMW: Culture collection of Mike Wingfield, housed at FABI, Pretoria, South Africa

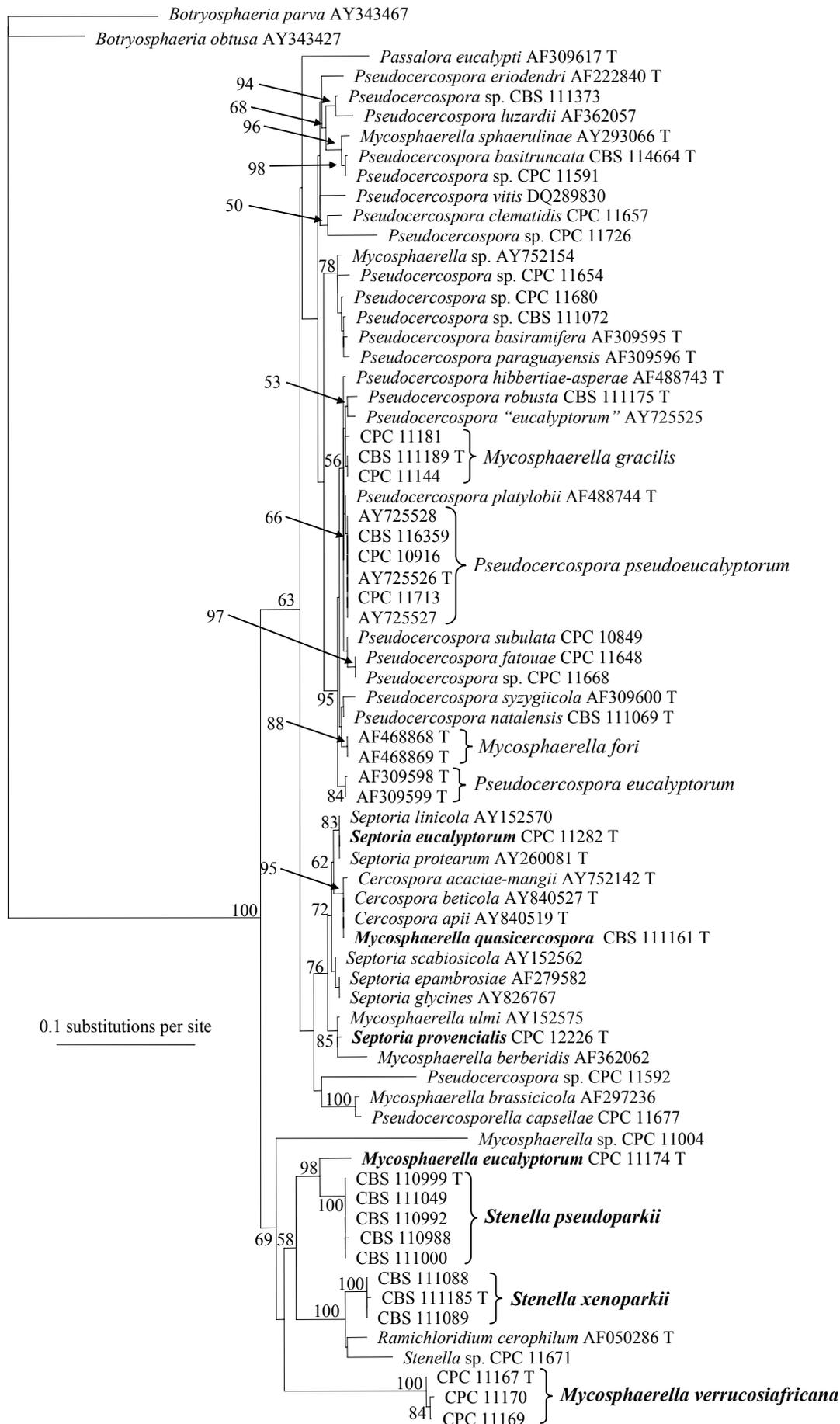




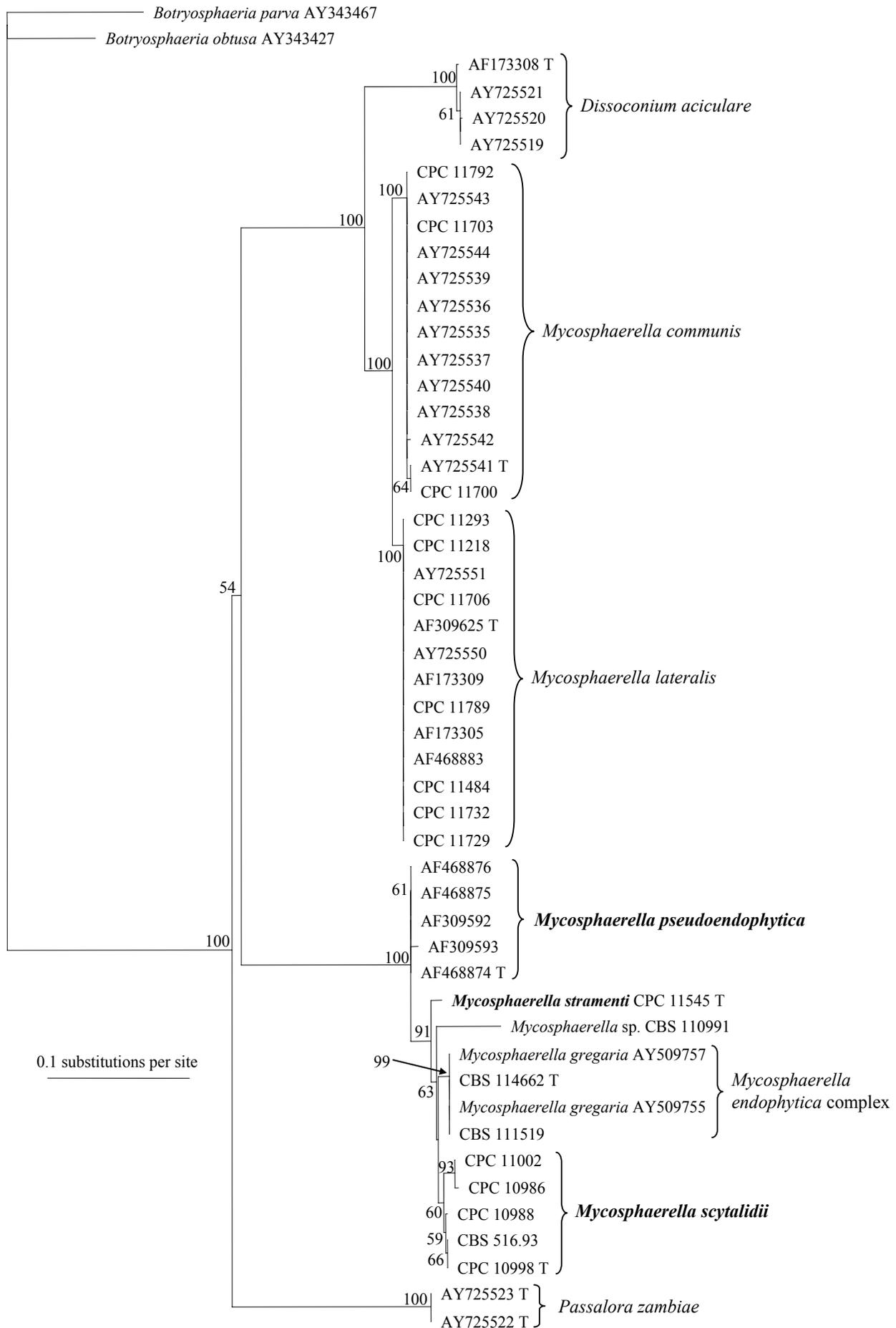
**Fig. 1.** Distance tree obtained from a neighbour-joining analysis using the HKY85 substitution model on the ITS sequence alignments. The scale bar shows the number of substitutions per site and bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to two *Botryosphaeria* species. New species are indicated in **bold**, and ex-type strains with a T.



**Fig. 2.** Distance tree obtained from a neighbour-joining analysis using the HKY85 substitution model on the ITS sequence alignments. The scale bar shows the number of substitutions per site and bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to two *Botryosphaeria* species. New species are indicated in **bold**, and ex-type strains with a T.



**Fig. 3.** Distance tree obtained from a neighbour-joining analysis using the HKY85 substitution model on the ITS sequence alignments. The scale bar shows the number of substitutions per site and bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to two *Botryosphaeria* species. New species are indicated in **bold**, and ex-type strains with a T.



**Fig.4.** Distance tree obtained from a neighbour-joining analysis using the HKY85 substitution model on the ITS sequence alignments. The scale bar shows the number of substitutions per site and bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to two *Botryosphaeria* species. New species are indicated in **bold**, and ex-type strains with a T.



The genus *Mycosphaerella* includes species that are pathogens (primary, secondary or opportunistic), saprobes, endophytes (saprobic or plant-pathogenic), or have mutualistic (in lichen) associations (Crous *et al.* 2000, 2001). Several taxa have low levels of virulence, and appear to be secondary colonists of lesions caused by other pathogens including species of *Mycosphaerella* (Crous 1998). Some species of *Ramularia* also appear to be hyperparasites on pustules of various rust species (Braun 1998). Because several species can co-inhabit the same lesion, either as primary or secondary pathogens, saprobes or endophytes (Crous 1998, Crous *et al.* 2004b), species identification based on the host can be extremely difficult. Although ascospore germination patterns, anamorph morphology and cultures greatly facilitate species identification, co-inhabitancy (Crous & Groenewald 2005) makes it difficult to link these cultures and anamorphs to their correct teleomorphs (Crous 2002).

The present study presents the second in a series characterising the *Mycosphaerella* species occurring on eucalypts. A major aim of this study was to use comparisons of DNA sequence data to clarify as many as possible of the formerly published host and distribution records (Crous 1998). Furthermore, while previous descriptions focused on species associated with leaf spots, this study also includes species from eucalypt leaf litter.

## MATERIALS AND METHODS

### Isolates

*Eucalyptus* leaves bearing *Mycosphaerella* ascomata, or with *Mycosphaerella* leaf spots were chosen for study. Excised lesions were soaked in water for approximately 2 h, after which they were placed in the bottom of Petri dish lids, with the top half of the dish containing 2 % malt extract agar (MEA) (Biolab, Midrand, South Africa). Ascospore germination patterns were examined after 24 h, and single-ascospore and conidial cultures established as described by Crous (1998). Colonies were sub-cultured onto carnation leaf agar (CLA) [1 % water agar (Biolab) with autoclaved carnation leaves placed onto the surface of the solidified medium] and incubated at 25 °C under continuous near-ultraviolet light to promote sporulation.

### DNA phylogeny

The protocol of Lee & Taylor (1990) was used to isolate genomic DNA from fungal mycelium, grown on MEA in Petri dishes. The primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify part of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. The PCR reaction mixture and conditions were the same as those used by Crous *et al.* (2004b).

The ITS nucleotide sequences generated in this study were added to other sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov>) and

the alignment was assembled using Sequence Alignment Editor v. 2.0a11 (Rambaut 2002) with manual adjustments for visual improvement where necessary. Due to the size and the complexity of the original alignment, the sequences were split over four smaller alignments, each containing genetically similar sequences. The four datasets were each treated identically. Phylogenetic analyses of sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2002). Phylogenetic analysis of the aligned ITS sequence data consisted of neighbour-joining analysis with the uncorrected ("p"), the Kimura 2-parameter and the HKY85 substitution model in PAUP. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. When they were encountered, ties were broken randomly. Sequence data were deposited in GenBank (Table 1) and the alignments in TreeBASE.

### Taxonomy

Wherever possible, 30 measurements ( $\times 1000$  magnification) were made of structures mounted in lactic acid, with the extremes of spore measurements given in parentheses. Colony colours (surface and reverse) were assessed after 1 mo on MEA, oatmeal agar (OA) and potato-dextrose agar (PDA) (Gams *et al.* 1998) at 25 °C in the dark, using the colour charts of Rayner (1970). All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands (Table 1). Nomenclatural novelties and descriptions were deposited in MycoBank <[www.mycobank.org](http://www.mycobank.org)>.

## RESULTS

### DNA phylogeny

For the ITS region, approximately 500 to 560 bases were determined for all isolates (Table 1). The trees resulting from each of the four alignments are depicted in Figs 1–4. The first alignment contains 102 taxa (including the two outgroups) and 544 characters including alignment gaps. Of these characters, 295 are parsimony-informative, 37 are variable and parsimony-uninformative, and 212 are constant. Neighbour-joining analysis using the three substitution models yielded trees with similar topologies and bootstrap values. Parsimony analysis yielded 243 most parsimonious trees (TL = 1038 steps; CI = 0.620; RI = 0.893; RC = 0.554). The topology of the distance trees differed from the trees obtained using parsimony mainly at the deeper nodes (data not shown). Parts of the distance tree obtained using the HKY85 substitution model are shown in Figs 1–4. The first alignment and derived tree (Fig. 1) includes *M. nubilosa* (100 % bootstrap support), species of *Colletogloeopsis* Crous & M.J. Wingf., the *M. molleriana* (Thüm.) Lindau complex (95 % bootstrap support), the *M. suttonii* Crous & M.J. Wingf. complex (100 % bootstrap support) and the *M. suberosa* Crous, F.A. Ferreira, Alfenas & M.J. Wingf. complex (100 % bootstrap support). One new

species of *Colletogloeopsis*, and four new species of *Mycosphaerella* are indicated.

The second alignment (Fig. 2) contains 90 taxa (including the two outgroups) and 535 characters including alignment gaps. Of these characters, 246 are parsimony-informative, 51 are variable and parsimony-uninformative, and 238 are constant. Neighbour-joining analysis using the three substitution models yielded trees with identical topologies and similar bootstrap values. Parsimony analysis yielded 481 most parsimonious trees (TL = 862 steps; CI = 0.613; RI = 0.927; RC = 0.568). The topology of the distance trees differed from the trees obtained using parsimony only in the placement of the *Mycosphaerella* sp. CPC 11171 clade (data not shown). The second alignment and derived tree mainly includes the *M. marksii* Carnegie & Keane complex (100 % bootstrap support), the *M. heimii* Crous complex (60 % bootstrap support), the *M. walkeri* R.F. Park & Keane (100 % bootstrap support) and the *M. parva* R.F. Park & Keane complex (100 % bootstrap support). Five new species of *Mycosphaerella* are indicated in the tree.

The third alignment (Fig. 3) contains 71 taxa (including the two outgroups) and 529 characters including alignment gaps. Of these characters, 237 are parsimony-informative, 71 are variable and parsimony-uninformative, and 221 are constant. Neighbour-joining analysis using the three substitution models yielded trees with identical topologies and similar bootstrap values. Parsimony analysis yielded 4319 most parsimonious trees (TL = 853 steps; CI = 0.626; RI = 0.856; RC = 0.536). The topology of the distance trees differed from the trees obtained using parsimony mainly at the deeper nodes (data not shown). The third alignment and derived tree mainly includes species of *Pseudocercospora* Speg., *Cercospora* Fresen., *Septoria* Sacc. and *Stenella* Syd. New species indicated in the tree include three in *Mycosphaerella*, two in *Septoria*, and two in *Stenella*.

The fourth alignment (Fig. 4) contains 50 taxa (including the two outgroups) and 570 characters including alignment gaps. Of these characters, 293 are parsimony-informative, 25 are variable and parsimony-uninformative, and 252 are constant. Neighbour-joining analysis using the three substitution models yielded trees with identical topologies and similar bootstrap values. Parsimony analysis yielded eight most parsimonious trees (TL = 627 steps; CI = 0.864; RI = 0.973; RC = 0.841). The topology of the distance trees was similar to that of the topology of the trees obtained using parsimony (data not shown). The fourth alignment and derived tree includes species of *Dissoconium* de Hoog, Oorschot & Hijwegen, *Passalora zambiae* Crous & T. Coutinho and *Mycosphaerella*, with three new species.

### Taxonomy

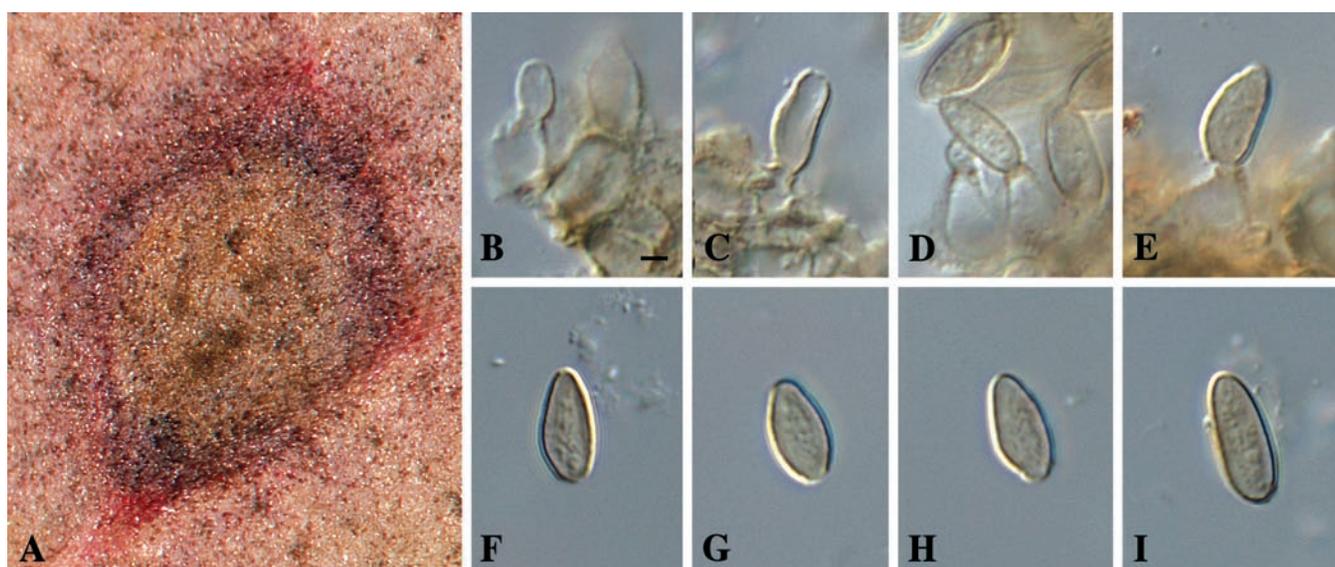
Several collections represented *Mycosphaerella* spp. morphologically and phylogenetically distinct from ex-type strains of the morphological species to which they had originally been assigned. These fungi are described as new taxa as follows:

***Colletogloeopsis stellenboschiana* Crous, sp. nov.**  
 MycoBank MB500833. Fig. 5.

***Etymology:*** Refers to Stellenbosch, where the fungus was collected.

*Coniothyrio ovato* similis sed conidiis minoribus, (6.5–)7–9(–10) × (3.5(–4) μm, distincta.

***Leaf spots*** amphigenous, circular to subcircular, 0.5–3 mm diam, pale brown, with a raised border and red-purple margin. ***Conidiomata*** amphigenous, pycnidial, medium brown, globose, 80–120 μm diam; wall of 3–4 layers of brown *textura angularis*. ***Conidiogenous cells*** discrete, ampulliform to subcylindrical, pale to medium brown, finely verruculose, proliferating 1–3 times percurrently near the apex, 3–6 × 3–4 μm. ***Conidia*** holoblastic, solitary, aseptate, ellipsoidal, with



**Fig. 5.** *Colletogloeopsis stellenboschiana* (CBS 116428). A. Leaf spot. B–E. conidiogenous cells giving rise to conidia. F–I. Conidia. Scale bar = 3.5 μm.

subobtuse apex and subtruncate base with minute marginal frill, medium brown, finely verruculose, widest below the middle, (6.5–)7–9(–10) × (3–)3.5(–4) µm.

**Holotype:** South Africa, Western Cape Province, Stellenbosch Mountain, on leaves of *Eucalyptus* sp., 4 Dec. 2004, P.W. Crous, CBS H-19688, **holotype**, culture ex-type CBS 116428 = CPC 10886.

**Cultures:** Colonies after 3 wk on MEA 15–40 mm diam; on PDA erumpent, spreading, producing copious amounts of slime, olivaceous-black at the centre, aerial mycelium olivaceous-grey, with a vinaceous-grey outer zone and wide olivaceous-black margin that is smooth but uneven; reverse olivaceous-black; on OA surface smoke-grey with a wide, grey-olivaceous border, forming a characteristic yellow pigment; on MEA grey-white on surface, with sectors of smoke-grey; margin thin, submerged, smoke-grey; reverse olivaceous-black; aerial mycelium sparse to moderate, grey-white; colonies fertile.

**Host:** *Eucalyptus* sp.

**Distribution:** South Africa.

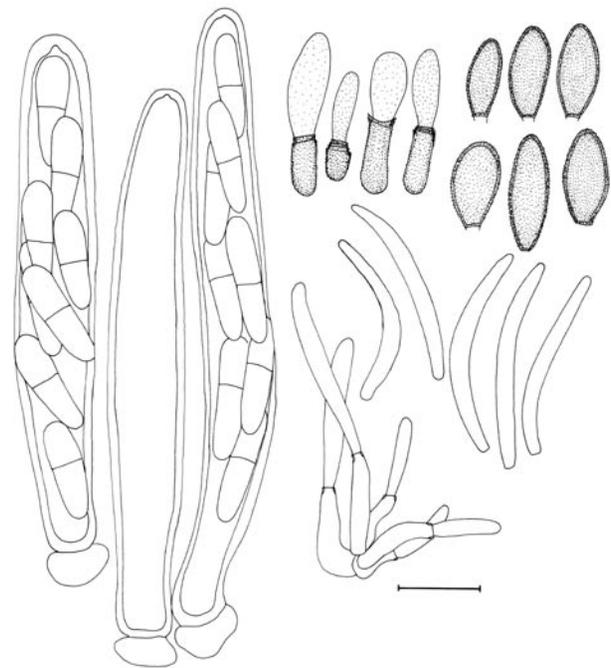
**Notes:** Numerous species of *Coniothyrium* Corda and several species of *Colletogloeopsis* cause spots on eucalypt leaves. *Colletogloeopsis stellenboschiana* is easily distinguished from the taxa occurring on eucalypt leaves (Crous 1998), and from representatives of the “*Coniothyrium ovatum*” species complex specifically, based on its conidial morphology. Phylogenetically it is closely related to members of the the *Colletogloeopsis* complex that cause stem cankers on eucalypt trees (Cortinas *et al.* 2006 – this volume)

***Mycosphaerella davisoniellae*** Crous, **sp. nov.**  
MycoBank MB500834. Fig. 6.

**Anamorph:** *Davisoniella eucalypti* H.J. Swart, Trans. Brit. Mycol. Soc. 90: 289. 1988.

Asci subcylindrici, subsessiles, 50–70 × 9–12 µm. Ascospores bi-vel triseriatae, tenuitunicatae, rectae, obovoideae, apicem versus latissimae, in medio uniseptatae, vix vel haud constrictae ad septum, 10–14 × 3–4 µm.

**Leaf spots** amphigenous, subcircular to irregular, 1–7 mm diam, discrete to confluent, medium brown, surrounded by raised, red-purple margin. **Ascomata** hypophyllous, embedded in a raised, black, subepidermal stroma, ostiolate, becoming erumpent up to 120 µm diam. **Asci** subcylindrical, subsessile, straight or slightly incurved, 8-spored, 50–70 × 9–12 µm. **Ascospores** bi- to triseriate, overlapping, hyaline, thin-walled, straight, obovoid with rounded ends, widest near the apex, medianly 1-septate, not to slightly constricted at the septum, tapering toward both ends, but more prominently toward the base, 10–14 × 3–4 µm. **Conidiomata** of *Davisoniella* embedded in the same black subepidermal stroma that contains ascomata, subepidermal, ostiolate, up to 450 µm diam; wall of 2–3 layers of brown *textura angularis*. **Conidiogenous cells** subcylindrical to ampulliform or doliiform, 5–15 × 3–4 µm, medium brown, verruculose, proliferating several times percurrently near the apex. **Conidia** solitary, brown, aseptate, verruculose, thick-walled, oval with an



**Fig. 6.** *Mycosphaerella davisoniellae* (anamorph *Davisoniella eucalypti*) (DAR 58999). A. Asci and ascospores. B. Conidiogenous cells and conidia of *D. eucalypti*. C. conidiogenous cells and conidia of synanamorph. Scale bar = 10 µm.

obtuse apex and a truncate to subtruncate base with a prominent basal frill, which can extend up to 2 µm from the brown basal rim of the conidium, (8–)10–12(–14) × 4.5–)5–6(–6.5) µm (av. 11 × 5.5 µm). **Synanamorph:** *Conidiomata* intermingled between that of *D. eucalypti* and ascomata of *M. davisoniellae*. **Conidiogenous cells** phialidic, hyaline, subcylindrical to ampulliform, with visible periclinal thickening, 8–15 × 2.5–3.5 µm. **Conidia** hyaline, curved, subcylindrical, widest in the middle, apex bluntly rounded, obtuse, base truncate, 17–30 × 2–1.5 µm.

**In vivo:** No cultures available.

**Specimen examined:** Australia, Darling Ranges W.A., Mundlimup Block, on leaves of *Eucalyptus marginata*, 24 Nov. 1981, F. Tay, DAR 58999, **holotype** of *D. eucalypti* and *M. davisoniellae*.

**Notes:** Swart (1988) reported that this fungus is associated with abundant leaf spots on saplings and the foliage of recently felled trees. *Conidiomata* of *D. eucalypti* were described as unilocular and subepidermal, occurring in a stroma which could result in some of them appearing as multilocular. Swart (1988) considered the fungus to be the stromatic counterpart of *Coniothyrium*. *Davisoniella eucalypti* is clearly related to species in the *Colletogloeopsis* complex that occurs on eucalypts, having characteristic aseptate, brown, verruculose conidia that arise from percurrently proliferating conidiogenous cells. *Davisoniella* is unique by virtue of its stroma, that gives rise to the uni- or multilocular conidiomata. Conidia of *D. eucalypti* exude in slimy masses. In many cases, the exudates included aseptate, hyaline, curved, subcylindrical conidia of a synanamorph. The latter anamorph was produced from unilocular conidiomata that formed in the same stromata that gave rise to *D. eucalypti*. Surprisingly, many of the

stromata investigated also contained ascomata of a *Mycosphaerella* species, which most likely also belong to the same fungus. The latter state is described here as *M. davisoniellae*.

***Mycosphaerella eucalyptorum*** Crous & M.J. Wingf., sp. nov. MycoBank MB500835. Fig. 7.

**Etymology:** Referring to its host, *Eucalyptus*.

*Mycosphaerellae parkii* similis, sed ascosporis maioribus, 12–17 × 3.5–4.5 µm, modo B germinantibus, distinguenda.

**Leaf spots** amphigenous, irregular to sub-circular, 2–20 mm diam, medium brown, with raised, brown borders, and thin, red-purple margins. **Ascomata** pseudothecial, amphigenous but predominantly epiphyllous, single, black, erumpent, globose, up to 120 µm diam; apical ostiole 10–15 µm diam, with prominent periphyses lining the ostiolar channel; wall of 2–3 layers of medium brown *textura angularis*. **Asci** aparaphysate, fasciculate, bitunicate, sessile, obovoid to ellipsoid, straight or slightly incurved, 8-spored, 35–50 × 8–12 µm. **Ascospores** tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid–ellipsoidal with obtuse ends, medianly 1-septate, widest in middle of apical cell, not constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (12–)14–15(–17) ×

(3.5–)4(–4.5) µm *in vivo*; some ascospores with slightly asymmetrical apical cells, as commonly observed in *M. marksii*.

**Holotype:** Indonesia, on leaves of *Eucalyptus* sp., Mar. 2004, M.J. Wingfield, CBS H-19689 **holotype**, culture ex-type CBS 118496 = CPC 11174.

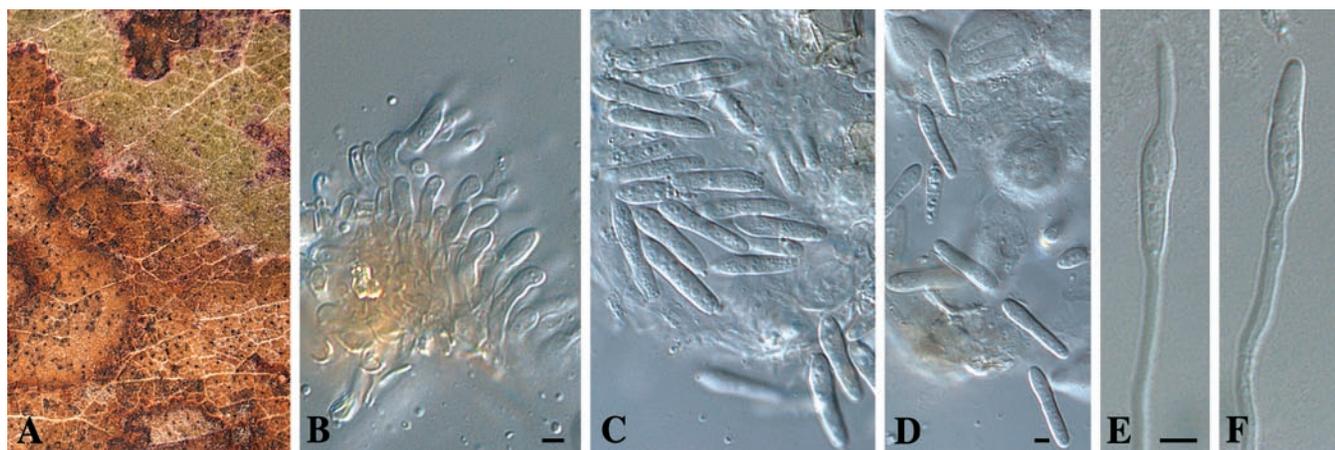
**Ascospore germination on MEA after 24 h:** Type B. Ascospores not darkening on MEA, and germinating from both ends, with germ tubes parallel to the long axis of the spore, not distorting, becoming slightly constricted upon germination, becoming up to 4 µm diam.

**Cultures:** Colonies on MEA after 3 wk 25–30 mm diam; on MEA flat, spreading, folding, with sparse aerial mycelium, olivaceous-grey, margins smooth, regular, reverse iron-grey; on PDA slightly erumpent, centre olivaceous-grey; outer zone pale olivaceous-grey; reverse iron-grey; on OA with sparse to moderate pale olivaceous-grey aerial mycelium and patches of olivaceous-grey.

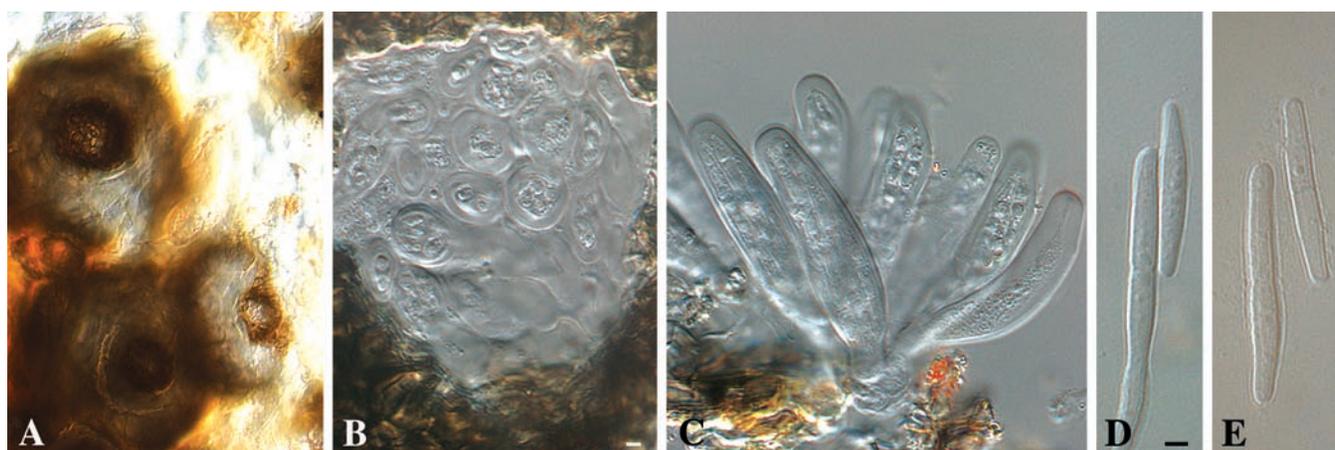
**Host:** *Eucalyptus* sp.

**Distribution:** Indonesia.

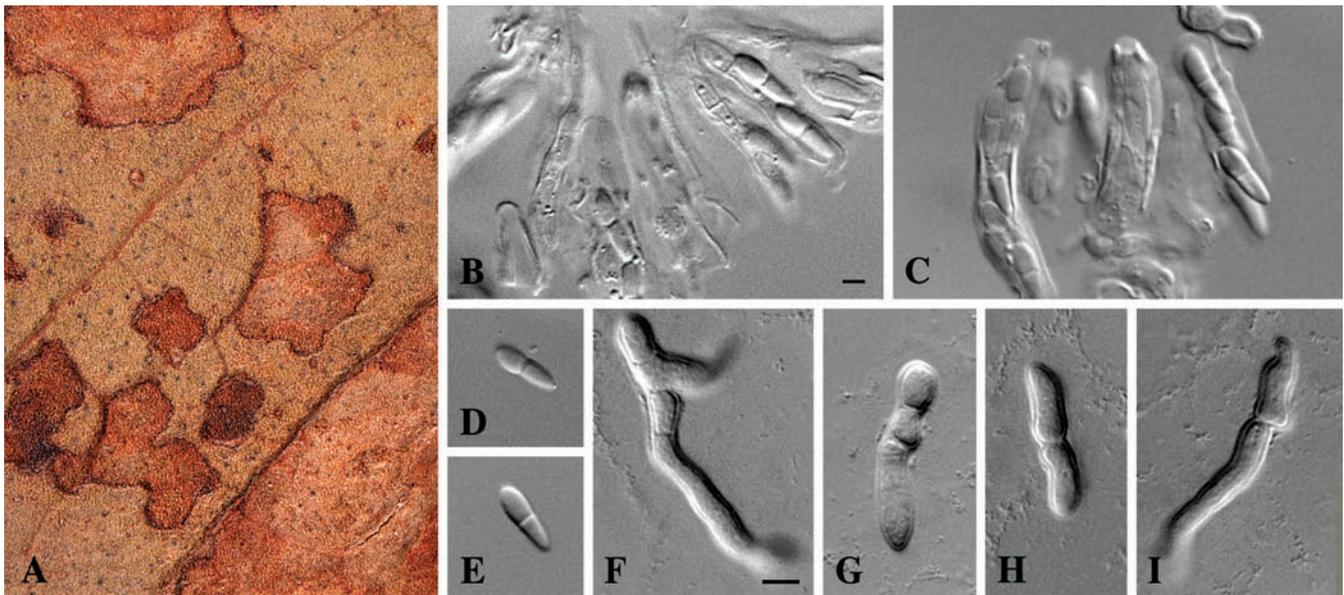
**Notes:** Conidia of a *Stenella* anamorph were found on some lesions. This link is, however, unconfirmed, and



**Fig. 7.** *Mycosphaerella eucalyptorum* (CBS 118496). A. Leaf spot. B. Ostiolar periphysoids. C–D. Ascospores. E–F. Germinating ascospores. Scale bars = 4 µm.



**Fig. 8.** *Mycosphaerella gracilis*. A. Ascomata on in leaf tissue. B. Ostiolar region of ascoma. C. Asci. D–E. Germinating ascospores. Scale bar = 3 µm.



**Fig. 9.** *Mycosphaerella gamsii* (CBS 118495). A. Leaf spot. B–C. Asci. D–E. Ascospores. F–I. Germinating ascospores. Scale bars: B = 3  $\mu$ m, F =  $\mu$ m.

isolates did not produce anamorph structures in culture. *Mycosphaerella eucalyptorum* is phylogenetically closely related to a *Mycosphaerella* sp. from Colombia that forms *Stenella pseudoparkii* in culture. Ascospores of *M. eucalyptorum* (12–17  $\times$  3.5–4.5  $\mu$ m) germinate with a Type B germination pattern as observed in *M. gracilis* (10–20  $\times$  2–3  $\mu$ m) (Fig. 8) and *M. marksii* (11–22.5  $\times$  2–3.5). It is easily distinguished from these taxa, however, based on its ascospore morphology and growth characteristics in culture (Crous 1998).

***Mycosphaerella gamsii* Crous, sp. nov.** MycoBank MB500836. Fig. 9.

**Etymology:** Named after the collector, well-known mycologist and friend, Prof. dr Walter Gams.

*Mycosphaerellae stramenticolae similis, sed ascosporis minoribus, (8–)9–10  $\times$  (2–)3  $\mu$ m, modo C germinantibus, distinguenda.*

**Leaf spots** amphigenous, irregular, 1–20 mm diam, medium brown, with a raised, dark brown border. **Ascomata** pseudothecial, amphigenous, but predominantly hypophyllous, single, black, subepidermal, becoming erumpent, globose, up to 90  $\mu$ m diam; apical ostiole 5–10  $\mu$ m diam; wall of 2–3 layers of medium brown *textura angularis*. **Asci** aparaphysate, fasciculate, bitunicate, subsessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 25–35  $\times$  7–9  $\mu$ m. **Ascospores** tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid–ellipsoidal, medianly 1-septate, widest in the middle of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)9–10  $\times$  (2–)3  $\mu$ m *in vivo*.

**Holotype:** India, Palampur, on leaves of *Eucalyptus* sp., Mar. 2004, W. Gams & M. Arzanlou, CBS H-19690, **holotype**, culture ex-type CBS 118495 = CPC 11138–11140. 5/6-6

**Ascospore germination on MEA after 24 h:** Type C. Ascospores not darkening on MEA, and germinating

from both ends, with germ tubes parallel to the long axis of the spore, but also variable in direction; becoming constricted upon germination, up to 5  $\mu$ m diam.

**Cultures:** Colonies on MEA 28–35 mm diam after 3 wk; on MEA spreading, folding, flat, with moderate smoke-grey aerial mycelium in the centre; outer region olivaceous-grey; margins smooth, regular; reverse iron-grey; on PDA with moderate aerial mycelium, pale olivaceous-grey, outer region olivaceous-grey with drops of slime; reverse iron-grey; on OA with moderate aerial mycelium, pale olivaceous-grey, with patches of olivaceous-grey.

**Host:** *Eucalyptus* sp.

**Distribution:** India.

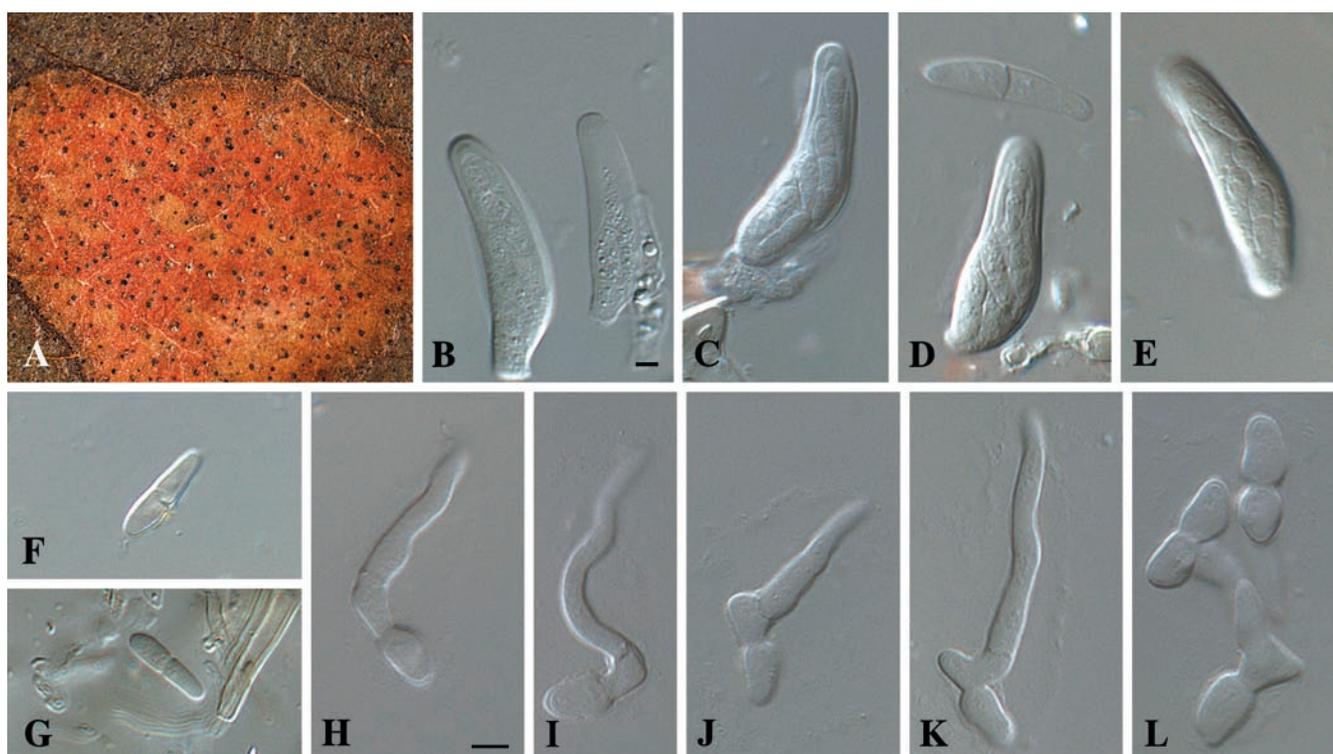
**Notes:** *Mycosphaerella gamsii* is phylogenetically closely related to *M. stramenticola*, but is distinguishable in having a Type C ascospore germination pattern, as is found in species such as *M. heimii*, *M. gregaria*, *M. molleriana*, *M. nubilosa* and *M. walkeri*. *Mycosphaerella gamsii* has ascospores that are 8–10  $\times$  2–3  $\mu$ m, thus shorter than those of the species listed above, and it also lacks an anamorph in culture.

***Mycosphaerella perpendicularis* Crous & M.J. Wingf., sp. nov.** MycoBank MB500837. Fig. 10.

**Etymology:** Referring to ascospores that germinate with germ tubes growing 90° to the long axis of the spore.

*Mycosphaerellae heimioide similis, sed ascosporis longioribus, (8–)9–10(–12)  $\times$  (2.5–)3  $\mu$ m, modo M germinantibus distinguenda.*

**Leaf spots** amphigenous, irregular to sub-circular, 5–15 mm diam, medium brown, frequently with a orange-red discoloration in the central part; border raised, dark brown. **Ascomata** pseudothecial, epiphyllous, single, black, subepidermal, globose, up to 90  $\mu$ m diam; apical ostiole 10–15  $\mu$ m diam; wall of 2–3 layers of medium brown *textura angularis*. **Asci** aparaphysate,



**Fig. 10.** *Mycosphaerella perpendicularis* (CBS 118367). A. Leaf spot. B–E. Asci. F–G. Ascospores. H–L. Germinating ascospores. Scale bars: B = 3  $\mu$ m, H = 5  $\mu$ m.

fasciculate, bitunicate, sessile, obovoid to broadly ellipsoid, slightly incurved, 8-spored, 25–35  $\times$  7–8  $\mu$ m. *Ascospores* multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid–ellipsoidal with obtuse ends, medianly 1-septate, widest in the middle of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)9–10(–12)  $\times$  (2.5–)3  $\mu$ m *in vivo*.

*Holotype*: Colombia, Suiza, on leaves of *Eucalyptus eurograndis*, Jan. 2004, M.J. Wingfield, CBS H-19691, **holotype**, culture ex-type CBS 118367 = CPC 10983–10985.

*Ascospore germination on MEA after 24 h*: Type M. Ascospores not darkening on MEA, and germinating from both ends, with germ tubes 90° to the long axis of the spore, and distorting upon germination, becoming up to 5  $\mu$ m wide.

*Cultures*: Colonies on MEA reaching 28–37 mm diam after 3 wk; colonies folding, spreading, flat, with sparse aerial mycelium, which is olivaceous-grey on the agar surface, and with smoke-grey aerial mycelium; margins are smooth, regular; reverse iron-grey at the centre, olivaceous-grey in the outer zone; on OA with moderate aerial mycelium, olivaceous-grey at centre, greenish black in outer zone; on PDA olivaceous-grey with some drops of slime, iron-grey in reverse.

*Host*: *Eucalyptus eurograndis*.

*Distribution*: Colombia.

*Notes*: Germinating ascospores of *M. perpendicularis* have a characteristic Type M germination pattern, similar to that of *M. heimioides*. *Mycosphaerella perpendicularis* can easily be distinguished from *M.*

*heimioides*, however, by virtue of the fact that the ascospores distort at germination. In addition, the germ tubes of *M. heimioides* never quite reach 90° to the long axis of the spore, whereas those of *M. perpendicularis* are at right angles.

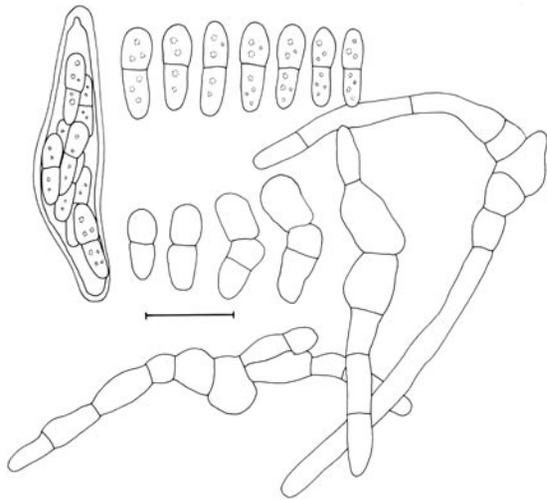
***Mycosphaerella pluritubularis*** Crous & J.P. Mansilla, **sp. nov.** MycoBank MB500838. Figs 11–12.

*Etymology*: Refers to the ascospores that have multiple germ tubes when they germinate.

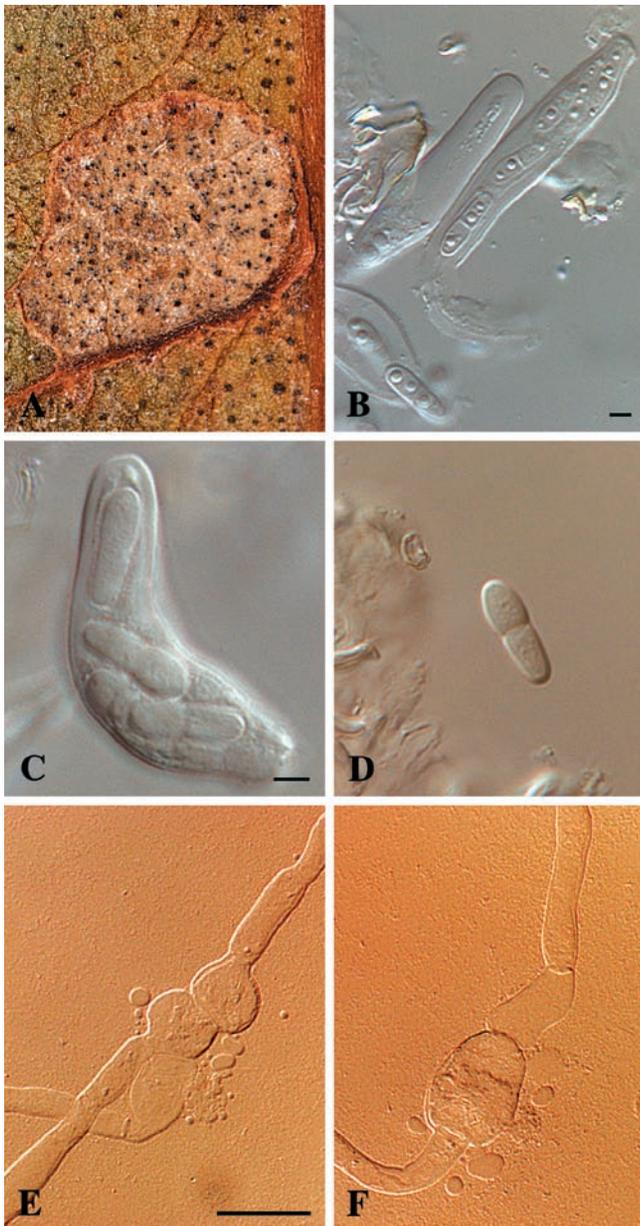
*Mycosphaerellae nubilosae* similis, set ascosporis brevioribus, (8–)9–10(–11)  $\times$  3(–4)  $\mu$ m, saepe plures quam 2 tubos germinationis preferentibus, distinguenda.

*Leaf spots* amphigenous, irregular to sub-circular, 5–15 mm diam, pale to medium brown, surrounded by a thin, raised, dark brown border. *Ascomata* pseudothecial, hypophyllous, single, black, immersed becoming erumpent, globose, up to 100  $\mu$ m diam; apical ostiole 10–15  $\mu$ m diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, sessile, obovoid to subcylindrical, straight to slightly incurved, 8-spored, 30–45  $\times$  7–10  $\mu$ m. *Ascospores* multiseriate, overlapping, hyaline, prominently guttulate, thin-walled, straight, obovoid with subobtuse ends, medianly 1-septate, widest at the middle of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)9–10(–11)  $\times$  3(–4)  $\mu$ m *in vivo*.

*Holotype*: Spain, on leaves of *E. globulus*, Nov. 2004, J.P. Mansilla, CBS H-19692 **holotype**, culture ex-type CBS 118508 = CPC 11697).



**Fig. 11.** *Mycosphaerella pluritubularis* (CBS 118508). A. Ascus and ascospores. B. Sequence of germinating ascospores, with those after 24 h at the right of the plate. Scale bar = 10  $\mu$ m.



**Fig. 12.** *Mycosphaerella pluritubularis* (CBS 118508). A. Leaf spot. B–C. Asci. D. ascospore. E–F. Germinating ascospores. Scale bars: B–C = 3  $\mu$ m, E = 11  $\mu$ m.

**Ascospore germination on MEA after 24 h:** Type F. Ascospores not darkening on MEA, and germinating from both ends, with germ tubes parallel to the long axis of the spore, and distorting prominently upon germination, becoming up to 11  $\mu$ m diam; frequently germinating with more than two germ tubes.

**Cultures:** Colonies after 3 wk 17–22 mm diam on MEA; on PDA colonies forming copious amounts of slime; surface olivaceous-black with patches of olivaceous-grey and pale olivaceous-grey; aerial mycelium sparse; margins feathery, uneven; reverse iron-grey; on OA surface smoke-grey with patches of olivaceous-grey; on MEA with sparse aerial mycelium, colonies erumpent, iron-grey, margins feathery, irregular; reverse olivaceous-black; colonies sterile.

**Host:** *E. globulus*.

**Distribution:** Spain.

**Notes:** *Mycosphaerella pluritubularis* is characterised by its distinct ascospore germination pattern (Type F), but where ascospores form more than two germ tubes, thus distinguishing it from other species like *M. nubilosa* that have more typical type F germination patterns.

***Mycosphaerella pseudaficana*** Crous & T. Coutinho, sp. nov. MycoBank MB500839. Fig. 13.

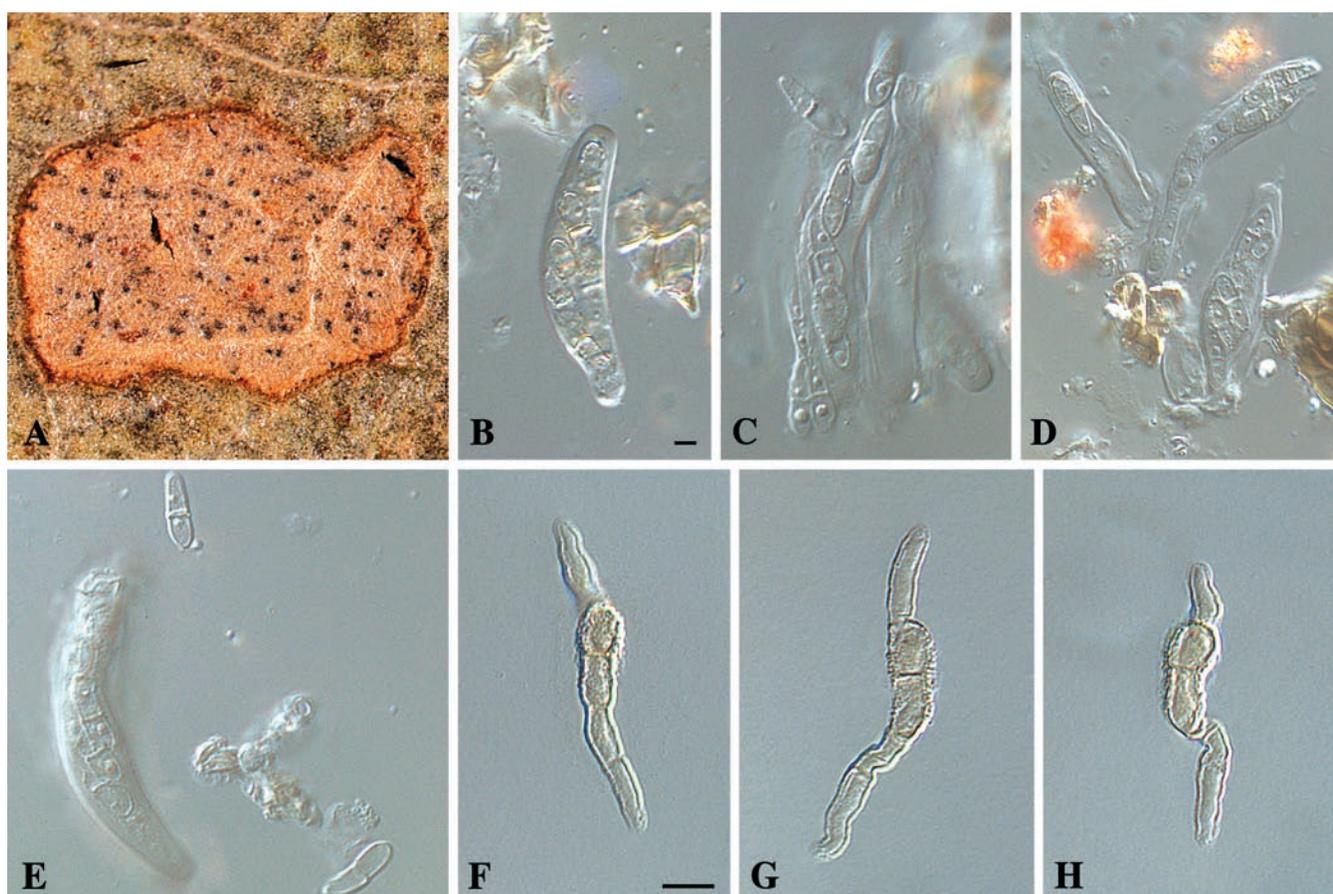
**Etymology:** Referring to its morphological similarity to *M. africana*.

*Mycosphaerellae africanae* similis, sed ascosporis maioribus, 8–11  $\times$  2.5–3  $\mu$ m, distinguenda.

**Leaf spots** amphigenous, irregular to sub-circular, 2–7 mm diam, medium brown, surrounded by a thin, raised, concolorous border. **Ascomata** pseudothecial, hypophyllous, single, black, immersed becoming erumpent, globose, up to 120  $\mu$ m diam; apical ostiole 10–15  $\mu$ m diam; wall of 2–3 cell layers of medium brown *textura angularis*. **Asci** aparaphysate, fasciculate, bitunicate, sessile, narrowly ellipsoid to subcylindrical, slightly incurved, 8-spored, 35–45  $\times$  7–9  $\mu$ m. **Ascospores** tri- to multiseriate, overlapping, hyaline to pale brown, guttulate, thin-walled, straight to slightly curved, smooth to finely roughened, fusoid–ellipsoidal with subobtuse ends, medianly 1-septate, widest in the middle of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)9–10(–11)  $\times$  (2.5–)3  $\mu$ m *in vivo*. **Spermatogonia** similar to the ascomata in morphology. **Spermatia** hyaline, smooth, rod-shaped with bluntly rounded ends, 3–4  $\times$  1–1.5  $\mu$ m.

**Holotype:** Zambia, on leaves of *E. globulus*, Aug. 1995, T. Coutinho, PREM 54973 **holotype**, culture ex-type CBS 114782 = CPC 1230; 1229–1231.

**Ascospore germination on MEA after 24 h:** Type G. Ascospores darkening and becoming verruculose on MEA; germinating from both ends as observed in *M. africana*, with germ tubes irregular to the long axis of the spore, and distorting prominently upon germination, becoming up to 8  $\mu$ m wide.



**Fig. 13.** *Mycosphaerella pseudaficana* (CBS 114782). A. Leaf spot. B–E. Asci and ascospores. F–H. Germinating ascospores. Scale bars: B = 3  $\mu$ m, F = 7  $\mu$ m.

**Cultures:** Colonies reaching 12–17 mm diam after 3 wk on MEA; colonies erumpent, irregular, surface iron-grey with olivaceous-grey, sparse aerial mycelium in central part; margins catenate, smooth; reverse greenish black; on PDA colonies erumpent, olivaceous-black with sparse olivaceous-grey aerial mycelium in the central part, margins smooth, catenate; reverse greenish black; on OA olivaceous-grey with smooth, catenate margins and green-olivaceous central part.

**Host:** *E. globulus*.

**Distribution:** Zambia.

**Notes:** Ascospores of *M. pseudaficana* (8–11  $\times$  2.5–3  $\mu$ m) germinate with a Type G pattern similar to that observed in *M. africana* (7–11  $\times$  2–3  $\mu$ m). Ascospores of *M. pseudaficana* are more verrucose than those of *M. africana*, but both taxa have very similar ascospore dimensions and germination patterns. They do differ, in the symptoms with which they are associated. Lesions of *M. pseudaficana* are generally larger, and they lack the red-purple margin found in *M. africana*. The easiest means to distinguish these taxa from each other is to compare their growth in culture: colonies of *M. africana* are black, produce a brown pigment in MEA, and form clusters of chlamydospores, whereas cultures of *M. pseudaficana* also produce clusters of chlamydospores on MEA, but are iron-grey, and lack the diffuse brown pigment observed in colonies of *M. africana*.

***Mycosphaerella pseudocryptica* Crous, sp. nov.**

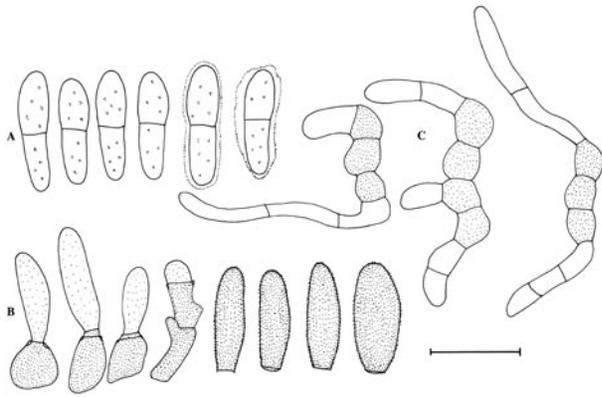
Mycobank MB500840. Figs 14–15.

**Anamorph:** *Colletogloeopsis* sp.

**Etymology:** Morphologically similar to *M. cryptica*.

*Mycosphaerellae crypticae* similis, sed ascosporis minoribus, (11–)12–14(–15)  $\times$  (3–)3.5(–4)  $\mu$ m, saepe utrinque germinantibus, distinguenda.

**Leaf spots** amphigenous, irregular to subcircular, 0.5–2 mm diam, pale brown, with a raised, red-brown margin. **Ascomata** pseudothecial, hypophyllous, arranged in dense clusters in pale brown areas next to the leaf spots associated with conidiomata of the anamorph, black, immersed, globose, up to 70  $\mu$ m diam; apical ostiole 10–15  $\mu$ m diam; wall of 2–3 layers of medium brown *textura angularis*. **Asci** paraphysate, fasciculate, bitunicate, subsessile, narrowly ellipsoid to subcylindrical, straight or slightly incurved, 8-spored, 35–45  $\times$  9–11  $\mu$ m. **Ascospores** multiseriate, overlapping, hyaline, granular, thin-walled, straight, fusoid–ellipsoidal with obtuse ends, medianly 1-septate, widest at the middle of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (11–)12–14(–15)  $\times$  (3–)3.5(–4)  $\mu$ m, *in vivo*; frequently encased in an irregular mucous sheath. **Mycelium** internal, consisting of branched, septate, medium brown, smooth, 3–4  $\mu$ m wide hyphae. **Conidiomata** intermixed among ascomata or separate, predominantly on the lower



**Fig. 14.** *Mycosphaerella pseudocryptica* (anamorph *Colletogloeopsis* sp.) (CBS 118504). A. Ascospores, some with sheath. B. Germinating ascospores. C. Conidiogenous cells and conidia. Scale bar = 10 µm.

leaf surface, pycnidial, substromatal, up to 120 µm diam; wall of 3–4 layers of brown *textura angularis*. *Conidiophores* 0–1-septate, but mostly reduced to conidiogenous cells. *Conidiogenous cells* discrete, ampulliform to subcylindrical, medium brown, smooth to finely verruculose, proliferating 1–3 times percurrently near apex, but also intercalary and sympodially, 5–15 × 3–5 µm. *Conidia* holoblastic, solitary, aseptate, fusoid with obtuse to subobtuse apices and truncate bases, medium brown, finely verruculose, (10–)12–14(–17) × (3.5–)4(–6) µm; inconspicuous basal marginal frill present.

**Holotype:** New Zealand, Wellington Botanical Garden, on leaves of *Eucalyptus* sp., Mar. 2004, J.A. Stalpers, CBS H-19693, **holotype**, culture ex-type CBS 118504 = CPC 11267; 11267–11269 (teleomorph), CPC 11264–11266 (anamorph).

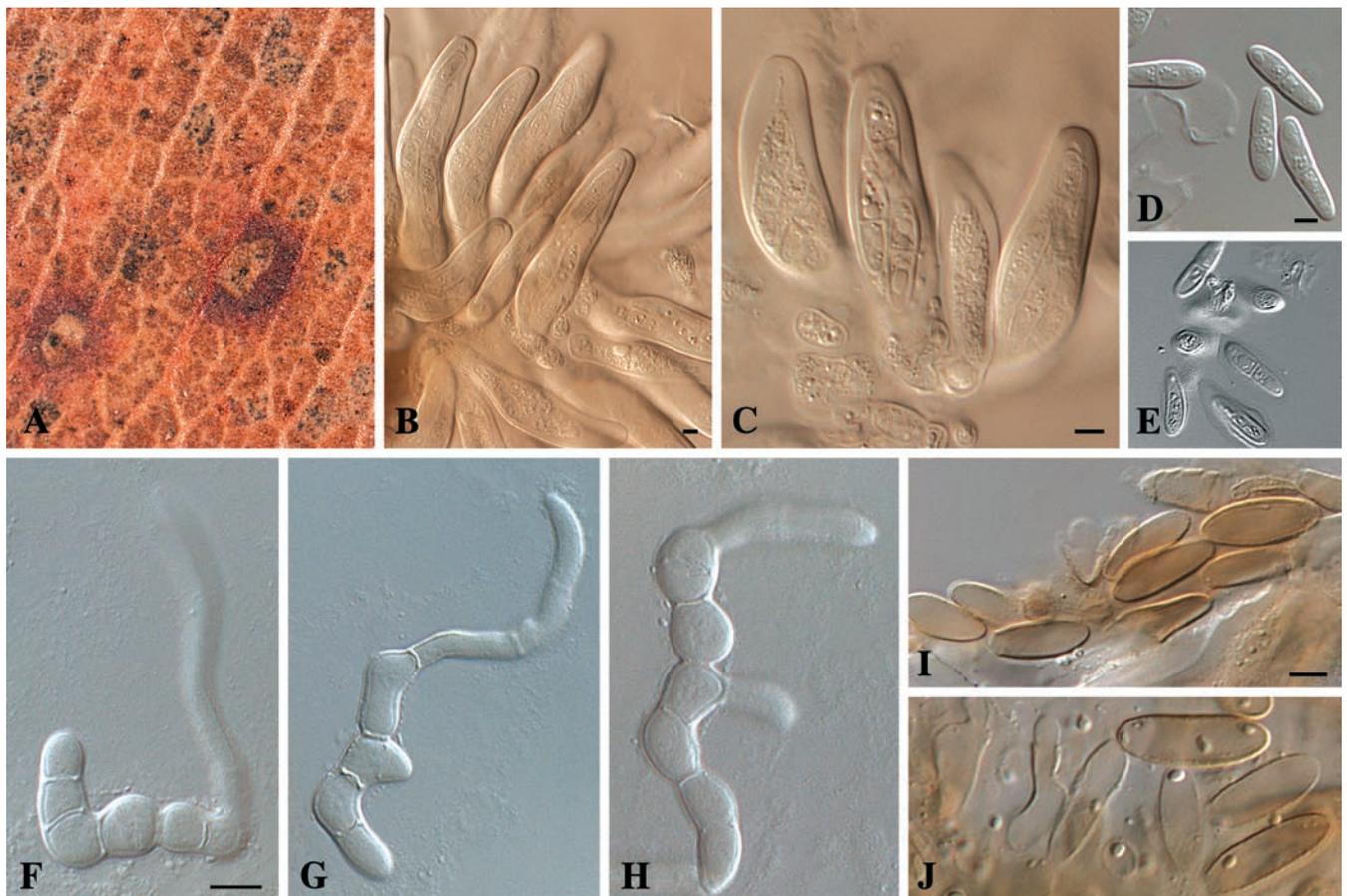
**Ascospore germination on MEA after 24 h:** Type A. Ascospores smooth, becoming olivaceous on MEA, germinating predominantly from both ends, with germ tubes at some angle to the long axis of the spore, and with a constriction at the ascospore septum; ascospores becoming up to 7 µm wide.

**Cultures:** Colonies slow growing, 3–8 mm diam after 3 wk on MEA; on MEA colonies erumpent, aerial mycelium sparse to absent, margins smooth, surface white-grey to smoke-grey, or with a reddish tinge in patches; reverse fuscous-black; on PDA erumpent, white to smoke-grey with patches of vinaceous-grey; reverse vinaceous-grey, with a diffuse red pigment visible in the agar, up to 2 cm from colony margins; on OA pale grey-olivaceous with a pale vinaceous grey pigment diffusing into the agar.

**Host:** *Eucalyptus* sp.

**Distribution:** New Zealand.

**Notes:** Ascospores of *M. pseudocryptica* germinate with a Type A pattern (as observed in *M. cryptica*), except that they tend to germinate from both ends. It is possible, therefore, that collections of *M. pseudocryptica* have in the past been confused with those of *M. cryptica*.



**Fig. 15.** *Mycosphaerella pseudocryptica* (anamorph *Colletogloeopsis* sp.) (CBS 118504). A. Leaf spot. B–C. Asci. D–E. Ascospores. F–H. Germinating ascospores. I–J. Conidia and conidiogenous cells. Scale bars: B–C, I = 4 µm, D = 3.5 µm, F = 7 µm.

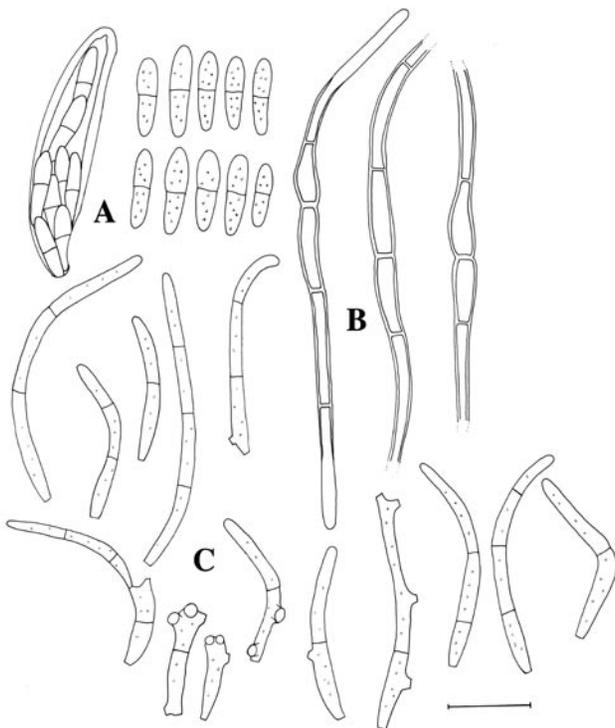
Isolates also form a *Colletogloeopsis* anamorph in culture, which is similar to *M. cryptica*. Ascospores of *M. pseudocryptica* are 11–15 × 3–4 µm, and conidia 10–17 × 3.5–6 µm, while ascospores of *M. cryptica* are 9–17.5 × 2–5.5 µm, and conidia are 8.5–18 × 4–6 µm. Phylogenetically *M. pseudocryptica* is closely related to the *M. molleriana* complex (Fig. 1), and distinct from *M. cryptica*.

***Mycosphaerella pseudoendophytica*** Crous & G. Hunter, **sp. nov.** MycoBank MB500841. Figs 16–17.  
**Anamorph:** *Pseudocercospora* sp.

**Etymology:** Named after its morphological similarity to *M. endophytica*.

*Mycosphaerellae endophyticae* similis, sed ascosporis modo C germinantibus distinguenda.

**Leaf spots** amphigenous, irregular to subcircular or angular, 2–5 mm diam, brown, with a raised, dark brown margin. **Ascomata** pseudothecial, amphigenous, black, subepidermal, erumpent to superficial, globose, up to 120 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown *textura angularis*. **Asci** aparaphysate, fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoid, straight or slightly incurved, 8-spored, 30–40 × 8–10 µm. **Ascospores** multiseriate, overlapping, hyaline, sparsely guttulate, thin-walled, straight to slightly curved, fusoid–ellipsoidal with obtuse ends, medianly 1-septate, widest in the middle of the apical cell, not to slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)9–10(–11) × (2–)2.5–3 µm, *in vivo*. **Mycelium** internal, consisting of branched, septate, pale to medium brown, smooth, 3–4 µm wide hyphae. **Conidiomata** *in vitro* sporodochial,



**Fig. 16.** *Mycosphaerella pseudoendophytica* (anamorph *Pseudocercospora* sp.) (CBS 113288). A. Ascus and ascospores. B. Germinating ascospores. C. Conidia. Scale bar = 10 µm



**Fig. 17.** Leaf spot associated with *Mycosphaerella pseudoendophytica* (holotype)

hyaline. **Conidiogenous cells** aggregated, unbranched or branched, hyaline, smooth, tapering to flat-tipped apical and lateral loci, proliferating sympodially, 8–15 × 2–3.5 µm. **Conidia** holoblastic, solitary, but frequently undergoing microcyclic conidiation, giving rise to one or several additional conidia, smooth, hyaline, obclavate, apex subobtuse, base long obconically subtruncate to truncate, irregularly curved, 0–3-septate, 12–40 × 1.5–2 µm; hila inconspicuous.

**Holotype:** South Africa, KwaZulu-Natal, Enon, Richmond, on leaves of *E. nitens*, 3 May 2000, G. Hunter, CBS H-19694, **holotype**, culture ex-type CBS 113288 = CMW 9098.

**Ascospore germination on MEA after 24 h:** Type C. Ascospores smooth, not darkening on MEA, germinating from both ends, with germ tubes parallel to the long axis of the spore, and with a constriction at the ascospore septum; ascospores becoming up to 3.5 µm wide.

**Cultures:** Similar to those of *M. endophytica* (Crous 1998).

**Host:** *E. nitens*.

**Distribution:** South Africa.

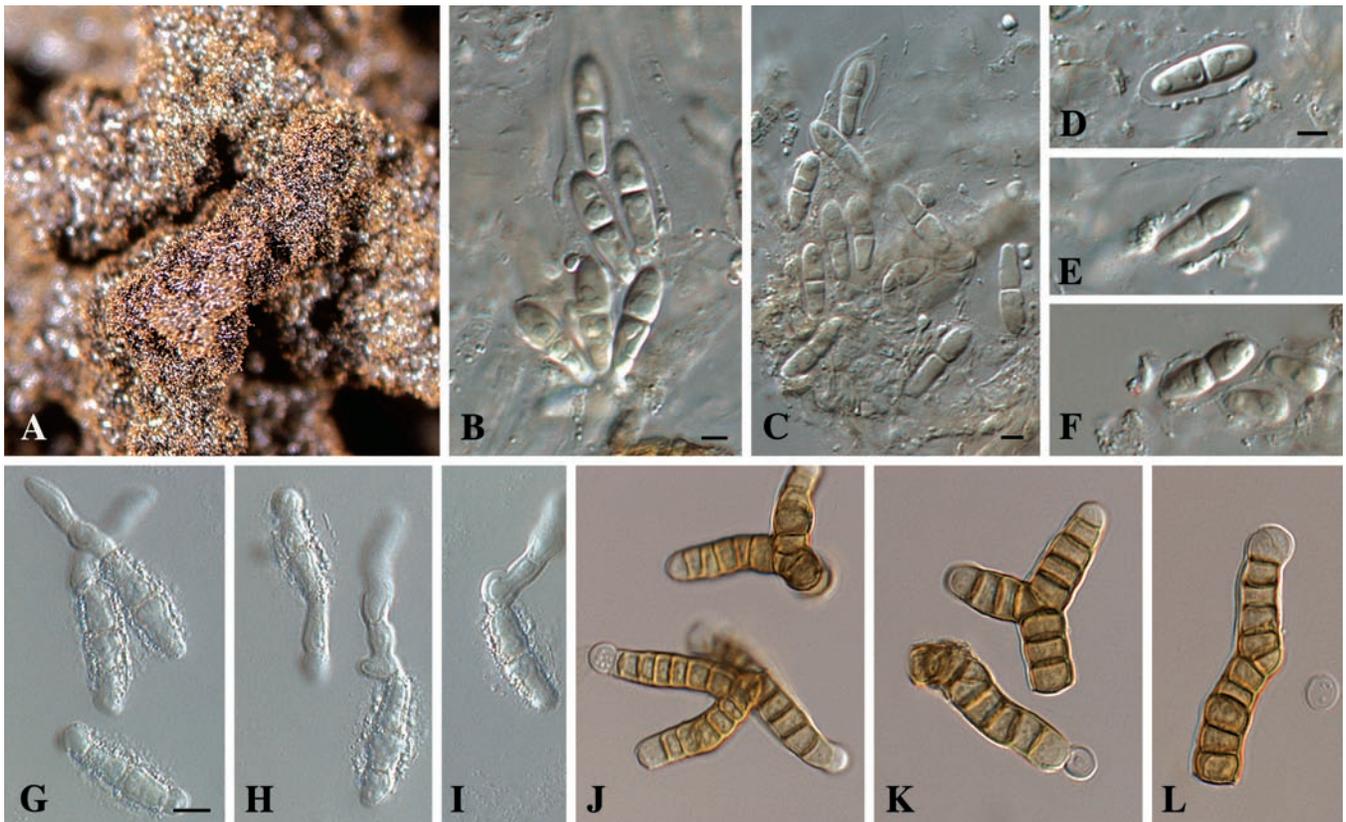
**Notes:** *Mycosphaerella pseudoendophytica* has been known to us for some time, but its formal description required a molecular comparison with ex-type strains of *M. endophytica* (which it resembles in anamorph morphology), and *M. ellipsoidea* (which it resembles in ascospore germination pattern). As can be seen here, *M. pseudoendophytica* (Fig. 4) is clearly a distinct species, sharing features of both of these taxa.

***Mycosphaerella pseudosuberosa*** Crous & M.J. Wingf., **sp. nov.** MycoBank MB500842. Fig. 18.  
**Anamorph:** *Trimmatostroma* sp.

**Etymology:** Morphologically similar to *M. suberosa*.

*Mycosphaerellae suberosae* similis, sed ascosporis minoribus, (11–)12–14(–15) × (3–)3.5(–4) µm, distinguenda.

**Leaf spots** amphigenous, associated with brown, corky spots on leaf petioles. **Ascomata** pseudothecial, single



**Fig. 18.** *Mycosphaerella pseudosuberosa* (anamorph *Trimmatostroma* sp.) (CBS 118911). A. Colony on MEA. B–C. Broken asci. D–F. Ascospores (note sheath). G–I. Germinating ascospores. J–L. *Trimmatostroma* conidia produced in culture. Scale bars: B–D = 4  $\mu$ m, G = 8  $\mu$ m.

to aggregated, black, immersed becoming erumpent, globose, up to 120  $\mu$ m diam; apical ostiole 10–20  $\mu$ m diam; wall of 3–6 layers of brown *textura angularis*. Asci aparaphysate, fasciculate, bitunicate, sessile, obovoid to broadly ellipsoid, straight or slightly incurved, 8-spored, 35–45  $\times$  12–16  $\mu$ m. Ascospores tri- to multiseriate, overlapping, hyaline, guttulate, thick-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, medianly 1-septate, widest at the middle of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (11–)12–14(–15)  $\times$  (3–)3.5(–4)  $\mu$ m *in vivo*; frequently surrounded by an irregular mucous sheath.

**Holotype:** Uruguay, on leaves and petioles of *Eucalyptus* sp., Apr. 2005, M.J. Wingfield, CBS H-19695, **holotype**, culture ex-type CBS 118911 = CPC 12085.

**Ascospore germination on MEA after 24 h:** Type H. Ascospores darkening and becoming verruculose on MEA, germinating from both ends, with germ tubes primarily parallel to the long axis of the spore, and distorting prominently upon germination, becoming up to 11  $\mu$ m wide.

**Cultures:** Colonies extremely slow growing, erumpent, uneven, black; aerial mycelium absent; colonies powdery, producing a *Trimmatostroma* anamorph.

**Host:** *Eucalyptus* sp.

**Distribution:** Uruguay.

**Notes:** *Mycosphaerella pseudosuberosa* is morphologically similar, and phylogenetically closely

related to *M. suberosa*. It can be distinguished by its ascospores that are slightly narrower (3–4  $\mu$ m vs. 3–6  $\mu$ m), having a mucous sheath, and germinating via two germ tubes (predominantly) that originate from the ends of the spore. Germinating spores exude mucus, and become pale brown and verruculose, which differs from the numerous germ tubes and dark brown ascospores observed in *M. suberosa*. Furthermore, cultures of *M. suberosa* are hard and resistant to being cut, while those of *M. pseudosuberosa* are powdery, producing a *Trimmatostroma* anamorph in culture. From the phylogenetic data available, it appears that there may be more species within the *M. suberosa* complex awaiting description (Fig. 1).

***Mycosphaerella quasircospora*** Crous & T. Coutinho, **sp. nov.** MycoBank MB500843. Fig. 19.

**Etymology:** Refers to the fact that this fungus is phylogenetically closely related to species of *Cercospora*.

*Mycosphaerellae nubilosae* similis, sed ascosporis brevioribus, 10–14  $\times$  3–4  $\mu$ m, distinguenda.

**Leaf spots** amphigenous, irregular to sub-circular, 2–10 mm diam, pale brown, surrounded by a thin, raised, dark brown border; spots becoming confluent with age. **Ascomata** pseudothecial, hypophyllous, single, black, immersed becoming erumpent, globose, up to 100  $\mu$ m diam; wall of 2–3 cell layers of medium brown *textura angularis*. Asci aparaphysate, fasciculate, bitunicate, sessile, obovoid to broadly ellipsoid, straight to slightly incurved, 8-spored, 35–50  $\times$  10–12  $\mu$ m.



Fig. 19. *Mycosphaerella quasircospora* (CBS 111161). A. Ascomata on leaf. B–C. Asci. D. Ascospores. Scale bars = 4  $\mu$ m.

*Ascospores* tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, obovoid with subobtuse ends, unequally 1-septate, widest close to the apex of the apical cell, not constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (10–)12–13(–14)  $\times$  (3–)3.5(–4)  $\mu$ m *in vivo*; apical cell 4–6  $\mu$ m long, basal cell 6–8  $\mu$ m long.

*Holotype*: Tanzania, on leaves of *E. maidenii*, May 1995, T. Coutinho, PREM 54971, *holotype*, culture ex-type CBS 111161 = CPC 1098.

*Ascospore germination on MEA after 24 h*: Type F. Similar to *M. nubilosa*.

*Cultures*: Colonies after 3 wk on MEA reaching 6–15 mm diam; on MEA erumpent with sparse aerial mycelium, pale olivaceous-grey; margins smooth, regular; reverse ochraceous with patches of pale olivaceous-grey; on PDA erumpent, centres white to pale olivaceous-grey, outer zone olivaceous-grey, margins irregular, feathery; reverse smoke-grey in the central part, olivaceous-grey in the outer region; colonies sterile.

*Host*: *E. maidenii*.

*Distribution*: Tanzania.

*Notes*: Ascospores of *M. quasircospora* (10–14  $\times$  3–4  $\mu$ m) germinate with a Type F germination pattern, similar to that observed in *M. nubilosa* (11–16  $\times$  3–4.5  $\mu$ m), but are somewhat shorter, and also cluster phylogenetically apart (Fig. 3). Of particular interest is the fact that it aligns with sequences of *Cercospora apii*, for which no teleomorph is known. Cultures are sterile, and a re-examination of the original specimen also failed to reveal the presence of a *Cercospora* state. This is presently the only *Mycosphaerella* teleomorph clustering with *Cercospora apii* other than *C. acaciae-mangii* (Crous et al. 2004b) (Fig. 3).

***Mycosphaerella scytalidii*** Crous & M.J. Wingf., *sp. nov.* MycoBank MB500844. Fig. 20.

*Anamorph*: *Stenella* sp.

*Synanamorph*: *Scytalidium*-like.

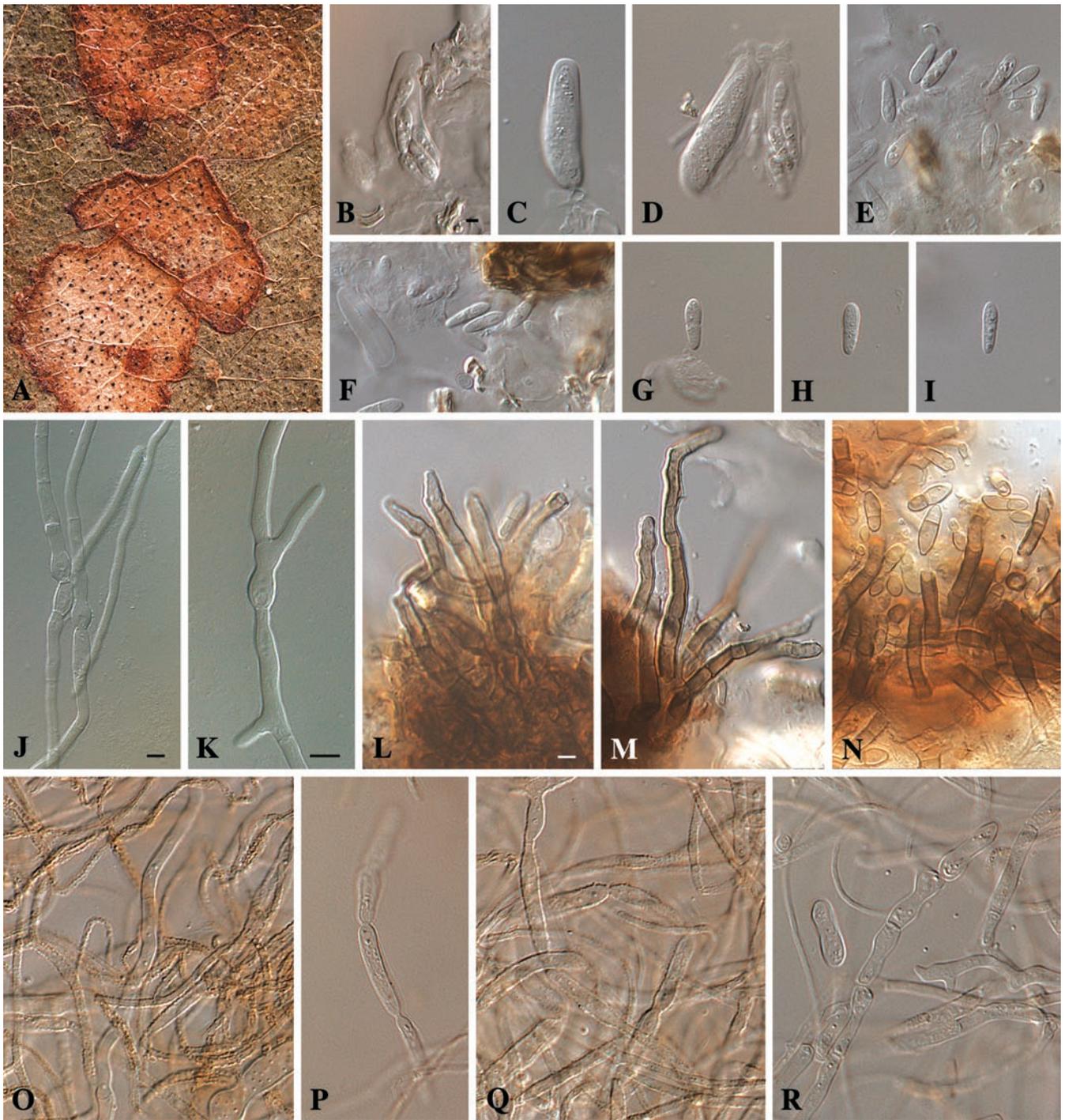
*Etymology*: Referring to the *Scytalidium*-like synanamorph.

*Mycosphaerellae parkii* similis, sed ascosporis minoribus, 8–10  $\times$  (2.5–)3  $\mu$ m, modi I germinantibus, distinguenda.

*Leaf spots* amphigenous, irregular to sub-circular, 1–8 mm diam, grey to medium brown, with a raised, dark brown border. *Ascomata* pseudothecial, amphigenous, single, black, immersed becoming erumpent, globose, up to 90  $\mu$ m diam; apical ostiole 5–10  $\mu$ m diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, obovoid to ellipsoid, straight or slightly incurved, 8-spored, 25–30  $\times$  7–9  $\mu$ m. *Ascospores* tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with subobtuse ends, medianly 1-septate, widest in the middle of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, 8–10  $\times$  (2.5–)3  $\mu$ m *in vivo*. *Mycelium* internal and external, consisting of septate, branched, verruculose hyphae, 2–3  $\mu$ m wide. *Caespituli* fasciculate, amphigenous on the leaves, brown, up to 50  $\mu$ m wide and 60  $\mu$ m high. *Conidiophores* aggregated in loose fascicles arising from the upper cells of a brown stroma up to 50  $\mu$ m wide and 30  $\mu$ m high, or situated on the top of the ascomata; conidiophores medium brown, finely verruculose, 1–4-septate, subcylindrical, straight to geniculate-sinuous, unbranched, 20–40  $\times$  2–4  $\mu$ m. *Conidiogenous cells* terminal, unbranched, medium brown, smooth to verruculose, tapering to the flat-tipped apical loci, proliferating sympodially, 7–15  $\times$  2–3  $\mu$ m, with thickened, darkened, refractive scars. *Conidia* solitary, or in simple chains, medium brown, verruculose, subcylindrical to ellipsoidal, apex obtuse, base subtruncate, 1–2-septate, frequently constricted at the septa, 7–15  $\times$  3–3.5  $\mu$ m; hila thickened, darkened, refractive. Aerial mycelium disarticulating into hyaline, smooth arthroconidia that are *Scytalidium*-like, 12–35  $\times$  3–5  $\mu$ m.

*Holotype*: Colombia, Angela Maria, on leaves of *Eucalyptus urophylla*, Jan. 2004, M.J. Wingfield, CBS H-19696 *holotype*, culture ex-type CBS 118493 = CPC 10998.

*Ascospore germination on MEA after 24 h*: Type I. Ascospores not darkening on MEA, and germinating from both ends, with germ tubes parallel to the long axis of the spore, lateral branches present, and spore distorting upon germination, becoming up to 5  $\mu$ m wide.



**Fig. 20.** *Mycosphaerella scytalidii* (anamorph *Stenella* sp., synanamorph, *Scytalidium*-like.) (CBS 118493). A. Leaf spot. B–D. Asci. E–I. Ascospores. J–K. Germinating ascospores. L–M. Conidiophores. N. Conidia. O–R. Mycelium in culture. Scale bars: B, L = 3  $\mu$ m, J–K = 5  $\mu$ m.

**Cultures:** Colonies on MEA reaching 18–30 mm diam after 3 wk; colonies erumpent, folding, margin smooth, irregular, aerial mycelium moderate, pale olivaceous-grey; reverse iron-grey; on PDA with moderate aerial mycelium, olivaceous-grey with patches of pale olivaceous-grey; reverse olivaceous-black; on OA pale olivaceous-grey with patches of olivaceous-grey and iron-grey.

**Host:** *Eucalyptus urophylla*.

**Distribution:** Colombia.

**Notes:** Several other as yet undescribed species occur

on *Eucalyptus* leaves in Colombia, and some, such as *M. longibasalis* Crous & M.J. Wingf. (Crous 1998) (Fig. 21), is still not known from culture. Isolate CPC 10986 clusters with CPC 11002, and in culture they are distinct from CPC 11004. We were, however, unable to trace these isolates back to ascomata due to several species being present on the same leaf spots. Thus, further collections will be required before these taxa can be named.

*Mycosphaerella scytalidii* is phylogenetically closely related to the *Mycosphaerella* sp. represented by CPC 11002 and CPC 10986 (Fig. 4). For reasons explained above, however, we presently cannot name the latter

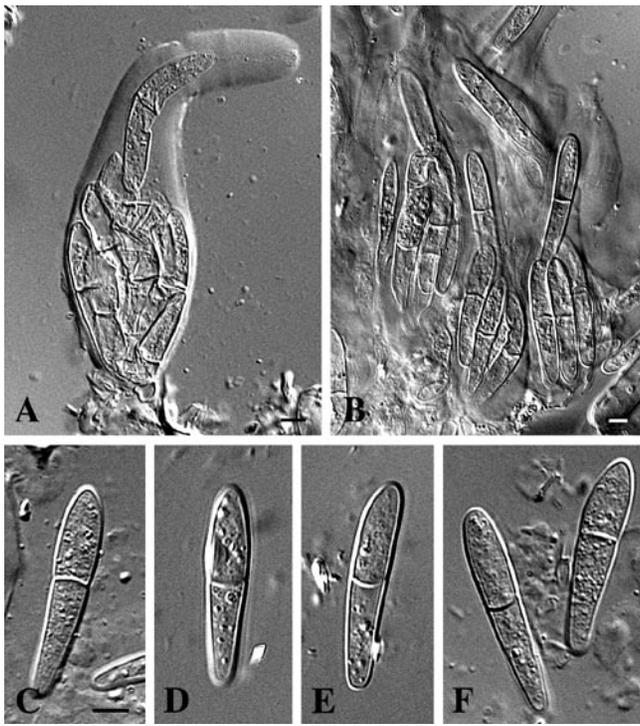


Fig. 21. *Mycosphaerella longibasalis*. A–B. Broken asci. C–F. Ascospores. Scale bars = 4  $\mu$ m.

species. *Mycosphaerella scytalidii* is also noteworthy based on the fact that it forms a *Stenella* anamorph, as well as a *Scytalidium*-like synanamorph in culture. Numerous species of *Mycosphaerella* form clusters of chlamydospores on their hyphal tips in culture (*M. bellula*, *M. jonkershoekensis*) (Crous *et al.* 2004a), leading to the impression that they could develop into *Trimmatostroma*-like anamorphs. None, however, have been reported to form *Scytalidium* anamorphs. Many species of *Mycosphaerella* form aerial mycelium that remain hyaline, with wide, disarticulating cells, suggesting that this anamorph morphology may be more prevalent in species of *Mycosphaerella* than previously realised. Ascospores of *M. scytalidii* germinate with a Type I pattern, but none of the species on *Eucalyptus* with this germination pattern form a *Stenella* anamorph in culture.

***Mycosphaerella secundaria*** Crous & A.C. Alfenas, sp. nov. MycoBank MB500845. Fig. 22.

*Etymology*: Referring to the ecology of this fungus as a secondary coloniser on lesions of *M. suberosa*.

*Mycosphaerellae parkii* similis, sed ascosporis minoribus, 8–10  $\times$  2.5–3  $\mu$ m, distinguenda.

Occurring as a secondary colonist on leaf spots caused by *M. suberosa*, or *M. perpendicularis*. *Ascomata* pseudothecial, amphigenous, single, inconspicuous, sparsely distributed, black, subepidermal, rarely erumpent, globose, up to 90  $\mu$ m diam. *Asci* paraphysate, fasciculate, bitunicate, subsessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 20–30  $\times$  7–9  $\mu$ m. *Ascospores* tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, ellipsoidal with subobtuse ends, medianly 1-septate, widest close to the apex of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, 8–10  $\times$  2.5–3  $\mu$ m *in vivo*.

*Holotype*: Brazil, Bahia, Teixeira de Freitas, on leaves of *Eucalyptus* sp., 8 Jun. 2004, A.C. Alfenas, CBS H-19697, *holotype*, culture ex-type CBS 118507 = CPC 11551–11553.

*Ascospore germination on MEA after 24 h*: Type D. Similar to *M. parkii*.

*Cultures*: Colonies on MEA after 3 wk reaching 25–35 mm diam; on MEA olivaceous-grey, flat, spreading, folding, with sparse aerial mycelium and smooth, even margins; reverse iron-grey; on PDA iron-grey with olivaceous-grey aerial mycelium in central part, and drops of slime throughout; reverse iron-grey; on OA flat, spreading, olivaceous-grey.

*Host*: *Eucalyptus* spp.

*Distribution*: Brazil, Colombia.

*Notes*: When this species was initially collected in 1992 (CPC 504), it was noted that it occurred in lesions ascribed to *M. suberosa*, presumably as a secondary pathogen. We have now been able to recollect this fungus where it had colonised lesions caused by *M. suberosa*, as well as those of *Cryptosporiopsis eucalypti* Sankaran & B. Sutton on eucalypts in Brazil. In the same

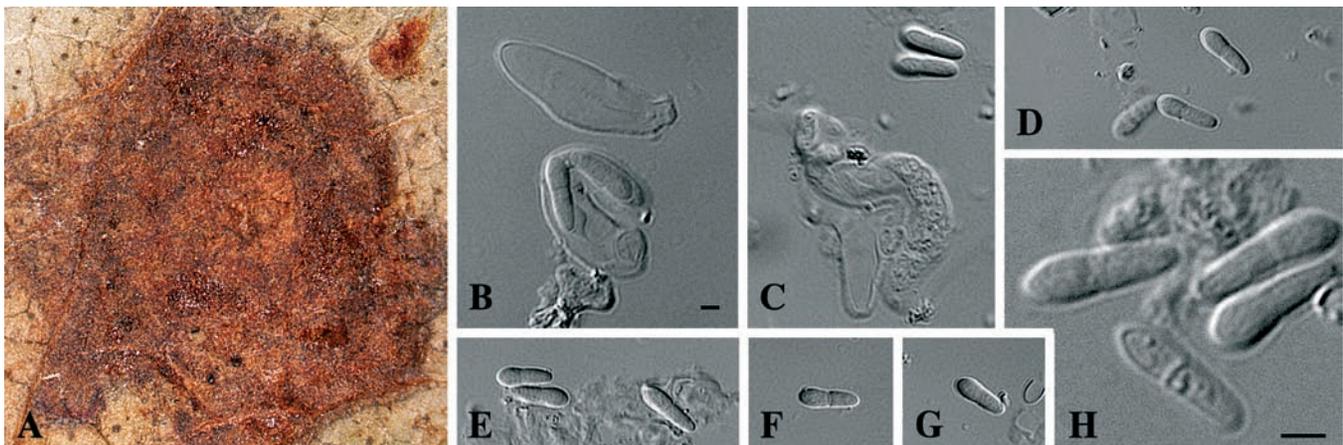


Fig. 22. *Mycosphaerella secundaria* (CBS 118507). A. Leaf spot. B–C. Broken asci. D–H. Ascospores. Scale bars: B, H = 3  $\mu$ m.



Fig. 23. *Mycosphaerella stramentii* (CBS 118909). A–B. Asci. C–F. Ascospores. G. Germinating ascospore. Scale bar = 3  $\mu$ m.

phylogenetic clade accommodating *M. secundaria* from Brazil, isolates collected in Colombia were also found which were apparently associated with lesions caused by *M. perpendicularis* (Fig. 2). *Mycosphaerella secundaria* has thus far only been collected in association with other species of *Mycosphaerella* that we believe are the primary pathogens. *Mycosphaerella perpendicularis* (ascospores 8–10  $\times$  2.5–3  $\mu$ m) was originally treated as *M. parkii* (ascospores 8–15  $\times$  2–3.5  $\mu$ m) (Crous 1998).

**Additional culture examined:** Brazil, Picadao, Conceicao da Barra, on leaves of *E. grandis*, 27 Apr. 1992, A.C. Alfenas, CBS 115608 = CPC 504.

***Mycosphaerella stramentii* Crous & A.C. Alfenas, sp. nov.** MycoBank MB500846. Fig. 23.

**Etymology:** Refers to the occurrence of this fungus on leaf litter.

*Mycosphaerellae parkii* similis, sed ascosporis minoribus, (8–)10–12(–13)  $\times$  3(–3.5)  $\mu$ m, modo I germinantibus, distinguenda.

**Leaf spots** absent, ascomata associated with leaf litter. **Ascomata** pseudothecial, amphigenous, but predominantly hypophyllous, single, black, immersed becoming erumpent, globose, up to 120  $\mu$ m diam. **Asci** aparaphysate, fasciculate, bitunicate, sessile, narrowly ellipsoid to subcylindrical, straight or slightly incurved, 8-spored, 25–40  $\times$  7–8  $\mu$ m. **Ascospores** tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid-ellipsoidal with subobtuse ends, medianly 1-septate, widest in middle of apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)10–12(–13)  $\times$  3(–3.5)  $\mu$ m, *in vivo*.

**Holotype:** Brazil, Minas Gerais, Belo Oriente, on leaf litter of *Eucalyptus* sp., 24 Jan. 2004, A.C. Alfenas, CBS H-19698, **holotype**, culture ex-type CBS 118909 = CPC 11545–11547.

**Ascospore germination on MEA after 24 h:** Type I. Ascospores not darkening on MEA, and germinating from both ends, with germ tubes parallel to the long axis of the spore, and lateral branches also present; ascospore constricting at the septum, becoming up to 5  $\mu$ m wide.

**Cultures:** Colonies on MEA reaching 20–27 mm diam after 3 wk; on MEA colonies erumpent, spreading, aerial mycelium sparse, surface folding, pale olivaceous-grey, with central part having patches of smoke-grey; margin feathery, irregular, reverse greenish black; on PDA surface olivaceous-black with patches of smoke-grey aerial mycelium in central part; margins feathery, irregular, reverse greenish black; on OA olivaceous-black with smoke-grey aerial mycelium; margins irregular, feathery.

**Host:** *Eucalyptus* sp.

**Distribution:** Brazil.

**Notes:** Ascospores of *M. stramentii* germinate with a Type I pattern. Several taxa are known to have this pattern of ascospore germination (Crous 1998), from which *M. stramentii* can be distinguished by its ascospore dimensions and cultural characteristics. Phylogenetically it is closely related to *M. endophytica* (Fig. 4).

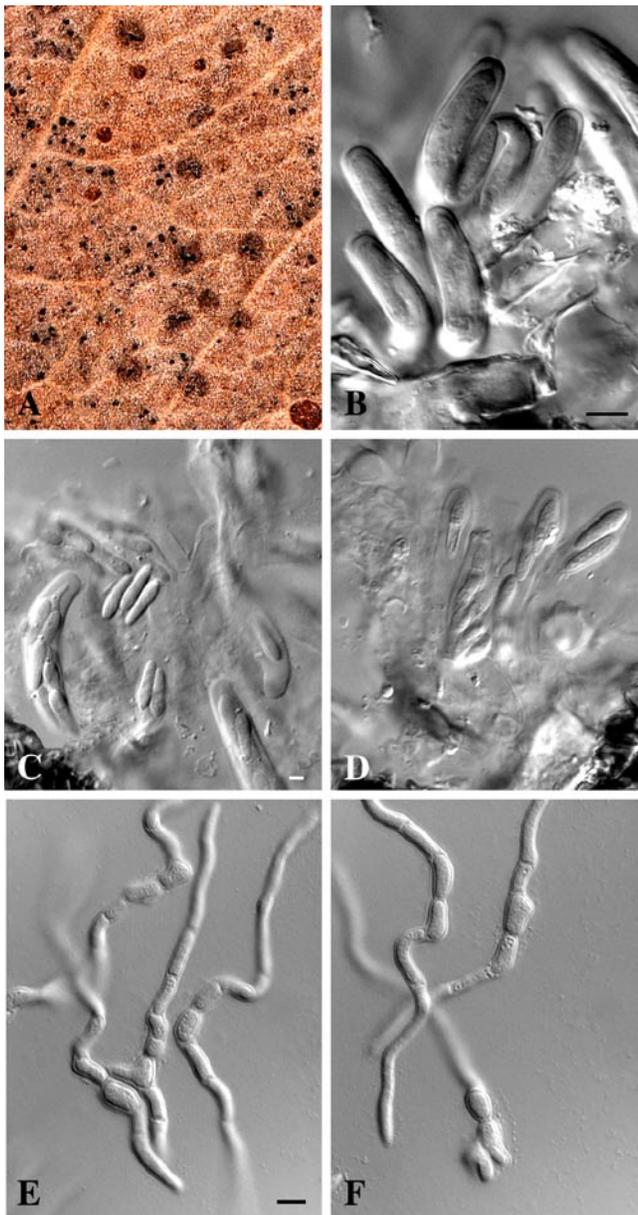
***Mycosphaerella stramenticola* Crous & A.C. Alfenas, sp. nov.** MycoBank MB500847. Fig. 24.

**Etymology:** Latin *stramentum* = leaf litter, the substrate from which this fungus was collected.

*Mycosphaerellae crystallinae* similis, sed ascosporis minoribus, 8–11  $\times$  3–3.5  $\mu$ m, distinguenda.

**Leaf spots** absent, associated with leaf litter. **Ascomata** pseudothecial, amphigenous, single, black, immersed becoming erumpent, globose, up to 90  $\mu$ m diam; apical ostiole 5–10  $\mu$ m diam; wall of 2–3 layers of medium brown *textura angularis*. **Asci** aparaphysate, fasciculate, bitunicate, sessile, narrowly ellipsoid to subcylindrical, straight or slightly incurved, 8-spored, 30–35  $\times$  7–9  $\mu$ m. **Ascospores** tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with subobtuse ends, medianly 1-septate, widest in the middle of the apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)9–10(–11)  $\times$  3(–3.5)  $\mu$ m, *in vivo*.

**Holotype:** Brazil, Bahia, Eunapolis, on leaf litter of *Eucalyptus* sp., 23 May 2004, A.C. Alfenas, CBS H-19699 **holotype**, culture ex-type CBS 118506 = CPC 11438–11440.



**Fig. 24.** *Mycosphaerella stramenticola* (CBS 118506). A. Ascomata on leaf. B. Asci. C–D. Ascospores. E–F. Germinating ascospores. Scale bars: B = 8  $\mu$ m, C = 3  $\mu$ m, E = 6  $\mu$ m.

*Ascospore germination on MEA after 24 h:* Type I. Ascospores not darkening on MEA, and germinating from both ends, with germ tubes parallel to the long axis of the spore, and distorting prominently upon germination, becoming up to 6  $\mu$ m wide; lateral branches also present.

*Cultures:* Colonies on MEA reaching 22–38 mm diam after 3 wk; colonies flat, spreading; aerial mycelium sparse; margins smooth, regular, surface olivaceous-grey with drops of slime; reverse iron-grey; on OA pale olivaceous-grey in the centre due to moderate aerial mycelium; olivaceous-grey in the outer region; on PDA olivaceous-grey with drops of slime, margin thin, iron-grey on surface and reverse.

*Host:* *Eucalyptus* sp.

*Distribution:* Brazil.

*Notes:* *Mycosphaerella stramenticola* is phylogenetically closely related to isolates CPC 727–728 (Fig. 2), which represent an undescribed taxon from Indonesia. *Mycosphaerella stramenticola* has ascospores that germinate with a Type I pattern, thus being similar to those of *M. crystallina* (11–15  $\times$  3–4  $\mu$ m), *M. ellipsoidea* (8–11  $\times$  2–3  $\mu$ m), *M. endophytica* (8–11  $\times$  2–3  $\mu$ m), *M. lateralis* (7–16  $\times$  2–3  $\mu$ m), *M. irregulariramosa* (7–10  $\times$  1.5–2.5  $\mu$ m) and *M. tasmaniensis* (10–13  $\times$  2.5–4  $\mu$ m). Ascospores of *M. stramenticola* are 8–11  $\times$  3–3.5  $\mu$ m, and thus being wider than those of *M. ellipsoidea*, *M. endophytica*, and *M. irregulariramosa*. Furthermore, cultures of *M. stramenticola* are sterile, while all the other species listed here produce anamorphs in culture.

***Mycosphaerella sumatrensis*** Crous & M.J. Wingf., sp. nov. MycoBank MB500848. Fig. 25.

*Etymology:* Refers to Sumatra, where this fungus was collected.

*Mycosphaerellae kenienensis* similis, sed ascosporis maioribus, 12–16  $\times$  3–4  $\mu$ m, distinguenda.

*Leaf spots* amphigenous, irregular to subcircular, 2–10 mm diam, pale brown with a dark brown, raised border, and thin, red-purple margin. *Ascomata* pseudothecial, amphigenous but predominantly epiphyllous, single, black, subepidermal to erumpent, globose, up to 80  $\mu$ m diam; apical ostiole 15–20  $\mu$ m diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* paraphysate, fasciculate, bitunicate, sessile, obovoid, straight or slightly incurved, 8-spored, 30–40  $\times$  9–11  $\mu$ m. *Ascospores* multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid–ellipsoidal with obtuse ends, medianly 1-septate, widest in middle of apical cell, not constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (12–)13–15(–16)  $\times$  (3–)4  $\mu$ m, *in vivo*.

*Holotype:* Indonesia, Northern Sumatra, on leaves of *Eucalyptus* sp., Feb. 2004, M.J. Wingfield, CBS H-19704, **holotype**, culture ex-type CBS 118499 = CPC 11171, CBS 118501 = CPC 11175, CBS 118502 = CPC 11178.

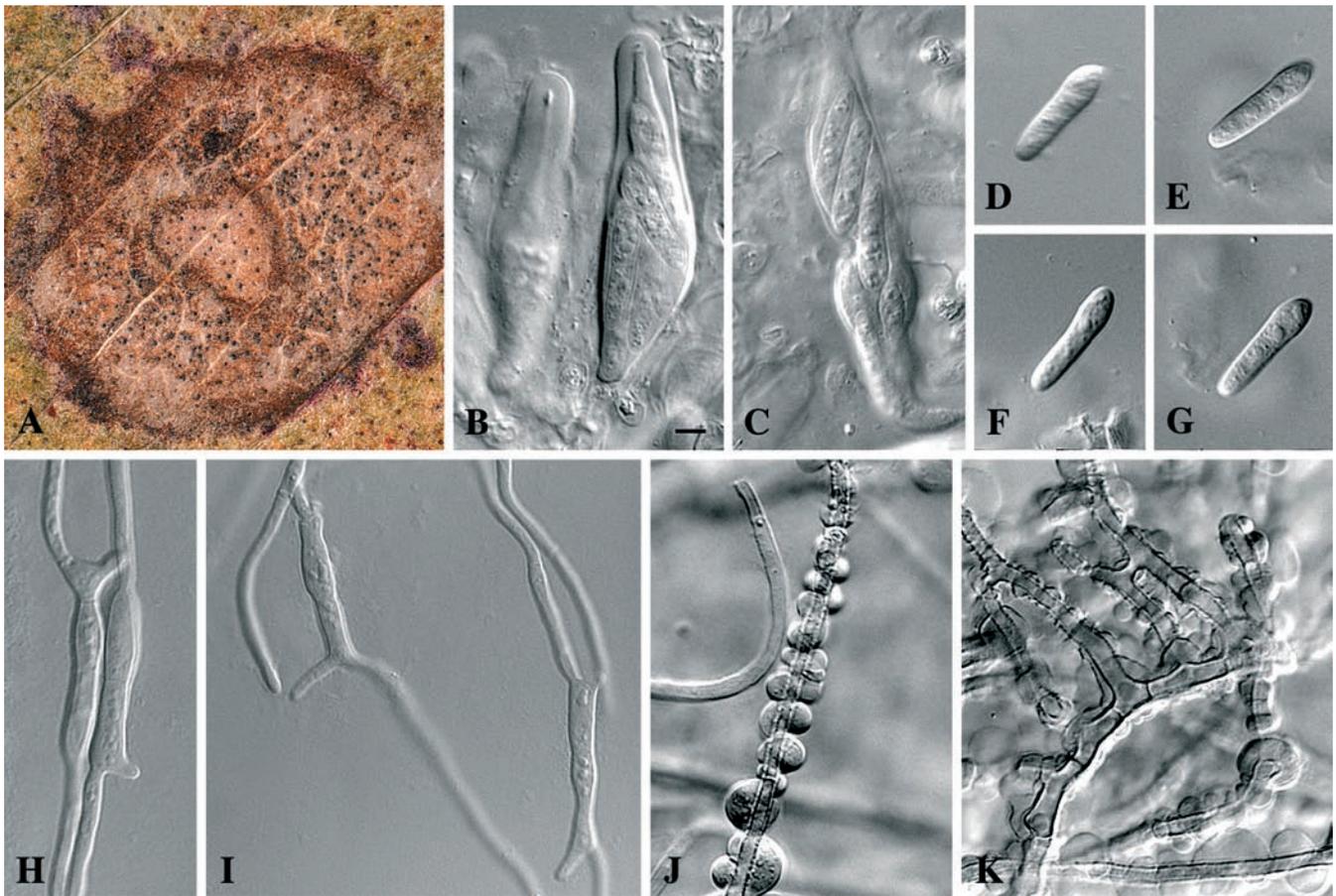
*Ascospore germination on MEA after 24 h:* Type J. Ascospores not darkening on MEA, and germinating from both ends, with germ tubes parallel to the long axis of the spore, but also with one or two lateral branches forming at the spore ends; ascospores becoming slightly constricted and up to 4  $\mu$ m wide.

*Cultures:* Colonies 8–19 mm diam on MEA after 3 wk; erumpent, with sparse aerial mycelium, smoke-grey; margin smooth, but irregular; reverse olivaceous-black; on PDA erumpent, olivaceous-grey with a thin whitish border; iron-grey in reverse; on OA smoke-grey, appearing olivaceous-black in the centre due to collapse of the aerial in copious amounts of slime.

*Host:* *Eucalyptus* sp.

*Distribution:* Indonesia.

*Notes:* *Mycosphaerella sumatrensis* is phylogenetically



**Fig. 25.** *Mycosphaerella sumatrensis* (CBS 118499). A. Leaf spot. B–C. Asci. D–G. Ascospores. H–I. Germinating ascospores. J–K. Hyphae with exudate droplets. Scale bar = 4  $\mu$ m.

distinct from other species occurring on *Eucalyptus* (Fig. 2). Ascospores (12–16  $\times$  3–4  $\mu$ m) germinate with Type J germination patterns, as do *M. colombienseis* (11–15  $\times$  3–4  $\mu$ m) and *M. kenienseis* (7–11  $\times$  2.5–3  $\mu$ m). However, ascospores of *M. sumatrensis* are larger than those of *M. kenienseis*, and it has no anamorph, while *M. colombienseis* occurs in close association with its *Pseudocercospora* anamorph (Crous 1998).

***Mycosphaerella verrucosiafricana*** Crous & M.J. Wingf., **sp. nov.** MycoBank MB500849. Fig. 26.

**Etymology:** Refers to *M. africana*, to which it is morphologically similar.

*Mycosphaerellae africanae similis, sed ascosporis latoribus, verruculosis, (7–)8–9(–10)  $\times$  3(–3.5)  $\mu$ m, distinguenda.*

**Leaf spots** amphigenous, irregular to sub-circular, 5–15 mm diam, pale brown to grey, surrounded by a raised, dark brown border, and a thin, red-purple margin. **Ascوماتa** pseudothecial, amphigenous but chiefly hypophyllous, single, black, immersed becoming erumpent, globose, up to 60  $\mu$ m diam; apical ostiole 10–15  $\mu$ m diam; wall of 2–3 layers of medium brown *textura angularis*. **Asci** aparaphysate, fasciculate, bitunicate, sessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 18–27  $\times$  7–8  $\mu$ m. **Ascospores** tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, ellipsoid with obtuse ends, medianly 1-septate, widest in the middle of the

apical cell, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (7–)8–9(–10)  $\times$  3(–3.5)  $\mu$ m *in vivo*.

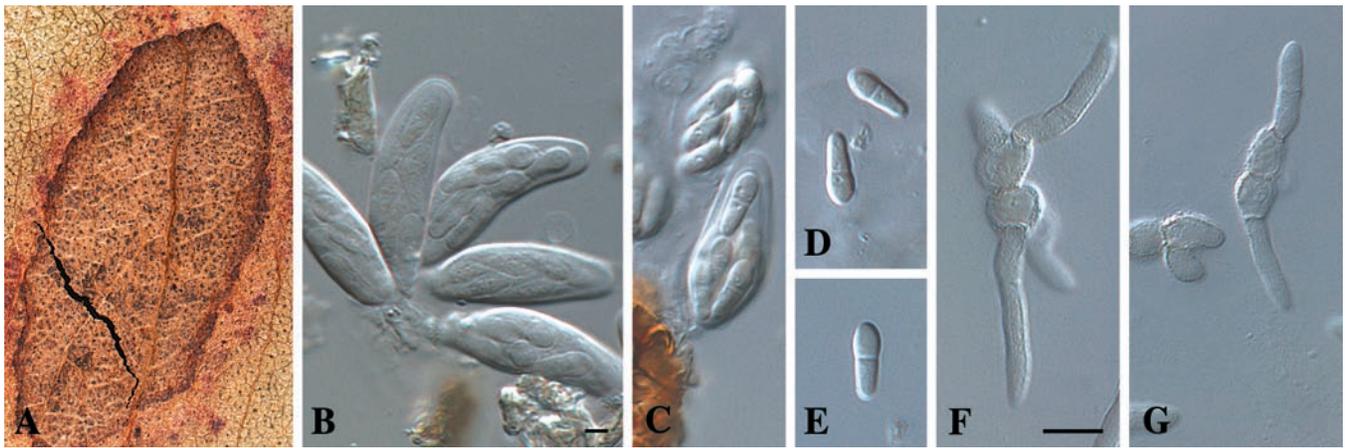
**Holotype:** Indonesia, Northern Sumatra, on leaves of *Eucalyptus* sp., Feb. 2004, M.J. Wingfield, CBS H-19705 **holotype**, culture ex-type CBS 118496 = CPC 11167, CBS 118497 = CPC 11169, CBS 118498 = CPC 11170).

**Ascospore germination on MEA after 24 h:** Type E. Ascospores becoming dark brown and verruculose on MEA, and germinating from both ends, with germ tubes irregular to the long axis of the spore; frequently with more than two germ tubes, and distorting prominently upon germination, becoming up to 9  $\mu$ m diam.

**Cultures:** Colonies on MEA 12–22 mm diam after 3 wk; erumpent, spreading, with smooth, uneven margins; upper surface cracking open; aerial mycelium sparse to absent; colonies sectoring, olivaceous-grey; margin thin, iron-grey; reverse greenish-black; on PDA with moderate aerial mycelium, and spots of slime appearing spread over the iron-grey surface; reverse greenish black; on OA colonies submerged; aerial mycelium almost completely absent, greenish black; forming chains of dark brown, thick-walled chlamydospores that aggregate into small microsclerotia (on all media); colonies sterile.

**Host:** *Eucalyptus* sp.

**Distribution:** Indonesia.



**Fig. 26.** *Mycosphaerella verrucosiafricana* (CBS 118496). A. Leaf spot. B–C. Asci. D–E. Ascospores. F–G. Germinating ascospores. Scale bars: B = 3  $\mu$ m, F = 8  $\mu$ m.



**Fig. 27.** *Pseudocercospora subulata* (CBS 118489). A. Leaf spot. B. Conidiophores. C–E. Conidia. Scale bars = 5  $\mu$ m.

**Notes:** *Mycosphaerella verrucosiafricana* is distinguished from other taxa currently known from *Eucalyptus* in that it has a characteristic ascospore germination pattern. Germinating ascospores turn brown and verruculose, but germinate with more than two germ tubes, which grow irregular to the long axis of the spore (Type G, becoming type E with age). Young ascospores just beginning to germinate can be confused with those of *M. africana*, as they initially also have only two germ tubes, though the ascospores are more distinctly verruculose than those of *M. africana*. Within a few hours of germination, additional germ tubes appear, and the pattern is more similar to that of Type E, which is seen in *M. suberosa*. *Mycosphaerella verrucosiafricana* is distinguished from *M. suberosa* in that the germ tubes remain hyaline, and ascospores and leaf spots are quite distinct from those of *M. suberosa*.

***Pseudocercospora subulata*** Z.Q. Yuan, de Little & Mohammed, *Nova Hedwigia* 71: 416. 2000. Fig. 27.

= *Pseudocercospora pseudobasitruncata* U. Braun & M. Dick, *New Zealand J. For. Res.* 32: 228. 2002.

**Specimen examined:** **New Zealand**, North Island, KeriKeri, on leaves of *E. botryoides*, 17 Oct. 2003, M.A. Dick, CBS 118489 = CPC 10849.

**Cultures:** Colonies reaching 25–35 mm diam after

3 wk on MEA; pale olivaceous-grey, erumpent, with moderate to extensive aerial mycelium; margin regular, smooth, reverse iron-grey; on PDA pale olivaceous-grey, margin thin, olivaceous-grey, reverse iron-grey; on OA central part erumpent, pale olivaceous-grey, outer zone olivaceous-grey, flat and spreading.

**Host:** *E. botryoides*.

**Distribution:** New Zealand.

**Notes:** *Pseudocercospora subulata* is morphologically similar to *P. pseudobasitruncata*, and hence they are listed here as synonyms. The culture used in this study was obtained from lesions colonised by both *P. crousii* U. Braun & M. Dick and *P. subulata*. Although the culture was obtained from a single germinating conidium, it is sterile, and we were unable to rule out the possibility that it may represent *P. crousii* and not *P. subulata*. Further collections and cultures are required to undertake DNA sequence comparisons with the *Pseudocercospora* Speg. species recently described from eucalypts by Braun & Dick (2002).

***Septoria eucalyptorum*** Crous, **sp. nov.** MycoBank MB500850. Figs 28–29.

**Etymology:** Refers to its host, *Eucalyptus*.

Septoriae linicolae similis, sed conidiis brevioribus,  $8\text{--}22 \times 2\text{--}2.5 \mu\text{m}$ , distinguenda.

**Leaf spots** absent, conidiomata associated with leaf litter. **Mycelium** internal, consisting of smooth, branched, septate, pale brown,  $2\text{--}2.5 \mu\text{m}$  wide hyphae. **Conidiomata** pycnidial, immersed, brown, globose on leaves, up to  $160 \mu\text{m}$  diam; wall consisting of 3–6 cell layers of *textura angularis*. **Conidiophores** lining the inner layer of the conidioma, dense aggregated, subcylindrical, straight to curved, 0–1-septate, mostly reduced to conidiogenous cells. **Conidiogenous cells** terminal, unbranched, hyaline, smooth, subcylindrical, proliferating sympodially near the apex,  $5\text{--}10 \times 2\text{--}2.5 \mu\text{m}$ . **Conidia** solitary *in vivo*, but undergoing microcyclic conidiation *in vitro*, finely guttulate, subcylindrical to narrowly obclavate, with obtuse to subobtuse apex, and long subtruncate base, straight to curved, 1(–3)-septate,  $(8\text{--})12\text{--}16\text{--}(22) \times 2\text{--}(2.5) \mu\text{m}$ ; hila inconspicuous,  $0.5\text{--}1 \mu\text{m}$  diam.

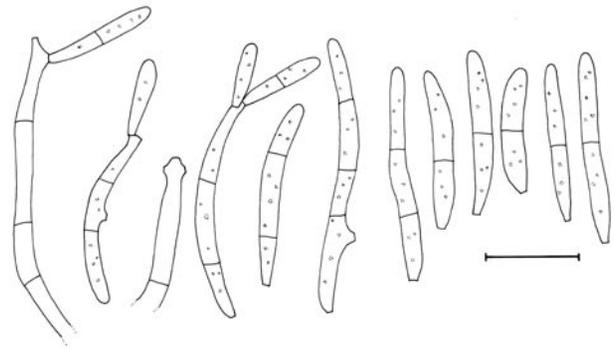
**Holotype:** India, Palampur, on *Eucalyptus* leaf litter, Feb. 2004, W. Gams & M. Arzanlou, CBS H-19700, **holotype**, cultures ex-type CBS 118505 = CPC 11282, CPC 11283.

**Cultures:** Colonies after 3 wk on MEA  $30\text{--}40 \text{ mm}$  diam; on MEA pale white to smoke-grey; aerial mycelium sparse; colonies spreading, margins even, smooth; reverse fuscous-black with patches of vinaceous-grey; on PDA producing large amounts of slime, with thread-like tufts of aerial mycelium; surface pale purplish grey (centre) with a zone of vinaceous-grey, and a pale vinaceous-grey, flat, spreading marginal region; reverse vinaceous-grey with patches of pale vinaceous-grey; on OA pale vinaceous-grey (centre) with a zone of purplish grey, a wide, flat margin concolorous with the medium; conidiomata frequently formed along circadian growth lines.

**Host:** *Eucalyptus* sp.

**Distribution:** India.

**Notes:** Sankaran *et al.* (1995) listed several species of *Septoria* on *Eucalyptus*, most of which have been redispersed to other genera. The exceptions are *S. eucalypti* G. winter & Roum. (conidia filiform–acicular,



**Fig. 28.** Conidiophores and conidia of *Septoria eucalyptorum* (CBS 118505). Scale bar =  $10 \mu\text{m}$ .

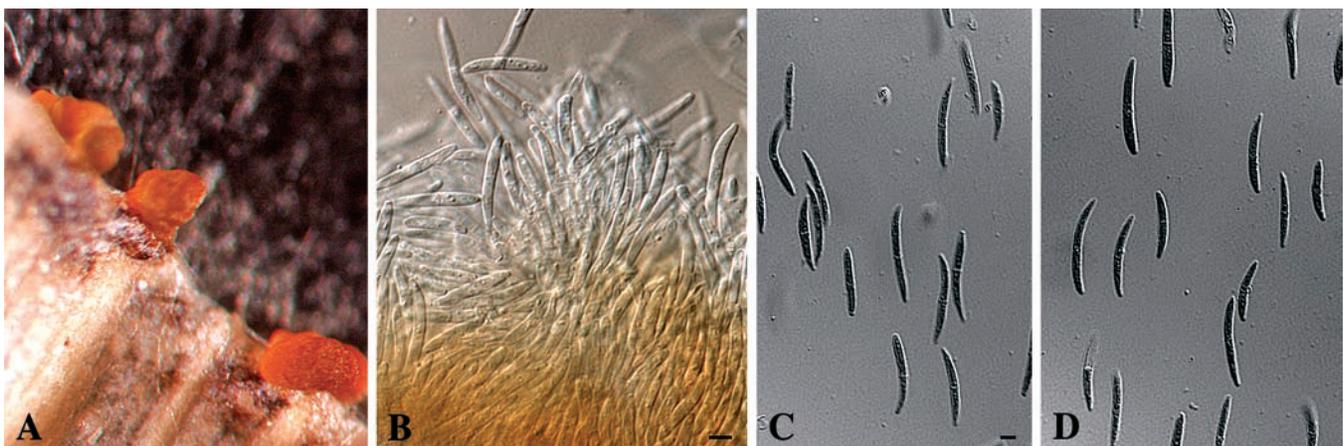
1-septate,  $14\text{--}18 \times 1.5 \mu\text{m}$ ) and *S. mortolensis* Penz. & Sacc. (conidia 0–2-septate,  $50\text{--}55 \times 3\text{--}3.5 \mu\text{m}$ ). Gadgil & Dick (1999) recently described *S. typica* Gadgil & M. Dick, which is characterised by having filiform, sigmoid or falcate, 1-septate conidia,  $65\text{--}70 \times 2\text{--}3 \mu\text{m}$ . *Septoria eucalyptorum* is distinct from this species in having conidia that are subcylindrical to narrowly obclavate, 1(–3)-septate,  $8\text{--}22 \times 2\text{--}2.5 \mu\text{m}$ . DNA sequence data in the present study (Fig. 3), show that *Septoria eucalyptorum* is closely allied to *S. linicola* (on *Linum*, conidia filiform, 1–3-septate,  $17\text{--}40 \times 1.5\text{--}3 \mu\text{m}$ ) and *S. protearum* (on *Protea*, conidia subcylindrical to narrowly obclavate, (0–)1–3(–4)-septate,  $6\text{--}30 \times 1.5\text{--}2 \mu\text{m}$ ). To fully resolve this relationship, however, other loci will need to be sequenced, as the ITS domain is insufficient to distinguish species complexes in *Septoria*.

***Septoria provincialis*** Crous, **sp. nov.** MycoBank MB500851. Fig. 30.

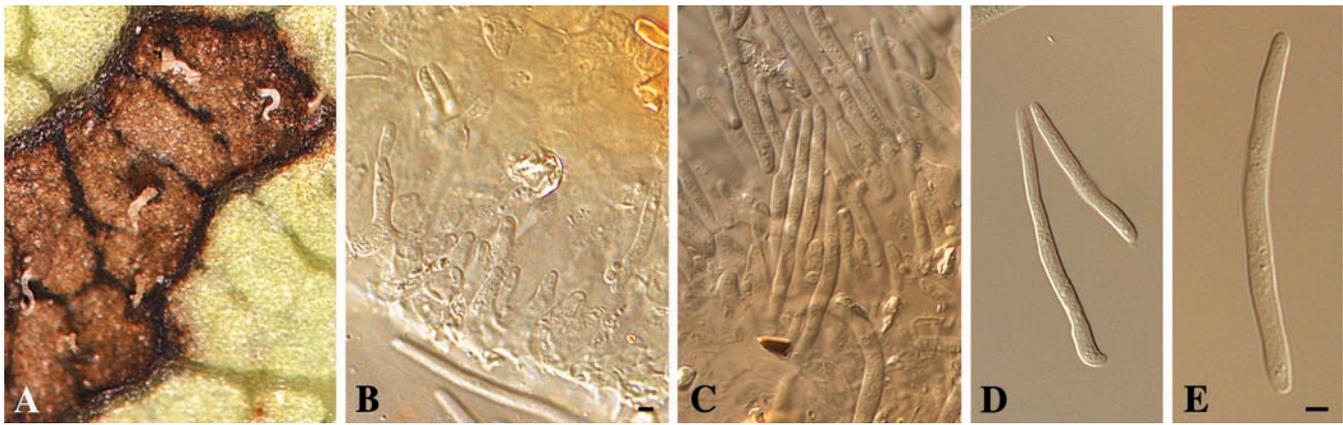
**Etymology:** Refers to the Provence in France where the fungus was collected.

Septoriae mortolensi similis, sed conidiis brevioribus,  $12\text{--}45 \times 2.5\text{--}4 \mu\text{m}$ , distinguenda.

**Leaf spots** amphigenous, dark brown, angular, confined by leaf veins, 1–6 mm diam, becoming confluent with age. **Mycelium** internal, consisting of smooth, branched, septate, hyaline,  $3\text{--}4 \mu\text{m}$  wide hyphae. **Conidiomata**



**Fig. 29.** *Septoria eucalyptorum* (CBS 118505). A. Conidiomata forming on CLA, with conidial masses. B. Conidial mass. C–D. Conidia. Scale bars =  $4 \mu\text{m}$ .



**Fig. 30.** *Septoria provencialis* (CBS 118910). A. Leaf spot. B–C. Conidiogenous cells. D–E. Conidia. Scale bars = 3 µm.

amphigenous on leaves, pycnidial, immersed, brown, globose, up to 200 µm diam; wall consisting of 2–4 cell layers of *textura angularis*. *Conidiophores* lining the inner surface of the conidioma, densely aggregated, subcylindrical to ampulliform, straight to slightly curved, 0–2-septate, 6–25 × 3–5 µm. *Conidiogenous cells* terminal, unbranched, hyaline, smooth, subcylindrical to ampulliform, proliferating sympodially or several times percurrently near the apex, 6–10 × 3–5 µm. *Conidia* solitary *in vivo*, finely guttulate, subcylindrical to narrowly obclavate, with subobtuse apex, and obconically subtruncate base, variously curved to irregular, mostly widest in the middle of the basal cell, tapering towards the apex, (1–)2(–3)-septate, (12–)30–40(–45) × 2.5–3(–4) µm.

*Holotype*: France, Provence, Cheval Blanc camping site, on juvenile *Eucalyptus* leaves, 29 Jul. 2005, P.W. Crous, CBS H-19701, *holotype*, cultures ex-type CBS 118910 = CPC 12226, CPC 12227–12228.

*Cultures*: Colonies 10–15 mm diam after 3 wk on MEA; colonies erumpent, surface irregular, catenate, olivaceous-grey with cream to pale rosy-buff spore masses; aerial mycelium absent; margins smooth, regular, with a thin outer zone that is pale olivaceous-grey to slightly rosy-buff; colonies olivaceous-black in reverse.

*Host*: *Eucalyptus* sp.

*Distribution*: France.

*Note*: Conidia of *S. provencialis* (12–45 × 2.5–4 µm) are most similar to *S. mortolensis* (50–55 × 3–3.5 µm), although on average, they are much shorter.

***Stenella pseudoparkii*** Crous & M.J. Wingf., *sp. nov.*  
 MycoBank MB500852. Fig. 31.

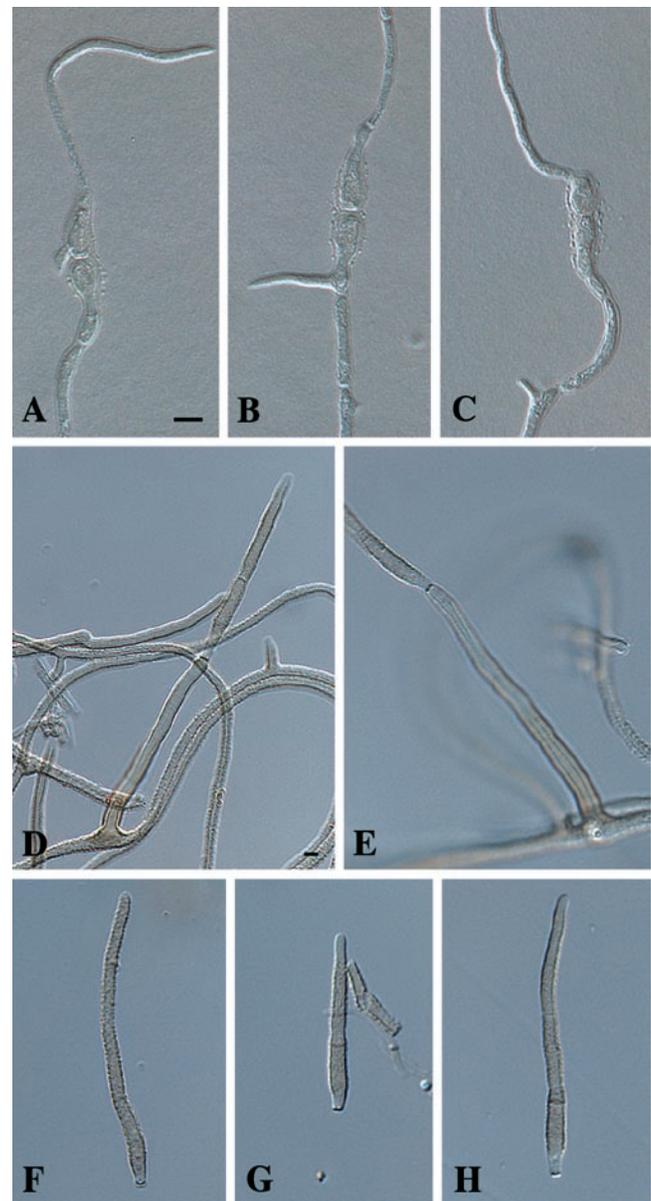
*Teleomorph*: *Mycosphaerella* sp.

*Etymology*: Morphologically similar to *M. parkii* and its anamorph, *S. parkii*.

*Stenellae parkii* similis, sed conidiis brevioribus, 20–50 × 2.5–3 µm, distinguenda.

*Leaf spots* amphigenous, irregular to subcircular, 3–7 mm diam, pale brown, with a raised border. *Conidiophores* arising singly from superficial mycelium,

brown, smooth to finely verruculose, 1–4-septate, subcylindrical, straight to variously curved, unbranched, 15–60 × 3–4 µm. *Conidiogenous cells* terminal, unbranched, medium brown, smooth, tapering to flat-tipped apical loci that are darkened and refractive,



**Fig. 31.** *Stenella pseudoparkii* (teleomorph *Mycosphaerella* sp.) (CBS 19702). A–C. Germinating ascospores. D–E. Conidiophores. F–H. Conidia. Scale bars: A = 5 µm, D = 3 µm.

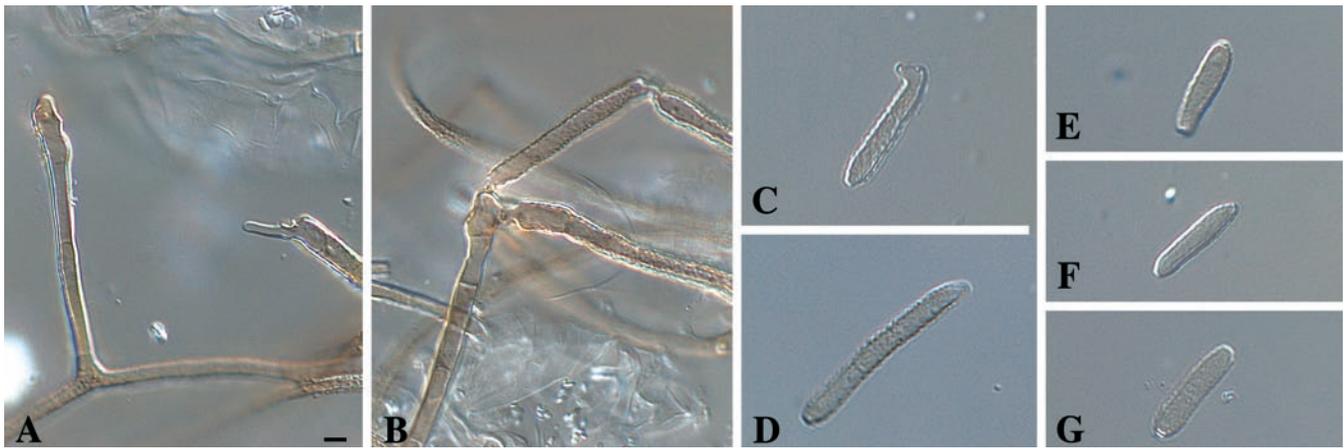


Fig. 32. *Stenella xenoparkii* (teleomorph *Mycosphaerella* sp.) (CBS 111185). A–B. Conidiophores. C–G. Conidia. Scale bar = 4  $\mu$ m.

proliferating sympodially, 15–25  $\times$  2–3  $\mu$ m. *Conidia* solitary to catenulate in simple chains, medium brown, verruculose, cylindrical or narrowly obclavate, with subobtuse apex, and long obconically subtruncate base, straight to curved, 1–5-septate, 20–50  $\times$  2.5–3  $\mu$ m; hila thickened, darkened and refractive.

*Holotype*: Colombia, on leaves of *Eucalyptus* sp., 1995, M.J. Wingfield, CBS H-19702 *holotype*, culture ex-type CBS 110999 = CPC 1087; 1088–1092.

*Ascospore germination on MEA after 24 h*: Type D. Ascospores smooth, not darkening on MEA, germinating from both ends, with germ tubes parallel to the long axis of the spore, and some lateral branches; ascospores distorting, becoming up to 5  $\mu$ m wide.

*Cultures*: Colonies after 3 wk on MEA 23–30 mm diam, pale olivaceous-grey, spreading, with moderate aerial mycelium, and smooth, irregular margins; colonies folding, erumpent; reverse olivaceous-black; on PDA pale olivaceous-grey with moderate aerial mycelium and copious amounts of slime; margins submerged in the agar; reverse olivaceous-grey; on OA pale olivaceous-grey, colonies folding with moderate aerial mycelium, and a thin olivaceous-grey margin.

*Host*: *Eucalyptus* sp.

*Distribution*: Colombia.

*Notes*: Several species of *Mycosphaerella* were present on the lesions from which *S. pseudoparkii* was isolated, and it was not possible to trace the ascospores back to the specific ascomata. The description of the *Mycosphaerella* teleomorph thus has to await further collections. The ascospores that shot out onto MEA germinated with a Type D pattern, which together with its *Stenella* anamorph, resulted in it being identified as *M. parkii* (Crous 1998). Phylogenetically, *S. pseudoparkii* is distinct from *M. parkii*, and most closely related to *M. scytalidii*, which has a Type I germination pattern. *Stenella pseudoparkii* has shorter conidia (20–50  $\times$  2.5–3  $\mu$ m) than *Stenella parkii* (25–200  $\times$  2–2.5  $\mu$ m) (Crous & Alfenas 1995, Crous 1998).

*Stenella xenoparkii* Crous & M.J. Wingf., *sp. nov.* MycoBank MB500853. Fig. 32.

*Teleomorph*: *Mycosphaerella* sp.

*Etymology*: refers to the morphological similarity with *M. parkii* and its anamorph, *S. parkii*.

*Stenellae parkii* similis, sed conidiis brevioribus, 12–50  $\times$  3–5  $\mu$ m, distinguenda.

*Leaf spots* amphigenous, irregular to subcircular, 2–10 mm diam, pale brown, with a raised border and thin, red-purple margin. *Conidiophores* arising singly from superficial mycelium, medium brown, finely verruculose, 1–2-septate, subcylindrical, straight to variously curved, unbranched, 30–60  $\times$  3–4  $\mu$ m. *Conidiogenous cells* terminal, unbranched, medium brown, verruculose, tapering to flat-tipped apical loci that are darkened and refractive, proliferating sympodially, 10–25  $\times$  3–4  $\mu$ m. *Conidia* catenulate in branched chains, medium brown, verruculose, cylindrical or narrowly obclavate, with subobtuse apex, and subtruncate base, straight to curved, 0–2-septate, 12–50  $\times$  3–5  $\mu$ m; hila thickened, darkened and refractive.

*Holotype*: Indonesia, on leaves of *E. grandis*, Mar. 1996, M.J. Wingfield, *holotype* PREM 54968, *isotype* in CBS H-19703, cultures ex-type CBS 111185 = CPC 1300; 1299–1301.

*Ascospore germination on MEA after 24 h*: Type D. Similar to *M. parkii*.

*Cultures*: Colonies after 3 wk on MEA 25–35 mm diam; on MEA spreading, slightly erumpent, margins smooth but irregular; aerial mycelium sparse to moderate; surface olivaceous-black, but central part grey due to aerial mycelium; reverse olivaceous-black; on PDA olivaceous-black with mucous droplets and aerial mycelium that is olivaceous-grey in the central part, but has a reddish tinge in the outer region; reverse greenish black; on OA iron-grey with sparse to moderate olivaceous-grey aerial mycelium.

*Host*: *Eucalyptus grandis*.

*Distribution*: Indonesia.

*Notes*: The specimen on which this species is based was originally identified as representing *M. parkii*. The original identification was based on its characteristic leaf spots, ascospore germination patterns and

dimensions, and the presence of a *Stenella* anamorph. Teleomorph material was not retained, and hence only the anamorph, which forms in culture, can be named. Conidia of *S. xenoparkii* (12–50 × 3–5 µm) are shorter and wider than those of *S. pseudoparkii* (20–50 × 2.5–3 µm) and *S. parkii* (25–200 × 2–2.5 µm) (Crous & Alfenas 1995, Crous 1998).

## DISCUSSION

In this study we have described 21 new species of *Mycosphaerella* or its anamorphs from *Eucalyptus* leaves. Some of these new species arise from a re-examination of specimens and cultures treated previously (Crous 1998). The species in this earlier study had been described primarily on the basis of morphology and without the support of DNA sequence comparisons.

Results of this study are similar to those of Crous *et al.* (2004b) showing that there are several species of *Mycosphaerella* on eucalypts that have distinct cultural characteristics and can be separated based on phylogenetic analyses, but that share the same symptoms, morphological characteristics and ascospore germination patterns. It is clearly very difficult to accurately identify *Mycosphaerella* species on eucalypts in the absence of DNA sequence analyses. Identifications or species described based solely on morphological characteristics must consequently be viewed with some circumspection.

A good example of the confusion arising from identifications based solely on phenotypic characters is found in the case of *M. parkii*. In the present study, we reconsidered several collections originally identified as *M. parkii* based on symptoms, ascospore dimensions, germination patterns, and the presence of a *Stenella* anamorph in culture. The “*M. parkii*”-like isolates were consequently shown to represent several species. Because of insufficient material being available, only two anamorph species *S. xenoparkii* and *S. pseudoparkii*, could be named.

Cryptic species were also found among isolates originally identified as *M. africana*. These identifications were based on the presence of fusoid–ellipsoidal ascospores that are constricted at the septum, that darken upon germination, and that produce colonies that are relatively slow-growing. These isolates are described here as *M. verrucosiafricana* and *M. pseudofrancana*. Other examples of cryptic species were found in the case of *M. pseudoendophytica*, which is morphologically similar to *M. endophytica*, *M. pseudosuberosa*, which is similar to *M. suberosa*, and *M. pseudocryptica*, which is similar to *M. cryptica*.

In this study we have applied only DNA sequences of the ITS region. Although this locus has been very useful in delimiting species of *Mycosphaerella* from *Eucalyptus*, it is not always sufficient to derive conclusions for all species complexes (Crous *et al.* 2004c, Hunter *et al.* 2006 – this volume). For example, it is not suitable for distinguishing species in anamorph

genera such as *Cercospora* and *Septoria*. In contrast, sequences of the ITS region appear to be useful for distinguishing species with *Pseudocercospora*, *Ramularia* and most other *Mycosphaerella* anamorph genera that we have considered (Crous & Groenewald, unpubl. data). It appears, therefore, that the ITS region has evolved at different rates in different anamorph genera associated with *Mycosphaerella*, and that it is more conserved in *Cercospora* and *Septoria*, two genera that always cluster together.

In this study, we have described several new *Mycosphaerella* species from leaf litter. This suggests that there are numerous endophytic *Mycosphaerella* species that sporulate once leaves have died. The biology of *Mycosphaerella* species suggests that these fungi are probably not saprobes but rather that they infect living leaf tissue and only sporulate after leaf fall. Virtually nothing is known of this life-habit of *Mycosphaerella* species, and it would be intriguing to follow the infection patterns of species that are not primary pathogens.

*Mycosphaerella secundaria* was one of the more intriguing fungi arising from this study. This fungus has been collected on several occasions, but its unique nature was not confirmed previously. *Mycosphaerella secundaria* is always found on leaf spots caused by *M. suberosa*. This is an unusual habit for a species of *Mycosphaerella*, and its ecological role deserves further study.

*Dissoconium dekkeri* (teleomorph: *M. lateralis*) was originally described as a potential hyperparasite of powdery mildew (De Hoog *et al.* 1983, 1991), and has since been isolated from many different hosts (Crous *et al.* 2004b). Jackson *et al.* (2004) showed that *M. lateralis* is not a hyperparasite of *M. nubilosa* and *M. cryptica*, the two species with which *M. lateralis* frequently co-occurs. Jackson *et al.* (2004) also showed that *D. dekkeri* can infect *Eucalyptus* leaves. *Mycosphaerella lateralis* and *M. communis* occur on leaves of numerous *Eucalyptus* spp., and they are frequently found on leaf spots caused by other *Mycosphaerella* species, as well as unrelated fungi (Crous unpubl. data). The ecological role of *M. lateralis*, however, remains to be determined.

The linking of yet another anamorph genus to *Mycosphaerella*, namely *Davisoniella*, draws an interesting parallel to morphologically similar coelomycete genera. Of particular interest, are the taxa currently accommodated in *Colletogloeopsis*, particularly those that are *Coniothyrium*-like and to which *D. eucalypti* is definitely closely related if not congeneric. Presently no cultures are available, the relationship between these taxa remains to be proven, and hence the anamorph is best retained in *Davisoniella*.

In this study, we have added 21 species to the number of *Mycosphaerella* spp. presently known to occur on *Eucalyptus* leaves and stems. We suggested that there could easily be at least as many *Mycosphaerella* spp. on eucalypts as there are species of that genus. This would imply that only 14 % of the species of *Mycosphaerella* from eucalypts



have presently been described. This means that significant challenges face the taxonomists who wish to distinguish *Mycosphaerella* spp. from eucalypts in future. Most likely, in future studies, DNA sequence comparisons based on multiple genes will be required to accurately identify these fungi. Given the enormity of this task, focus will clearly need to be directed to those species that are primary pathogens. However, the primary pathogens are so easily confused with other less important species, that all material will ultimately have to be thoroughly studied and understood.

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## Foliicolous microfungi occurring on *Encephalartos*

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### Key words

*Catenulostroma*  
*Cladophialophora*  
*Dactylaria*  
ITS nrDNA  
LSU nrDNA  
*Ochroconis*  
*Phaeomoniella*  
*Saccharata*  
systematics  
*Teratosphaeria*

**Abstract** Species of *Encephalartos*, commonly known as bread trees, bread palms or cycads are native to Africa; the genus encompasses more than 60 species and represents an important component of the indigenous African flora. Recently, a leaf blight disease was noted on several *E. altensteinii* plants growing at the foot of Table Mountain in the Kirstenbosch Botanical Gardens of South Africa. Preliminary isolations from dead and dying leaves of *E. altensteinii*, *E. lebomboensis* and *E. princeps*, collected from South Africa, revealed the presence of several novel microfungi on this host. Novelty include *Phaeomoniella capensis*, *Saccharata kirstenboschensis*, *Teratosphaeria altensteinii* and *T. encephalarti*. New host records of species previously only known to occur on Proteaceae include *Cladophialophora proteae* and *Catenulostroma microsporium*, as well as a hyperparasite, *Dactylaria leptosphaericola*, occurring on ascomata of *T. encephalarti*.

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### INTRODUCTION

*Encephalartos* (Zamiaceae) is a genus of cycads indigenous to Africa. Due to its edible pith, species of *Encephalartos* are commonly referred to as bread trees or bread palms ([www.kew.org/plants/](http://www.kew.org/plants/)). Another interesting aspect that makes *Encephalartos* noteworthy is the fact that it could represent one of the oldest pot-plants in the world. A specimen of *E. altensteinii* was collected in the Eastern Cape Province of South Africa in the early 1770s, and taken to Kew Botanic Gardens in the UK by Francis Masson in 1775, where it is still to be seen in the Palm House today. Although this plant genus is endangered and known to suffer from trunk and root parasites, as well as fungal infections, very few fungi have been described from this host (Doidge 1950, Nag Raj 1993, [nt.ars-grin.gov/fungal-databases/](http://nt.ars-grin.gov/fungal-databases/)).

Fungal biodiversity has been poorly studied from most African countries, which could explain why so few fungal taxa have thus far been reported from *Encephalartos*. In a recent attempt to estimate how many species of fungi could occur at the tip of Africa, Crous et al. (2006a) concluded that the 1.5 M estimate suggested by Hawksworth (1991) was clearly too conservative. Based on available data, South Africa alone should have at least 200 000 fungal species associated with plant species, without taking into account the number associated with insects, or other ecological habitats such as water and soil.

Because of its extremely hard, leathery leaves, microfungi are not readily observed to colonise foliage of *Encephalartos* species. In January 2008, however, a tip blight disease was

observed on several *Encephalartos* palms growing in the Kirstenbosch Botanical Gardens of South Africa, as well as in the KwaZulu-Natal Province. The aim of the present study was therefore to determine if any microfungi could be isolated from these diseased leaves and also investigate symptomatic *Encephalartos* leaf samples collected from elsewhere.

### MATERIALS AND METHODS

#### Isolates

Dead *Encephalartos* leaves, or leaves with tip blight symptoms, were chosen for study. As none of the collections had leaves that were visibly colonised, leaves were incubated in moist chambers for up to 2 wk, and inspected daily for fungi. Leaf pieces bearing ascomata were subsequently soaked in water for approximately 2 h, after which they were placed in the bottom of Petri dish lids, with the top half of the dish containing 2 % malt extract agar (MEA; Oxoid, Hampshire, England). Ascospore germination patterns were examined after 24 h, and single ascospore and conidial cultures established as described by Crous (1998). Colonies were subcultured onto 2 % potato-dextrose agar (PDA), synthetic nutrient-poor agar (SNA), MEA, and oatmeal agar (OA) (Gams et al. 2007), and incubated under continuous near-ultraviolet light at 25 °C to promote sporulation. All cultures obtained in this study are maintained in the culture collection of the CBS (Table 1). Nomenclatural novelties and descriptions were deposited in MycoBank (Crous et al. 2004b).

#### DNA phylogeny

Genomic DNA was isolated from fungal mycelium grown on MEA, using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) according to the manufacturer's protocols. The Primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990) were used to amplify part of the nuclear rDNA operon spanning

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<sup>4</sup> Microbe Division / Japan Collection of Microorganisms, RIKEN BioResource Center, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.

**Table 1** Collection details and GenBank accession numbers for fungal species isolated from *Encephalartos* spp.

Species	Strain no. <sup>1</sup>	Substrate	Collector(s)	GenBank Accession number	
				ITS <sup>2</sup>	LSU <sup>2</sup>
<i>Catenulostroma abietis</i>	CPC 14996	Dead leaf tissue of <i>E. altensteinii</i>	P.W. Crous	FJ372387	FJ372404
<i>Cladophialophora proteae</i>	CPC 14902	Dead leaf tissue of <i>E. altensteinii</i>	P.W. Crous	FJ372388	FJ372405
<i>Lophiostoma</i> sp.	CPC 15000; CBS 123543	Living leaves of <i>E. altensteinii</i>	P.W. Crous et al.	FJ372389	FJ372406
<i>Ochroconis</i> sp.	CPC 15461; CBS 123536	Living leaves of <i>E. lebomboensis</i>	A.R. Wood	FJ372390	FJ372407
<i>Phaeomoniella capensis</i>	CPC 15416; CBS 123535	Living leaves of <i>E. altensteinii</i>	A.R. Wood	FJ372391	FJ372408
<i>Saccharata kirstenboschensis</i>	CPC 15275; CBS 123537	Living leaves of <i>E. princeps</i>	A.R. Wood	FJ372392	FJ372409
<i>Teratosphaeria altensteinii</i>	CPC 15133; CBS 123539	Living leaves of <i>E. altensteinii</i>	P.W. Crous et al.	FJ372394	FJ372411
<i>Teratosphaeria encephalarti</i>	CPC 14886; CBS 123540	Living leaves of <i>E. altensteinii</i>	P.W. Crous et al.	FJ372395	FJ372412
	CPC 15281; CBS 123544	Living leaves of <i>E. altensteinii</i>	A.R. Wood	FJ372396	FJ372413
	CPC 15362; CBS 123541	Living leaves of <i>E. altensteinii</i>	A.R. Wood	FJ372397	FJ372414
	CPC 15413; CBS 123545	Living leaves of <i>E. altensteinii</i>	A.R. Wood	FJ372398	FJ372415
	CPC 15464; CBS 123546	Living leaves of <i>E. lebomboensis</i>	A.R. Wood	FJ372399	FJ372416
	CPC 15465	Living leaves of <i>E. lebomboensis</i>	A.R. Wood	FJ372400	FJ372417
	CPC 15466	Living leaves of <i>E. lebomboensis</i>	A.R. Wood	FJ372401	FJ372418
<i>Teratosphaeria</i> sp.	CPC 14997	Living leaves of <i>E. altensteinii</i>	P.W. Crous et al.	FJ372402	FJ372419

<sup>1</sup> CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.

<sup>2</sup> ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; LSU: 28S nrDNA.

the 3' end of the 18S rRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region (ITS2) and the first 900 bases at the 5' end of the 28S rRNA gene (LSU). The primers ITS4 (White et al. 1990) and LR0R (Rehner & Samuels 1994) were used as internal sequence primers to ensure good quality sequences over the entire length of the amplicon. The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Crous et al. (2006b). Alignment gaps were treated as new character states. Sequence data were deposited in GenBank (Table 1) and alignments in TreeBASE ([www.treebase.org](http://www.treebase.org)). The ITS sequences were compared with the sequences available in NCBI's GenBank nucleotide database using a megablast search.

### Morphology

Colony growth characteristics (surface and reverse) were assessed on MEA, PDA, OA and SNA (Gams et al. 2007), and colours determined using the colour charts of Rayner (1970). Microscopic observations were made from fungal colonies cultivated on different media, as stated with each fungus. Preparations were mounted in lactic acid and studied by means of a light microscope ( $\times 1000$  magnification). Microscopic observations were made from hyphomycetes by using the transparent tape or slide culture technique, as respectively explained by Schubert et al. (2007) and Arzanlou et al. (2007). The 95 % confidence intervals were derived from 30 observations of spores formed in culture, with extremes given in parentheses. All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands, or the working collection (CPC) of P.W. Crous (Table 1).

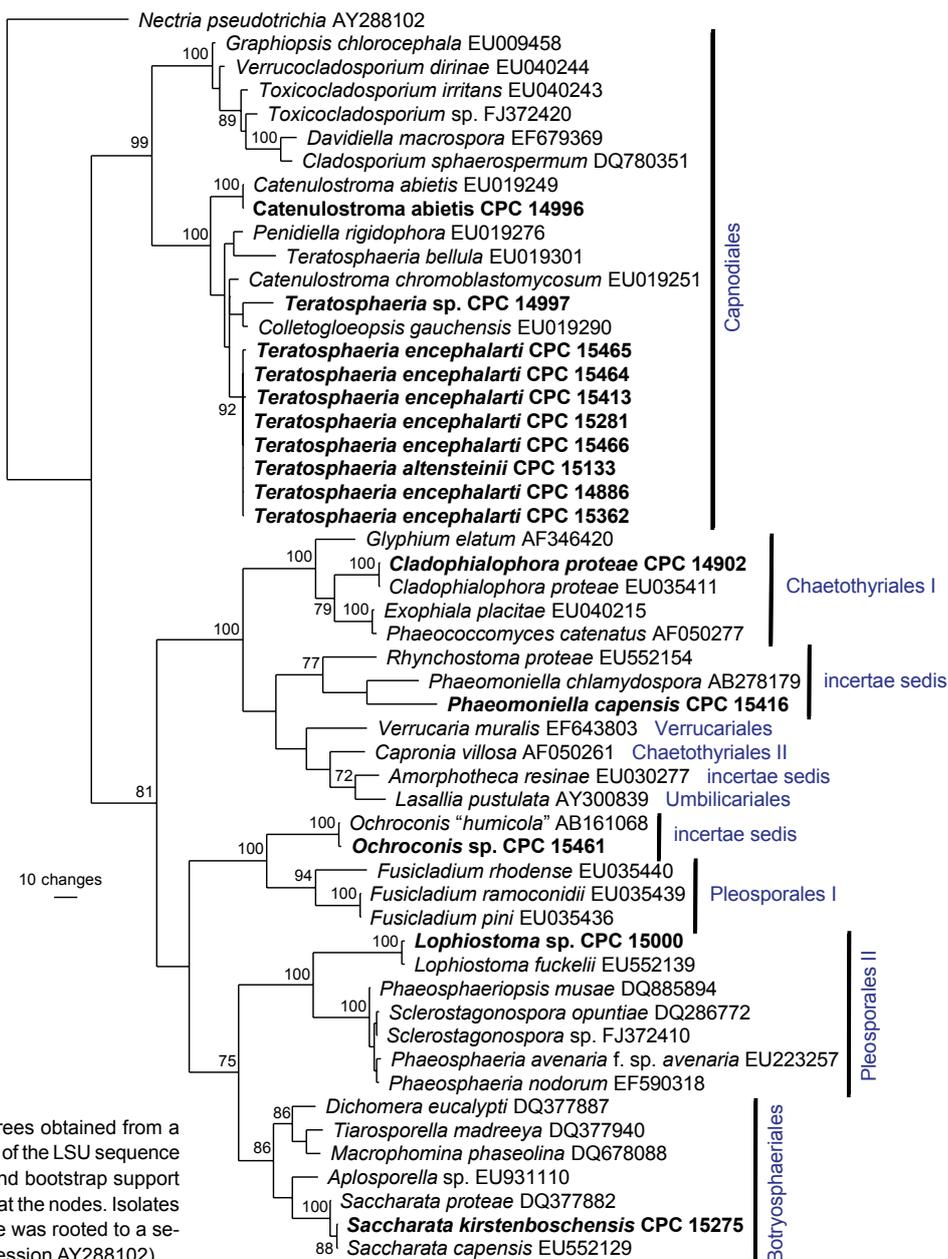
## RESULTS

### DNA phylogeny

Amplification products of approximately 1 700 bases were obtained for the isolates listed in Table 1. The LSU region of the sequences was used to obtain additional sequences from GenBank, which were added to the alignment. Due to the inclusion of the shorter *Phaeomoniella chlamydospora* (GenBank AB278179) and *Ochroconis 'humicola'* (GenBank AB161068) sequences in the alignment, it was not possible to subject the full length of the determined LSU sequences (Table 1) to the analyses. The manually adjusted alignment contained 53 sequences (including the outgroup sequence) and, of the 563 characters used in the phylogenetic analyses, 253 were parsimony-informative, 24 were variable and parsimony-uninformative, and 286 were constant. Neighbour-joining analyses using three substitution models on the sequence data yielded trees supporting the same tree topology to one another but differed from the most parsimonious tree shown in Fig. 1 with regard to the placement of the clade containing *Ochroconis* and *Fusicladium* (in the distance analyses, this clade moves to a more basal position). Forty equally most parsimonious trees (TL = 1039 steps, CI = 0.477, RI = 0.833, RC = 0.397), one of which is shown in Fig. 1, were obtained from the parsimony analysis of the LSU alignment. The isolates from *Encephalartos* are distributed across several families and orders and taxonomic novelties are described below and specific taxa are highlighted in the Discussion. Results obtained from the BLAST searches of the ITS sequences are discussed where applicable.

### Taxonomy

Several species of fungi which are believed to be new were collected, and are described in genera such as *Phaeomoniella*, *Saccharata* and *Teratosphaeria*. New records for *Encephalartos* include *Catenulostroma microsporum*, *Cladophialophora proteae*, *Dactylaria leptosphaeriicola*, and undescribed species of *Teratosphaeria*, *Lophiostoma* and *Ochroconis*.



**Fig. 1** One of 40 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the LSU sequence alignment. The scale bar shows 10 changes, and bootstrap support values (>70 %) from 1 000 replicates are shown at the nodes. Isolates from *Encephalartos* are shown in **bold**. The tree was rooted to a sequence of *Nectria pseudotrachia* (GenBank accession AY288102).

***Phaeomoniella capensis*** Crous & A.R. Wood, *sp. nov.* — MycoBank MB508007; Fig. 2

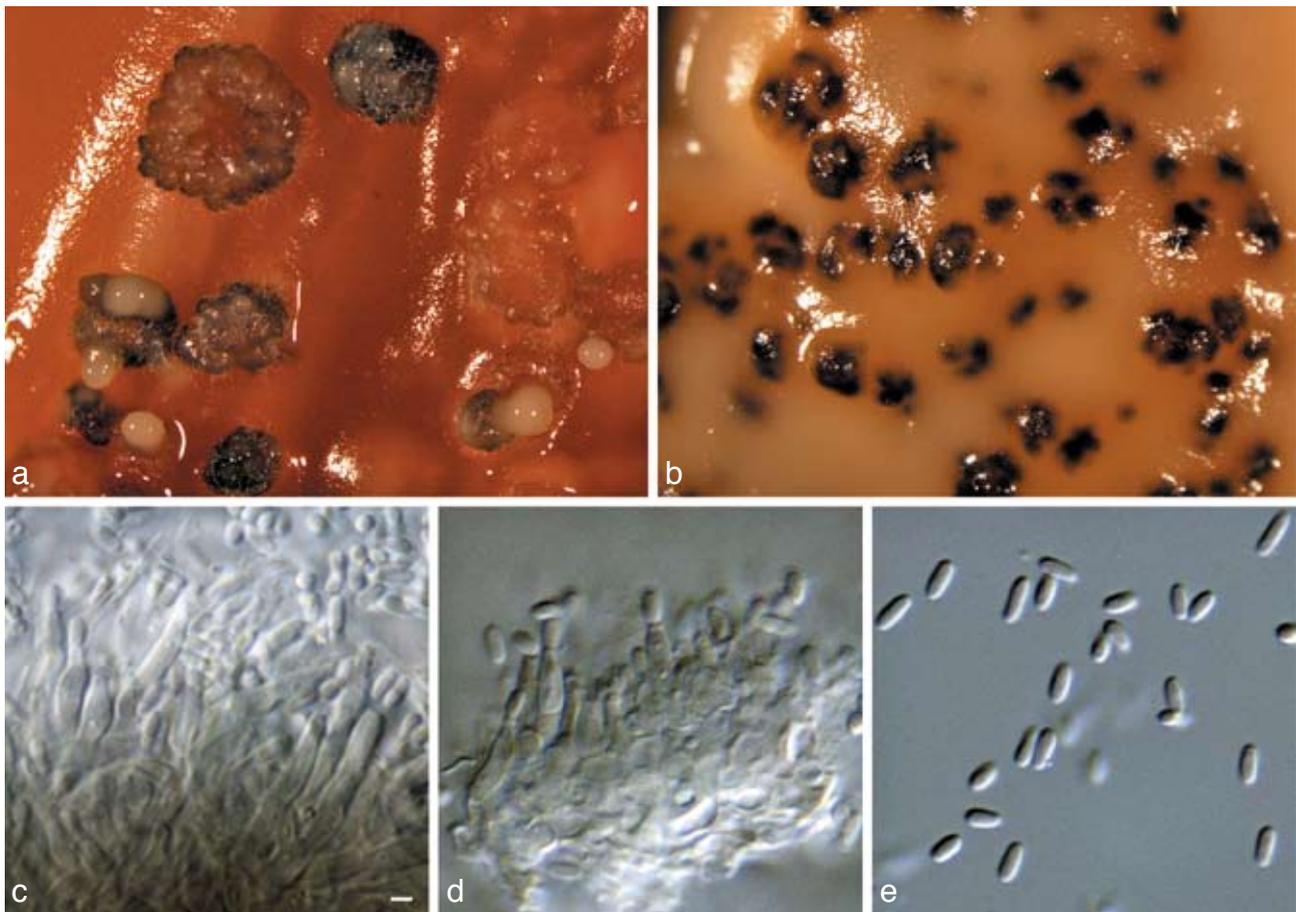
*Phaeomoniellae chlamydosporae* similis, sed conidiis majoribus, (2–)3(–4) × 1–1.5 µm.

**Etymology.** Name refers to the Cape Province of South Africa, where this fungus was collected.

On SNA. *Mycelium* consisting of septate, branched, hyaline to pale brown, thick-walled hyphae, 1.5–2 µm; developing hyaline, thin-walled, swollen, globose structures. *Conidiomata* pycnidial to acervular, opening by irregular rupture, erumpent, brown, up to 250 µm diam; wall of 3–6 layers of brown *textura*

*angularis*. *Conidiophores* hyaline, smooth, highly variable in morphology, occurring in branched structures, 2–4-septate, or solitary, ampulliform, reduced to phialides. *Conidiogenous cells* 3–10 × 2–3 µm; apical opening with minute periclinal thickening. *Conidia* hyaline, smooth, narrowly ellipsoid, straight, (2–)3(–4) × 1–1.5 µm.

Cultural characteristics — *Colonies* erumpent, spreading, lacking aerial mycelium, slimy, with folded surface and smooth, catenulate margin; on PDA salmon with patches of apricot, and apricot in reverse, reaching 10 mm diam after 1 mo; on OA salmon to flesh with brown patches due to conidiomatal formation, reaching 12 mm diam after 1 mo; on MEA salmon



**Fig. 2** *Phaeomoniella capensis* in vitro (CBS 123535). a. Colony on OA; b. colony on PDA; c, d. conidiogenous cells and conidia; e. conidia. — Scale bar = 10  $\mu$ m.

with patches of apricot and flesh, apricot in reverse, reaching 15 mm diam after 1 mo.

*Specimen examined.* SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 22 May 2008, A.R. Wood, CBS H-20159, culture ex-type CPC 15416 = CBS 123535, CPC 15417–15418.

**Notes** — Two fungal species that have previously been described from *Encephalartos* need to be compared with *P. capensis*. *Leptothyrium evansii* forms hypophylous pycnidia with oblong, hyaline conidia,  $3.5\text{--}5 \times 1.5\text{--}2 \mu\text{m}$ , thus larger than observed in *P. capensis* (Sydow & Sydow 1912). The second species, *Phoma encephalarti*, is distinct in having larger, biguttulate conidia,  $6.3\text{--}7.2 \times 2.7\text{--}3.6 \mu\text{m}$  (Negodi 1932).

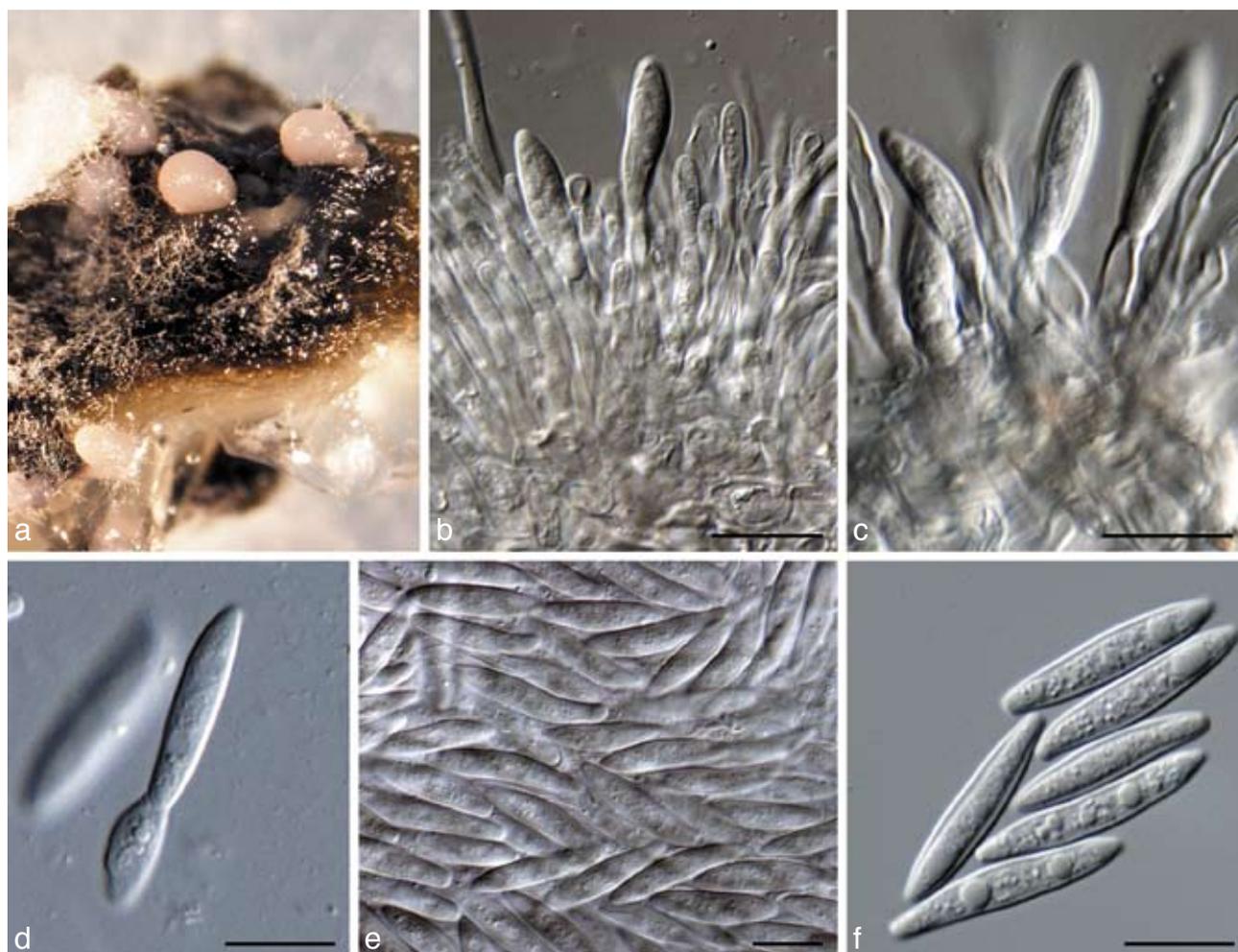
The fact that the present collection clusters in *Phaeomoniella* (hyphomycetous genus) is somewhat surprising. However, this genus also has a phoma-like synanamorph and a yeast-like growth in culture (Crous & Gams 2000), similar to *P. capensis*. Although further collections may eventually show this complex to represent more than one genus, we presently consider it best to place the *Encephalartos* fungus in *Phaeomoniella* based on current data. BLAST results of the ITS sequence revealed an identity of 89 % with *Phaeomoniella chlamydospora* (GenBank accession AY772237).

***Saccharata kirstenboschensis*** Crous & A.R. Wood, *sp. nov.*  
— MycoBank MB508008; Fig. 3

*Saccharatae proteae* similis, sed conidiis minoribus,  $(16\text{--})18\text{--}22(\text{--}24) \times 3.5\text{--}4(\text{--}5) \mu\text{m}$ .

*Etymology.* Name refers to Kirstenbosch Botanical Gardens, South Africa, where this fungus was collected.

On WA with sterile pine needles. *Conidiomata* pycnidial, black, up to 350  $\mu\text{m}$  diam, with a single, central ostiole; wall consisting of 2–3 layers of brown *textura angularis*. *Conidiophores* subcylindrical, hyaline, smooth, frequently reduced to conidiogenous cells or branched in apical part, 1–2-septate,  $10\text{--}45 \times 2\text{--}3.5 \mu\text{m}$ . *Conidiogenous cells* terminal, subcylindrical, hyaline,  $15\text{--}20 \times 2\text{--}3 \mu\text{m}$ ; apex with periclinal thickening, or with 1–3 percurrent proliferations. *Paraphyses* intermingled among conidiophores, at times arising as lateral branches from conidiophores, or separate, unbranched or branched above, hyaline, smooth, 0–3-septate, 2–3  $\mu\text{m}$  wide, extending above conidiophores. *Conidia* hyaline, smooth, fusiform to narrowly ellipsoid, apex subobtuse, base truncate with minute marginal frill, guttulate, thin-walled,  $(16\text{--})18\text{--}22(\text{--}24) \times 3.5\text{--}4(\text{--}5) \mu\text{m}$ , base 2–3  $\mu\text{m}$  wide.



**Fig. 3** *Saccharata kirstenboschensis* in vitro (CBS 123537). a. Conidiomata on WA with sterile pine needles; b, c. conidiogenous cells giving rise to conidia; d. conidium attached to conidiogenous cell; e, f. conidia. — Scale bars = 10  $\mu\text{m}$ .

**Cultural characteristics** — Colonies on MEA, PDA and OA spreading, erumpent, with moderate aerial mycelium and uneven, catenulate margins; pale olivaceous-grey with patches of grey and olivaceous-grey; reverse olivaceous-grey; reaching 6 cm diam after 1 mo.

**Specimen examined.** SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos princeps*, 22 May 2008, A.R. Wood, holotype CBS H-20160, culture ex-type CPC 15275 = CBS 123537, CPC 15276–15277.

**Notes** — The genus *Saccharata* presently consists of two species, namely *S. proteae* (conidia  $20\text{--}30 \times 4.5\text{--}6 \mu\text{m}$ ; Denman et al. 1999, Crous et al. 2006b) and *S. capensis* (conidia  $13\text{--}18 \times 3.5\text{--}5.5 \mu\text{m}$ ; Marinowitz et al. 2008). *Saccharata kirstenboschensis* represents an intermediate species, having conidia  $16\text{--}24 \times 3.5\text{--}5 \mu\text{m}$ . Furthermore, it is the first species of *Saccharata* known to occur on a host other than Proteaceae, although all taxa described thus far appear to be endemic to South Africa. BLAST results of the ITS sequence revealed an identity of 98 % with *S. proteae* (GenBank accession EU552145; 819 of 830 bases) and *S. capensis* (GenBank accession EU552130; 803 of 816 bases).

***Teratosphaeria altensteinii* Crous, sp. nov.** — MycoBank MB508010; Fig. 4

*Teratosphaeriae bellulae* similis, sed ascosporis minoribus,  $7\text{--}8\text{--}(9) \times 2.5\text{--}3\text{--}(3.5) \mu\text{m}$ .

**Etymology.** Name refers to its host species, *Encephalartos altensteinii*.

**Leaves** with tip-blight symptoms; necrotic tissue grey-brown, separated from healthy tissue by a narrow, dark-brown border. **Ascomata** hypophyllous, black, immersed, substomatal, up to 90  $\mu\text{m}$  diam; ostiole lined with periphyses; wall consisting of 2–3 layers of medium brown *textura angularis*. **Asci** aparaphysate, fasciculate, bitunicate, sessile, obovoid, straight to slightly curved, 8-spored,  $35\text{--}37 \times 8\text{--}9 \mu\text{m}$ . **Ascospores** bi- to triseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, prominently constricted at the septum, tapering towards both ends, but more prominently towards the lower end,  $7\text{--}8\text{--}(9) \times 2.5\text{--}3\text{--}(3.5) \mu\text{m}$ ; germinating ascospores on MEA become brown and verruculose, germinating with multiple germ tubes irregular to the long axis of the spore, constricted at septum and distorting, up to 8  $\mu\text{m}$  wide.



Fig. 4 *Teratosphaeria altensteinii* in vitro (CBS 123539). a, b. Asci; c, d. ascospores; e–g. germinating ascospores on MEA. — Scale bars = 10  $\mu$ m.

Cultural characteristics — Colonies on MEA spreading, somewhat erumpent, with moderate aerial mycelium, and even, catenulate margins; surface iron-grey; reverse greenish black; reaching 20 mm diam after 1 mo; on PDA and OA similar, but olivaceous-grey on surface, and iron-grey in reverse; on MEA and PDA hyphae form terminal clusters of chlamydospore-like cells, which are catenulostroma-like in appearance, and frequently detach under squash mounts.

*Specimen examined.* SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 6 Jan. 2008, P.W. Crous, M.K. Crous, M. Crous & K. Raath, holotype CBS H-20162, culture ex-type CPC 15133 = CBS 123539, CPC 15134–15135.

*Notes* — *Teratosphaeria altensteinii* is phylogenetically closely related to *T. bellula* (593 of 601 bases when the ITS sequence is compared to GenBank accession EU707861), which is a pathogen of Proteaceae (Crous & Wingfield 1993, Crous et al. 2004a, 2008). Morphologically it has ascospores that are similar in shape, but are distinct in that they lack a prominent sheath and are somewhat smaller (7–9  $\times$  2.5–3.5  $\mu$ m) than those of *T. bellula* (8–11  $\times$  2–3.5  $\mu$ m; Crous & Wingfield 1993).

***Teratosphaeria encephalarti*** Crous & A.R. Wood, *sp. nov.*  
— MycoBank MB508011; Fig. 5

*Anamorph.* *Penidiella* sp.

*Teratosphaeriae bellulae* similis, sed ascosporis majoribus, (9–)10–11(–14)  $\times$  (3–)3.5–4  $\mu$ m.

*Etymology.* Name refers to its host genus, *Encephalartos*.

*Leaves* with tip-blight symptoms; necrotic tissue grey-brown. *Ascomata* hypophyllous, black, immersed, substomatal, up to 90  $\mu$ m diam; ostiole lined with periphyses; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoid, straight to curved, 8-spored, 30–40  $\times$  10–13  $\mu$ m. *Pseudoparaphyses* intermingled among asci, branched, septate, hyaline, 2–3  $\mu$ m wide. *Ascospores* bi- to triseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, prominently constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (9–)10–11(–14)  $\times$  (3–)3.5–4  $\mu$ m; turning brown and verruculose in older asci; germinating ascospores on



**Fig. 5** *Teratosphaeria encephalarti* (CBS 123540). a. Diseased *Encephalartos altensteinii* palms in Kirstenbosch Botanical Gardens, South Africa; b. leaf blight symptoms; c. ascomata on leaves (arrows); d, e. asci; f. ascospores; g–k. germinating ascospores on MEA; l–o. *Penidiella* anamorph with branched conidial chains. — Scale bars = 10 µm.

MEA become brown and verruculose, germinating with several germ tubes irregular to the long axis of the spore, constricted at septum and distorting, up to 7 µm wide. On OA. *Mycelium* consisting of creeping, branched, septate, brown, smooth, 2–3.5 µm wide hyphae. *Conidiophores* solitary, erect, subcylindrical, arising from creeping hyphae, medium brown, thick-walled, smooth to finely verruculose, 1–6-septate, 15–50 × 3–4.5 µm. *Conidiogenous cells* terminal, subcylindrical, medium brown, smooth, up to 4 µm wide; scars somewhat thickened and darkened, up to 2.5 µm wide. *Ramoconidia* 0–1-septate, subcylindrical to elongate-ellipsoid, medium brown, smooth, thick-walled, with 1–3 apical loci, 10–15 × 3–4 µm. *Secondary ramoconidia* 0–1-septate, narrowly ellipsoid, 7–10 × 3–3.5 µm. *Intercalary conidia* in chains of up to 15, aseptate, fusoid-ellipsoid, medium brown, smooth, (5–)6–7(–8) × 2–3(–2.5) µm. *Terminal conidia* aseptate, ellipsoid, pale to medium brown, with truncate base, 3–4 × 2–3 µm; hila slightly thickened and darkened, 0.5–1 µm wide.

Cultural characteristics — *Colonies* on OA, MEA and PDA spreading with moderate aerial mycelium and smooth, catenulate margins; centre olivaceous-grey, outer region and reverse iron-grey; reaching 30 mm diam after 1 mo.

*Specimens examined.* SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 6 Jan. 2008, P.W. Crous, M.K. Crous, M. Crous & K. Raath, holotype CBS H-20163, culture ex-type CPC 14886 = CBS 123540, CPC 14887–14888; 22 May 2008, A.R. Wood, culture CPC 15413 = CBS 123545, CPC 15414–15415; CPC 15362 = CBS 123541, CPC 15363–15364; CPC 15281 = CBS 123544, CPC 15282–15283; KwaZulu-Natal, South Coast, Uvongo, Skyline Nature Reserve, arboretum, living leaves of *Encephalartos lebomboensis*, 29 May 2008, A.R. Wood, culture CPC 15464 = CBS 123546, CPC 15465–15466.

Notes — *Teratosphaeria encephalarti* appeared to be quite dominant on the dying leaves of *E. altensteinii* in the Western Cape Province and it is possible that this species plays a role in the recently observed leaf blight disease. Inoculation studies are required, however, to confirm its potential role in this disease. Phylogenetically *T. encephalarti* and *T. altensteinii* are distantly related (88 % based on ITS) to *T. associata*, which occurs on *Eucalyptus* and *Protea* spp. (Crous et al. 2007a, 2008). The ITS sequences of the ex-type strains of *T. altensteinii* and *T. encephalarti* have an identity of 91 % with each other (430 of 468 bases).

### Undetermined species

#### *Lophiostoma* sp.

Cultural characteristics — *Colonies* on MEA, PDA and OA spreading with moderate aerial mycelium, and smooth, catenulate margins; surface olivaceous-grey; reverse iron-grey; reaching 25 mm diam after 1 mo.

*Specimen examined.* SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 6 Jan. 2008, P.W. Crous, M.K. Crous, M. Crous & K. Raath, culture CPC 15000 = CBS 123543, CPC 15001–15002.

Notes — Isolate CBS 123543 is representative of a species of *Lophiostoma* (based on ITS DNA sequence similarity to *L. macrostomum* GenBank accession EU552140). It could not

be described, however, due to paucity of material. Ascospores remained hyaline upon germination on MEA, but distort prominently (up to 10 µm wide), becoming constricted, with germ tubes growing down into the agar.

#### *Ochroconis* sp. — Fig. 6

On OA. *Colonies* moderately fast-growing, flat with predominantly submerged mycelium. *Mycelium* consisting of branched, septate, hyaline to pale brown, smooth, 2–2.5 µm wide hyphae. *Conidiophores* erect, arising from creeping hyphae, unbranched, 1–6-septate, straight to flexuous, brown, thick-walled, 10–50 × 2.5–3.5 µm. *Conidiogenous cells* terminal, integrated, 10–35 µm long, polyblastic, cylindrical, straight to flexuous, pale to medium brown, with scattered pimple-shaped, subhyaline denticles, 0.5 µm wide and long. *Conidia* (5–)7–9(–10) × (2.5–)3(–3.5) µm, solitary, subhyaline, smooth to verruculose, 1-septate, thin-walled, obovoid to fusiform, apex subobtuse, base narrowly truncate with minute marginal frill, 0.5 µm wide; conidial secession rhexolytic.

Cultural characteristics — *Colonies* on MEA, PDA and OA spreading, flat, with even, smooth margins, and sparse aerial mycelium; surface olivaceous-grey, reverse iron-grey; colonies reaching 25 mm diam after 1 mo.

*Specimen examined.* SOUTH AFRICA, KwaZulu-Natal, South Coast, Uvongo, Skyline Nature Reserve, arboretum, living leaves of *Encephalartos lebomboensis*, 29 May 2008, A.R. Wood, culture CPC 15461 = CBS 123536, CPC 15462–15463.

Notes — Species of *Ochroconis* are known to infect cold blooded vertebrates, or to occur as saprobes on different plant substrates and in soil (de Hoog et al. 2000), suggesting that the species from *Encephalartos* is probably saprobic. Phylogenetically the present collection clusters with a strain identified as *Ochroconis humicola* (CBS 780.83), though conidia of the ex-type strain of *O. humicola* (CBS 116655) are larger and it clusters distant from these strains. Preliminary DNA sequence data suggest that many species of *Ochroconis* in fact represent species complexes, and hence it would be best to treat the *Encephalartos* collection as part of a generic revision (de Hoog et al. in prep).

#### *Teratosphaeria* sp.

Cultural characteristics — *Colonies* on MEA, PDA and OA erumpent, fluffy, with abundant aerial mycelium and even, catenulate margins; surface olivaceous-grey with patches of iron-grey and pale olivaceous-grey; reverse iron-grey; reaching 30 mm diam after 1 mo.

*Specimen examined.* SOUTH AFRICA, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 6 Jan. 2008, P.W. Crous, M.K. Crous, M. Crous & K. Raath, culture CPC 14997–14999.

Notes — Isolate CPC 14997 could not be described due to paucity of material. Based on the DNA similarity to an ITS sequence of *Batcheloromyces leucadendri* (accession EU552103; 739 of 801 bases identity) deposited in GenBank, however, it appears to represent a species of *Teratosphaeria*. Ascospores germinated from both polar ends with germ tubes growing parallel to the long axis of the spore. Germinating spores became



**Fig. 6** *Ochroconis* sp. in vitro (CBS 123536). a, b. Conidial fascicles on MEA with light from above and below, respectively; c–j. conidiophores giving rise to conidia, with visible denticles (arrows); k. conidia. — Scale bars = 10 µm.

prominently constricted and distorted, up to 7 µm wide, pale brown, and somewhat verruculose.

## DISCUSSION

Prior to the present study only four fungal species had been described from *Encephalartos*, namely *Leptothyrium evansii*, *Pestalotia encephalartos*, *Phoma encephalarti* and *Phyllosticta encephalarti* (<http://nt.ars-grin.gov/fungaldatabases/>). A very preliminary examination of four collections during the present study has added a further four species in genera such as *Phaeo-*moniella**, *Saccharata* and *Teratosphaeria*. Furthermore, due to paucity of fungal material, several other species remain to be described in future studies. At present none of these fungi are confirmed as being pathogenic, and further work is required to determine which species are pathogens of *Encephalartos* and what impact they have on the population dynamics of these

plant species. Considering that many of these cycad species are endangered this could have important consequences for their conservation.

What is interesting to note, however, is that some species known from indigenous Proteaceae were also observed for the first time on *Encephalartos*. *Dactylaria leptosphaeriicola* (Fig. 7) was initially described as a hyperparasite of ascomata of *Leptosphaeria protearum* on leaves of *Protea repens*. It is interesting that this fungus was found occurring on ascomata of *Teratosphaeria encephalarti* on *Encephalartos altensteinii* in the present study. As found by Braun & Crous (1992), conidia of this species failed to germinate on MEA or PDA, stressing its close hyperparasitic relationship with its ascomycetous host. It is possible, however, that *D. leptosphaeriicola* is not a true member of *Dactylaria*, but represents yet another undescribed genus resembling *Dactylaria* in morphology. To confirm this, however, DNA will have to be isolated from fresh collections,



Fig. 7 *Dactylaria leptosphaericola* in vivo. a. Conidial fascicles on leaf; b. conidiogenous cells giving rise to conidia; c–e. conidia. — Scale bars = 10  $\mu$ m.

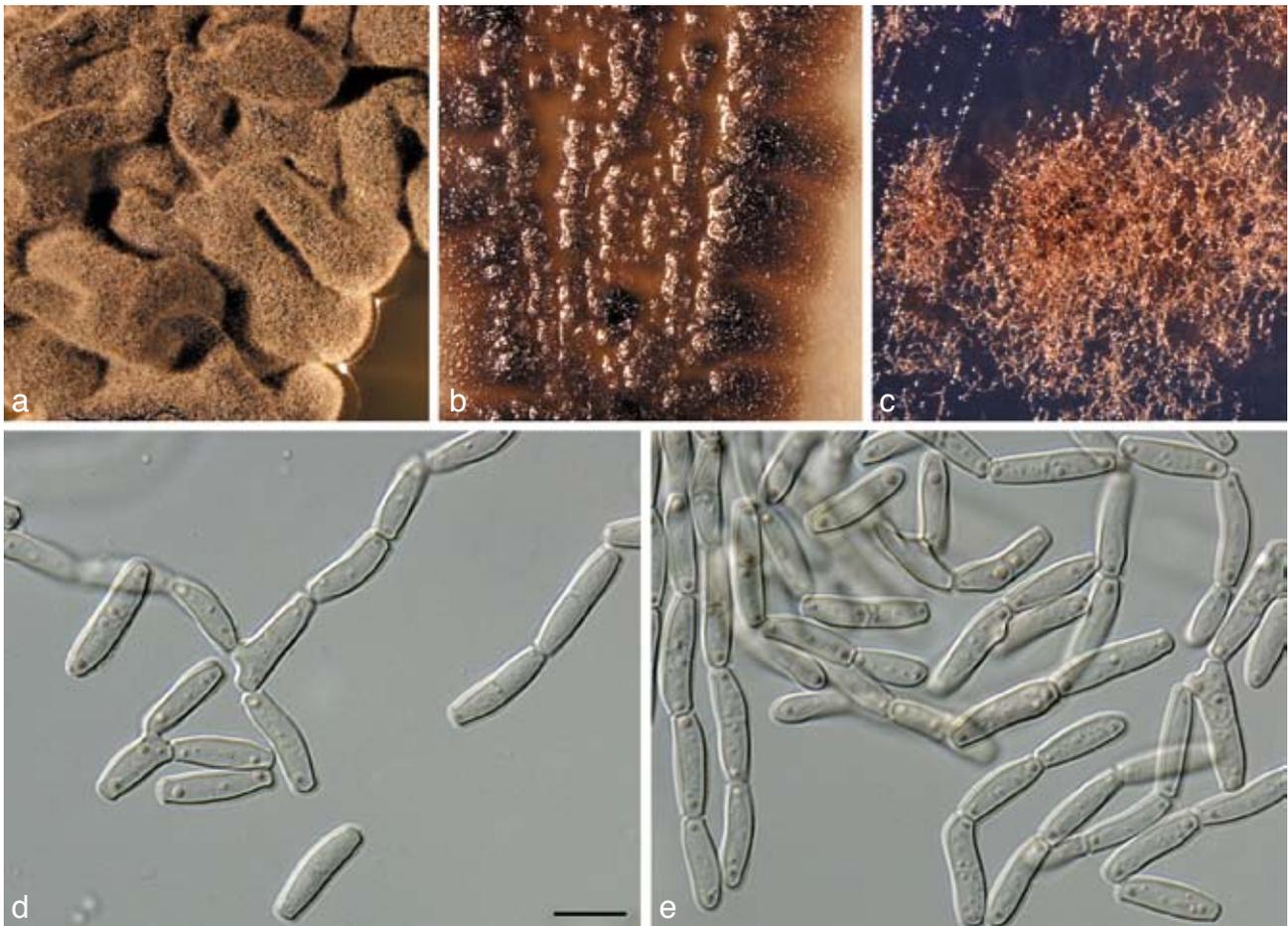


Fig. 8 *Cladophialophora proteae* in vitro (CPC 14902). a–c. Colony on MEA, OA and PDA, respectively; d, e. conidial chains. — Scale bar = 10  $\mu$ m.

which would be difficult, as fascicles occur in conjunction with ascomata of other fungi, and attempts to cultivate the fungus have thus far proven to be unsuccessful.

*Cladophialophora proteae* was initially isolated from lesions of *Batcheloromyces proteae* on *Protea cynaroides*, to which it was assumed to be pathogenic, though no inoculation tests have ever been conducted to confirm this hypothesis (Swart et al. 1998). The status of *Cladophialophora* and *Pseudocladosporium* has been an issue of debate, and as *Cladophialophora* was used for taxa pathogenic to humans, Crous et al. (2004a) allocated the species isolated from *Protea* to *Pseudocladosporium*. However, as shown in a subsequent molecular study (Crous et al. 2007b), *Pseudocladosporium* is a synonym of *Fusicladium* (Venturiaceae) while species of *Cladophialophora* (Herpotrichiellaceae) were shown to occur on humans and plant hosts, and thus the name *Cladophialophora proteae* can be used for this fungus (Fig. 8). The fact that this species could also occur on dead leaf tissue of *Encephalartos altensteinii* (CPC 14902–14904) in the Western Cape Province is surprising, however, and again questions its possible ecological role and its potential wider host range.

The link of '*Trimmatostroma*' to '*Mycosphaerella*' was first reported on leaf spots of *Teratosphaeria maculiformis* from *Protea cynaroides* leaves collected in South Africa by Taylor & Crous (2000). After initial data suggesting that *Teratosphaeria* and *Mycosphaerella* represented a single genus (Taylor et al. 2003), a subsequent study demonstrated that these were in fact from two different families and that species of *Teratosphaeria* belonged to the Teratosphaeriaceae, in which the anamorph genus *Catenulostroma* was established for these trimmatostroma-like anamorphs (Crous et al. 2007a). Within *Catenulostroma* there is a species complex surrounding *C. abietis*, which based on DNA sequence data solely of the ITS gene region, is very difficult to distinguish. It is quite possible, therefore, that the *Encephalartos* isolates (CPC 14996), although phylogenetically similar to *Catenulostroma microsporum* (*Teratosphaeria microspora*), may very well still be shown to represent yet another cryptic species in this complex.

Africa is well known to have a high level of botanical diversity. As shown here after an initial cursory look at a few *Encephalartos* leaves, these plants were found to host numerous undescribed species of fungi. Given the high level of endemism found in African flora, it can be expected that an equally high number of these fungal species will be unique species. Unfortunately, indigenous African fungal biodiversity has never been regarded as a research priority and as such this research topic has never been well supported financially. Given the current importance placed on ecotourism and the preservation of unique African flora and fauna, it is clearly timely that more research focus and financial resources be channelled towards documenting, studying ecological roles and impacts, and conserving African mycoflora.

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# Characterization and Distribution of Mating Type Genes in the Dothistroma Needle Blight Pathogens

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## ABSTRACT

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*Dothistroma septosporum* and *D. pini* are the two causal agents of Dothistroma needle blight of *Pinus* spp. in natural forests and plantations. Degenerate primers amplified portions of mating type genes (*MAT1-1-1* and *MAT1-2*) and chromosome walking was applied to obtain the full-length genes in both species. The mating-type-specific primers designed in this study could distinguish between the morphologically similar *D. pini* and *D. septosporum* and between the different mating types of these species. Screening of isolates from global collections of *D. septosporum* showed that only MAT2 isolates are present in Australian and New Zea-

land collections, where only the asexual form of the fungus has been found. In contrast, both mating types of *D. septosporum* were present in collections from Canada and Europe, where the sexual state is known. Intriguingly, collections from South Africa and the United Kingdom, where the sexual state of the fungus is unknown, included both mating types. In *D. pini*, for which no teleomorph is known, both mating types were present in collections from the United States. These results provided new insights into the biology and global distribution of two of the world's most important pine pathogens and should facilitate management of the diseases caused by these fungi.

*Additional keywords:* ascomycetes, heterothallic, *Mycosphaerella*, sexual reproduction.

Dothistroma needle blight, also known as red band needle blight, is one of the most important diseases of *Pinus* spp., both in natural forest ecosystems and particularly in plantations of non-native pines (9,19,20,27). The disease owes its international notoriety to the fact that it has been one of the most important constraints to the development of plantation forestry in many countries of Africa as well as in New Zealand, Australia, Chile, and other South American countries (19,20,27). The disease is particularly severe on *Pinus radiata* D. Don. This species is highly desirable for its rapid growth and exceptional timber and, consequently, it was one of the first nonnative tree species established in intensively managed plantations in the tropics and Southern Hemisphere. Outbreaks of Dothistroma needle blight on *P. radiata* led to devastating losses and resulted in the abandonment of *P. radiata* from plantation forestry in many countries (11,31,51).

The main causal agent of Dothistroma needle blight has been a matter of considerable taxonomic confusion. Thus, in different parts of the world, the disease has been attributed to either a single pathogen, different species of a pathogen, or varieties of a species. This also has differed depending on whether the pathogen was considered introduced or native in areas where the disease has been studied. In a recent study based on DNA sequence comparisons, two distinct phylogenetic lineages for *Dothistroma* isolates were identified (2). These clearly separated *Dothistroma*

*septosporum*, which has a worldwide distribution, and *D. pini*, until recently found only in the north-central United States. This study also showed that the disease which devastated plantations of *P. radiata* in the Southern Hemisphere is caused by *D. septosporum*. Recently, *D. pini* has been found infecting *P. palassiana* D. Don. in the Ukraine (I. Barnes, *unpublished data*) and it clearly has a distribution much wider than was believed at the time of the study of Barnes et al. (2).

Dothistroma needle blight, now known to have been caused by *D. septosporum*, resulted in huge damage to *P. radiata* plantations in the Southern Hemisphere in the 1950s and 1960s (9,19,20,27). Consequently, considerable research was conducted on the disease and great efforts were made to minimize its impact (8,19,20,41,46). These included selection of alternative species, tree breeding, agricultural practices, and the first examples of aerial applications of chemical fungicides in forest plantations (19). Although the disease has continued to be important, it generally is considered to be under reasonable control. There has, however, been a recent resurgence of the disease in various Northern Hemisphere countries and this has raised concern that a new wave of losses might occur elsewhere in the world (5,53).

Almost nothing is known regarding the genetic diversity among isolates of *D. septosporum* and *D. pini*. *D. septosporum* first was identified in New Zealand in 1964 (21). A study by Hirst et al. (26) applied random amplified polymorphic DNA (RAPD) markers to a population of *D. septosporum* (previously described as *D. pini*) from New Zealand and the results showed no genetic variation. These results support the hypothesis that it is an introduced pathogen that has been spreading asexually ever since its introduction into that country.

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The sexual state of *D. septosporum* is a species of *Mycosphaerella* known as *Mycosphaerella pini* Rostr. (17). In most countries of the Southern Hemisphere where *D. septosporum* has long been an important forest pathogen, only the anamorph has been reported (2,5,14; M. J. Wingfield, unpublished data). In contrast, no sexual state has ever been reported for *D. pini*. The absence or rarity of a sexual state for either of these fungi could be the result of selection pressure and a reduced need for sexual reproduction (14). Likewise, lower frequency and limited distribution of the teleomorph compared with the anamorph suggests that the primary method of dispersal of the fungus could be an asexual cycle. Here, conidia rather than ascospores would represent the inoculum of primary epidemiological importance (10,28).

Mating type genes play an important part in the biology and evolution of fungal species. Thus, knowledge of these genes can provide insight into the potential prevalence of sexual reproduction in different species. Some heterothallic Pyrenomycetes and Discomycetes can contain up to four genes at the mating type 1 idiomorph (*MATI-1*) of the *MAT* locus (40,43,44,55). These include the *MATI-1-1* encoding an  $\alpha$  domain protein, the *MATI-1-2* encoding an amphipathic  $\alpha$  helix protein, the *MATI-1-3* gene encoding a high mobility group (HMG) domain protein, and the *MATI-1-4* gene encoding a metallothionein protein. Only one gene has been characterized for the mating type 2 idiomorph (*MATI-2*) and it encodes a regulatory protein with an HMG domain. The DNA sequences of the idiomorphs, located at the *MAT* locus of individuals of two different mating types, are unrelated and, therefore, cannot be called alleles; however, these sequences are flanked by conserved regions (32). The formal nomenclature that is proposed for mating type genes of heterothallic ascomycetes is used here for the *MATI-1-1* and, because only a single *MATI-2* gene has been identified for filamentous ascomycetes, this gene is referred to as *MATI-2* (49).

DNA and amino acid sequences of the *MATI-1-1* and *MATI-2* genes in fungi show no obvious similarities, although the mating type locus has common flanking regions (48). Except for the HMG and  $\alpha$  domains, the similarity of homologous mating type genes usually is very low between different species (47). The direct target genes of the mating type proteins have not yet been described, although there is evidence for the control of some genes, such as pheromone genes (4). Mating type genes have been described from various sexual and presumably asexual fungi that are close relatives of the genus *Dothistroma* (Mycosphaerellaceae). Detailed analyses have been done on the distribution of the mating types of the sexually reproducing *M. graminicola* (50,56) and the presumably asexual species *Septoria passerinii* (23), *Cercospora beticola*, *C. zeae-maydis*, and *C. zeina* (25). Equal distribution of the mating types was found in most of the populations from these five species sampled from different geographical scales, indicating that sexual stages probably exist for the latter four apparently asexual species.

*D. septosporum* first was described from Idaho (United States) but now is seen in many parts of the world (2). In most of the areas where this species has been introduced and causes serious disease, only the asexual state of the fungus is ever seen. This raises the interesting question as to whether this could be attributed to the introduction of only one mating type into these new environments. Thus, the aims of this study were to characterize the mating type gene or genes of the causal agents of *Dothistroma* needle blight and to ascertain which mating types are present in the different countries where diseases caused by these fungi occur. To achieve this objective, the full-length *MATI-1-1* and *MATI-2* genes of *D. septosporum* and *D. pini* were isolated and sequenced using polymerase chain reaction (PCR)-based techniques. This made it possible to develop a multiplex PCR method for the rapid screening of *MATI-1-1* and *MATI-2* in isolates of the pathogens. A global collection of isolates subsequently was screened to determine which mating types are present in these collections.

**Fungal isolates.** In all, 230 *Dothistroma* isolates obtained from various locations in 15 countries were chosen to represent a global distribution of *Dothistroma* spp. (Table 1). Countries for which more than one isolate was screened included Austria ( $n = 10$ ), Canada ( $n = 106$ ), Chile ( $n = 10$ ), New Zealand ( $n = 38$ ), Poland ( $n = 11$ ), South Africa ( $n = 11$ ), Ukraine ( $n = 4$ ), the United Kingdom ( $n = 10$ ), and the United States ( $n = 17$ ). Isolates were obtained from different culture collections and standard protocols were used to isolate the genomic DNA.

The initial screening of the mating type genes was undertaken for *D. septosporum* using two isolates. These included CBS 116489 obtained from *P. radiata* in Tzaneen, South Africa and American Type Culture Collection (ATCC) MYA-605 obtained from *P. radiata* in Rotorua, New Zealand. For *D. pini*, four isolates were used: CBS 116485, obtained from *P. nigra* in Crystal Township, MI; CBS 116487, obtained from *P. nigra* in Evergreen Township, MI; CBS 116483, obtained from River Township, MI; and CBS 117609, obtained from *P. palassiana* in Tsyurupinsk, Ukraine. The identities of the six isolates used for the screening of the mating types previously had been confirmed using comparisons of DNA sequence data for the internal transcribed spacer (ITS) regions of the ribosomal DNA (2; J. Z. Groenewald, unpublished data).

**Isolation and characterization of *MATI-1-1* of *Dothistroma* spp.** The *MATI-1-1*-specific degenerate primers (MgMfSpMat1-1f1 and MgMfSpMat1-1r2) (Table 2), designed by Groenewald et al. (25), were used to screen and amplify a partial region of the *MATI-1-1* genes of the *Dothistroma* isolates.

The PCR mixtures and amplification reactions were the same as described by Groenewald et al. (25) for the amplification of the partial *MATI-1-1* in *Cercospora* spp. The PCR products obtained were separated by electrophoresis at 80 V for 1 h on a 1% (wt/vol) agarose gel containing ethidium bromide at 0.1  $\mu$ g/ml in 1 $\times$  Tris-acetate-EDTA buffer (0.4 M Tris, 0.05 M sodium acetate, and 0.01 M EDTA, pH 7.85) and visualized under UV light. Amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, Netherlands) following the manufacturer's recommendations. The products were analyzed on an ABI Prism 3730 DNA Sequencer (Applied Biosystems, Foster City, CA). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNA-STAR, Madison, WI).

Internal primers were designed in the partially sequenced *MATI-1-1* genes for each of the species (CBS 116489 for *D. septosporum* and CBS 116487 for *D. pini*). In order to obtain the full-length genes, these internal primers were used together with the appropriate primers from the DNA walking speedup kit (Seegene Inc., Rockville, MD) to determine additional sequences upstream and downstream of the partial *MATI-1-1* sequences. The Blastx algorithm (1) was used to compare the sequences obtained from the two *Dothistroma* spp. with protein sequences of other fungi present in the National Center for Biotechnology Information (NCBI) nonredundant protein database. The geneid web server (v1.2; Research Unit on Biomedical Informatics of IMIM, Barcelona, Spain) was used to predict the gene and intron or exon boundaries using the genetic code of *Neurospora crassa*. The conversion of DNA sequences to putative amino acid sequences was done using the translation tool of the proteomics server ExpASY (18). The percentage of identities between the predicted *MATI-1-1* gene sequences for the *Dothistroma* spp. was calculated using the alignment tool of ALIGN (37).

**Isolation and characterization of *MATI-2* of *Dothistroma* spp.** The *MATI-2*-specific degenerate primers (MgMfSpMat1-2f2 and MgMfSpMat1-2fr1) (Table 2), designed by Groenewald et al. (25), were used to screen isolates of *D. septosporum* and *D. pini*

TABLE 1. Origins of the *Dothistroma septosporum* and *D. pini* strains used during this study and the distribution of their mating types

Country, area, site	Collector	Species	Number of strains	MAT1-1-1	MAT1-2
Australia					
A.C.T. Canberra	K. Old	<i>D. septosporum</i>	10	0	10
Austria					
Thenneberg	T. Kirisits	<i>D. septosporum</i>	10	6	4
Brazil					
São Paulo	T. Namekata	<i>D. septosporum</i>	1	0	1
Canada					
Northwest British Columbia (BC)					
Brown Bear Road	K. Lewis & A. Dale	<i>D. septosporum</i>	10	5	5
Bell Irving River	K. Lewis & A. Dale	<i>D. septosporum</i>	1	0	1
Bulkley Canyon	K. Lewis & A. Dale	<i>D. septosporum</i>	9	5	4
Evelyn Pasture	K. Lewis & A. Dale	<i>D. septosporum</i>	1	0	1
Jonas Creek	K. Lewis & A. Dale	<i>D. septosporum</i>	2	0	2
Kinskutch Road	K. Lewis & A. Dale	<i>D. septosporum</i>	8	7	1
Kuldo Creek	K. Lewis & A. Dale	<i>D. septosporum</i>	7	2	5
Kisgegas Canyon	K. Lewis & A. Dale	<i>D. septosporum</i>	5	2	3
Squingula River Mine	K. Lewis & A. Dale	<i>D. septosporum</i>	8	1	7
Mosque River	K. Lewis & A. Dale	<i>D. septosporum</i>	6	1	5
Mitten Road	K. Lewis & A. Dale	<i>D. septosporum</i>	7	4	3
Nangeese Road	K. Lewis & A. Dale	<i>D. septosporum</i>	8	4	4
North Kuldo Road	K. Lewis & A. Dale	<i>D. septosporum</i>	4	1	3
Sanyam River	K. Lewis & A. Dale	<i>D. septosporum</i>	1	0	1
Nash Y	K. Lewis & A. Dale	<i>D. septosporum</i>	9	7	2
Orendo	K. Lewis & A. Dale	<i>D. septosporum</i>	7	6	1
Motaze Lake & Squingula River	K. Lewis & A. Dale	<i>D. septosporum</i>	8	6	2
Sunday Lake	K. Lewis & A. Dale	<i>D. septosporum</i>	4	1	3
Goldstream River, BC	D. Morrison	<i>D. septosporum</i>	1	0	1
Chile					
Valdivia	M. J. Wingfield	<i>D. septosporum</i>	10	0	10
France					
Meurthe-et-Moselle	M. Morelet	<i>D. septosporum</i>	1	0	1
Germany					
Bavarian Alps	L. Pehl	<i>D. septosporum</i>	1	0	1
Guatemala					
Sierra de Chuacús	Unknown	<i>D. septosporum</i>	1	0	1
New Zealand					
Bay of Plenty	M. A. Dick	<i>D. septosporum</i>	1	0	1
Golden Downs sites 1/2/3	P. Hirst	<i>D. septosporum</i>	4	0	4
Kaingora Forest	M. J. Wingfield	<i>D. septosporum</i>	10	0	10
Kaingora sites 1/2/3	P. Hirst	<i>D. septosporum</i>	11	0	11
Kinleith	P. Hirst	<i>D. septosporum</i>	5	0	5
Mt. Maunganui	K. Dobbie	<i>D. septosporum</i>	1	0	1
Rotorua	M. E. Buchanan	<i>D. septosporum</i>	2	0	2
Tongariro	J. W. Gilmour	<i>D. septosporum</i>	1	0	1
West Coast South Island	B. Doherty	<i>D. septosporum</i>	1	0	1
Poland					
Miechow Forest, Cracow	T. Kowalski	<i>D. septosporum</i>	11	3	8
Slovakia	E. Foffova	<i>D. septosporum</i>	1	1	0
South Africa					
Hogsback	J. Roux	<i>D. septosporum</i>	10	3	7
Tzaneen	I. Barnes	<i>D. septosporum</i>	1	1	0
Ukraine					
Tsyurupinsk	A. C. Usichenko	<i>D. pini</i>	4	4	0
United Kingdom					
West Midlands	A. Coggin	<i>D. septosporum</i>	1	0	1
South East England	A. V. Brown	<i>D. septosporum</i>	1	0	1
Forest of Dean	R. Beasley	<i>D. septosporum</i>	1	1	0
New Forest	A. V. Brown	<i>D. septosporum</i>	7	1	6
United States					
Bandon, Oregon	S. Cooley	<i>D. septosporum</i>	1	0	1
Michigan					
Crystal Township	G. Adams	<i>D. pini</i>	10	4	6
Evergreen Township	G. Adams	<i>D. pini</i>	1	1	0
River Township	G. Adams	<i>D. pini</i>	1	0	1
Central Minnesota	T. Nicholls	<i>D. pini</i>	1	1	0
Lincoln, Nebraska	G. Peterson	<i>D. pini</i>	3	2	1
Total	...	...	230	80	150

to obtain a partial region of the *MAT1-2* genes. The same PCR conditions described above were used to amplify the partial *MAT1-2* regions. Twelve internal primers were designed in the partially sequenced *MAT1-2* sequences for both species (ATCC MYA-605 for *D. septosporum* and CBS 116485 for *D. pini*) and the chromosome walking method also was used to obtain the full-length *MAT1-2* genes. The same procedure and programs described for the characterization and analyses of the *MAT1-1-1* sequences were used to characterize and analyze the *Dothistroma MAT1-2* sequences.

**Development and screening of *D. pini* and *D. septosporum* mating-type-specific primers.** *Dothistroma MAT1-1-1*-specific primers (Table 2) were designed from the aligned *MAT1-1-1* sequences of *D. pini* and *D. septosporum* (GenBank accession nos. DQ915449 and DQ915450, respectively). The forward primers were designed to be specific for *D. septosporum* (DseptMat1f) or *D. pini* (DpiniMat1f2) and, therefore, are both species and mating type specific. The reverse primer (DotMat1r) was designed from homologous regions within the *MAT1-1-1* genes and, therefore, is only mating type specific.

*Dothistroma MAT1-2*-specific primers (Table 2) were designed from the aligned *MAT1-2* sequences of *D. pini* and *D. septosporum* (GenBank accession nos. DQ915451 and DQ915452, respectively). The two forward primers were designed in regions of the genes that were variable between the two species. DseptMat2f was designed to be specific for *D. septosporum* and DpiniMat2f for *D. pini*, and both, therefore, are species and mating type specific. The reverse primer (DotMat2r) was designed from homologous regions within both the *MAT1-2* genes and, thus, is only mating type specific.

Multiplex PCR was used to screen for the *MAT1-1-1* or the *MAT1-2* of *D. pini* and *D. septosporum* in two separate reactions. The reaction mixtures had a total volume of 12.5 µl and contained 0.7 µl of diluted genomic DNA, 1× PCR buffer (Bioline, Randolph, MA), 48 µM each of the dNTPs, 4 pmol of each primer, 1 mM MgCl<sub>2</sub>, and 0.7 units of *Taq* polymerase (Bioline, Randolph, MA). The amplification reactions were done on a GeneAmp PCR System 9600 (Applied Biosystems). The initial denaturation step was done at 94°C for 5 min, followed by 40 cycles of 94°C (20 s), 65°C (20 s), and 72°C (40 s). A final elongation step at 72°C (5 min) was included in the run. The resulting PCR products were visualized as described above.

**Phylogenetic analyses.** The nucleotide sequences of the α domain (*MAT1-1-1*) and HMG domain (*MAT1-2*) of *D. septosporum* and *D. pini* determined in this study and additional mating type sequences for other species representing different fungal orders downloaded from NCBI's GenBank database were used for phylogenetic analyses. These sequences were analyzed using the mating type gene sequences of *Magnaporthe grisea* (GenBank accession nos. AB080672 and AB080673, respectively) as the outgroup. All phylogenetic analyses were done using Phylogenetic Analysis Using Parsimony (PAUP) v4.0b 10 (Swofford, D. L. 2003. Sinauer Associates, Sunderland, MA). Maximum parsimony analyses were conducted as described by Groenewald et al.

(24). All sequences generated were deposited in GenBank, and the alignments and trees were deposited in TreeBASE (TreeBASE accession no. SN3047).

## RESULTS

**Isolation and characterization of *MAT1-1-1* in *Dothistroma* spp.** The degenerate primers MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 amplified a fragment of 914 bp for three of the six *Dothistroma* isolates tested (Fig. 1). The fragments obtained from strains CBS 116489, CBS 117609, and CBS 116487 were sequenced. The translated sequence of the fragment obtained from strain CBS 116489 (*D. septosporum*) showed 39 and 46% identity to a 229- and 63-amino-acid (aa) region of the *M. graminicola* MAT1 protein and 32% identity to a 213-aa region of the *S. passerinii* MAT1 protein using Blastx on the GenBank database. This confirmed that the 914-bp fragment is part of the *MAT1-1-1* gene of *D. septosporum*.

Sequences for the fragments obtained from the *D. pini* strains (CBS 117609 and CBS 116487) showed 100% identity to each other in this region. The translated sequences showed 39% identity to a 226-aa ( $E = 2 \times 10^{-30}$ ) and 37% identity to a 78-aa region ( $E = 2 \times 10^{-30}$ ) of the *M. graminicola* mating type 1-1 protein (GenBank accession no. AAL30838). It also showed 32% identity to a 218-aa region ( $E = 5 \times 10^{-18}$ ) of the *S. passerinii* MAT-1 protein (GenBank accession no. AAO49357). This confirmed that the 914-bp fragment is part of the *MAT1-1-1* gene of *D. pini*.

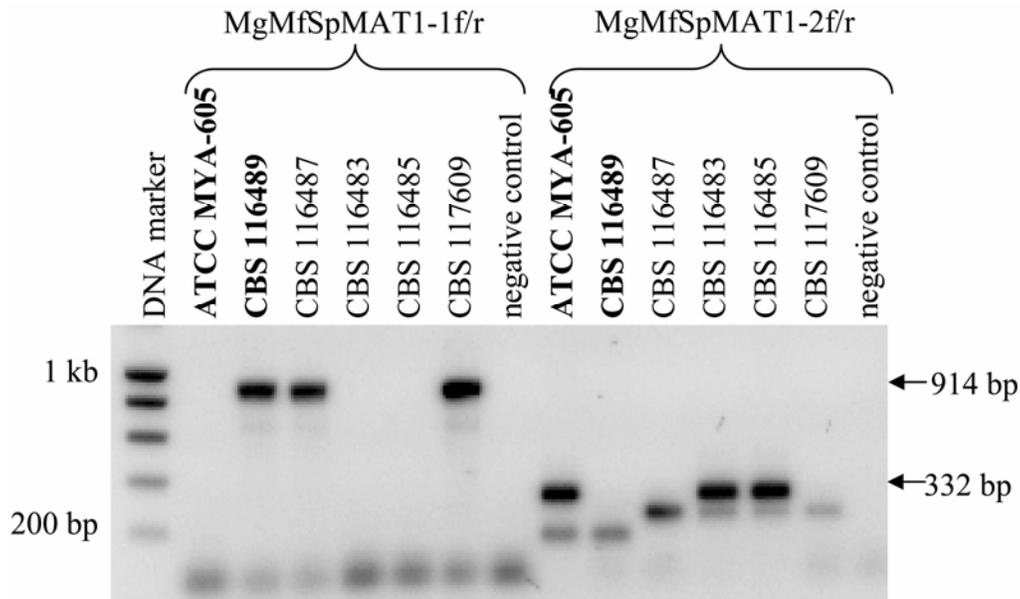
Four chromosome walking steps were used to obtain the full-length *MAT1-1-1* gene sequences for *D. septosporum* and *D. pini*. The geneid software predicted that the *MAT1-1-1* genes of both species contained four exons. The predicted length of the genes and the exon and intron positions are illustrated in Figure 2. Although the number of nucleotide and amino acid residues was the same for the *MAT1-1-1* of *D. septosporum* and *D. pini*, an identity of 94.1 and 94.3% was found between the 1,311-nucleotide and the 387-aa residues, respectively. All introns of the *MAT1-1-1* from both species contained a perfect lariat sequence (RCTRAC), except for the second intron of the *MAT1-1-1* of *D. septosporum*. When this intron is included in the coding region, an early stop codon is introduced in the reading frame, indicating that this is a true intron. The positions of the three predicted introns in the *Dothistroma* spp. studied correlate with those found for *Cercospora* spp. (25). The number of predicted introns (two) in the conserved α domain of the *Dothistroma* spp. correlated with the number predicted for the same region in *M. graminicola* (50) and *S. passerinii* (23).

**Isolation and characterization of *MAT1-2* of *Dothistroma* spp.** The degenerate primers MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1 amplified a fragment of 332 bp for the *Dothistroma* isolates that did not amplify the 914-bp fragment using the *MAT1-1-1* degenerate primers (Fig. 1). An extra 180-bp fragment also was obtained from the two *D. septosporum* strains and an extra 280-bp fragment from the four *D. pini* strains. The 332-bp fragment obtained from strain ATCC MYA-605 (*D. septo-*

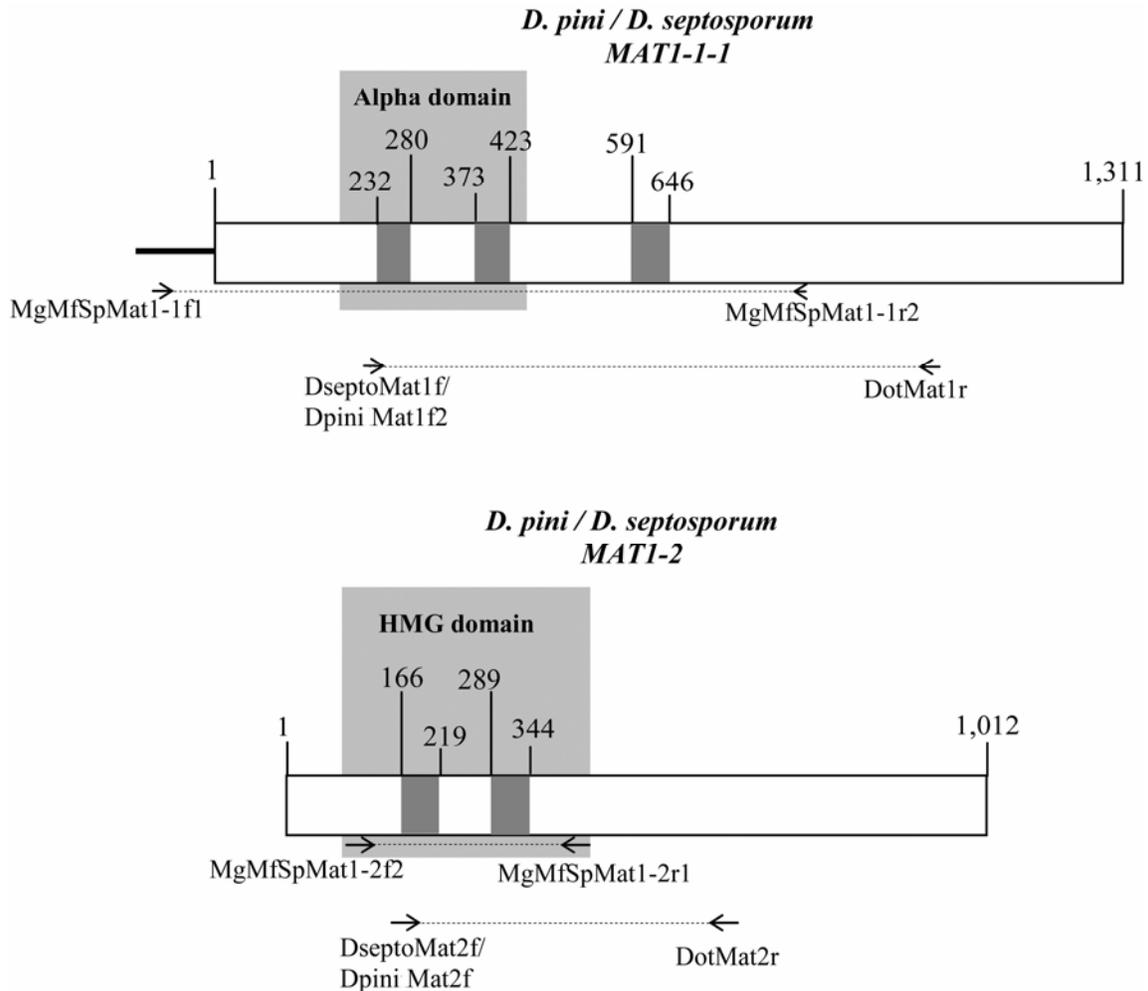
TABLE 2. Primers used during this study<sup>a</sup>

Primer	5'–3'	Description
MgMfSpMat1-1f1	CATNGCNCATCCCTTTG	<i>MAT1-1-1</i> -specific degenerate primer
MgMfSpMat1-1r2	GGCTTNGANACCATGGTGAG	<i>MAT1-1-1</i> -specific degenerate primer
MgMfSpMat1-2f2	CAAAGAANCNTTCNTGATCT	<i>MAT1-2</i> -specific degenerate primer
MgMfSpMat1-2r1	TTCTTCTCNGATGGCTTGC	<i>MAT1-2</i> -specific degenerate primer
DseptMat1f	<u>CGCAGTAAGTGA</u> <u>TGCCCTGAC</u>	<i>Dothistroma septosporum MAT1-1-1</i> -specific primer
DpiniMat1f2	<u>AGTAAGCGA</u> <u>CGCGCTCCCATG</u>	<i>D. pini MAT1-1-1</i> <i>MAT1</i> -specific primer
DotMat1r	<u>TTGCTGACCGGCTGCTGGTG</u>	<i>Dothistroma MAT1-1-1</i> -specific primer
DseptMat2f	<u>GTGAGTGA</u> <u>ACGCCGCACATGG</u>	<i>D. septosporum MAT1-2</i> -specific primer
DpiniMat2f	<u>GT</u> <u>AAGTGA</u> <u>TCTG</u> <u>TAACATGC</u>	<i>D. pini MAT1-2</i> -specific primer
DotMat2r	<u>CTGGTCGTGAAGTCCATCGTC</u>	<i>Dothistroma MAT1-2</i> -specific primer

<sup>a</sup> Nucleotides specific to the given *Dothistroma* sp. are underlined.



**Fig. 1.** Amplification products obtained from *Dothistroma septosporum* (in bold face) and *D. pini* isolates containing the partial *MAT1-1-1* (914-bp) and *MAT1-2* (332-bp) genes using the degenerate primer pairs MgMfSpMAT1-1 and MgMfSpMAT1-2, respectively.



**Fig. 2.** Diagrammatic representation of the full-length *MAT1-1-1* and *MAT1-2* genes of *Dothistroma septosporum* and *D. pini*. The predicted sites of exons (white bars), and introns (black bars) are shown, and their locations (nucleotide position) are indicated. The areas amplified by the MgMfSpMAT1-1 and MgMfSpMAT1-2 primer sets as well as the mating-type-specific primers for each species are indicated.

*sporum*) was sequenced, and the translated sequence showed 55% identity to a 65-aa ( $E = 1 \times 10^{-19}$ ) and 70% identity to a 27-aa region ( $E = 1 \times 10^{-19}$ ) of the *M. graminicola* mating type 1-2 protein (GenBank accession no. AAL30836) as well as 50% identity to a 65-aa region ( $E = 7 \times 10^{-17}$ ) of the *S. passerinii* MAT-2 protein (GenBank accession no. AAO49358) using Blastx on the GenBank database. This confirmed that the 332-bp fragment is part of the *MAT1-2* gene of *D. septosporum*. The 332-bp translated sequences for the fragments obtained from the two *D. pini* strains (CBS 116483 and CBS 116485) showed 52% identity to a 65-aa ( $E = 1 \times 10^{-19}$ ) and 68% identity to a 29-aa region ( $E = 1 \times 10^{-19}$ ) of the *M. graminicola* mating type 1-2 protein (GenBank accession no. AAL30836) as well as a 47% ( $E = 7 \times 10^{-17}$ ) and 68% identity ( $E = 7 \times 10^{-17}$ ) to the same amino acid regions of the *S. passerinii* MAT-2 protein (GenBank accession no. AAO49358). This confirmed that the 332-bp fragment is part of the *MAT1-2* gene of *D. pini*. Sequences for the 180-bp (*D. septosporum*) and 280-bp (*D. pini*) fragments showed no homology to protein sequences available in GenBank.

For both of the species, four chromosome walking steps were used to obtain the full-length *MAT1-2* gene sequences. The geneid software predicted that the *MAT1-2* sequences of both species contain three exons. The predicted length of the genes, as well as exon and intron positions, is illustrated in Figure 2. Although the number of nucleotide and amino acid residues was the same for the *MAT1-2* of the two *Dothistroma* spp., an identity of 94.4 and 92.7% was found between the 1,012-nucleotide and the 302-aa residues, respectively. All the introns found for both species contained a perfect lariat sequence. The number of predicted introns (two) of the *Dothistroma* spp. studied correlates with the number predicted for *Cercospora* spp. (25), but the specific locations of these introns within the gene differed. Only one predicted intron was found in the HMG domain of species of *Cercospora* (25), *M. graminicola* (51), and *S. passerinii* (23), whereas two predicted introns were found in the same region of the *Dothistroma* spp. studied.

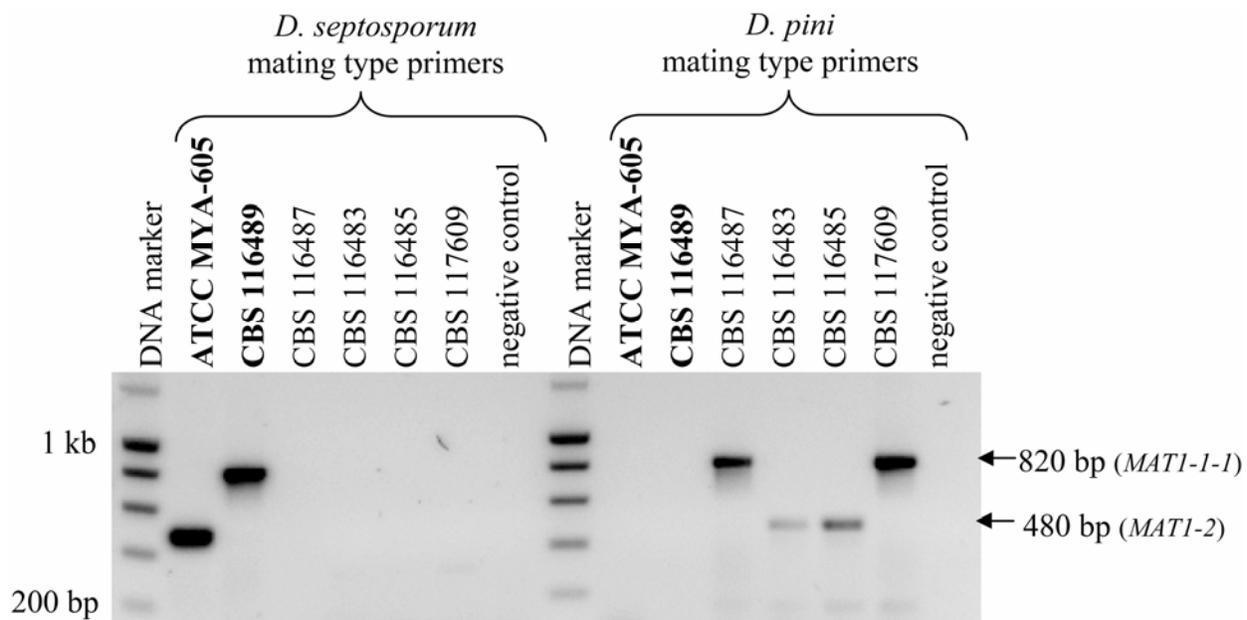
**Screening with *D. pini* and *D. septosporum* mating-type-specific primers.** In the *D. pini* MAT1 isolates, DpiniMat1f2 and DotMat1r amplified an 820-bp fragment and, in the *D. pini* MAT2 isolates, DpiniMat2f and DotMat2r amplified a 480-bp fragment (Fig. 3). Each isolate tested showed either the 820- or 480-bp fragment of the *MAT1-1-1* or *MAT1-2* genes, respectively. None

of the isolates contained both fragments. The *D. pini* mating-type-specific primers did not amplify the *MAT1-1-1* and *MAT1-2* fragments in any of the *D. septosporum* isolates (Fig. 3). The majority of the *D. pini* isolates were from areas in the United States where both mating types are known to exist. Eight isolates of each mating type were found for these *D. pini* isolates, whereas only MAT1 isolates were found for the *D. pini* collection from the Ukraine (Table 1). In the *D. septosporum* MAT1 isolates, DseptoMat1f2 and DotMat1r amplified an 820-bp fragment; in the *D. septosporum* MAT2 isolates, DseptoMat2f and DotMat2r amplified a 480-bp fragment (Fig. 3). Each isolate tested showed either the 820- or 480-bp fragment of the *MAT1-1-1* or *MAT1-2* genes, respectively. None of the isolates amplified both fragments.

The *D. septosporum* mating-type-specific primers did not amplify the *MAT1-1-1* and *MAT1-2* fragments of the *D. pini* isolates (Fig. 3). In all, 20 *D. pini* and 210 *D. septosporum* isolates (Table 1) were screened with the two mating-type-specific primer sets to determine the mating type and to confirm the identity of each isolate. All *D. septosporum* isolates obtained from Chile, Australia, and New Zealand contained only the *MAT1-2*. In contrast, isolates representing both mating types were present in the Austria, Canada, Poland, South Africa, and United Kingdom collections. Only one isolate was available each from Germany, Brazil, France, Guatemala, Slovakia, and the United States. All of these isolates contained the *MAT1-2* gene, except for the isolate from Slovakia that contained *MAT1-1-1*.

**Phylogenetic analyses.** The alignment of partial *MAT1-1-1* nucleotide sequences ( $\alpha$  domain) contained 21 strains, including *M. grisea* as the outgroup, and had a total length of 174 characters. Of the 174 characters, 23 were constant, 15 were variable and uninformative, and 136 were parsimony informative. The alignment of partial *MAT1-2* nucleotide sequences (HMG domain) contained 21 strains, including *M. grisea* as outgroup, and had a total length of 253 characters. Of the 249 characters, 37 were constant, 13 were variable and uninformative, and 199 were parsimony informative. Two equally parsimonious trees were obtained from each of the *MAT1-1* alignments (Fig. 4A; tree length of 638 steps; CI = 0.498, RI = 0.649, RC = 0.324) and from the *MAT1-2* alignment (Fig. 4B; tree length of 886 steps; CI = 0.512, RI = 0.659, RC = 0.338).

The topology of the phylogenetic trees using the  $\alpha$  domain (Fig. 4A) and HMG domain (Fig. 4B) sequences were similar.



**Fig. 3.** *Dothistroma septosporum* (bold face) and *D. pini* isolates screened using the Dsepto/Dpini/DotMat1 primer set (820-bp fragment) and the same *Dothistroma* isolates screened with the Dsepto/Dpini/DotMat2 primer set (480-bp fragment).

The Capnodiales, Hypocreales, and Pleosporales clades showed high bootstrap support (92 to 97%) in both trees. The phylogenetic analysis using the DNA sequences in the HMG-box and  $\alpha$  domain showed that *D. pini* and *D. septosporum*, respectively, are phylogenetically closely related to *Cercospora* spp., *M. graminicola*, and *S. passerinii* as illustrated by the 92% (*MAT1-1-1*) and 97% (*MAT1-2*) bootstrap support values.

## DISCUSSION

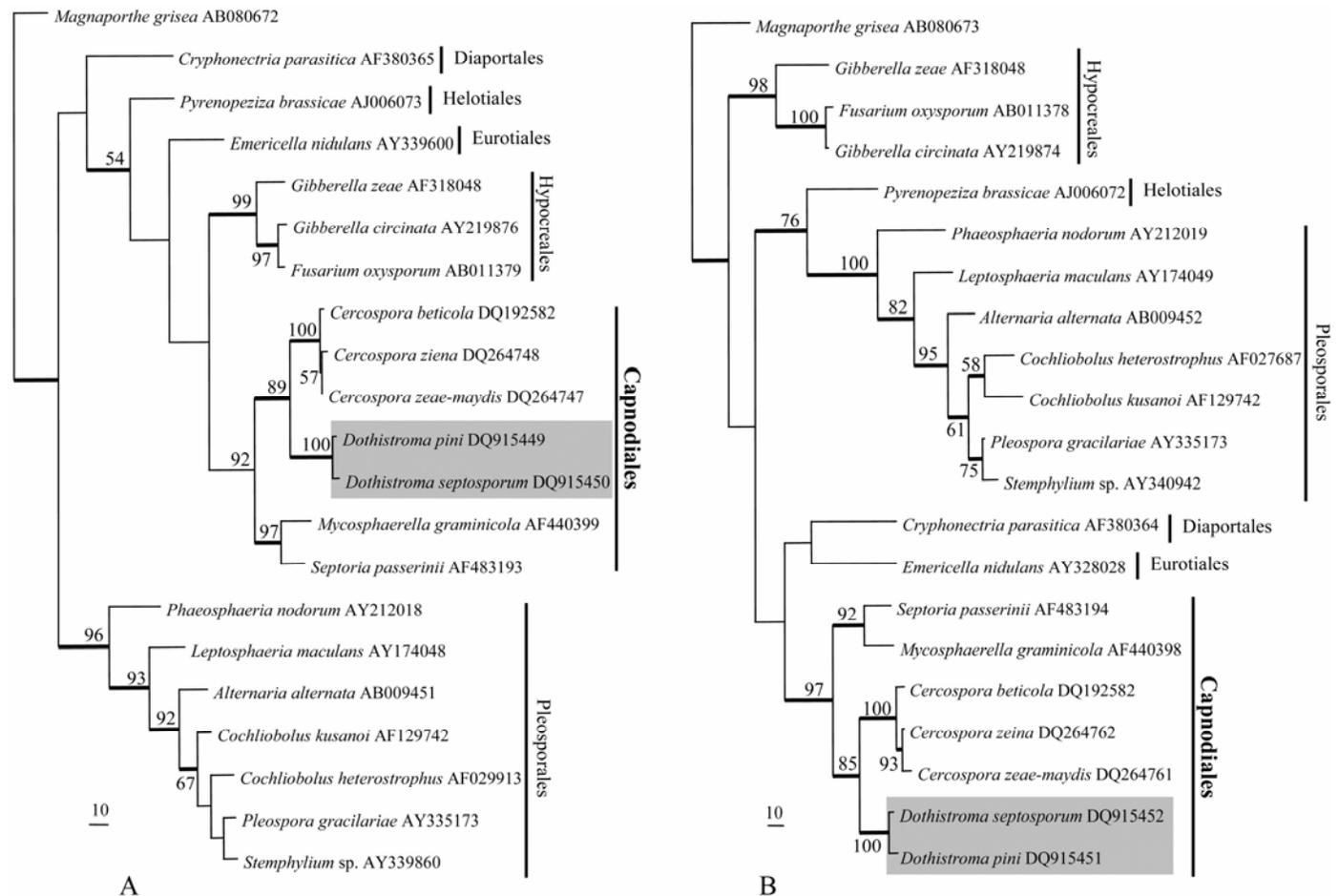
This study represents the first attempt to ascertain which mating types are present in the different countries where diseases caused by *D. septosporum* and *D. pini* occur. In this regard, emphasis is on *D. septosporum*, because it has been introduced into numerous countries, where it has caused very damaging disease problems. Thus, the degenerate primer sets MgMfSpMAT1-1 and MgMfSpMAT1-2 (25) were used successfully to amplify portions of the mating type genes of *D. septosporum* and *D. pini*. This made it possible to characterize the full-length *MAT1-1-1* or *MAT1-2* genes of both species.

The *MAT1-1-1* and *MAT1-2* genes characterized for *D. septosporum* and *D. pini* in this study contained areas that correspond to a putative  $\alpha$  domain and an HMG domain also described for the *MAT1-1-1* and *MAT1-2* of other ascomycetes. The two putative introns in the  $\alpha$  domains of the *Dothistroma MAT1-1-1* also have been found in corresponding areas in *M. graminicola* (50), *S. passerinii* (23), and several *Cercospora* spp. (25). However, the third predicted intron in the downstream area flanking the  $\alpha$  domain of the *MAT1-1-1* of both *Dothistroma* spp. is present only

in the *Cercospora* sp., and not in *M. graminicola* or *S. passerinii*. The number of introns found in the HMG domain of the *MAT1-2* in both *Dothistroma* spp. differed from that of closely related species. The first predicted intron also is present in *M. graminicola* (50), *S. passerinii* (23), and *Cercospora* spp. (25). In contrast, the second predicted intron is present only in the *MAT1-2* of the *Dothistroma* spp., and not in any other members of the Mycosphaerellaceae thus far studied. These data indicate that clear differences can be found even within the conserved regions of the corresponding genes in different *Mycosphaerella* spp.

The predicted length of the encoded proteins among different *MAT1-1-1* and *MAT1-2* genes of ascomycetes varies greatly (23,25,40). In most species, the MAT1 protein is much larger than the MAT2. Results of this study have shown that this also is the case for the *Dothistroma* spp., where 387 aa were found for MAT1 and 302 aa for MAT2. Expression studies have not been done on the mating type genes of any of the above-mentioned members of the Mycosphaerellaceae. Additional studies at the mRNA and protein levels would be necessary to confirm the exact length of the coding regions and the intron and exon boundaries for the mating type genes of the *Dothistroma* spp.

Results of this study showed substantial differences between the nucleotide as well as amino acid sequences of the corresponding mating type genes and proteins of *D. septosporum* and *D. pini*. Using nucleotide sequences for phylogenetic inference in these fungi is consistent with previous studies where conserved domains within the mating type genes have been used to study the phylogenetic relationships among different fungal species and families (12,25,34,35,52). Differences in mating type sequences



**Fig. 4.** One of two equally parsimonious trees obtained from each of the **A**, *MAT1-1-1* sequence alignment rooted to *Magnaporthe grisea* (AB080672) and **B**, *MAT1-2* sequence alignment rooted to *M. grisea* (AB080673). In both trees, bootstrap support values from 1,000 replicates are shown at the nodes, whereas thickened lines indicate strict consensus branches.

for *D. septosporum* and *D. pini* show that these species are distinct genetic entities and provides strong support for the results of Barnes et al. (2), who provided the first DNA-based evidence that the species are distinct.

Based on morphological characteristics, Barr (3) attempted to reclassify *Mycosphaerella pini* in a new genus outside of *Mycosphaerella*. However, molecular phylogenetic analyses have shown that *Mycosphaerella* is the most appropriate designation for this fungus classification (2,22). Phylogenetic analyses, based on the sequences of the HMG and  $\alpha$  domains, also confirm that *Dothistroma* spp. are members of the Mycosphaerellaceae. All remaining species also grouped within their corresponding families; however, the relationship between different families is unresolved.

The mating-type-specific primer sets developed in this study, DpiniMat1 and DpiniMat2 as well as DseptoMat1 and DseptoMat2, can be used effectively in multiplex PCR assays to amplify areas within the mating type genes for *D. pini* and *D. septosporum* populations, respectively. These primers also can be used to distinguish between the two *Dothistroma* spp., making them useful tools for rapid and accurate diagnoses of two important pathogens that are morphologically similar. Prior to this study, the only diagnostic tool available to distinguish between *D. pini* and *D. septosporum*, was to amplify the ITS of the ribosomal DNA region with universal primers and then to digest the amplicon with the restriction endonuclease *AluI* (2). Although the latter technique is useful, the ITS amplicon of *D. pini* is digested into two fragments whereas that of *D. septosporum* is not. Therefore, to prevent a false positive result for *D. septosporum*, a prior confirmation that the fungus is a *Dothistroma* sp. is required. The mating-type-specific primer sets emerging from this study are species specific and do not require a prior view on the identity of unknown isolates. They are, therefore, multifunctional and can be used for the rapid identification of the species as well as its mating type.

Although results of this study have shown that *D. pini* is probably heterothallic with a single isolate containing only one of the two mating type genes, no teleomorph has yet been linked to this species. Where both mating types were observed for the isolates from the United States, the sexual state most likely is present, but has not been observed. In contrast, the *M. pini* teleomorph of *D. septosporum* previously has been observed in some parts of the United States (9,38,39) where *D. pini* is predominantly found. Given that the anamorphs of these fungi are morphologically similar and have been confused in the past, it is possible that teleomorph structures reported for *D. septosporum* could have been linked to *D. pini* and not to *D. septosporum*.

Although a small number of isolates were screened for most countries, this study shows that *D. septosporum* probably are heterothallic and that one mating type (MAT2) seems to be more prevalent in several of the collections studied (e.g., New Zealand). Although sexual reproduction has been confirmed in *D. septosporum*, asexual reproduction happens more frequently, and the absence or rarity of the opposite mating type (MAT1) in most of the collections can explain the common occurrence of the asexual stage. Therefore, it also is possible that the teleomorph is not as rare as first believed. We found that both mating types exist within *D. septosporum* populations from Europe (Poland and Austria) and Canada, where the sexual stage (*M. pini*) has been reported in the past (7,15,17,28,29). However, the teleomorph has never been found in countries in the Southern Hemisphere such as Chile, Australia, and New Zealand, where these pathogens have long been a major problem (14,31). These are also the countries for which only one mating type (MAT2) has been observed, and this might explain the absence or rarity of the sexual stage.

Discovery in this study of only a single mating type of *D. septosporum* in New Zealand, Australian, and Chilean collections can be explained by the fact that the fungus is an introduced

pathogen in those countries. For New Zealand, Hirst et al. (26) also found that no genetic variation exist among isolates of a *D. septosporum* population, which is strongly supported by the results of the present study. Dothistroma needle blight was introduced in Australia in the 1970s and it was suggested that this occurred by natural means, with conidia being blown across the Tasman Sea from New Zealand. This view was supported by the fact that the strict quarantine regulations in Australia would have made it unlikely that infected plant material entered the country (13,31,33). The presence of only one mating type shown in this study and the fact that no genetic diversity has been found yet for the pathogen in New Zealand (26) supports the view that only one genotype was introduced into or became established in Australia and New Zealand. Asexual reproduction evidently has perpetuated the spread of the fungus subsequently. We suspect that the same situation will have been true for Chile.

An intriguing result of this study has been the discovery that both mating types of *D. septosporum* exist in the South African and United Kingdom collections. This is especially interesting because the pathogen is non-native in these countries and it might have been expected that the situation would have been similar to that in other countries such as New Zealand, where the pathogen also is an alien invasive. In addition, the teleomorph of *D. septosporum* has never been observed in South Africa (M. J. Wingfield, unpublished data) and the United Kingdom (A. V. Brown, unpublished data), despite concerted efforts to detect it.

It is important to recognize that the presence of both mating types of *D. septosporum* in these two countries could indicate the presence of clandestine sex in the fungus. This would indicate the potential for the pathogen to evolve more effectively in these countries than would be true elsewhere in the world, where only a single mating type exists. Such change in the fungus could complicate efforts to develop trees resistant to Dothistroma needle blight infection in South Africa and the United Kingdom. In this regard, it has been shown previously that the introduction of the second mating type of a pathogen can cause rapid increase in virulence, gene transfer, and genetic variation, such as in *Phytophthora infestans* (16,30,42,45) and *Ophiostoma novo-ulmi* (36). This implies that the accidental introduction of the opposite mating type of *D. septosporum* into countries such as New Zealand, Australia, and Chile could seriously exacerbate red band needle disease in those countries. Thus, every effort must be made to ensure that new mating types of *D. septosporum* do not enter these countries.

There has been a dramatic increase in the impact of Dothistroma needle blight caused by *D. septosporum* in western Canada, the United States, and the United Kingdom in recent years (5,6,53). Possible reasons for this change in the disease situation in these countries are an abundance of host material or a directional climate change, as suggested by Woods et al. (54). The discovery that both mating types exist in these countries is another factor that can contribute to the change in the disease situation. The presence of both mating types increases the possibility for sexual reproduction. This, in turn, can lead to the exchange of genetic material between different strains, resulting in a possible increase in the viability of this species. Therefore, further investigation is necessary to determine whether the presence of both mating types, which could increase genetic diversity, a dramatic climate change, or possibly a combination of both these factors might account for the drastic increase in the severity of this disease.

Because only one mating type of *D. septosporum* appears to be present in most countries of the Southern Hemisphere, it is important to restrict the MAT1 isolates to their present locations. This can be achieved through refining quarantine regulations based on the knowledge that only one mating type of the pathogen is present in the country. The mating-type-specific PCR developed during this study could be implemented easily as a control method to

test for the presence of the mating types for *Dothistroma* spp. in pine plantations. One of the weaknesses of quarantine regulations internationally is that they typically rely on lists of names of pathogens rather than on knowledge of their biology and population genetics. Results of this study have provided valuable new insights into the distribution of mating types of *D. septosporum* and *D. pini* that should enhance the quality of quarantine regulations in the future.

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## Host range of *Cercospora apii* and *C. beticola* and description of *C. apiicola*, a novel species from celery

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**Abstract:** The genus *Cercospora* is one of the largest and most heterogeneous genera of hyphomycetes. *Cercospora* species are distributed worldwide and cause *Cercospora* leaf spot on most of the major plant families. Numerous species described from diverse hosts and locations are morphologically indistinguishable from *C. apii* and subsequently are referred to as *C. apii sensu lato*. The importance and ecological role that different hosts play in taxon delimitation and recognition within this complex remains unclear. It has been shown that *Cercospora* leaf spot on celery and sugar beet are caused respectively by *C. apii* and *C. beticola*, both of which are part of the *C. apii* complex. During this study we characterized a new *Cercospora* species, *C. apiicola*, which was isolated from celery in Venezuela, Korea and Greece. The phylogenetic relationship between *C. apiicola* and other closely related *Cercospora* species was studied with five different gene areas. These analyses revealed that the *C. apiicola* isolates cluster together in a well defined clade. Both *C. apii* and *C. beticola sensu stricto* form well defined clades and are shown to have wider host ranges and to represent distinct species.

**Key words:** Ascomycetes, *Cercospora apii* complex,

*Cercospora* leaf spot, molecular phylogeny, species boundaries, taxonomy

### INTRODUCTION

The genus *Cercospora* Fresen. first was described in 1863 by Fresenius (Fuckel 1863) and currently is one of the largest and most heterogeneous genera of hyphomycetes (Crous and Braun 2003). Species belonging to this plant pathogenic genus are distributed worldwide and cause *Cercospora* leaf spot on most of the major plant families (Crous and Braun 2003). Since the description of the genus, the taxonomy of its species has become difficult because *Cercospora* for many years has been a dumping ground for all dematiaceous hyphomycetes with filiform conidia (Pons and Sutton 1988). Johnson and Valteau (1949) stated that most of the morphologically uniform *Cercospora* isolates belong to a single *Cercospora* species that occurs on a wide host range and morphologically is indistinguishable from *C. apii* Fresen. *Cercospora apii* is the oldest available name for this large complex of morphologically indistinguishable *Cercospora* taxa. This approach was questioned by Chupp (1954), who stated in his monograph that species of *Cercospora* are generally host specific. Chupp subsequently formulated the concept of “one host species, genus or family equals one *Cercospora* species”. Chupp’s concept led to the description of a large number of species based on host substrate, with more than 3000 names being listed by Pollack (1987). Crous and Braun (2003) revised these species and redispersed many of them. A total of 659 *Cercospora* species were recognized, with a further 281 being referred to synonymy under *C. apii s.l.* This decision was substantiated by the various inoculation experiments that have been conducted on the *C. apii* complex (Vestal 1933, Johnston and Valteau 1949, Fajola 1978) and that raised doubts whether host specificity existed within this complex.

To date only a few species belonging to *C. apii s.l.* have been cultured, and molecular data addressing host specificity within this complex is still lacking (Crous et al 2004). Three scenarios are possible when examining the host-species association of taxa belonging to the *C. apii* complex. The first scenario is that a single species of *Cercospora* occurs on a wide host range; the second is that several species exist with overlapping host ranges; the third is that some

*Cercospora* species are host specific whereas others are not.

The first evidence that distinct species exist within the *C. apii* morphotype recently was published by Groenewald et al (2005). The latter study focused on *Cercospora* species isolated from sugar beet (*Beta vulgaris*) and celery (*Apium graveolens*). Characteristics examined for these isolates included morphology, cultural characteristics and cardinal temperature requirements for growth. These data were supplemented with amplified fragment length polymorphism analyses and phylogenetic analyses with five different genes. Groenewald et al (2005) showed that three distinct *Cercospora* species exist on sugar beet and/or celery, namely *C. beticola* on sugar beet, *C. apii* on both celery and sugar beet and a third that was isolated from celery in Venezuela and Korea.

The ability to infect different hosts during artificial inoculation is of questionable value as a character in species delimitation. For instance, a recent study revealed that *C. beticola* could infect safflower during artificial inoculation experiments (Lartey et al 2005). However *C. beticola* has yet to be isolated from this host in the field. Only a few taxa that belong to the *C. apii* complex have been studied in the past in an attempt to elucidate the relationship between fungal species and host. The first objective of this study, therefore, was to name the new *Cercospora* species from celery. The second objective was to use DNA sequence data to examine the host range of this species, including *C. apii* s.s. and *C. beticola* s.s. as defined by Groenewald et al (2005).

#### MATERIALS AND METHODS

*Isolates.*—Those used in this study were obtained from the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands, as well as the working collection of Pedro Crous (CPC) that is housed at CBS (TABLE I). Single conidial isolates also were obtained from symptomatic material as explained in Crous (1998). Isolates were plated onto 2% malt-extract agar (MEA) and oatmeal agar (OA) (Gams et al 1998) and incubated at 24 C for 8 d.

*DNA isolation, amplification and sequencing.*—The Fast-DNA kit (BIO 101, Carlsbad, California) was used according to the manufacturer's instructions to isolate genomic DNA of 200–400 mg fungal mycelia grown on MEA plates. A sterile blade was used to scrape the mycelia from the surface of the plate. For the phylogenetic analyses, parts of these gene areas were used: the internal transcribed spacers and 5.8S rRNA gene (ITS), the actin gene (ACT), the translation elongation factor 1- $\alpha$  gene (EF), the calmodulin gene (CAL) and the histone H3 gene (HIS). PCR primers and amplification conditions followed the protocols

outlined by Groenewald et al (2005). PCR products were separated by electrophoresis at 80 V for 40 min on a 0.8% (w/v) agarose gel containing 0.1  $\mu$ g/mL ethidium bromide in 1 $\times$  TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and viewed under UV-light.

Amplicons were sequenced in both directions with the PCR primers and a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Roosendaal, the Netherlands) according to the manufacturer's recommendations. The products were analyzed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Foster City, California). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNASTar, Madison, Wisconsin).

*Data analysis.*—The consensus sequences were assembled and added to alignment (TreeBASE matrix number M2242) of Groenewald et al (2005) with Sequence Alignment Editor 2.0a11 (Rambaut 2002), and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were done in PAUP (phylogenetic analysis using parsimony) 4.0b10 (Swofford 2003) and consisted of neighbor joining analysis with the uncorrected "p", the Jukes-Cantor and the HKY85 substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analysis, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all datasets with the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees was evaluated by 1000 bootstrap replications (Hillis and Bull 1993). Other measures calculated included tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC). The resulting trees were printed with TreeView 1.6.6 (p 1996). A partition homogeneity test was done in PAUP to test whether the different loci can be used in a combined analysis (Farris et al 1994). Sequences were deposited in GenBank (accession numbers listed in TABLE I) and the alignment and trees in TreeBASE (accession number SN2512).

*Morphology.*—Fungal structures were mounted in lactic acid and examined under a light microscope (1000 $\times$ ). The extremes of spore measurements (30 observations) are given in parentheses. Colony colors were rated after 8 d on MEA and OA at 24 C in the dark with the color charts of Rayner (1970).

#### RESULTS

*Sequence data analyses.*—A partition homogeneity test showed that all five datasets were not combin-

TABLE I. *Cercospora* isolates included in the study

Strains and accession numbers ( <sup>a</sup> CBS; <sup>b</sup> CPC)	Host	Origin	Collector	GenBank no. (ITS, EF, ACT, CAL, HIS)
<b><i>C. apii</i> Fresen.</b>				
CBS 119.25; CPC 5086	<i>Apium graveolens</i>	—	L. J. Klotz	AY840512, AY840479, AY840443, AY840410, AY840377
CBS 121.31; CPC 5073	<i>Beta vulgaris</i>	Austria	—	AY840513, AY840480, AY840444, AY840411, AY840378
CBS 127.31; CPC 5119	<i>B. vulgaris</i>	Hungary	—	AY840514, AY840481, AY840445, AY840412, AY840379
CBS 152.52; CPC 5063	<i>B. vulgaris</i>	Netherlands	G. van den Ende	AY840515, AY840482, AY840446, AY840413, AY840380
CBS 252.67; CPC 5084	<i>Plantago lanceolata</i>	Rumania	O. Constantinescu	DQ233318, DQ233342, DQ233368, DQ233394, DQ233420
CBS 257.67; CPC 5057	<i>Helianthemum</i> sp.	Romania	O. Constantinescu	DQ233319, DQ233343, DQ233369, DQ233395, DQ233421
CBS 536.71; CPC 5087	<i>A. graveolens</i>	Romania	O. Constantinescu	AY752133, AY752166, AY752194, AY752225, AY752256
CBS 553.71; CPC 5083	<i>Plumbago europaea</i>	Romania	O. Constantinescu	DQ233320, DQ233344, DQ233370, DQ233396, DQ233422
CBS 110813; CPC 5110	<i>Moluccella laevis</i>	USA	S. T. Koike	AY156918, DQ233345, DQ233371, DQ233397, DQ233423
CBS 110816; CPC 5111	<i>M. laevis</i>	USA	S. T. Koike	AY156919, DQ233346, DQ233372, DQ233398, DQ233424
CBS 114416; CPC 10925	<i>Apium</i> sp.	Austria	—	AY840516, AY840483, AY840447, AY840414, AY840381
CBS 114418; CPC 10924	<i>A. graveolens</i>	Italy	Meutri	AY840517, AY840484, AY840448, AY840415, AY840382
CBS 114485; CPC 10923	<i>A. graveolens</i>	Italy	Meutri	AY840518, AY840485, AY840449, AY840416, AY840383
<sup>c</sup> CBS 116455; CPC 11556	<i>A. graveolens</i>	Germany	K. Schrameyer	AY840519, AY840486, AY840450, AY840417, AY840384
CBS 116504; CPC 11579	<i>A. graveolens</i>	Germany	K. Schrameyer	AY840520, AY840487, AY840451, AY840418, AY840385
CBS 116507; CPC 11582	<i>A. graveolens</i>	Germany	K. Schrameyer	AY840521, AY840488, AY840452, AY840419, AY840386
CPC 5112	<i>M. laevis</i>	New Zealand	C. F. Hill	DQ233321, DQ233347, DQ233373, DQ233399, DQ233425
<b><i>C. beticola</i> Sacc.</b>				
CBS 116.47; CPC 5074	<i>B. vulgaris</i>	Netherlands	G. E. Bunschoten	AY752135, AY752168, AY752196, AY752227, AY752258
CBS 117.47	<i>B. vulgaris</i>	Czechia	G. E. Bunschoten	DQ233322, DQ233348, DQ233374, DQ233400, DQ233426
CBS 122.31; CPC 5072	<i>B. vulgaris</i>	Germany	—	AY752136, AY752169, AY752197, AY752228, AY752259
CBS 123.31; CPC 5071	<i>B. vulgaris</i>	Spain	—	AY840522, AY840489, AY840453, AY840420, AY840387
CBS 124.31; CPC 5070	<i>B. vulgaris</i>	Romania	—	AY840523, AY840490, AY840454, AY840421, AY840388
CBS 125.31; CPC 5069	<i>B. vulgaris</i>	Japan	—	AY840524, AY840491, AY840455, AY840422, AY840389

TABLE I. Continued

Strains and accession numbers ( <sup>a</sup> CBS; <sup>b</sup> CPC)	Host	Origin	Collector	GenBank no. (ITS, EF, ACT, CAL, HIS)
CBS 126.31; CPC 5064	<i>B. vulgaris</i>	Germany	—	AY840525, AY840492, AY840456, AY840423, AY840390
CBS 539.71; CPC 5062	<i>B. vulgaris</i>	Romania	O. Constantinescu	DQ233323, DQ233349, DQ233375, DQ233401, DQ233427
CBS 548.71; CPC 5065	<i>Malva pusilla</i>	Romania	O. Constantinescu	DQ233324, DQ233350, DQ233376, DQ233402, DQ233428
CBS 113069; CPC 5369	<i>Spinacia</i> sp.	Botswana	L. Lebogang	DQ233325, DQ233351, DQ233377, DQ233403, DQ233429
CBS 116454; CPC 11558	<i>B. vulgaris</i>	Germany	S. Mittler	AY840526, AY840493, AY840457, AY840424, AY840391
<sup>c</sup> CBS 116456; CPC 11557	<i>B. vulgaris</i>	Italy	V. Rossi	AY840527, AY840494, AY840458, AY840425, AY840392
CBS 116501; CPC 11576	<i>B. vulgaris</i>	Iran	A. A. Ravanlou	AY840528, AY840495, AY840459, AY840426, AY840393
CBS 116502; CPC 11577	<i>B. vulgaris</i>	Germany	S. Mittler	AY840529, AY840496, AY840460, AY840427, AY840394
CBS 116503; CPC 11578	<i>B. vulgaris</i>	Italy	—	AY840530, AY840497, AY840461, AY840428, AY840395
CBS 116505; CPC 11580	<i>B. vulgaris</i>	France	S. Garressus	AY840531, AY840498, AY840462, AY840429, AY840396
CBS 116506; CPC 11581	<i>B. vulgaris</i>	Netherlands	—	AY840532, AY840499, AY840463, AY840430, AY840397
CPC 5113	<i>Limonium sinuatum</i>	New Zealand	C. F. Hill	DQ233326, DQ233352, DQ233378, DQ233404, DQ233430
CPC 5123	<i>A. graveolens</i>	New Zealand	C. F. Hill	DQ233327, DQ233353, DQ233379, DQ233405, DQ233431
CPC 5125	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY752137, AY752170, AY752198, AY752229, AY752260
CPC 5128	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY752138, AY752171, AY752199, AY752230, AY752261
CPC 5370	<i>Spinacia</i> sp.	Botswana	L. Lebogang	DQ233328, DQ233354, DQ233380, DQ233406, DQ233432
CPC 10166	<i>B. vulgaris</i>	New Zealand	C. F. Hill	DQ233329, DQ233355, DQ233381, DQ233407, DQ026471
CPC 10168	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY840533, AY840500, AY840464, AY840431, AY840398
CPC 10171	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY840534, AY840501, AY840465, AY840432, AY840399
CPC 10195	<i>B. vulgaris</i>	New Zealand	C. F. Hill	DQ233330, DQ233356, DQ233382, DQ233408, DQ026472
CPC 10197	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY840535, AY840502, AY840466, AY840433, AY840400
CPC 10204	<i>B. vulgaris</i>	New Zealand	C. F. Hill	DQ233331, DQ233357, DQ233383, DQ233409, DQ233433

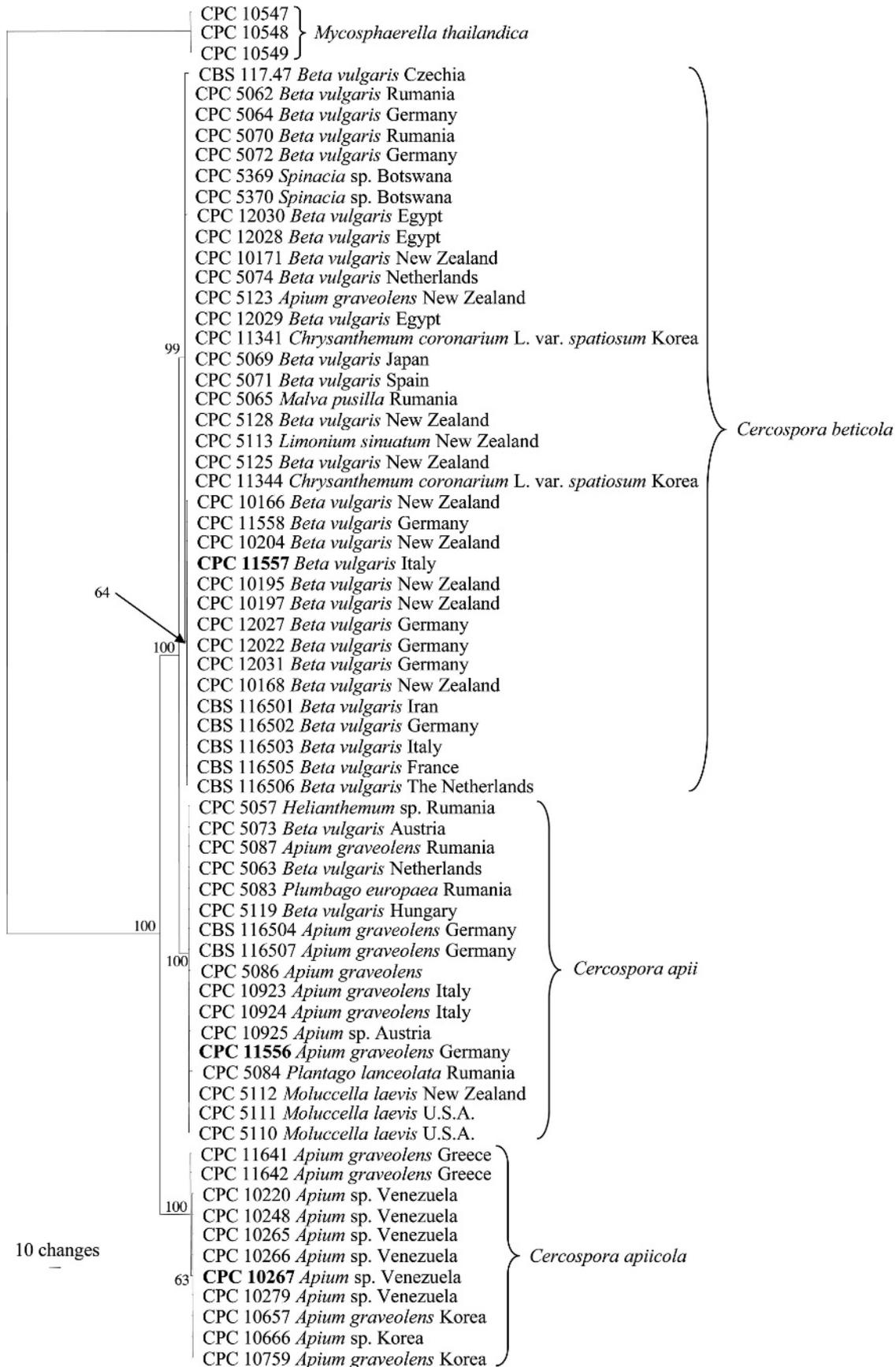
TABLE I. Continued

Strains and accession numbers ( <sup>a</sup> CBS; <sup>b</sup> CPC)	Host	Origin	Collector	GenBank no. (ITS, EF, ACT, CAL, HIS)
CPC 11341	<i>Chrysanthemum coronarium</i>	Korea	H. D. Shin	DQ233332, DQ233358, DQ233384, DQ233410, DQ233434
CPC 11344	<i>Chrysanthemum coronarium</i>	Korea	H. D. Shin	DQ233333, DQ233359, DQ233385, DQ233411, DQ233435
CPC 12022	<i>B. vulgaris</i>	Germany	S. Mittler	DQ233334, DQ233360, DQ233386, DQ233412, DQ233436
CPC 12027	<i>B. vulgaris</i>	Germany	S. Mittler	DQ233335, DQ233361, DQ233387, DQ233413, DQ026468
CPC 12028	<i>B. vulgaris</i>	Egypt	M. Hasem	DQ233336, DQ233362, DQ233388, DQ233414, DQ233437
CPC 12029	<i>B. vulgaris</i>	Egypt	M. Hasem	DQ233337, DQ233363, DQ233389, DQ233415, DQ233438
CPC 12030	<i>B. vulgaris</i>	Egypt	M. Hasem	DQ233338, DQ233364, DQ233390, DQ233416, DQ233439
CPC 12031	<i>B. vulgaris</i>	Germany	S. Mittler	DQ233339, DQ233365, DQ233391, DQ233417, DQ026470
<b><i>C. apiicola</i></b>				
<sup>c</sup> CBS 116457; CPC 10267	<i>Apium</i> sp.	Venezuela	N. Pons	AY840536, AY840503, AY840467, AY840434, AY840401
CBS 116458; CPC 10657	<i>Apium</i> sp.	Korea	H. D. Shin	AY840537, AY840504, AY840468, AY840435, AY840402
CPC 10220	<i>Apium</i> sp.	Venezuela	N. Pons	AY840538, AY840505, AY840469, AY840436, AY840403
CPC 10248	<i>Apium</i> sp.	Venezuela	N. Pons	AY840539, AY840506, AY840470, AY840437, AY840404
CPC 10265	<i>Apium</i> sp.	Venezuela	N. Pons	AY840540, AY840507, AY840471, AY840438, AY840405
CPC 10266	<i>Apium</i> sp.	Venezuela	N. Pons	AY840541, AY840508, AY840472, AY840439, AY840406
CPC 10279	<i>Apium</i> sp.	Venezuela	N. Pons	AY840542, AY840509, AY840473, AY840440, AY840407
CPC 10666	<i>Apium</i> sp.	Korea	H. D. Shin	AY840543, AY840510, AY840474, AY840441, AY840408
CPC 10759	<i>A. graveolens</i>	Korea	H. D. Shin	AY840544, AY840511, AY840475, AY840442, AY840409
CPC 11641	<i>A. graveolens</i>	Greece	A. N. Jama	DQ233340, DQ233366, DQ233392, DQ233418, DQ233440
CPC 11642	<i>A. graveolens</i>	Greece	A. N. Jama	DQ233341, DQ233367, DQ233393, DQ233419, DQ233441

<sup>a</sup> CBS strain numbers, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

<sup>b</sup> CPC strain numbers, Collection of Pedro Crous, housed at CBS, The Netherlands.

<sup>c</sup> Type strains of the different *Cercospora* species.



able ( $P = 0.001$ ) but that four of the data sets (ITS, EF, ACT and CAL) could be combined ( $P = 1.000$ ) and these therefore were analyzed as one combined set. The combined alignment contained 67 strains, including the three outgroups, and had a total length of 1262 characters, of which 935 were constant, six were parsimony uninformative and 321 were parsimony informative. The topology of the neighbor joining trees obtained with the different substitution models was the same. A similar topology was found for the most parsimonious trees. Parsimony analysis of the combined data resulted in a single parsimonious trees (FIG. 1) (TL = 350 steps; CI = 0.997; RI = 0.999; RC = 0.996). From the phylogenetic analysis (FIG. 1), three distinct and well supported clades were obtained. The first clade (99% bootstrap support) contains *Cercospora* isolates belonging to the *C. beticola* s.s. clade. Twenty-nine of these isolates were obtained from *Beta* species, but several isolates in this group also were obtained from five additional hosts (two from *Chrysanthemum*, one from *Apium*, one from *Limonium*, one from *Malva* and two from *Spinacia*). The isolates were obtained from Europe, Africa, Asia and New Zealand). The second clade (100% bootstrap support) contains *C. apii* s.s. isolates. These isolates also were obtained from a diverse range of hosts (three from *Beta*, three from *Moluccella*, one from *Plantago*, one from *Plumbago* and one from *Helianthemum*), but the primary host infected by isolates in this group appears to be *Apium* (eight isolates). Isolates from the second clade were from Europe, America and New Zealand. The third clade (100% bootstrap support) contains isolates of *C. apiicola* that thus far have been isolated only from *Apium* species in Venezuela, Korea and Greece.

Because the HIS dataset was not combinable with other sequence data, it was analyzed separately. The HIS alignment contained 67 strains including the three outgroups, and had a total length of 380 characters, of which 319 were constant, one was parsimony uninformative and 60 were parsimony informative. The topology of the neighbor joining trees obtained with the different substitution models was the same and was identical to the topology of the most parsimonious tree. Parsimony analysis of the HIS data resulted in the single most parsimonious

tree (FIG. 2) (TL = 73 steps; CI = 0.986; RI = 0.998; RC = 0.984). From the phylogenetic analysis (FIG. 2), three well supported clades with 100% bootstrap values were obtained. The first clade contained eight isolates (seven from *Beta* species from different countries and one from *Helianthemum* in Rumania) that were present in the *C. beticola* s.s. clade obtained from the first analysis, except for the *Helianthemum* isolate which grouped in the *C. apii* s.s. clade (FIG. 1). The second clade contained the remaining *C. beticola* s.s. and *C. apii* s.s. isolates. The third clade consisted only of the *C. apiicola* isolates, which is consistent with the first analysis using the other four loci.

*Taxonomy.*—*Cercospora apii* and *C. beticola* s.s. were circumscribed by Groenewald et al (2005). During the present study several *Cercospora* isolates were obtained from celery exhibiting *Cercospora* leaf spot. A population of 47 plants collected in Venezuela by N. Pons, as well as individual diseased plants collected in Greece and Korea, were found to be associated with a novel species of *Cercospora*. The latter species is morphologically distinct from the *C. apii* s.l. complex. Its conidiophores are relatively short, 25–70 × 4–6 μm, and the conidia are obclavate-cylindrical, not acicular, measuring (50–)80–120(–150) × (3–)4–5 μm and being 1–6-septate (FIGS. 3, 4). This species therefore is described as new:

***Cercospora apiicola*** M. Groenewald, Crous & U. Braun, sp. nov.

Differt a *C. apii* (s.s. et s.l.) conidiophoris relative brevibus, 25–70 × 4–6 μm, conidiis obclavatis-cylindricalibus, nonacicularibus, tantum 1–6-septatis.

*Specimen examined.* VENEZUELA. La Guanota, Caripe, Edo. Monagas, 1050 m.s.n.m., *Apium* sp., 23 Jul 2002, N. Pons, HOLOTYPE herb. CBS 18473, culture ex-type CBS 116457 MycoBank MB500768.

*Leaf spots* amphigenous, subcircular to irregular, 3–10 mm diam, medium brown, with a raised or inconspicuous, indefinite margin, not surrounded by a border of different color. *Caespituli* amphigenous, but primarily hypophyllous. *Stromata* lacking to well developed, 30–60 μm diam, medium brown. *Conidiophores* arising in fascicles of 4–10, moderately dense, arising from stromata, emerging through stomata or erumpent through the cuticle, subcylindrical, upper part geniculate-sinuuous, unbranched, 1–

←

FIG. 1. Single most parsimonious tree obtained from a heuristic search with 100 random taxon additions of the combined ITS, EF, ACT and CAL sequence alignment. The scale bar shows ten changes and bootstrap support values from 1000 replicates are shown at the nodes. Type strains are shown in bold print. The tree was rooted to three *Mycosphaerella thailandica* strains.



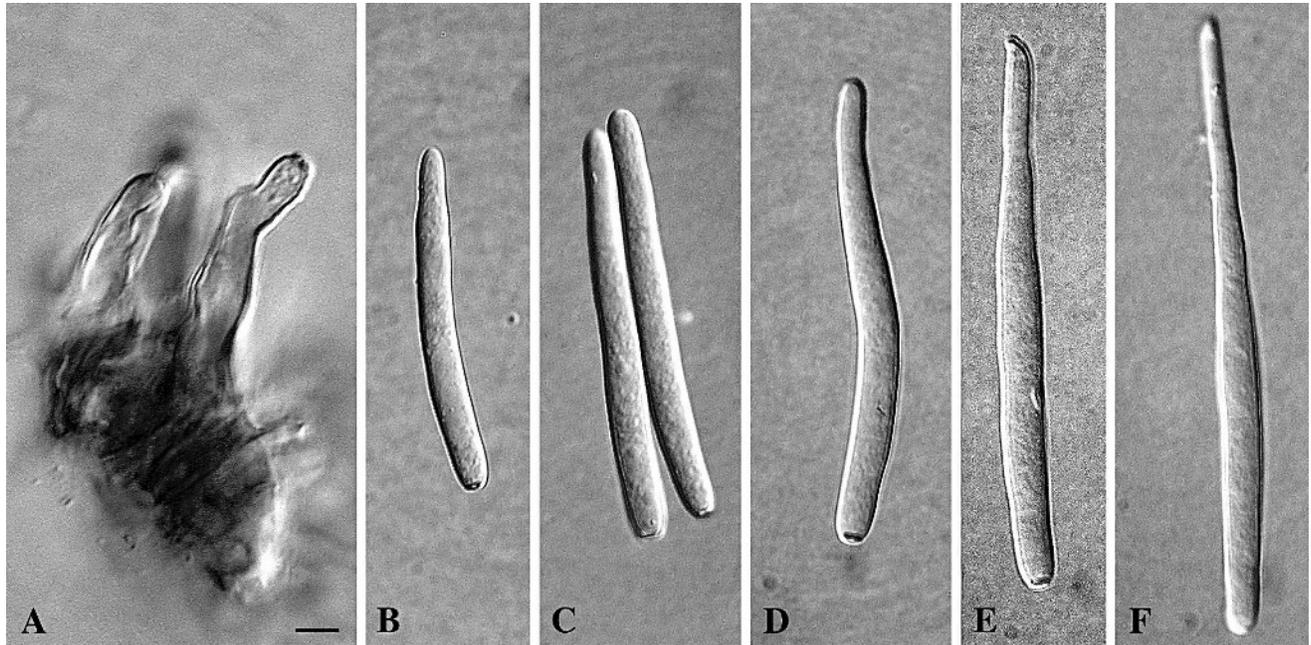


FIG. 3. *Cercospora apiicola* (holotype). A. Conidiophore. B–F. Conidia. Bar = 5  $\mu$ m.

3-septate, 25–70  $\times$  4–6  $\mu$ m, medium brown, becoming pale brown toward the apex, smooth, wall somewhat thickened. *Conidiogenous cells* integrated, terminal, 15–30  $\times$  4–5  $\mu$ m, occasionally unilocal, usually multilocal, sympodial; loci subcircular, planate, thickened, darkened, refractive, 2.5–3  $\mu$ m wide. *Conidia* solitary, cylindrical when small, obclavate-cylindrical when mature, not acicular, (50–)80–120 (–150)  $\times$  (3–)4–5  $\mu$ m, 1–6-septate; apex subobtuse, base obconically subtruncate; hila 2–2.5  $\mu$ m wide, thickened, darkened, refractive.

*Cultural characteristics.* Colonies are smooth to folded, erumpent with smooth, even to uneven margins and sparse to moderate aerial mycelium; white to smoke-gray on MEA (surface), and olivaceous-gray to iron-gray beneath; on OA colonies are white to olivaceous-gray on the surface. Cardinal temperature requirements for growth, min 6 C, opt 24 C, max 30 C.

*Host range and distribution.* *Apium graveolens*, *Apium* sp., Greece, Korea, Venezuela.

#### DISCUSSION

During a recent study in which we circumscribed *C. apii* and *C. beticola* s.s., we collected isolates of several

*Cercospora* spp. that are part of the *C. apii* s.l. species complex. A whole population of “*C. apii*” collected on celery from Venezuela was revealed to be a distinct species. Several months later we isolated the same species on celery collected from Korea. At that time it was thought that this species had not yet invaded European celery fields because it was absent from European *Cercospora* isolates from this crop (Groenewald et al 2005). However in the present study we report the presence of this species on celery from Greece and describe it as *C. apiicola* sp. nov. Cultural and morphological examination of the *C. apiicola* strains support the observation made by Groenewald et al (2005) that this new *Cercospora* species is distinct from the two closely related species, *C. beticola* and *C. apii*, that previously have been isolated from celery. The isolation of this new *Cercospora* species on a well known crop such as celery is an indication that there may still be many other undescribed cercosporoid species on well known crops and ornamental plants awaiting description.

Chupp (1954) associated *Cercospora* leaf spot on sugar beet with infections of *C. beticola*, and that of celery with *C. apii*. Ellis (1971) discussed the *C. apii* s.l. isolates in detail and described a wide host range for this species, but five years later he changed his

←

FIG. 2. The single most parsimonious tree obtained from a heuristic search with 100 random taxon additions of the histone H3 sequence alignment. The scale bar shows a single change and bootstrap support values from 1000 replicates are shown at the nodes. Type strains are shown in boldface. The tree was rooted to three *Mycosphaerella thailandica* strains.

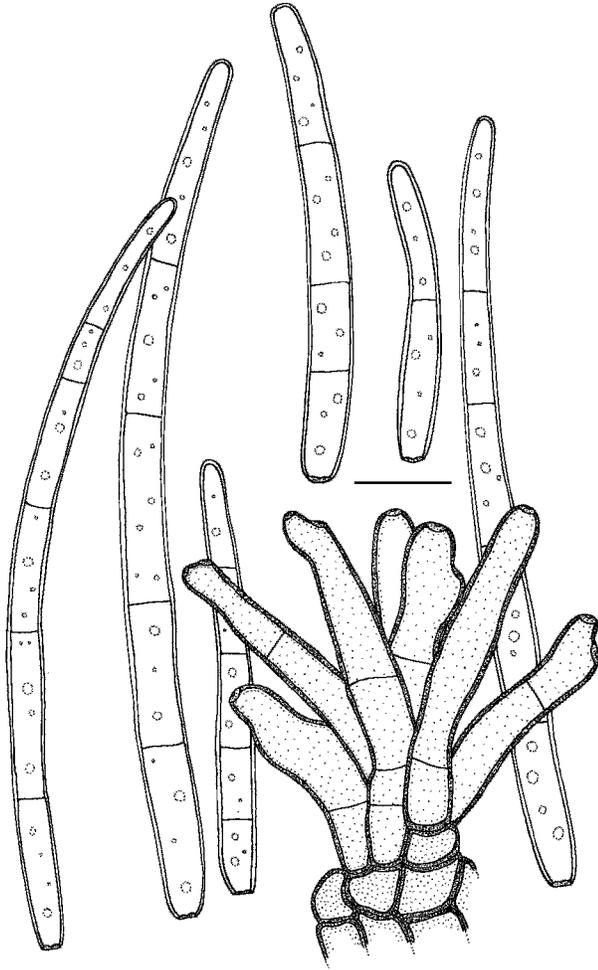


FIG. 4. Line drawing of conidiophores and conidia of the *C. apii* holotype (CBS 116457). Bar = 10  $\mu$ m.

opinion and narrowed the host range of *C. apii* to celery and *C. beticola* to sugar beet (Ellis 1976). Crous and Braun (2003) linked 83 host genera to *C. apii* and nine host genera to *C. beticola* infections. Groenewald et al (2005) again cast doubt on the purported wide host ranges of these species. In the present study a survey of *Cercospora* isolates from 10 host genera identified several additional hosts for both *C. apii* s.s. and *C. beticola* s.s. From these data we can confirm four additional host genera for *C. apii* (*Helianthemum*, *Moluccella*, *Plantago*, *Plumbago*) and five additional host genera for *C. beticola* (*Apium*, *Chrysanthemum*, *Limonium*, *Malva*, *Spinacia*). According to Crous and Braun (2003) several *Cercospora* species (listed in parentheses) are associated with these hosts: *Apium* (*C. apii*), *Beta* (*C. beticola*), *Helianthemum* (*C. cistinearum*, *C. helianthemii*), *Moluccella* (*C. moluccellae*), *Plantago* (*C. pantoleuca*, *C. plantaginis*), *Plumbago* (*C. apii*, *C. plumbaginea*), *Limonium* (*C. apii*, *C. insulana*, *C. statices*), *Malva*

(*C. althaeina*, *C. beticola*, *C. hyalospora*, *C. malvae*, *C. malvarum*) and *Spinacia* (*C. bertrandii*, *C. beticola*, *C. spinaciicola*). In the treatment of Crous and Braun (2003) neither *Apium*, *Chrysanthemum* or *Limonium* are listed as hosts of *C. beticola* nor *Beta*, *Helianthemum*, *Moluccella* and *Plantago* as hosts of *C. apii*. This study provides the first molecular evidence that these two species have wider host ranges than had been accepted by Chupp (1954) and Ellis (1976). However from the present study it appears that both species have narrower host ranges than that proposed by Crous and Braun (2003), but this has to be investigated further by conducting pathogenicity studies on all the hosts previously listed for these species.

The host range data obtained in the present study illustrate that *C. beticola* s.s. and *C. apii* s.s. are not entirely host specific and that it is not possible to identify these two species solely based on host. Despite of the additional host genera that were found for *C. apii* and *C. beticola*, it is clear that *C. apii* s.s. is mainly isolated from celery, whereas *C. beticola* is mainly isolated from sugar beet, even though both of these species have been isolated from the other's primary host in the past.

Crous and Groenewald (2005) introduced the pogo stick hypothesis to explain the colonization of necrotic *Mycosphaerella* lesions by other species of *Mycosphaerella* that jump hosts in the process of reaching their real hosts. The possibility that this process of substrate colonization and host jumps also occurs in asexual *Mycosphaerella* species could explain the isolation of specific *Cercospora* species from "atypical" hosts and needs to be investigated further. It would be especially interesting to determine whether *Cercospora* species occurring on "atypical" hosts are able to cause disease on these hosts or not.

As illustrated in this study, morphology, host specificity and geographic location are not suitable characters for the identification of species of the *Cercospora apii* complex. Groenewald et al (2005) used sequence data in combination with other features such as growth rate to establish species boundaries for *C. apii*, *C. apii* (as *Cercospora* sp.) and *C. beticola*. From these established species boundaries, species-specific primers were designed in polymorphic areas of the calmodulin gene for the three species. This combined approach probably represents the most reliable way to characterize and identify species within this complex.

Five loci were used in this study for phylogenetic analyses, although all five loci sequenced were not congruent and therefore could not be used in a combined phylogenetic analysis. Two separate analyses thus were performed, the first combining ITS, EF, ACT and CAL sequences and the second

using only HIS sequences. The first analysis separated the *C. apii* s.s., *C. beticola* s.s. and *C. apiicola* isolates. Although the second analysis also was able to separate the *C. apiicola* isolates from the *C. apii* s.s./*C. beticola* s.s. isolates, it was unable to distinguish between *C. apii* s.s. and *C. beticola* s.s. isolates. Using HIS data a small cluster representing seven *C. beticola* s.s. and one *C. apii* s.s. isolate grouped separately from other *C. apii* s.s./*C. beticola* s.s. isolates. The unique polymorphisms (10 in total) observed in the histone H3 sequences of these isolates were identical and were not present in the other isolates or in our *Cercospora* sequence database. A possible explanation might be host jumping by the *Helianthemum* isolate, followed by recombination with the *Beta* isolates. However more *Helianthemum* isolates need to be studied to confirm whether this allele is unique to *Helianthemum* before one can address this issue. Caution therefore should be taken when using histone H3 sequence data for *Cercospora* phylogeny because variation in the histone H3 sequence may not indicate species differences.

It can be concluded from this study that strains belonging to the *C. apii* s.s. and *C. beticola* s.s. clades can be isolated from other hosts and, although these species are mainly isolated from celery and sugar beet, they are not host specific. It seems that the new species from celery described in this paper (viz. *C. apiicola*) is host specific because no other *Cercospora* strain isolated from other hosts and available in our sequence database has similar sequences. The reasons why host jumping by *C. apii* and *C. beticola* is so common remains unknown. However it is not unlikely that under stress—a shortage of host tissue or unsuitable weather—the new species might be able to jump from celery onto other hosts.

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# Distinct Species Exist Within the *Cercospora apii* Morphotype

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## ABSTRACT

Groenewald, M., Groenewald, J. Z., and Crous, P. W. 2005. Distinct species exist within the *Cercospora apii* morphotype. *Phytopathology* 95:951-959.

The genus *Cercospora* is one of the largest genera of hyphomycetes. *Cercospora apii* sensu lato is the oldest name for a large complex of morphologically indistinguishable *Cercospora* spp. occurring on a wide host range. There are currently 659 recognized *Cercospora* spp., and names of another 281 morphologically identical species are included in the synonymy of *C. apii* sensu lato. Two of the species that belong to the *C. apii* complex, *C. apii* and *C. beticola*, cause *Cercospora* leaf spot on *Apium graveolens* (celery) and *Beta vulgaris* (sugar beet), respectively. In

the present study, multilocus sequence data, amplified fragment length polymorphism analysis, and cultural characteristics were used as additional features to characterize morphologically similar *Cercospora* strains occurring on celery and sugar beet. From the data obtained, it is shown that *C. apii* and *C. beticola*, although morphologically similar and able to cross-infect each others' hosts, are distinct functional species that should be retained as separate entities. Furthermore, a third, as yet undescribed species of *Cercospora* was detected in celery fields in Korea and Venezuela, suggesting that additional undescribed species also may be found to cause *Cercospora* leaf spot on celery. A polymerase chain reaction-based diagnostic protocol distinguishes all three *Cercospora* spp.

In his monograph of the genus *Cercospora* Fresen., Chupp (6) accepted 1,419 species. In total, more than 3,000 species of *Cercospora* have been described, of which 659 presently are recognized (7). Generally, species of *Cercospora* are considered to be host specific (6) at the level of the plant genus or family; this concept has led to the description of a large number of species. Several *Cercospora* spp., which are morphologically indistinguishable from *Cercospora apii* Fresen., were placed in the *C. apii* complex (13). Cross-inoculation studies revealed that isolates in the *C. apii* complex can infect an extremely wide host range, including *Apium graveolens* (celery) and *Beta vulgaris* (sugar beet) (1,2,22,23,38,42). In their revision of the genus *Cercospora*, Crous and Braun (7) referred 281 morphologically indistinguishable species to the *C. apii* sensu lato complex. Recent genetic analyses of *Cercospora* spp. have relied mainly on DNA sequences of the internal transcribed spacers (ITSs) and the 5.8S ribosomal (r)RNA gene. These studies have revealed that most species of *Cercospora*, in particular the members of the *C. apii* complex, are identical or very closely related (18,30,36, 37). Judging from their morphological similarity as well as their proven cross-infectiveness, it is probable that the species in the *C. apii* complex should be considered synonymous.

Species seen as representative of *C. apii* sensu lato lack a known teleomorph. Although the genus *Cercospora* is a well-established anamorph of the genus *Mycosphaerella* (11,18), only a few teleomorphs have been elicited via cultural studies (7,9). Phylogenetic analyses of all *Cercospora* isolates to date have placed them as a well-defined clade in the genus *Mycosphaerella*. Therefore, if a teleomorph were to be found for *C. apii*, it should be a species of *Mycosphaerella* (11,18,30,36).

*C. beticola*, causal agent of *Cercospora* leaf spot on *B. vulgaris*, originally was described by Saccardo (34), and is assumed to have originated in central Europe and the Mediterranean area. *C. apii*, which causes *Cercospora* leaf spot on *A. graveolens*, was described from the region between The Netherlands and Germany (15), and is assumed to have originated in Western Europe. *C. beticola* is seen as part of the *C. apii* complex (7,13). Several studies so far have suggested that *C. beticola* on sugar beet should be treated as a synonym of *C. apii* (2,13,22,38,42).

*Cercospora* leaf spot on sugar beet is a serious problem wherever this crop is grown. It is one of the most common and destructive sugar beet diseases, affecting more than a third of all fields worldwide (20,35). A whole sugar beet field can be destroyed by an outbreak of *C. beticola*, resulting in complete loss of the crop (12,32,41).

The similarity in disease symptoms and pathogen morphology seen in celery and sugar beet *Cercospora* leaf spot diseases led Crous and Braun (7) to conclude that *C. beticola* should be treated as a synonym of *C. apii* sensu lato. Although *Cercospora* leaf spot is no longer considered the most destructive disease on celery (26), in some parts of the world (e.g., Florida), *C. apii* is still seen as a serious pathogen of this crop (27).

The main objective of the present study was to confirm or reject the synonymy of *C. apii* and *C. beticola*. It was felt that the same study would provide some indication as to the status of a large number of the purported synonyms of *C. apii*. To address these matters, 38 *Cercospora* isolates were collected from sugar beet and celery; representing a total of 13 countries. Isolates were subjected to multigene sequence analysis and amplified fragment length polymorphism (AFLP) analysis, as well as cultural and morphological comparisons. Here, we show that both celery and sugar beet are hosts to two species of *Cercospora*, with one of these species infecting both hosts. Although *C. apii* and *C. beticola* are able to cross-infect each other's hosts and are morphologically similar to one another, they still appear to operate as functional species on their respective primary namesake hosts in nature.

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## MATERIALS AND METHODS

**Fungal isolates.** Single-spore isolations were obtained from symptomatic celery and sugar beet leaves, and cultures were established on 2% malt extract agar (MEA) (16) (Table 1). The *Cercospora* isolates were examined morphologically to confirm their identity as *C. apii* sensu stricto as described by Crous and Braun (7). Some reference isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Utrecht, The Netherlands.

**Morphological and cultural characterization.** *Cercospora* reference strains were selected from celery and sugar beet for morphological and cultural characterization (Table 1). Strains were plated onto 2% MEA and oatmeal agar (OA) (16) and incubated at 24°C in the dark for 8 days. Colony characteristics were determined and colors rated on the different growth media using a color chart (31). Cardinal growth temperatures were determined on MEA (8). These plates were incubated in the dark for 8 days at temperatures beginning at 6°C and progressing to 36°C in 3°C intervals; in addition, growth at 40°C was studied. Several isolates taken from each of the three different groups were used (Table 1). The experiments featured three simultaneous replicates for each isolate; the whole trial was repeated once.

**DNA extraction and sequencing.** DNA analysis was done on all isolates listed in Table 1. The FastDNA kit (BIO 101, Carlsbad, CA) was used according to the manufacturer's instructions to isolate genomic (g)DNA of 200 to 400 mg of fungal mycelia grown on MEA plates for 8 days at 24°C. A sterile blade was used to scrape the mycelia from the surface of the plate. The primers ITS1 and ITS4 (43) were used to amplify the ITS areas as well as the 5.8S rRNA gene (ITS). Part of the actin (ACT) gene was amplified using the ACT512F and ACT783R primers (4) and part of the translation elongation factor (EF) 1- $\alpha$  gene using the primers EF728F and EF986R (4). The CAL228F and CAL737R primers (4) were used to amplify part of the calmodulin (CAL) gene, and the primers CylH3F and CylH3R (10) to amplify part of the histone H3 (HIST) gene. The polymerase chain reaction (PCR) conditions were the same for all regions, except for the MgCl<sub>2</sub> concentration, which was 2 mM for the CAL region and 1.5 mM for the remaining areas. The reaction mixture had a total volume of 12.5  $\mu$ l and contained 1  $\mu$ l of diluted gDNA, 1 $\times$  PCR buffer, 48  $\mu$ M each of the dNTPs, 2.5 pmol of each primer, and 0.7 units *Taq* polymerase (Bioline GmbH, Luckenwalde, Germany). The amplification reactions were done on a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). The initial denaturation step was done at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C (30 s), annealing at 52°C (30 s), and elongation at 72°C (30 s). A final elongation step at 72°C (7 min) was included in the run. The PCR products were separated by electrophoresis at 80 V for 40 min on a 0.8% (wt/vol) agarose gel containing ethidium bromide at 0.1  $\mu$ g/ml in 1 $\times$  Tris-acetate-EDTA buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light.

The amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, The Netherlands) according to the manufacturer's recommendations. The products were analyzed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Foster City, CA). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNASTAR, Madison, WI).

**Phylogenetic analysis.** The sequences were assembled and added to the outgroups using Sequence Alignment Editor (version 2.0a11; Department of Zoology, University of Oxford, Oxford, UK), and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were done in Phylogenetic Analysis Using Parsimony (PAUP; version 4.0b10; Sinauer Associates, Sunderland, MA) and con-

sisted of neighbor-joining analysis with the uncorrected ("p"), Jukes-Cantor, and Kimura 2-parameter substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analysis, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1,000 bootstrap replications (19). Other measures calculated included tree length, consistency index, retention index, and rescaled consistency index (TL, CI, RI, and RC, respectively). The resulting trees were printed with TreeView version 1.6.6 (29). A partition homogeneity test was done in PAUP to test whether the different loci can be used in a combined analysis (14). Sequences were deposited in GenBank (accession numbers listed in Table 1) and the alignments in TreeBASE (accession no. S1285).

**AFLP analysis.** Restriction enzyme digestion and adaptor ligation were done using 30 ng of gDNA, 1 $\times$  T4 DNA ligase buffer, 50 mM NaCl, 2 U of *MseI*, 2 units of *EcoRI*, 40 U of T4 DNA ligase, 10  $\mu$ g of bovine serum albumin, 50 pmol of *MseI* adaptor, and 5 of pmol *EcoRI* adaptor made up to a final volume of 11  $\mu$ l (39). All enzymes were obtained from New England BioLabs (Beverly, MA). This reaction was carried out at 37°C for 12 h. A 1:1 dilution was made with dH<sub>2</sub>O and 4  $\mu$ l was used in the preselective PCR. The preselective PCR was performed in a 20- $\mu$ l volume containing 25 pmol of primer *EcoRI*-0 (39), 25 pmol of primer *MseI*-0 (39), 1.5 mM MgCl<sub>2</sub>, 1 $\times$  Bioline *Taq* reaction buffer, 0.1 mM each dNTP and, 0.75 units of Bioline *Taq* polymerase. An initial 72°C step was done for 2 min, followed by 20 cycles of denaturation at 94°C (20 s), annealing at 56°C (40 s), and elongation at 72°C (1 min). The preselective amplification was confirmed by electrophoresis on a 0.8% (wt/vol) agarose gel as described above. The preamplified DNA was diluted 1:1 with dH<sub>2</sub>O and used as template for selective amplification. Primers used in the selective amplification were *EcoRI*-A [FAM]/*MseI*-CT, *EcoRI*-AT [JOE]/*MseI*-C, and *EcoRI*-AG [NED]/*MseI*-C (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). The reactions contained 1.5 mM MgCl, 0.5 units of Bioline *Taq* polymerase, 1 $\times$  Bioline *Taq* polymerase buffer, 0.1 mM each dNTP, 0.5  $\mu$ l of *EcoRI* primer, and 0.5  $\mu$ l of *MseI* primer made up to a final volume of 10  $\mu$ l. Selective PCR products (2  $\mu$ l), amplified with the different primer combinations for each of the isolates, were mixed together with 0.5  $\mu$ l of GeneScan 500 (labeled with 6-carboxy-X-rhodamine) (Applied Biosystems) and made up to a final volume of 25  $\mu$ l with formamide. The products were denatured at 100°C for 5 min, followed by 30-min runs on an ABI 310 genetic analyzer. The AFLP data were analyzed using Bionumerics software (version 2.5; Applied Maths, Kortrijk, Belgium).

**Development of a species-specific diagnostic test.** The CAL gene was found to be very effective for separating the three species described in the present study; therefore, this area was targeted for the development of a species-specific diagnostic test. Primers *Cercocal-F* and *Cercocal-R* (Table 2) were designed from regions of the CAL gene that are conserved for the *Cercospora* spp. in our database. They act as outer primers and their amplification functions as a positive control. Three internal primers (*Cercocal-beta*, *Cercocal-*apii**, and *Cercocal-sp*), each specific for one of the three *Cercospora* spp. described in this study, were designed. The species-specific primers were used in separate PCRs together with the outer control primers. Strains of *C. beticola*, *C. apii*, the undescribed *Cercospora* sp., and 13 other species of *Cercospora* (Table 1) were screened with these primers. The sequences and specific nucleotide binding sites of the primers

TABLE 1. *Cercospora* isolates included in the study

Strain, accession no. <sup>a</sup>	Host	Origin	Collector	GenBank number <sup>b</sup>				
				ITS	EF	ACT	CAL	HIST
<i>Cercospora achyranthis</i> CPC 10091*	<i>Achyranthes japonica</i>	Korea	H. D. Shin	...	...	...	...	...
<i>C. apii</i>								
CBS 119.25; CPC 5086	<i>Apium graveolens</i>	...	L. J. Klotz	AY840512	AY840479	AY840443	AY840410	AY840377
CBS 121.31; CPC 5073	<i>Beta vulgaris</i>	Austria	...	AY840513	AY840480	AY840444	AY840411	AY840378
CBS 127.31; CPC 5119	<i>B. vulgaris</i>	Hungary	...	AY840514	AY840481	AY840445	AY840412	AY840379
CBS 152.52; CPC 5063	<i>B. vulgaris</i>	Netherlands	G. van den Ende	AY840515	AY840482	AY840446	AY840413	AY840380
CBS 536.71; CPC 5087	<i>A. graveolens</i>	Romania	O. Constantinescu	AY752133	AY752166	AY752194	AY752225	AY752256
CBS 114416; CPC 10925	<i>Apium</i> sp.	Austria	...	AY840516	AY840483	AY840447	AY840414	AY840381
CBS 114418; CPC 10924	<i>A. graveolens</i>	Italy	Meutri	AY840517	AY840484	AY840448	AY840415	AY840382
CBS 114485; CPC 10923	<i>A. graveolens</i>	Italy	Meutri	AY840518	AY840485	AY840449	AY840416	AY840383
CBS 116455; CPC 11556**	<i>A. graveolens</i>	Germany	K. Schrameyer	AY840519	AY840486	AY840450	AY840417	AY840384
CBS 116504; CPC 11579	<i>A. graveolens</i>	Germany	K. Schrameyer	AY840520	AY840487	AY840451	AY840418	AY840385
CBS 116507; CPC 11582	<i>A. graveolens</i>	Germany	K. Schrameyer	AY840521	AY840488	AY840452	AY840419	AY840386
<i>C. beticola</i>								
CBS 116.47; CPC 5074	<i>B. vulgaris</i>	Netherlands	G. E. Bunschoten	AY752135	AY752168	AY752196	AY752227	AY752258
CBS 122.31; CPC 5072	<i>B. vulgaris</i>	Germany	...	AY752136	AY752169	AY752197	AY752228	AY752259
CBS 123.31; CPC 5071	<i>B. vulgaris</i>	Spain	...	AY840522	AY840489	AY840453	AY840420	AY840387
CBS 124.31; CPC 5070	<i>B. vulgaris</i>	Romania	...	AY840523	AY840490	AY840454	AY840421	AY840388
CBS 125.31; CPC 5069	<i>B. vulgaris</i>	Japan	...	AY840524	AY840491	AY840455	AY840422	AY840389
CBS 126.31; CPC 5064	<i>B. vulgaris</i>	Germany	...	AY840525	AY840492	AY840456	AY840423	AY840390
CBS 116454; CPC 11558	<i>B. vulgaris</i>	Germany	S. Mittler	AY840526	AY840493	AY840457	AY840424	AY840391
CBS 116456; CPC 11557**	<i>B. vulgaris</i>	Italy	V. Rossi	AY840527	AY840494	AY840458	AY840425	AY840392
CBS 116501; CPC 11576	<i>B. vulgaris</i>	Iran	A. A. Ravanlou	AY840528	AY840495	AY840459	AY840426	AY840393
CBS 116502; CPC 11577	<i>B. vulgaris</i>	Germany	S. Mittler	AY840529	AY840496	AY840460	AY840427	AY840394
CBS 116503; CPC 11578	<i>B. vulgaris</i>	Italy	...	AY840530	AY840497	AY840461	AY840428	AY840395
CBS 116505; CPC 11580	<i>B. vulgaris</i>	France	S. Garressus	AY840531	AY840498	AY840462	AY840429	AY840396
CBS 116506; CPC 11581	<i>B. vulgaris</i>	Netherlands	...	AY840532	AY840499	AY840463	AY840430	AY840397
CPC 5125	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY752137	AY752170	AY752198	AY752229	AY752260
CPC 5128	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY752138	AY752171	AY752199	AY752230	AY752261
CPC 10168	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY840533	AY840500	AY840464	AY840431	AY840398
CPC 10171	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY840534	AY840501	AY840465	AY840432	AY840399
CPC 10197	<i>B. vulgaris</i>	New Zealand	C. F. Hill	AY840535	AY840502	AY840466	AY840433	AY840400
<i>C. bizzozeriana</i>								
CBS 258.67; CPC 5061*	<i>Cardaria draba</i>	Romania	O. Constantinescu	...	...	...	...	...
<i>C. canescens</i>								
CPC 1138*	<i>Vigna</i> sp.	South Africa	S. van Wyk	...	...	...	...	...
<i>C. flagellaris</i>								
CPC 10124*	<i>Phytolacca americana</i>	Korea	H. D. Shin	...	...	...	...	...
<i>C. kikuchii</i>								
CBS 135.28; CPC 5067*	<i>Glycine soja</i>	Japan	H. W. Wollenweber	...	...	...	...	...
<i>C. malvacearum</i>								
CBS 126.26; CPC 5066*	<i>Malva</i> sp.	...	...	...	...	...	...	...
<i>C. penzigii</i>								
CPC 3950*	<i>Citrus</i> sp.	South Africa	...	...	...	...	...	...
<i>C. piaropi</i>								
CBS 113127*	<i>Eichhornia crassipes</i>	United States	R. Charudattan	...	...	...	...	...
<i>C. polygonacea</i>								
CPC 10117*	<i>Persicaria</i> sp.	Korea	H. D. Shin	...	...	...	...	...
<i>C. rautensis</i>								
CBS 555.71; CPC 5082*	<i>Coronilla varia</i>	Romania	O. Constantinescu	...	...	...	...	...
<i>C. ricinella</i>								
CPC 10104*	<i>Ricinus communis</i>	Korea	H. D. Shin	...	...	...	...	...
<i>C. rodmanii</i>								
CBS 113130*	<i>Eichhornia crassipes</i>	United States	R. Charudattan	...	...	...	...	...
<i>Cercospora</i> sp.								
CBS 116457; CPC 10267**	<i>Apium</i> sp.	Venezuela	N. Pons	AY840536	AY840503	AY840467	AY840434	AY840401
CBS 116458; CPC 10657	<i>Apium</i> sp.	Korea	H. D. Shin	AY840537	AY840504	AY840468	AY840435	AY840402
CPC 10220	<i>Apium</i> sp.	Venezuela	N. Pons	AY840538	AY840505	AY840469	AY840436	AY840403
CPC 10248	<i>Apium</i> sp.	Venezuela	N. Pons	AY840539	AY840506	AY840470	AY840437	AY840404
CPC 10265	<i>Apium</i> sp.	Venezuela	N. Pons	AY840540	AY840507	AY840471	AY840438	AY840405
CPC 10266	<i>Apium</i> sp.	Venezuela	N. Pons	AY840541	AY840508	AY840472	AY840439	AY840406
CPC 10279	<i>Apium</i> sp.	Venezuela	N. Pons	AY840542	AY840509	AY840473	AY840440	AY840407
CPC 10666	<i>Apium</i> sp.	Korea	H. D. Shin	AY840543	AY840510	AY840474	AY840441	AY840408
CPC 10759	<i>A. graveolens</i>	Korea	H. D. Shin	AY840544	AY840511	AY840475	AY840442	AY840409
<i>C. violae</i>								
CPC 10725*	<i>Viola mondshivica</i>	Korea	H. D. Shin	...	...	...	...	...

<sup>a</sup> Origin of strain numbers: CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands and CPC = Collection of Pedro Crous, The Netherlands; \* indicates additional *Cercospora* spp. tested with the species-specific primers; \*\* indicates *C. apii*, *C. beticola*, and *Cercospora* sp. isolates used for colony characteristics as well as growth rate measurements.

<sup>b</sup> ITS = internal transcribed spacer, EF = elongation factor, ACT = actin, CAL = calmodulin, HIST = histone H3.

are listed in Table 2. The same PCR conditions were used for the detection of all three species. The reaction mixture had a total volume of 12.5 µl and contained 1 µl of diluted gDNA, 1× PCR buffer, 1.5 mM MgCl<sub>2</sub>, 48 µM each of the dNTPs, 1 pmol of CercoCal-F, 3 pmol of each of CercoCal-R and the specific internal primer, and 0.7 units (Bioline) of *Taq* polymerase. The amplification reactions were done on a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). The initial denaturation step was done at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C (30 s), annealing at 58°C (30 s), and elongation at 72°C (30 s). A final elongation step at 72°C (7 min) was included to ensure that full-length products were obtained. The PCR products were separated on a 1.5% agarose gel and visualized as described above.

## RESULTS

**Morphological and cultural characterization.** The morphological characteristics of the conidia and conidiophores for all isolates obtained from celery and sugar beet (Table 1) were the same as described for *C. apii* sensu lato by Crous and Brown (7). Isolates from celery obtained from Venezuela and Korea were distinct, however, in that conidiophores were relatively short, 25 to 70 by 4 to 6 µm, and conidia were obclavate-cylindrical, not acicular. They measured (minimum length, 50) 80 to 120 (maximum length, 150) by (minimum width, 3) 4 to 5 µm and were one to six septate.

To facilitate the standardization of further genotypic studies on the *C. apii* complex, we herewith designate new epitype (a specimen selected to serve as an interpretative type in support of other type material, to facilitate the precise application of the published name) materials with cultures for *C. apii* and *C. beticola*. For *C. apii*, the original herbarium material used for the type (“holotype”) has been lost, but some of the original material might have been distributed and a lectotype, therefore, can be designated from these duplicates. Isolectotypes are duplicate specimens of the same lectotype. All of the material originally associated with the publication of the name *C. beticola* has been lost; therefore, a specimen has to be designated to serve as if it were the holotype of the species (“neotype”). Isonetypes are duplicate specimens of the neotype and ex-epitype cultures (to facilitate molecular studies) are derived from the epitype material.

*Cercospora apii* Fresen., Beitr. Mykol. 3:91. 1863.

Lectotype (proposed here): on *Apium graveolens*, Germany, Oestrich, garden, Fuckel, Fungi rhen. 117, in HAL. Fresenius (15) cited material of *C. apii* obtained from Fuckel. This is an indirect reference to the material distributed by Fuckel as Fungi rhen. 117. Original material in the herbarium of Fresenius could not be traced, and probably is not preserved; therefore, we prefer to select one of the duplicates distributed by Fuckel to serve as lectotype. Isolectotypes: Fuckel, Fungi rhen. 117. Epitype (proposed here): on *Apium graveolens*, Germany, Landwirtschaftsamt Heilbronn, 10.08.2004, K. Schrameyer, culture ex-epitype CBS 116455.

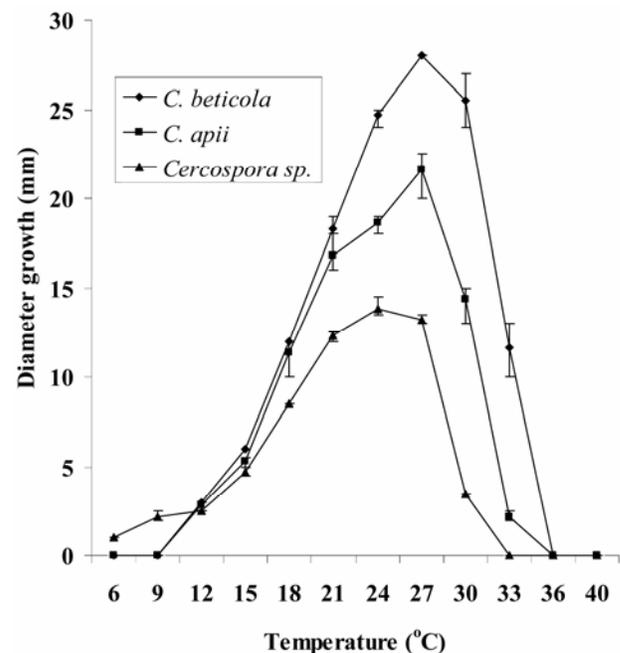
*Cercospora beticola* Sacc., Nuovo Giorn. Bot. Ital. 8:189. 1876.

Neotype (proposed here): on *Beta vulgaris*, Italy, Vittorio (Treviso), Sept. 1897, Sacc., Fungi ital. 197 (PAD). Isonetypes: Sacc., Fungi ital. 197. Epitype (proposed here): on *Beta vulgaris*,

Italy, Ravenna, 10.7.2003, Rossi V., culture ex-epitype CBS 116456.

Colonies of *C. beticola* and *C. apii* are smooth, erumpent, and regular, with smooth, even margins, and sparse to moderate aerial mycelium. *C. beticola* colonies on MEA are greenish-gray on the surface and dark mouse-gray beneath. On OA, colonies are white to green-olivaceous. *C. apii* colonies on MEA are pale greenish-gray on the surface and dark mouse-gray beneath. The surfaces of the colonies are white to green-olivaceous on OA. Morphologically divergent isolates from Venezuela and Korea are smooth to folded, erumpent with smooth, even to uneven margins, and sparse to moderate aerial mycelium. On MEA, colonies are white to smoke-gray on the surface, and olivaceous-gray to iron-gray beneath. On OA, colonies are white to olivaceous-gray on the surface.

The temperature ranges and colony diameters of three reference isolates (CBS 116455, CBS 116456, and CBS 116457), representing each of the three different species, are given in Figure 1. The Venezuela and Korea isolates can grow at lower temperatures (6°C) than *C. beticola* and *C. apii* (12°C), whereas *C. beticola* and *C. apii* have a higher maximum temperature tolerance (33°C) than the *Cercospora* sp. (30°C). The optimal temperature for growth of the *Cercospora* sp. was observed to be 24°C, whereas the optimal growth temperature for *C. apii* and *C. beticola* is 27°C. The *Cercospora* sp. grows much more slowly than the other two species, growing only 1.72 mm/day at its optimum temperature, whereas *C. beticola* and *C. apii* grew 3.5 and 2.7 mm/day at their respective optimal temperatures. Differences in growth rate between *C. apii* and *C. beticola* were observed for most of the



**Fig. 1.** Colony diameters at different temperatures ranging from 6 to 40°C for 8 days on 2% malt extract agar were calculated for *Cercospora apii* (CBS 116455), *C. beticola* (CBS 116456), and *Cercospora* sp. from Venezuela (CBS 116457).

**TABLE 2.** Primers designed from calmodulin sequences for the species identification amplifications

Primer	Sequence (5'–3')	Nucleotide position <sup>a</sup>	Description
CercoCal-F	CGCGAGGCAGAGCTAACGA	61–79	Positive control forward primer
CercoCal-beta	GCCACCCTCTGCGAATGTA	117–137	<i>Cercospora beticola</i> -specific primer
CercoCal- <i>apii</i>	GACCACCCTCTGCAACTGCG	117–137	<i>C. apii</i> -specific primer
CercoCal-sp	GCCACTTCTGTGACTGCA	117–137	<i>Cercospora</i> sp.-specific primer
CercoCal-R	GTGAGGAATTCGGGGAAATC	275–294	Reverse primer

<sup>a</sup> The calmodulin sequence of *C. apii* strain CBS 116455 (GenBank accession no. AY840417) was used to derive the nucleotide positions of the primers.

temperatures tested. *C. beticola* grew faster than *C. apii* (Fig. 1). *C. beticola* was more tolerant of temperatures higher than 30°C (1.46 versus 0.26 mm/day at 33°C).

**Phylogenetic analysis.** A partition homogeneity test showed that the five data sets were combinable ( $P = 0.834$ ); therefore, the sequence data were analyzed as one combined set. The combined alignment of ITS, ACT, EF, CAL, and HIST contained 41 strains including the three outgroups, and had a total length of 1,611 characters, of which 1,183 were constant, 3 were parsimony uninformative, and 425 were parsimony informative. The topology of the neighbor-joining trees obtained using the different substitution models was the same. A similar topology was found for the most parsimonious trees. Parsimony analysis of the combined data resulted in 12 parsimonious trees, one of which is shown in Figure 2 (TL = 465 steps, CI = 0.989, RI = 0.997, and RC = 0.986). From the phylogenetic analysis (Fig. 2), three distinct and well-supported clades were obtained. The first clade contained isolates of the new *Cercospora* sp. from *Apium* spp. (100% bootstrap support), the second clade contained only *Cercospora* isolates from *B. vulgaris* (91% bootstrap support), and the third clade contained *Cercospora* isolates from both *B. vulgaris* and *Apium* spp. (100% bootstrap support). All the isolates from the third clade were isolated in Europe. The ITS and ACT data sets showed no variation among the isolates from the second and the third clade and no significant variation could be observed between the isolates of these two clades with the EF and HIST data sets. The amount of variation observed within the CAL region of the *C. beticola* and *C. apii* isolates (96% similarity) was significant and placed these species into two distinct phylogenetic clades, each with a high bootstrap support in the combined analysis.

**AFLP analysis.** Genetic differences between isolates of the different clades also were confirmed using AFLP analysis. Banding patterns obtained with the *EcoRI*-A [FAM]/*MseI*-CT and *EcoRI*-AT [JOE]/*MseI*-C primer combinations are shown in Figure 3. The number and sizes of the polymorphic bands obtained for isolates of the *Cercospora* sp., using the *EcoRI*-A [FAM]/*MseI*-CT primer combination, show major differences with the profiles obtained for the other two species (Fig. 3A). Although isolates from the *C. apii* and *C. beticola* clades are more similar to each other than to the *Cercospora* sp., several bands are specific to each of the species, as seen using the *EcoRI*-A [FAM]/*MseI*-CT and *EcoRI*-AT [JOE]/*MseI*-C primer combinations (Fig. 3). The primer combination *EcoRI*-AG [NED]/*MseI*-C also was tested on isolates from the three *Cercospora* spp. and the banding patterns obtained showed results similar to those obtained with the other two primer combinations (data not shown).

**Species identification.** Easy and rapid identification of *C. beticola*, *C. apii*, and the new *Cercospora* sp. was possible using three multiplex PCR amplifications, each specific for one of the species. A 234-bp fragment, which serves as the positive control, was present for all three species, whereas a 176-bp fragment was observed only for the *Cercospora* sp. elucidated by the specific internal primer (Fig. 4). Only the 234-bp fragment was present for all other *Cercospora* spp. tested in our database representing 13 *Cercospora* spp. (data not shown). Therefore, primers CercoCal-beta, CercoCal-*apii*, and CercoCal-sp are specific for *C. beticola*, *C. apii*, and the *Cercospora* sp., respectively, and can be used for their identification and detection.

## DISCUSSION

Although morphological characteristics frequently are used to identify newly isolated fungi, it is not possible to distinguish *C. apii* (celery) from *C. beticola* (sugar beet) based solely on morphology. At the onset of this study, these species were considered to be synonymous as part of the *C. apii* sensu lato complex. Our data, however, refute the hypothesis that all morphologically indistinguishable *Cercospora* forms represent one species (7,13).

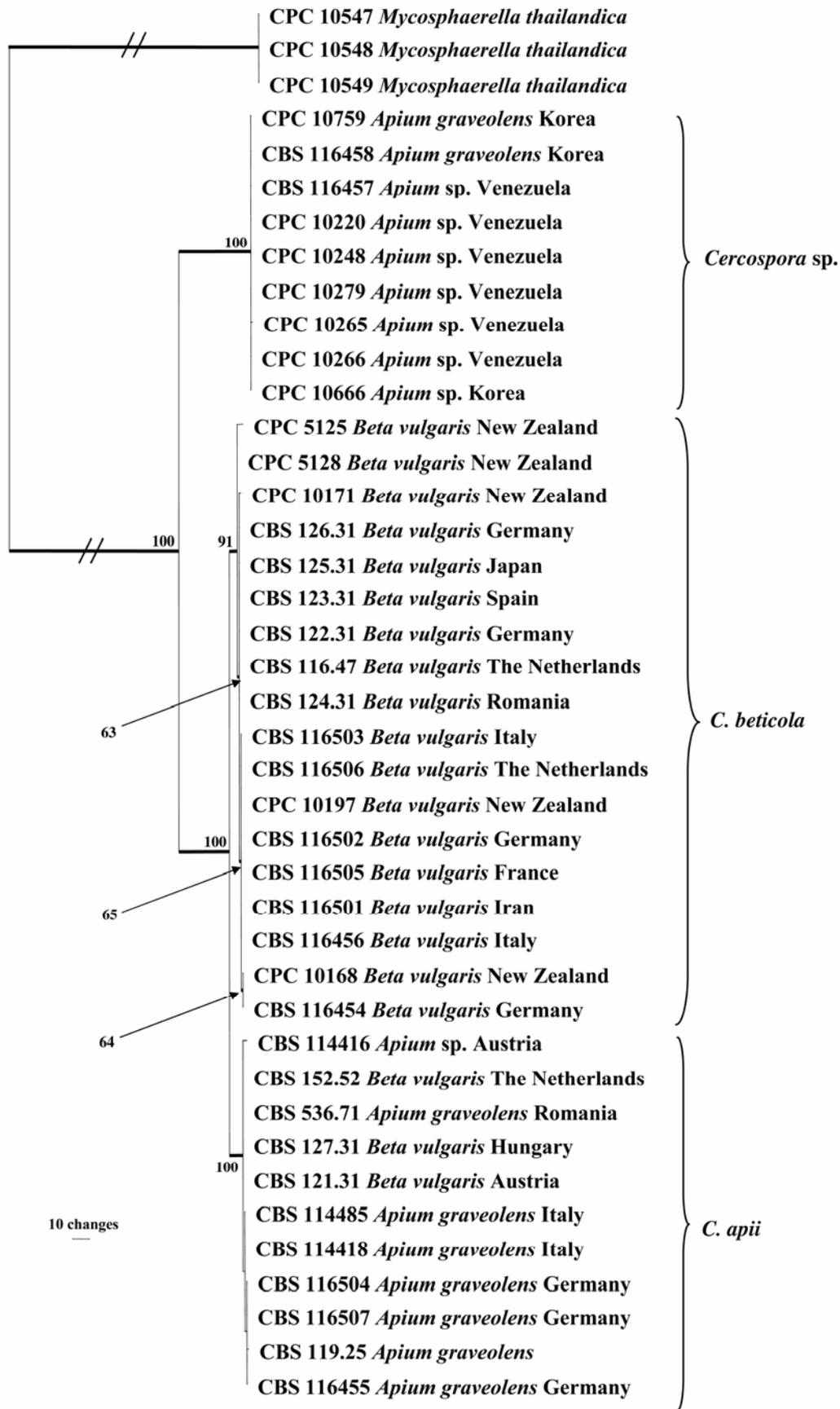
*C. apii* sensu stricto, which typify the *C. apii* sensu lato complex, including *C. beticola*, which is a morphologically similar fungus originally described from sugar beet, are shown to differ genetically and with some cultural characteristics from one another to an extent confirming species-level separation. It is now possible to identify the studied species using these characteristics.

Among the sequence types studied, only CAL strongly supports the split of *C. apii* and *C. beticola* into two distinct phylogenetic groups. This grouping, however, is confirmed in the growth studies as well as in AFLP analysis. This study shows that the choice and number of loci sequenced can be crucial in elucidating phylogenetic relationships of very closely related species and that using the wrong or an insufficient number of sequence loci could result in erroneous synonymies being proposed. It also shows that phenotypic characteristics, such as growth rates and temperature thresholds, can be very important parameters in the identification of species that are morphologically identical.

From the phylogenetic data obtained, it is clear that *C. apii* occurs mainly on celery, whereas *C. beticola* occurs on sugar beet, and that cross-infection of each other's hosts is rare. We did, however, study three isolates, revealed molecularly as *C. apii* sensu stricto, that were obtained from sugar beet in Europe (CBS 121.31 and CBS 127.31, deposited in 1931, and CBS 152.52 in 1952). The origin of *C. apii* is suspected to be Western Europe, and certainly the species was first described from celery collected in Germany. Because all of the *C. apii* isolates available in this study were from European countries, we do not know whether *C. apii* has been introduced on *Apium* spp. in non-European countries. It has been reported that *C. apii* sensu lato isolates can infect hosts other than the ones they were isolated from (7,22, 38,42). Therefore, it is quite possible that *C. apii*, which grows much more slowly than *C. beticola* at high temperatures (Fig. 1), originally was able to infect sugar beet and compete with *C. beticola* in the early 1900s, when Europe was considerably colder than is currently the case (28). Without doubt, *C. beticola* has been introduced from Europe to many other parts of the world, and this species now can be found on almost every continent (7; current study). The absence of *C. apii* on fresh diseased leaf material of *B. vulgaris* obtained for the purposes of this study can be ascribed to the unique growth properties of *C. beticola*. It is very probable that the faster growth rate and its ability to easily grow at higher temperatures allow *C. beticola* to out-compete *C. apii* for infection sites on *B. vulgaris*. It is clear that environmental factors, such as temperature and availability of specific plant species, play an important role in the survival and infection ability of the fungus. Thus, it seems that genotype-environment interactions (24) may play a role in the fitness of species in the *C. apii* complex.

We illustrated an easy PCR-based method which can be used in laboratories that use basic PCR techniques as a diagnostic tool. Although three PCRs are necessary to distinguish between the three *Cercospora* spp. affecting celery and sugar beet, it is possible to limit the number of reactions according to the crop from which the pathogen was isolated. Thus far, the new *Cercospora* sp. has never been isolated from *B. vulgaris*; however, because both *C. beticola* and *C. apii* have been isolated from sugar beet, it is important to test isolates from that source as possible representatives of both these species. None of the *C. beticola* isolates confirmed as such with molecular data have been isolated from *Apium* spp.; therefore, it remains possible that *C. beticola* might not infect celery under field conditions.

Because of the major loss in sugar beet production due to *Cercospora* leaf spot, naturally derived fungicides and synthetic fungicides with broad chemistries are currently being used to control *Cercospora* spp. infections in this crop (21). Several studies have indicated that *C. beticola* has become resistant to fungicides in the benzimidazole class (17,33,40) and has developed increased tolerance to fungicides in the organotin and triazole classes (3,5,

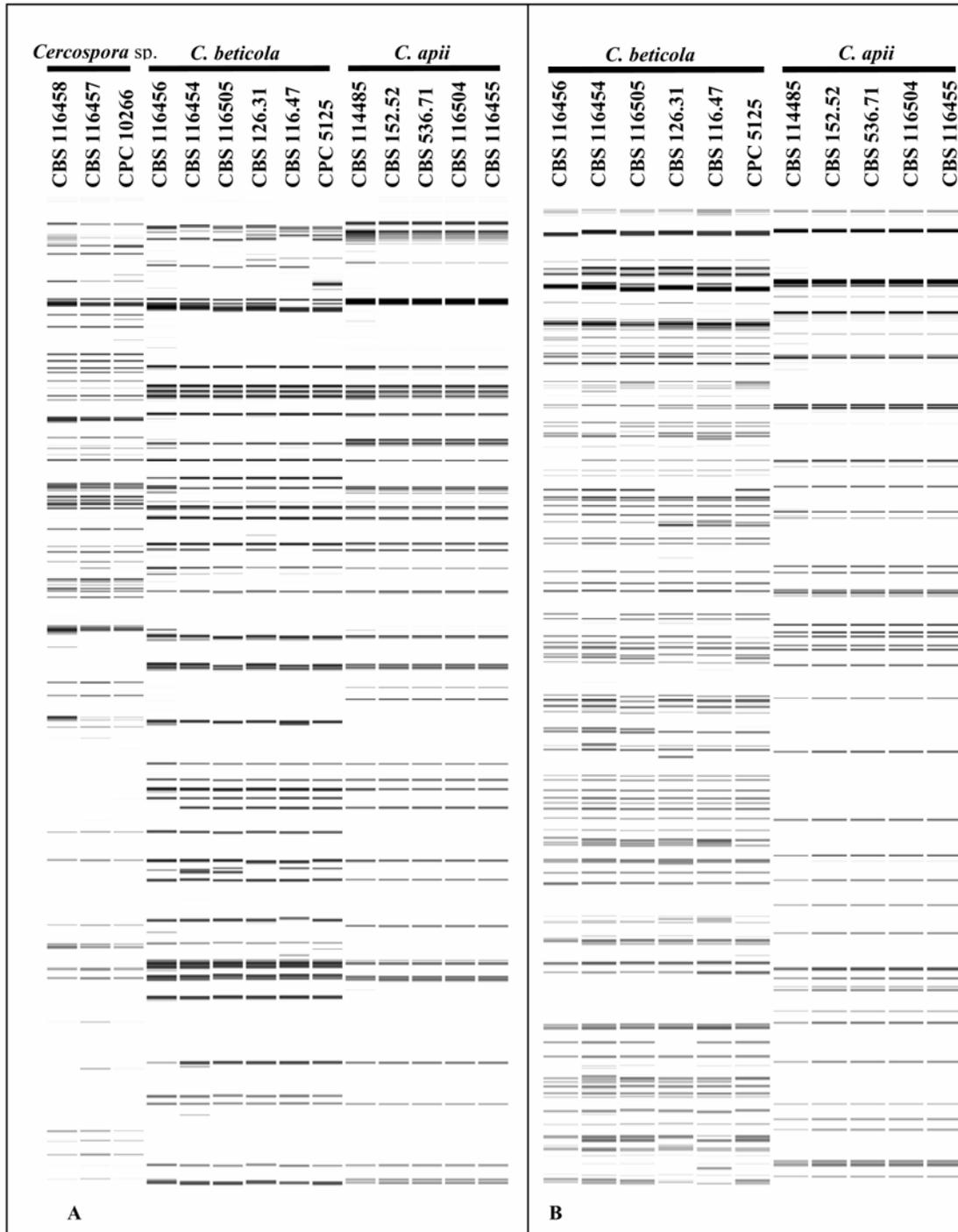


**Fig. 2.** One of the 12 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined internal transcribed spacer, elongation factor 1- $\alpha$ , actin, calmodulin, and histone H3 sequences alignment. The scale bar shows 10 changes and bootstrap support values from 1,000 replicates are shown in percentages at the nodes. Thickened lines indicate the strict consensus branches. The tree was rooted with three *Mycosphaerella thailandica* isolates.

25,41). In order to reduce fungicide tolerance of *Cercospora* spp. and to control the severity of *Cercospora* leaf spot disease of sugar beet, the frequent rotation of fungicide chemistries as well as the development of crops resistant to *Cercospora* infections have been implemented (21,41). Although *C. beticola* seems to be the main agent of *Cercospora* leaf spot on sugar beet, this study shows that *C. apii* also can be isolated from *Cercospora* leaf spot lesions on sugar beet. Fungicide trials must be done on these two species to determine their respective resistance levels against different fungicides. If there is a significant difference in their resistance levels, it might provide an explanation for the buildup

of fungicide resistance of *Cercospora* leaf spot in sugar beet. This also can have major implications for the use of fungicides in other crops to which *Cercospora* spp. are pathogenic.

The relationships of all the other species that have been ascribed to the *C. apii* complex need to be studied in detail. Knowledge of whether species names previously synonymized with *C. apii* are correctly considered superfluous will enable us to better understand the diversity and host specificity of species in this complex, and will enable us to delineate the functional species units that operate in nature. The three species described in this study can be separated from one another not only on the genetic level but also



**Fig. 3.** Visualization of the amplified fragment length polymorphism (AFLP) band patterns were done using Bionumerics software. **A**, AFLP fingerprints of different isolates of the *Cercospora* sp., *Cercospora beticola*, and *C. apii* using primer combination *EcoRI*-A [FAM]/*MseI*-CT. **B**, AFLP fingerprints of *C. beticola* and *C. apii* isolates using primer combination *EcoRI*-AT [JOE]/*MseI*-C.

by the ecological niche of each of the species. The genotypic differences observed for the three *Cercospora* spp. can be linked most of the time to the ecological differences between them; for example, cardinal temperature ranges and host identity.

From our data, it is clear that Chupp (6) was not totally incorrect when he proposed that *Cercospora* spp. were restricted to specific host genera or families. If this concept could be used for all the *Cercospora* spp.–host combinations, it would be easy to identify *Cercospora* spp. based on their hosts. Unfortunately, the present study confirms that this concept is not applicable to the genus as whole. For instance, the *Cercospora* sp. present on typical *Cercospora* leaf spot symptoms of celery in Venezuela and Korea is a distinct species that matches none of the 200 *Cercospora* sequences in our database. This species grows much more slowly than *C. apii*, and is unable to grow at 33°C or above, but can grow at much lower temperatures than *C. apii*; for example, at 6 to 10°C. Based on phylogenetic and AFLP analyses, this species is different from *C. apii* as well as *C. beticola*. A population representing more than 50 celery plants was collected of this species in Venezuela, indicating that it obviously is well established on this host. The fact that this species also occurs on celery in Korea suggests that, rather than representing a pathogen that normally grows on another host but occasionally occurs on celery by chance alone, it is instead an established pathogen of celery. It probably has been overlooked in the past due to its morphological similarity to *C. apii* and similar host symptomatology. This discovery of such a widespread cryptic species on a well-studied host like celery, however, does stimulate one to question whether similar cryptic species could exist within additional “common” pathogens that we currently accept as having wide host ranges. The present study illustrates how important it is to the plant pathology community to lodge reference strains of the pathogens they are working with in long-term storage in publicly accessible collections. Had it not been for the plant pathologists who lodged their *C. apii* strains in the early 1900s, it would not have been possible to prove the presence of different *Cercospora* spp. on celery, or the natural occurrence of *C. apii* on sugar beet. This

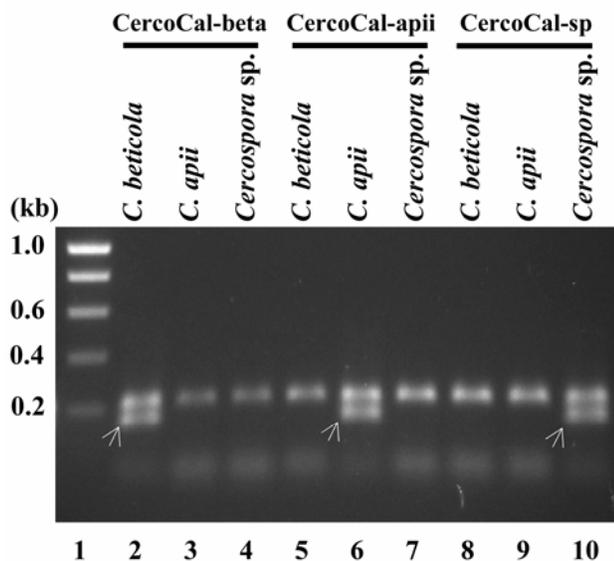
riddle, in spite of the advanced techniques employed here, remains unresolved to this day.

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**Fig. 4.** Identification of *Cercospora beticola*, *C. apii*, and the new *Cercospora* sp. using the different species-specific (CercoCal) primers. Lane 1 contains the DNA marker. The 234-bp fragment, the positive control, is present for all the polymerase chain reaction amplifications done (lanes 2 to 10). The species-specific fragment (176 bp, indicated with an arrow) can be observed only when the amplification reaction contains *C. beticola* (CBS 116456) DNA with primer CercoCal-beta (lane 2), *C. apii* (CBS 116455) with primer CercoCal-apii (lane 6), or *Cercospora* sp. (CBS 116457) with primer CercoCal-sp (lane 10).



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# Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex

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## Abstract

The genus *Cercospora* consists of numerous important, apparently asexual plant pathogens. We designed degenerate primers from homologous sequences in related species to amplify part of the *C. apii*, *C. apiicola*, *C. beticola*, *C. zae-maydis* and *C. zeina* mating type genes. Chromosome walking was used to determine the full length mating type genes of these species. Primers were developed to amplify and sequence homologous portions of the mating type genes of additional species. Phylogenetic analyses of these sequences revealed little variation among members of the *C. apii* complex, whereas *C. zae-maydis* and *C. zeina* were found to be dissimilar. The presence of both mating types in approximately even proportions in *C. beticola*, *C. zae-maydis* and *C. zeina* populations, in contrast to single mating types in *C. apii* (MAT1) and *C. apiicola* (MAT2), suggests that a sexual cycle may be active in some of these species.

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**Keywords:** Allele frequency; *Cercospora* leaf spot; Geographic distribution; Mating types; *Mycosphaerella*; PCR amplification

## 1. Introduction

The genus *Cercospora* was described by Fresenius (Fuekel, 1863) and is one of the largest genera of hyphomycetes. More than 3000 names were listed by Pollack (1987), but Crous and Braun (2003) revised the genus and reduced many species to synonymy, leaving a total of 659 *Cercospora* species. There are 281 morphologically indistinguishable *Cercospora* species, infecting a wide range of plant genera and families, listed as synonyms under *C. apii sensu lato* (Crous and Braun, 2003).

*Cercospora apii* is the main causal agent of *Cercospora* leaf spot on celery, although it has also been confirmed to occur on additional host genera such as *Beta*, *Helianthemum*, *Mohuccella*, *Plantago* and *Plumbago* (Crous and Braun, 2003; Groenewald et al., 2005, 2006). A second

*Cercospora* species, *C. apiicola*, has also been found to cause *Cercospora* leaf spot on celery (Groenewald et al., 2005, 2006). A multi-gene phylogeny revealed *C. apiicola* to be distinct from *C. apii* (Groenewald et al., 2005, 2006). This species is morphologically similar, but not identical, to *C. apii*, and has thus far only been isolated from celery in Venezuela, Korea and Greece.

*Cercospora beticola*, which causes *Cercospora* leaf spot on sugar beet (Groenewald et al., 2005; Saccardo, 1876), is morphologically identical to *C. apii*. Although these two species were considered to be synonymous in the past (Crous and Braun, 2003), a multi-gene phylogenetic comparison and cultural characteristics revealed them to be distinct species (Groenewald et al., 2005). *C. beticola* has also been confirmed from additional host genera such as *Apium*, *Chrysanthemum*, *Limonium*, *Malva*, and *Spinacia* (Crous and Braun, 2003; Groenewald et al., 2006).

Three *Cercospora* species have been linked to grey leaf spot on maize, namely *C. zae-maydis*, *C. zeina*, and an

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unnamed *Cercospora* sp. (Crous et al., 2006), though it appears that other *Cercospora* species may also occur on this host (Wang et al., 1998). The unnamed *Cercospora* sp. reported by Crous et al. (2006) appeared to be morphologically and phylogenetically more similar to isolates in the *C. apii* complex than to *C. zaeae-maydis* and *C. zeina*. The description of *C. zeina* (Crous et al., 2006) has resolved some of the taxonomic uncertainty surrounding groups in *C. zaeae-maydis*. The previously described *C. zaeae-maydis* group II is now *C. zeina*, whereas group I is *C. zaeae-maydis sensu stricto* (Crous et al., 2006; Dunkle and Levy, 2000; Goodwin et al., 2001).

No teleomorphs are known for the *Cercospora* species causing leaf spot on celery, sugar beet or maize, although there was an unconfirmed report of a teleomorph for *C. zaeae-maydis* (Latterell and Rossi, 1977). Wang et al. (1998) were unable to find evidence of the *MAT-2* idiomorph in isolates of *C. zaeae-maydis*, and *in vitro* pairing studies with isolates of *C. zaeae-maydis* and *C. zeina* have thus far proven unsuccessful in producing a teleomorph (Crous et al., 2006). Wang et al. (1998) reported that there is little genotypic variation in populations of Group I and Group II (*C. zaeae-maydis* and *C. zeina*, respectively), which might be expected for asexual species. In contrast, high levels of genetic variation have been reported within and among *C. beticola* field populations, as well as among isolates from the same leaf lesion (Große-Herrenthey, 2001; Moretti et al., 2004). Phylogenetic analyses using the ITS sequences of a variety of *Cercospora* species have resolved *Cercospora* as a well-defined monophyletic clade within the teleomorph genus *Mycosphaerella* (Crous et al., 2000, 2001, 2004; Goodwin et al., 2001; Pretorius et al., 2003; Stewart et al., 1999). Based on these data, it is clear that if sexual states do exist for these species, they would reside in *Mycosphaerella*.

In the absence of a known sexual stage, several approaches can be used to test for evidence of sexual reproduction. Populations that regularly undergo sexual reproduction should have many more genotypes that result in higher levels of genotypic diversity compared to those with only asexual reproduction (Milgroom, 1996). This type of genetic structure is seen in most populations of *M. graminicola* (Linde et al., 2002; Zhan and McDonald, 2004; Zhan et al., 2003). Another method to test for the possibility of sexual reproduction is to establish the occurrence and frequency of the mating type genes. Both mating types have been characterized for filamentous ascomycetes such as *Alternaria alternata* and *Fusarium oxysporum*, for which only asexual reproduction have been observed (Arie et al., 1997, 2000). Therefore, the presence of the mating type idiomorphs in a given species alone is insufficient to prove that a sexual stage exists. However, it is probable that sexual recombination does take place if the two mating types occur in approximately equal frequencies within a given population (Halliday et al., 1999; Linde et al., 2003; Milgroom, 1996; Waalwijk et al., 2002).

The fact that different mating types are necessary for sexual reproduction was first recognized for the genus

*Rhizopus* by Blakeslee (1904); and the first molecular characterization of the mating type idiomorphs was achieved for the yeast *Saccharomyces cerevisiae* (Astell et al., 1981). *Neurospora crassa* was the first filamentous ascomycete for which the mating type genes (*MAT1-1-1* and *MAT1-2*) were cloned and sequenced (Glass et al., 1988). The direct target genes of the mating type proteins have not yet been described, although there is evidence for the control of some genes such as pheromone genes (Bobrowicz et al., 2002). The DNA and amino acid sequences of mating type genes show no obvious similarities, although the mating type locus is surrounded by common flanking regions (Turgeon et al., 1993). Except for the high mobility group (HMG)- and the alpha domains, the similarity of homologous mating type genes is usually very low between different species (Turgeon, 1998). Regions with similarities of up to 90% can be found in the HMG domain, and these homologous regions have been used to design degenerative primers for amplification and cloning of the *MAT1-2* gene (Arie et al., 1997).

Four *MAT1-1* genes have been observed in ascomycetes (Pöggeler, 2001). Three of these genes can be distinguished from one another by the specific domain they contain. The *MAT1-1-1* gene contains an alpha domain, the *MAT1-1-2* gene has a MAT A-2 domain, and the *MAT1-1-3* gene has a HMG domain, whereas the *MAT1-1-4* encodes for a metallothionein protein (Kronstad and Staben, 1997; Turgeon, 1998). Only a single gene, *MAT1-2*, is known to confer the MAT2 phenotype. The formal mating type gene nomenclature proposed by Turgeon and Yoder (2000) will be used to define the mating type locus and genes from the *Cercospora* species.

The *MAT1-2* nucleotide sequences show high variability among species but low variability within species (Du et al., 2005; Paoletti et al., 2005). Sequences of the HMG domain of the *MAT1-2* gene have been used to investigate the phylogenetic relationships among closely related species in the *Gibberella fujikuroi* complex (Steenkamp et al., 2000), the *Ceratocystis coerulea* complex (Witthuhn et al., 2000), *Fusarium graminearum* (O'Donnell et al., 2004), the *Ophiostoma ulmi* complex (Paoletti et al., 2005), and *Colletotrichum* species (Du et al., 2005). Most of these studies concluded that sequences of the HMG domain gave the same and sometimes even greater resolution and stronger support for most branches in a phylogenetic tree than the sequences of the more frequently used internal transcribed spacer regions of nuclear ribosomal DNA.

Sexual reproduction frequently results in genetic recombination and this has a major impact on the dynamics and fitness of a species. The teleomorphs of the *Cercospora* leaf spot pathogens are unknown, and have thus far not been successfully induced by crosses in the laboratory. As a first step to understanding the reproduction cycle in the apparently asexual species of the genus *Cercospora*, our objectives are to identify which mating type(s) are present in *Cercospora* species and to characterize the mating type gene(s). To achieve this objective, we (1) sequence and char-

acterize the full-length mating type genes of *C. apii*, *C. apii-cola*, *C. beticola*, *C. zea-maydis*, and *C. zeina* using PCR-based techniques, (2) amplify and sequence portions of the *MAT1-1-1* and *MAT1-2* genes of other *Cercospora* species for comparison, and (3) develop a multiplex PCR method for rapid identification of the *MAT1-1-1* and *MAT1-2* genes to determine the frequencies of the mating types in different *Cercospora* populations.

## 2. Materials and methods

### 2.1. Fungal isolation and DNA extraction

Single conidial cultures were established from *Cercospora* leaf spots associated with celery leaves collected in Venezuela (*C. apii-cola*) on 23 June 2002 and in Germany (*C. apii*) on 10 August 2004. Isolations were also made from symptomatic sugar beet leaves obtained from The Netherlands, Germany, Italy, France and New Zealand in 2003 and from Iran in 2004. Symptomatic maize leaves were collected from fields in South Africa (*C. zeina*) in the beginning of 2005 and from Pioneer 3394, a gray leaf spot susceptible hybrid of *Zea mays*, in the USA (*C. zea-maydis*) on 2 August 2005. Sampling was done in an X figure across each field to ensure consistency. For each population, 50 symptomatic leaves were collected: 10 of each leg and 10 from the center plant. Isolates collected were used to screen for mating type distribution. Additional isolates used during this study were obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Utrecht, the Netherlands. DNA analyses were done on all isolates listed in Table 1. The FastDNA kit (BIO 101, Carlsbad, California) was used according to the manufacturer's instructions to isolate genomic DNA from 200 to 400 mg fungal mycelia grown on MEA plates for 8 days at 24 °C.

### 2.2. Degenerate primer development and screening of *Cercospora* isolates

The primer pairs, MAT1-1F/R, and MAT1-2F/R, described by Waalwijk et al. (2002) for the screening of the *MAT1-1-1* and *MAT1-2* genes, respectively, of *M. graminicola*, as well as the degenerate *MAT1-2* primers, ChHMG1 and ChHMG2 described by Arie et al. (1997), were used in an attempt to amplify part of the mating type genes of *C. beticola*. The amplifications were done according to the authors' instructions, and additional annealing temperatures (47 and 50 °C) were tested.

The *MAT1-1-1* sequences of *M. graminicola* (GenBank Accession No. AF440399), *S. passerinii* (GenBank Accession No. AF483193) and *M. fijiensis* (Abeln, unpublished data) and the *MAT1-2* sequences of *M. graminicola* (GenBank Accession No. AF440398), *S. passerinii* (GenBank Accession No. AF483194) and *M. fijiensis* (Abeln, unpublished data) were aligned using MegAlign from the Lasergene package (DNA-STAR, Madison, WI). Two sets of degenerate primers were

designed from this alignment, one set in a conserved region of the *MAT1-1-1* (MgMfSpMat1-1f1 5'-CATTNGCNCATCCCTTTG-3' and MgMfSpMat1-1r2 5'-GGCTTNGANACCATGGTGAG-3') and the other in a conserved region of the *MAT1-2* (MgMfSpMat1-2f2 5'-CAAAGAANGCNTTCNTGATCT-3' and MgMfSpMat1-2r1 5'-TTCTTCTCNGATGGCTTGC-3') gene. Initially, five randomly selected *C. beticola* isolates from the German population were screened with these two primer sets in order to amplify a partial region of the *MAT1-1-1* or *MAT1-2* genes.

The same PCR conditions were used for the amplification of both partial mating type genes. The reaction mixtures had a total volume of 12.5 µl and contained 0.7 µl of diluted gDNA, 1× PCR buffer (Bioline, London, UK), 48 µM of each of the dNTPs, 8 pmol of each degenerate primer, 1.5 mM MgCl<sub>2</sub> and 0.7 units *Taq* polymerase (Bioline). The amplification reactions were done on a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). The initial denaturation step was done at 94 °C for 5 min, followed by 15 cycles of 94 °C (20 s), 52 °C (20 s) and 72 °C (50 s), followed by 25 cycles of 94 °C (20 s), 50 °C (20 s), and 72 °C (50 s). A final elongation step at 72 °C (5 min) was included in the run. The PCR products obtained were separated by electrophoresis at 80 V for 1 h on a 1% (w/v) agarose gel containing 0.1 µg/ml ethidium bromide in 1× TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV-light. Amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, The Netherlands) according to the manufacturer's recommendations. The products were analyzed on an ABI Prism 3700 DNA Sequencer (Applied Biosystems). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package.

The degenerate primers and the amplification and sequencing conditions described above were also used to screen *C. apii*, *C. apii-cola*, *C. zea-maydis* and *C. zeina* isolates to obtain portions of their mating type genes.

### 2.3. Isolation and characterization of *Cercospora MAT1-1-1* and *MAT1-2* genes

Internal primers were designed in the partially sequenced *MAT1-1-1* and *MAT1-2* genes for each of the species. These internal primers were used together with the appropriate primers from the DNA walking speedup kit (Seegene Inc., Rockville, USA) to determine additional sequences upstream and downstream of the partial sequences in order to obtain the full-length genes. In total, 57 primers were designed and used for the chromosome walking. Blastx (Altschul et al., 1997) was used to compare the sequences obtained from the five *Cercospora* species with protein sequences of other fungi present in the NCBI non-redundant protein database. The geneid v1.2 web server (<http://www1.imim.es/geneid.html>—Research Unit on Biomedical Informatics of IMIM, Barcelona, Spain)

Table 1  
*Cercospora* isolates included in this study

Accession Nos. <sup>a</sup>	Host genus	Origin	Collector	GenBank No. <i>MAT1-1-1</i> ; <i>MAT1-2</i>
<i>C. acaciae-mangii</i> CPC 10527	<i>Acacia</i>	Thailand	K. Pongpanich	—;DQ264749
<i>C. achyranthis</i> CPC 10091	<i>Achyranthes</i>	Korea	H.D. Shin	DQ264733; —
<i>C. apii</i> CPC 5057; CBS 257.67	<i>Helianthemum</i>	Romania	O. Constantinescu	DQ264734; —
CPC 5086; CBS 119.25	<i>Apium</i>	—	G.H. Coons	DQ264735; —
<sup>b</sup> CPC 11556; CBS 116455	<i>Apium</i>	Germany	K. Schrameyer	DQ264736; —
<i>C. “apii”</i> CPC 5329; CBS 115536	<i>Cajanus</i>	South Africa	L. van Jaarsveld	—;DQ264750
CPC 5365; CBS 114817	<i>Fuchsia</i>	New Zealand	C.F. Hill	DQ264737; —
CPC 5366; CBS 115060	<i>Gaura</i>	New Zealand	C.F. Hill	—;DQ264751
<i>C. apiicola</i> CPC 10266	<i>Apium</i>	Venezuela	N. Pons	—;DQ264753
<sup>b</sup> CPC 10267; CBS 116457	<i>Apium</i>	Venezuela	N. Pons	—;DQ264752
<i>C. berteroeae</i> CPC 5090; CBS 538.71	<i>Berteroea</i>	Romania	O. Constantinescu	—;DQ264754
<i>C. beticola</i> CPC 5065; CBS 548.71	<i>Malva</i>	Romania	O. Constantinescu	—;DQ264755
CPC 5069; CBS 125.31	<i>Beta</i>	Japan	—	—;DQ264756
CPC 5128	<i>Beta</i>	New Zealand	C.F. Hill	—;DQ264757
CPC 5125	<i>Beta</i>	New Zealand	C.F. Hill	DQ264738; —
<sup>b</sup> CPC 12190	<i>Beta</i>	Germany	S. Mittler	—;DQ192582
<sup>b</sup> CPC 12191	<i>Beta</i>	Germany	S. Mittler	DQ192581; —
<i>C. canescens</i> CPC 1138; CBS 111134	<i>Vigna</i>	South Africa	S. van Wyk	DQ264739; —
<i>C. erysimi</i> CPC 5361; CBS 115059	<i>Erysimum</i>	New Zealand	C.F. Hill	DQ264740; —
<i>C. ipomoeae-pedis-caprae</i> CPC 10094	<i>Ipomoea</i>	Korea	H.D. Shin	—;DQ264758
<i>C. kikuchii</i> CPC 5067; CBS 135.28	<i>Glycine</i>	Japan	H.W. Wollenweber	DQ264741; —
<i>C. lactucae-sativae</i> CPC 10082	<i>Ixeris</i>	Korea	H.D. Shin	—;DQ264759
<i>C. malvacearum</i> CPC 5066; CBS 126.26	<i>Malva</i>	—	C. Killian	DQ264742; —
<i>C. modiolae</i> CPC 5115	<i>Modiola</i>	New Zealand	C.F. Hill	—;DQ264760
<i>C. penzigii</i> CPC 4001	<i>Citrus</i>	Swaziland	M.C. Pretorius	DQ264743; —
CPC 4410; CBS 115482	<i>Citrus</i>	South Africa	M.C. Pretorius	DQ264744; —
<i>C. polygonaceae</i> CPC 10117	<i>Persicaria</i>	Korea	H.D. Shin	DQ264745; —
<i>C. violae</i> CPC 5079; CBS 251.67	<i>Viola</i>	Romania	O. Constantinescu	DQ264746; —
<i>C. zae-maydis</i> <sup>b</sup> CBS 117758	<i>Zea</i>	Iowa, U.S.A.	B. Fleener	DQ264747; —
<sup>b</sup> CBS 117760	<i>Zea</i>	Pennsylvania, U.S.A.	B. Fleener	—;DQ264761
<i>C. zeina</i> <sup>b</sup> CPC 11995	<i>Zea</i>	South Africa	P. Caldwell	—;DQ264762
<sup>b</sup> CPC 11998	<i>Zea</i>	South Africa	P. Caldwell	DQ264748; —

Table 1 (continued)

Accession Nos. <sup>a</sup>	Host genus	Origin	Collector	GenBank No. <i>MAT1-1-1</i> ; <i>MAT1-2</i>
<i>Cercospora</i> sp.				
CPC 5126	<i>Oenothera</i>	New Zealand	C.F. Hill	—;DQ264763
CPC 10627	<i>Delairea</i>	South Africa	C.L. Lennox	—;DQ264764
CPC 12062	<i>Zea</i>	South Africa	—	—;DQ264765

<sup>a</sup> CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Collection of Pedro Crous, housed at CBS.

<sup>b</sup> Strains used for characterization of full-length *MAT1-1-1* and *MAT1-2* sequences.

was used to predict the gene and intron/exon boundaries using the genetic code of *Neurospora crassa*. The conversion of DNA sequences to putative amino acid sequences was done using the translate tool of ExPASy (Gasteiger et al., 2003). The percentage identities between the predicted *MAT1-1-1* and *MAT1-2* gene sequences for the different *Cercospora* species were calculated using the alignment tool of ALIGN (Pearson et al., 1997).

#### 2.4. Obtaining partial MAT sequences of additional *Cercospora* isolates

*Cercospora*-specific primers for the mating type genes were designed from the aligned sequences of *C. apii*, *C. apiicola*, *C. beticola*, *C. zea-maydis*, and *C. zeina*. The aligned *MAT1-1-1* sequences included *C. beticola*, *C. apii*, *C. zea-maydis* and *C. zeina* (GenBank Accession Nos. DQ192581, DQ264736, DQ264747 and DQ264748, respectively). The aligned *MAT1-2* sequences included those of *C. beticola*, *C. apiicola*, *C. zea-maydis* and *C. zeina* (GenBank Accession Nos. DQ192582, DQ264752, DQ264761 and DQ264762, respectively). The sequences of each gene were aligned using MegAlign from the Lasergene package (DNASTAR). To robustly amplify partial *Cercospora* mating type genes, the primers CercosporaMat1f (5'-CTTGCACTGAGGACATGG-3') and CercosporaMat1r (5'-GAGGCCATGGTGAGTGAG-3') were designed from the conserved regions of the *MAT1-1-1* gene, and primers CercosporaMat2f (5'-GATNTACCNTCTCGA CCTC-3') and CercosporaMat2r (5'-CTGTGGAGCAGTG GTCTC-3') were designed from the conserved regions of the *MAT1-2* gene. Twenty-six additional *Cercospora* isolates representing species that belong to the *C. apii* complex (Table 1) were screened with the CercosporaMat1 and CercosporaMat2 primer sets in two separate amplification reactions.

For amplification of the *MAT1-1-1* and *MAT1-2* gene regions, primer concentrations were halved and the other reagent concentrations were as described above. The initial denaturation was done at 94 °C for 5 min, followed by 20 cycles of 94 °C (20 s), 58 °C (20 s) and 72 °C (50 s), followed by 20 cycles of 94 °C (20 s), 55 °C (20 s) and 72 °C (50 s). A final elongation step at 72 °C (5 min) was included. The obtained PCR products were visualized and sequenced as described above.

#### 2.5. Phylogenetic analyses and protein alignment

The partial *MAT1-1-1* and *MAT1-2* sequences of the *Cercospora* isolates were analyzed using the mating type

gene sequences of *M. graminicola* (GenBank Accession Nos. AF440399 and AF440398, respectively) and *S. passerinii* (GenBank Accession Nos. AF483193 and AF483194, respectively) as outgroup taxa. All phylogenetic analyses were done in PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford, 2003). Maximum parsimony and neighbor joining analyses were conducted as described by Groenewald et al. (2005). All sequences generated were deposited in GenBank (Table 1), and the alignments and trees were deposited in TreeBASE (TreeBASE Accession No. SN2529).

Amino acid sequences of the alpha domain (MAT1) and/or HMG domain (MAT2) of *M. graminicola* and *S. passerinii* were downloaded from NCBI's GenBank database. The downloaded amino acid sequences of both of the mating type proteins were aligned to that of the five *Cercospora* species using Sequence Alignment Editor v2.0a11 (Rambaut, 2002).

#### 2.6. Mating type distribution in *Cercospora* populations

The two primer sets, CercosporaMat1 and CercosporaMat2, were used in a multiplex PCR to screen for the presence of the two mating type genes in the *C. apii*, *C. apiicola*, *C. beticola*, *C. zea-maydis* and *C. zeina* populations. Reagent concentrations were as described above and all four primers were present at equal concentrations. The initial denaturation step was done at 94 °C for 5 min, followed by 40 cycles of 94 °C (20 s), 60 °C (30 s) and 72 °C (50 s); a final elongation step at 72 °C (5 min) was included. The products were separated on a 1% agarose gel and visualized as described above. The mating type frequency and the *MAT1-1/MAT1-2* ratios were calculated for each population.

### 3. Results

#### 3.1. *MAT1-1-1* isolation and characterization in *Cercospora* species

The MAT1-1F and MAT1-1R primers that were designed to amplify part of the *MAT1-1-1* of *M. graminicola* (Waalwijk et al., 2002) were not successful in amplifying the mating type 1 region of *C. beticola*. The degenerate primers, MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2, designed from the *M. graminicola*, *S. passerinii* and *M. fijiensis* sequences, amplified a fragment of 922 bp for three of the five *C. beticola* isolates tested (Fig. 1). The fragment obtained from strain CPC 12191 was sequenced,

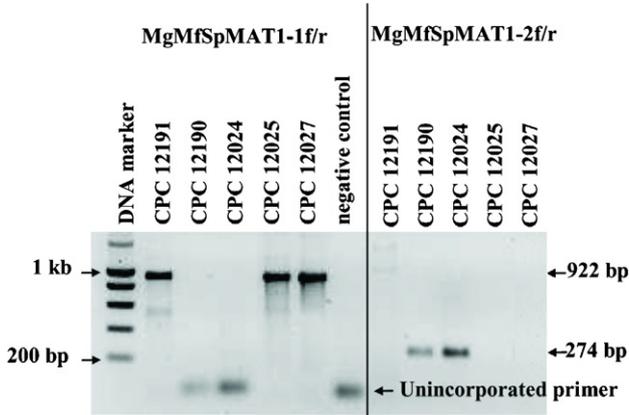


Fig. 1. Amplification products obtained from *Cercospora beticola* isolates containing the *MAT1-1-1* (922 bp) and *MAT1-2* (274 bp) genes using the degenerate primer pairs MgMfSpMAT1-1 and MgMfSpMAT1-2, respectively.

and the translated sequence showed 77% identity to a 57 amino acid region of the *S. passerinii* MAT1 protein and 54% identity to a 57 amino acid region as well as 34% identity to a 82 amino acid region of the *M. graminicola* MAT1 protein using Blastx on the GenBank database. This confirmed that the 922 bp fragment is part of the *MAT1-1-1* gene of *C. beticola*. A homologous fragment was also obtained from *C. apii*, *C. zea-maydis* and *C. zeina* isolates during the first round of amplification using the MgMfSpMAT11f1 and MgMfSpMAT11r2 degener-

Table 2

Percentage nucleotide identity across the whole *MAT1-1-1* (upper right triangle) and *MAT1-2* (lower left triangle) genes between the *Cercospora* species studied

	<i>C. zea-maydis</i>	<i>C. zeina</i>	<i>C. apiicola</i>	<i>C. apii</i>	<i>C. beticola</i>
<i>C. zea-maydis</i>	—	92.6	NA	87.4	87.3
<i>C. zeina</i>	74.5	—	NA	87.3	87.2
<i>C. apiicola</i>	70.3	90.8	—	NA	NA
<i>C. apii</i>	NA	NA	NA	—	99.9
<i>C. beticola</i>	90.2	70.6	76.4	NA	—

NA = not available due to the absence of the specific gene in the isolates tested.

ate primers. The *C. apiicola* population of 47 isolates, as well as 11 additional *C. apiicola* isolates, that were obtained from Greece, Korea and Venezuela and used in previous studies by Groenewald et al. (2005, 2006), were screened for the presence of the mating type genes, but all isolates were found to only contain the *MAT1-2* gene.

The full-length *MAT1-1-1* gene sequences for all four *Cercospora* species were obtained by chromosome walking. The geneid software predicted that the *MAT1-1-1* sequences of all four species contain four exons (Fig. 2). Although the number of amino acids was the same for all three species (335 aa), several differences were observed between the *MAT1-1-1* of the two maize pathogens and that of *C. apii* and *C. beticola*. The predicted length of the genes, as well as exon and intron positions are illustrated in Fig. 2 and the percentage sequence similarities between the different *Cercospora* species are listed in Table 2. Perfect

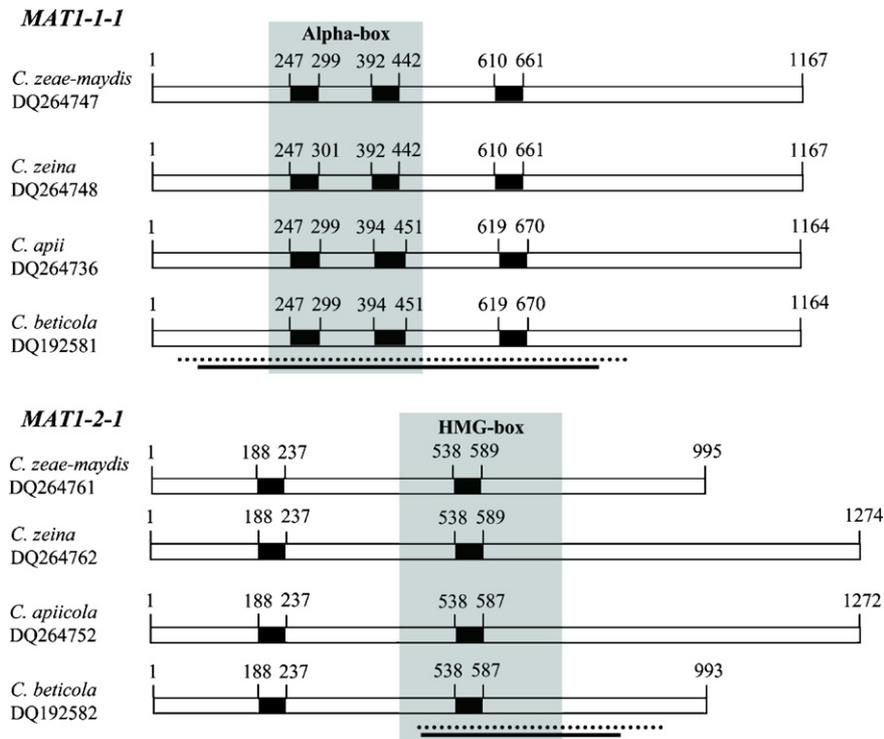


Fig. 2. Diagrammatic representation of the full-length mating type genes of *Cercospora zea-maydis*, *C. zeina*, *C. apiicola*, *C. apii* and *C. beticola*. The predicted sites of exons (white bars), and introns (black bars) are shown, and their locations (nucleotide position) are indicated. The lines at the bottom of each diagram indicate the area amplified by the *CercosporaMat1* and *CercosporaMat2* primer sets (dotted line) and the area used for the phylogenetic analyses (solid black line).

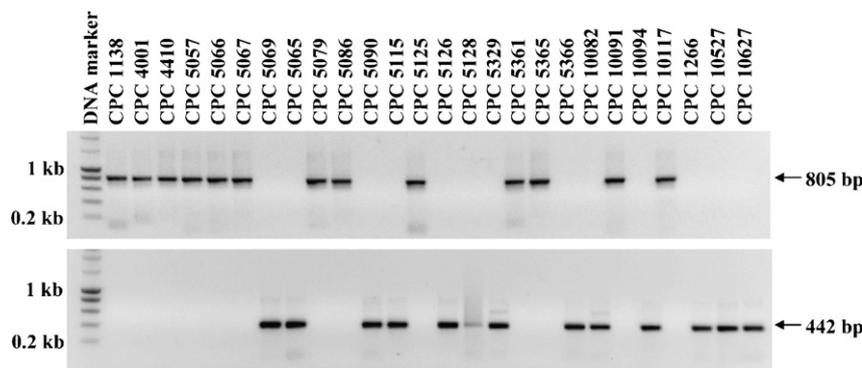


Fig. 3. Different *Cercospora* species screened using the CercosporaMat1 primer set (805 bp fragment; top part of photo) and the same *Cercospora* isolates screened with the CercosporaMat2 primer set (442 bp fragment; lower part of photo).

ariat sequences (RCTRAC) (Bruchez et al., 1993) were present in the introns of all four *Cercospora* species, except in the first intron of *C. beticola* and *C. apii*, that contained a GCTGAT sequence starting at 16 nt upstream from the likely 3' splice site. The number of predicted introns (two) in the conserved alpha domain region of the *Cercospora* species studied correlates with the number predicted for the same region in *M. graminicola* (Waalwijk et al., 2002) and *S. passerinii* (Goodwin et al., 2003).

### 3.2. MAT1-2 isolation and characterization in *Cercospora* species

The MAT1-2 region in the *C. beticola* genome could not be amplified using the MAT1-2F and MAT1-2R primers of *M. graminicola* (Wang et al., 1998) nor using the degenerate ChHMG1 and ChHMG2 primers of Arie et al. (1997). The degenerate primers (MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1) designed in this study resulted in a 274 bp PCR product in those *C. beticola* isolates of the test panel which did not amplify with the MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 primers (Fig. 1). The fragment obtained from CPC 12190 was sequenced and the translated sequence showed 59% identity to a 76 amino acid region of the *S. passerinii* MAT2 protein and 61% identity to a 76 amino acid region of the *M. graminicola* MAT2 protein using Blastx. This confirmed that a part of the MAT1-2 gene of *C. beticola* had been amplified using the newly developed degenerate primers.

A 274 bp fragment was also amplified in three of the additional four *Cercospora* species (*C. apiicola*, *C. zeaemaydis* and *C. zeina*) using the degenerate primers. A *C. apii* population of 32 isolates as well as 17 additional *C. apii* isolates, that were obtained from different countries and used in previous studies by Groenewald et al. (2005, 2006), were screened for the presence of the mating type genes, but only the MAT1-1-1 gene was found. The sequence of these products corresponded with the MAT1-2 sequence found for *C. beticola*. Chromosome walking enabled us to obtain the full-length MAT1-2 genes of *C. apiicola*, *C. beticola*, *C. zeaemaydis* and *C. zeina*. The predicted length of the genes, as well as exon and intron positions are illustrated in Fig. 2.

Both introns in all four MAT1-2 genes contain a perfect lariat sequence (RCTRAC). The predicted presence of a single intron in the conserved HMG domain region of the *Cercospora* species corresponded with the predicted intron for the same region in *M. graminicola* (Waalwijk et al., 2002) and *S. passerinii* (Goodwin et al., 2003).

The percentage sequence identities between the different *Cercospora* species are listed in Table 2. Because the putative MAT1-2 gene of *C. beticola* and *C. zeaemaydis* is much shorter than that of the other species, the similarities among the MAT1-2 sequences vary greatly. The high similarity (90.2%) between *C. zeaemaydis* and *C. beticola* is largely due to their similarity in number of nucleotides. The number of amino acids predicted for the MAT2 protein of *C. beticola* and *C. zeaemaydis* was 299, whereas for *C. zeina* and *C. apiicola* it was 392 amino acids.

### 3.3. Partial MAT1-1-1 and MAT1-2 sequences from additional *Cercospora* species

The *Cercospora*-specific mating type primer sets CercosporaMat1 and CercosporaMat2 were successful in amplifying a portion (location indicated with a dashed black line in Fig. 2) of the MAT1-1-1 or the MAT1-2 genes, respectively, of 26 additional *Cercospora* isolates representing 17 putative species. The primer pair CercosporaMat1f and CercosporaMat1r amplified a fragment of approximately 805 bp in half of the isolates tested, and the CercosporaMat2f and CercosporaMat2r primer set a 442 bp fragment in the rest of the isolates (Fig. 3). These sequences, which included the alpha and the HMG domain, respectively, were aligned with the corresponding MAT regions of the *Cercospora* species characterized in this study. The sequences were of relatively high similarity, even in the variable regions flanking the conserved domains (alignments available in TreeBASE Accession No. SN2529).

### 3.4. Phylogenetic analyses of nucleic acid sequences

The MAT1-1-1 alignment (TreeBASE Accession No. SN2529) contained 19 taxa, including the two outgroups, and 702 characters, including alignment gaps. Of these characters,

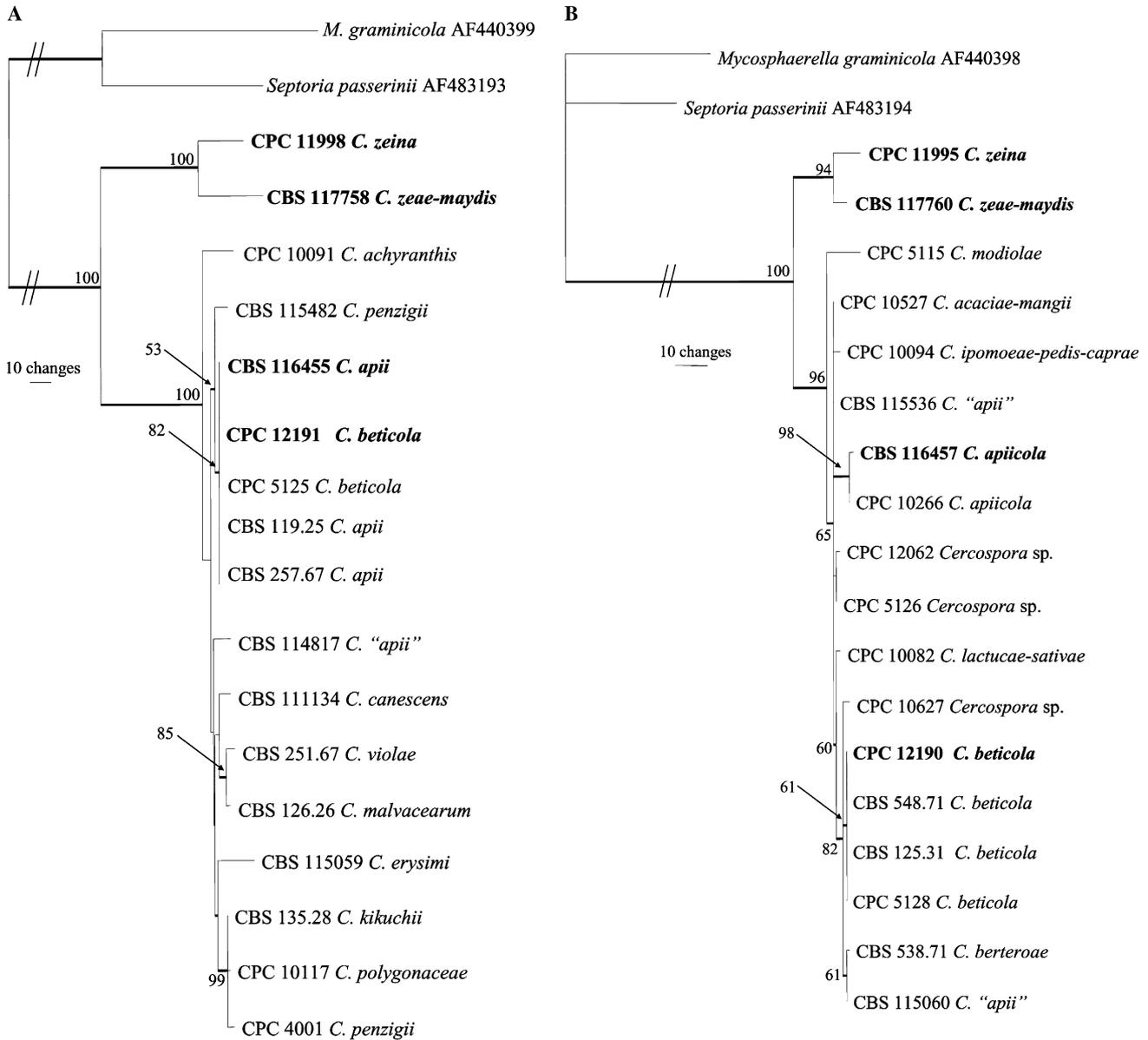


Fig. 4. (A) One of five most parsimonious trees obtained from the *MATI-1-1* sequence alignment. Bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to *Mycosphaerella graminicola* (AF440399) and *Septoria passerinii* (AF483193) (tree length = 622 steps; CI = 0.904; RI = 0.857 and RC = 0.774). (B) One of three most parsimonious trees obtained from the *MATI-2* sequence alignment. Bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to *M. graminicola* (AF440398) and *S. passerinii* (AF483194) (tree length = 247 steps; CI = 0.943; RI = 0.917 and RC = 0.865). Thickened lines indicate the strict consensus branches. Labels in bold represent species for which full-length genes were sequenced.

290 were constant, 139 were variable and parsimony-uninformative, and 273 characters were parsimony-informative. The *MATI-2* alignment (TreeBASE Accession No. SN2529) contained 20 taxa, including the two outgroups, and 362 characters, including alignment gaps. Of these characters, 181 were constant, 68 were variable and parsimony-uninformative, and 113 characters were parsimony-informative.

Similar trees were obtained irrespective of whether neighbor joining or parsimony was used. Five most parsimonious trees were obtained from the *MATI-1-1* sequences, and three most parsimonious trees were obtained from the *MATI-2* sequences. The most parsimonious trees differed somewhat in the arrangement of the

taxa within the clade containing the *C. apii* complex (Fig. 4). Limited variation was observed among the isolates belonging to the *C. apii* complex, and these isolates clustered together with bootstrap support values of 100% (*MATI-1-1*) and 96% (*MATI-2*). The trees obtained for both the *MATI-1-1* and *MATI-2* datasets showed that the two isolates that do not belong to the *C. apii* complex, namely *C. zae-maydis* and *C. zeina*, group together with a 100% bootstrap support for *MATI-1-1* and 94% bootstrap support for *MATI-2*. The phylogenetic trees obtained from these sequences are congruent with the main groupings of the housekeeping gene trees published for the *Cercospora* species (Crous et al., 2006; Groenewald et al., 2005, 2006).

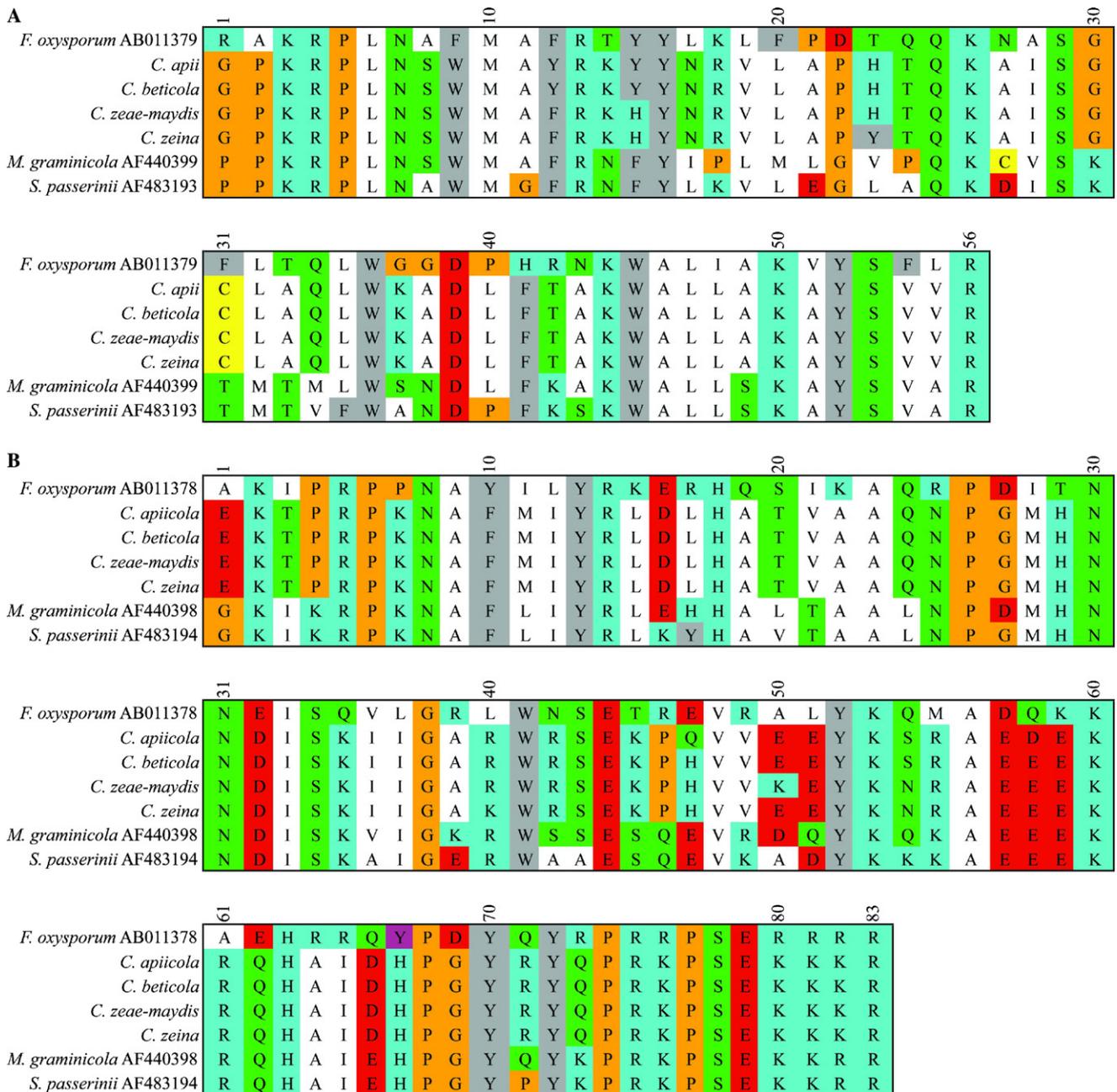


Fig. 5. Protein sequence alignments of the conserved A, alpha domain and B, HMG domain of the mating type genes of *Cercospora* species and closely related fungi.

The *MATI-1-1* phylogeny showed that all the isolates from *C. apii* (CBS 116455, CBS 119.25 and CBS 257.67) and *C. beticola* (CPC 5125 and CPC 12191) group together with a bootstrap support value of 82% (Fig. 4A). The unnamed *Cercospora* sp. from maize (CPC 12062) did not group with the other maize isolates in the *MATI-2* analysis, but it did group with the rest of the *Cercospora* isolates with a bootstrap support value of 96% (Fig. 4A). The analyses of the *MATI-1-1* sequences showed that the isolate from *Helianthemum* (CBS 257.67) identified as *C. apii* in an earlier study (Groenewald et al., 2006) grouped together with the other *C. apii* isolates obtained from celery (CBS 116455 and CBS 119.25) (Fig. 4B). The analysis

using the *MATI-2* dataset showed that the isolate from *Malva* (CBS 548.71) and identified as *C. beticola* using sequence data (Groenewald et al., 2006) grouped with the *C. beticola* isolates (CBS 125.31, CPC 5128, CPC 12190) from sugar beet (Fig. 4B).

### 3.5. Comparison of predicted amino acid sequences

The predicted amino acid sequences in the alpha (MAT1) and HMG (MAT2) domain showed very high similarity among the four *Cercospora* species (Fig. 5A). For the alpha domain only three amino acid changes were detected between *C. beticola* and *C. zeina*, and only two

between *C. beticola* and *C. zeae-maydis*. The amino acid compositions of the alpha domain of *C. beticola* and *C. apii* were identical. For the HMG domain, two amino acid changes were predicted between *C. beticola* and each of *C. zeae-maydis*, *C. apiicola* and *C. zeina* (Fig. 5B). The *C. beticola* predicted amino acid sequences showed moderate identity (Fig. 5) to the alpha domain (MAT2) and HMG domain (MAT2) regions of *S. passerinii* (53.6% and 67.5%, respectively) and *M. graminicola* (57.1% and 67.5%, respectively).

### 3.6. Distribution of *MATI-1-1* and *MATI-2* in *Cercospora* populations

A total of 255 *C. beticola* isolates (46 from France, 41 from Germany, 33 from Italy, 48 from The Netherlands, 50 from Iran and 37 from New Zealand) were screened with a multiplex PCR assay using primer pairs *CercosporaMAT1* (805 bp fragment) and *CercosporaMAT2* (442 bp fragment). Each tested isolate showed either the 442 bp fragment or the 805 bp fragment of the respective *MATI-1-1* or *MATI-2* genes, and no isolate showed both fragments. The *MATI-1-1* and *MATI-2* genes were equally distributed in most of the *C. beticola* populations. The ratios were in most cases near to 1.00 (0.85–1.19), except for the Italian population, in which a ratio of 0.50 was found (Table 3). There was no significant deviation ( $P < 0.05$ ) from a 1:1 ratio for the *MATI-1-1*:*MATI-2* ratio calculated for each of the populations tested.

A total of 43 *C. zeae-maydis*, 49 *C. zeina*, 32 *C. apii* and 47 *C. apiicola* isolates were screened for the presence of the mating type genes, and no isolate showed both fragments. The *MATI-1-1* and *MATI-2* genes were distributed in the *C. zeae-maydis* and *C. zeina* populations at observed *MATI-1-1*:*MATI-2* ratios of 0.95 and 1.58, respectively, which did not differ ( $P < 0.05$ ) from the expected 1:1 ratio based on Chi-square analyses (Table 3). All of the *C. apiicola* isolates obtained from Venezuela were found to be

*MATI-2*, whereas all the *C. apii* isolates obtained from Germany were found to be *MATI-1-1*.

## 4. Discussion

Very little is known about the occurrence or importance of sex in apparently asexual species of *Cercospora*. During this study the mating type genes of a sugar beet pathogen, *C. beticola*, two celery pathogens, *C. apii* and *C. apiicola*, and two maize pathogens, *C. zeae-maydis* and *C. zeina*, were sequenced and characterized. The degenerate primer sets MgMfSpMAT1-1 and MgMfSpMAT1-2 successfully amplified a portion of the mating type genes, and these sequences led to the characterization of the full-length *MATI-1-1* and/or *MATI-2* sequences of *Cercospora* species. Preliminary data reveal that these degenerate primer sets can also amplify the corresponding areas within the mating type genes of other species belonging to the *Mycosphaerellaceae* and allied *Davidiellaceae*. These species include some important pathogens of pines (*Dothistroma pini*, *D. septosporum*), tomatoes (*Passalora fulva*), bananas (*M. musicola*, *M. musae*), eucalypts (*M. marksii*, *M. thailandica*), or are important as agents in human health or food spoilage (*Cladosporium herbarum*), and will be treated elsewhere in future studies.

The *MATI-1-1* gene characterized during this study contains an area that corresponds to a putative alpha domain of *MATI-1-1*, and DNA sequences in the *MATI-2* gene correspond to the HMG domain described from other ascomycetes. As illustrated in this and other studies, these two domains are also found in the mating type genes of a wide range of ascomycetes. The putative introns in these domains of the *Cercospora* mating type genes are also found in *M. graminicola* and *S. passerinii* (Goodwin et al., 2003; Waalwijk et al., 2002). However, additional introns are predicted in the areas flanking the conserved boxes of each of the respective genes for *Cercospora*. The number of putative introns also varies for the *MATI-1-1* and *MATI-2* genes of other ascomycetes. Species containing only one

Table 3  
Occurrence and frequency of the *MATI-1-1* and *MATI-2* genes in *Cercospora* populations

Populations (country; region)	N <sup>a</sup>	<i>MATI-1-1</i>	<i>MATI-2</i>	Ratio <sup>b</sup>	$\chi^2$ <sup>c</sup>	P <sup>d</sup>
<i>C. beticola</i> (France; Longvic)	46	25 (0.54)	21 (0.46)	1.19	0.35	0.55
<i>C. beticola</i> (Germany; Niedersachsen)	41	22 (0.54)	19 (0.46)	1.16	0.22	0.64
<i>C. beticola</i> (Italy; Ravenna)	33	11 (0.33)	22 (0.67)	0.50	3.77	0.05
<i>C. beticola</i> (Netherlands; Bergen op Zoom)	48	22 (0.46)	26 (0.54)	0.85	0.33	0.57
<i>C. beticola</i> (Iran; Pakajik)	50	26 (0.52)	24 (0.48)	1.08	0.08	0.78
<i>C. beticola</i> (New Zealand; Unknown)	37	19 (0.51)	18 (0.49)	1.06	0.03	0.86
<i>C. zeae-maydis</i> (USA; Iowa)	43	21 (0.49)	22 (0.51)	0.95	0.02	0.89
<i>C. zeina</i> (South Africa; KwaZulu-Natal)	49	30 (0.61)	19 (0.39)	1.58	2.5	0.11
<i>C. apiicola</i> (Venezuela; Caripe)	47	0 (0)	47 (1)	e	62.67	<0.001
<i>C. apii</i> (Germany; Baden Württemberg)	32	32 (1)	0 (0)	f	58.33	<0.001

The numbers in brackets represent the frequency of the gene.

<sup>a</sup> Number of isolates analyzed.

<sup>b</sup> *MATI-1-1*:*MATI-2* ratio.

<sup>c</sup>  $\chi^2$  value for the deviation from the expected 1:1 ratio.

<sup>d</sup> Probability of a greater  $\chi^2$  value under the null hypothesis of 1:1 ratio (1 degree of freedom).

<sup>e</sup> *MATI-1-1* was not detected in *C. apiicola*.

<sup>f</sup> *MATI-2* was not detected in *C. apii*.



putative intron in both of these genes include *Alternaria alternata* (Arie et al., 2000), *Ascochyta rabiei* (Barve et al., 2003), *Cochliobolus heterostrophus* (Turgeon et al., 1993) and *Pyrenopeziza brassicae* (Singh and Ashby, 1998; Singh and Ashby, 1999). *Fusarium oxysporum* (Arie et al., 2000), *Giberella fujikuroi* and *G. zeae* (Yun et al., 2000) have two introns in the *MAT1-2* region, whereas *Ophiostoma novo-ulmi* has one intron in the *MAT1-2* gene (Paoletti et al., 2005). The putative intron splicing sites and gene predictions of only a few filamentous ascomycetes, e.g., *A. alternata* (*MAT1-1-1* and *MAT1-2*), *F. oxysporum* (*MAT1-1-1* and *MAT1-2*) and *O. novo-ulmi* (*MAT1-2*), have been confirmed by mRNA studies. Further studies at the mRNA and protein level are necessary to confirm the exact length of the coding regions as well as the intron and exon boundaries for the mating type genes of the *Cercospora* species.

The predicted length of the encoded proteins among different *MAT1-1-1* and *MAT1-2* genes of ascomycetes varies greatly (Goodwin et al., 2003; Pöggeler, 2001). Usually the MAT1 protein is much larger than the MAT2 protein of the same species. However, this is not the case for *M. graminicola*, where the predicted MAT1 protein (296 amino acids) is smaller than the predicted MAT2 protein (394 amino acids) (Waalwijk et al., 2002), and for *C. zeina* (predicted MAT1 = 339 amino acids and MAT2 protein = 392 amino acids).

Most protein coding genes used in previous taxonomic studies of *Cercospora* lack resolution to distinguish closely related *Cercospora* species (Groenewald et al., 2005, 2006). This study is the first to conduct phylogenetic analyses of partial mating type genes to determine whether they have sufficient discriminatory resolution between closely related *Cercospora* species, particularly those included in the *C. apii* complex. The *Cercospora* mating type-specific primer sets (CercosporaMat1 and CercosporaMat2) amplifies the three introns of *MAT1-1-1* and the intron that is present in the HMG domain of the *MAT1-2*. One of the biggest problems encountered when using *MAT* genes in phylogenetic analyses is that sometimes only one mating type is known in the species, or only one isolate of a species is available, and this isolate carries only one of the two mating type genes. This was the case for most of the *Cercospora* species tested, and these taxa could only be compared to taxa with sequences of the same mating type. Another problem is that the *MAT* gene sequences differ a great deal among different genera and even among species of the same genus. This may restrict analyses to related species and to only a small portion of the gene, specifically, to the more conserved regions (alpha or HMG domains) of these genes. The conserved regions may lack the resolution to distinguish among closely related species, as was the case within the group of isolates belonging to the *C. apii* complex and it is clear that the *MAT1-1-1* sequences cannot separate *C. apii* and *C. beticola*. Mating type genes therefore do not appear to represent promising loci for phylogenetic studies aimed at distinguishing cryptic species belonging to the *C. apii* complex.

Both mating type genes have been isolated from strains of *C. beticola*, *C. zeae-maydis* and *C. zeina*. The *Cercospora* mating type-specific primer sets (CercosporaMat1 and CercosporaMat2) can be used in a multiplex PCR assay for amplification of these two genes in *Cercospora* populations. The two mating types are approximately evenly distributed within the six sampled populations of *C. beticola* as well as in the *C. zeae-maydis* population in the USA and in the *C. zeina* population in South Africa, suggesting that the genes may be functional in these populations. If *C. beticola*, *C. zeae-maydis* and *C. zeina* were strictly asexual, we would expect that with time there would be a skewed distribution of the mating types, or perhaps only a single mating type would be found. Also, if these populations arose from a human introduction of a single genotype, we might expect only one mating type to be present, as was found for the *C. apii* and *C. apiicola* populations. The presence of both mating type genes in the USA population of *C. zeae-maydis* and the South African population of *C. zeina* further strengthens the hypothesis (Crous et al., 2006; Dunkle and Levy, 2000) that these species are native to North America and Africa, respectively. Though the teleomorph has not been confirmed for these three *Cercospora* species, we would expect their teleomorphs to be in the genus *Mycosphaerella*. Detailed analyses have been done on the distribution of the mating types of *M. graminicola* and an equal distribution of the mating types were found in different populations of this sexually reproducing fungus (Waalwijk et al., 2002; Zhan et al., 2002). It is therefore probable that these *Cercospora* species that contain both mating types, are also able to reproduce sexually, but that the teleomorph is not readily observed in nature nor induced under laboratory conditions. However, Halliday and Carter (2003) found segregation of the mating types in natural populations of *Cryptococcus gattii* but, on studying the population structure using AFLP fingerprinting, did not find any evidence supporting genetic exchange between members of the population. These results indicated a clonal population structure even though both mating types were present. All attempts to obtain successful matings between these isolates failed, and the authors concluded that heterogeneity in genome composition resulted in mating incompatibility which gave rise to the clonal population structure (Halliday and Carter, 2003). Contrary to Halliday et al. (1999), who found severely skewed distributions of up to 30:1 for the mating types of some *Cryptococcus gattii* populations, all the *Cercospora* populations we sampled containing both mating types favored a 1:1 ratio, being more consistent with the distribution pattern observed for the sexually reproducing *M. graminicola*. A detailed study on the genetic population structure and the genome composition (for example chromosome number and genome size) of the *Cercospora* species characterized in this study is needed to further evaluate the effect of mating type distribution in these species.

Only the *MAT1-2* gene was present in the *C. apiicola* isolates tested, including isolates from Korea and Greece that were used in previous studies (Groenewald et al., 2005, 2006), as well as a field population of 47 isolates from Venezuela. Although it is possible that a *MAT1-1* gene may exist for this species, these data suggest that it would rarely occur, if it were to be present. Without sexual recombination, a species may not be able to rapidly evolve, and it is subsequently more difficult for these species to easily adapt to different environmental conditions. Alternatively, *C. apiicola* may be native to another part of the world, and the sampled populations may be introductions of a single mating type. The tested isolates of *C. apii sensu stricto* contained only the *MAT1-1* gene. Based on our current sampling, we predict that *C. apii* is asexual. However, more populations need to be studied, but due to the cultivation of celery under controlled greenhouse conditions we were unsuccessful in obtaining more populations. Unlike *C. apiicola*, *C. apii* has an extremely wide host range (Crous and Braun, 2003; Groenewald et al., 2006). The geographic origin of *C. apii* is Western Europe, whereas *C. apiicola* was originally described from Korea and Venezuela (Groenewald et al., 2005). Recently, Groenewald et al. (2006) showed that *C. apiicola* also occurs in Europe (i.e. Greece). As only one mating type has until now been found for *C. apii* (MAT1) and *C. apiicola* (MAT2), it is possible that these two species lack the ability to reproduce sexually due to the absence of the opposite mating type. If these species are homothallic, they will still be able to reproduce sexually. Our attempts to induce mating between isolates of *C. apii* have failed. In the sexually reproducing basidiomycetous yeast *Cryptococcus neoformans*, laboratory matings produce offspring with an equal distribution of the mating types (Kwon-Chung, 1976). However, in environmental and clinical isolates the majority of isolates belong to one mating type; yet they still retain their sexual reproductive potential by means of fruiting, a process of diploidization followed by reduction to haploid basidiospores which results in a high rate of recombination (Lin et al., 2005). Similar methods of sexual recombination have not yet been observed or reported for the *Cercospora* species characterized here, and strictly asexual reproduction can not be ruled out.

Mating type genes play an important part in the biology and evolution of fungal species. Knowledge of these genes can provide insight in the potential prevalence of sex in species of *Cercospora*, the majority of which are currently thought to be asexual. The primers that were developed during this study allowed us to determine and characterize the mating type genes of several agronomically important *Cercospora* species. The even distribution of the mating types for most species studied here do not favor asexual reproduction; however, further studies are needed to determine whether recombination is taking place. The primers designed here will allow the identification and characterization of mating type genes, or portions thereof, of other important *Cercospora* species and other members of the *Mycosphaerellaceae*.

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## Indirect evidence for sexual reproduction in *Cercospora beticola* populations from sugar beet

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*Cercospora beticola* is the main causal agent of cercospora leaf spot on sugar beet and has a large negative impact on the yield and quality of sugar beet production worldwide. Previous studies have shown that both mating type idiomorphs of *C. beticola* are present in natural populations, suggesting that *C. beticola* is heterothallic and may be reproducing sexually. *Cercospora beticola* isolates are diverse in the morphology of their conidia, onset of disease symptoms and fungicide resistance. To find the source of this diversity and to determine if sexual reproduction occurs in this fungus, *C. beticola* populations were collected from Western Europe, Iran and New Zealand. The mating types of these isolates were determined and AFLP analyses were used to study the genetic diversity in these populations. The mating type ratios did not deviate significantly from a 1:1 ratio in most of the populations and AFLP analyses showed high levels of genetic variation within and between the populations, with 86.4% of the isolates having unique genotypes. All populations were in significant linkage disequilibrium but levels of disequilibrium were low, and loci from only one primer pair were in significant gametic equilibrium in populations from the Netherlands and Italy. From these results there is the possibility that *C. beticola* reproduces sexually. High levels of gene flow among the samples from Europe demonstrated a single panmictic European population. This study confirms *C. beticola* to be a genetically highly diverse species, supporting the assumption that some populations are reproducing sexually.

**Keywords:** AFLP, *Beta vulgaris*, gene flow, genetic diversity, mating type idiomorphs, population structure

### Introduction

More than 3000 species have been named in the genus *Cercospora* (Pollack, 1987), which is currently regarded as one of the largest genera of hyphomycetes. Following the recent revision by Crous & Braun (2003), this number was significantly reduced to 659 species, with a further 281 species that are treated as morphologically indistinguishable from *C. apii sensu lato*. *Cercospora beticola* belongs to the *C. apii* complex (Crous & Braun, 2003) and is the main causal agent of cercospora leaf spot of sugar beet (Saccardo, 1876; Groenewald *et al.*, 2005, 2006a). Some confusion existed in the past about whether *C. beticola* and *C. apii*, the main leaf spot causing agent of *Apium* species, are synonymous. Groenewald *et al.* (2005) conducted a detailed study of the cultural characteristics, cardinal temperature requirements for growth

and molecular analyses to demonstrate that these two *Cercospora* species are indeed distinct.

*Cercospora beticola* is considered to be one of the most destructive foliar pathogens of sugar beet, causing yield losses of up to 40% (Shane & Teng, 1992; Holschulte, 2000). For most *Cercospora* species, including *C. beticola*, no sexual stage is known from nature and *in vitro* pairing studies have not been successful in producing a teleomorph for *C. beticola* (unpublished data). The genus *Cercospora* is a well-established anamorph of *Mycosphaerella* (Crous & Braun, 2003), and phylogenetic analyses on a variety of *Cercospora* species have placed them as a well-defined clade within *Mycosphaerella* (Crous *et al.*, 2001, 2006a, 2006b; Goodwin *et al.*, 2001). Therefore, if a sexual stage does exist for *C. beticola*, it would be a species of *Mycosphaerella*.

A wide array of phenotypic diversity has been described for *C. beticola* that includes variation in spore morphology and production, cultural characteristics, pathogenicity and fungicide resistance (Rossi, 1995; Moretti *et al.*, 2004). In fungi, gene diversity is not necessarily affected by the mating structure (McDonald, 1997), but sexually

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reproducing fungi usually have high levels of genotypic diversity and alleles among loci should be randomly associated (Milgroom, 1996). Even though phenotypic markers indicate high levels of variation, little is known about the genetic structure of *C. beticola* populations.

Recently a few studies attempted to determine the population genetic structure of *C. beticola* and a substantial amount of genetic variation was found within *Cercospora* strains isolated from sugar beet fields in Italy (Moretti *et al.*, 2006), and genetic variation was also observed in *C. beticola* isolates from lesions of the same plant (Moretti *et al.*, 2004). This is in contrast to the data available for other *Cercospora* species which have low levels of genetic diversity, e.g. *C. sorghii* (Okori *et al.*, 2004). This species also shows low genetic differentiation between populations from Uganda, suggesting a close genetic relatedness among populations (Okori *et al.*, 2004). Similarly, genetic variation among isolates of *C. zea-maydis* from Africa (Okori *et al.*, 2003) and the United States (Wang *et al.*, 1998; Crous *et al.*, 2006a) was also found to be low, with little genetic differentiation either within or between populations.

Mating type genes are often under frequency-dependent selection in randomly mating populations (Milgroom, 1996; May *et al.*, 1999). Mating type genes (*MAT1-1-1* and *MAT1-2*) of *C. beticola* were isolated and characterized to show that the fungus has a bipolar mating system (Groenewald *et al.*, 2006b). However, the putative intron splicing sites, gene predictions and functionality of these genes in *C. beticola* have not yet been confirmed and additional studies are necessary to show whether these genes are functional. Ascomycetes that are heterothallic have a single locus, two allele mating system which requires two nuclei of opposite mating types to fuse in order for sexual reproduction to occur (Kronstad & Staben, 1997). *Cercospora* mating type-specific primers were developed for use in a multiplex PCR to determine the frequencies of these idiomorphs in field populations (Groenewald *et al.*, 2006b). They found that mating types occurred in similar frequencies in *C. beticola* field populations, a phenomenon that is commonly accepted as indicative of random mating, such as in *Mycosphaerella graminicola* (Waalwijk *et al.*, 2002; Zhan *et al.*, 2002). Groenewald *et al.* (2006b) therefore suggested that some *Cercospora* species cannot be strictly asexual and that another method of reproduction has to occur to account for the frequency-dependent selection of the mating type genes observed within field populations.

Although previous studies showed that high levels of genotypic variation could be found in populations of *C. beticola* (Moretti *et al.*, 2004, 2006), these studies were all based on small sample sizes ( $N \leq 13$  per population). Knowledge of the distribution of the mating types, together with the amount of genotypic variation observed within a specific fungal population, can provide a strong indication whether or not sexual reproduction is likely to occur. The main objectives of this study were therefore to (i) determine the genetic structure of *C. beticola* populations with AFLPs, including genotypic diversity and

gametic disequilibrium, and (ii) to determine whether there is frequency-dependent selection on mating types. This knowledge will provide indirect evidence for the possible presence of a sexual cycle occurring in this fungus. In order to achieve these objectives populations from Western Europe, Iran and New Zealand were analysed.

## Materials and methods

### Fungal isolation and DNA extraction

*Beta vulgaris* leaves were sampled during the 2003 growing season from single sugar beet fields in four European countries (Netherlands, Germany, France and Italy) as well as in New Zealand (Table 1). The samples from Iran were collected during the 2004 growing season. The sampling was done in an X figure across each field. For each population, leaves with symptoms were collected from 10 plants in each leg of the cross. Single-spore isolations were made and cultures were established on 2% malt extract agar (MEA). The isolates were examined morphologically to confirm their identity as *C. apii sensu lato* as described by Crous & Braun (2003). All isolates were also screened with *C. beticola*-specific primers to confirm that they were truly *C. beticola* before being included in the analyses (Groenewald *et al.*, 2005). Isolates were cultured on MEA plates for 8 days at 24°C, and 200–400 mg mycelium were used in the DNA extraction using the FastDNA kit (BIO 101, Carlsbad) according to the manufacturer's instructions.

### Screening of markers

Degenerate mating type idiomorph primers designed by Groenewald *et al.* (2006b) were used to screen all isolates from the six *C. beticola* populations as described previously. AFLP analyses were performed according to Vos *et al.* (1995), with minor modifications as described by Groenewald *et al.* (2005). Genomic DNA (30 ng) from 250 isolates was digested with the restriction enzymes *EcoRI* and *MseI* and ligated to the corresponding adaptors. Four selective primer combinations were used, namely *EcoRI*-A-[FAM]/*MseI*-CT, *EcoRI*-AT-[JOE]/*MseI*-C, *EcoRI*-AG-[NED]/*MseI*-C and *EcoRI*-G-[JOE]/*MseI*-CG (Applied Biosystems), for the final amplification step. To test the reproducibility of the AFLP profiles, separate DNA extractions, PCR amplifications and AFLP analyses were performed in duplicate on 10 isolates (using the four

Table 1 *Cercospora beticola* populations included in this study

Country of origin	Sample size	Location	Collector
France (Fr)	46	Longvic	S. Garressus
Germany (Ger)	39	Niedersachsen	S. Mittler
Italy (It)	32	Ravenna	V. Rossi
Netherlands (Neth)	48	Bergen op Zoom	Unknown
New Zealand (NZ)	35	Unknown	C.F. Hill
Iran (Ir)	50	Pakajik	A.A. Ravanlou

primer combinations). An error rate of 1% (1 to 2 bands difference per isolate among 206 loci) was observed. Only polymorphic loci (78) were included in the analyses.

### Data analyses

The presence and absence of bands obtained from AFLP analyses were scored as 1 and 0, respectively, and these results were combined for the statistical analyses. Isolates were considered members of the same clone or clonal lineage if they had 99% similar bands. Clones identified with AFLPs which had different mating type idiomorphs were considered different haplotypes. To quantify genotypic variation within populations, the genotype richness was measured with a Shannon-Wiener index (Grünwald & Hoheisel, 2006).

To evaluate the associations among loci in each sample, the index of association ( $I_A$ ) and an unbiased estimate of multilocus linkage disequilibrium ( $\bar{r}_d$ ) were used.  $I_A$  and  $\bar{r}_d$  values were calculated by using Multilocus 1.3 software, and 1000 artificially recombined data sets were used to determine the statistical values of the test (Agapow & Burt, 2001). Significant departures from an expected 1:1 ratio in mating type frequencies were tested with a chi-squared test.

TFPGA (Miller, 1997) and POPGENE v1.32 (Yeh *et al.*, 1997) were used to analyse the 0/1 matrix. The population genetic analyses program TFPGA was used to calculate the gene diversity (Nei, 1978), percentage of polymorphic loci,  $F$ -statistics, genetic distances and the exact tests. The percentage polymorphic loci were based on 99% criteria. The population differentiation was calculated using the method of Weir & Cockerham (1984), jackknife over loci was done with 10 000 iterations using a confidence interval (C.I.) of 95%. Genetic distances between the populations were calculated using Wright's (1978) modification of Rogers' (1972) distance. For this study, a value of < 0.1 indicates small genetic distances, 0.10–0.15 indicates moderate genetic distances, 0.15–0.2 indicates high genetic distances and > 0.2 indicates very large genetic distances. A graphical representation of the genetic distance data (Nei, 1978) was done using the UPGMA algorithm. Bootstrap support values were calculated over all the loci using 1000 repetitions. The exact test was used to determine if significant differences in allele frequencies exist between populations (Sokal & Rohlf, 1995). The Markov Chain Monte Carlo approach that was used to calculate the exact test values gives an approximation of the exact probability of the observed differences in allele frequencies (Raymond & Rousset, 1995).

POPGENE was used to calculate the gene flow ( $Nm$ ) between any two populations, between the four Western European populations, between the five Eurasian populations and between all six populations. The grouping of populations into major geographic areas of Asia (Iran), Europe (Netherlands, France, Italy and Germany) and New Zealand allowed the analysis of variation (analysis of molecular variance, or AMOVA) at three levels: within

individual populations, between populations within geographic regions, and between geographic regions. All calculations, including random-permutation procedures to assess statistical significance, were performed using the GenALEX 6 package (Peakall & Smouse, 2005).

## Results

### AFLP markers

Moderate levels of polymorphism were obtained from the four AFLP primer combinations used in this study (Table 2). In total, 208 bands could be scored unambiguously. The number of polymorphic bands obtained from all six populations varied from 15 to 22 (Table 2) and the band sizes ranged from 50 to 500 base pairs. The AFLP primer sets *EcoRI*-AG/*MseI*-C amplified the largest number of polymorphic bands (22) whereas AFLP primer pair *EcoRI*-G/*MseI*-CG amplified the lowest number of polymorphic bands (15) (Table 2). The percentage polymorphic loci ranged from 20.9% in the New Zealand population to 30.6% in the German population (Table 2).

### Population genetic analyses

Genotypic diversity ( $H$ ) ranged from 3.25 (New Zealand) to 3.82 (France, Table 3). Among 250 isolates, 217 (86.4%) unique genotypes were obtained. Unique genotypes refer to isolates with dissimilar AFLP profiles, but also to isolates with identical AFLP profiles but different mating types.

Gene diversity ( $H$ ) is lowest in the New Zealand population (0.19) and highest in the German and Italian populations (0.27) (Table 3). The theta value shows high population differentiation (0.17) across the six populations, and moderate population differentiation across the four European populations (0.07) and five Eurasian populations (0.07) (Table 4). The pairwise comparisons of population differentiation between the New Zealand population and other populations was high (theta = 0.33–0.41), even though the New Zealand population had only two private alleles. The theta values from pairwise comparisons between the remaining populations varied between 0.02 (Dutch/Italian) and 0.13 (French/German).

**Table 2** The number of polymorphic bands analysed with four AFLP primer combinations on 250 *Cercospora beticola* isolates

Primer pair	No. of bands	No. of polymorphic bands <sup>a</sup>						All
		NZ	Fr	Ger	Ir	It	Neth	
<i>EcoRI</i> -A/ <i>MseI</i> -CT	54	14	14	16	14	16	16	21
<i>EcoRI</i> -AG/ <i>MseI</i> -C	52	11	16	17	17	16	15	22
<i>EcoRI</i> -G/ <i>MseI</i> -CG	52	8	11	13	12	12	12	15
<i>EcoRI</i> -AT/ <i>MseI</i> -C	48	10	11	17	9	14	16	21
Total	206	43	52	63	52	58	59	79
% Polymorphic loci		20.9	25.2	30.6	25.2	28.6	28.6	38.3

<sup>a</sup>NZ = New Zealand, Fr = France, Ger = Germany, Ir = Iran, It = Italy, Neth = Netherlands, All = total of all six populations.

Population	<i>n</i>	<i>H</i> <sup>a</sup>	<i>H</i> <sup>b</sup>	MAT frequency		$\chi^2$ <sup>c</sup>	<i>I</i> <sub>A</sub> <sup>d</sup>	$\bar{r}_d$ <sup>d</sup>
				Mat1-1-1	Mat1-2			
NZ	35 (27)	3.25	0.19	0.52	0.48	0.037	1.530*	0.037*
Fr	46 (46)	3.82	0.23	0.54	0.46	0.347	0.729*	0.015*
Ger	39 (32)	3.39	0.27	0.59	0.41	1.125	1.934*	0.032*
Ir	50 (43)	3.67	0.24	0.49	0.51	0.023	1.025*	0.021*
It	32 (32)	3.47	0.27	0.31	0.69	4.500*	0.377*	0.006*
Neth	48 (37)	3.29	0.25	0.57	0.43	0.675	0.214*	0.004*
Total	250 (217)	–	–	0.51	0.49	6.668	1.135*	0.016*

<sup>a</sup>Shannon – Wiener index for genotype richness.

<sup>b</sup>Gene diversity (Nei, 1978).

<sup>c</sup> $\chi^2$  value based on 1:1 ratio and 1 degree of freedom for clone corrected populations and 5 degrees of freedom for the contingency  $\chi^2$  analyses of the total data set. \*Indicates mating type frequencies which are significantly different at  $P < 0.05$ .

<sup>d</sup>\*indicates significant *I*<sub>A</sub> and  $\bar{r}_d$  values at  $P < 0.01$ .

**Table 4** Gene flow (*Nm*) (below diagonal) and theta (population differentiation, above diagonal) for pair-wise comparisons among the six *Cercospora beticola* populations, among all combined populations, and among populations from Europe or Eurasia

	NZ	Fr	Ger	Ir	It	Neth
NZ	–	0.41*	0.33*	0.40*	0.36*	0.36*
Fr	1.3	–	0.13*	0.08*	0.06	0.06
Ger	1.8	5.7	–	0.10	0.05	0.06
Ir	1.4	8.5	6.6	–	0.06	0.09
It	1.6	11.1	10.9	11.3	–	0.02
Neth	1.6	11.4	10.4	8.0	19.1	–
<i>Nm</i> (all populations)	2.2					
<i>Nm</i> (European)	6.8					
<i>Nm</i> (Eurasian)	5.8					
theta (all populations)	0.17*					
theta (European)	0.07*					
theta (Eurasian)	0.07*					

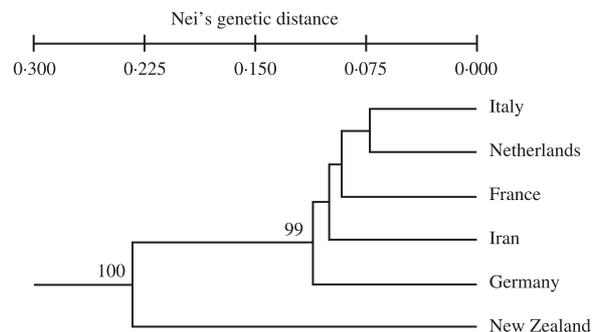
\* $P \leq 0.01$ , *P*-values obtained with 1000 randomizations in Multilocus v1.3.

The high gene flow (*Nm*) values of 6.8 and 5.8 across the four European and five Eurasian populations, respectively, indicate high genetic exchange between these populations, but *Nm* was low when the New Zealand population was included in the calculation (*Nm* = 2.2) (Table 4). Low *Nm* values (1.3–1.8) were observed between the New Zealand population and every other population analysed. The highest *Nm* values were obtained in pairwise comparisons between Italy and the Netherlands (*Nm* = 19.1), followed by Netherlands/France (*Nm* = 11.4) (Table 4).

AMOVA analyses revealed that the percentage of genetic variation among individuals within populations was 75%. Only 4% of the variation was due to differences among populations within a region (European populations) and 21% to differences among geographic regions.

In 14 cases, isolates with the same multilocus AFLP haplotype had different mating type idiomorphs. Mating type ratios did not deviate significantly from a 1:1 ratio

**Table 3** The number of isolates, (number of haplotypes), genotypic and gene diversity, tests of multilocus association and mating type frequencies of *Cercospora beticola* clone corrected populations



**Figure 1** Graphical representation of the genetic distance data (Nei, 1978) generated by UPGMA clustering in the software TFPGA. The scale bar shows the genetic distance, and bootstrap support values in percentage from 1000 replicates are shown at the nodes (only bootstrap support values of 70% and higher are shown).

suggesting frequency-dependent selection, except in the population from Italy where MAT1-2 isolates were more predominant (Table 3). Multilocus measures of association (*I*<sub>A</sub> = index of association and  $\bar{r}_d$  = multilocus linkage disequilibrium) were significant ( $P < 0.01$ ) for all populations (Table 3). All four loci showed significant ( $P < 0.01$ ) *I*<sub>A</sub> and  $\bar{r}_d$  values for the New Zealand, German and Iranian populations. Loci from only two primer combinations were in gametic disequilibrium for the population from France, and only one primer combination A/CT was in significant ( $P < 0.01$ ) gametic disequilibrium for the *C. beticola* populations from Italy and the Netherlands (data not shown).

### Cluster analysis

Figure 1 represents the genetic distance data obtained between populations using the TFPGA program with UPGMA clustering, and bootstrap support values from 1000 replicates are shown. Genetic distances between the New Zealand population and all other populations were high (0.22–0.25) (Fig. 1). The genetic distance values between the remaining populations were lower and varied

between 0.07 and 0.13. The Exact test showed significant differences between the New Zealand populations and the rest ( $P < 0.001$ ) as well as for the pair-wise comparison between populations of France/Germany ( $P = 0.02$ ).

## Discussion

This study is the first to report on the genetic structure and mating type distribution of *C. beticola* populations from different geographic localities. The results obtained from population differentiation, gene flow and genetic distance analyses suggest that the populations from Europe and Iran are genetically similar, whereas the New Zealand population is significantly different. High levels of genetic variation were found among the *C. beticola* isolates tested. This variation, illustrated by the high number of distinct haplotypes obtained with the AFLP analyses, compares well with earlier studies that also reported high levels of genetic variation among isolates obtained from the same lesion on a sugar beet plant in Italy (Moretti *et al.*, 2004), and between isolates from Italy (Moretti *et al.*, 2006). Most of the isolates that were obtained from one plant during the present study also had a distinct multilocus AFLP haplotype (data not shown). The sampling allowed partitioning of genetic variation and showed that most variation could be found within populations (75%), whereas only 4% of the variation was due to differences among populations within a region (European populations) and 21% to differences among geographic regions.

To date no teleomorph has been found for *C. beticola* (Groenewald *et al.*, 2006b) and the reproductive structure of this pathogen has been considered clonal. However, this study found high levels of genotypic diversity in all six populations analysed. It is known that populations that regularly undergo sexual reproduction should have many genotypes that result in higher levels of genotypic diversity compared to those that reproduce only asexually (Milgroom, 1996). This type of genetic structure is seen in most populations of *M. graminicola* (Linde *et al.*, 2002; Zhan *et al.*, 2003; Zhan & McDonald, 2004). Thus, the genotypic diversity observed for *C. beticola* is exceptionally high for a presumed asexually reproducing organism.

Milgroom (1996) and Zhan *et al.* (2002) found that a combination of high levels of genetic diversity and the equal distribution of mating types in a given population indicates that sexual recombination occurs. This study therefore screened for the presence and frequency of the mating type idiomorphs in the populations. The equal distribution of mating types in most populations (except Italy) suggests frequency-dependent selection and thus random mating. Both mating types could also be found on the same plant (data not shown), providing opportunity for genetic exchange. Thus, the high levels of genotypic diversity together with equal mating type ratios indicate that this fungus reproduces sexually. If *C. beticola* was strictly asexual, one would expect that, over time, there would be a skewed distribution of the mating types, or that only one mating type would be present, as was found for other *Cercospora* species such as *C. apii* and *C. apiicola*

(Groenewald *et al.*, 2006b). *Cercospora beticola* has been observed to form spermatogonia on leaf tissues collected during this study, which is also indicative of a possible sexual cycle, although any sexual stage that may exist is, so far, not readily observed in nature nor induced under laboratory conditions.

Tests for multilocus associations ( $I_A$  and  $\bar{r}_d$ ) showed that all six populations were in gametic disequilibrium. This suggests that asexual production is predominant and that random mating occurs only rarely, if at all. However, although significant, the values of  $I_A$  and  $\bar{r}_d$  were low for populations from Italy, France and the Netherlands. Furthermore,  $\bar{r}_d$  was similar or even lower in *C. beticola* (0.004–0.037) than that estimated for *Pyrenophora teres* f.sp. *teres* (0.037–0.039), which is known to undergo regular sexual recombination (Rau *et al.*, 2003). Furthermore, only one primer combination was in significant gametic disequilibrium in the population from Italy and the Netherlands. This contradicts results on frequency-dependent selection and levels of genotypic diversity which suggest populations undergo regular sexual recombination. There are two possible explanations for gametic disequilibrium in these populations. First, frequent population expansions during epidemics can result in populations dominated by closely related individuals (Maynard-Smith *et al.*, 2000). During epidemics, even though populations are recombining, genotypes may arise that are strongly favoured by selection. These genotypes will increase in frequency, generating disequilibrium until recombination has had time to randomize the genetic background (Maynard-Smith *et al.*, 2000), presumably at the end of the growing season when sexual reproduction is known to occur as a survival mechanism for many plant pathogens. Unless mating type idiomorphs are linked to pathogenicity factors or fungicide resistance, their frequency should by chance follow a 1:1 ratio during the epidemic. However, the AFLP loci used in this study were selectively neutral.

A second explanation for the observed gametic disequilibrium lies with the type of marker used. AFLPs often represent hypervariable regions that include dispersed repetitive elements (reviewed in Wong *et al.*, 2001), resulting in a co-dominant marker. Thus, conventional population genetic approaches to analyse AFLP data will underestimate the variability at each locus and overestimate the number of loci analysed, since each allele will be taken as an independent locus (Wong *et al.*, 2001). In a comparison between RFLP and hypervariable AFLP markers, Yan *et al.* (1999) showed that heterozygosity was underestimated in the yellow fever mosquito by AFLP markers, resulting in Hardy-Weinberg disequilibrium. The present results suggest that at least one AFLP primer pair (A/CT) amplified hypervariable regions since it was the only primer combination that showed significant gametic disequilibrium in all *C. beticola* populations analysed. Furthermore, in populations from Italy and the Netherlands, this was the only primer combination that resulted in loci (20 out of 78) in gametic disequilibrium. It is therefore suggested that at least the *C. beticola* populations from Italy and the Netherlands are in gametic equilibrium.

The high level of genotypic variation in *C. beticola* can also be explained by other factors. First, it is possible that *C. beticola* reproduced sexually prior to modern agricultural practices (e.g. burying of plant material during soil cultivation) which prevents sexual reproduction at the end of the growing season. Secondly, Weiland & Koch (2004) showed that the genome of *C. beticola* can undergo chromosome changes after repetitive subculturing. These changes were observed after chromosome separation by gel electrophoreses. Although the authors studied only two isolates and did not mention the number of times the sub-culturing was repeated before these rearrangements were observed, the possibility that such rearrangements can influence results obtained using marker systems, such as AFLPs, has to be taken into account. In order to limit these chromosomal rearrangements in isolates, sub-culturing during this study was kept to a minimum and the DNA was extracted from the cultures directly after the original isolation. It is therefore concluded that the genetic variation observed in the populations screened during this study occurred during the life cycle of the fungus in its natural field environment.

Genetic diversity within a species can also be caused by asexual events that include hyphal anastomosis (Molnar *et al.*, 1990), selfing (Anderson & Kohn, 1995), normal mutations (Koenig *et al.*, 1997; Bentley *et al.*, 1998; O'Donnell *et al.*, 1999) and events occurring during parasexual cycles (Kuhn *et al.*, 1995; Taylor *et al.*, 1999). There is no evidence for parasexual recombination as an important generator of genetic diversity *in vivo* for any fungal system. The high levels of genetic diversity observed in *C. beticola* cannot be explained by mutation only, thus it is proposed that, apart from asexual recombination, a sexual cycle must be present for this pathogen.

No geographic boundaries could be enforced on the European populations based on the country of isolation because of the low population subdivision and low genetic distances between them, and because of shared haplotypes. Also, the Iranian population was not differentiated from the European populations. Sharing of haplotypes among geographic populations could be explained by man-mediated dispersal, as import and export of host material between countries in the European Union readily occurs because of the open borders. The high gene flow and low genetic distance and differentiation values observed between European populations and Iran indicate that genotype transfer also readily takes place between these countries. Based on genetic distance analysis, the Iranian genotypes are intermingled with European isolates, but this was not found for the New Zealand isolates. Therefore, it can be concluded that the European populations and the population from Iran are panmictic.

Pennycook (1989) recorded *C. beticola* on sugar beet in New Zealand, and during the last few years it has been isolated from different localities in New Zealand (New Zealand Fungi Database, 2002). The population from New Zealand is readily distinguished from other populations because of its low gene diversity, high genetic distances and population subdivision. This genetic differentiation

could either be due to a founder event, or the New Zealand populations might represent a different species of *Cercospora*. Groenewald *et al.* (2006a) included New Zealand isolates in a multi-gene phylogeny and could not distinguish them from the other *C. beticola* isolates. Also, the *C. beticola*-specific primers (Groenewald *et al.*, 2005) amplified a product of the correct size for the New Zealand isolates. Only two private AFLP alleles and two null alleles were found to be specific to the New Zealand populations during this study. From these data it is concluded that the *Cercospora* isolates obtained from sugar beet in New Zealand are indeed *C. beticola*. Small population sizes and genetic drift during founder events could have resulted in genetic subdivision, as has been found for other *Mycosphaerella* populations (Boileau *et al.*, 1992; Hayden *et al.*, 2003). However, the specific origin of *C. beticola* in New Zealand is unknown. The first strain that was designated a type of *C. beticola* was described from *Beta cicla* in Italy in 1875 (Saccardo, 1876) and it is most likely that earlier sugar beet trade introduced *C. beticola* to New Zealand from Europe.

Several studies have reported high levels of variation during the onset and progression of cercospora leaf spot on sugar beet (Wolf & Verreet, 2002, 2005), and that *C. beticola* has become resistant or has developed an increased tolerance to fungicides (Karaoglanidis *et al.*, 2000; Weiland & Koch, 2004). Variation in fungicide resistance and variability in disease symptoms on resistant sugar beet plants make effective disease management difficult. It is likely that the high levels of genetic variation that exists within *C. beticola* plays a role in the variation in pathogenicity that has been reported.

Previous studies showed that some genetic variation exists within *C. beticola*, but it was not known whether this variation was due to sexual recombination. The results here indicate that the genetic variation observed in the isolates studied was most likely caused by recombination events. It is suggested that *C. beticola* has both an asexual and sexual reproduction system and that it is unlikely that only asexual reproduction occurs in *C. beticola*. The high levels of genotypic variation and the equal distribution of the mating types within populations suggest that sexual recombination events most likely play an important role in the reproductive cycle of this species.

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# A multi-gene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves

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**Abstract:** Species of the ascomycete genus *Mycosphaerella* are regarded as some of the most destructive leaf pathogens of a large number of economically important crop plants. Amongst these, approximately 60 *Mycosphaerella* spp. have been identified from various *Eucalyptus* spp. where they cause leaf diseases collectively known as *Mycosphaerella* Leaf Disease (MLD). Species concepts for this group of fungi remain confused, and hence their species identification is notoriously difficult. Thus, the introduction of DNA sequence comparisons has become the definitive characteristic used to distinguish species of *Mycosphaerella*. Sequences of the Internal Transcribed Spacer (ITS) region of the ribosomal RNA operon have most commonly been used to consider species boundaries in *Mycosphaerella*. However, sequences for this gene region do not always provide sufficient resolution for cryptic taxa. The aim of this study was, therefore, to use DNA sequences for three loci, ITS, Elongation factor 1-alpha (EF-1 $\alpha$ ) and Actin (ACT) to reconsider species boundaries for *Mycosphaerella* spp. from *Eucalyptus*. A further aim was to study the anamorph concepts and resolve the deeper nodes of *Mycosphaerella*, for which part of the Large Subunit (LSU) of the nuclear rRNA operon was sequenced. The ITS and EF-1 $\alpha$  gene regions were found to be useful, but the ACT gene region did not provide species-level resolution in *Mycosphaerella*. A phylogeny of the combined DNA datasets showed that species of *Mycosphaerella* from *Eucalyptus* cluster in two distinct groups, which might ultimately represent discrete genera.

**Key words:** Actin, Ascomycetes, Translation Elongation factor 1-alpha, Multi-gene phylogeny, *Mycosphaerella*, *Mycosphaerella* Leaf Disease, ribosomal RNA operon.

## INTRODUCTION

Species of *Eucalyptus* are native to Australia with isolated pockets of native *Eucalyptus* forests also occurring in Papua New Guinea and the Philippines (Turnbull 2000). Many *Eucalyptus* spp. have been removed from these centres of origin to new environments where they are typically propagated in plantations for the production of paper, pulp and other wood products (Wingfield 1999, Turnbull 2000, Wingfield *et al.* 2001). In these non-native environments, *Eucalyptus* trees are susceptible to many pests and diseases including those known in their areas of origin and others that have undergone host shifts (Wingfield 2003, Slippers *et al.* 2005). These pests and diseases cause significant annual losses to *Eucalyptus* plantations resulting in decreased revenue for commercial forestry companies.

*Mycosphaerella* Johanson is one of the largest genera of the ascomycetes, accommodating more than 2000 species. Approximately 60 *Mycosphaerella* spp. have been associated with leaf diseases of many *Eucalyptus* spp., and these are collectively referred to as *Mycosphaerella* Leaf Disease (MLD) (Crous 1998, Maxwell *et al.* 2003, Crous *et al.* 2004a). The disease is particularly prevalent on the juvenile leaves and shoots of *Eucalyptus* trees, where infection results in premature defoliation, twig cankers and stunting of tree growth (Lundquist & Purnell 1987, Crous 1998, Park *et al.* 2000). However, several *Mycosphaerella* spp. can also infect adult *Eucalyptus* foliage, and this has been attributed to their ability to produce a proto-appressorium that enables direct cuticle penetration (Ganapathi 1979, Park & Keane 1982b). In some

situations, trees may thus be subjected to infection by a suite of different *Mycosphaerella* spp.

Identification of *Mycosphaerella* spp. based on morphology is known to be difficult. This is because these fungi tend to produce very small fruiting structures with highly conserved morphology, and they are host-specific pathogens that grow poorly in culture. Traditionally, morphological characters of the teleomorph and anamorph have been used in species delimitation (Crous 1998). Park & Keane (1982a) introduced ascospore germination patterns as an additional characteristic to identify *Mycosphaerella* spp., and Crous (1998) subsequently identified 14 different ascospore germination patterns for the *Mycosphaerella* spp. occurring on *Eucalyptus*. Crous (1998) and Crous *et al.* (2000) also introduced features of these fungi growing in culture and especially anamorph morphology as important and useful characteristics on which to base species delimitation. DNA-based methods such as RAPDs and species-specific primers have also been employed to distinguish between *Mycosphaerella* species occurring on *Eucalyptus* (Carnegie *et al.* 2001, Maxwell *et al.* 2005).

Comparisons of DNA sequence data have emerged as the most reliable technique to identify *Mycosphaerella* spp. The majority of studies employing DNA sequence data for species identification have relied on sequence data from the Internal Transcribed Spacer (ITS) region of the ribosomal RNA operon (Crous *et al.* 1999, 2001, 2004a, b, Hunter *et al.* 2004a, b). Although comparisons of gene sequences for this region have been useful, the resolution provided by this region is not uniformly adequate to discriminate between individuals

of a species complex or to effectively detect cryptic species (Crous *et al.* 2004b). Thus, recent studies have shown the importance of employing Multi-Locus Sequence Typing (MLST) to effectively identify cryptic fungal species and to study species concepts (Taylor & Fischer 2003).

A single morphological species does not always reflect a single phylogenetic unit (Taylor *et al.* 2000). Within *Mycosphaerella*, teleomorph morphology is conserved and the anamorph morphology provides additional characteristics to discriminate between taxa (Crous *et al.* 2000). Yet the collective teleomorph and anamorph morphology is often not congruent with phylogenetic data. Thus, recent phylogenetic studies have led to the recognition of several species complexes within *Mycosphaerella* (Crous *et al.* 2001, 2004b, Braun *et al.* 2003). Most of these studies have been based on comparisons of sequences for the ITS regions of the ribosomal DNA operon. Given the important data that have emerged from them, it is well recognised that greater phylogenetic resolution will be required for future taxonomic studies on *Mycosphaerella* species.

The aim of this study was to use MLST to consider species and anamorph concepts in *Mycosphaerella* spp. occurring on *Eucalyptus*. This was achieved by sequencing four nuclear gene regions, namely part of the Large Subunit (D1–D3 of LSU) and ITS region of the nuclear rRNA operon, and a portion of the Actin (ACT) and Elongation Factor 1-alpha (EF-1 $\alpha$ ) gene regions.

## MATERIALS AND METHODS

### *Mycosphaerella* isolates

For this study, an attempt was made to obtain cultures of as many *Mycosphaerella* spp. known to infect *Eucalyptus* leaves as possible. All cultures used in the investigation are housed in culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1). All cultures were grown on 2 % (w/v) malt extract agar (MEA) (Biolab, South Africa), at 25 °C for approximately 2–3 mo to obtain sufficient mycelial growth for DNA extraction.

### DNA isolation

Mycelium from actively growing cultures was scraped from the surface of cultures, freeze-dried for 24 h and then ground to a fine powder using liquid nitrogen. DNA was isolated using the phenol : chloroform (1:1) extraction protocol as described in Hunter *et al.* (2004a, b). DNA was precipitated by the addition of absolute ethanol (100 % EtOH). Isolated DNA was cleaned by washing with 70 % Ethanol (70 % EtOH) and dried under vacuum. SABAX water was used to resuspend the isolated DNA. RNaseA (10  $\mu$ g/ $\mu$ L) was added to the resuspended DNA and incubated at 37 °C for approximately 2 h to digest any residual RNA. Isolated DNA was visualised in a 1 % agarose gel (w/v) (Roche Diagnostics, Mannheim), stained with ethidium

bromide and visualised under ultra-violet light.

### PCR amplification and purification

DNA (*ca.* 20 ng) isolated from the *Mycosphaerella* spp. used in this study was used as a template for amplification using the Polymerase Chain Reaction (PCR). All PCR reactions were mixed in a total volume of 25  $\mu$ L containing 10 $\times$  PCR Buffer (5 mM Tris-HCl, 0.75 mM MgCl<sub>2</sub>, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP) (Roche Diagnostics, South Africa), 0.2  $\mu$ M of forward and reverse primers (Inqaba Biotech, South Africa) and 1.25 U Taq DNA Polymerase (Roche Diagnostics, South Africa) and DNA (20 ng/ $\mu$ L). Sterilised distilled water was added to obtain a final volume of 25  $\mu$ L.

The ITS-1, ITS-2 and the 5.8 S gene regions of the ITS region of the rRNA operon were amplified using primers ITS-1 (5'–TCC GTA GGT GAA CCT GCG G–3') and LR-1 (5'–GGT TGG TTT CTT TTC CT–3') (Vilgalys & Hester 1990, White *et al.* 1990). Reaction conditions for the ITS gene regions followed those of Crous *et al.* (2004a) and Hunter *et al.* (2004a, b).

A portion of the LSU (including domains D1–D3) of the rRNA operon was amplified using primers LR0R (5'–ACC CGC TGA ACT TAA GC–3') (Moncalvo *et al.* 1995) and LR7 (5'–TAC TAC CAC CAA GAT CT–3') (Vilgalys & Hester 1990). PCR cycling conditions were as follows: an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 62 °C for 30 s, primer extension at 72 °C for 1 min and a final elongation step at 72 °C for 7 min.

A portion of the EF-1 $\alpha$  was amplified using the primers EF1-728F (5'–CAT CGA GAA GTT CGA GAA GG–3') and EF1-986R (5'–TAC TTG AAG GAA CCC TTA CC–3') (Carbone & Kohn 1999). Reaction conditions were: an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s and primer extension at 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 7 min.

A portion of the ACT gene was amplified using the primers ACT-512F (5'–ATG TGC AAG GCC GGT TTC GC–3') and ACT-783R (5'–TAC GAG TCC TTC TGG CCC AT–3') (Carbone & Kohn 1999). PCR reaction conditions were: an initial denaturation step at 96 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s and extension at 72 °C for 45 s. This was followed by 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C and elongation at 72 °C for 45 s with an increase of 5 s per cycle. The reaction was completed with a final elongation step at 72 °C for 7 min.

All PCR products were visualised in 1.5 % agarose gels (wt/v) stained with ethidium bromide and viewed under ultra-violet light. Sizes of PCR amplicons were estimated by comparison against a 100 bp molecular weight marker (O' RangeRuler™ 100 bp DNA ladder) (Fermentas Life Sciences, U.S.A.). Prior to DNA sequencing, PCR products were purified through Centri-sep spin columns (Princeton Separations, Adelphia, NJ) containing Sephadex G-50 (Sigma Aldrich, St. Louis, MO) as outlined by the manufacturer.

### DNA sequencing and phylogenetic analysis

Purified PCR products were used as template DNA for sequencing reactions on an ABI PRISM™ 3100 Automated DNA sequencer (Applied Biosystems, Foster City, CA). The ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) was used for sequencing reactions following the manufacturer's instructions. Most sequencing reactions were performed with the same primers used for PCR reactions. Exceptions were in the case of the ITS region where two internal primers ITS-2 (5'-GCT GCG TTC TTC ATC GAT GC-3') and ITS-3 (5'-GCA TCG ATG AAG AAC GCA GC-3') (White *et al.* 1990) were included for the sequencing reactions. Similarly, for the LSU region two internal primers LR3R (5'-GTC TTG AAA CAC GGA CC-3') and LR-16 (5'-TTC CAC CCA AAC ACT CG-3') were used for the sequencing reactions.

All resulting sequences were analysed with Sequence Navigator v. 1.0.1 (Applied Biosystems, Foster City, CA). Sequence alignments were done using MAFFT (Multiple alignment program for amino acid or nucleotide sequences) v. 5.667 (Kato *et al.* 2005) and manually adjusted where necessary. Phylogenetic analyses and most parsimonious trees were generated in PAUP v. 4.0b10 (Swofford 2002) by heuristic searches with starting trees obtained through stepwise addition with simple addition sequence and with the MULPAR function enabled. Tree Bisection Reconnection (TBR) was employed as the swapping algorithm. All gaps were coded as missing data and characters were assigned equal weight. Branch support for nodes was obtained by performing 1000 bootstrap replicates of the aligned sequences. For parsimony analyses, measures that were calculated include tree length (TL), retention index (RI), consistency index (CI), rescaled consistency index (RC) and homoplasy index (HI). *Botryosphaeria ribis* Grossenb. & Duggar was used as the outgroup to root all trees.

A Partition Homogeneity Test (Farris *et al.* 1994), of all possible combinations, consisting of 1000 replicates on all informative characters was conducted in PAUP to determine if the LSU, ITS and EF-1 $\alpha$  data sets were combinable. All sequences of *Mycosphaerella* spp. used in this study have been deposited in GenBank (Table. 1). Sequence alignments and trees of the LSU, ITS, EF-1 $\alpha$  and ACT have been deposited in TreeBASE (accession numbers: LSU = SN2535, ITS = SN2534, EF-1 $\alpha$  = SN2536, ACT = SN2537).

Parsimony and distance analyses of combined DNA sequence alignments were conducted in PAUP. Parsimony analyses of all DNA sequence alignments were identical to those described earlier. For distance analyses, Modeltest v. 3.04 (Posada & Crandall 1998) was used to determine the best evolutionary model to fit the combined DNA sequence alignment. A neighbour-joining analysis with an evolutionary model was conducted in PAUP. Here, the distance measure was a general time-reversible (GTR) and the proportion of sites assumed to be invariable (I) was 0.4919, identical sites were removed proportionally to base frequencies estimated from all sites, rates of variable

sites assumed to follow a gamma distribution (G) with shape parameter of 0.6198. Ties (if encountered) were broken randomly.

## RESULTS

### DNA sequencing and phylogenetic analysis

**Large Subunit (LSU) phylogeny:** The LSU alignment had a total length of 1714 characters. An indel of 383 bp present in *M. ohnowa* Crous & M.J. Wingf. (CBS 112973) and *Mycosphaerella mexicana* Crous (CBS 110502) was excluded from the analyses. In the LSU data set, 1075 characters were constant while 77 characters were parsimony-uninformative and 179 characters were parsimony-informative. Parsimony analysis of the LSU data set resulted in the retention of thirty most parsimonious trees (TL = 663, CI = 0.519, RI = 0.878, RC = 0.456). One of these trees (Fig. 1) could be resolved into two clades (Clades 1–2). Clade 1, supported with a bootstrap value of 70 %, included *Mycosphaerella* isolates characterised by *Phaeophleospora* Rangel (*M. ambiphylla* A. Maxwell, *M. suttoniae* Crous & M.J. Wingf.), *Colletogloeopsis* Crous & M.J. Wingf. [*M. molleriana* (Thüm.) Lindau, *M. vespa* Carnegie & Keane, *M. cryptica* (Cooke) Hansf.], *Uwebraunia* Crous & M.J. Wingf. [*M. nubilosa* (Cooke) Hansf.], *M. ohnowa*, *Readeriella* Syd. & P. Syd. (*M. readeriellophora* Crous & J.P. Mansilla), and *Passalora* Fr. (*M. tasmaniensis* Crous & M.J. Wingf.) anamorphs.

The second major clade (Clade 2) resolved in the LSU tree was well-supported with a bootstrap value of 98 %. *Mycosphaerella* species in this clade also grouped strongly following their anamorph associations. Here *Mycosphaerella* isolates could be resolved into several sub-clades also characterised by their anamorph associations. These were *Sonderhenia* (*M. walkerii* R.F. Park & Keane.), *Pseudocercospora* Speg. [*M. heimioides* Crous & M.J. Wingf., *M. heimii* Crous, *M. crystallina* Crous & M.J. Wingf., *M. irregulariramosa* Crous & M.J. Wingf., *M. colombiensis* Crous & M.J. Wingf., *M. gracilis* Crous & Alfenas, *Pseudocercospora robusta* Crous & M.J. Wingf., *Ps. natalensis* Crous & T. Coutinho, *M. fori* G.C. Hunter, Crous & M.J. Wingf., *Ps. basitruncata* Crous, *Ps. pseudoeucalyptorum* Crous, *Ps. eucalyptorum* Crous, M.J. Wingf., Marasas & B. Sutton., *Ps. paraguayensis* (Koboyashi) Crous, *Ps. basiramifera* Crous] *Passalora* [*Pass. eucalypti* (Crous & Alfenas) Crous & U. Braun, *Pass. zambiae* Crous & T. Coutinho], and *Dissoconium* (*M. lateralis* Crous & M.J. Wingf., *M. communis* Crous & J.P. Mansilla).

**Internal Transcribed Spacer Region (ITS) phylogeny:** The ITS sequence alignment consisted of a total of 793 characters. Of these 499 characters were constant, 62 characters were variable and parsimony-uninformative and 232 characters were parsimony-informative. A 185 bp indel was observed in isolates of *M. gregaria* Carnegie & Keane (CBS 110501), *M. endophytica* Crous & H. Smith (CBS 111519) and *M. endophytica* (CMW 5225) and was excluded in the phylogenetic analysis.

**Table 1.** Isolates of *Mycosphaerella* used in this study for DNA sequencing and phylogenetic analysis.

Teleomorph	Anamorph	Isolate No. <sup>a</sup>			Host	Country	Collector	GenBank Accession No.			
		CMW	CBS	STEU				LSU	ITS	ACT	EF-1a
<i>M. africana</i>	Unknown	3026	116155	795	<i>E. viminalis</i>	South Africa	P.W. Crous	DQ246258	DQ267577	DQ147608	DQ235098
		4945	116154	794	<i>E. viminalis</i>	South Africa	P.W. Crous	DQ246257	AF309602	DQ147609	DQ235099
<i>M. ambiphylla</i>	<i>Phaeophleospora</i> sp.	14180	110499	N/A	<i>E. globulus</i>	Australia	A. Maxwell	DQ246219	AY725530	DQ147669	DQ235103
<i>M. aurantia</i>	Unknown	14460	110500	N/A	<i>E. globulus</i>	Australia	A. Maxwell	DQ246256	AY725531	DQ147610	DQ235097
<i>M. colombiensis</i>	<i>Pseudocercospora colombiensis</i>	4944	110969	1106	<i>E. urophylla</i>	Colombia	M.J. Wingfield	DQ204744	AY752149	DQ147639	DQ211660
		11255	110967	1104	<i>E. urophylla</i>	Colombia	M.J. Wingfield	DQ204745	AY752147	DQ147640	DQ211661
<i>M. communis</i>	<i>Dissoconium commune</i>	14672	114238	10440	<i>E. globulus</i>	Spain	J.P. Mansilla	DQ246262	AY725541	DQ147655	DQ235141
		14673	110976	849	<i>E. cladocalyx</i>	South Africa	P.W. Crous	DQ246261	AY725537	DQ147654	DQ235140
<i>M. cryptica</i>	<i>Colletogloeopsis nubilosum</i>	3279	110975	936	<i>E. globulus</i>	Australia	A.J. Carnegie	DQ246222	AF309623	DQ147674	DQ235119
		2732	N/A	355	<i>Eucalyptus</i> sp.	Chile	M.J. Wingfield	N/A	AF309622	N/A	N/A
<i>M. crystallina</i>	<i>Pseudocercospora crystallina</i>	3042	N/A	800	<i>E. bicostata</i>	South Africa	M.J. Wingfield	DQ204746	DQ267578	DQ147637	DQ211662
		3033	681.95	802	<i>E. bicostata</i>	South Africa	M.J. Wingfield	DQ204747	AY490757	DQ147636	DQ211663
<i>M. ellipsoidea</i>	<i>Uwebraunia ellipsoidea</i>	4934	N/A	1224	<i>Eucalyptus</i> sp.	South Africa	Unknown	DQ246253	AF309592	DQ147647	DQ235129
		5166	N/A	1225	<i>Eucalyptus</i> sp.	South Africa	Unknown	DQ246254	AF309593	DQ147648	DQ235127
<i>M. endophytica</i>	<i>Pseudocercosporella endophytica</i>	14912	111519	1191	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ246255	DQ267579	DQ147646	DQ235131
		5225	N/A	1192	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ246252	DQ267580	DQ147649	DQ235128
<i>M. flexuosa</i>	Unknown	5224	111012	1109	<i>E. globulus</i>	Colombia	M.J. Wingfield	DQ246232	AF309603	DQ147653	DQ235126
<i>M. fori</i>	<i>Pseudocercospora</i> sp.	9095	N/A	N/A	<i>E. grandis</i>	South Africa	G.C. Hunter	DQ204748	AF468869	DQ147618	DQ211664
		9096	N/A	N/A	<i>E. grandis</i>	South Africa	G.C. Hunter	DQ204749	DQ267581	DQ147619	DQ211665
<i>M. gracilis</i>	<i>Pseudocercospora gracilis</i>	14455	243.94	730	<i>E. urophylla</i>	Indonesia	A.C. Alfenas	DQ204750	DQ267582	DQ147616	DQ211666
<i>M. grandis</i>	Unknown	8557	N/A	N/A	<i>E. globulus</i>	Chile	A. Rotella	DQ246241	DQ267583	DQ147644	DQ235108
		8554	N/A	N/A	<i>E. globulus</i>	Chile	M.J. Wingfield	DQ246240	DQ267584	DQ147643	DQ235107
<i>M. gregaria</i>	Unknown	14462	110501	N/A	<i>E. globulus</i>	Australia	A. Maxwell	DQ246251	DQ267585	DQ147650	DQ235130
<i>M. heimii</i>	<i>Pseudocercospora heimii</i>	4942	110682	760	<i>Eucalyptus</i> sp.	Madagascar	P.W. Crous	DQ204751	AF309606	DQ147638	DQ211667
<i>M. heimioides</i>	<i>Pseudocercospora heimioides</i>	14776	111364	N/A	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ204752	DQ267586	DQ147632	DQ211668
		3046	111190	1312	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ204753	AF309609	DQ147633	DQ211669
<i>M. intermedia</i>	Unknown	7163	114356	10902	<i>E. saligna</i>	New Zealand	K. Dobbie	DQ246247	AY725546	N/A	N/A
		7164	114415	10922	<i>E. saligna</i>	New Zealand	K. Dobbie	DQ246248	AY725547	DQ147627	DQ235132
<i>M. irregulariramosa</i>	<i>Pseudocercospora irregulariramosa</i>	4943	114774	1360	<i>E. saligna</i>	South Africa	M.J. Wingfield	DQ204754	AF309607	DQ147634	DQ211670
		5223	N/A	1362	<i>E. saligna</i>	South Africa	M.J. Wingfield	DQ204755	AF309608	DQ147635	DQ211671
<i>M. ohnowa</i>	Unknown	4937	112896	1004	<i>E. grandis</i>	South Africa	M.J. Wingfield	N/A	AF309604	DQ147662	DQ235125
		4936	112973	1005	<i>E. grandis</i>	South Africa	M.J. Wingfield	DQ246231	AF309605	DQ147661	DQ235124
<i>M. keniensis</i>	Unknown	5147	111001	1084	<i>E. grandis</i>	Kenya	T. Coutinho	DQ246259	AF309601	DQ147611	DQ235100



Table 1. (Continued).

Teleomorph	Anamorph	Isolate No. <sup>a</sup>			Host	Country	Collector	GenBank Accession No.			
		CMW	CBS	STEU				LSU	ITS	ACT	EF-1a
<i>M. lateralis</i>	<i>Dissoconium dekkeri</i>	14906	110748	825	<i>E. grandis</i> × <i>E. saligna</i>	South Africa	G. Kemp	DQ204768	AF173315	DQ147651	DQ211684
		5164	111169	1232	<i>E. globulus</i>	Zambia	T. Coutinho	DQ246260	AY25550	DQ147652	DQ235139
<i>M. madeirae</i>	<i>Pseudocercospora</i> sp.	14458	112895	3745	<i>E. globulus</i>	Madeira	S. Denman	DQ204756	AY725553	DQ147641	DQ211672
<i>M. marksii</i>	Unknown	14781	682.95	842	<i>E. grandis</i>	South Africa	G. Kemp	DQ246249	DQ267587	DQ147624	DQ235133
		5150	110920	935	<i>E. botryoides</i>	Australia	A.J. Carnegie	DQ246250	AF309588	DQ147625	DQ235134
		5230	N/A	782	<i>E. botryoides</i>	Australia	A.J. Carnegie	DQ246246	DQ267588	DQ147626	DQ235135
<i>M. mexicana</i>	Unknown	14461	110502	N/A	<i>E. globulus</i>	Australia	A. Maxwell	DQ246237	AY725558	DQ147660	DQ235123
<i>M. readeriellophora</i>	<i>Readeriella readeriellophora</i>	14233	114240	10375	<i>E. globulus</i>	Spain	J.P. Mansilla	DQ246238	AY725577	DQ147658	DQ235117
<i>M. molleriana</i>	<i>Colletogloeopsis molleriana</i>	4940	111164	1214	<i>E. globulus</i>	Portugal	S. McCrae	DQ246220	AF309620	DQ147671	DQ235104
		2734	111132	784	<i>E. globulus</i>	U. S. A.	M.J. Wingfield	DQ246223	AF309619	DQ147670	DQ235105
<i>M. nubilosa</i>	<i>Uwebraunia juvenis</i>	3282	116005	937	<i>E. globulus</i>	Australia	A.J. Carnegie	DQ246228	AF309618	DQ147666	DQ235111
		9003	114708	N/A	<i>E. nitens</i>	South Africa	G.C. Hunter	DQ246229	AF449099	DQ147667	DQ235112
<i>M. parkii</i>	<i>Stenella parkii</i>	14775	387.92	353	<i>E. grandis</i>	Brazil	M.J. Wingfield	DQ246245	AY626979	DQ147612	DQ235137
<i>M. parva</i>	Unknown	14459	110503	N/A	<i>E. globulus</i>	Australia	A. Maxwell	DQ246243	AY626980	DQ147645	DQ235110
		14917	116289	10935	<i>Eucalyptus</i> sp.	South Africa	P.W. Crous	DQ246242	AY725576	DQ147642	DQ235109
<i>M. suberosa</i>	Unknown	5226	436.92	515	<i>E. dunnii</i>	Brazil	M.J. Wingfield	DQ246235	AY626985	DQ147656	DQ235101
		7165	N/A	N/A	<i>E. muelleriana</i>	New Zealand	Unknown	DQ246236	DQ267589	DQ147657	DQ235102
<i>M. suttonii</i>	<i>Phaeophleospora epicoccoides</i>	5348	N/A	1346	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	DQ246227	AF309621	DQ147673	DQ235116
<i>M. vespa</i>	<i>Colletogloeopsis</i> sp.	11558	117924	N/A	<i>E. globulus</i>	Tasmania	Unknown	DQ246221	DQ267590	DQ147668	DQ235106
<i>M. tasmaniensis</i>	<i>Passalora tasmaniensis</i>	14780	111687	1555	<i>E. nitens</i>	Tasmania	M.J. Wingfield	DQ246233	DQ267591	DQ147676	DQ235121
		14663	114556	N/A	<i>E. nitens</i>	Tasmania	M.J. Wingfield	DQ246234	DQ267592	DQ147677	DQ235122
<i>M. toledana</i>	<i>Phaeophleospora toledana</i>	14457	113313	N/A	<i>Eucalyptus</i> sp.	Spain	P.W. Crous	DQ246230	AY725580	DQ147672	DQ235120
<i>M. walkerii</i>	<i>Sonderhenia eucalypticola</i>	20333	N/A	N/A	<i>E. globulus</i>	Chile	M.J. Wingfield	DQ267574	DQ267593	DQ147630	DQ235095
		20334	N/A	N/A	<i>E. globulus</i>	Chile	M.J. Wingfield	DQ267575	DQ267594	DQ147631	DQ235096
Unknown	<i>Passalora eucalypti</i>	14907	111306	1457	<i>E. saligna</i>	Brazil	P.W. Crous	DQ246244	AF309617	DQ147678	DQ235138
Unknown	<i>Passalora zambiae</i>	14782	112971	1227	<i>E. globulus</i>	Zambia	T. Coutinho	DQ246264	AF725523	DQ147675	DQ235136
Unknown	<i>Pseudocercospora epispermogonia</i>	14778	110750	822	<i>E. grandis</i> × <i>E. saligna</i>	South Africa	G. Kemp	DQ204757	DQ267596	DQ147629	DQ211673
		14786	110693	823	<i>E. grandis</i> × <i>E. saligna</i>	South Africa	G. Kemp	DQ204758	DQ267597	DQ147628	DQ211674
Unknown	<i>Phaeophleospora eucalypti</i>	11687	113992	N/A	<i>E. nitens</i>	New Zealand	M. Dick	DQ246225	DQ267598	DQ147664	DQ235115



Table 1. (Continued).

Teleomorph	Anamorph	Isolate No. <sup>a</sup>			Host	Country	Collector	GenBank Accession No.			
		CMW	CBS	STEU				LSU	ITS	ACT	EF-1a
Unknown	<i>Pseudocercospora basitruncata</i>	14910	111692	1582	<i>Eucalyptus</i> sp.	New Zealand	M.J. Wingfield	DQ246224	DQ267599	DQ147663	DQ235114
		14914	114664	1202	<i>E. grandis</i>	Colombia	M.J. Wingfield	DQ204759	DQ267600	DQ147622	DQ211675
		14785	111280	1203	<i>E. grandis</i>	Colombia	M.J. Wingfield	DQ204760	DQ267601	DQ147621	DQ211676
Unknown	<i>Pseudocercospora basiramifera</i>	5148	N/A	N/A	<i>E. pellita</i>	Thailand	M.J. Wingfield	DQ204761	AF309595	DQ147607	DQ211677
Unknown	<i>Pseudocercospora eucalyptorum</i>	5228	110777	16	<i>E. nitens</i>	South Africa	P.W. Crous	DQ204762	AF309598	DQ147614	DQ211678
Unknown	<i>Pseudocercospora natalensis</i>	14777	111069	1263	<i>E. nitens</i>	South Africa	T. Coutinho	DQ267576	N/A	DQ147620	N/A
		14784	111070	1264	<i>E. nitens</i>	South Africa	T. Coutinho	DQ204763	AF309594	DQ147623	DQ211679
Unknown	<i>Pseudocercospora paraguayensis</i>	14779	111286	1459	<i>E. nitens</i>	Brazil	P.W. Crous	DQ204764	DQ267602	DQ147606	DQ211680
Unknown	<i>Pseudocercospora pseudoecalyptorum</i>	14908	114242	10390	<i>E. globulus</i>	Spain	J.P. Mansilla	DQ204765	AY725526	DQ147613	DQ211681
Unknown	<i>Pseudocercospora robusta</i>	14911	114243	10500	<i>E. nitens</i>	New Zealand	W. Gams	DQ204766	AY725527	DQ147615	DQ211682
		5151	111175	1269	<i>E. robusta</i>	Malaysia	M.J. Wingfield	DQ204767	AF309597	DQ147617	DQ211683
Unknown	<i>Readeriella novaezealandiae</i>	14913	114357	10895	<i>E. botryoides</i>	New Zealand	M.A. Dick	DQ246239	DQ267603	DQ147659	DQ235118
<i>Botryosphaeria ribis</i>	<i>Fusicoccum ribis</i>	7773	N/A	N/A	<i>Ribus</i> sp.	U. S. A.	G. Hudler.	DQ246263	DQ267604	DQ267605	DQ235142

<sup>a</sup>CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

STEU: Culture collection of Stellenbosch University, South Africa. Isolate numbers from Crous (1998).

N/A: Not available



A heuristic search of the ITS data set resulted in the retention of four most parsimonious trees (TL = 871, RI = 0.782, CI = 0.358, RC = 0.280). One of these phylogenetic trees (Fig. 2) generated by parsimony analysis of the ITS alignment could be resolved into two monophyletic clades (Clades 1–2). Clade 1 was only weakly supported with a bootstrap value of 50 % after 1000 bootstrap replicates. Clade 1 could be further resolved into several smaller sub-clades where isolates grouped strongly based on their anamorph affiliations. These included *Sonderhenia*, *Pseudocercospora*, *Passalora*, *Uwebraunia/Pseudocercospora*, *Stenella*, *Readeriella*, *Phaeophleospora* and *Colletogloeopsis*. The second monophyletic clade (Clade 2) grouped sister to the first larger monophyletic clade and

contained isolates of *M. lateralis* and *M. communis* (*Dissoconium* anamorphs). This clade was well-supported with a bootstrap value of 100 % after 1000 bootstrap replicates.

*Translation Elongation factor 1-alpha (EF -1 $\alpha$ ) phylogeny:* The EF-1 $\alpha$  alignment contained 373 characters. Of these, 41 characters were constant, 23 characters were variable and parsimony-uninformative and 309 characters were parsimony-informative. Heuristic searches resulted in the retention of six most parsimonious trees (TL = 3194, RI = 0.777, CI = 0.345, RC = 0.268), one of which is shown (Fig. 3). Species of *Mycosphaerella* could be resolved into three clades (Clades 1–3).

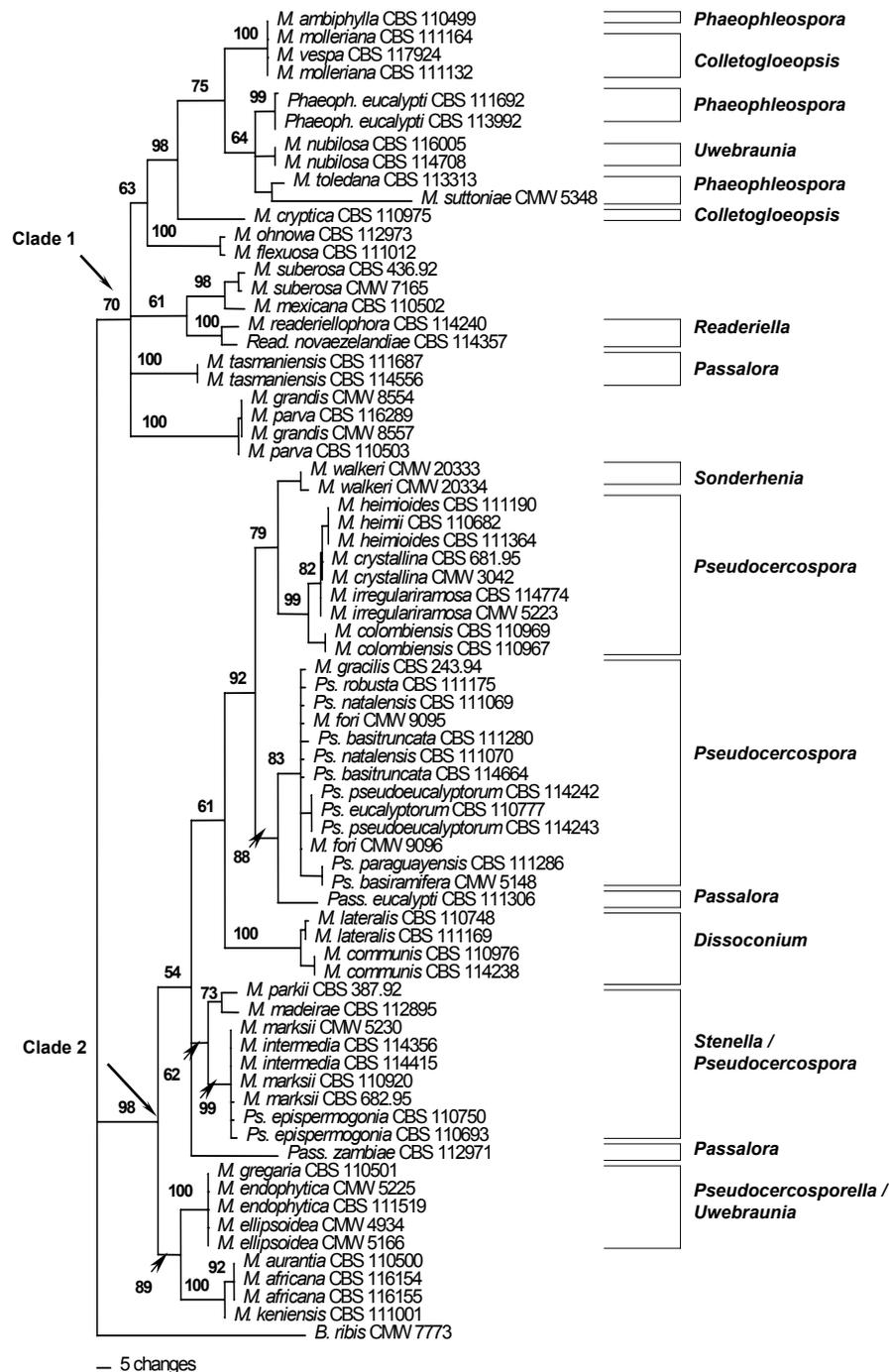
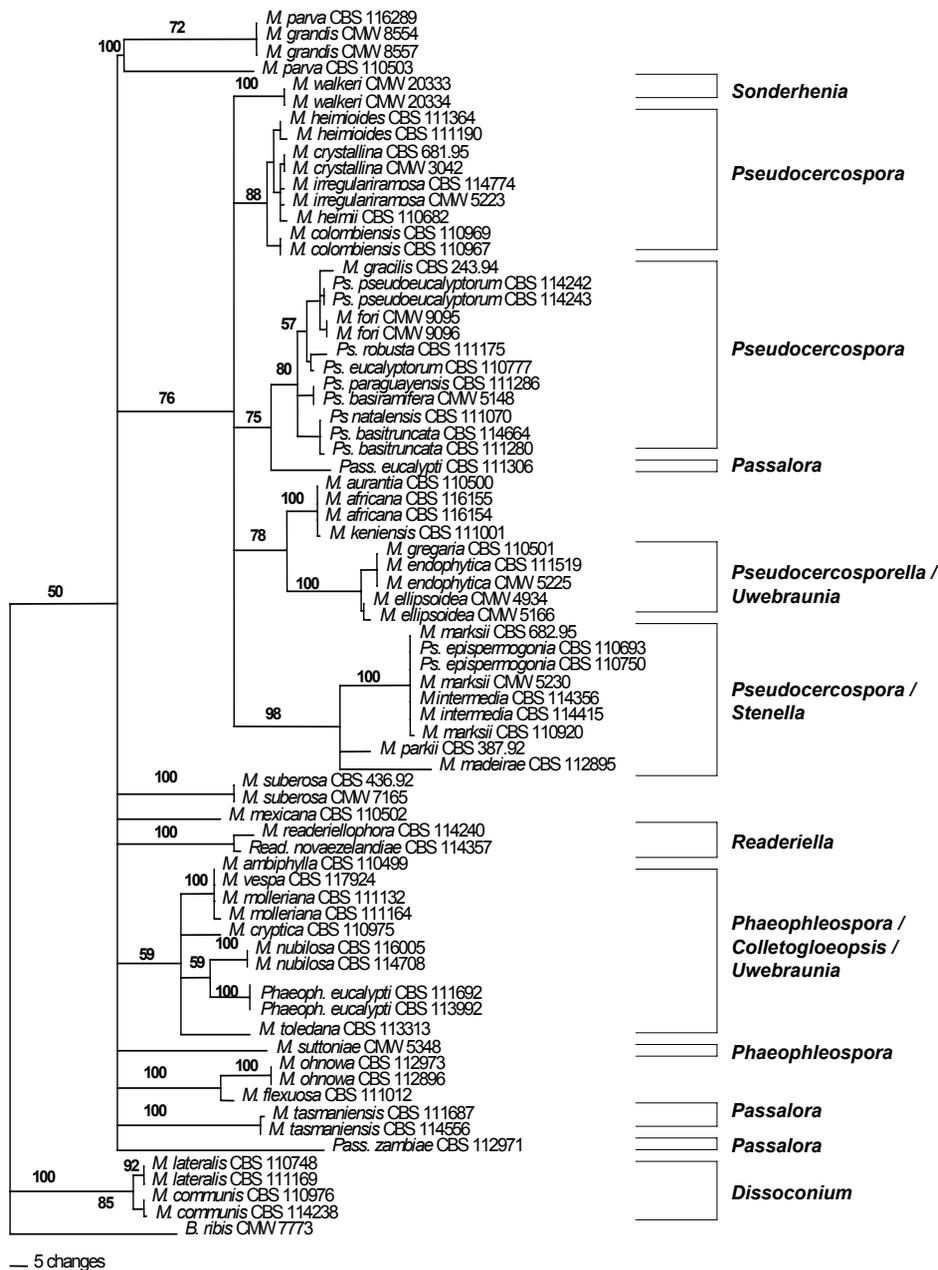


Fig. 1. Phylogram obtained from the Large Subunit (LSU) rDNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing two well-supported main clades (Clades 1–2). Tree length = 663, CI = 0.519, RI = 0.878, RC = 0.456. Bootstrap values based on 1000 replicates are indicated above branches. Anamorph affinities are indicated next to the vertical lines.

Clade 1 was weakly supported with a bootstrap value of 67 %. This clade contained *Mycosphaerella* isolates represented by *Pseudocercospora*, *Sonderhenia*, *Phaeophleospora*, *Colletogloeopsis*, *Uwebraunia*, *Readeriella* and *Passalora* anamorphs. Clade 2 was sister to Clade 1 and had a higher bootstrap support of 80 %. Within this clade, *Mycosphaerella* isolates could be separated into three sub-clades that were well-supported. These three sub-clades contained species of *Mycosphaerella* that produced *Pseudocercospora*, *Uwebraunia*, *Pseudocercospora*, *Passalora* and *Stenella* anamorphs. Clade 3 with bootstrap support of 80 % included isolates of *M. lateralis* and *M. communis* and was basal to Clades 1 and 2.

*Actin (ACT) phylogeny:* The aligned ACT sequence dataset contained a total of 294 characters. Of these, 135 characters were constant, 30 characters were variable and parsimony-uninformative and 129 characters were parsimony-informative. Heuristic

searches of the aligned ACT dataset resulted in the retention of six most parsimonious trees (TL = 1007, RI = 0.682, CI = 0.235, RC = 0.160). One of these trees, shown in Fig. 4, was very poorly resolved and all deeper nodes were present in a basal polytomy. However, certain smaller clades were resolved and these included a clade including *M. fori*, *M. gracilis*, *Ps. eucalyptorum*, *Ps. pseudoeucalyptorum*, *Ps. robusta*, *Ps. basitruncata*, *Ps. natalensis*, *Ps. basiramifera* and *Ps. paraguayensis*. This clade was supported with a bootstrap value of only 67 %. Another clade supported with a bootstrap value of 100 % contained isolates of *M. ellipsoidea* Crous & M.J. Wingf., *M. endophytica* and *M. gregaria*. Isolates of *M. amphiphylla*, *M. molleriana* and *M. vespa* also clustered together with 100 % bootstrap support. Isolates of *M. intermedia* M.A. Dick & Dobbie, *M. marksii* Carnegie & Keane and *Pseudocercospora epispermogonia* Crous & M. J. Wingf. grouped together in a clade that was supported with a bootstrap value of

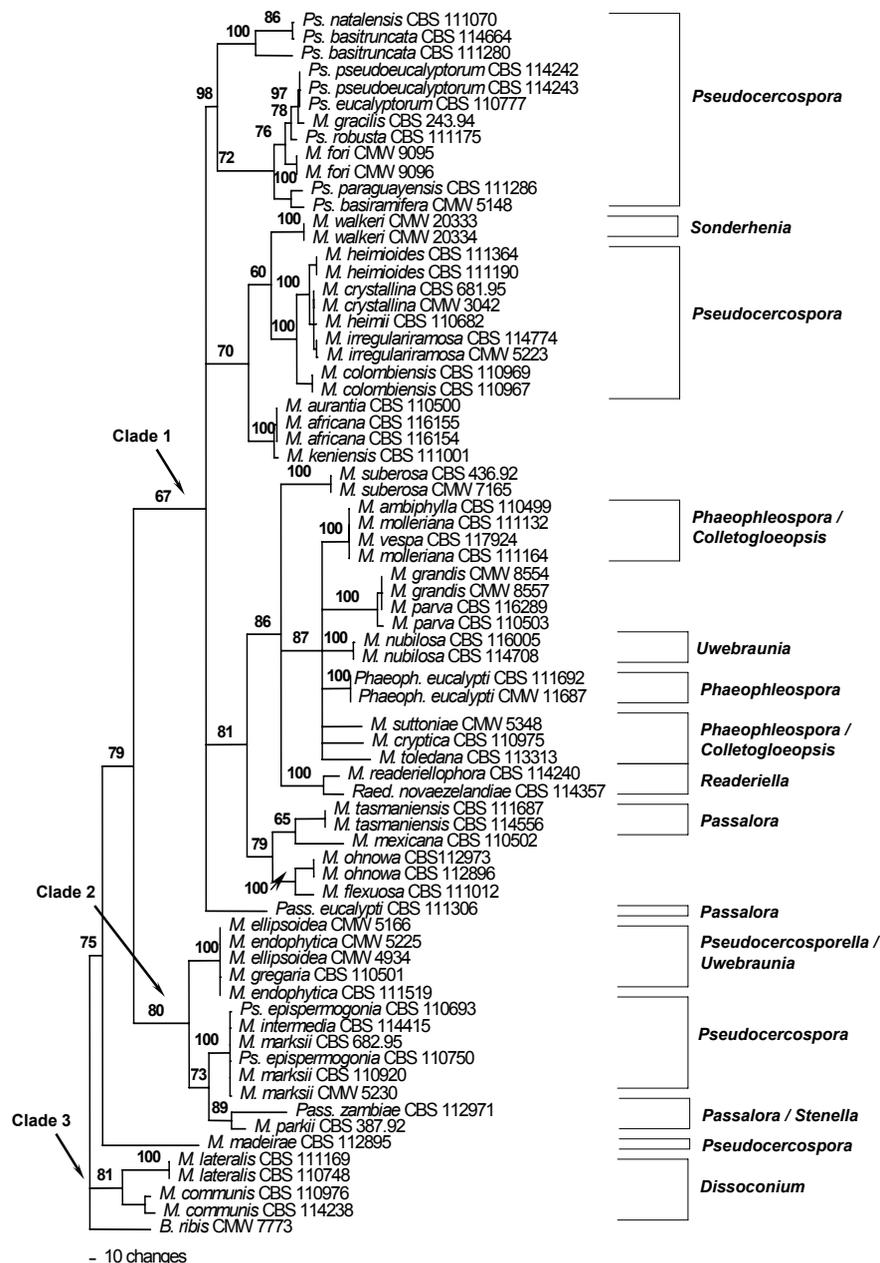


**Fig. 2.** Phylogram obtained from the Internal Transcribed Spacer (ITS) DNA sequence alignment of *Mycosphaerella* spp. occurring on Eucalyptus leaves indicating two monophyletic clades (Clades 1–2). Tree length = 871, CI = 0.358, RI = 0.782, RC = 0.280.

84 %. Isolates of *M. flexuosa* Crous & M.J. Wingf., *M. lateralis* and *M. communis* were also accommodated in a well-supported clade with a bootstrap value of 99 %. Isolates of *M. grandis* Carnegie & Keane and *M. parva* R.F. Park & Keane were also resolved into a clade with a bootstrap value of 99 %.

**Phylogeny of combined data set:** A partition homogeneity test of the combined LSU, ITS and EF-1 $\alpha$  alignment conducted in PAUP resulted in a P-value of 0.001 for all possible combinations of the LSU, ITS and EF-1 $\alpha$  DNA alignments. This value is less than the conventionally accepted P-value of  $P > 0.05$  required to combine data. However, several studies have accepted a P-value of 0.001 or greater and have further stated that the conventional P-value of 0.005 is inordinately conservative (Cunningham 1997, Darlu & Lecointre 2002, Dettman *et al.* 2003). Thus, the LSU, ITS and EF-1 $\alpha$  DNA sequence data sets were combined. The ACT dataset was omitted from the combined data set due to

the lack of resolution among species of *Mycosphaerella*. Therefore, the combined LSU, ITS and EF-1 $\alpha$  data set had a total length of 2880 characters. Of these, 1459 were constant, 150 were variable and parsimony-uninformative and 701 characters were parsimony-informative. An indel of 382 bp was excluded for *M. ohnowa* CBS 112973 and *M. mexicana* CBS 110502 and another indel of 186 bp was excluded for *M. gregaria* CBS 110501 and *M. endophytica* CMW 5225 and CBS 111519. A parsimony analysis resulted in the retention of ten most parsimonious trees (TL = 1677, CI = 0.384, RI = 0.817, RC = 0.314, HI = 0.616). One of these trees (Fig. 5) exhibited a similar topology to that obtained from the LSU alignment. From the analysis of the combined data set, isolates of *Mycosphaerella* could again be resolved into two clades (Clades 1–2) (Fig. 5). Clade 1 was poorly supported with a bootstrap value of only 66 % and the same isolates were contained in this clade as in the LSU Clade 1



**Fig. 3.** Phylogram obtained from the Elongation factor 1-alpha (EF-1 $\alpha$ ) DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing three main clades. Tree length = 3194, CI = 0.345, RI = 0.777, RC = 0.268.

(Fig. 1). Clade 2 of the combined phylogenetic tree was well-supported with a bootstrap value of 81 %. This clade could be further resolved into several smaller well-supported sub-clades containing *Mycosphaerella* isolates that grouped according to their anamorph associations (Fig. 5). Neighbour-joining analysis yielded a phylogenetic tree with the same topology as the most parsimonious trees (data not shown). Here, all *Mycosphaerella* spp. could be resolved into two main clades (Clade 1–2), similar to those of the parsimony analysis (Fig. 5). *Mycosphaerella* spp. could be further sub-divided into several sub-clades corresponding to their anamorph associations, similar to those observed for the parsimony analysis.

## DISCUSSION

Results of this study represent the first attempt to employ DNA sequence data from a relatively large number of nuclear gene regions in order to consider the phylogenetic relationships for *Mycosphaerella* spp. occurring on *Eucalyptus* leaves. Other similar studies have relied entirely on sequence data of the ITS region (Crous *et al.* 1999, 2001, 2004a, and 2006 – this volume, Hunter *et al.* 2004b). Although the ITS region offers sufficient resolution to distinguish most taxa, it has not been adequate to separate cryptic taxa in *Mycosphaerella* (Crous *et al.* 2004b). Results of the present study showed that combined DNA sequence

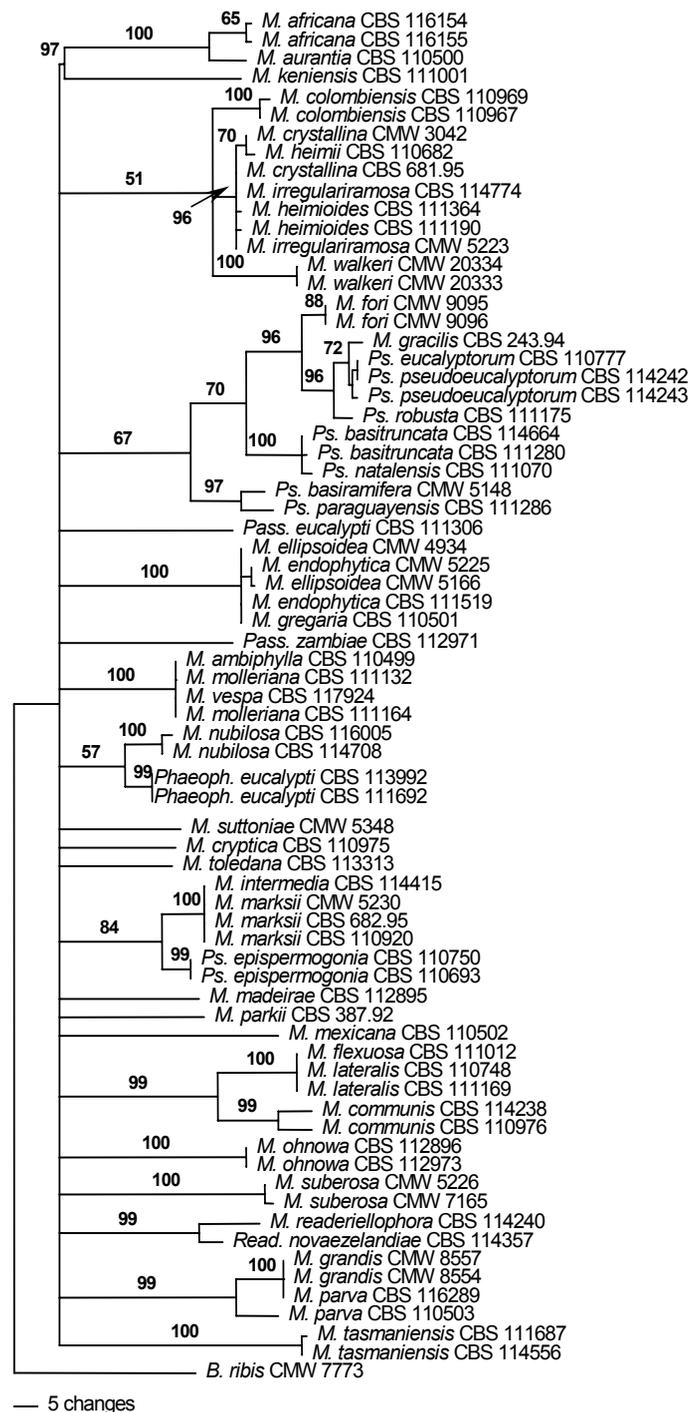
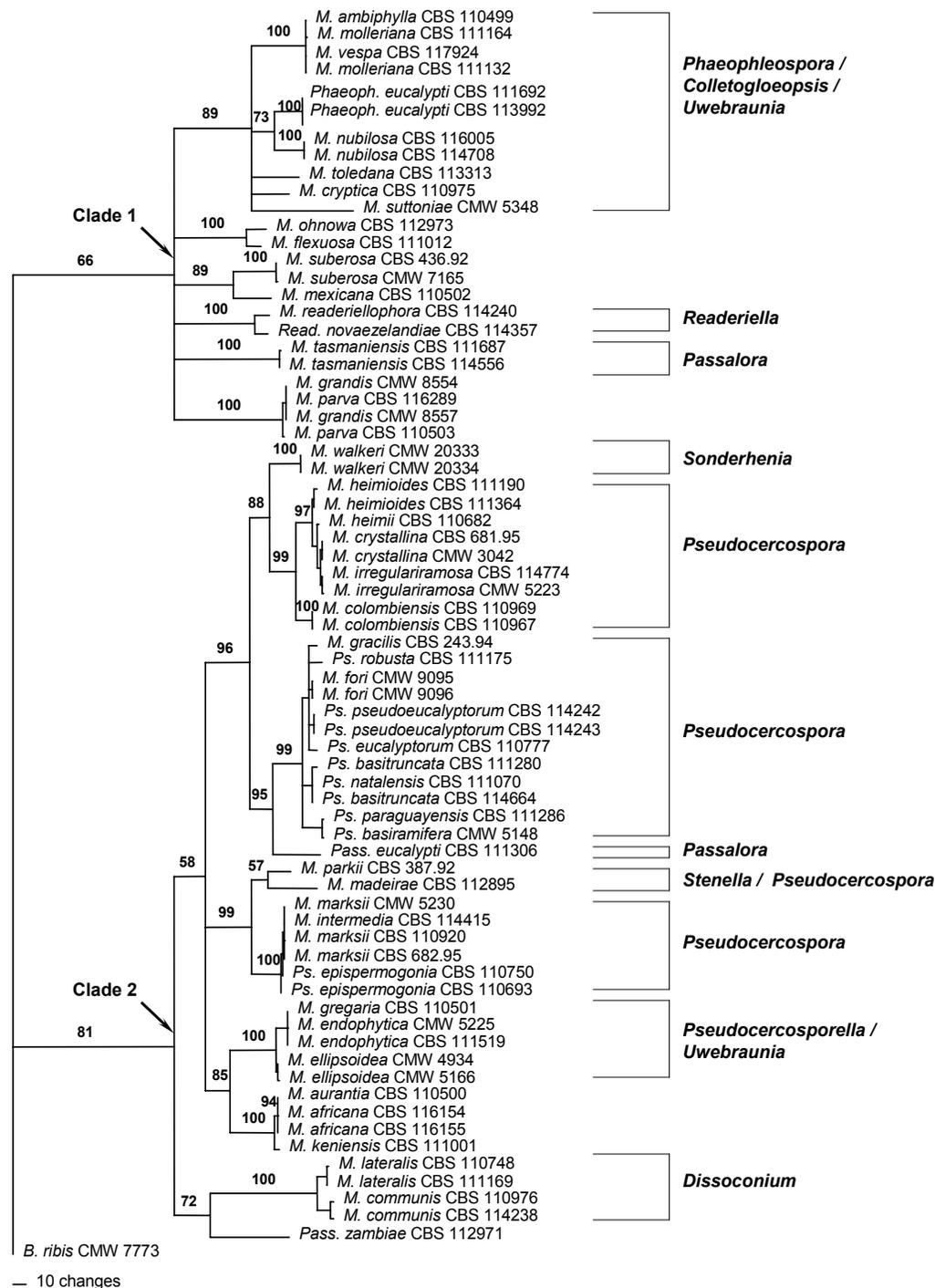


Fig. 4. Phylogram obtained from the Actin (ACT) DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves. Tree length = 1007, CI = 0.235, RI = 0.682, RC = 0.160.

data from the LSU, ITS, EF-1 $\alpha$  gene regions offer increased genetic resolution to study species concepts in *Mycosphaerella*. However, genes such as the ACT, did not support data emerging from the other loci sequenced, and indicated variation within some clades that were well supported by sequences of other loci and morphological characteristics. These observations led us to exclude ACT data from the final analyses. A similar finding has also emerged from other studies including greater numbers of *Mycosphaerella* species (Crous & Groenewald, unpubl. data).

*Mycosphaerella ambiphylla*, *M. molleriana* and *M. vespa* grouped together in a well-supported clade in the phylogeny emerging from the combined alignment. This

was also true for the ITS, EF-1 $\alpha$  and ACT phylogenies where these isolates grouped in a distinct clade with a 100 % bootstrap support. *Mycosphaerella molleriana* and *M. vespa* both have *Colletogloeopsis* anamorphs, however, *M. ambiphylla* produces a *Phaeophleospora* anamorph (Crous & Wingfield 1997a, Maxwell *et al.* 2003). Interestingly, the *Phaeophleospora* anamorph of *M. ambiphylla* was differentiated from *Colletogloeopsis* only by the fact that conidia are produced in a pycnidium as opposed to an acervulus (Maxwell *et al.* 2003). Application of conidiomatal structure to differentiate anamorphs of *Mycosphaerella* has previously been viewed with circumspection especially because *Mycosphaerella* anamorphs can produce different



**Fig. 5.** Phylogram obtained from the combined LSU, ITS and EF-1 $\alpha$  DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing two main clades. Tree length = 1677, CI = 0.384, RI = 0.817, RC = 0.314.

conidiomatal forms under differing environmental conditions (Crous *et al.* 2000, Cortinas *et al.* 2006 – this volume). Therefore, the placement of the *M. ambiphylla* anamorph in *Phaeophleospora* is questioned and it should be re-evaluated in terms of its morphological similarities to *Colletogloeopsis*.

Ascospore germination patterns of *M. ambiphylla*, *M. molleriana* and *M. vespa* are all similar, with germ tubes that grow parallel to the long axis of the spore and ascospores with a slight constriction at the median septum, typical of a type C ascospore germination pattern (Crous 1998, Carnegie & Keane 1998, Maxwell *et al.* 2003). Furthermore, overlap is seen in ascospore dimensions of the three species where those of *M. molleriana* are (11–)12–14(–17) × (2.5–)3.5–4(–4.5) µm, those of *M. ambiphylla* are (12–)14–15(–22) × (3.5–)4.5–5(–6) µm and those of *M. vespa* 9.5–16.5 × 2.5–4 µm (Crous 1998, Carnegie & Keane 1998, Maxwell *et al.* 2003). Leaf lesions of the three species are also similar, pale brown to dark red-brown with lesions of *M. vespa* and *M. ambiphylla* often producing a red margin that was, however, not observed in *M. molleriana* (Crous 1998, Carnegie & Keane 1998, Maxwell *et al.* 2003). Morphological features of *M. ambiphylla*, *M. molleriana* and *M. vespa* are also very similar. This is supported in the DNA phylogeny of the present study where these three species appear to represent a single taxon and therefore suggest that *M. ambiphylla*, *M. molleriana* and *M. vespa* should be synonymised under *M. molleriana*, which is the oldest epithet. We therefore reduce *M. ambiphylla* and *M. vespa* to synonymy with *M. molleriana* as follows:

***Mycosphaerella molleriana*** (Thüm.) Lindau, *Natürliche Pflanzenfamilie*, 1: 424. 1897.

= *Sphaerella molleriana* Thüm., *Revista Inst. Sci. Lit. Coimbra* 28: 31. 1881.

= *Mycosphaerella vespa* Carnegie & Keane, *Mycol. Res.* 102: 1275. 1998.

= *Mycosphaerella ambiphylla* A. Maxwell, *Mycol. Res.* 107: 354. 2003.

*Anamorph: Colletogloeopsis molleriana* Crous & M.J. Wingf., *Canad. J. Bot.* 75: 670. 1997.

*Mycosphaerella flexuosa* has no known anamorph (Crous 1998). An isolate of this fungus included in the present study grouped together with isolates of *M. ohnowa* in the LSU, ITS, EF-1α and combined data set with high bootstrap support. This similarity was also observed in a recent study of *Mycosphaerella* spp. on *Eucalyptus* based on ITS sequence data (Crous *et al.* 2004a). *Mycosphaerella ohnowa* is also not known to produce an anamorph (Crous *et al.* 2004a). Although these two species are phylogenetically similar, they can be distinguished from one another based on different ascus and ascospore dimensions, ascospore germination patterns and cultural characteristics (Crous 1998, Crous *et al.* 2004a). Although morphologically distinct, it is interesting that these two taxa are phylogenetically so closely related and might suggest a recent speciation event.

Isolates of *M. grandis* and *M. parva* consistently grouped together in a separate clade in all of the DNA

sequence data sets in this study. This has also been shown by Crous *et al.* (2004a), where isolates of these two species grouped together in a distinct clade based on ITS DNA sequences. *Mycosphaerella grandis* was originally described from *E. grandis* in Australia, and recognised as a distinct species of *Mycosphaerella* due to its lesion characteristics, and ascospore morphology (Carnegie & Keane 1994). However, Crous (1998) examined the type of *M. grandis* and *M. parva* and found the two species to be congeneric, and reduced them to synonymy under *M. parva*. Results from the present study support the synonymy.

*Mycosphaerella lateralis* and *M. communis*, both known to have *Dissoconium* anamorphs, showed various phylogenetic placements in this study. From the LSU phylogeny, *M. lateralis* and *M. communis* were situated within a large *Mycosphaerella* clade sister to a *Pseudocercospora* sub-clade. However, in the ITS and EF-1α phylogenies the *Dissoconium* clade was situated basal to the larger *Mycosphaerella* clade. This is consistent with findings of Crous *et al.* (1999, 2000) where the *Dissoconium* clade also resided outside the larger monophyletic *Mycosphaerella* clade. The LSU gene region is well-known to be conserved and to show less nucleotide differences than the ITS and EF-1α gene regions. Although the house-keeping genes investigated here lead to the conclusion that *Dissoconium* could be different from *Mycosphaerella s. str.*, this proved not to be the case when LSU data were considered. *Dissoconium* is morphologically identical to *Uwebraunia*, and the separation of these two genera no longer seems tenable. Only two species, *M. ellipsoidea* and *M. nubilosa*, have *Uwebraunia* anamorphs (Crous *et al.* 2004a). However, cultures of both species produced these anamorphs only upon initial isolation, and those that are currently available are sterile. In contrast, strains with *Dissoconium* anamorphs readily produce those in culture, and they usually sporulate profusely. It appears that the status of *Uwebraunia* will only be resolved once fresh, sporulating collections of either *M. ellipsoidea* or *M. nubilosa* can be obtained.

*Mycosphaerella* spp. with *Pseudocercospora* anamorphs grouped into three clades in all of the phylogenies generated in this study. Species in the *Pseudocercospora* clades have short branch lengths arising from a common internode, suggesting that they have speciated relatively recently from a common ancestor (Ávila *et al.* 2005) and, most likely have co-evolved with their *Eucalyptus* hosts as suggested by Crous *et al.* (2000). Ávila *et al.* (2005) suggested that *Pseudocercospora* may represent a monophyletic lineage. But, results of this and other studies (Ayala-Escobar *et al.* 2006) have shown that *Pseudocercospora* is paraphyletic in *Mycosphaerella* and has evolved more than once in the genus. The availability of new DNA datasets for several gene regions are likely to resolve cryptic species and species complexes within *Pseudocercospora*, as has already been shown for the *M. heimii* and the *P. eucalyptorum* species complexes (Crous *et al.* 2000, 2004a).

*Mycosphaerella heimioides*, *M. heimii*, *M. crystallina* and *M. irregulariramosa* are all morphologically similar

and are regarded as members of the *M. heimii* species complex (Crous & Wingfield 1997b, Crous *et al.* 2001). Previous studies based on ITS DNA sequence data have demonstrated the phylogenetic relatedness of these four species (Crous *et al.* 2001, Crous *et al.* 2004a). However, bootstrap support for their phylogenetic placement was low (Crous *et al.* 2004a). The phylogeny of combined DNA sequence data in this study showed that the four species in the *M. heimii* complex reside in a well-supported clade (bootstrap support 97 %). The short branch lengths indicate that the four species have also recently diverged from a common ancestor.

In the phylogeny based on the combined sequence data sets, *M. gracilis* grouped in a well-supported *Pseudocercospora* clade that also included isolates of *Ps. robusta*, *M. fori*, *Ps. pseudoeucalyptorum*, *Ps. eucalyptorum*, *Ps. basitruncata*, *Ps. natalensis*, *Ps. paraguayensis* and *Ps. basiramifera*. This is the first study in which DNA sequence data for *M. gracilis* have been incorporated into a phylogeny. In the ITS, EF-1 $\alpha$  and ACT phylogenies, *M. gracilis* was phylogenetically most closely related to *Ps. pseudoeucalyptorum*. However, *M. gracilis* (anamorph: *Pseudocercospora gracilis* Crous & Alfenas) can be distinguished from *Ps. pseudoeucalyptorum* by its single conidiophores arising exclusively from secondary mycelium, which is different to *Ps. pseudoeucalyptorum* in which conidiophores arise from loose or dense fascicles of a stroma (Crous 1998, Crous *et al.* 2004a). Furthermore, conidia of *Ps. gracilis* are more septate, longer, and more uniformly cylindrical in shape than those of *Ps. pseudoeucalyptorum* (Crous 1998, Crous *et al.* 2004a). Results of the present study clearly emphasise the fact that species which are morphologically distinct, can be very closely related.

An interesting result emerging from the phylogenetic analyses in this study was the placement of *Pseudocercospora epispermogonia* in relation to *Mycosphaerella marksii* and *Mycosphaerella intermedia*. Sequences for all but the ACT gene region showed that these three taxa represent the same phylogenetic species. Although it has previously been suggested that *M. marksii* should have a *Stenella* anamorph because of its proximity to *M. parkii* (Crous *et al.* 2001), the current data suggest that this anamorph could be *Ps. epispermogonia*. Crous & Wingfield (1996) described *Ps. epispermogonia* from spermatogonia on lesions colonised by *M. marksii*, but failed to link the two states because single-ascospore cultures did not form an anamorph in culture. *Mycosphaerella intermedia* is morphologically similar to *M. marksii*, and probably represents the same taxon. We therefore reduce *M. intermedia* to synonymy with *M. marksii* as follows:

***Mycosphaerella marksii*** Carnegie & Keane, Mycol. Res. 98: 413–416. 1994.

= *Mycosphaerella intermedia* M. A. Dick & Dobbie, New Zealand J. Bot. 39: 270. 2001.

Anamorph: *Pseudocercospora epispermogonia* Crous & M.J. Wingf., Mycologia 88: 456. 1996.

*Mycosphaerella africana*, *M. aurantia* and *M. keniensis*

have no known anamorphs. Previous studies based on ITS sequence data have suggested that *M. africana* and *M. keniensis* grouped close to *Mycosphaerella* spp. with *Passalora* anamorphs. It has thus been assumed that *M. africana* and *M. keniensis* would have *Passalora* anamorphs if they were to be found (Crous *et al.* 2000). However, the phylogenies emerging from LSU, ITS and EF-1 $\alpha$  sequences and the combined data for the three regions showed that *M. africana*, *M. keniensis* and *M. aurantia* consistently group separately from the *Passalora* anamorphs, close to a clade of isolates with *Uwebraunia* and *Pseudocercospora* anamorphs. The association of these three taxa to *Passalora* is thus doubted. Furthermore, the clade containing *M. africana*, *M. aurantia* and *M. keniensis* is also well-supported and seems to represent a single evolving lineage.

Moreover, results of the present study show that *M. aurantia* and *M. africana* represent a single phylogenetic species. These two species consistently grouped together in all phylogenies with *M. keniensis* grouping as a sister. *Mycosphaerella aurantia* was described from leaves of *E. globulus* in south-western Australia and is known only from this location (Maxwell *et al.* 2003). Morphologically, *M. aurantia* produces asci and ascospores that are similar in size and morphology to *M. africana*. However, the ascospores of *M. aurantia* are not constricted at the median septum whereas those of *M. africana* had such constrictions, and ascospores of *M. aurantia* are longer (9–)11–12(–15)  $\mu\text{m}$  than those of *M. africana* (7–)8–10(–11)  $\mu\text{m}$  (Crous 1998, Maxwell *et al.* 2003). Furthermore, *M. aurantia* produces lateral hyaline germ tubes that grow parallel to the long axis of the ascospore and become slightly verrucose to produce lateral branches upon prolonged incubation (Maxwell *et al.* 2003). This is in contrast to ascospores of *M. africana* that germinate in an irregular fashion producing distinctly dark verrucose germ tubes from different positions of the distorted ascospore (Crous 1998). It is intriguing that these two species, which are morphologically quite distinct, would represent a single phylogenetic species. Additional isolates of these species are required to determine whether they represent two distinct taxa or are conspecific.

*Mycosphaerella gregaria* was described from leaves of *E. grandis* in Victoria, Australia (Carnegie & Keane 1997). No anamorph has been observed for this species (Carnegie & Keane 1997, Crous 1998). An isolate of *M. gregaria*, collected from *E. globulus* in Australia, consistently grouped in a clade with isolates of *M. endophytica* and *M. ellipsoidea*. *Mycosphaerella endophytica* and *M. ellipsoidea* are known to have *Pseudocercospora* and *Uwebraunia* anamorphs, respectively (Crous 1998). Based on previous studies employing ITS sequence data, isolates of *M. endophytica* grouped sister to isolates of *M. aurantia*, *M. ellipsoidea* and *M. africana* (Crous *et al.* 2004a). However, based on sequence data from the four gene regions employed in this study, isolates of *M. endophytica* grouped in a distinct well-supported clade with *M. ellipsoidea*. This is interesting because *M. ellipsoidea* has an *Uwebraunia* anamorph (Crous & Wingfield 1996). *Mycosphaerella endophytica* and *M.*

*pseudoendophytica* Crous & G. Hunter are the only *Mycosphaerella* spp. occurring on *Eucalyptus* that are known to have *Pseudocercospora* anamorphs (Crous 1998, Crous et al. 2006 – this volume).

Phylogenies emerging from analyses of sequences for the four gene regions considered in this study suggest that *Mycosphaerella* constitutes heterogeneous groups of which only a few are closely linked to certain anamorph genera. It is evident that for the larger part the evolution of the anamorph genera within *Mycosphaerella* has been polyphyletic, and not monophyletic as previously suggested. This can be seen by the multiple evolution of anamorph genera such as *Passalora*, *Pseudocercospora*, *Phaeophleospora* and *Stenella* within *Mycosphaerella* (Crous et al. 2006). It would thus not be advisable to predict anamorph relationships based on the phylogenetic position within *Mycosphaerella*. Not only has the same morphology evolved more than once in the group, but disjunct anamorph morphologies also frequently cluster together (Crous et al. 2000, 2004a, 2006). This makes the interpretation difficult, and predictions based on position in clades unreliable.

The production of four nucleotide sequence data sets for species of *Mycosphaerella* occurring on *Eucalyptus* leaves should serve as a framework for the more accurate taxonomic placement of these fungi in future. The importance of species complexes in *Mycosphaerella* has become more evident in this genus in recent years (Crous et al. 2004a, b, 2006 – this volume). To study species complexes, variable gene regions must be studied and the generation of greater numbers of data sets should allow for increased resolution at the species level. This in turn will aid in the resolution of species complexes and cryptic speciation. Studies of the deeper branches for groups in *Mycosphaerella* can in future utilise sequence data for the LSU region that have not previously been available. These should provide a more lucid indication and support for phenotypic characters that are phylogenetically informative.

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## A multigene phylogeny of the Dothideomycetes using four nuclear loci

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**Abstract:** We present an expanded multigene phylogeny of the Dothideomycetes. The final data matrix consisted of four loci (nuc SSU rDNA, nuc LSU rDNA, *TEF1*, *RPB2*) for 96 taxa, representing five of the seven orders in the current classification of Dothideomycetes and several outgroup taxa representative of the major clades in the Pezizomycotina. The resulting phylogeny differentiated two main dothideomycete lineages comprising the pseudoparaphysate Pleosporales and paraphysate Dothideales. We propose the subclasses Pleosporomycetidae (order Pleosporales) and Dothideomycetidae (orders Dothideales, Capnodiales and Myriangiales). Furthermore we provide strong molecular support for the placement of Mycosphaerellaceae and Piedraiaceae within the Capnodiales and introduce Davidiellaceae as a new family to accommodate species of *Davidiella* with *Cladosporium* anamorphs. Some taxa could not be placed with certainty (e.g. Hysteriales), but there was strong support for new groupings. The clade containing members of the genera *Botryosphaeria* and *Guignardia* resolved well but without support for any relationship to any other described orders and we hereby propose the new order Botryosphaeriales. These data also are consistent with the removal of Chaetothyriales and Coryneliales from the Dothideomycetes and strongly support their placement in the Eurotiomycetes.

**Key words:** bitunicate asci, hamathecium, loculoascomycetes, pseudoparaphyses

### INTRODUCTION

Members of the Dothideomycetes often are found as pathogens, endophytes or epiphytes of living plants and also as saprobes degrading cellulose and other complex carbohydrates in dead or partially digested plant matter in leaf litter or dung. Combinations of these niches can be occupied by a single fungus as it passes through its life cycle; for example several fungi initiate their life cycles on living plants and switch to saprobic states when the plant dies or leaves are lost. The nutritional modes are not limited to associations with plants and several species are lichenized, while others occur as parasites on fungi or members of the kingdom animalia.

Although to a casual observer there is little to distinguish the flask-, spherical- or disk-shaped fruiting bodies of the Dothideomycetes from several other ascomycete groups, they share a distinctive pattern of development. The asci bearing the sexual spores develop in locules already formed lysigenously within vegetative hyphae. This, defined as ascolocular development, is in contrast to ascohymenial development found in the majority of other fungal classes. Ascohymenial development generates asci in a broad hymenium interspersed with apically free paraphyses and the reproductive structure is derived from cells after fertilization.

Building on earlier descriptions of ascolocular development Nannfeldt (1932) proposed the group “Ascoloculares” and in 1955 this was formally proposed as a class “Loculoascomycetes” by Luttrell (1955). The importance of ascus morphology and dehiscence, in addition to the presence of surrounding elements inside the ascostromata, was emphasized (Luttrell 1951). The bitunicate ascus remains a defining character in modern dothideomycete taxonomy. It consists of a thick extensible inner layer (endotunica) and a thin inextensible outer layer (ectotunica). Most species release their ascospores by the extension of the inner ascus wall and the rupture of the outer wall, similar to a jack-in-the-box (fissitunicate), but variations are numerous. Another character of note, the centrum, defined as the tissues and cells occupying the cavity of the sexual structure, was expanded by Luttrell when he described three different ascostromatal developmental types exemplified by the genera *Dothidea*, *Pleospora* and *Elsinoë* forming part of the currently accepted orders, Dothideales, Pleosporales and Myriangiales (see [tolweb.org/Dothideomycetes](http://tolweb.org/Dothideomycetes) for details). The ha-

mathecium (Eriksson 1981) (i.e. the sterile centrum tissues existing between the asci) is one of the most reliable characters used to delineate ordinal classifications within the Dothideomycetes. The presence of pseudoparaphyses (sterile cells extending down from the upper portion of the ascoma, initially attached at both ends, although the upper part may become free) is a notable character for the Pleosporales, together with mainly ostiolate flask-shaped pseudothecia. Conversely the absence of pseudoparaphyses and the presence of fascicles of asci are important in the Dothideales. The Myriangiales also do not have pseudoparaphyses but produce single globose asci in multiple locules. Several additional orders currently accepted are defined by combinations of centrum and ascomal characters. For a summary of different centrum types and features see Kirk et al (2001 p 224–225).

The different classification systems proposed thus far exhibited an emphasis on varying characters. For instance, the presence and morphology of characters in the hamathecium, together with ascostroma shape were used as the main characters to define ordinal groups by Luttrell (1955), while von Arx and Müller (1975) emphasized the form of the ascus and the specific opening of the ascoma. Although basing her classification on the work of Luttrell, Barr (1987) employed additional characters such as the morphology of pseudoparaphyses.

The best studied species in this group tend to be plant pathogens on important agricultural crops. Therefore a large body of work in dothideomycete taxonomy and systematics concerns descriptions of anamorphs, the predominant morphological state encountered on agricultural crops; in fact several families in this class (e.g. Pleosporaceae, Mycosphaerellaceae, Tubeufiaceae) include a high proportion of anamorphic species. These include both hyphomycetes and coelomycetes. Many of the hyphomycetes have sympodially proliferating conidiogenous cells. *Phoma*-like and other coelomycetes occur in several families (e.g. Leptosphaeriaceae, Lophiostomataceae); these have ostiolate pycnidia lined with phialidic, annellidic or holoblastic conidiogenous cells and produce small, aseptate conidia in slime. Other important species include the group now informally referred to as the “black yeasts” (some of which also belong to the Eurotiomycetes) characterized by the production of dark, slimy colonies and sporulation patterns that resemble the budding of true yeasts but actually are reduced versions of phialidic, annellidic or sympodially proliferating conidiogenous cells (de Hoog 1974). A selection of the variety of morphological structures exhibited by teleomorph and anamorph forms in the Dothideomycetes is shown (FIG. 1).

The refinement of character state homologies and the development of morphology-based classifications into a phylogenetic classification system are accelerating with the advent of molecular data. Initial analyses using DNA sequence data from the small subunit ribosomal RNA gene did not support the monophyly of the Loculoascomycetes (Spatafora et al 1995, Berbee 1996). A more recent phylogeny produced from protein gene coding data (Liu and Hall 2004) was inferred as supporting the taxonomic concepts for a monophyletic lineage for ascostromatic taxa, but the ontogenetic designations were considered oversimplified by some (Lumbsch et al 2005). Other studies combining data from protein-coding genes and the ribosomal operon have shown the paraphyly of ascostromatic, bitunicate lineages (Lutzoni et al 2004, Reeb et al 2004). An example is the group of fungi that recently were transferred to the Eurotiomycetes based on nuclear small subunit ribosomal sequences, the “black yeasts” of the Chaetothyriales (Winka et al 1998). Together with the Verrucariales and Pyrenulales these bitunicate taxa have been placed within a separate subclass, the Chaetothyriomycetidae (Miadlikowska and Lutzoni 2004), which is sister of the Eurotiomycetidae (Lutzoni et al 2004, Reeb et al 2004) in the class Eurotiomycetes (also see Geiser et al in this issue).

Several studies provide the groundwork for a phylogenetically based classification for the Dothideomycetes. Most have used nuclear small subunit ribosomal data, but nuclear large subunit ribosomal and mitochondrial small subunit sequences also were used (Lindemuth et al 2001, Lumbsch and Lindemuth 2001). This allowed for the reassessment of specific morphological characters proposed in earlier work. Specifically, poor support for phylogenetic groups based on the morphology of pseudoparaphyses was found while phylogenetic correlation of their presence or absence was well supported (Liew et al 2000, Lumbsch and Lindemuth 2001), although a single exception to this was noted (Silva-Hanlin and Hanlin 2000). In spite of these recent examples of interordinal, molecular-based phylogenetic studies, a large number of species within the ascostromatic Ascomycota remain listed as Dothideomycetes or Chaetothyriomycetes incertae sedis (Eriksson 2006). Furthermore the question of whether Dothideomycetes represents a natural group derived from a single ancestor is not settled and the need to investigate its relationships to a number of the bitunicate lichen species such as the currently separate class Arthoniomycetes remains essential. The main focus of this study however is to provide an extension of previous ribosomal DNA-based phylogenetic studies and combine a number of smaller phylogenetic analyses

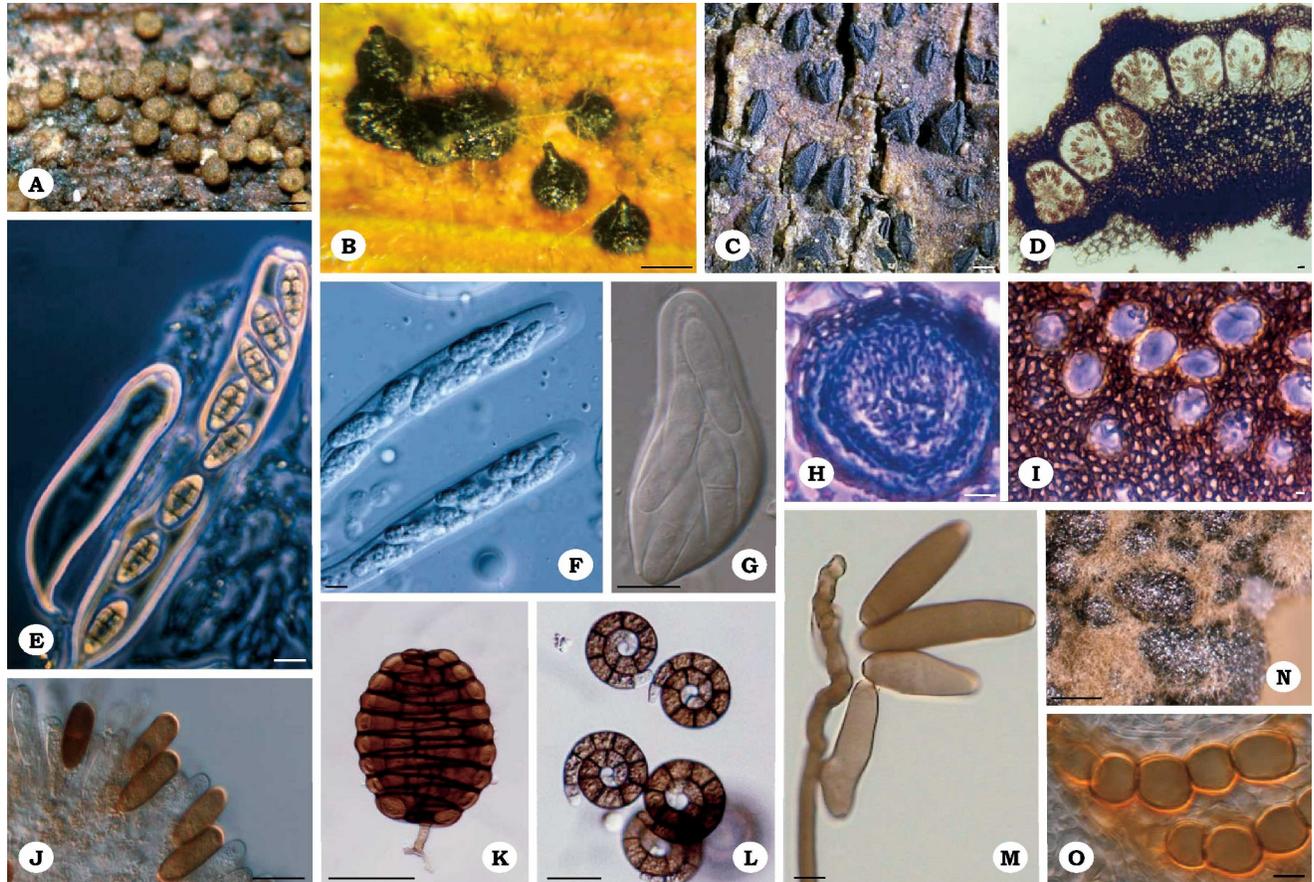


FIG. 1. A selection of dothideomycete morphological forms. Teleomorphs, ascostromata: A. Light-colored, flask-shaped pseudothecia of *Tubeufia cerea* (Tubeufiaceae) on wood. B. Dark pseudothecia of *Cochliobolus heterostrophus* (Pleosporales) on corn leaf. C. Hysterothecia of *Hysteropatella prostii* (Hysteriales), with slit-like openings. Teleomorphs, asci and locules: D. *Stylodothis puccinioides* (Dothideales), multiascus locules. E. *Pyrenophora brizae* (Pleosporales) bitunicate asci, one with broken ectotunica. F. *Guignardia magniferae* (Botryosphaerales) asci with ascospores. G. Bitunicate ascus of *Davidiella tassiana* (Capnodiales). H. *Phaeosphaeria avenaria*, juvenile ascoma with pseudoparaphyses. I. *Myriangiium duriaei* (Myriangiales), monascus locules in stroma. Anamorphs: J. Conidia borne in pycnidium of *Dothiorella* sp. (Botryosphaerales). K, L. Helical conidia, in two different dimensions, of *Helicoon* and *Helicoma* spp. (Tubeufiaceae). M. Conidia and conidiophore of *Bipolaris* sp. (Pleosporales). N. Stroma of *Trimmatostroma abietis* (Capnodiales) bearing conidia in culture. O. Chlamydozoospores of *Trimmatostroma abietis* (Capnodiales). Scale bars are approximations obtained from published sources; the bar indicates 10 µm except in A, B, C and N where it indicates 200 µm. Photo credits, courtesy of: Jean-Paul Priou (A), B. Gillian Turgeon (B), Hans-Otto Baral (C), Robert A. Shoemaker (D, E, H, I), Gary Samuels (F), Pedro W. Crous (G, J, N, O), Clement K.M. Tsui (K, L), Keith A. Seifert (M).

within the framework of a multiple gene analysis showing intraordinal relationships in the Dothideomycetes.

#### MATERIALS AND METHODS

*Sampling and alignments.*—Sequence data were obtained from GenBank and the Assembling the Fungal Tree of Life Project (AFTOL; <http://ocid.nacse.org/research/aftol/>). All strains and sequences used in this study are listed (SUPPLEMENTARY TABLE I). DNA alignments are available from the AFTOL Web site and TreeBASE (SN2913-11828). A number of sequences generated by the AFTOL project and available from the AFTOL Web site as well as from

GenBank were used. Newly generated DNA sequences were deposited at GenBank (TABLE I supplement). Genes used were nuclear small subunit ribosomal RNA gene DNA (nuc SSU), nuclear large subunit rDNA (nuc LSU), elongation factor la gene (*TEF1*), and the second largest subunits of RNA polymerase II gene (*RPB2*). Herbaria and culture collections where strains and specimens used in this study are deposited are listed (TABLE I supplement).

*Phylogenetic analysis.*—Maximum and weighted parsimony (MP and WP) analyses were performed on a combined dataset with a total of 117 taxa that included 96 Dothideomycetes. Nineteen taxa contained data for only three loci to maximize taxon sampling. The majority of the missing data were in the terminal branches of the tree, and care was

taken to include complete data sampling for taxa on branches underpinned by the more basal nodes. Two taxa with only ribosomal data (AFTOL ID 1856 *Phoma herbarum* and AFTOL ID 1864, *Didymella cucurbitacearum*) also were included to clarify the position of the clade surrounding *Phoma herbarum*. Removal of these taxa did not significantly affect support values in other parts of the tree. Likewise a comparison of a parsimony and Bayesian analysis with and without complete sets of characters yielded trees with congruent topologies. DNA sequences from a single strain (*Leptosphaeria maculans* DAOM 229267) inadvertently were included twice in the final analysis but were left in the final tree to ensure correct comparison across all approaches. We rooted the tree with three taxa from the class Pezizomycetes as outgroups (*Pyronema domesticum*, *Caloscypha fulgens* *Gyromitra californica*) (not shown in figure).

For the WP analyses the unambiguously aligned regions were subjected to symmetric step matrices for eight partitions (i.e. nuc SSU rDNA, nuc LSU rDNA and six codon positions of *TEF1* and *RPB2*) to incorporate the differences in substitution rates and patterns as described in Lutzoni et al (2004). MP and WP analyses were performed with only parsimony informative characters with these settings: 100 replicates of random sequence addition, TBR branch swapping and MULTREES in effect. Maximum likelihood was performed with PHYML (Guindon and Gascuel 2003) using a GTR+I+ $\Gamma$  model of evolution. In all preceding cases nodal support was verified by nonparametric bootstrapping under the conditions mentioned, using 500 replicates.

Initial incongruence in the single gene trees for the taxa used was tested by examining single gene analyses with WP under the conditions previously mentioned for a set of taxa containing data for all four loci. A 70% majority rule consensus tree was compared in each case. Phylogenetic analysis using Bayesian inference of maximum likelihood was performed with a parallelized version of MrBayes v 3.1.2 across four processors (Altekar et al 2004). MrBayes was run with these parameters: a general time reversible model of DNA substitution (GTR) with gamma-distributed rate variation across sites (invariance, partitioning across genes and codons). A Markov chain Monte Carlo (MCMC) analysis with metropolis coupling was run starting from a random tree for  $5 \times 10^6$  generations, sampling every 100th cycle. Four chains were run simultaneously with the initial 1000 cycles discarded as burn-in. Two additional runs with  $5 \times 10^6$  generations were compared to confirm that stationarity in likelihood values was reached and compared. The phylogenies obtained in all cases were congruent. A 50% majority rule tree from a total of 45 000 trees obtained from a single run is presented (FIG. 2).

#### RESULTS AND DISCUSSION

**Data analyses.**—The alignment for the phylogenetic analyses, after excluding introns and ambiguously aligned regions, consisted of 5098 base pairs, 1882 of which were parsimony informative. The reciprocal comparisons of 70% bootstrap trees from each gene

with 61 core taxa did not reveal any incongruence (data not shown). Therefore all of 109 taxa in the current taxon sampling were used in the combined analyses. The heuristic search in MP and WP analyses yielded six MPTs with 20 917 steps (CI = 0.204, RI = 0.535) and three MPTs with 34 319.54 steps, respectively. In model-based methods, ML heuristic search analysis resulted in a tree of  $-94 457.67$  log likelihood and resulted after the GTR model was applied with a gamma value of 0.395 across four rate categories with a proportion of invariant sites equal to 0.287. The Bayesian analysis converged on the plateau of the log-likelihood on a mean value of  $-93 955$ . The tree from Bayesian analyses is shown (FIG. 2) with all of the bootstrap proportions as well as the Bayesian posterior probabilities. Internodes were considered strongly supported if they received all of bootstrap proportions  $\geq 70\%$  and posterior probabilities  $\geq 95\%$  (Lutzoni et al 2004).

**Overview.**—The tree (FIG. 2) contains representatives of the major classes in the Ascomycota, as defined previously (Eriksson 2001). The supraclass relationships in our analysis indicated no support for a close relationship between the Dothideomycetes and Sordariomycetes, alluded to in an earlier study (Lutzoni et al 2004) and the sister relationships of the Sordariomycetes and Leotiomycetes are supported in agreement with recent data (Lumbsch et al 2005). A few taxon pairs containing isolates used in previous works have remarkably high similarity to each other over all four loci. Two examples noted in this analysis were incorrectly identified strains, namely “*Clathrospora diplospora*” CBS 174.52 = *Alternaria alternata* and “*Epipolaeum longisetosum*=*Raciborskiomyces longisetosum*” CBS 180.53 = *Cladosporium herbarum*.

**Non-Dothideomycete bitunicate groups.** Several lineages historically associated with the loculoascomycetes, such as the two species representing the Coryneliales, also were included. The placement of *Caliciopsis orientalis* together with *Caliciopsis pinea* (FIG. 2) indicates a close relationship with the Eurotiomycetidae (Geiser et al this issue). Other ordinal groups traditionally associated with the Dothideomycetes and now placed in the Eurotiomycetes were mentioned earlier. These groups share a number of centrum characters with members of the Dothideomycetes, such as the presence of periphysoids (Verrucariales, Chaetothyriales) and periphysate ostioles (Verrucariales, Chaetothyriales, Pyrenulales). The phylogeny (FIG. 2) confirms the separation of the Chaetothyriales and Verrucariales from the Dothideomycetes.

**Dothideomycetes-Arthoniomycetes clade.** The relationship of the Dothideomycetes and Arthoniomycetes (node A) is well supported by Bayesian and

maximum likelihood but not parsimony, although in an analysis without third codon positions, support by MP bootstrap and WP bootstrap increased. The internal node supporting the monophyly of the Dothideomycetes (node B) also had higher support in maximum likelihood and the two parsimony processes when the more saturated third codon positions were omitted. In more complete analyses containing characters from the RPB1 locus, this node was moderately supported and the *Trypethelium* strain is shown inside the Dothideomycetes (see Spatafora et al this issue).

Although taxon sampling for the Arthoniomycetes is sparse in our dataset, these levels of support (FIG. 2) largely agree with other recent large analyses where the Dothideomycetes is resolved as monophyletic but with low statistical support (Lumbsch et al 2005). A possible sister relationship of Dothideomycete/Arthoniomycetes has been proposed (Barr 1987, Tehler 1990) and there is some phylogenetic support for this (Lumbsch et al 2005, Lutzoni et al 2004). Clear differences between the groups exist, such as the ascohymental type development of the Arthoniomycetes apothecium (Henssen and Thor 1994). More thorough sampling of Arthoniomycetes will test the monophyly of its relationship with the Dothideomycetes. It is premature to comment on the ultimate monophyly of the Dothideomycetes, but it seems quite reasonable that increased sampling of taxa and genes could increase support for this node. As pointed out by Lumbsch et al (2005), most of the large scale interclass relationships have been in conflict in recent publications and taxon sampling should be an important consideration before making major classification changes.

*Dothideomycetes.* The addition of protein gene data illustrates that the lineages clustering around the core orders Pleosporales and Dothideales correlate with the presence or absence of pseudoparaphyses and other centrum characteristics. The node supporting the Dothideales, Capnodiales, Myriangiales and Mycosphaerellaceae (C) is strongly supported. This node was unaffected when third base codon positions were removed, but a small increase in parsimony bootstrap support was noted at node M, combining the Dothideales and Myriangiales, although ML bootstrap decreased. Saturation and the specific evolutionary model applied might have influenced this. Node C might indicate a single loss of pseudoparaphyses in all the terminal clades. However previous molecular phylogenies based on nuc SSU rDNA data have shown the presence of members of the aparaphysate genus *Leptosphaerulina* nested within the Pleosporales (Silva-Hanlin and Hanlin 2000), which could imply multiple, isolated losses of this character in other parts of the tree.

Anamorphs play an important role in the life cycles of many orders of Dothideomycetes. Many are coelomycetes, especially phialidic, *Phoma*-like anamorphs, which may be a plesiomorphic anamorph character in the class, perhaps serving some kind of spermatial function. In the Pleosporaceae and Mycosphaerellaceae hyphomycetes with sympodially proliferating conidiogenous cells with scars, and dry conidia, are particularly common and strictly anamorphic species may comprise the majority in these families. The Capnodiales, with their multitude of hyphomycete and coelomycete synanamorphs, and the helicoconidial anamorphs of the Tubeufiaceae, contain particularly distinctive anamorph groups. The anamorph genera of both hyphomycetes and coelomycetes, lacking teleomorph connections, continue to be examined for their phylogenetic relationships, many of them undoubtedly will be found to be associated with the Dothideomycetes. Several clades are well supported (FIG. 2) and will be discussed in more detail below.

*Aparaphysate Dothideomycetes.*—We hereby propose an emendation of the subclass Dothideomycetidae (*nom. nud.*) (Kirk et al 2001), which has been superceded by the Dothideomycetes O.E. Erikss. and Winka (2000). Dothideomycetidae *sensu* Lutzoni et al (2004) also was included in the Sordariomycetes as subclass Dothideomycetidae along with the subclass Sordariomycetidae (*syn. Sordariomycetes s. str.*) and Arthoniomycetidae (*syn. Arthoniomycetes*), although there was no strong statistical support for this broadened concept of Sordariomycetes. We validate and emend the concept of Dothideomycetidae *sensu* Kirk et al (2001) to include the bitunicate orders Dothideales, Capnodiales and Myriangiales, which lack paraphyses, pseudoparaphyses and paraphysoids. This emended subclass overlaps with the Loculoparenchymatomycetidae (Barr 1983) but differs by including the Myriangiales and excluding the Asterinales, now listed under its constituent families as Dothideomycetes et Chaetothyriomycetes incertae sedis by Eriksson (2006).

**Dothideomycetidae** P.M. Kirk, P.F. Cannon, J.C. David & J.A. Stalpers, ex Schoch, Spatafora, Crous et Shoemaker, **subclass nov.**

≡ *Dothideomycetidae* P.M. Kirk, P.F. Cannon & J.C. David & J.A. Stalpers, in Kirk et al, Dictionary of Fungi, 9th ed., p 165, 572. 2001 (*nom. nud.*).

Ascomata immersa vel erumpentia vel superficialia, minuta vel magnitudine media, separata vel in stromate basilari aggregata, unilocularia vel plurilocularia, ostiolata, nonnumquam periphysata. Pseudoparaphyses absentes, periphysoideae nonnumquam praesentes. Asci globosi vel ellipsoidei vel clavati vel



subcylindrici. Ascospores hyalinae vel subhyalinae vel fuscae, unicellulares vel pluriseptatae vel muriformes. Anamorphoses seu coelomycetes seu hyphomycetes.

Ascomata immersed, erumpent or sometimes superficial, minute, small or medium-sized; separate or merged or grouped on basal stroma, uni- to multi-loculate apical pore mostly present, when present ostiolar canal at times periphysate, stromatic tissues may contain pseudoparenchymatous cells. Pseudoparaphyses lacking, periphysoids may be present; Asci globose, subglobose, ovoid to ellipsoid, saccate, oblong, clavate or subcylindrical, Ascospores hyaline, subhyaline or dark brown, variable in shape and size, one celled or one to several septate or muriform.

Anamorphs coelomycetous and/or hyphomycetous.

*Type order.* Dothideales (1897) Lindau in Engler & Prantl, Nat. Pflanzenfam. 1(1):373. 1897.

*Represented orders.* Dothideales Lindau 1897, Capnodiales Woron. 1925, Myriangiiales Starbäck 1899.

*Dothideales.* Species from this order generally have smaller ascomata and fewer asci than the pseudoparaphysate Pleosporales (node D) and traditionally have been segregated because of the absence of pseudoparaphyses in their pseudothecia. The species included in this order encompass saprotrophs, hemibiotrophs and biotrophs. It is represented by eight species in our analysis, including the recent epitype isolate of *Dothidea sambuci*, the type of the genus *Dothidea* (Shoemaker et al 2003). The family Dothideaceae includes biotrophs, necrotrophs and saprobes on plant tissue. *Stylothis puccinoides* was redescribed as a separate species from *Dothidea* but remains closely associated with the genus in our phylogeny.

Three members of the Dothioraceae are polyphetic in the tree. The so-called black yeast anamorphs associated with Dothideomycetes tend to occur in this family, with *Aureobasidium pullulans* (probably an anamorph species complex based on the ITS sequences deposited in GenBank), and the micro-morphologically similar *Hormonema dematioides* (teleomorph *Sydowia polyspora*, perhaps also a complex of anamorph species) (de Hoog 1974). These species are found commonly on moist surfaces of plants and can convert from yeast to meristematic growth under

nutritional stress. Some progress in the resolution of the nature of *Aureobasidium pullulans* has been made here with the linkage of *Columnosphaeria fagi* (H.J. Hudson) M.E. Barr to a “neotype” culture CBS 584.75 of *A. pullulans* var. *pullulans* (SUPPLEMENTARY TABLE I).

*Capnodiales.* The node I is well supported in this multigene analysis. This same node is present in a ribosomal rDNA phylogeny containing “*Raciborskiomyces longisetosus*” as erroneous name for a *Cladosporium* species with *Capnodium citri* (Lumbsch and Lindemuth 2001). Synapomorphies are limited in this expanded order and these taxa have not been grouped together before. The presence of short, paraphyses-like cells in the ostiolar pore of some genera of the Capnodiales such as *Capnodium* also are reported from other families, including the Mycosphaerellaceae (von Arx and Müller 1975) and might be a synapomorphy uniting these taxa. We hereby propose an expansion of the current Capnodiales to include the Mycosphaerellaceae and Piedriaceae. The constituent families are discussed below.

*Capnodiaceae.* An ascostromatal family without pseudoparaphyses, the Capnodiaceae are leaf epiphytes associated with the honeydew of insects. Also known as sooty molds, they tend to live in complex communities, with multiple species, and often multiple fungal parasites of those species, inhabiting a common, sooty mass. They are noted for the production of darkly pigmented hyphae, often of very characteristic morphology (Hughes 1976, Reynolds 1998). The members of this order have superficial ascostromata with ovoid asci in fascicles and hyaline to dark, one to multiseptate ascospores. The sooty molds are highly pleomorphic and often highly pleoanamorphic. The order includes many anamorphic species, all dematiaceous, including several conidiomatal, mycelial (often with dry-spored, blastic phragmo- or dictyoconidia) or presumably spermatial (usually phialidic) hyphomycete genera or pycnidial synanamorphs (Hughes 1976).

*Mycosphaerellaceae.* The *Mycosphaerellaceae* is characterized by small pseudothecial ascomata that are immersed in host tissue, single and superficial, or imbedded in a pseudoparenchymatal stroma, papil-

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FIG. 2. Dothideomycete phylogeny. 50% majority rule consensus tree of 45 000 trees obtained by Bayesian inference and MCMCMC under GTR+I+ $\Gamma$  applied across seven partitions. Only orders and families with more than two members under the current classification of Eriksson (2005) are shown in shadow. Bar indicates the nucleotide substitutions per site. Nodes of interest are labeled alphabetically and support values are shown above and below. Bayesian PP = posterior probability, ML BP = maximum likelihood bootstrap, MP BP = maximum parsimony bootstrap, WP = weighted parsimony bootstrap. Gaps (–) show a collapsed node and asterisks show the presence of a differently resolved node under the specific statistical sampling method used.

late, ostiolate, lacking interascal tissue. Asci vary from ovoid to saccate to subcylindrical, usually stipitate, with or without an apical chamber, lacking any other apical mechanism. Ascospores are hyaline to slightly pigmented, 1-septate, but in some cases also 3-septate, and sometimes are enclosed in a sheath. *Mycosphaerella* has close to 30 anamorph genera associated with it, most of which have cicatrized, sympodially proliferating conidiogenous cells and single or acropetally catenate, dry conidia. The two clades delineated within *Mycosphaerella* here also were recognized in a separate study employing multiple genes to resolve relationships in *Mycosphaerella* (Hunter et al 2006). Node II contains the type of *Mycosphaerella*, *M. punctiformis*, and the bulk of *Mycosphaerella* species, while the second clade (above I4) appears to contain more extremotolerant species (Crous et al unpubl data).

*Mycosphaerella* is distinguished from *Davidiella* (*Cladosporium* anamorphs) by lacking irregular lumens or inclusions in its ascospores and not having anamorphs with protruberant, thickened, darkened, *Cladosporium*-like scars (Braun et al 2003, Aptroot 2006). As shown in this study *Davidiella* with its *Cladosporium* anamorphs (type species *Davidiella tassiana*, anamorph *Cladosporium herbarum*) clusters in a well supported clade apart from *Mycosphaerella* s.str. (*Mycosphaerellaceae*), and thus a new family is proposed for clade II.

**Davidiellaceae** Schoch, Spatafora, Crous et Shoemaker, **fam. nov.**

Ascomata *Mycosphaerellae* similia, sed lumen ascoporum forma variabile et anamorphe *Cladosporium*.

Ascomata immersed to erumpent, small or medium-sized; separate or aggregated, uniloculate, apical pore present, periphysate; wall of several layers of brown, thickened, pseudoparenchymatal cells. Pseudoparaphyses lacking. Asci bitunicate, 8-spored, ovoid to ellipsoid or subcylindrical, fasciculate, with or without apical chamber. Ascospores hyaline to pale brown, smooth to somewhat roughened, mucous sheath sometimes present, one-septate, thick-walled, with irregular lumens. Anamorphs are species of *Cladosporium*.

*Typus.* *Davidiella tassiana* (De Not.) Crous & U. Braun, Mycol. Prog. 2:8. 2003.

The position of a single representative of the Piedraiaceae, *Piedraia hortae*, is refined here as associated with the Capnodiales and allies but not the Myriangiales as reported earlier (Lindemuth et al 2001). This species was described with an ascus containing only one thin wall (Shoemaker and Egger 1982). The specialized parasites in this family are

almost exclusively associated with human hair in tropical regions. It is shown with low parsimony bootstrap support (I3) with *Trimmatostroma abietis*, a meristematic anamorph species isolated from conifer needles and rock surfaces. This species was shown to be closely related to *Mycosphaerella* and its allies in a recently published molecular phylogeny (Selbman et al 2005).

*Myriangiales.* The Myriangiales are reported to be related to the Dothideales (node M), although without any significant bootstrap support for this placement. They generally have ascostromata without ostioles in monoascal locules. The species of the type genus, *Myriangium*, has globose asci scattered at many levels in an undifferentiated stromatic mass (Sivanesan 1984). The order includes saprobic, epiphytic or biotrophic organisms. The anamorphs of this order, when known, generally are acervular coelomycetes with polyphialidic conidiogenous cells, such as the *Sphaceloma* anamorphs of *Elsinoë* species (Kirk et al 2001).

*Paraphysate Dothideomycetes.*

We hereby propose a new subclass for the pseudoparaphysate taxa supported by node D1.

**Pleosporomycetidae** Schoch, Spatafora, Crous et Shoemaker, **subclass nov.**

Ascomata perithecialia vel hysterothecialia vel cleistothecialia, immersa vel erumpentia. Hamathecii pseudoparaphyses cellulares vel trabeculatae, maturae nonnumquam deliquescentes. Asci bitunicati, plerumque basillares, nonnumquam lateraliter extendentes, cylindrici vel clavati vel oblongi vel saccati. Ascosporae colore, forma septisque variabiles, plerumque heteropolares sed nonnumquam etiam symmetricae.

Ascomata perithecioid, hysterothecioid or cleistothecioid, conchate or dolabrate, immersed, erumpent or superficial; globose, sphaeroid, turbinate, ovoid, obpyriform, conoid, doliiform, dimidiate. Hamathecium of wide to narrow cellular or trabeculate pseudoparaphyses, deliquescing at maturity in some. Asci bitunicate, usually basal, at times extending laterally, cylindric, clavate, oblong or saccate. Ascospores variable in pigmentation, shape and septation, usually with bipolar asymmetry, but some symmetrical.

*Type order.* Pleosporales Luttrell ex M.E. Barr.

*Represented order.* Pleosporales Luttrell ex M.E. Barr.

*Pleosporales.* The Pleosporales is the largest order in the Dothideomycetes. It contains several well known plant pathogens such as *Cochliobolus heterostrophus*, the causative agent for southern blight on corn, *Leptosphaeria maculans*, causing black leg on rape seed and

*Phaeosphaeria nodorum* causing stagonospora blotch in cereals. In this analysis a strain of *Delitschia winterti* is placed above node D, supporting the rest of the Pleosporales according to Eriksson's broad concept (2001). *Delitschia* shares features common to several bitunicate species occurring on dung; they are darkly pigmented, usually strongly constricted ascospores with germ slits (Barr 2000). The family Delitschiaceae was described by Barr (2000) for species previously placed in the Sporormiaceae. The delineation is based on an ostiole containing periphyses and asci with wide outer ascus walls and an ocular chamber containing refractive rods. This placement was confirmed with nuc SSU rDNA sequence comparisons (Kruys 2005). A combined nuc SSU analysis of *Delitschia winterti* grouped it close to another species of the genus, *D. didyma* (AF242264), confirming the identification of the strain used (results not shown). Members of this family are hypersaprotrophic on old dung and exposed wood.

There was also strong support for the monophyly of Pleosporales, with *Lophium mytilinum* branching at its most basal node (D1). This species is found as a saprobe on wood and on cones of conifers and is listed incertae sedis as part of the Mytiliniaceae (Eriksson 2006). The family contains species with characteristic conch shaped ascomata. Analyzing additional taxa from the Mytiliniaceae and related groups also will be important to investigate ancestral character states for the Pleosporales but they should be placed as Pleosporomycetidae incertae sedis for now.

The morphology of ascospores has played an important role in delimiting families in the Pleosporales. However, as noted from some of the first molecular based phylogenies of the Dothideomycetes, several family relationships might be poorly supported (Lindemuth et al 2001). Perhaps the strains chosen are not good exemplars for their families or are derived from misidentified specimens. However it seems unlikely that this can account for all the relationships (FIG. 2) and a reassessment at this level of classification seems urgent. Here we will discuss only briefly a selection of highlighted families (FIG. 2).

The most basal node inside the Pleosporales (D2) supports two members of the Testudinaceae, provisionally included among Ascomycota incertae sedis by Eriksson (2006). Members of this family are mainly isolated from soil and produce reduced, cleistothecoid ascostromata. This clade unexpectedly contains the ostiolate marine species, *Verruculina enalia* (Didymosphaeriaceae) as also noted in an earlier phylogenetic analysis (Kruys 2005). The next well supported clade above node D3 supports the Spor-

ormiaceae. These fungi are found commonly on dung but some occur on other substrates (e.g. wood, soil and plant debris). A large number of species in this group have germ slits. This morphological variability was confirmed in a phylogenetic study using DNA sequences from multiple ribosomal loci (Kruys 2005).

The Lophiostomataceae and Melanommataceae are inferred as paraphyletic in the next set of clades (above D4 and D5), with one clade including two species of *Lophiostoma* (Lophiostomataceae 1). This clade also contains one species of *Trematosphaeria heterospora*, which was classified as *Lophiostoma heterosporum* (Barr 1992). The second clade (Lophiostomataceae 2) includes members of the Lophiostomataceae and Pleomassariaceae as well as Melanommataceae. Node D5 contains a diverse group of species isolated from diseased and decaying plants as well as soil (each currently classified under a different family). This overlapped with relationships reported before, using molecular-based phylogenies (Liew et al 2000, 2002), but like many of the other clades will require more intense sampling to address family and genus descriptions.

The more terminal branches in the Pleosporales (D6) include well studied families containing important plant pathogens, saprobes and animal pathogens with numerous anamorphs. *Didymella cucurbitacearum* forms a clade with the anamorphs *Ascochyta pisi* and *Phoma herbarum* (D8), parasites on agricultural crops. *Leptosphaeria* (Leptosphaeriaceae), shown on a single branch, is a large genus with pale to dark brown and septate ascospores. Members of this family have flask-shaped pseudothecia with narrow asci and a characteristic thin apex. Many species are associated with coelomycetous anamorphs. *Phoma* anamorphs are particularly common (Camara et al 2001, Verkley et al 2004). The Phaeosphaeriaceae (D9) are distinguished from the Leptosphaeriaceae by ascomal wall morphology and all have pycnidial coelomycetes, mostly classified in *Stagonospora*, characterized by holoblastic or sometimes annellidic conidiogenesis and the production of phragmoconidia. Unnamed pycnidial microconidial anamorphs also are reported in some species (Leuchtman 1984). In a poorly supported clade a trio of species without any clear phylogenetic placement are noted. Two of these species are anamorphs, *Coniothyrium palmarum* and *Pyrenochaeta nobilis*, linked to the teleomorphs *Leptosphaeria* and *Herpotrichia*.

The next well supported node (D10) contains the Pleosporaceae, which have ascostromata that are mainly flask-shaped pseudothecia embedded in the substrate with 1-septate to muriform ascospores. In

addition to species found in marine environments and as parasites on animals a number of important grass and cereal crop parasite genera, *Cochliobolus*, *Pyrenophora* and *Lewia*, are included in this family. The sexual states are normally well linked with single anamorph genera. Important anamorph species include the well known genera *Alternaria* (with *Ulocladium* paraphyletic within it), *Stemphylium*, the so-called helminthosporia (*Bipolaris*, *Curvularia*, *Drechslera*, *Exserohilum*) and a few other genera such as *Dendryphon* and *Dendryphiopsis*.

*Dothideomycetes incertae sedis*.—A number of orders could not be placed in any of the two subclasses defined and will be discussed in more detail. Two orders, Jahnulales and Patellariales, currently listed by Eriksson (2006) are not included in this analysis but a separate comparison using deposited sequences from nuc SSU obtained from GenBank combined with our complete taxa revealed them to be separate from the groups referred to in this paper (data not shown).

Members of Hysteriales have been reported with pseudoparaphyses in apotheciid ascomata with elongated openings (von Arx and Müller 1975, Barr 1987, Luttrell 1974) and are often saprobes on wood or weak parasites of woody plants. Four members of the Hysteriales agreeing mainly with Luttrell's original definitions are included (FIG. 2) and it is clear that these are not a monophyletic group, a proposition also mentioned by Luttrell (1973). *Farlowiella carmichaeliana* could not be resolved with any certainty.

The phylogeny also supports a relationship between the dung fungus *Phaeotrichum benjaminii* and *Tyrannosorus pinicola* (FIG. 2). *Phaeotrichum* is characterized by dark-brown, septate spores and cleistotheciid ascostromata. *T. pinicola* produces ostiolate ascostromata with characteristic long, sharp spines and have been isolated from wood and plant material. The multiple germ slits that were described for *T. pinicola* may be linked to the terminal germ pores characteristic of *P. benjaminii*.

Node E supports *Kirschsteiniothelia aethiops* with its *Dendryphiopsis atra* anamorph. These two species also appear unrelated to other species in the genus (Shearer 1993) based on nuc SSU rDNA data and the genus is reportedly heterogenous (Hawksworth and Eriksson 2003). *K. aethiops* does not have close associations with the Pleosporaceae and should be placed in a separate family.

The Tubeufiaceae clade (above node G) contains species with a variety of nutritional modes. They often are reported as saprobes from terrestrial and freshwater environments, but some species are hyperparasites and others can parasitize insects. Teleomorphs consist of brightly colored ascostromata, with long,

hyaline, multiseptate ascospores (Rossman 1987). The best-known anamorphs of the Tubeufiaceae are helicosporous hyphomycetes and well known genera include *Helicodendron*, *Helicomycetes* and *Helicoon*. Recent DNA sequence-based comparisons did not find strong correlation between these anamorph forms and phylogenetic groups. (Tsui et al 2006). Combining recent focused phylogenies into a large scale dataset is required before placement of this group in the current classification.

*Botryosphaeriaceae*. The position of the Botryosphaeriaceae (H) within the Dothideomycetes has been enigmatic. The taxonomy of this group of plant-associated fungi has relied mostly on anamorph descriptions; sequence data recently have linked several anamorph genera to the genus *Botryosphaeria* (Jacobs and Rehner 1998). Associated anamorphs were divided into two groups, those with thin-walled, hyaline conidia (*Fusicoccum*), and those with thick-walled, pigmented conidia (*Diplodia*) (Denman et al 2000). In a recent phylogenetic study employing LSU sequence data to resolve relationships among members of the Botryosphaeriaceae, Crous et al (2006) segregated *Botryosphaeria* into several genera, supported by morphological differences of their anamorphs. From the phylogenetic results obtained in this study, it is clear that the Botryosphaeriaceae deserves an order separate from the Pleosporales and Dothideales, which is introduced below.

**Botryosphaeriales** Schoch, Crous & Shoemaker, **ord. nov.**

*Family. Botryosphaeriaceae* Theiss. & P. Syd., Ann. Mycol. 16:16 (1918).

*Type. Botryosphaeria* Ces. & De Not., Comment Soc. crittog. Ital. 1:211 (1863)..

*Type species. B. dothidea* (Moug.:Fr.) Ces. & De Not., Comment Soc. crittog. Ital. 1:212 (1863).

Ascomata unilocularia vel plurilocularia, pariete multistratosa fusca inclusa, singularia vel aggregata, raro in stromate submersa. Asci bitunicati, endotunica crassa, stipitati vel sessiles, clavati, camera apicali distincta, pseudoparaphysibus hyalinis, septatis, ramosis vel simplicibus intermixti. Ascosporae hyalinae vel pigmentatae, unicellulares vel septatae, ellipsoideae vel ovoideae, nonnumquam appendicibus vel tunica gelatinosis praeditae. Anamorphoses: conidiomata pycnidialia, unilocularia vel multilocularia, saepe in stromate submersa, cellulis conidiogenis phialidicis, conidia hyalina vel pigmentata, tenui- vel crassitunicata proferentibus, quae nonnumquam appendicibus vel tunica gelatinosis praedita sunt.

*Ascomata* uni- to multilocular with multilayered dark brown walls, occurring singularly or in clusters, frequently embedded in stromatic tissue. *Asci* bituni-

cate, with a thick endotunica, stalked or sessile, clavate, with a well developed apical chamber, intermixed with hyaline, septate pseudoparaphyses, branched or not. *Ascospores* hyaline to pigmented, septate or not, ellipsoid to ovoid, with or without mucoid appendages or sheath. *Anamorphs* have uni- to multilocular pycnidial conidiomata, frequently embedded in stromatic tissue, with hyaline, phialidic conidiogenous cells, and hyaline to pigmented, thin- to thick-walled conidia, which sometimes have mucoid appendages or sheaths.

*Conclusion.*—This multigene phylogeny contributes to the overall phylogenetic classification of the Dothideomycetes. We emend a previously proposed subclass, the Dothideomycetidae, and propose a new one, the Pleosporomycetidae, based on the presence or absence of pseudoparaphyses as defined by Barr (1987) based on Luttrell (1955). The orders according to Eriksson (2006) are largely upheld with the exception of the Hysteriales, but we also expand this classification with an additional order, the Botryosphaerales, and redefine the Capnodiales to include the currently defined Mycosphaerellaceae and Piedraiaceae. A new family, the Davidiellaceae, is proposed to accommodate *Davidiella* species with *Cladosporium* anamorphs. Several clades did not correlate with familial relationships under Eriksson's classification (2006) and should be addressed in subsequent analyses. Similarly a number of small clades are incertae sedis and remain to be addressed in the future. The strains used in this study, although validated by morphological examinations in previous publications (e.g. Berbee 1996) as well as by comparisons with sequences from GenBank, should continue to be validated by more intensive taxon sampling in a number of clades. The value of additive sampling in this study, where two strains used in previous studies could be shown to be misidentified, supports this.

One large gap in this analysis is the absence of lichenized lineages. A single unidentified *Trypethelium* species was included, but numerous lichenized ascostromatic bitunicate species (such as those in the Pyrenulales) remain candidates for placement in the Dothideomycetes. In fact a study by Del Prado et al (2006) shows good support for a placement of the lichenized Trypetheliaceae within the Dothideomycetes. In addition, numerous lineages remain unresolved in this class. For example the current classification of Eriksson (2006) contains 23 families placed in orders but more than 40 families remain listed as Chaetothyriomycetes et Dothideomycetes incertae sedis. It appears likely that, in the process of combining the comprehensive body of work already done on the biology, ontogeny and morphol-

ogy of these fungi within a molecular-based phylogenetic context, they will continue to surprise and challenge us well into the future.

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# Biodiversity in the *Cladosporium herbarum* complex (*Davidiellaceae*, *Capnodiales*), with standardisation of methods for *Cladosporium* taxonomy and diagnostics

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**Abstract:** The *Cladosporium herbarum* complex comprises five species for which *Davidiella* teleomorphs are known. *Cladosporium herbarum* s. str. (*D. tassiana*), *C. macrocarpum* (*D. macrocarpa*) and *C. bruhnei* (*D. alicina*) are distinguishable by having conidia of different width, and by teleomorph characters. *Davidiella variabile* is introduced as teleomorph of *C. variabile*, a homothallic species occurring on *Spinacia*, and *D. macrospora* is known to be the teleomorph of *C. iridis* on *Iris* spp. The *C. herbarum* complex combines low molecular distance with a high degree of clonal or inbreeding diversity. Entities differ from each other by multilocus sequence data and by phenetic differences, and thus can be interpreted to represent individual taxa. Isolates of the *C. herbarum* complex that were formerly associated with opportunistic human infections, cluster with *C. bruhnei*. Several species are newly described from hypersaline water, namely *C. ramotenellum*, *C. tenellum*, *C. subinflatum*, and *C. herbaroides*. *Cladosporium pseudiridis* collected from *Iris* sp. in New Zealand, is also a member of this species complex and shown to be distinct from *C. iridis* that occurs on this host elsewhere in the world. A further new species from New Zealand is *C. sinuosum* on *Fuchsia excorticata*. *Cladosporium antarcticum* is newly described from a lichen, *Caloplaca regalis*, collected in Antarctica, and *C. subtilissimum* from grape berries in the U.S.A., while the new combination *C. ossifragi*, the oldest valid name of the *Cladosporium* known from *Narthecium* in Europe, is proposed. Standard protocols and media are herewith proposed to facilitate future morphological examination of *Cladosporium* spp. in culture, and neotypes or epitypes are proposed for all species treated.

**Taxonomic novelties:** *Cladosporium antarcticum* K. Schub., Crous & U. Braun, sp. nov., *C. herbaroides* K. Schub., Zalar, Crous & U. Braun, sp. nov., *C. ossifragi* (Rostr.) U. Braun & K. Schub., comb. nov., *C. pseudiridis* K. Schub., C.F. Hill, Crous & U. Braun, sp. nov., *C. ramotenellum* K. Schub., Zalar, Crous & U. Braun, sp. nov., *C. sinuosum* K. Schub., C.F. Hill, Crous & U. Braun, sp. nov., *C. subinflatum* K. Schub., Zalar, Crous & U. Braun, sp. nov., *C. subtilissimum* K. Schub., Dugan, Crous & U. Braun, sp. nov., *C. tenellum* K. Schub., Zalar, Crous & U. Braun sp. nov., *Davidiella macrocarpa* Crous, K. Schub. & U. Braun, sp. nov., *D. variabile* Crous, K. Schub. & U. Braun, sp. nov.

**Key words:** Clonality, *Davidiella*, homothallism, new species, phylogeny, recombination, taxonomy.

## INTRODUCTION

*Cladosporium herbarum* (Pers.: Fr.) Link, type species of the genus *Cladosporium* Link, is one of the most common environmental fungi to be isolated worldwide. It abundantly occurs on fading or dead leaves of herbaceous and woody plants, as secondary invader on necrotic leaf spots, and has frequently been isolated from air (Samson *et al.* 2000), soil (Domsch *et al.* 1980), foodstuffs, paints, textiles, humans (de Hoog *et al.* 2000) and numerous other substrates. It is also known to occur on old carpophores of mushrooms and other fungi (Heuchert *et al.* 2005) and to be a common endophyte (Riesen & Sieber 1985, Brown *et al.* 1998, El-Morsy 2000), especially in temperate regions. Under favourable climatic conditions *C. herbarum* also germinates and grows as an epiphyte on the surface of green, healthy leaves (Schubert 2005).

Persoon (1794) introduced *C. herbarum* as *Dematium herbarum* Pers., which was later reclassified by Link (1809) as *Acladium herbarum* (Pers.) Link. In 1816, Link included *C. herbarum* together with three additional species in his newly described genus *Cladosporium*. Clements & Shear (1931) proposed *C. herbarum* as lectotype species of the latter genus, a decision followed by de Vries (1952) and Hughes (1958). Several authors provided detailed treatments of *C. herbarum* (de Vries 1952, Ellis 1971, Domsch *et al.* 1980, Prasil & de Hoog 1988), and there are literally thousands of records of this species in the literature. McKemy & Morgan-Jones (1991) and Ho *et al.* (1999) examined *C. herbarum* in culture and published detailed descriptions of its features *in vitro*.

*Cladosporium macrocarpum* Preuss, a second component within the herbarum complex, has hitherto been known and treated as an allied, but morphologically distinct species on the basis of its

wider and somewhat larger, frequently 2–3-septate, more regularly verrucose conidia, shorter conidial chains and more pronounced prolongations of the conidiophores. Dugan & Roberts (1994) carried out examinations of morphological and reproductive aspects of both species, and in so doing demonstrated a morphological continuum between *C. macrocarpum* and *C. herbarum*, concluding that the name *herbarum* should have preference. Therefore, Ho *et al.* (1999) introduced the new combination *C. herbarum* var. *macrocarpum* (Preuss) M.H.M. Ho & Dugan. Although transitional forms have been discussed to occur between the two species, several authors still prefer to retain *C. macrocarpum* as a separate species.

In an attempt to elucidate the species within the *C. herbarum* complex, therefore, a multilocus DNA sequence typing approach was used, employing five genes, namely the internal transcribed spacers of the rDNA genes (ITS), actin, calmodulin, translation elongation factor 1- $\alpha$ , and histone H3. These data were supplemented with morphological examinations under standardised conditions, using light and scanning electron microscopy, as well as cultural characteristics and growth studies.

## MATERIAL AND METHODS

### Isolates

Isolates included in this study were obtained from the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands, or were freshly isolated from a range of different substrates. Single-conidial and ascospore isolates were obtained using the techniques as explained in Crous (1998) for species of *Mycosphaerella* Johanson and its anamorphs. Isolates

were inoculated onto 2 % potato-dextrose agar (PDA), synthetic nutrient-poor agar (SNA), 2 % malt extract agar (MEA) and oatmeal agar (OA) (Gams *et al.* 2007), and incubated under continuous near-ultraviolet light at 25 °C to promote sporulation. All cultures obtained in this study are maintained in the culture collection of the CBS (Table 1). Nomenclatural novelties and descriptions were deposited in MycoBank ([www.MycoBank.org](http://www.MycoBank.org)).

### DNA isolation, amplification and sequence analysis

Fungal colonies were established on agar plates, and genomic DNA was isolated as described in Gams *et al.* (2007). Partial gene sequences were determined as described by Crous *et al.* (2006) for actin (ACT), calmodulin (CAL), translation elongation factor 1- $\alpha$  (EF), histone H3 (HIS) and part (ITS) of the nuclear rDNA operon spanning the 3' end of the 18S rRNA gene (SSU), the first internal transcribed spacer, the 5.8S rRNA gene, the second internal transcribed spacer and the 5' end of the 28S rRNA gene (LSU). The nucleotide sequences were generated using both PCR primers to ensure good quality sequences over the entire length of the amplicon. Subsequent sequence alignment and phylogenetic analysis followed the methods of Crous *et al.* (2006). Gaps longer than 10 bases were coded as single events for the phylogenetic analyses; the remaining gaps were treated as new character states. Sequence data were deposited in GenBank (Table 1) and the alignment and tree in TreeBASE ([www.treebase.org](http://www.treebase.org)).

### Data analysis

The number of entities in the dataset of 79 strains was inferred with STRUCTURE v. 2.2 software (Pritchard *et al.* 2000, Falush *et al.* 2003) using an UPGMA tree of data of the ACT gene compared with CAL, EF and HIS with the exclusion of the nearly invariant ITS region. For this analysis group indications were derived from a tree produced with MrAIC (Nylander 2004). The length of the burn-in period was set to 1 000 000, number of MCMC repeats after burn-in 10 000, with admixture ancestry and allele frequencies correlated models, assuming that all groups diverged from a recent ancestral population and that allele frequencies are due to drift. Uniform prior for ALPHA was set to 1.0 (default) and allele frequencies with  $\lambda$  set to 1.0 (default). The numbers of MCMC repetitions after burn-in were set as 10 000 and 100 000. The number of clusters (K) in STRUCTURE was assumed from 5 to 7. Population differentiation  $F_{ST}$  (index:  $\theta$ ) was calculated with 1–6 runs using the same software. The null hypothesis for this analysis is no population differentiation. When observed theta ( $\theta$ ) is significantly different from those of random data sets ( $p < 0.05$ ), population differentiation is considered.

Association of multilocus genotypes was screened with the multilocus option in BioNUMERICS v. 4.5. To test for reproductive mode in each population, the standardised index of association ( $I^S_A$ ; Haubold *et al.* 1998) was calculated with START2 software (Jolley *et al.* 2001). The null hypothesis for this analysis is complete panmixia. The values of  $I^S_A$  were compared between observed and randomised datasets. The hypothesis would be rejected when  $p < 0.05$ . Mean genetic diversity (H) and diversities of individual loci were calculated with LIAN v. 3.5 (Haubold & Hudson 2000). Degrees of recombination or horizontal gene transfer were also visualised using SPLITS TREE v. 4.8 software (Huson & Bryant 2006). Split decomposition was carried out with default settings, i.e., character transformation using uncorrected (observed, "P") distances, splits transformation using "equal angle", and optimise boxes iteration set to 2.

### Morphology

As the present study represents the first in a series dealing with *Cladosporium* spp. and their *Davidiella* Crous & U. Braun teleomorphs in culture, a specific, standardised protocol was established by which all species complexes will be treated in future.

**Morphology of the anamorph:** Microscopic observations were made from colonies cultivated for 7 d under continuous near-ultraviolet light at 25 °C on SNA. Preparations were mounted in Shear's solution (Gams *et al.* 2007). To study conidial development and branching patterns, squares of transparent adhesive tape (Titan Ultra Clear Tape, Conglom Inc., Toronto, Canada) were placed on conidiophores growing in the zone between the colony margin and 2 cm inwards, and mounted between two drops of Shear's solution under a glass coverslip. Different types of conidia are formed by *Cladosporium* species for which different terms need to be adopted.

**Ramoconidia** are conidia with usually more than one (mostly 2 or 3) conidial hilum, which typically accumulate at the tip of these conidia. Conidiogenous cells with more than one conidiogenous locus are first formed as apical parts of conidiophores. Such apical

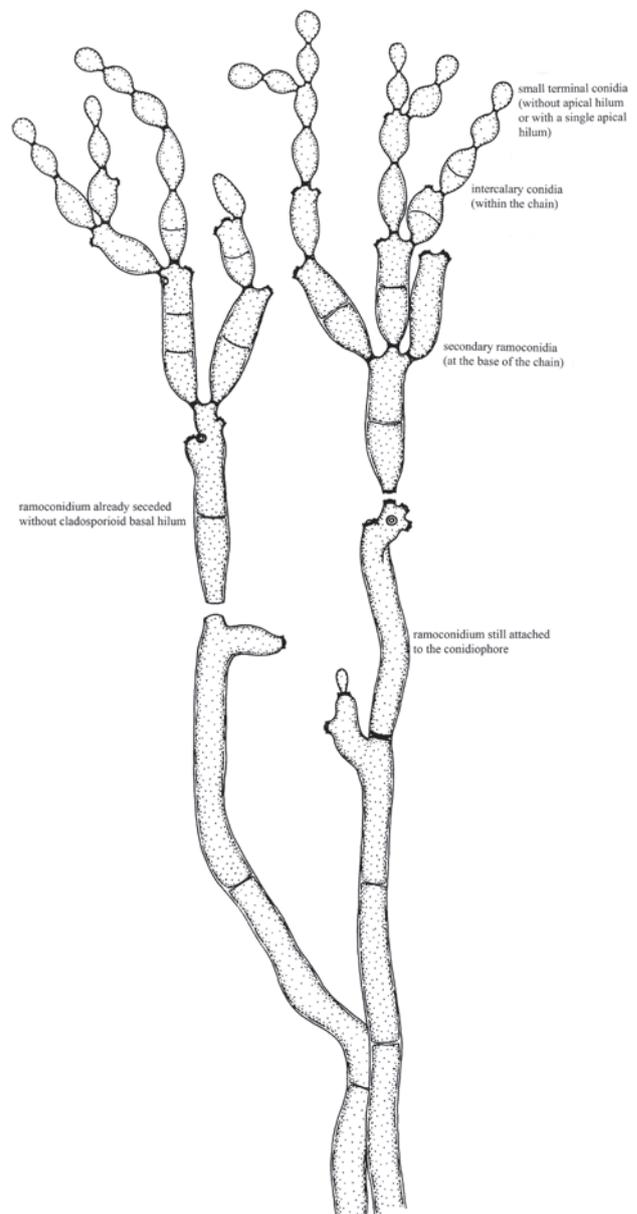


Fig. 1. *Cladosporium* conidiophore with ramoconidia, secondary ramoconidia, intercalary conidia, and small, terminal conidia. Scale bar = 10  $\mu$ m. K. Schubert del.

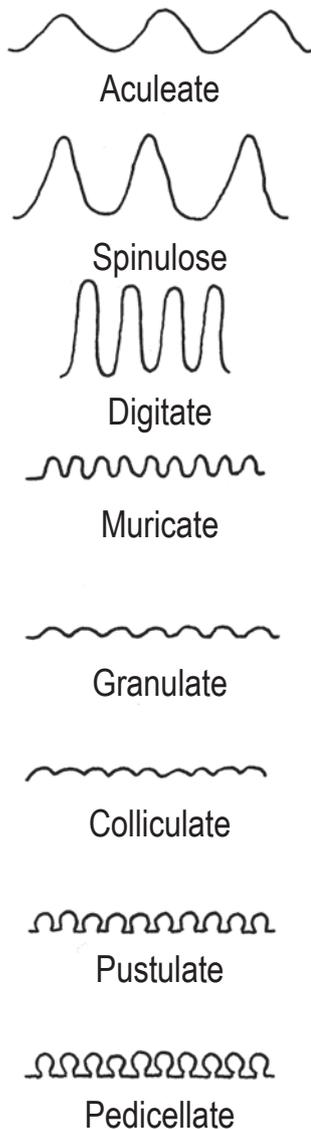


Fig. 2. Terms used to describe conidium wall ornamentation under the cryo-electron microscope. Adapted from David (1997).

parts of conidiophores are called **ramoconidia** if they secede at a septum from the conidiophore (Kirk *et al.* 2001). The septum at which the ramoconidium secedes often appears to be somewhat refractive or darkened. Ramoconidia are characterised by having a truncate, undifferentiated base (thus they lack a differentiated, coronate basal hilum formed in the context of conidiogenesis) and they can be very long, aseptate to sometimes multi-septate. Although they were formed initially as part of the conidiophore, they function as propagules. Only few of the species known until now have the ability to form true ramoconidia. **Secondary ramoconidia** also have more than one distal conidial hilum but they always derive from a conidiogenous locus of an earlier formed cell, which can be either a conidiogenous cell or a ramoconidium. Secondary ramoconidia are often shorter but somewhat wider than ramoconidia; they are often septate, and typically have a narrowed base with a coronate hilum (Fig. 1). Conidia in *Cladosporium* are cells with a coronate basal hilum, which is formed in the context of conidiogenesis and with either a single (when formed as intercalary units in unbranched parts of chains) or without any distal conidial hilum (when formed at the tip of conidial chains). For the first, the term “**intercalary conidium**” and for the latter, “**small terminal conidium**” is used. Intercalary conidia typically are larger and more pigmented and have a more differentiated surface ornamentation

than the small terminal conidia. In older literature true ramoconidia were often cited as “ramoconidia *s. str.*”, whereas secondary ramoconidia have been referred to as “ramoconidia *s. lat.*”

*Morphology of the teleomorph:* Teleomorphs were induced by inoculating plates of 2 % tap water agar onto which autoclaved stem pieces of *Urtica dioica* (European stinging nettle) were placed. Inoculated plates were incubated on the laboratory bench for 7 d, after that period they were further incubated at 10 °C in the dark for 1–2 mo to stimulate teleomorph development. Wherever possible, 30 measurements ( $\times 1\,000$  magnification) were made of conidia and ascospores, with the extremes of spore measurements given in parentheses. Cultural characteristics: Colonies were cultivated on PDA, MEA and OA plates for 14 d at 25 °C in the dark, after which the surface and reverse colours were rated using the charts of Rayner (1970). Linear growth was determined on MEA, PDA and OA plates by inoculating three plates per isolate for each medium, and incubating them for 14 d at 25 °C, after that period colony diameters were determined.

### Low-temperature scanning electron microscopy

Isolates of *Cladosporium* spp. were grown on SNA with 30 g agar/L for 3–4 d at room temperature under black light. Relevant parts of the small colonies with conidiophores and conidia were selected under a binocular, excised with a surgical blade as small agar (3  $\times$  3 mm) blocks, and transferred to a copper cup for snap-freezing in nitrogen slush. Agar blocks were glued to the copper surface with frozen tissue medium (KP-Cryoblock, Klinipath, Duiven, Netherlands) mixed with 1 part colloidal graphite (Agar Scientific, Stansted, U.K.). Samples were examined in a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 Cryostation for cryo-electron microscopy (cryoSEM). Electron micrographs were acquired from uncoated frozen samples, or after sputter-coating by means of a gold/palladium target for 3 times during 30 s (Fig. 2). Micrographs of uncoated samples were taken at an acceleration voltage of 3 kV, and consisted out of 30 averaged fast scans (SCAN 2 mode), and at 5 kV in case of the coated samples (PHOTO mode).

## RESULTS

### Phylogeny and differentiation

The manually adjusted alignment contained 80 sequences (including the outgroup sequence) and the five loci were represented by a total of 1 516 characters including alignment gaps which were used in the analysis. Of the 1 516 characters, 369 were parsimony-informative, 259 were variable and parsimony-uninformative, and 888 were constant.

Forty equally most parsimonious trees (TL = 1 933 steps; CI = 0.569; RI = 0.786; RC = 0.447), one of which is shown in Fig. 3, were obtained from the parsimony analysis of the combined genes. Neighbour-joining analysis using three substitution models (uncorrected “p”, Kimura 2-parameter and HKY85) on the sequence data yielded trees with identical topologies. These differed from the tree presented in Fig. 3 with regard to the placement of *C. macrocarpum* strain CPC 12054 which was placed as a sister branch to the *C. bruhnei* Linder clade in the distance analyses (results not shown) because it shares an identical CAL sequence. All cryptic species consisting of multiple strains are clustering in well-supported clades with bootstrap support values ranging from 71 % (*C. herbarum*) to 100 % [e.g. *C. ramotenellum* K. Schub.,

Table 1. Isolates subjected to DNA sequence analyses and morphological examinations.

Anamorph	Teleomorph	Accession number <sup>1</sup>	Host	Country	Collector	GenBank numbers <sup>2</sup> (ITS, EF, ACT, CAL, HIS)
<i>Cladosporium antarcticum</i>	—	CBS 690.92* (ex-type)	<i>Caloplaca regalis</i>	Antarctica	C. Möller	EF679334, EF679405, EF679484, EF679560, EF679636
<i>Cladosporium bruhnei</i>	<i>Davidiella allicina</i>	CBS 134.31 = ATCC 11283	—	Germany	—	EF679335, EF679406, EF679485, EF679561, EF679637
		CBS 157.82	<i>Quercus robur</i>	Belgium	—	EF679336, EF679407, EF679486, EF679562, EF679638
		CBS 159.54 = ATCC 36948	Man, skin	The Netherlands	—	EF679337, EF679408, EF679487, EF679563, EF679639
		CBS 161.55	Man, sputum	The Netherlands	—	EF679338, EF679409, EF679488, EF679564, EF679640
		CBS 177.71	<i>Thuja tincture</i>	The Netherlands	—	EF679339, EF679410, EF679489, EF679565, EF679641
		CBS 188.54 = ATCC 11290 = IMI 049638 = CPC 3686	—	—	—	AY251077, EF679411, EF679490, EF679566, EF679642
		CBS 366.80	Man, skin	The Netherlands	—	EF679340, EF679412, EF679491, EF679567, EF679643
		CBS 399.80	Man, skin	The Netherlands	—	AJ244227, EF679413, EF679492, EF679568, EF679644
		CBS 521.68	Air	The Netherlands	—	EF679341, EF679414, EF679493, EF679569, EF679645
		CBS 572.78	<i>Polyporus radiatus</i>	Russia	V.K. Melnik	DQ289799, EF679415, DQ289866, DQ289831, EF679646
		CBS 813.71	<i>Polygonatum odoratum</i>	Czech Republic	—	EF679342, EF679416, EF679494, EF679570, EF679647
		CBS 110024	Industrial water	Germany, Nordrhein-Westfalen	—	EF679343, EF679417, EF679495, EF679571, EF679648
		CBS 115683 = ATCC 66670 = CPC 5101	CCA-treated Douglas-fire pole	U.S.A., New York	C.J. Wang	AY361959, EF679418, AY752193, AY752224, AY752255
		CBS 121624* = CPC 12211 (neotype)	<i>Hordeum vulgare</i>	Belgium	J.Z. Groenewald	EF679350, EF679425, EF679502, EF679578, EF679655
		CPC 11386	<i>Tilia cordata</i>	Germany, Sachsen-Anhalt	K. Schubert	EF679344, EF679419, EF679496, EF679572, EF679649
		CPC 11840	<i>Ourisia macrophylla</i>	New Zealand	A. Blouin	EF679345, EF679420, EF679497, EF679573, EF679650
		CPC 12042 = EXF-389	Hypersaline water from salterns	Slovenia	P. Zalar	EF679346, EF679421, EF679498, EF679574, EF679651
		CPC 12045 = EXF-594	Hypersaline water from salterns	Spain	P. Zalar	EF679347, EF679422, EF679499, EF679575, EF679652
		CPC 12046 = EXF-680	Air conditioning system	Slovenia	P. Zalar	EF679348, EF679423, EF679500, EF679576, EF679653
		CPC 12139	<i>Hordeum vulgare</i>	The Netherlands	—	EF679349, EF679424, EF679501, EF679577, EF679654
CPC 12212	<i>Hordeum vulgare</i>	Belgium	J.Z. Groenewald	EF679351, EF679426, EF679503, EF679579, EF679656		
CPC 12921	<i>Eucalyptus</i> sp.	Australia	—	EF679352, EF679427, EF679504, EF679580, EF679657		
<i>Cladosporium cladosporioides</i> complex	—	CBS 673.69	Air	The Netherlands	—	EF679353, EF679428, EF679505, EF679581, EF679658
	<i>Davidiella</i> sp.	CBS 109082	<i>Silene maritima</i>	United Kingdom	A. Aptroot	EF679354, EF679429, EF679506, EF679582, EF679659
	—	CPC 11606	<i>Musa</i> sp.	India	M. Arzanlou	EF679355, EF679430, EF679507, EF679583, EF679660
	—	CPC 11609	<i>Musa</i> sp.	India	M. Arzanlou	EF679356, EF679431, EF679508, EF679584, EF679661
<i>Cladosporium herbaroides</i>	—	CBS 121626* = CPC 12052 = EXF-1733 (ex-type)	Hypersaline water from salterns	Israel	P. Zalar	EF679357, EF679432, EF679509, EF679585, EF679662
<i>Cladosporium herbarum</i>	<i>Davidiella tassiana</i>	CBS 111.82	<i>Arctostaphylos uva-ursi</i>	Switzerland	E. Müller	AJ238469, EF679433, EF679510, EF679586, EF679663
		CBS 300.49	<i>Biscutella laevigata</i>	Switzerland	J.A. von Arx	EF679358, EF679434, EF679511, EF679587, EF679664
		CBS 121621* = CPC 12177 (epitype)	<i>Hordeum vulgare</i>	The Netherlands	—	EF679363, EF679440, EF679516, EF679592, EF679670
		CPC 11600	<i>Delphinium barbeyi</i>	U.S.A., Colorado	A. Ramalay	DQ289800, EF679435, DQ289867, DQ289832, EF679665
		CPC 11601	<i>Delphinium barbeyi</i>	U.S.A., Colorado	A. Ramalay	EF679359, EF679436, EF679512, EF679588, EF679666
		CPC 11602	<i>Delphinium barbeyi</i>	U.S.A., Colorado	A. Ramalay	EF679360, EF679437, EF679513, EF679589, EF679667
		CPC 11603	<i>Delphinium barbeyi</i>	U.S.A., Colorado	A. Ramalay	EF679361, EF679438, EF679514, EF679590, EF679668



		CPC 11604	<i>Delphinium barbeyi</i>	U.S.A., Colorado	A. Ramalay	EF679362, EF679439, EF679515, EF679591, EF679669
		CPC 12178	<i>Hordeum vulgare</i>	The Netherlands	—	EF679364, EF679441, EF679517, EF679593, EF679671
		CPC 12179	<i>Hordeum vulgare</i>	The Netherlands	—	EF679365, EF679442, EF679518, EF679594, EF679672
		CPC 12180	<i>Hordeum vulgare</i>	The Netherlands	—	EF679366, EF679443, EF679519, EF679595, EF679673
		CPC 12181	<i>Hordeum vulgare</i>	The Netherlands	—	EF679367, EF679444, EF679520, EF679596, EF679674
		CPC 12183	<i>Hordeum vulgare</i>	The Netherlands	—	EF679368, EF679445, EF679521, EF679597, EF679675
<i>Cladosporium iridis</i>	<i>Davidiella macrospora</i>	CBS 107.20	<i>Iris</i> sp.	—	—	EF679369, EF679446, EF679522, EF679598, EF679676
		CBS 138.40* (epitype)	<i>Iris</i> sp.	The Netherlands	—	EF679370, EF679447, EF679523, EF679599, EF679677
<i>Cladosporium macrocarpum</i>	<i>Davidiella macrocarpa</i>	CBS 175.82	Water	Romania	—	EF679371, EF679448, EF679524, EF679600, EF679678
		CBS 223.31 = ATCC 11287	<i>Mycosphaerella tulasnei</i>	—	—	AF222830, EF679449, EF679525, EF679601, EF679679
		CBS 299.67	<i>Triticum aestivum</i>	Turkey	—	EF679372, EF679450, EF679526, EF679602, EF679680
		CBS 121811* = CPC 12755 (neotype)	<i>Spinacia oleracea</i>	U.S.A.	—	EF679376, EF679454, EF679530, EF679606, EF679684
		CPC 11817	<i>Corylus</i> sp.	U.S.A.	—	EF679373, EF679451, EF679527, EF679603, EF679681
		CPC 12054 = EXF-2287	Hypersaline water from salterns	Slovenia	P. Zalar	EF679374, EF679452, EF679528, EF679604, EF679682
		CBS H-19855 = CPC 12752 = CBS 121623	<i>Spinacia oleracea</i>	U.S.A.	—	EF679375, EF679453, EF679529, EF679605, EF679683
		CPC 12756	<i>Spinacia oleracea</i>	U.S.A.	—	EF679377, EF679455, EF679531, EF679607, EF679685
		CPC 12757	<i>Spinacia oleracea</i>	U.S.A.	—	EF679378, EF679456, EF679532, EF679608, EF679686
		CPC 12758	<i>Spinacia oleracea</i>	U.S.A.	—	EF679379, EF679457, EF679533, EF679609, EF679687
		CPC 12759	<i>Spinacia oleracea</i>	U.S.A.	—	EF679380, EF679458, EF679534, EF679610, EF679688
<i>Cladosporium ossifragi</i>	—	CBS 842.91* (epitype)	<i>Narthecium ossifragum</i>	Norway	M. di Menna	EF679381, EF679459, EF679535, EF679611, EF679689
		CBS 843.91	<i>Narthecium ossifragum</i>	Norway	M. di Menna	EF679382, EF679460, EF679536, EF679612, EF679690
<i>Cladosporium pseudiridis</i>	—	CBS 116463* = LYN 1065 = ICMP 15579 (ex-type)	<i>Iris</i> sp.	New Zealand	C.F. Hill	EF679383, EF679461, EF679537, EF679613, EF679691
<i>Cladosporium ramotenellum</i>	—	CBS 121628* = CPC 12043 = EXF-454 (ex-type)	Hypersaline water from salterns	Slovenia	P. Zalar	EF679384, EF679462, EF679538, EF679614, EF679692
		CPC 12047 = EXF-967	Air conditioning system	Slovenia	P. Zalar	EF679385, EF679463, EF679539, EF679615, EF679693
<i>Cladosporium sinuosum</i>	—	CBS 121629* = CPC 11839 = ICMP 15819 (ex-type)	<i>Fuchsia excorticata</i>	New Zealand	A. Blouin	EF679386, EF679464, EF679540, EF679616, EF679694
<i>Cladosporium spinulosum</i>	—	CBS 102044	Hypersaline water from salterns	Slovenia	S. Soujak	EF679387, EF679465, EF679541, EF679617, EF679695
		CBS 119907* = CPC 12040 = EXF-334 (ex-type)	Hypersaline water from salterns	Slovenia	P. Zalar	EF679388, EF679466, EF679542, EF679618, EF679696
<i>Cladosporium subinflatum</i>	—	CBS 121630* = CPC 12041 = EXF-343 (ex-type)	Hypersaline water from salterns	Slovenia	P. Zalar	EF679389, EF679467, EF679543, EF679619, EF679697
<i>Cladosporium</i> sp.	—	CBS 172.52 = ATCC 11320	<i>Carya illinoensis</i>	U.S.A.	—	EF679390, EF679468, EF679544, EF679620, EF679698
		CBS 113741	Grape berry	U.S.A.	—	EF679391, EF679469, EF679545, EF679621, EF679699
		CBS 113742	Grape berry	U.S.A.	—	EF679392, EF679470, EF679546, EF679622, EF679700
		CBS 113744	Grape bud	U.S.A.	—	EF679393, EF679471, EF679547, EF679623, EF679701
		CPC 12484	<i>Pinus ponderosa</i>	Argentina	A. Greslebin	EF679394, EF679472, EF679548, EF679624, EF679702
		CPC 12485	<i>Pinus ponderosa</i>	Argentina	A. Greslebin	EF679395, EF679473, EF679549, EF679625, EF679703
<i>Cladosporium subtilissimum</i>	—	CBS 113753	Bing cherry fruits	U.S.A.	—	EF679396, EF679474, EF679550, EF679626, EF679704
		CBS 113754*	Grape berry	U.S.A.	—	EF679397, EF679475, EF679551, EF679627, EF679705
		CPC 12044 = EXF-462	Hypersaline water from salterns	Slovenia	P. Zalar	EF679398, EF679476, EF679552, EF679628, EF679706
<i>Cladosporium tenellum</i>	—	CBS 121634* = CPC 12053 = EXF-1735 (ex-type)	Hypersaline water from salterns	Israel	P. Zalar	EF679401, EF679479, EF679555, EF679631, EF679709

Table 1. (Continued).

Anamorph	Teleomorph	Accession number <sup>1</sup>	Host	Country	Collector	GenBank numbers <sup>2</sup> (ITS, EF, ACT, CAL, HIS)
		CPC 11813	<i>Phyllactinia</i> sp. on <i>Corylus</i> sp.	U.S.A.	D. Glawe	EF679399, EF679477, EF679553, EF679629, EF679707
		CPC 12051 = EXF-1083	Hypersaline water from saltens	Israel	P. Zalar	EF679400, EF679478, EF679554, EF679630, EF679708
<i>Cladosporium variabile</i>	<i>Davidiella variabile</i>	CBS 121636* = CPC 12751 (epitype)	<i>Spinacia oleracea</i>	U.S.A.	—	EF679402, EF679480, EF679556, EF679632, EF679710
—	<i>Davidiella</i> sp.	CPC 12753	<i>Spinacia oleracea</i>	U.S.A.	—	EF679403, EF679481, EF679557, EF679633, EF679711
		CBS 289.49	<i>Allium schoenoprasum</i>	Switzerland	E. Müller	AY152552, EF679482, EF679558, EF679634, EF679712
		CBS 290.49	<i>Trisetum distichophyllum</i>	Switzerland	E. Müller	EF679404, EF679483, EF679559, EF679635, EF679713

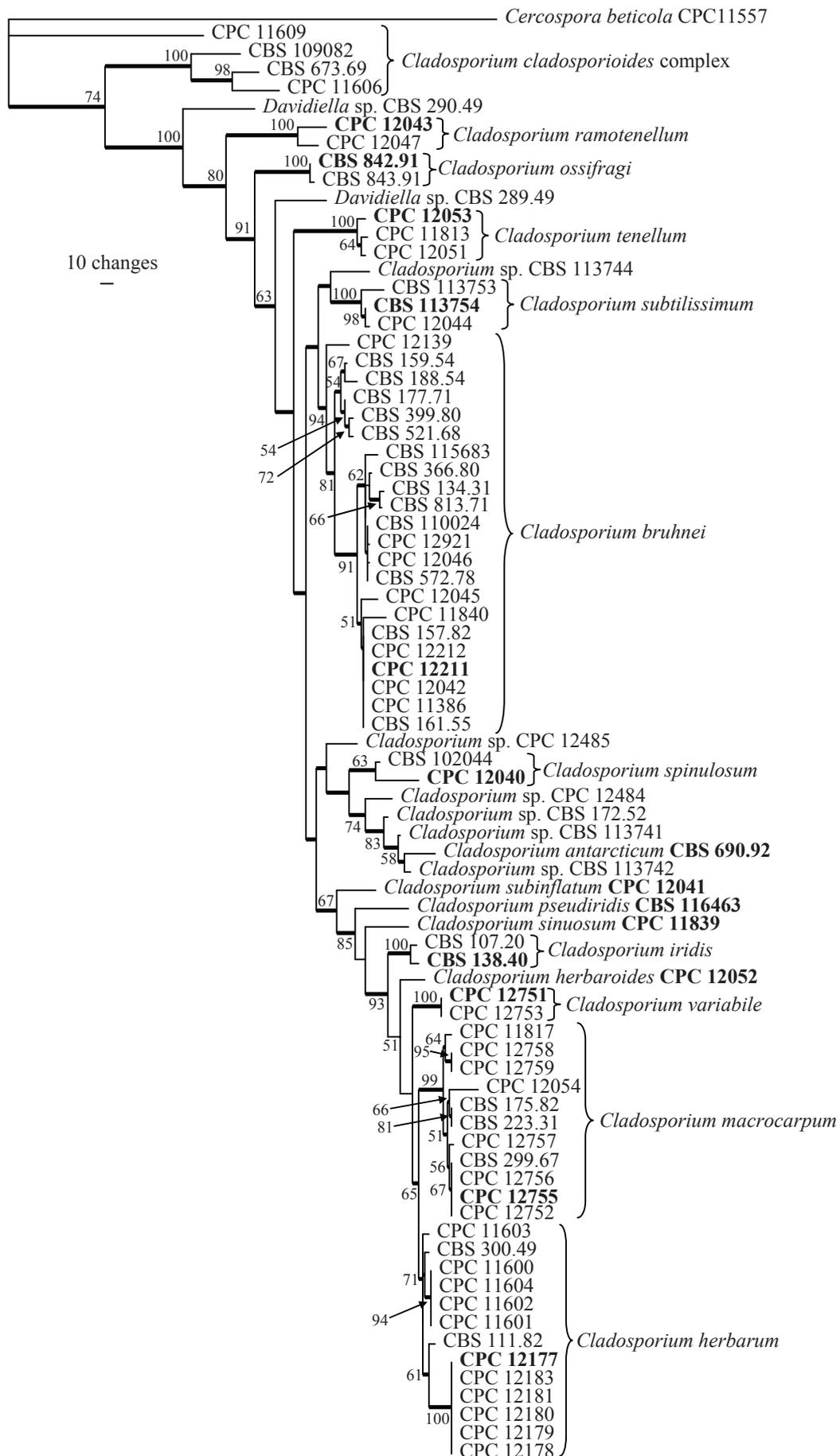
<sup>1</sup>ATCC: American Type Culture Collection, Virginia, U.S.A.; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; EXF: Extremophilic Fungi Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia; ICMP: International Collection of Micro-organisms from Plants, Landcare Research, Private Bag 92170, Auckland, New Zealand; IMI: International Mycological Institute, CAB International, Egham, Basingstoke Lane, U.K.

<sup>2</sup>ACT: partial actin gene, CAL: partial calmodulin gene, EF: partial elongation factor 1-alpha gene, HIS: partial histone H3 gene, ITS: internal transcribed spacer region.

<sup>3</sup>Ex-type cultures.

Zalar, Crous & U. Braun and *C. ossifragi* (Rostr.) U. Braun & K. Schub.]. The intraspecific variation in the *C. bruhnei* clade is due to genetic variation present in the sequence data of all loci except for ITS, those in the *C. macrocarpum* clade in all loci except for ITS and ACT, and those in the *C. herbarum* clade in all loci except for ITS and CAL (data not shown). However, none of the variation for these species could be linked to host specificity or morphological differences. In general, ITS data did not provide any resolution within the *C. herbarum* complex, whereas EF data provided species clades with very little intraspecific variation and ACT, CAL and HIS revealed increasing intraspecific variation (ACT the least and HIS the most).

The mean genetic diversity (H) of the entire data set excluding the nearly invariant ITS region was 0.9307, with little difference between genes (ACT = 0.9257, CAL = 0.9289, EF = 0.9322, HIS = 0.9361). The loci showed different numbers of alleles (ACT: 22, CAL: 16, EF: 21, HIS: 20, ITS: 6). Differentiation of entities when calculated with STRUCTURE software using the admixture/correlated model showed highest value with K = 6. At this value  $F_{ST}$  varied between 0.1362 and 0.3381. Linkage disequilibrium calculated using the standardised index of association ( $I_{SA}^S$ ) for the entire dataset (observed variance  $V_o = 0.5602$ , expected variance  $V_e = 0.2576$ ) was 0.3914 ( $P = 0.0001$ ), consistent with a small amount of recombination that did not destroy the linkage between alleles. Only few groups appeared to be separated for all alleles; degrees of gene flow are indicated in Fig. 4. SPLITSTREE software produced unresolved star-shaped structures for all genes, without any sign of reticulation (Fig. 5).



**Fig. 3.** One of 40 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined sequence alignment (ITS, ACT, CAL, EF, HIS). The scale bar shows ten changes, and bootstrap support values from 1 000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and strain numbers in bold represent ex-type sequences. The tree was rooted to sequences of *Cercospora beticola* strain CPC 11557 (GenBank accession numbers AY840527, AY840458, AY840425, AY840494, AY840392, respectively).



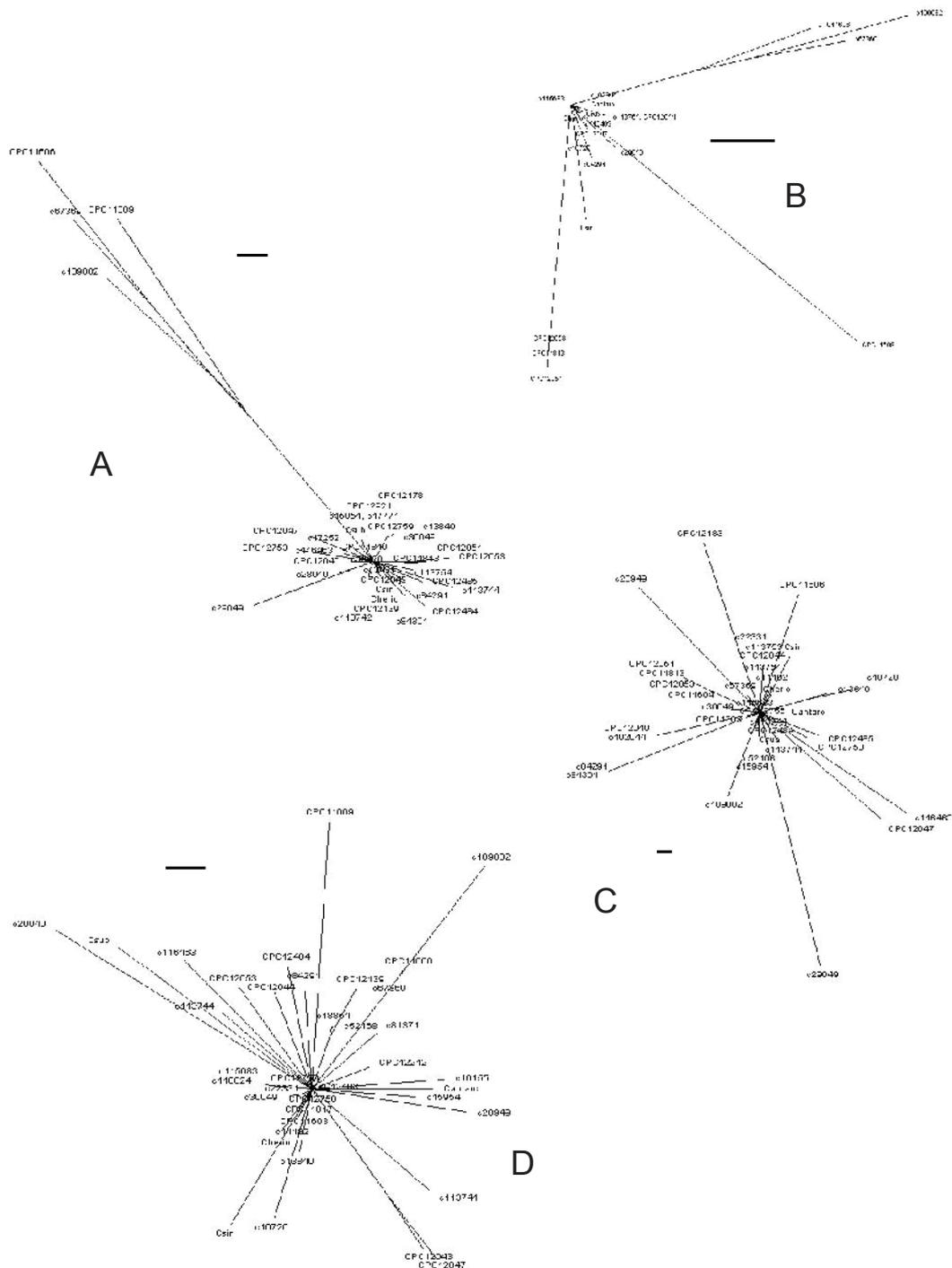


Fig. 5. Split decomposition of the *Cladosporium herbarum* complex using SPLITSTREE of 16–22 unique alleles obtained from 79 *Cladosporium* isolates for four loci. The star-like structures suggest clonal development. A = ACT, B = CAL, C = HIS, D = EF. Scale bars = 0.01 nucleotide substitutions per site.

## Taxonomy

### Key to the *Cladosporium* species treated

Morphological features used in the key to distinguish the species treated in this study were determined after 7 d growth at 25 °C on SNA using light microscopy, and cultural characteristics after 14 d incubation on PDA.

1. Conidia usually smooth, rarely minutely verruculose ..... ***C. cladosporioides*** (species complex)
1. Conidia with different surface ornamentation, minutely to distinctly verruculose, verruculose to echinulate or spiny ..... 2
2. Conidiophores uniform, macronematous; conidia solitary, sometimes formed in short unbranched chains ..... 3
2. Conidiophores both macronematous and micronematous; conidia always catenate, usually formed in branched chains ..... 5

3. Conidiophores due to geniculations often growing zigzag-like, (4–)5–7  $\mu\text{m}$  wide; conidia 9–21  $\times$  (5–)6–8  $\mu\text{m}$ , 0–1-septate; conidiogenous loci and conidial hila 1.2–2(–2.2)  $\mu\text{m}$  diam ..... **C. sinuosum**
3. Conidiophores not growing zigzag-like, wider, 6–11  $\mu\text{m}$ ; conidia very large and wide, 15–75(–87)  $\times$  (7–)10–19(–21)  $\mu\text{m}$ , often with more septa; conidiogenous loci and hila wider, (2–)2.5–4  $\mu\text{m}$  diam ..... 4
4. Conidia (18–)30–75(–87)  $\times$  (7–)10–16(–18)  $\mu\text{m}$ , (0–)2–6(–7)-septate, walls thickened, especially in older conidia, up to 1  $\mu\text{m}$  thick ..... **C. iridis**
4. Conidia shorter and wider, 15–55  $\times$  (9–)11–19(–21)  $\mu\text{m}$ , 0–3-septate, walls distinctly thickened, up to 2  $\mu\text{m}$ , usually appearing zonate ..... **C. pseudiridis**
- 5(2) Macronematous conidiophores nodulose or nodose with conidiogenous loci usually confined to swellings ..... 6
5. Macronematous conidiophores non-nodulose or only occasionally subnodulose due to geniculate proliferation, but conidiogenous loci not confined to swellings ..... 11
6. Macronematous conidiophores 3–6  $\mu\text{m}$  wide, swellings 5–11  $\mu\text{m}$  wide ..... 7
6. Macronematous conidiophores somewhat narrower, (1.5–)2.5–5  $\mu\text{m}$  wide, swellings 3–8  $\mu\text{m}$  wide ..... 8
7. Aerial mycelium twisted; conidial septa often distinctly darkened, becoming sinuous with age, apex and base of the conidia often appear to be distinctly darkened; slower growing in culture (29 mm after 14 d on PDA) ..... **C. variable**
7. Aerial mycelium not twisted; conidial septa as well as apex and base not distinctly darkened, septa not sinuous with age; faster growing in culture (on average 38 mm after 14 d on PDA) ..... **C. macrocarpum**
8. Macronematous conidiophores (1.5–)2.5–4.5(–5.5)  $\mu\text{m}$  wide, swellings 3–6.5  $\mu\text{m}$  wide; conidia 4–17(–22)  $\mu\text{m}$  long, ornamentation variable, but usually densely echinulate, spines up to 0.8  $\mu\text{m}$  long ..... **C. subinflatum**
8. Macronematous conidiophores slightly wider, 3–5  $\mu\text{m}$ , swellings (4–)5–8(–9)  $\mu\text{m}$  wide; conidia longer, up to 25(–35)  $\mu\text{m}$ , ornamentation minutely verruculose to verrucose, but not echinulate or spiny ..... 9
9. Conidia formed by macronematous conidiophores 3–33  $\times$  (2–)3–6(–7)  $\mu\text{m}$ , with age becoming wider, (3.5–)5–9(–11)  $\mu\text{m}$ , darker and more thick-walled ..... **C. herbaroides**
9. Conidia formed by macronematous conidiophores not becoming wider and darker with age, usually up to 7  $\mu\text{m}$  wide ..... 10
10. Conidiophores usually with small head-like swellings, sometimes also with a second intercalary nodule; small terminal conidia 4–9  $\times$  2.5–3.5  $\mu\text{m}$ , secondary ramoconidia and occasionally formed ramoconidia 10–24(–31)  $\times$  3–5(–7)  $\mu\text{m}$  ..... **C. bruhnei**
10. Conidiophores with a single or often numerous swellings in short succession giving the stalk a knotty/gnarled appearance; conidia wider, small terminal conidia 4–10  $\times$  3–5(–6)  $\mu\text{m}$ , intercalary conidia 6–16  $\times$  4–6  $\mu\text{m}$ , secondary ramoconidia 12–25(–35)  $\times$  (3–)5–7(–9)  $\mu\text{m}$  ..... **C. herbarum**
- 11(5) Small terminal and intercalary conidia 4–15  $\times$  3–5  $\mu\text{m}$ , secondary ramoconidia 16–36(–40)  $\times$  (4–)5–8  $\mu\text{m}$ , 0–3(–4)-septate, ramoconidia absent ..... **C. ossifragi**
11. Small terminal conidia, ramoconidia and secondary ramoconidia distinctly narrower, 2–5(–6)  $\mu\text{m}$  wide, 0–2(–3)-septate ..... 12
12. Mycelium dimorphic, narrow hyphae 1–3  $\mu\text{m}$  wide, hyaline to subhyaline, thin-walled, hyphae of the second type wider, 3.5–8(–9)  $\mu\text{m}$ , pale to dark greyish olivaceous or olivaceous-brown, thick-walled, sometimes even two-layered, 1(–1.5)  $\mu\text{m}$  thick, hyphae appearing consistently enveloped in polysaccharide-like material or covered by a slime coat; conidiophores usually several times slightly to distinctly geniculate towards the apex, with numerous conidiogenous loci crowded towards the apex, up to 14 per conidiogenous cell ..... **C. antarcticum**
12. Mycelium not dimorphic, neither enveloped in polysaccharide-like material nor covered by a slime coat; conidiophores usually not geniculate, occasionally only slightly so ..... 13
13. Conidial ornamentation distinctly echinulate, spiny (baculate, digitate or capitate under SEM), spines 0.5–1.3  $\mu\text{m}$  long, loose to moderately dense, conidial hila usually situated on small peg-like prolongations or denticles ..... **C. spinulosum**
13. Conidial ornamentation different, minutely verruculose to verruculose, conidial hila not situated on peg-like prolongations ..... 14
14. Small terminal conidia narrowly obovoid, limoniform or fusiform, but neither globose nor subglobose; conidiogenous loci and conidial hila 0.5–2(–2.5)  $\mu\text{m}$  diam ..... **C. subtilissimum** (species complex)
14. Numerous small globose or subglobose terminal conidia formed, also ovoid or limoniform; conidiogenous loci and conidial hila somewhat smaller, 0.5–1.5(–2)  $\mu\text{m}$  diam ..... 15

15. Conidiophores usually with numerous conidiogenous loci forming sympodial clusters of pronounced scars at the apex, sometimes up to 10 or even more denticulate loci; conidia 3–20(–28) × 2.5–5(–6) μm, 0–1(–2)-septate, often with several apically crowded hila, up to 7(–9) ..... **C. tenellum**
15. Conidiophores usually only with few conidiogenous loci, mostly 1–3; conidia longer and narrower, 2.5–35 × 2–4(–5) μm, 0–3-septate, usually with up to three distal conidial hila ..... **C. ramotenellum**

### Key to the *Davidiella* species treated

1. Ascospores frequently wider than 7 μm when mounted in Shear's solution or lactic acid, apical cell obtusely rounded ..... 2
1. Ascospores not wider than 7 μm when mounted in Shear's solution or lactic acid, apical cell acutely rounded, ascospores (20–)25–27 (–30) × (5.5–)6–7 μm ..... **D. allicina**
2. Pseudoparaphyses prominent; asci frequently >95 μm; ascospores (22–)23–26(–28) × (6–)6.5–7(–8) μm ..... **D. macrocarpa**
2. Pseudoparaphyses mostly absent in older ascomata; asci <95 μm ..... 3
3. Ascospores (22–)26–30(–35) × (7–)7.5–8(–9) μm; asci wider than 18 μm ..... **D. variabile**
3. Ascospores (17–)20–23(–25) × (6–)7(–8) μm; asci not wider than 18 μm ..... **D. tassiana**

### Generic concept of the teleomorph

The introduction of the teleomorph genus *Davidiella* was mainly based on phylogenetic studies within the *Mycosphaerellaceae* (Braun *et al.* 2003), where it could be demonstrated that “*Mycosphaerella*” species with *Cladosporium* anamorphs formed a sister clade to *Mycosphaerella* (Crous *et al.* 2000, 2001). Braun *et al.* (2003) transferred five species to *Davidiella* based on prior established anamorph-teleomorph connections, though no details were provided pertaining to morphological differences between *Davidiella* and *Mycosphaerella*. Aptroot (2006) transferred several additional species to *Davidiella*, and distinguished them from true *Mycosphaerella* species by the presence of distinct, irregular cellular inclusions (lumina) in their ascospores. Furthermore, Schoch *et al.* (2006) placed *Davidiella* in a separate family (*Davidiellaceae*) in the *Capnodiales*. During the course of the present study, several fresh specimens of *Davidiella* spp. were collected or induced in culture, making it possible to circumscribe the genus as follows:

***Davidiella*** Crous & U. Braun, Mycol. Progr. 2: 8. 2003, **emend.**

*Ascomata* pseudothecial, black to red-brown, globose, inconspicuous and immersed beneath stomata to superficial, situated on a reduced stroma, with 1(–3) short, periphysate ostiolar necks; periphysoids frequently growing down into cavity; wall consisting of 3–6 layers of *textura angularis*. *Asci* fasciculate, short-stalked or not, bitunicate, sessile, obovoid to broadly ellipsoid or subcylindrical, straight to slightly curved, 8-spored. *Pseudoparaphyses* frequently present in mature ascomata, hyaline, septate, subcylindrical. *Ascospores* bi- to multiseriate, hyaline, obovoid to ellipsoid-fusiform, with irregular lumina inclusions, mostly thick-walled, straight to slightly curved; frequently becoming brown and verruculose in asci; at times covered in mucoid sheath. *Cladosporium* anamorph usually produced in culture, but not in all taxa.

*Type species: Davidiella tassiana* (De Not.) Crous & U. Braun, Mycol. Progr. 2: 8. 2003.

### Description of *Cladosporium* species

Based on morphological examinations (David 1997) and phylogenetic studies employing DNA sequence data (Crous *et al.* 2000, 2001,

2007 – this volume, Braun *et al.* 2003), the generic concept of the genus *Cladosporium* has been stabilised. *Cladosporium* is confined to *Davidiella* (*Davidiellaceae*, *Capnodiales*) anamorphs with coronate conidiogenous loci and conidial hila consisting of a central convex dome and a raised periclinal rim.

***Cladosporium antarcticum*** K. Schub., Crous & U. Braun, **sp. nov.** MycoBank MB504573. Figs 6–8.

*Etymology:* Refers to Antarctica, where the fungus was collected.

Differt a *Cladosporio* licheniphilo conidiophoris saepe non-ramosis, frequentibus geniculatis, angustioribus, (2–)3–4.5 μm, conidiis longioribus et angustioribus, 4–30 × 2.5–5 μm, 0–3-septatis, verruculosis vel verrucosis.

*Mycelium* immersed and superficial, dimorphic, branched, often with short lateral outgrowths, narrow hyphae 1–3 μm wide, hyaline to subhyaline, thin-walled, hyphae of the second type wider, 3.5–8(–9) μm, pluriseptate, often somewhat constricted at the septa, sometimes swollen, pale to dark greyish olivaceous or olivaceous-brown, smooth or verruculose, thick-walled, sometimes even two-layered (two distinct wall layers visible), 1(–1.5) μm thick, hyphae appearing consistently enveloped in polysaccharide-like material or covered by a slime coat. *Conidiophores* micronematous and macronematous, solitary or in loose groups, arising from plagiotropous or ascending hyphae, terminally or usually laterally. *Macronematous conidiophores* erect to somewhat decumbent, straight to somewhat flexuous or bent, cylindrical, once or several times slightly to distinctly geniculate towards the apex due to sympodial proliferation, unbranched or once branched, up to 120 μm long, 3–4.5 μm wide, sometimes slightly attenuated towards the apex, pluriseptate, up to eight septa, occasionally slightly constricted at the septa, pale to medium or even dark olivaceous-brown or greyish brown, paler towards apices, smooth to somewhat rough-walled, walls thickened but thinner-walled towards apices, sometimes slightly swollen at the base, up to 6 μm wide. *Conidiogenous cells* integrated, terminal and intercalary, once or several times slightly to distinctly geniculate, 10–33 μm long, proliferation sympodial, with several or numerous conidiogenous loci, at first terminal, later turning to one side of the stalk and situated on small lateral shoulders, up to 14 per cell, protuberant, denticulate, 1–1.5(–2) μm diam, thickened and darkened-refractive. *Micronematous conidiophores* as short lateral, peg-like outgrowths with a single apical scar or somewhat longer, occasionally once

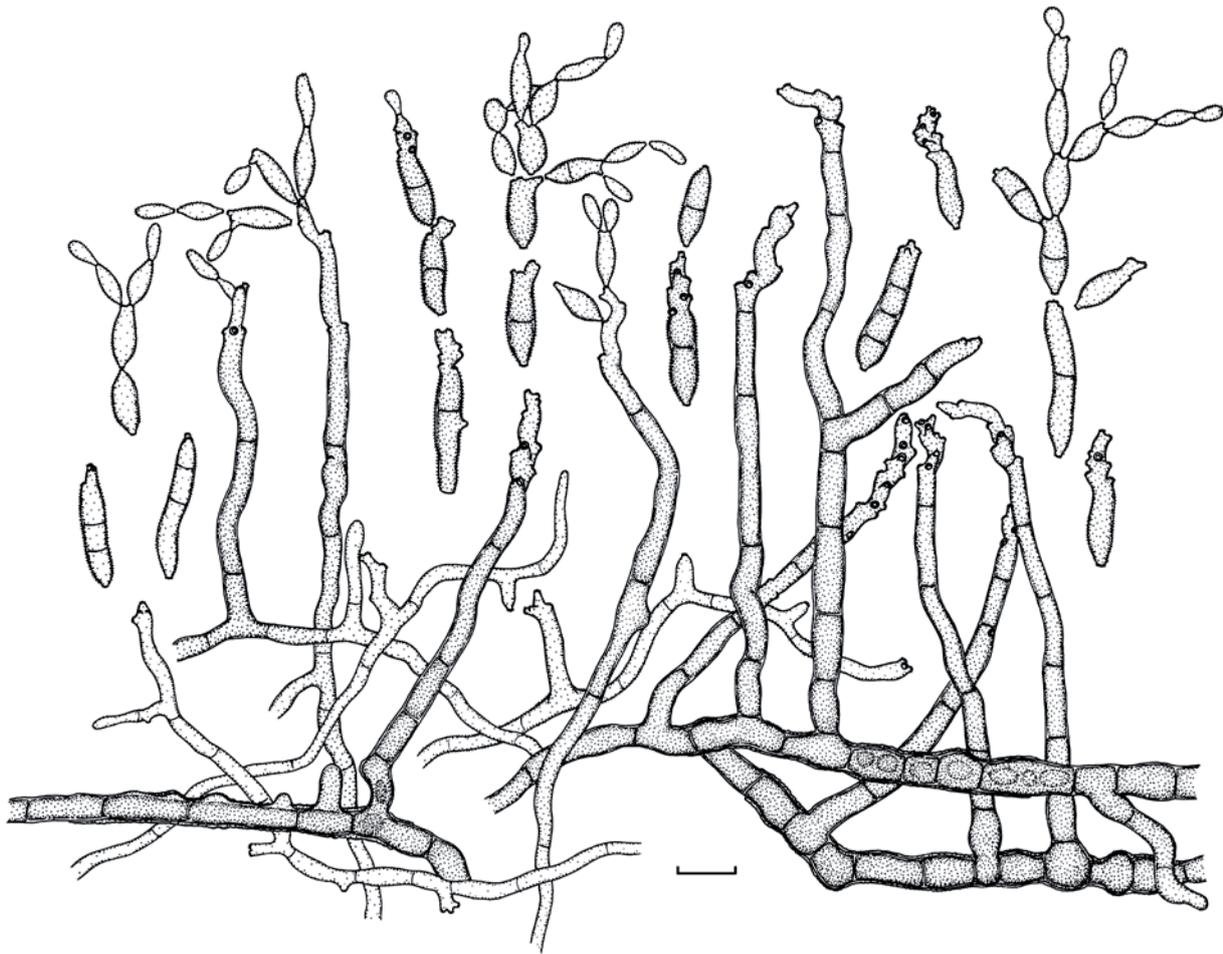


Fig. 6. *Cladosporium antarcticum* (CBS 690.92). Macro- and micronematous conidiophores and conidia. Scale bar = 10  $\mu$ m. K. Schubert del.

geniculate with several conidiogenous loci at the apex, 2–22  $\times$  2–3  $\mu$ m, pale greyish olivaceous, loci denticulate. *Ramoconidia* occasionally occurring, cylindrical, up to 30  $\mu$ m long, 4–5  $\mu$ m wide, 0–1-septate, concolorous with the tips of conidiophores, with a broadly truncate, unthickened and not darkened base, without dome and rim, 2.5  $\mu$ m wide. *Conidia* catenate, in branched chains, straight, small terminal conidia obovoid, limoniform or narrowly ellipsoid, 4–14  $\times$  2.5–4  $\mu$ m [av.  $\pm$  SD, 8.5 ( $\pm$  3.3)  $\times$  3.5 ( $\pm$  0.6)], 0(–1)-septate, secondary ramoconidia ellipsoid to cylindrical, often with several or numerous conidial hila crowded at the distal end, up to 12, 13–30  $\times$  4–5  $\mu$ m [av.  $\pm$  SD, 20.1 ( $\pm$  5.8)  $\times$  4.3 ( $\pm$  0.5)  $\mu$ m], 0–3-septate, sometimes slightly constricted at the median septum, pale olivaceous-brown or greyish brown, minutely verruculose to verrucose (granulate under SEM), walls more or less thickened, rounded or slightly attenuated towards apex and base, hila protuberant, denticulate, 0.8–1.5(–2)  $\mu$ m diam, thickened and darkened-refractive; microcyclic conidiogenesis occurring.

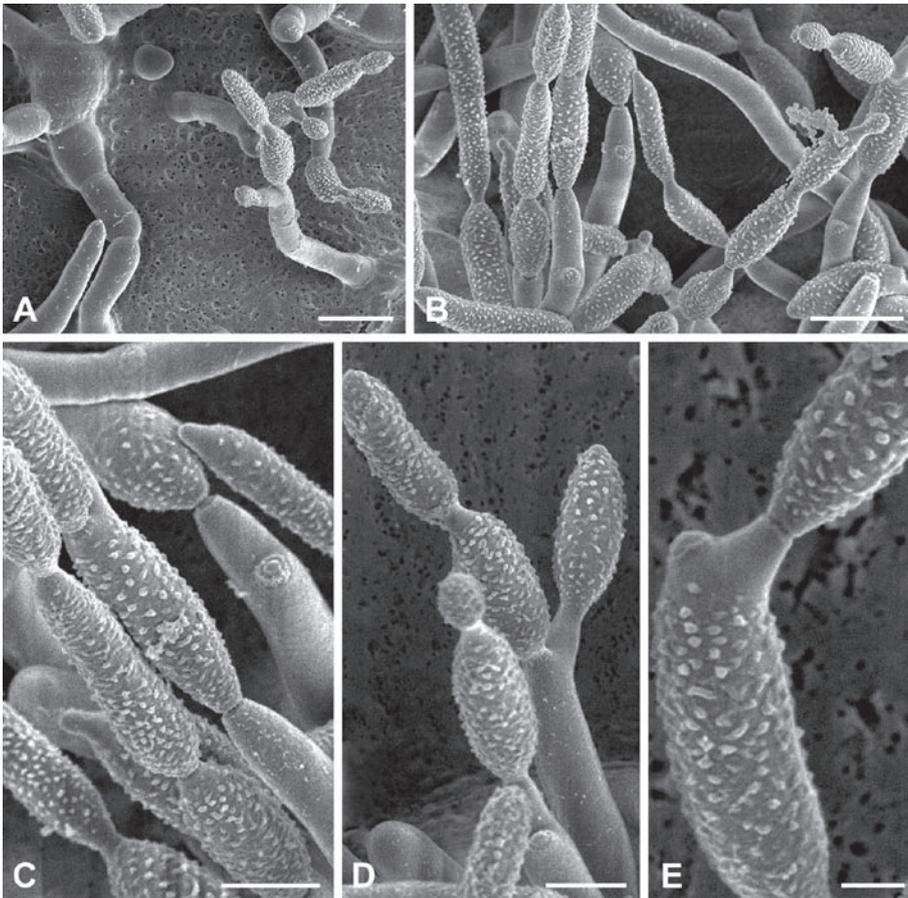
**Cultural characteristics:** Colonies on PDA attaining 9 mm diam after 14 d at 25  $^{\circ}$ C, greenish olivaceous to grey-olivaceous, at the margin becoming dull green, reverse with a pale olivaceous-grey centre and a broad olivaceous-black margin, margin narrow, regular, entire edge, white, feathery, aerial mycelium sparse but colonies appearing felty, growth flat with somewhat elevated colony centre, prominent exudates not formed, sporulation dense, covering almost the whole colony. Colonies on MEA attaining 12 mm diam after 14 d at 25  $^{\circ}$ C, olivaceous-grey to iron-grey, iron-grey reverse, velvety to powdery, aerial mycelium sparse, sporulation profuse. Colonies

on OA attaining 4 mm after 14 d at 25  $^{\circ}$ C, olivaceous-grey, aerial mycelium sparse, diffuse, growth flat, without prominent exudates, sporulating.

**Specimen examined:** **Antarctica**, King George, Arctowski, isolated from the lichen *Caloplaca regalis* (Teloschistaceae), C. Möller, No. 32/12, 1991, CBS-H 19857, **holotype**, isotype HAL 2024 F, culture ex-type CBS 690.92.

**Substrate and distribution:** On the lichen *Caloplaca regalis*; Antarctica.

**Notes:** This is the second genuine lichenicolous species of the genus *Cladosporium*. *Cladosporium licheniphilum* Heuchert & U. Braun, occurring on apothecia of *Pertusaria alpina* in Russia, is quite distinct from *C. antarcticum* by having subcylindrical or only slightly geniculate-sinuous, wider conidiophores, 5–8  $\mu$ m, with numerous characteristic terminal branches and much shorter, 0–1-septate, smooth conidia, 3.5–13  $\times$  3–7  $\mu$ m (Heuchert & Braun 2006). *Cladosporium lichenicola* Linds. was invalidly published and *C. arthoniae* M.S. Christ. & D. Hawksw. as well as *C. lichenum* Keissl. are to be excluded from the genus *Cladosporium* since they do not possess the typical cladosporioid scar structure but inconspicuous, unthickened conidiogenous loci and conidial hila (Hawksworth 1979, Heuchert *et al.* 2005). The fungicolous species *C. uredinicola* Speg. and the follicolous species *C. alneum* Pass. ex K. Schub. and *C. psoraleae* M.B. Ellis are morphologically superficially similar. However, *C. uredinicola*, a widespread fungus on rust fungi, downy mildews and powdery mildew fungi, differs in having somewhat longer and wider, smooth conidia, 3–39  $\times$  2–6.5(–8)  $\mu$ m, and wider conidiogenous loci and conidial hila, 0.5–3



**Fig. 7.** *Cladosporium antarcticum* (CBS 690.92). A. Overview of the growth pattern on SNA. Note the very large bulbous cells formed at the base of different conidiophores. Other conidiophores sprout from the agar surface. B. Overview of conidiophores and conidia. Note the large distance of the scars on the conidiophore and the different stages of conidial formation on the tips of other conidia. The long secondary ramoconidia are also visible, and sparse aerial hyphae. C. Detail of B with details of the ornamentation and scars. The absence of ornamentation at the apical (spore-forming) end of the secondary ramoconidium is clearly visible. D–E. Tubular structures on conidiophore (D) and secondary ramoconidium (E). Scale bars: A–B = 10  $\mu$ m, C–D = 5  $\mu$ m, E = 2  $\mu$ m.



**Fig. 8.** *Cladosporium antarcticum* (CBS 690.92). A–B. Macronematous conidiophores. C, G. Mycelium enveloped by a polysaccharide-like layer. D, F. Conidia. E. Micronematous conidiophore. H. Ramoconidium with numerous distal scars. Scale bars = 10  $\mu$ m.

$\mu$ m (Heuchert *et al.* 2005); *C. alneum*, which causes leaf spots on *Alnus glutinosa*, possesses longer and wider conidiophores, 25–260  $\times$  (2–)3–7(–8.5)  $\mu$ m, and somewhat shorter, smooth conidia (Schubert 2005, Schubert *et al.* 2006); and *C. psoraleae*, known

from Myanmar on *Psoralea corylifolia*, can easily be distinguished from *C. antarcticum* by its smooth and wider conidia, 3.5–7  $\mu$ m, and wider conidiogenous loci and conidial hila, 1–3  $\mu$ m diam (Ellis 1972, Schubert 2005).

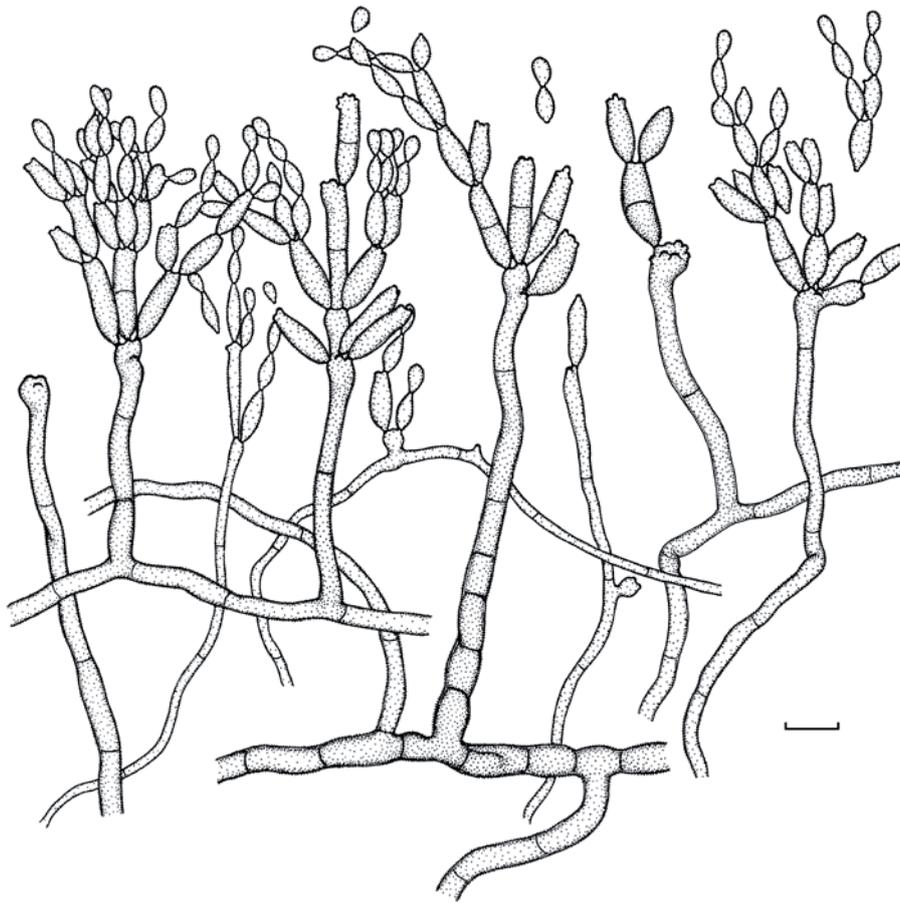


Fig. 9. *Cladosporium bruhnei* (CPC 12211). Macro- and micronematous conidiophores and conidia. Scale bar = 10  $\mu$ m. K. Schubert del.

***Cladosporium bruhnei*** Linder, Bull. Natl. Mus. Canada 97: 259. 1947. Figs 9–12.

≡ *Homodendrum hordei* Bruhne, in W. Zopf, Beitr. Physiol. Morph. nied. Org. 4: 1. 1894, non *C. hordei* Pass., 1887.

≡ *Cladosporium herbarum* (Pers.: Fr.) Link var. ( $\delta$ ) *cerealium* Sacc. f. *hordei* (Bruhne) Ferraris, Flora Ital. Crypt., Pars I, Fungi, Fasc.13: 882. 1914.

≡ *Cladosporium hordei* (Bruhne) Pidopl., Gribnaja Flora Grubych Kormov: 268. 1953, *nom. illeg.*, homonym, non *C. hordei* Pass., 1887.

**Teleomorph: *Davidiella allicina*** (Fr. : Fr.) Crous & Aptroot, in Aptroot, *Mycosphaerella* and its anamorphs: 2. *Conspectus of Mycosphaerella*. CBS Biodiversity Ser. 5: 30. 2006.

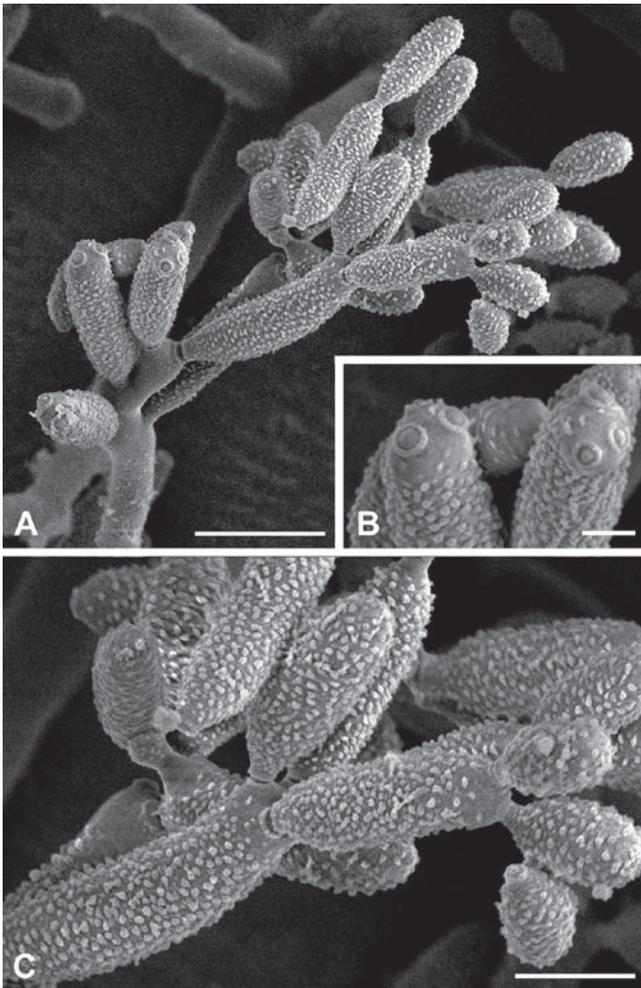
**Basionym: *Sphaeria allicina*** Fr., Kongl. Vetensk. Acad. Handl. 38: 247. 1817, sactioned by Fr., *Syst. Mycol.* 2: 437. 1823.

≡ *Sphaerella allicina* (Fr. : Fr.) Auersw., in Gonn. & Rabenh., *Mycol. Europaea* 5–6: 19. 1869.

**Ascomata** pseudothecial, black, superficial, situated on a small stroma, globose, up to 250  $\mu$ m diam; ostioles periphysate, with apical periphysoids present; wall consisting of 3–6 layers of reddish brown *textura angularis*. **Asci** fasciculate, bitunicate, sessile, obovoid to broadly ellipsoid, straight to slightly curved, 8-spored, 65–90  $\times$  16–25  $\mu$ m; with pseudoparenchymatal cells of the hamathecium persistent. **Ascospores** tri- to multiseriate, overlapping, hyaline, with irregular lumina, thick-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse basal end, and acutely rounded apical end, widest near the middle of the apical cell, medianly 1-septate, not to slightly constricted at the septum, (20–)25–27(–30)  $\times$  (5.5–)6–7  $\mu$ m.

**Mycelium** superficial, hyphae branched, 1.5–8  $\mu$ m wide, pluriseptate, broader hyphae usually slightly constricted at the septa and somewhat swollen, hyaline to subhyaline, almost smooth

to somewhat verruculose or irregularly rough-walled, sometimes appearing to have a slime coat, walls unthickened. **Conidiophores** macronematous, sometimes also micronematous, arising as lateral or terminal branches from plagiotropous or ascending hyphae, erect, straight to more or less flexuous, sometimes geniculate, nodulose, usually with small head-like swellings, sometimes also with intercalary nodules, sometimes swellings protruding and elongated to one side, unbranched, occasionally branched, (7–)20–330  $\mu$ m, sometimes even longer, (2–)3–5  $\mu$ m wide, swellings (4–)5–8  $\mu$ m wide, pluriseptate, not constricted at the septa, septa sometimes not very conspicuous, subhyaline to pale brown or pale olivaceous, smooth or somewhat verruculose, walls unthickened or almost so, more thickened with age. **Conidiogenous cells** integrated, usually terminal, cylindrical with a terminal head-like swelling, sometimes with a second swelling, 15–40  $\mu$ m long, proliferation sympodial, with few conidiogenous loci confined to swellings, up to five per swelling, loci protuberant, conspicuous, 1–2  $\mu$ m diam, thickened and darkened-refractive. **Conidia** catenate, formed in branched chains, straight to slightly curved, small terminal conidia subglobose, ovoid to obovoid or somewhat limoniform, 4–9  $\times$  2.5–3.5  $\mu$ m [av.  $\pm$  SD, 6.5 ( $\pm$  1.5)  $\times$  3.1 ( $\pm$  0.5)  $\mu$ m], aseptate; secondary ramoconidia and occasionally formed ramoconidia ellipsoid to subcylindrical or cylindrical, 10–24(–31)  $\times$  3–5(–7)  $\mu$ m [av.  $\pm$  SD, 16.1 ( $\pm$  4.1)  $\times$  4.1 ( $\pm$  0.8)  $\mu$ m], rarely up to 40  $\mu$ m long, 0–1(–3)-septate, very rarely 5-septate, subhyaline to pale brown or pale olivaceous, minutely verruculose to verruculose (mostly granulate with some muricate projections under SEM), walls unthickened or almost so, apex rounded or slightly attenuated towards apex and base, hila protuberant, conspicuous, 1–2  $\mu$ m wide, up to 1  $\mu$ m high, thickened and darkened-refractive; microcyclic conidiogenesis occurring.



**Fig. 10.** *Cladosporium bruhnei* (CPC 12211). A. Conidiophore with characteristic long secondary ramoconidium and complex conidiophore. B. Detail of hila on secondary ramoconidia. C. Details of prominent ornamentation on conidia. Scale bars: A = 10  $\mu\text{m}$ , B = 2  $\mu\text{m}$ , C = 5  $\mu\text{m}$ .

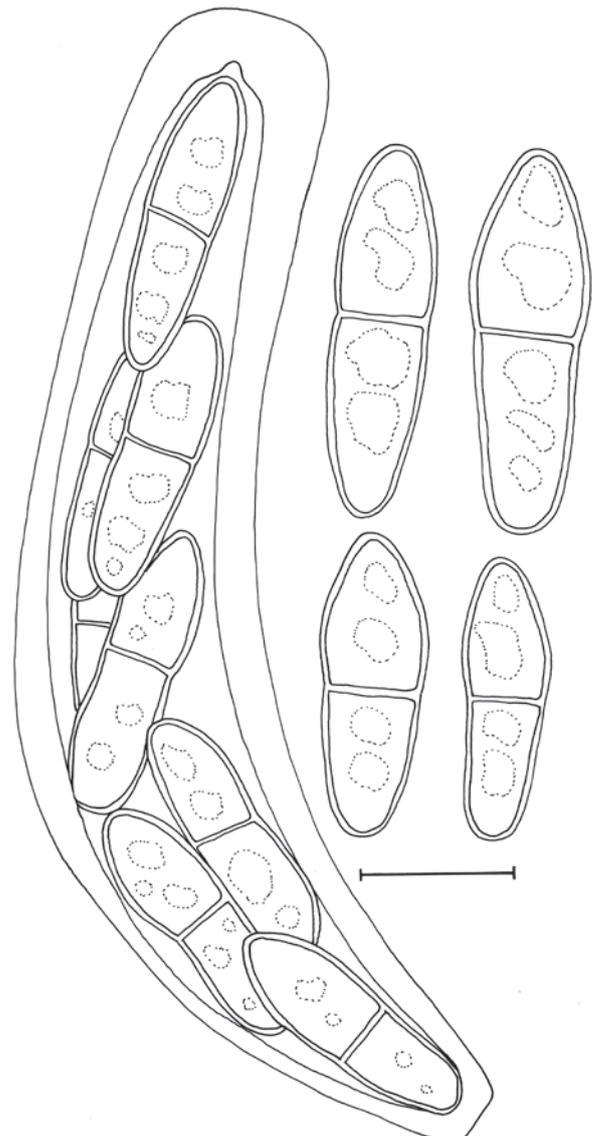
**Cultural characteristics:** Colonies on PDA reaching 22–32 mm diam after 14 d at 25 °C, olivaceous-grey to iron-grey, sometimes whitish, smoke-grey to pale olivaceous due to abundant aerial mycelium covering almost the whole colony, with age collapsing becoming olivaceous-grey, occasionally zonate, velvety to floccose, margin narrow, entire edge, white, glabrous to somewhat feathery, aerial mycelium sparse to abundant, white, fluffy, growth regular, flat to low convex, sometimes forming few exudates in the colony centre, sporulating. Colonies on MEA reaching 21–32 mm diam after 14 d at 25 °C, grey-olivaceous, olivaceous-grey to dull green or iron-grey, sometimes whitish to pale smoke-grey due to abundant aerial mycelium, olivaceous-grey to iron-grey reverse, velvety, margin narrow, entire edge to slightly undulate, white, radially furrowed, glabrous to slightly feathery, aerial mycelium sparse to abundant, mainly in the centre, white, fluffy, growth convex to raised, radially furrowed, distinctly wrinkled in the colony centre, without prominent exudates, sporulating. Colonies on OA reaching 20–32 mm diam after 14 d at 25 °C, smoke-grey, grey-olivaceous to olivaceous-grey, greenish black or iron-grey reverse, margin narrow, entire edge, colourless to white, glabrous, aerial mycelium sparse to abundant, dark smoke-grey, diffuse, high, later collapsed, felty, growth flat, without prominent exudates, sporulation profuse.

**Specimens examined:** *Sine loco et dato*, CBS 188.54 = ATCC 11290 = IMI 049638. **Australia**, N.S.W., Barrington Tops National Park, isolated from leaves of *Eucalyptus stellulata* (Myrtaceae), 3 Jan. 2006, B. Summerell, CPC 12921.

**Belgium**, isolated from *Quercus robur* (Fagaceae), CBS 157.82; Kampenhout, isolated from *Hordeum vulgare* (Poaceae), 26 June 2005, J.Z. Groenewald, CBS-H 19856, **neotype designated here** of *C. bruhnei*, isoneotype HAL 2023 F, cultures ex-type CBS 121624 = CPC 12211, CPC 12212. **Czech Republic**, Lisen, isolated from *Polygonatum odoratum* (Liliaceae), CBS 813.71, albino mutant of CBS 812.71. **Germany**, CBS 134.31 = ATCC 11283 = IMI 049632; Nordrhein-Westfalen, Mülheim an der Ruhr, isolated from industrial water, IWW 727, CBS 110024; Sachsen-Anhalt, Halle (Saale), Robert-Franz-Ring, isolated from leaves of *Tilia cordata* (Tiliaceae), 2004, K. Schubert, CPC 11386. **Netherlands**, isolated from air, CBS 521.68; isolated from *Hordeum vulgare*, 1 Jan. 2005, P.W. Crous, CPC 12139; isolated from man, skin, CBS 159.54 = ATCC 36948; Amsterdam, isolated from Thuja tincture, CBS 177.71; Geleen, St. Barbara Ziekenhuis, isolated from man, skin, CBS 366.80, CBS 399.80; isolated from man, sputum, Aug. 1955, CBS 161.55. **New Zealand**, Otago, Lake Harris, isolated from *Ourisia macrophylla* (Scrophulariaceae), 30 Jan. 2005, A. Blouin, Hill 1135, CPC 11840. **Russia**, Moscow region, isolated from *Polyporus radiatus* (Polyporaceae), Oct. 1978, CBS 572.78 = VKM F-405. **Slovenia**, Ljubljana, isolated from an air conditioning system, 2004, M. Butala, EXF-680 = CPC 12046; Sečovlje, isolated from hypersaline water from salterns (reserve pond), 2005, P. Zalar, EXF-389 = CPC 12042. **Spain**, Ebro Delta, isolated from hypersaline water from salterns (crystallisation pond), 2004, P. Zalar, EXF-594 = CPC 12045. **Sweden**, Skåne, on tip blight of living leaves of *Allium* sp. (Alliaceae), Fr. no. F-09810, UPS-FRIES, **holotype** of *Davidiella allicina*. **U.S.A.**, New York, Geneva, isolated from CCA-treated Douglas-fir pole, CBS 115683 = ATCC 66670 = CPC 5101.

**Substrate and distribution:** Living and decaying plant material, man, air, hypersaline and industrial water; widespread.

**Literature:** Saccardo (1899: 1076), Linder (1947: 289).



**Fig. 11.** *Davidiella allicina* (F-09810, UPS-FRIES, holotype). Ascus and ascospores. Scale bar = 10  $\mu\text{m}$ . P.W. Crous del.

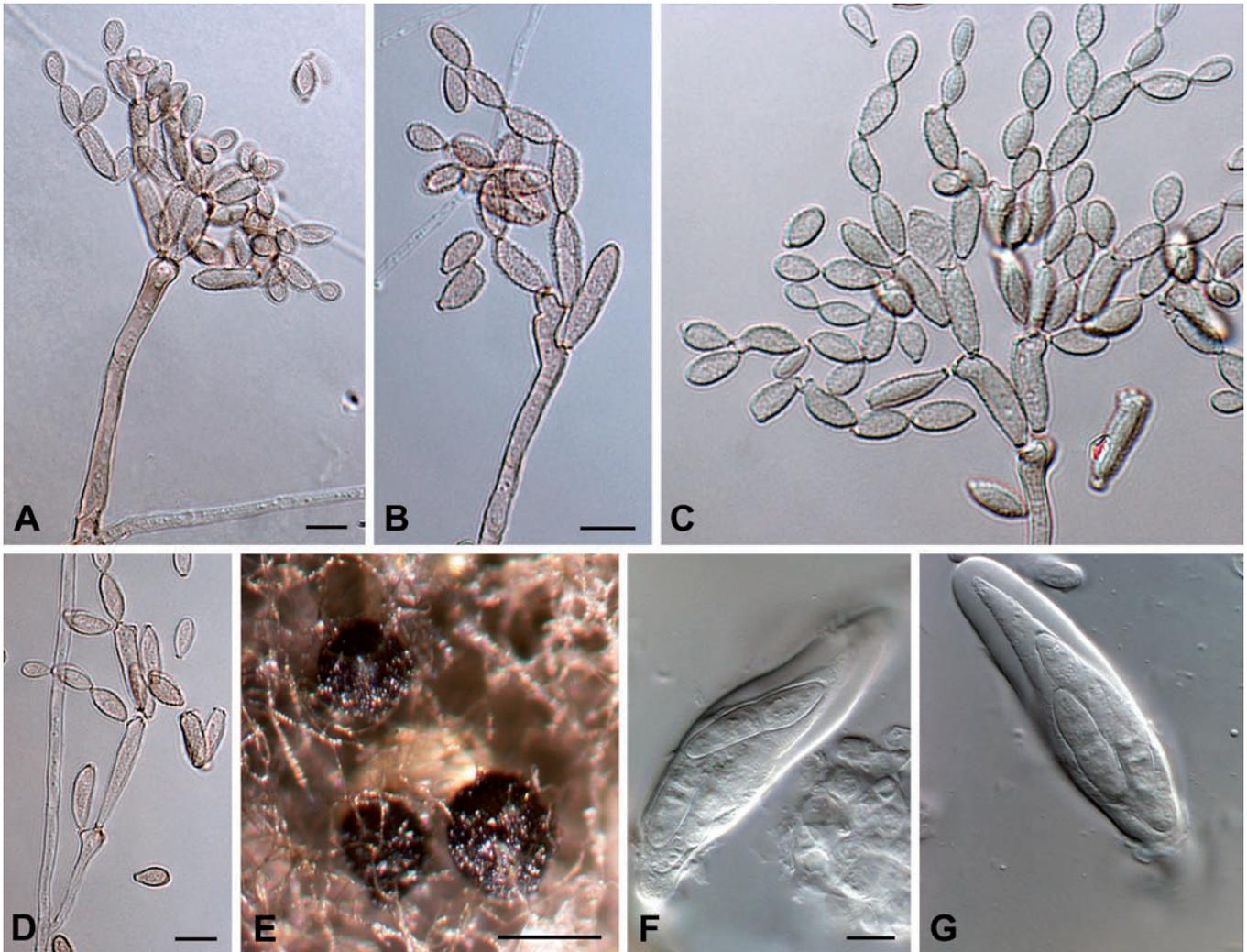


Fig. 12. *Cladosporium bruhnei* (CPC 12211) and its teleomorph *Davidiella allicina*. A–B. Macronematous conidiophores. C. Conidial chains. D. Micronematous conidiophore. E. Ascomata of the teleomorph formed on the host. F–G. Asci. Scale bars: A–B, D, F = 10  $\mu$ m, E = 200  $\mu$ m.

**Notes:** *Cladosporium bruhnei* proved to be an additional component of the herbarum complex. The species resembles *C. herbarum* s. str. as already stated by Linder (1947), but possesses consistently narrower conidia, usually 2.5–5  $\mu$ m wide, and the conidiophores often form only a single apical swelling. The species was described by Bruhne (*l.c.*) as *Hormodendrum hordei* from Germany but type material could not be located. Linder (1947) examined No. 1481a-5 (Canada, N. Quebec, Sugluk, on *Elymus arenarius* var. *villosus*, 31 Jul. 1936, E. Meyer), presumably in the National Museum, and stated that this specimen agreed well with the description and illustration given by Bruhne (*l.c.*). Although the species occurs on numerous substrates and is widely distributed, it has not yet been recognised as a distinct species since it has probably been interpreted as a narrow variant of *C. herbarum*.

Based on morphology and DNA sequence data, the CBS strain CBS 177.71 chosen by Prasil & de Hoog (1988) as representative living strain of *C. herbarum*, rather clusters together with isolates of *C. bruhnei*. The strain CBS 813.71 is an albino mutant of the latter species as it does not appear to contain colour pigment. Furthermore, all isolates from humans treated until now as *C. herbarum* proved to be conspecific with the narrow-spored *C. bruhnei*.

Although *Davidiella tassiana* (ascospores 17–25  $\times$  6–8.5  $\mu$ m, RO) was treated as synonymous to *D. allicina* (ascospores 20–27  $\times$  6–7  $\mu$ m, UPS) in Aptroot (2006), they differ in apical ascospore

taper, with ascospores of *D. allicina* being acutely rounded, while those of *D. tassiana* are obtusely rounded. The same ascospore taper was also observed in the teleomorph of *C. bruhnei*, and thus the name *D. allicina* is herewith linked to *C. bruhnei*, which is distinct from *C. herbarum*, having *D. tassiana* as teleomorph.

***Cladosporium herbaroides*** K. Schub., Zalar, Crous & U. Braun, sp. nov. MycoBank MB504574. Figs 13–15.

**Etymology:** Refers to its morphological similarity to *Cladosporium herbarum*.

Differt a *Cladosporio herbarum* conidiis polymorphis, 3–33  $\times$  (2–)3–6(–7)  $\mu$ m, postremo latioribus, (3.5–)5–9(–11)  $\mu$ m, fuscis et crassitunicatis; et a *Cladosporio macrocarpo* conidiophoris leniter angustioribus, 3–5  $\mu$ m latis, nodulis angustioribus, 5–8  $\mu$ m latis.

**Mycelium** branched, (1–)2–8  $\mu$ m wide, septate, often with small swellings and constrictions, subhyaline to pale brown or pale olivaceous-brown, smooth or almost so to somewhat verruculose, walls unthickened or almost so. **Conidiophores** macronematous and micronematous, arising lateral from plagiotropous hyphae or terminally from ascending hyphae. **Macronematous conidiophores** erect, straight to slightly flexuous, often geniculate, nodulose, with unilateral or multilateral swellings, often numerous swellings in short succession giving them a gnarled appearance, often forming somewhat protruding or prolonged lateral swellings or a branch-

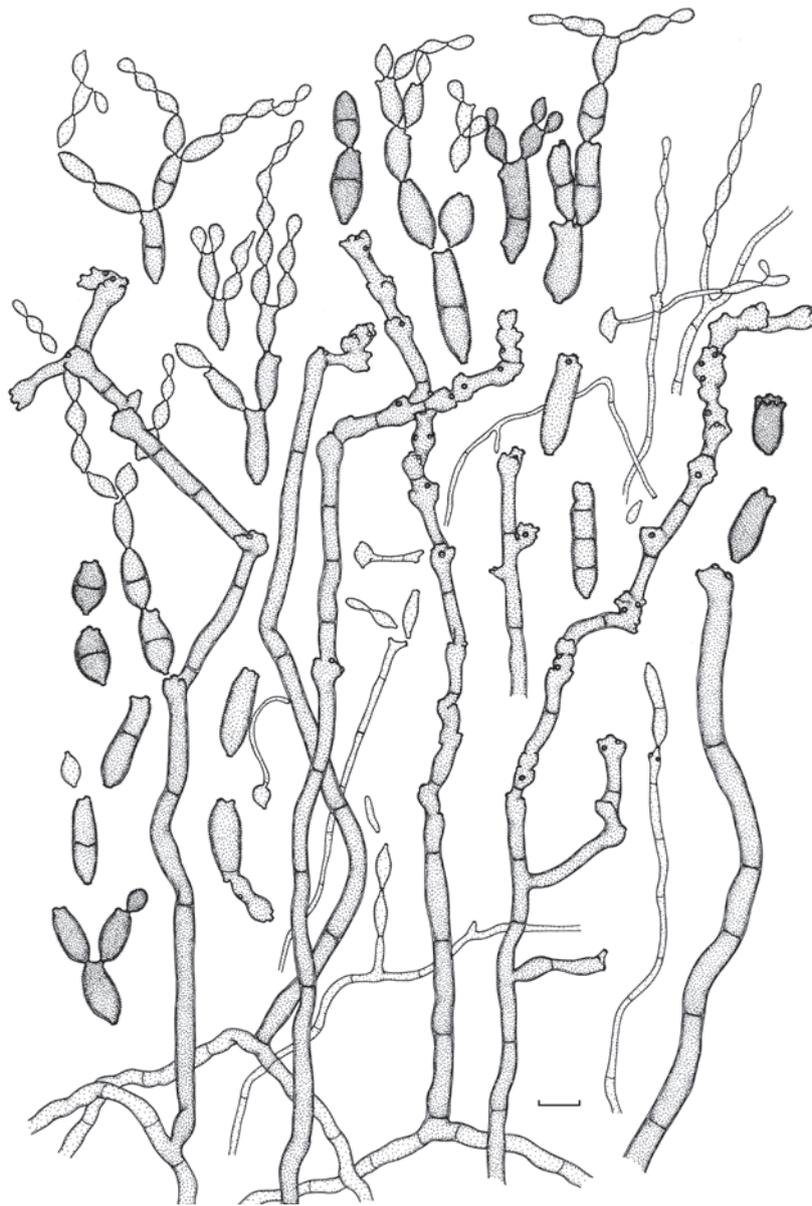
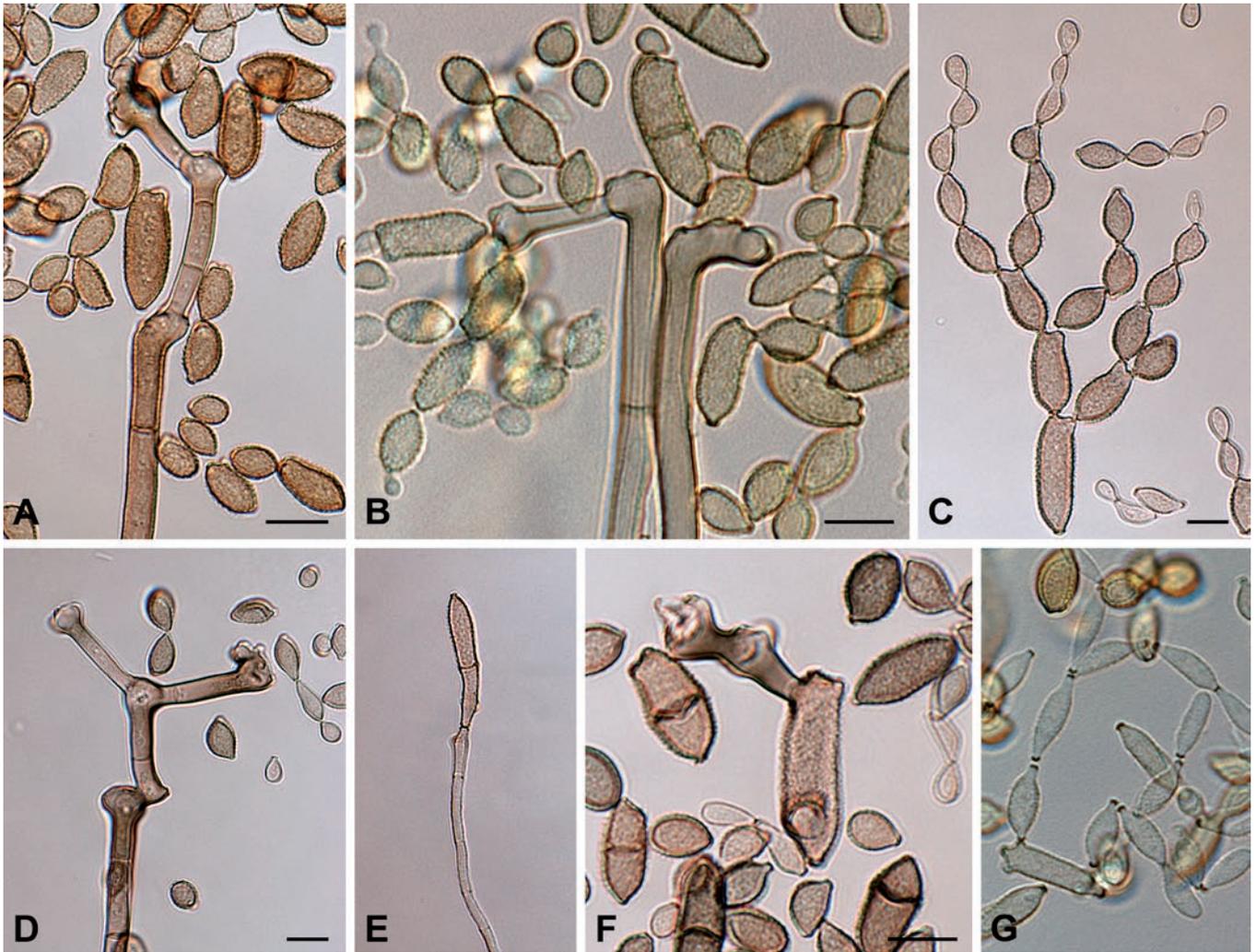


Fig. 13. *Cladosporium herbaroides* (CPC 12052). Macro- and micronematous conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert del.

like prolongation below the terminal swelling (due to sympodial proliferation), unbranched or sometimes branched, 30–230  $\mu\text{m}$  long or even longer, 3–5  $\mu\text{m}$  wide, swellings 5–8  $\mu\text{m}$  wide, septate, not constricted at septa, pale to medium olivaceous-brown, smooth or almost so, walls slightly thickened. *Conidiogenous cells* integrated, terminal or intercalary, cylindrical, usually nodulose to nodose forming distinct swellings, sometimes geniculate, 15–55  $\mu\text{m}$  long, with numerous conidiogenous loci usually confined to swellings or situated on small lateral shoulders, sometimes on the top of short peg-like prolongations or denticles, loci protuberant, 1–2  $\mu\text{m}$  diam, thickened and darkened-refractive. *Micronematous conidiophores* much shorter, narrower, paler, neither nodulose nor geniculate, arising laterally from plagiotropous hyphae, often only as short lateral denticles or branchlets of hyphae, erect, straight, conical to cylindrical, unbranched, 3–65  $\times$  2–3  $\mu\text{m}$ , mostly aseptate, sometimes up to five septa, subhyaline, smooth, walls unthickened. *Conidiogenous cells* integrated, terminal or conidiophores reduced to conidiogenous cells, conidiogenous loci solitary or sometimes as sympodial clusters of pronounced denticles, protuberant, 1–1.5  $\mu\text{m}$  diam, thickened and somewhat darkened-refractive. *Conidia* polymorphous, two main morphological types recognisable, formed

by the two different types of conidiophores, conidia formed by macronematous conidiophores catenate, in branched chains, straight to slightly curved, subglobose, obovoid, limoniform, ellipsoid to cylindrical, 3–33  $\times$  (2–)3–6(–7)  $\mu\text{m}$  [av.  $\pm$  SD, 14.5 ( $\pm$  7.9)  $\times$  5.2 ( $\pm$  1.2)  $\mu\text{m}$ ], 0–2(–3)-septate, sometimes slightly constricted at septa, septa median or somewhat in the lower half, pale to medium olivaceous-brown, verruculose to verrucose (granulate under SEM), walls slightly thickened, with up to three rarely four distal scars, with age becoming medium or even dark brown (chocolate brown), wider and more thick-walled, 5.5–33  $\times$  (3.5–)5–9(–11)  $\mu\text{m}$  [av.  $\pm$  SD, 14.4 ( $\pm$  6.9)  $\times$  7.2 ( $\pm$  1.9)  $\mu\text{m}$ ], walls up to 1  $\mu\text{m}$  thick, hila protuberant, 0.8–2(–2.5)  $\mu\text{m}$  diam, thickened and darkened-refractive; microcyclic conidiogenesis occurring. *Conidia* formed by micronematous conidiophores paler and narrower, mostly formed in unbranched chains, sometimes in branched chains with up to three distal hila, straight to slightly curved, limoniform, narrowly fusiform, almost filiform to subcylindrical, 10–26(–35)  $\times$  2–3.5  $\mu\text{m}$  [av.  $\pm$  SD, 15.6 ( $\pm$  6.2)  $\times$  2.9 ( $\pm$  0.5)  $\mu\text{m}$ ], 0–1(–3)-septate, subhyaline to pale brown, almost smooth to minutely verruculose, walls unthickened, hila protuberant, 1–1.5  $\mu\text{m}$  diam, thickened and somewhat darkened-refractive.



**Fig. 14.** *Cladosporium herbaroides* (CPC 12052). A–B, D. Macronematous conidiophores. C. Conidial chain. E. Micronematous conidiophore. F. Microcyclic conidiogenesis. G. Conidia formed by micronematous conidiophores. Scale bars = 10 µm.

**Cultural characteristics:** Colonies on PDA attaining 23 mm diam after 14 d at 25 °C, grey-olivaceous to olivaceous, olivaceous-grey reverse, velvety, margin regular, entire edge, narrow, feathery, aerial mycelium abundantly formed, loose, with age covering large parts of the colony, woolly, growth flat with somewhat elevated colony centre, folded, regular, deep into the agar, with few prominent exudates, sporulation profuse. Colonies on MEA attaining 24 mm diam after 14 d at 25 °C, grey- to greenish olivaceous, olivaceous-grey or iron-grey reverse, velvety to powdery, margin narrow, colourless, entire edge, somewhat feathery, aerial mycelium pale olivaceous-grey, sparse, growth convex, radially furrowed, folded in the colony centre, without prominent exudates, sporulating. Colonies on OA attaining 23 mm diam after 14 d at 25 °C, grey-olivaceous, margin more or less regular, entire edge, colourless, somewhat feathery, aerial mycelium whitish to smoke grey, at first sparse, later more abundantly formed, growth flat, without exudates, sporulation profuse.

**Specimen examined:** Israel, from hypersaline water of Eilat salterns, 2004, coll. N. Gunde-Cimerman, isol. M. Ota, CBS-H 19858, **holotype**, isotype HAL 2025 F, culture ex-type CBS 121626 = EXF-1733 = CPC 12052.

**Substrate and distribution:** Hypersaline water; Israel.

**Notes:** *Cladosporium herbaroides* is morphologically similar to *C. herbarum* but differs in having somewhat longer conidia becoming wider, darker and even more thick-walled with age [at first conidia 3–33 × (2–)3–6(–7) µm, with age (3.5–)5–9(–11) µm wide]. Besides

that, the species often produces a second conidial type formed on micronematous conidiophores, giving rise to unbranched conidial chains which are almost filiform, limoniform, narrowly fusiform to subcylindrical, much narrower and paler than the ones formed by macronematous conidiophores, 10–26(–35) × 2–3.5 µm. In *C. herbarum*, conidia formed by micronematous conidiophores do not occur as frequently as in *C. herbaroides*, and differ in being often clavate and somewhat wider, up to 4(–5) µm wide. *Cladosporium macrocarpum* is easily distinguishable by having somewhat wider conidiophores (3–)4–6 µm, with distinctly wider swellings, 5–10 µm wide, and the conidia are usually (3–)5–9(–10) µm wide.

***Cladosporium herbarum*** (Pers. : Fr.) Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 7: 37. 1816: Fr., Syst. mycol. 3(2): 370. 1832. Figs 16–19.

**Basionym:** *Dematium herbarum* Pers., Ann. Bot. (Usteri) 11: 32. 1794: Fr., Syst. mycol. 3(2): 370. 1832.

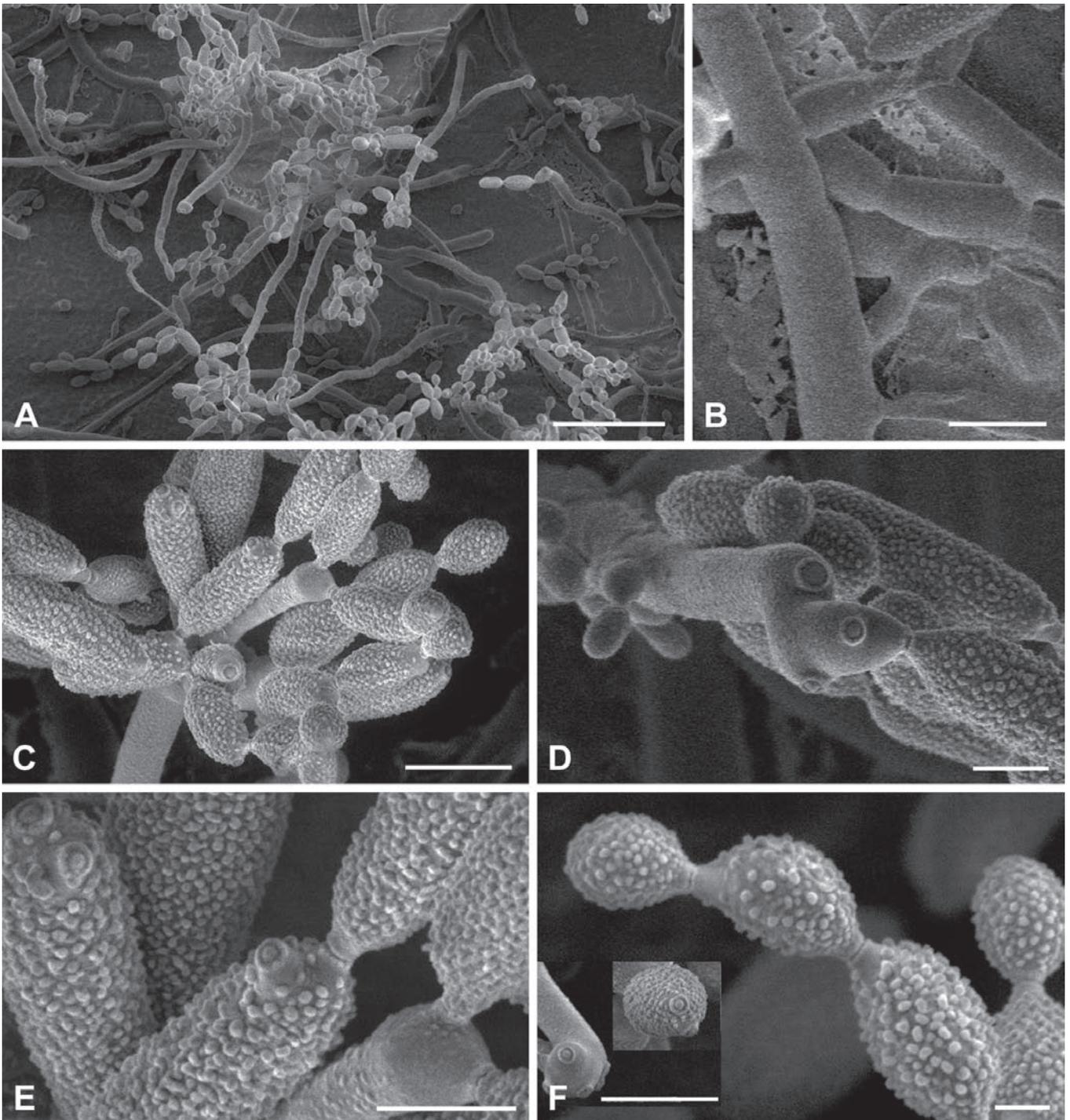
= *Dematium epiphyllum* var. (β) *chionanthi* Pers., Mycol. eur. 1: 16. 1822, **syn. nov.**

For additional synonyms see Dugan *et al.* (2004), Schubert (2005).

**Teleomorph:** *Davidiella tassiana* (De Not.) Crous & U. Braun, Mycol. Progr. 2: 8. 2003.

**Basionym:** *Sphaerella tassiana* De Not., Sferiacei Italici 1: 87. 1863.

= *Mycosphaerella tassiana* (De Not.) Johanson, Öfers. Förh. Kongl. Svenska Vetensk.-Akad. 41: 167. 1884.



**Fig. 15.** *Cladosporium herbaroides* (CPC 12052). A. Overview of the growth characteristics of this fungus. Broad hyphae run over the surface of the agar, and possibly give rise to conidiophore branches. The conidiophores of this fungus can be rather long, resembling aerial hyphae. Clusters of conidia are clearly visible in this micrograph. B. The very wide surface hyphae can anastomose. C. Conidiophore with secondary ramoconidia and conidia. Note the variation in scar size. D. A very elaborate, complex conidiophore with different scars of variable size, one being more than 2  $\mu\text{m}$  wide! E. Details of secondary ramoconidia and hila. Note the rather strong ornamentation in which smaller “particles” are between larger ones. F. Three conidia in a row. Note the scar formation in the chain and the reduction of the size of the cells throughout the spore-chain. The inset shows the resemblance of the scars on a conidiophore and on a secondary ramoconidium. Scale bars: A = 50  $\mu\text{m}$ , B–C, F (inset) = 10  $\mu\text{m}$ , D–E = 5  $\mu\text{m}$ , F = 2  $\mu\text{m}$ .

*Ascomata* pseudothecial, black, globose, erumpent to superficial, up to 200  $\mu\text{m}$  diam, with 1(–3) short, periphysate ostiolar necks; wall consisting of 3–6 layers of medium red-brown *textura angularis*. *Asci* fasciculate, bitunicate, sessile, obovoid to broadly ellipsoid, straight to slightly curved, 8-spored, 65–85  $\times$  13–17  $\mu\text{m}$ . *Pseudoparaphyses* absent in host material, but remnants observed when studied in culture, hyaline, septate, subcylindrical, anastomosing, 3–4  $\mu\text{m}$  wide. *Ascospores* tri- to multiseriate, overlapping, hyaline, with irregular luminal inclusions, thick-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse

ends, widest near middle of apical cell, medianly 1-septate, not to slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (17–)20–23(–25)  $\times$  (6–)7(–8)  $\mu\text{m}$ ; becoming brown and verruculose in asci. Ascospores germinating after 24 h on MEA from both ends, with spore body becoming prominently constricted at the septum, but not distorting, up to 7  $\mu\text{m}$  wide, hyaline to pale brown and appearing somewhat verruculose, enclosed in a mucoid sheath, with germ tubes being irregular, somewhat nodular.

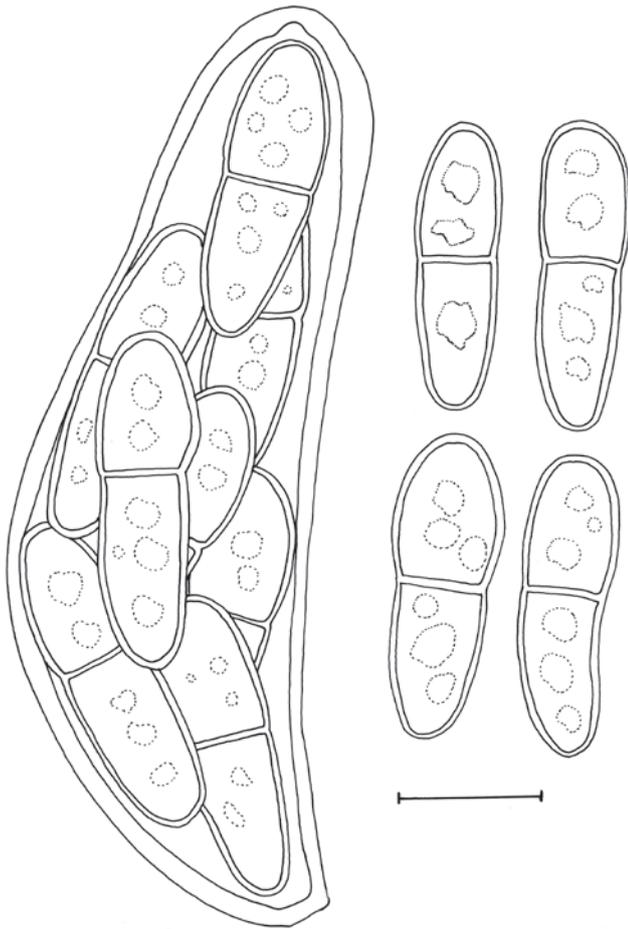


Fig. 16. *Davidiella tassiana* (RO, holotype). Ascus and ascospores. Scale bar = 10  $\mu\text{m}$ . P.W. Crous del.

*Mycelium* superficial, loosely branched, (0.5–)1–5  $\mu\text{m}$  wide, septate, sometimes constricted at septa, hyaline, subhyaline to pale brown, smooth or almost so to verruculose or irregularly rough-walled, sometimes appearing irregular in outline due to small swellings and constrictions, walls unthickened to somewhat thickened, cell lumen appearing to be granular. *Conidiophores* both macro- and micronematous, arising laterally from plagiotropous hyphae or terminally from ascending hyphae. *Macronematous conidiophores* erect, straight to flexuous, somewhat geniculate-sinuous, nodulose to nodose with unilateral or multilateral swellings, with a single to numerous swellings in short succession giving the stalk a knotty/gnarled appearance, unbranched or occasionally branched, up to three times, sometimes with a lateral branch-like proliferation below or at the apex, 10–320  $\times$  3.5–5  $\mu\text{m}$ , swellings 5–8(–9)  $\mu\text{m}$  wide, pluriseptate, septa sometimes constricted when formed after a node, pale to medium brown, older ones almost dark brown, paler towards the apex, smooth or minutely verruculose, walls thickened, sometimes even two-layered. *Conidiogenous cells* integrated, terminal or intercalary, nodulose to nodose, with a single or up to five swellings per cell, 10–24  $\mu\text{m}$  long, proliferation sympodial, with several conidiogenous loci confined to swellings, mostly situated on small lateral shoulders, more or less protuberant, broadly truncate to slightly convex, 1.5–2.5  $\mu\text{m}$  diam, thickened and somewhat darkened-refractive. *Micronematous conidiophores* hardly distinguishable from hyphae, sometimes only as short lateral outgrowth with a single apical scar, short, conical to almost filiform or narrowly cylindrical, non-nodulose, not geniculate, unbranched, 5–120  $\times$  1.5–3(–4)  $\mu\text{m}$ , pluriseptate, not constricted at septa, cells usually very short, 5–15  $\mu\text{m}$  long, subhyaline to pale brown,

almost smooth to minutely verruculose or irregularly rough-walled, sometimes forming clavate conidia, up to 33  $\mu\text{m}$  long, 0–2-septate. *Conidiogenous cells* integrated, terminal or conidiophores reduced to conidiogenous cells, narrowly cylindrical or filiform, with a single or two loci. *Conidia* catenate, in unbranched or loosely branched chains with branching mostly occurring in the lower part of the chain, straight to slightly curved, small terminal conidia without distal hilum obovoid, 4–10  $\times$  3–5(–6)  $\mu\text{m}$  [av.  $\pm$  SD, 7.8 ( $\pm$  1.9)  $\times$  4.7 ( $\pm$  0.9)  $\mu\text{m}$ ], aseptate, intercalary conidia with a single or sometimes up to three distal hila limoniform, ellipsoid to subcylindrical, 6–16  $\times$  4–6  $\mu\text{m}$  [av.  $\pm$  SD, 12.4 ( $\pm$  1.6)  $\times$  5.3 ( $\pm$  0.6)  $\mu\text{m}$ ], 0–1-septate, *secondary ramoconidia* with up to four distal hila, ellipsoid to cylindrical-oblong, 12–25(–35)  $\times$  (3–)5–7(–9)  $\mu\text{m}$  [av.  $\pm$  SD, 18.8 ( $\pm$  4.5)  $\times$  6.2 ( $\pm$  0.9)  $\mu\text{m}$ ], 0–1(–2)-septate, rarely with up to three septa, sometimes distinctly constricted at the septum, septum median or somewhat in the upper or lower half, pale greyish brown or brown to medium brown or greyish brown, minutely verruculose to verruculose, walls slightly to distinctly thickened, guttulate to somewhat granular, usually only slightly attenuated towards apex and base, apex obtuse or slightly truncate, towards the base sometimes distinctly attenuated with hila situated on short stalk-like prolongations, hila slightly to distinctly protuberant, truncate to slightly convex, (0.8–)1–2.5(–3)  $\mu\text{m}$  wide, 0.5–1  $\mu\text{m}$  high, somewhat thickened and darkened-refractive; microcyclic conidiogenesis occurring, conidia forming micro- and macronematous secondary conidiophores.

*Cultural characteristics*: Colonies on PDA reaching 19–37 mm diam after 14 d at 25  $^{\circ}\text{C}$ , grey-olivaceous to olivaceous-grey, whitish to smoke-grey or pale olivaceous-grey due to abundant aerial mycelium, velvety, reverse olivaceous-grey or iron-grey, margin almost colourless, regular, entire edge, glabrous to feathery, aerial mycelium abundant mainly in the colony centre, dense, felty, woolly, sometimes becoming somewhat reddish brown, fawn coloured, growth regular, flat to low convex with an elevated colony centre, sometimes forming few large prominent exudates, sporulation profuse. Colonies on MEA reaching 17–37 mm diam after 14 d at 25  $^{\circ}\text{C}$ , smoke-grey to pale olivaceous-grey towards margin, olivaceous-grey to iron-grey reverse, velvety, margin white, entire edge to slightly undulate, aerial mycelium abundant, dense, fluffy to felty, growth low convex or raised, radially furrowed, folded and wrinkled in the colony centre, without prominent exudates but sporulating. Colonies on OA reaching 12–28 mm diam after 14 d at 25  $^{\circ}\text{C}$ , olivaceous-grey to iron-grey, due to abundant aerial mycelium pale olivaceous-grey, olivaceous-grey reverse, margin narrow, more or less undulate, white, aerial mycelium white, loose to dense, high, fluffy to felty, covering large parts of the colony, growth flat to low convex, without prominent exudates, sporulating.

*Specimens examined*: *Sine loco, sine dato*, L 910.225-733, **lectotype** of *C. herbarum*, selected by Prasil & de Hoog, 1988. *Sine loco*, on leaves of *Chionanthus* sp. (Oleaceae), L 910.255-872 = L-0115833, **holotype** of *Dematium epiphyllum* var. ( $\beta$ ) *chionanthi*. **Netherlands**, Wageningen, isolated from *Hordeum vulgare* (Poaceae), 2005, P.W. Crous, CBS-H 19853, **epitype designated here** of *C. herbarum* and *D. tassiana*, isoeotype HAL 2022 F, ex-type cultures, CPC 12177 = CBS 121621, CPC 12178–12179, 12181, 12183. **Italy**, on upper and lower surface of dead leaves of *Carex nigra* ["fusca"] (Cyperaceae), Tassi no. 862, RO, **holotype** of *Davidiella tassiana*. **U.S.A.**, Colorado, San Juan Co., above Little Molas Lake, isolated from stems of *Delphinium barbeyi* (Ranunculaceae), 12 Sep. 2004, A. Ramaley, CBS-H 19868 (teleomorph), single ascospore isolates, CBS 121622 = CPC 11600, CPC 11601–11604.

*Substrate and distribution*: On fading and decaying plant material, on living leaves (phylloplane fungus), as secondary invader, as an endophyte, isolated from air, soil, foodstuffs, paints, textiles and numerous other materials; cosmopolitan.

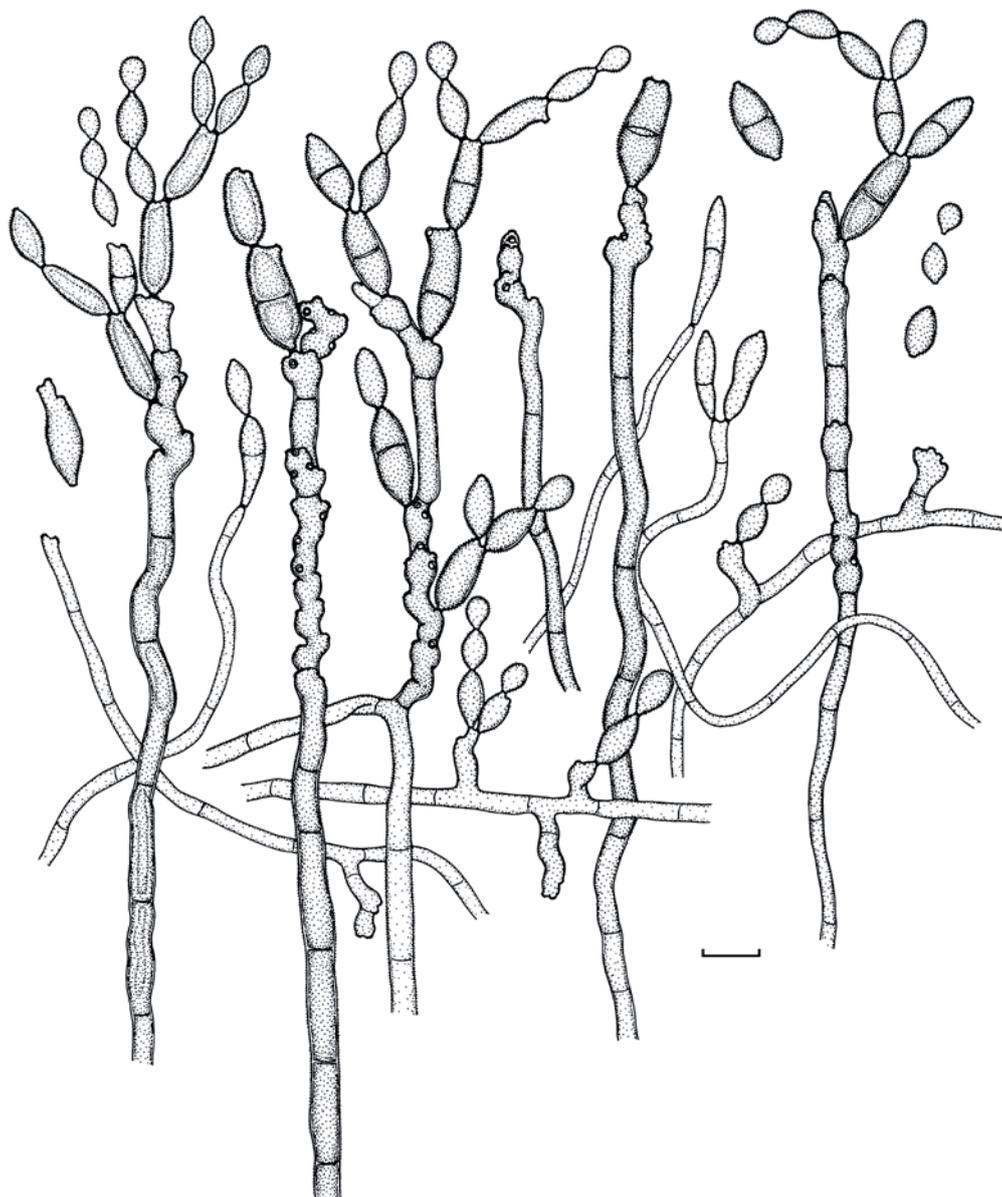


Fig. 17. *Cladosporium herbarum* (CPC 11600). Macro- and micronematous conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert del.

*Literature:* de Vries (1952: 71), Hughes (1958: 750), Ellis (1971: 313), Domsch *et al.* (1980: 204), Sivanesan (1984: 225), Ellis & Ellis (1985: 290, 468, 1988: 168), Prasil & de Hoog (1988), Wang & Zabel (1990: 202), McKemy & Morgan-Jones (1991), Dugan & Roberts (1994), David (1997: 59), Ho *et al.* (1999: 129), de Hoog *et al.* (2000: 587), Samson *et al.* (2000: 110), Samson *et al.* (2001).

*Notes:* De Vries (1952) incorrectly selected a specimen of Link's herbarium at herb. B as lectotype. Prasil & de Hoog (1988) discussed this typification and designated one of Persoon's original specimens as lectotype in which *C. herbarum* could be recognised. The latter material, which is in poor condition, could be re-examined within the course of these investigations and showed conidia agreeing with the current species concept of *C. herbarum* being (6–)9.5–14.5(–21)  $\times$  (5–)6–7(–8)  $\mu\text{m}$ . Since the identity of the strain CBS 177.71 chosen by Prasil & de Hoog (1988) as representative living strain of *C. herbarum* could not be corroborated, an epitype with a living ex-epitype culture is designated. The holotype specimen of *D. tassiana* (RO) is morphologically similar to that observed on the epitype of *C. herbarum*, having ascospores which are (17–)21–

23(–25)  $\times$  (6–)7–8(–8.5)  $\mu\text{m}$ , turning brown and verruculose in asci with age. However, no hamathecial remnants were observed in ascomata *in vivo*.

The connection to the teleomorph *D. tassiana* could be confirmed, which is in agreement with the findings of von Arx (1950) and Barr (1958). Ascospore isolates formed the typical *C. herbarum* anamorph in culture, and these anamorph cultures developed some immature fruiting bodies within the agar. When inoculated onto water agar plates with nettle stems, numerous ascomata with viable ascospores were formed in culture.

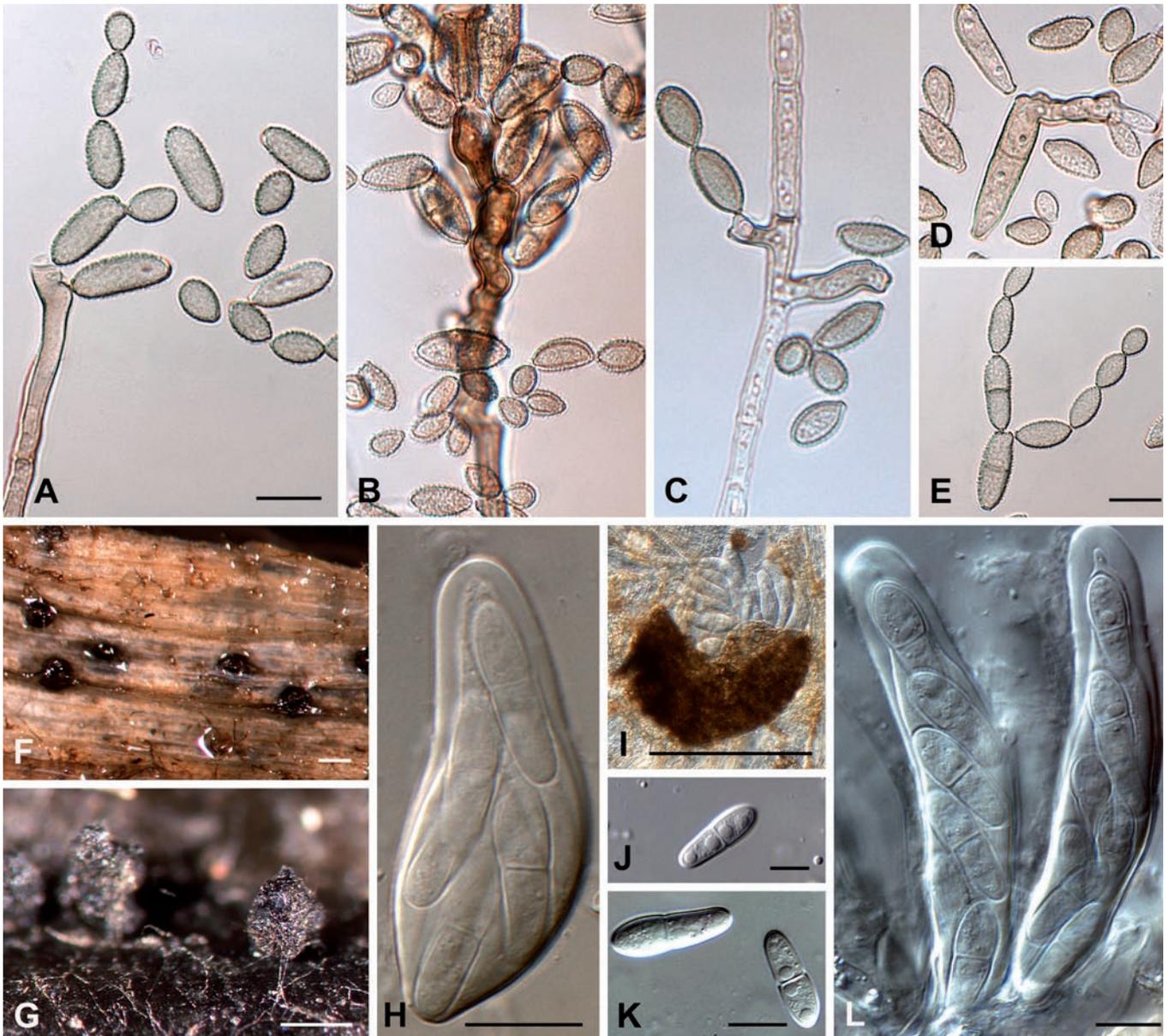
***Cladosporium iridis*** (Fautrey & Roum.) G.A. de Vries, *Contr. Knowl. Genus Cladosporium*: 49. 1952. Figs 20–21.

*Basionym:* *Scolicotrichum iridis* Fautrey & Roum., *Rev. Mycol. (Toulouse)* 13: 82. 1891.

$\equiv$  *Heterosporium iridis* (Fautrey & Roum.) J.E. Jacques, *Contr. Inst. Bot. Univ. Montréal* 39: 18. 1941.

For additional synonyms see Dugan *et al.* (2004).

*Teleomorph:* ***Davidiella macrospora*** (Kleb.) Crous & U. Braun, *Mycol. Progr.* 2: 10. 2003.



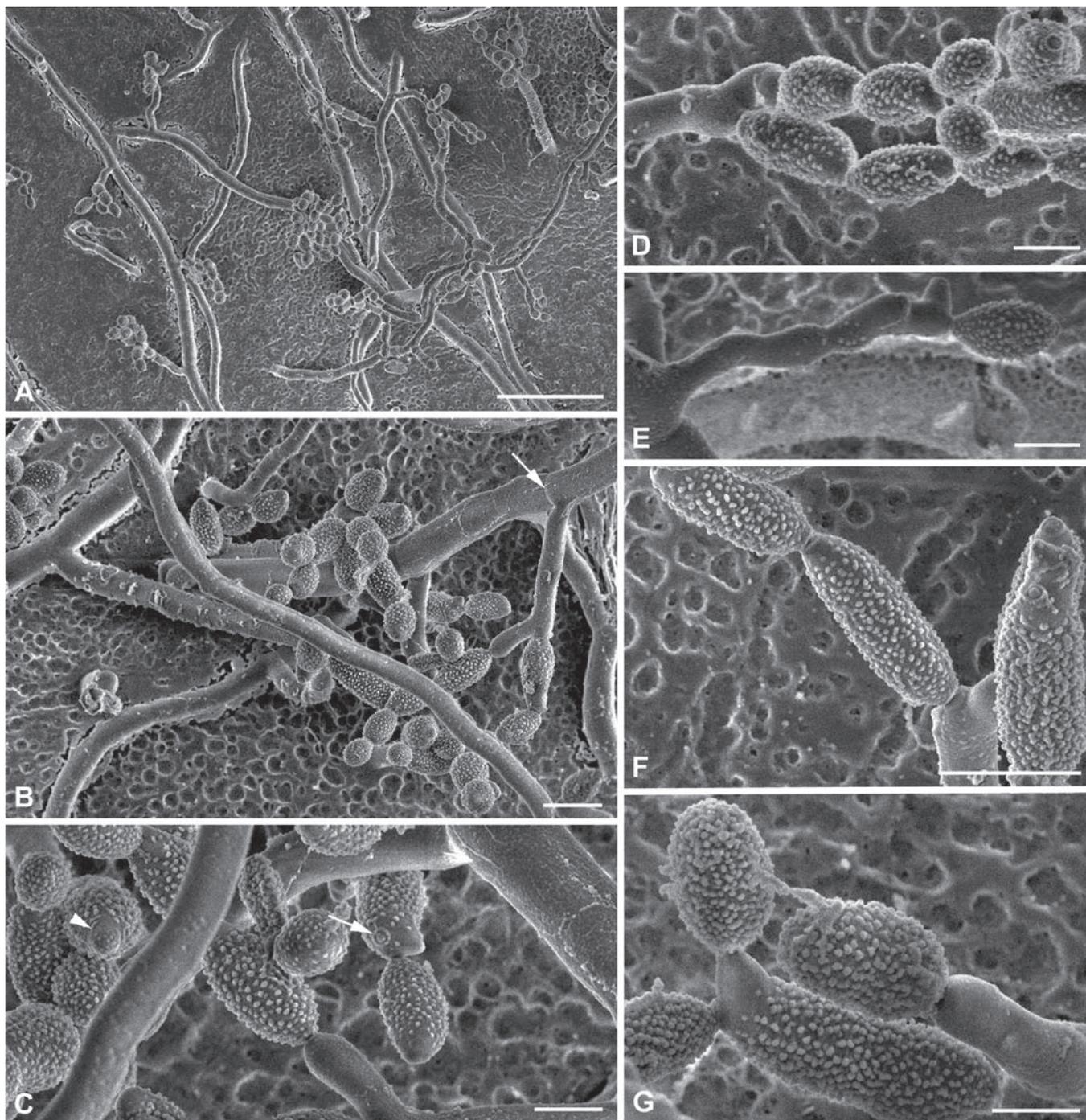
**Fig. 18.** *Cladosporium herbarum* (CPC 11600) and its teleomorph *Davidiella tassiana* (from the host and CPC 12181). A–B. Macronematous conidiophores. C. Micronematous conidiophore. D. Microcyclic conidiogenesis. E. Conidial chain. F. Ascomata on the leaf. G. Ascomata formed in culture on nettle stems. H–I. Asci on the host. J–K. Ascospores in culture. L. Asci in culture. Scale bars: A, E, H, J–L = 10  $\mu$ m, F–G, I = 200  $\mu$ m.

**Basionym:** *Didymellina macrospora* Kleb., Ber. Deutsch. Bot. Ges. 42: 60, 1924. 1925.

$\equiv$  *Mycosphaerella macrospora* (Kleb.) Jørst., Meld. Stat. Plantepatol. Inst. 1: 20. 1945.

*Mycelium* branched, 2–8  $\mu$ m wide, septate, not constricted at the septa, hyaline to pale brown, smooth, walls slightly thickened, sometimes guttulate. *Conidiophores* very long, usually terminally arising from ascending hyphae, erect to subdecumbent, slightly to distinctly flexuous, geniculate-sinuous, usually several times, subnodulose due to geniculate, sympodial proliferation forming swollen lateral shoulders, unbranched, rarely branched, up to 720  $\mu$ m long, 6–11  $\mu$ m wide, swellings 8–11(–14)  $\mu$ m wide, pluriseptate, often very regularly septate, not constricted at the septa, pale to medium olivaceous-brown, somewhat paler towards the apex, smooth to minutely verruculose, walls only slightly thickened. *Conidiogenous cells* integrated, terminal as well as intercalary, cylindrical-oblong, 15–55  $\mu$ m long, proliferation percurrent to sympodial, usually with a single geniculation forming laterally swollen shoulders often below a septum, conidiogenous loci confined to swellings, usually one locus per swelling, rarely

two, protuberant, (2–)2.5–4  $\mu$ m diam, somewhat thickened and darkened-refractive. *Conidia* solitary, sometimes in short, unbranched chains, straight to curved, young conidia pyriform to subcylindrical, connection between conidiophore and conidium being rather broad, subhyaline to pale olivaceous-brown, walls slightly thickened, then enlarging and becoming more thick-walled, cylindrical-oblong, soleiform with age, both ends rounded, usually with a slightly to distinctly bulbous base, visible from a very early stage, but broadest part often towards the apex not at the base, (18–)30–75(–87)  $\times$  (7–)10–16(–18)  $\mu$ m [av.  $\pm$  SD, 53.3 ( $\pm$  17.8)  $\times$  12.6 ( $\pm$  2.2)  $\mu$ m], (0–)2–6(–7)-septate, usually not constricted at the septa, rarely slightly constricted, septa often becoming sinuous with age, pale to medium olivaceous-brown, sometimes darker, verrucose to echinulate, walls thickened, especially in older conidia, up to 1  $\mu$ m thick, hila protuberant, often stalk-like or conically prolonged, up to 2  $\mu$ m long, (2–)2.5–3.5(–4)  $\mu$ m diam, with age becoming more sessile, sometimes just visible as a thickened plate just below the outer wall layer, especially in distal scars of branched conidia, periclinal rim often distinctly visible, hila somewhat thickened and darkened-refractive; microcyclic conidiogenesis not observed.



**Fig. 19.** *Cladosporium herbarum* (CPC 11600). A. Overview of hyphal growth and conidiophore formation of a colony on SNA. Conidiophores are often formed on very wide (approx. 10 µm), septate hyphae that often grow near the agar surface. B. A more detailed view on colony organisation reveals the ornamented conidia. Note the septum near the conidiophore (arrow). C. Detail of spore ornamentation and hila on a secondary ramoconidium (arrow). Ornamentation is visible during early stages of spore formation (arrow). D. Structure of the conidiophore, illustrating the complex morphology of the spore-forming apparatus. In addition, secondary ramoconidia, conidia, and a hilum on the conidium are visible. E. Complex structure of the spore-forming apparatus. F. Details of secondary ramoconidia with complex scar-pattern on the right cell. G. Details of a secondary ramoconidium giving rise to conidia. Note the lack of ornamentation at the location of spore formation. Scale bars: A = 50 µm, B, F = 10 µm, C–E, G = 5 µm.

**Cultural characteristics:** Colonies on PDA reaching 19–23 mm diam after 14 d at 25 °C, pale greenish olivaceous, smoke-grey to olivaceous-grey due to abundant aerial mycelium, greenish olivaceous to olivaceous reverse, margin broad, regular, entire edge to slightly undulate, feathery, aerial mycelium abundantly formed, felty, fluffy, covering large parts of the colony, mainly in the central parts, high, growth low convex with a somewhat raised colony centre. Colonies on MEA reaching 9–23 mm diam after 14 d at 25 °C, pale olivaceous-grey to olivaceous-grey, olivaceous-grey reverse, felty, margin slightly undulate, white, somewhat raised, aerial mycelium abundant, loose, diffuse, high, growth low convex,

radially furrowed, slightly folded. Colonies on OA reaching 10–19 mm diam after 14 d at 25 °C, olivaceous, margin broad, undulate, white, aerial mycelium white, very high, loose, diffuse, hairy, growth flat, due to the mycelium low convex, without prominent exudates and sporulating on all media.

**Specimens examined:** Isolated from *Iris* sp. (*Iridaceae*), CBS 107.20. **France**, Cote d'Or, Jardin de Noidan, on leaves of *Iris germanica*, Jul. 1880, F. Fautrey, Roumeguère, Fungi Sel. Gall. Exs. No. 5689, PC, **lectotype** of *C. iridis*, selected by David, 1997; K, isolectotype. **Netherlands**, Boterenbrood, isolated from leaves of *Iris* sp., Aug. 1940, CBS-H 19859, **epitype designated here** of *C. iridis*, culture ex-epitype CBS 138.40.

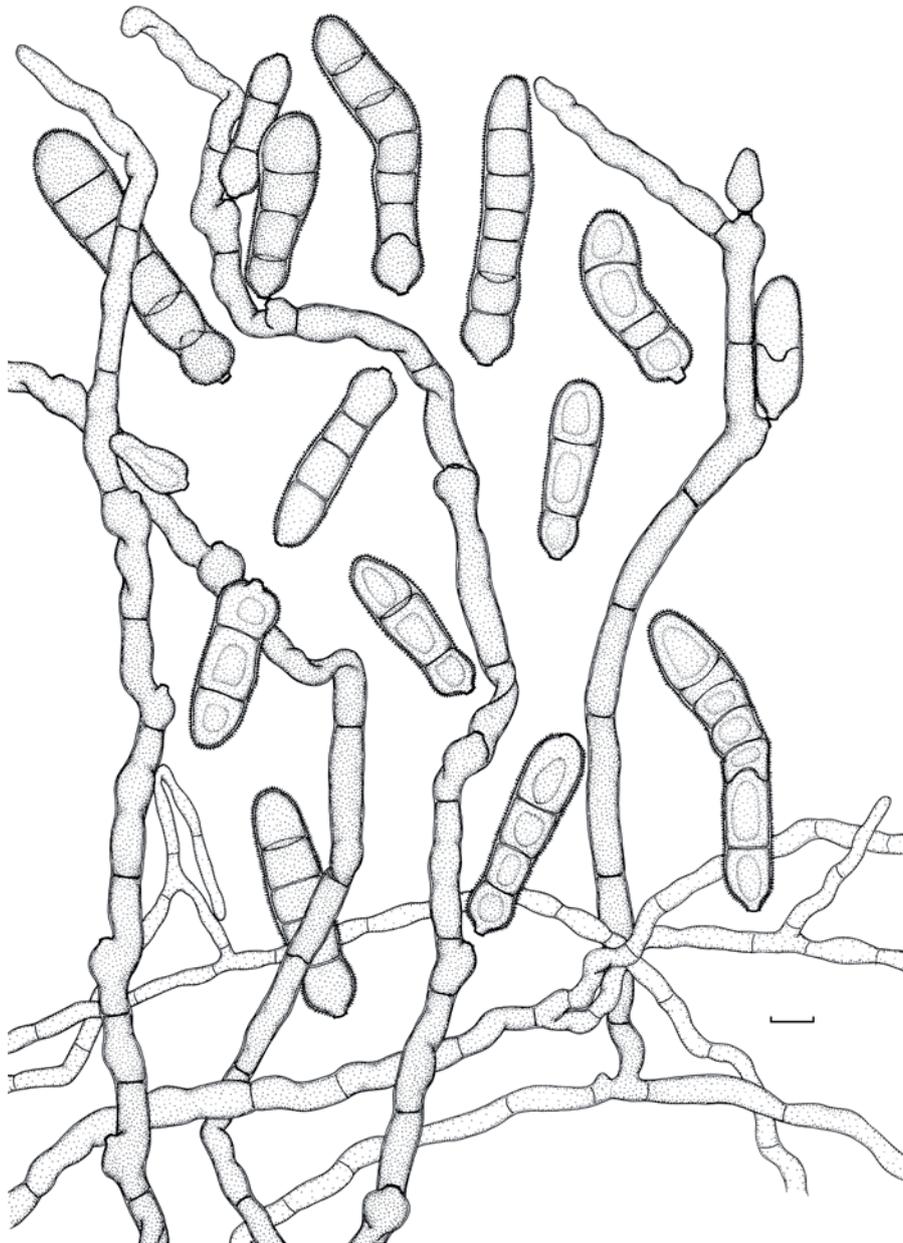


Fig. 20. *Cladosporium iridis* (CBS 138.40). Conidiophores and conidia. Scale bar = 10  $\mu$ m. K. Schubert *del.*

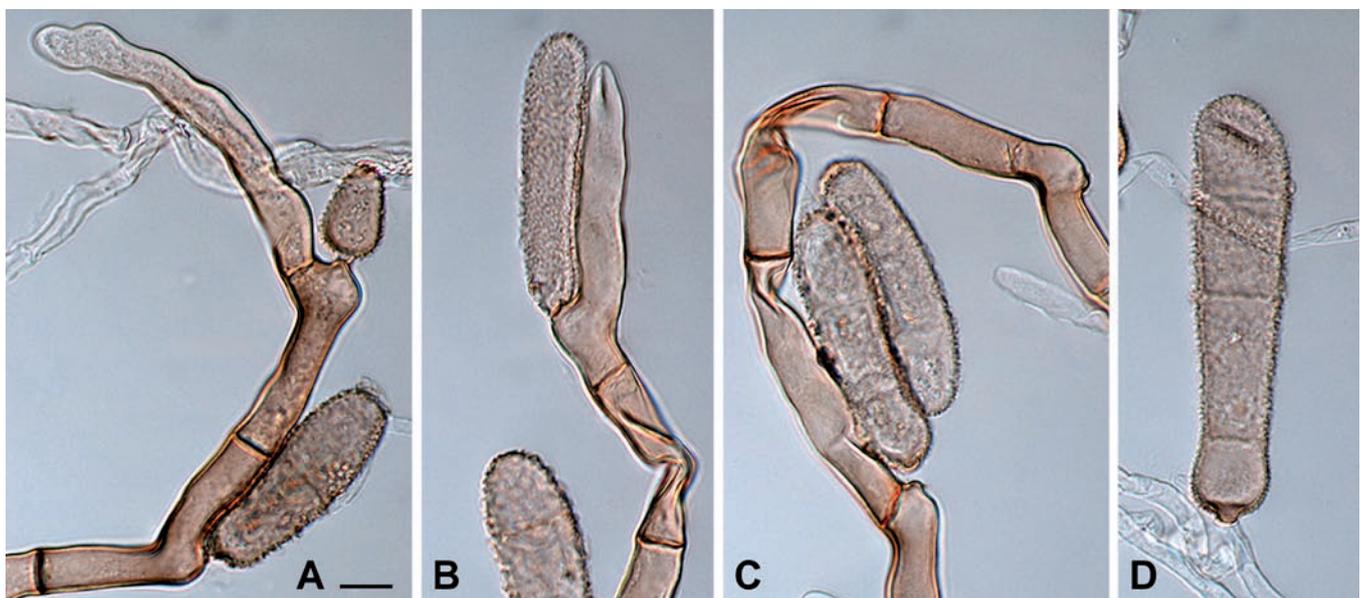


Fig. 21. *Cladosporium iridis* (teleomorph *Davidiella macrospora*) (CBS 138.40). A–C. Conidiophores with conidia. D. Conidium. Scale bar = 10  $\mu$ m.

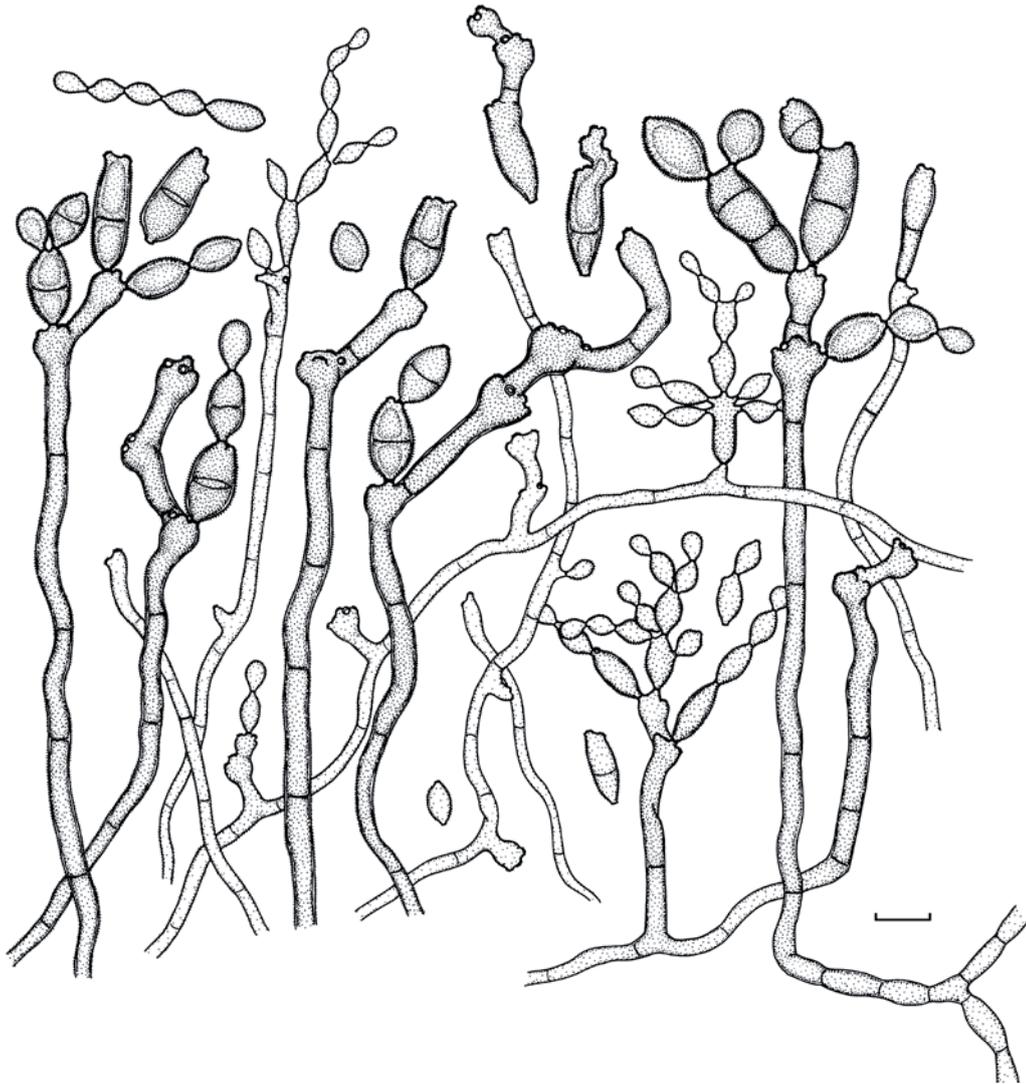


Fig. 22. *Cladosporium macrocarpum* (CBS 299.67). Macro- and micronematous conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert del.

**Substrates and distribution:** Leaf spot and blotch of *Iris* spp. including *I. crocea*, *I. florentina*, *I. foetidissima*, *I. germanica*, *I. gueldenstaediana*, *I. kamaonensis*, *I. pallida*, *I. plicata* (= *I. swertii* Hort.), *I. pseudacorus*, *I. pumila*, *I. spuria* ssp. *halophila*, and other species, also on *Belacamanda chinensis* (= *Gemmingia chinensis*), *Hemerocallis fulva*, *Gladiolus gandavensis*; Africa (Algeria, Morocco, South Africa, Zambia, Zimbabwe), Asia (Armenia, Azerbaijan, China, Georgia, India, Iran, Israel, Japan, Kazakhstan, Kirgizstan, Korea, Russia, Turkey, Turkmenistan, Uzbekistan), Australasia (Australia, New Zealand), Europe (Austria, Belgium, Belorussia, Cyprus, Czech Republic, Denmark, Estonia, France, Germany, Great Britain, Greece, Italy, Latvia, Lithuania, Malta, Moldavia, Montenegro, Netherlands, Norway, Poland, Romania, Russia, Serbia, Spain, Sweden, Ukraine), North America (Canada, U.S.A.), Central & South America (Argentina, Chile, Jamaica, Panama, Uruguay).

**Literature:** Ellis (1971: 312), Ellis & Waller (1974), Sivanesan (1984: 222), McKemy & Morgan-Jones (1990), David (1997: 43), Shin *et al.* (1999).

**Notes:** The description of the morphological parameters in culture is based on the isolate sporulating on PDA, since sporulation on SNA was not observed. The conidiophores and conidia *in vivo* are usually wider than in culture [conidiophores (6–)9–15(–17)  $\mu\text{m}$  wide, conidia (11–)15–23(–28)  $\mu\text{m}$ ].

***Cladosporium macrocarpum*** Preuss, in Sturm, Deutsch. Fl. 3(26): 27. 1848. Figs 22–25.

= *Cladosporium herbarum* var. *macrocarpum* (Preuss) M.H.M. Ho & Dugan, in Ho *et al.*, Mycotaxon 72: 131. 1999.

= *Dematium herbarum* var. ( $\beta$ ) *brassicae* Pers., Syn. meth. fung. 2: 699. 1801, **syn. nov.**

= *Dematium graminum* Pers., Mycol. eur. 1: 16. 1822, **syn. nov.**

= *Dematium vulgare* var. ( $\delta$ ) *typharum* Pers., Mycol. eur. 1: 14. 1822, **syn. nov.**

= *Dematium vulgare* var. ( $\beta$ ) *foliorum* Pers., Mycol. eur. 1: 14. 1822, **syn. nov.**

For additional synonyms see Dugan *et al.* (2004), Schubert (2005).

**Teleomorph:** *Davidiella macrocarpa* Crous, K. Schub. & U. Braun, **sp. nov.** MycoBank MB504582.

*Davidiellae tassianae* similis, sed pseudoparaphysibus prominentibus et ascosporis maioribus, (22–)23–26(–28)  $\times$  (6–)6.5–7(–8)  $\mu\text{m}$ .

**Ascomata** superficial on a small stroma, black, up to 200  $\mu\text{m}$  diam, globose, separate, but developing with 1–3 necks with age; ostioles consisting of pale brown to subhyaline cells, periphysate, with periphysoids growing into the cavity; wall consisting of 3–6 layers of medium brown *textura angularis*. *Pseudoparaphyses* present, hyaline, subcylindrical, septate, anastomosing, 3–4  $\mu\text{m}$  diam; hamathelial cells persistent in cavity. *Asci* fasciculate, bitunicate, subsessile, broadly ellipsoid with a long tapered stalk, straight to curved, 8-spored, 70–110  $\times$  15–20  $\mu\text{m}$ . *Ascospores* tri- to multiseriate, overlapping, hyaline, guttulate, irregular lumina

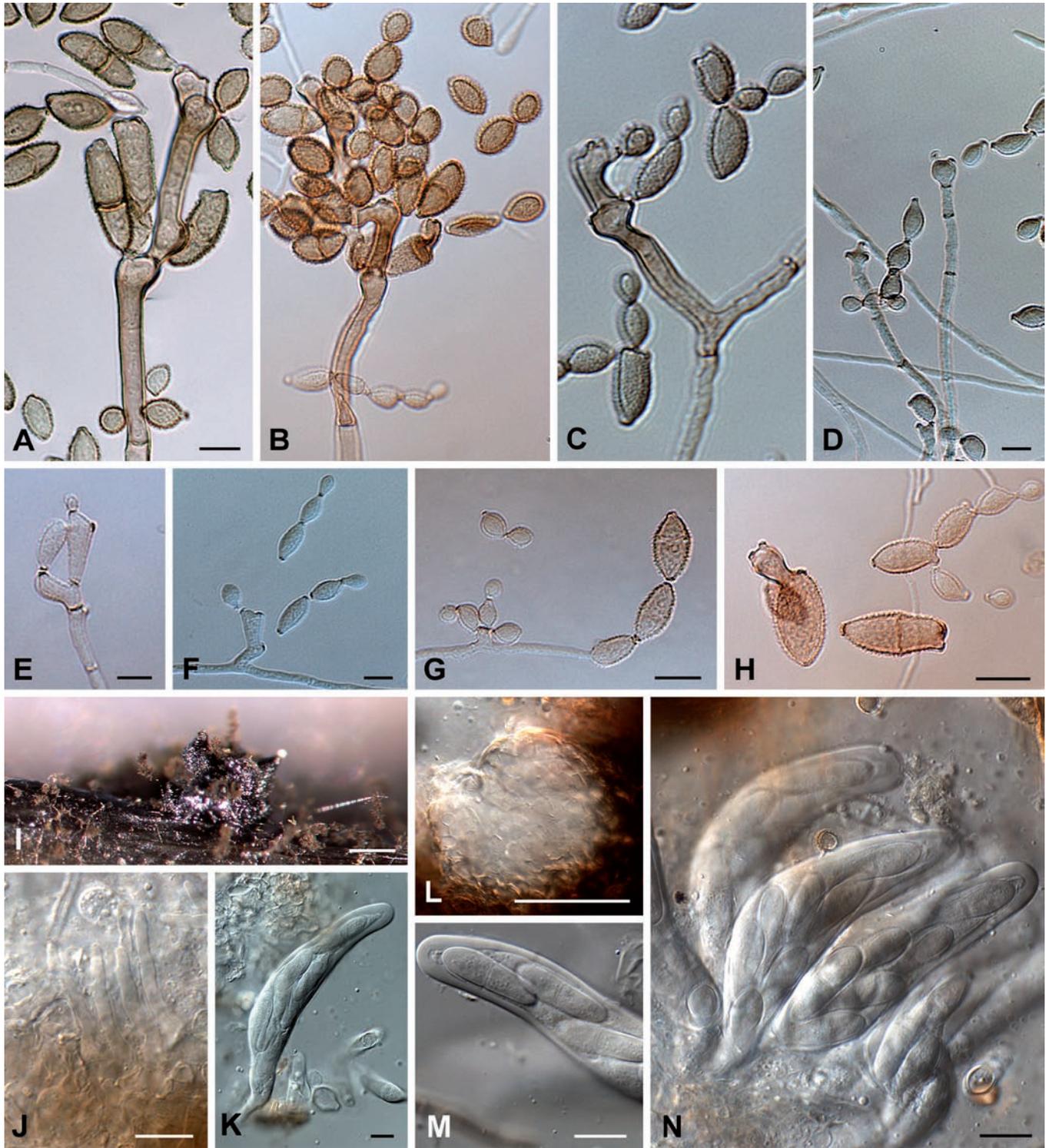
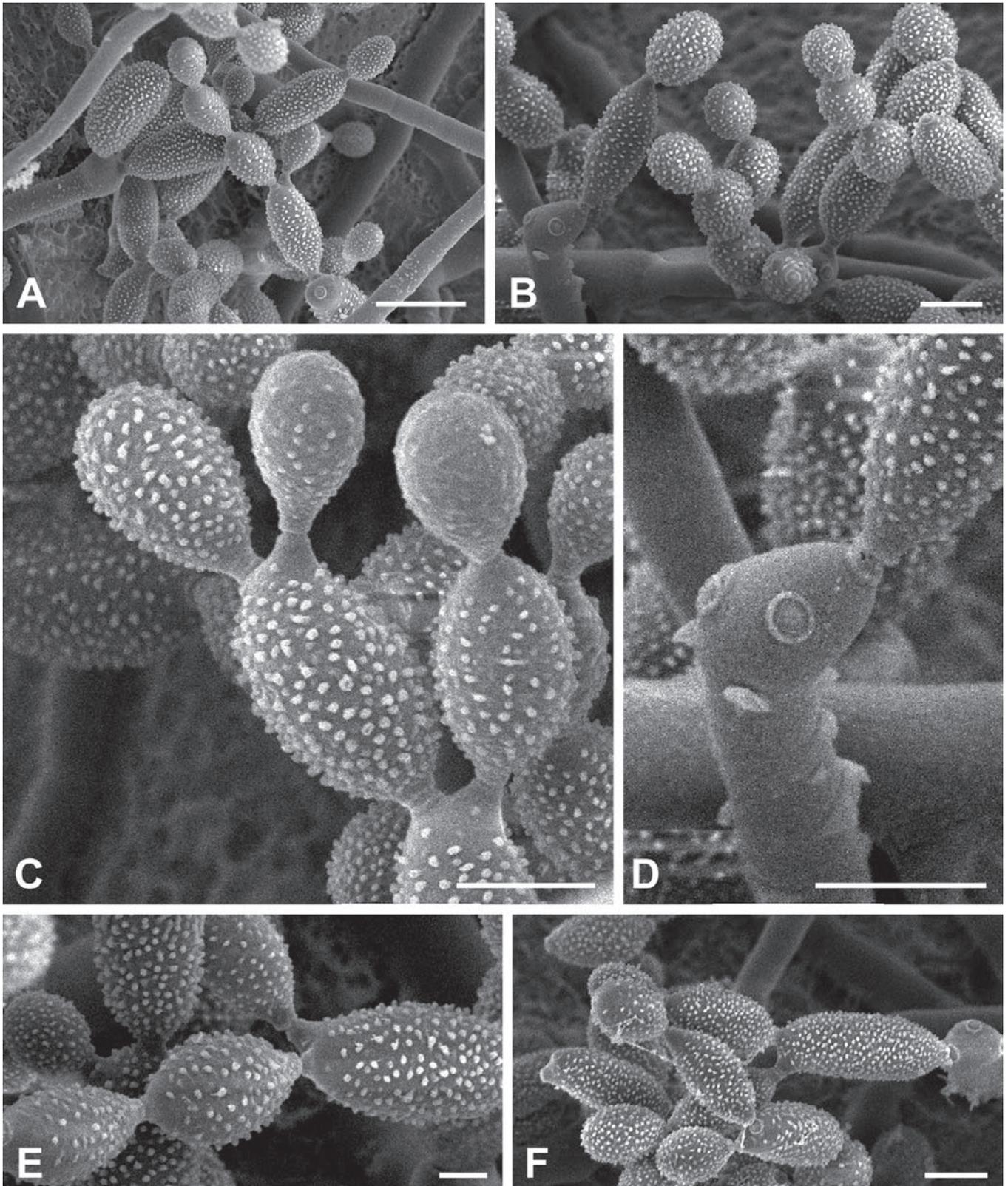


Fig. 23. *Cladosporium macrocarpum* (CBS 299.67) and its teleomorph *Davidiella macrocarpa* (CPC 12755). A–C. Macronematous conidiophores and conidia. D–G. Micronematous conidiophores. H. Microcyclic conidiogenesis. I. Ascomata formed on nettle stems in culture. J. Periphyses. K, M–N. Asci. L. Ostiole. Scale bars: A, D–H, J–N = 10  $\mu$ m, I = 200  $\mu$ m.

rarely observed, thick-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, widest in the middle of the apical cell, medianly 1-septate, not to slightly constricted at the septum, tapering towards both ends, but more prominently towards lower end, (22–)23–26(–28)  $\times$  (6–)6.5–7(–8)  $\mu$ m; mucoid sheath rarely observed, mostly absent.

*Mycelium* unbranched or loosely branched, 1–4.5(–5)  $\mu$ m wide, septate, sometimes slightly constricted at septa, hyaline to pale brown, smooth to minutely verruculose, walls unthickened or slightly thickened. *Conidiophores* micronematous and macronematous,

solitary, arising terminally from plagiotropous hyphae or terminally from ascending hyphae. *Macronematous conidiophores* erect, straight to somewhat flexuous, cylindrical-oblong, nodulose to nodose, with a single apical or usually several swellings either somewhat distinct from each other or often in short succession giving conidiophores a knotty appearance, swellings sometimes laterally elongated or formed at the top of a branch-like outgrowth below the apical swelling, sometimes distinctly geniculate, unbranched, sometimes branched, 12–260  $\times$  (3–)4–6  $\mu$ m, swellings 5–10  $\mu$ m wide, pluriseptate, sometimes slightly constricted at septa, pale to medium brown or olivaceous-brown, somewhat paler at apices,



**Fig. 24.** *Cladosporium macrocarpum* (CBS 299.67). A. Survey of a conidiophore that forms several secondary ramoconidia and conidia. Several aerial hyphae are also visible in this picture. B. Conidiophore with broadly ellipsoid secondary ramoconidia and obovoid conidia. Note the different scars on the conidiophore at the lower left. C. Ellipsoid or obovoid conidia with notable areas of scar formation. The ornamentation is relatively widely distributed over the body of the cell and similar to *C. variabile*. D. Detail of a conidiophore (see B) with scars. Note the relatively shallow rings of the scars. E. Details of conidia and a secondary ramoconidium. F. Conidiophore with a secondary ramoconidium and conidia. Note the hila on several spores and the lack of ornamentation at the site where spores are formed. Scale bars: A–C, = 10  $\mu\text{m}$ , D, F = 5  $\mu\text{m}$ , E = 2  $\mu\text{m}$ .

smooth to minutely verruculose or verruculose, walls somewhat thickened, sometimes even two-layered. *Conidiogenous cells* integrated, terminal or intercalary, cylindrical, nodulose with lateral shoulders or nodose with swellings round about the stalk, with conidiogenous loci confined to swellings, 12–37  $\mu\text{m}$  long, with up to

12 loci per cell, usually with up to six, loci conspicuous, protuberant, (1–)1.5–2  $\mu\text{m}$  diam, somewhat thickened and darkened-refractive. *Micronematous conidiophores* almost indistinguishable from hyphae, straight, narrowly filiform, non-nodulose or with a single or few swellings, mostly with small head-like swollen apices, usually

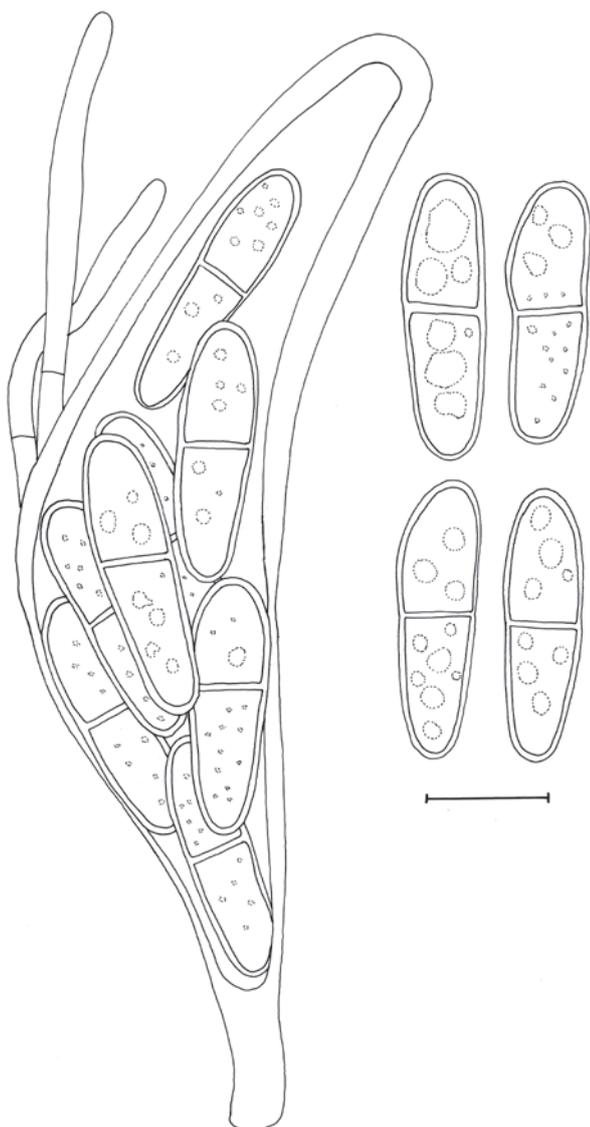


Fig. 25. *Davidiella macrocarpa* (CPC 12755). Ascus and ascospores. Scale bar = 10  $\mu\text{m}$ . P.W. Crous del.

only few micrometer long, 1.5–3  $\mu\text{m}$  wide, aseptate or with only few septa, subhyaline, smooth or almost so, walls unthickened, with a single or only few conidiogenous loci, narrow, 0.8–1.2  $\mu\text{m}$  diam, thickened and somewhat darkened-refractive. *Conidia* catenate, in branched chains, small terminal conidia subglobose, obovoid, oval, limoniform, 4–11  $\times$  (3–)4–6  $\mu\text{m}$  [av.  $\pm$  SD, 7.6 ( $\pm$  1.9)  $\times$  5.0 ( $\pm$  0.8)  $\mu\text{m}$ ], aseptate, intercalary conidia broadly ovoid-ellipsoid, 10–17  $\times$  (4.5–)5–9  $\mu\text{m}$  [av.  $\pm$  SD, 12.7 ( $\pm$  2.1)  $\times$  6.8 ( $\pm$  0.8)  $\mu\text{m}$ ], 0–1-septate; secondary ramoconidia broadly ellipsoid to subcylindrical, 14–25(–30)  $\times$  (5–)6–9(–10)  $\mu\text{m}$  [av.  $\pm$  SD, 19.4 ( $\pm$  3.5)  $\times$  7.6 ( $\pm$  1.0)  $\mu\text{m}$ ], 0–2(–3)-septate, sometimes slightly constricted at the septa, septa somewhat sinuous with age, pale brown to medium olivaceous-brown or brown, sometimes even dark brown, verruculose to echinulate (muricate under SEM), walls thickened, up to 1  $\mu\text{m}$  thick, mostly broadly rounded at apex and base, sometimes attenuated, sometimes guttulate by oil drops, with up to three apical hila, mostly 1–2, hila sessile (apparently somewhat immersed) to somewhat protuberant, 1–2(–2.5)  $\mu\text{m}$  diam, thickened and darkened-refractive; microcyclic conidiogenesis occurring with conidia forming secondary micro- and macronematous conidiophores, conidia often germinating with long hyphae. *Conidia* formed by micronematous conidiophores usually smaller, narrower and paler, catenate, in short unbranched

or branched chains, subglobose, obovoid to limoniform, ellipsoid or fusiform, 2.5–16  $\times$  1.5–5  $\mu\text{m}$ , 0(–1)-septate, few longer conidia subcylindrical to clavate, up to 37(–43)  $\mu\text{m}$  long, 0–2(–3)-septate, occasionally with up to four septa, sometimes slightly constricted at the septa, subhyaline to pale brown, almost smooth to minutely verruculose, walls unthickened, hila 0.8–1.2  $\mu\text{m}$  diam, thickened and darkened-refractive.

**Cultural characteristics:** Colonies on PDA reaching 30–43 mm in diam after 14 d at 25  $^{\circ}\text{C}$ , dark dull green to olivaceous-grey, olivaceous-grey, dark olivaceous- to iron-grey reverse, pulvinate, velvety, sometimes somewhat zonate, paler zones towards the margin, margin regular, entire edge, almost colourless to white, glabrous to feathery, aerial mycelium sparse to more abundant in the colony centre or covering large areas of the colony, hairy, fluffy or felty, whitish to smoke-grey, sometimes becoming reddish, livid red to vinaceous, growth flat, regular, sometimes forming few prominent exudates, exudates sometimes slightly reddish, sporulation profuse with two kinds of conidiophores, low and high. Colonies on MEA reaching 31–50 mm in diam after 14 d at 25  $^{\circ}\text{C}$ , grey-olivaceous to olivaceous-grey or iron-grey, sometimes pale olivaceous-grey to whitish due to abundant aerial mycelium, olivaceous-grey or iron-grey reverse, velvety or powdery, margin narrow, entire edge, colourless to white, glabrous, aerial mycelium sparse to abundant, hairy or felty, growth regular, flat to low convex, radially furrowed, without prominent exudates, sporulation profuse. Colonies on OA reaching 29–40 mm in diam after 14 d at 25  $^{\circ}\text{C}$ , grey-olivaceous, olivaceous-grey to dark smoke-grey, olivaceous-black or iron grey reverse, margin entire edge, narrow, colourless or white, glabrous, aerial mycelium sparse, mainly in the colony centre, felty, white to smoke-grey or grey-olivaceous, felty, growth flat, regular, without exudates, sporulating.

**Specimens examined:** *Sine loco et dato*, L 910.255-723 = L-0115836, **lectotype designated here** of *Dematium graminum*. *Sine loco*, on dead stems of *Brassica* sp. (*Brassicaceae*), No. 601, L 910.255-716 = L-0115849, **holotype** of *D. herbarum* var. ( $\beta$ ) *brassicae*. *Sine loco*, on leaves of *Iris* (*Iridaceae*), *Quercus* (*Fagaceae*), *Brassica* etc., L 910.255-736 = L-0115871, **holotype** of *D. vulgare* var. ( $\beta$ ) *foliorum*, isotype L 910.255-718 = L-0115872. *Sine loco et dato*, L 910.255-698 = L-0115852, **lectotype designated here** for *D. vulgare* var. ( $\delta$ ) *typharum*. Isolated from "*Mycosphaerella tulasnei*", CBS 223.32 = ATCC 11287 = IMI 049635. **Romania**, isolated from water, CBS 175.82. **Slovenia**, Sečovelje, isolated from hypersaline water from saltens (precristallisation pond), 2004, P. Zalar, EXF-2287 = CPC 12054. **Turkey**, Ankara, Tekeli, isolated from *Triticum aestivum* (*Poaceae*), isol. S. Tahsin, ident. A.C. Stolk, CBS 299.67. **U.S.A.**, Seattle, University of Washington Campus, 47.6263530, -122.3331440, isolated from cleistothecia of *Phyllactinia guttata* (*Erysiphaceae*) on leaves of *Corylus* sp. (*Corylaceae*), 16 Sep. 2004, D. Glawe, CPC 11817; Washington, isolated from *Spinacia oleracea* (*Chenopodiaceae*), 1 Jan. 2003, L. DuToit, CBS-H 19855, **neotype designated here** for *C. macrocarpum*, and **holotype** of *D. macrocarpa*, isoneotype HAL 2020 F, isotype HAL 2021 F, culture ex-type CPC 12752, 12756–12759, CPC 12755 = CBS 121623.

**Substrate and distribution:** Decaying plant material, human, hypersaline water, water; widespread.

**Literature:** de Vries (1952: 76), Ellis (1971: 315), Domsch *et al.* (1980: 208), Ellis & Ellis (1985: 290, 468), Matsushima (1985: 5), McKemy & Morgan-Jones (1991), Dugan & Roberts (1994), David (1997: 71), Samson *et al.* (2000: 112).

**Notes:** In the absence of Preuss's type material (not preserved) de Vries (1952) "lectotypified" *C. macrocarpum* by a specimen in Saccardo's herbarum (Herb. Myc. P.A. Saccardo no. 419, PAD). This material, subsequently distributed in Mycotheca Italica no. 1396, should correctly be regarded as neotype (David 1997). A single collection of Saccardo's Mycotheca Italica no. 1396 from herb. HBG, which can be considered as isoneotype material,

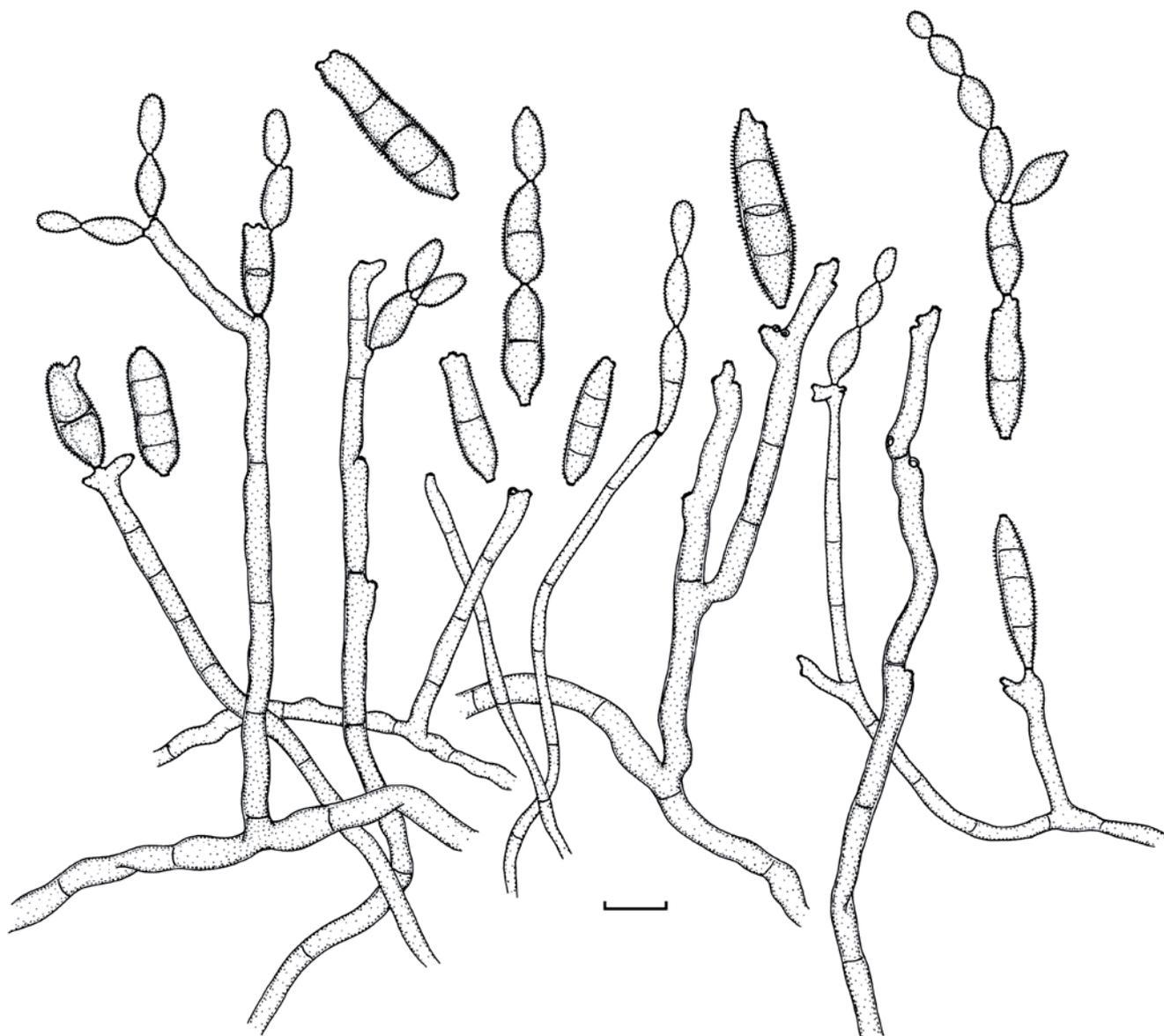


Fig. 26. *Cladosporium ossifragi* (CBS 842.91). Conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert del.

was re-examined and proved to rather agree with the species concept of *C. herbarum* s. str. The conidia were formed in simple, rarely branched chains, 6–26  $\times$  (4–)5.5–8(–9)  $\mu\text{m}$ , 0–3-septate, almost smooth or minutely to densely verruculose or verrucose (Schubert 2005). However, since de Vries' "lectotypification" was incorrect according to the code (ICBN, Art. 9.2, 9.17), a neotype is designated.

The delimitation of *C. macrocarpum* as a morphologically distinct species from *C. herbarum* has been controversially discussed by several authors (McKemy & Morgan-Jones 1991, Dugan & Robert 1994, Ho *et al.* 1999). Based on molecular as well morphological studies, it can be shown that *C. macrocarpum* is a well-defined species distinguishable from *C. herbarum* s. str. by forming conidiophores with wider nodes, 5–10  $\mu\text{m}$ , wider and more frequently septate conidia [small terminal conidia 4–11  $\times$  (3–)4–6  $\mu\text{m}$  versus 4–10  $\times$  3–5(–6)  $\mu\text{m}$  in *C. herbarum*, intercalary conidia 10–17  $\times$  (4.5–)5–9  $\mu\text{m}$  versus 6–16  $\times$  4–6  $\mu\text{m}$  in *C. herbarum*, secondary ramoconidia 14–25(–30)  $\times$  (5–)6–9(–10)  $\mu\text{m}$  versus 12–25(–35)  $\times$  (3–)5–7(–9)  $\mu\text{m}$  in *C. herbarum*] and by being connected to *Davidiella macrocarpa*. On natural substrates the conidiophores are usually somewhat wider than in culture, 4–8(–10)  $\mu\text{m}$  wide, and also the conidia can be somewhat wider, sometimes up to 13(–15)  $\mu\text{m}$ .

*Cladosporium graminum*, described by Persoon (1822), as well as *C. brunneum* and *C. gracile*, introduced by Corda (1837), are older synonyms of *C. macrocarpum* and, according to the code, would have priority. However, since *C. macrocarpum* is a well established, currently used name with numerous records in literature, a proposal to conserve the name against these older names is in preparation for formal publication in *Taxon*.

A characteristic difference between ascomata of *C. macrocarpum* in comparison to those of *C. herbarum*, are the smaller, globose pseudothecia, asci with longer stalks, prominence of pseudoparaphyses, and rather inconspicuous luminal ascospore inclusions.

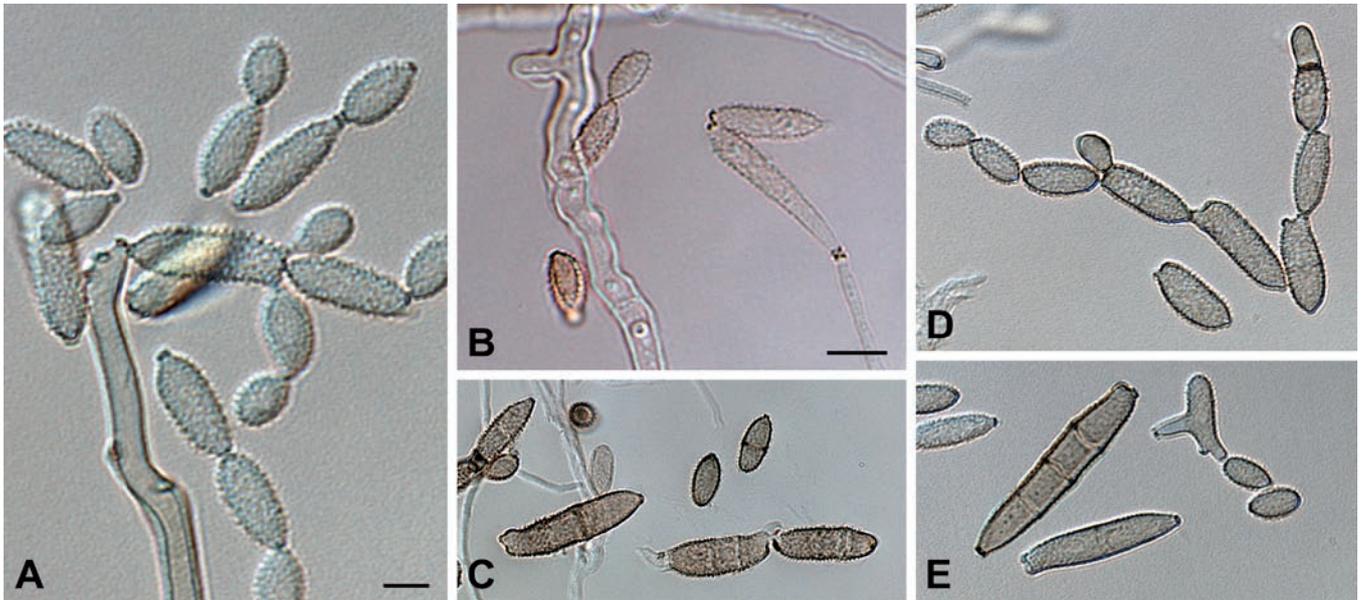
***Cladosporium ossifragi* (Rostr.) U. Braun & K. Schub., comb. nov.** MycoBank MB504575. Figs 26–28.

*Basionym:* *Napicladium ossifragi* Rostr., Bot. Færøes 1: 316. 1901.

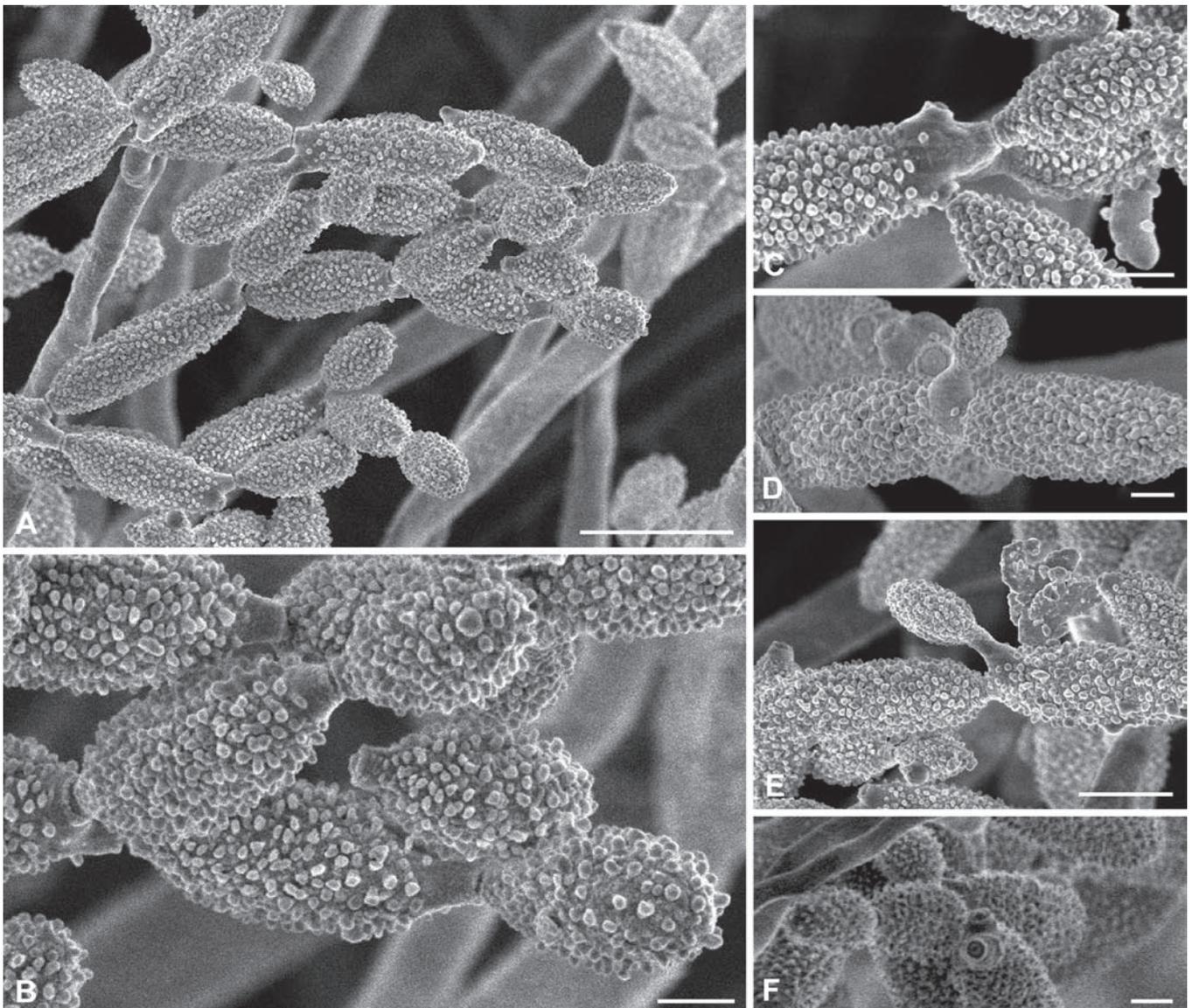
$\equiv$  *Heterosporium ossifragi* (Rostr.) Lind, Dan. fung.: 531. 1913.

$\equiv$  *Heterosporium magnusianum* Jaap, Schriften Naturwiss. Vereins Schleswig-Holstein 12: 346. 1902.

$\equiv$  *Cladosporium magnusianum* (Jaap) M.B. Ellis in Ellis, More Dematiaceous Hyphomycetes: 337. 1976.



**Fig. 27.** *Cladosporium ossifragi* (CBS 842.91). A. Macronematous conidiophore. B. Micronematous conidiophore. C–D. Conidia. E. Conidia and microcyclic conidiogenesis. Scale bars = 10  $\mu$ m.



**Fig. 28.** *Cladosporium ossifragi* (CBS 842.91). A. Survey on different secondary ramoconidia and conidia. B. Details of conidia and hilae. Note the very pronounced ornamentation and the absence of ornamentation near the site of spore formation. C. Detail of the end of a secondary ramoconidium with pronounced hilae. D. Formation of a new conidium. Note the broad scar behind it (> 1  $\mu$ m). E. Formation of a new conidium from a smooth-walled stalk. F. Hilae on a secondary ramoconidium. This micrograph is from the sample before coating with gold-palladium and shows similar features as the sample after sputter coating. Scale bars: A = 10  $\mu$ m, B–D, F = 2  $\mu$ m, E = 5  $\mu$ m.

*Mycelium* abundantly formed, twisted, often somewhat aggregated, forming ropes, branched, 1–5 µm wide, septate, often irregularly swollen and constricted, hyaline or subhyaline to pale brown, smooth, walls unthickened or only slightly thickened. *Conidiophores* macronematous and micronematous, arising from plagiotropous hyphae, terminally or laterally, erect to subdecumbent, more or less straight to flexuous, cylindrical, sometimes geniculate, subnodulose with loci often situated on small lateral shoulders, unbranched, sometimes branched, often very long, up to 350 µm long, 3–4.5(–5) µm wide, pluriseptate, shorter ones aseptate, not constricted at septa, pale to pale medium brown, paler towards apices, sometimes subhyaline, smooth to minutely verruculose, especially towards apices, walls somewhat thickened, up to 0.5 µm, sometimes appearing two-layered. *Conidiogenous cells* integrated, terminal as well as intercalary, cylindrical, sometimes geniculate, subnodulose, 5–31 µm long, proliferation sympodial, with few loci (1–3) per cell, loci usually confined to small lateral shoulders, protuberant, conspicuous, short cylindrical, 1–2 µm wide, up to 1 µm high, somewhat thickened, darkened-refractive. *Conidia* catenate, in short, unbranched or branched chains, straight, small terminal and intercalary conidia subglobose, obovoid to ellipsoid, 4–15 × 3–5 µm [av. ± SD, 9.3 (± 3.7) × 4.0 (± 0.7) µm], 0–1-septate, not constricted at the septa, pale brown, hila 0.8–1 µm diam, secondary ramoconidia cylindrical, sometimes ellipsoid or subfusiform, 16–36(–40) × (4–)5–8 µm [av. ± SD, 26.6 (± 7.4) × 6.0 (± 1.2) µm], (0–)1–3(–4)-septate [*in vivo* wider, (6–)7–9(–11) µm, and with up to five, rarely seven septa], not constricted at the septa, septa sometimes slightly sinuous, pale brown to pale medium brown, densely verruculose, verrucose to echinulate (densely muricate under SEM), walls unthickened to somewhat thickened, rounded or somewhat attenuated at apex and base, hila protuberant, conspicuous, sometimes situated on short, small prolongations, 1–2.5 µm diam, somewhat thickened and darkened-refractive; microcyclic conidiogenesis occasionally occurring.

**Cultural characteristics:** Colonies on PDA reaching 53 mm diam after 14 d at 25 °C, greenish olivaceous, grey-olivaceous to olivaceous-grey or iron-grey, appearing somewhat zonate, dull green to olivaceous-black reverse, margin colourless, regular, entire edge, aerial mycelium abundantly formed, covering at first the colony centre later most of the surface, dense, high, growth flat with elevated colony centre, somewhat folded. Colonies on MEA reaching 54 mm diam after 14 d at 25 °C, pale olivaceous-grey to olivaceous-grey in the centre, iron-grey reverse, velvety, margin colourless to white, entire edge, radially furrowed, aerial mycelium abundantly formed, fluffy to felty, growth flat with somewhat raised, folded colony centre. Colonies on OA attaining 52 mm diam after 14 d at 25 °C, olivaceous-grey to iron-grey, iron-grey to greenish black reverse, margin white, entire edge, aerial mycelium diffuse, loose, growth flat, prominent exudates absent, sporulation profuse on all media.

**Specimens examined:** **Denmark**, Undallslund, on leaves of *Nartheceum ossifragum* (*Melanthiaceae*), 13 Sep. 1885, E. Rostrup, CP, **neotype designated here** of *C. ossifragi*; Tønder, Rómó near Twismark, 19 Aug. 1911, H. Sydow, Sydow, Mycoth. Germ. 1047, M. **Germany**, Hamburg, Eppendorfer Moor, on leaves of *Nartheceum ossifragum*, 12 Sep. 1897, O. Jaap, HBG, **lectotype selected here** of *C. magnusianum*; 4 Sep. 1903, O. Jaap, Jaap, Fungi Sel. Exs. 49, M; Wernerwald near Cuxhaven, Aug. 1927, A. Ludwig, Petrak, Mycoth. Gen. 146, M. **Norway**, Bjerkreim County, isolated from leaves of *Nartheceum ossifragum*, M. di Menna, CBS-H 19860, **epitype designated here** of *C. ossifragi*, culture ex-epitype CBS 842.91 = ATCC 200946; Møre og Romsdal County, isolated from leaves of *Nartheceum ossifragum*, M. di Menna, CBS 843.91.

**Substrate and distribution:** Causing leaf spots on *Nartheceum ossifragum*; Europe (Austria, Denmark, Germany, Great Britain, Ireland, Norway).

**Literature:** Ellis & Ellis (1985: 390), David (1995a; 1997: 85–86, 88), Ho *et al.* (1999: 132).

**Notes:** Type material of *Napicladium ossifragi* is not preserved in Rostrup's herbarium (on *Nartheceum ossifragum*, Faeroe Islands, Viderö, Viderejde and Österö, Svinaa, *sine dato*, leg. Ostenfeld & Harz). However, other authentic collections seen and examined by Rostrup are deposited at CP. Lind (1913) re-examined these samples, synonymised *N. ossifragi* with *H. magnusianum* and correctly introduced the combination *H. ossifragi*. Nevertheless, the correct oldest name for this fungus has been ignored by most authors. David (1997), who clearly stated that *N. ossifragi* is the earliest name for this species, preferred to use the name *C. magnusianum* because the typification of Rostrup's name was still uncertain. Despite the lacking type material, there is no doubt about the correct identity of *N. ossifragi* since authentic material of this species, examined by and deposited in Rostrup's herbarium (CP), is preserved. Therefore, there is no reason to reject the oldest valid name for this species. The original collection of *C. magnusianum* cited by Jaap (1902) (on leaves of *Nartheceum ossifragum*, Denmark, Tønder, Rómó, peatbog by Twismark, Jul.–Aug. 1901, Jaap), but not designated as type, is not preserved (David 1997). It is neither deposited at B, HBG nor S. However, in the protologue Jaap (1902) also referred to material of this species found near Hamburg, which is, hence, syntype material available for lectotypification.

***Cladosporium pseudiridis*** K. Schub., C.F. Hill, Crous & U. Braun, **sp. nov.** MycoBank MB504576. Figs 29–30.

**Etymology:** Epithet derived from its similar morphology to *Cladosporium iridis*.

Differt a *Cladosporio iridis* conidiis 0–3-septatis, brevioribus et latoribus, 15–55 × (9–)11–19(–21) µm.

*Mycelium* sparingly branched, 2–7 µm wide, septate, not constricted at the septa, subhyaline to pale brown, smooth or almost so, walls somewhat thickened, guttulate or protoplasm appearing granular, sometimes enveloped by a slime coat. *Conidiophores* arising mostly terminally from ascending hyphae, sometimes also laterally from plagiotropous hyphae, erect, more or less straight, broadly cylindrical-oblong, once or several times slightly to distinctly geniculate-sinuous, forming more or less pronounced lateral shoulders, nodulose, unbranched, 100–320(–500) × 7–11 µm, swellings 10–14 µm wide, becoming narrower and paler towards the apex, septate, not constricted at the septa, septa mainly basal, apical cell often very long, pale to medium olivaceous-brown, subhyaline at the apex, smooth or almost so, sometimes minutely verruculose, walls usually distinctly thickened, sometimes even two-layered, up to 1(–2) µm thick, protoplasm granular, often clearly contrasting from the outer wall. *Conidiogenous cells* integrated, terminal and intercalary, cylindrical-oblong, slightly to distinctly geniculate-sinuous, nodulose with conidiogenous loci confined to swellings or lateral shoulders, 30–110 µm long, proliferation percurrent to sympodial, with a single or three, sometimes up to five geniculations per cell, usually only a single locus per swelling, protuberant, very prominent, short cylindrical, peg-like, clearly composed of a dome and surrounding rim, dome often higher than the periclinal rim, broad, somewhat paler than rim, conically narrowed, (2–)2.5–4 µm wide, up to 2 µm high, thickened and darkened-refractive.

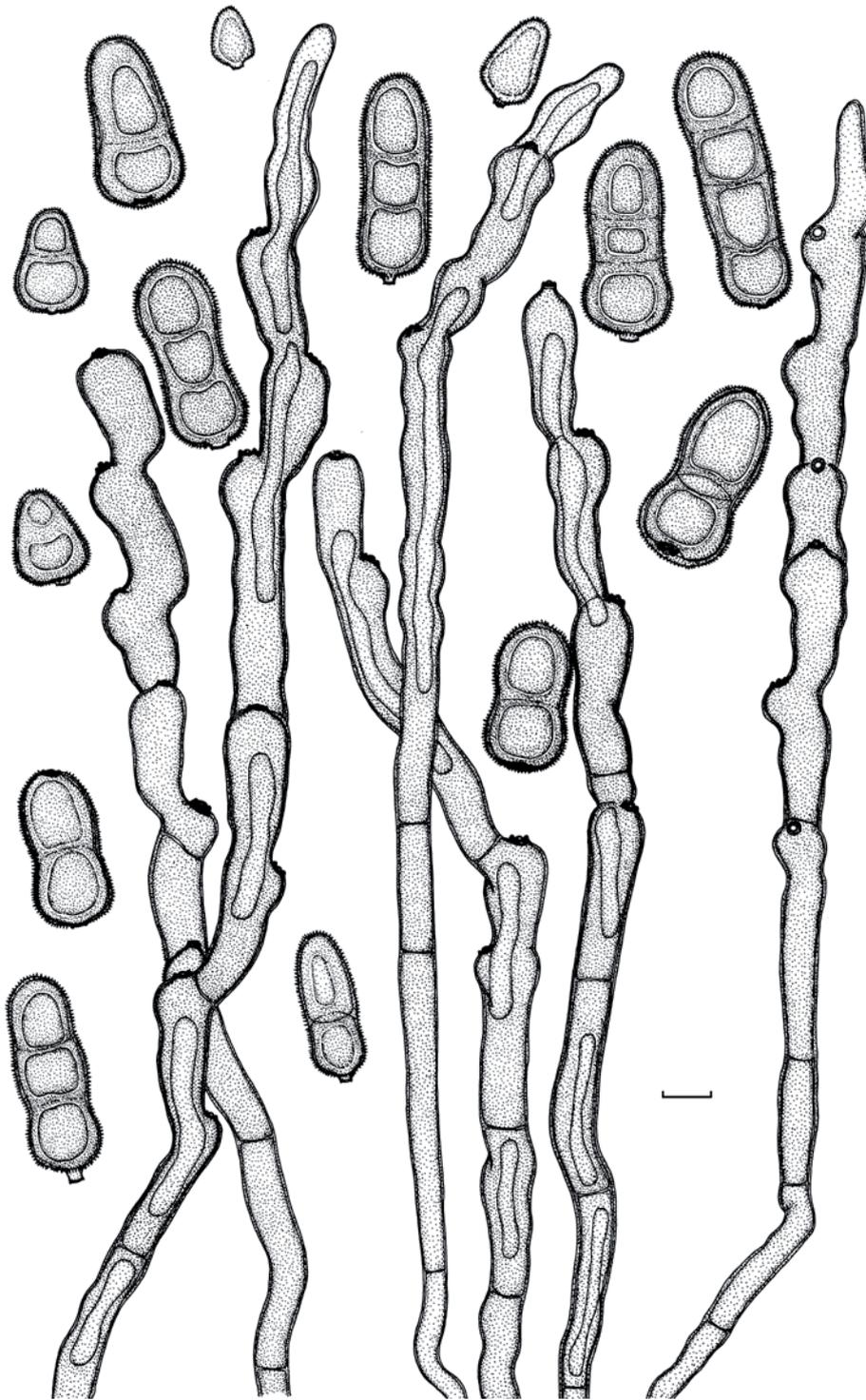


Fig. 29. *Cladosporium pseudiridis* (CBS 116463). Conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert del.

*Conidia* solitary, sometimes in short unbranched chains of two or three, straight to slightly curved, young conidia small, 0–1-septate, broadly ovoid to pyriform, 15–26  $\times$  (9–)11–16(–18)  $\mu\text{m}$  [av.  $\pm$  SD, 19.2 ( $\pm$  4.3)  $\times$  14.2 ( $\pm$  3)  $\mu\text{m}$ ], first septum somewhat in the upper half, the upper cell is much smaller but gradually extending as the conidium matures, mature conidia 1–3-septate, broadly pyriform, cylindrical-oblong or soleiform, usually with a distinctly bulbous base, 30–55  $\times$  12–19(–21)  $\mu\text{m}$  [av.  $\pm$  SD, 41.5 ( $\pm$  6.8)  $\times$  17.1 ( $\pm$  2.1)  $\mu\text{m}$ ], broadest part of conidia usually at the bulbous base, mostly attenuated towards the basal septum, septa becoming sinuous with age, pale to medium olivaceous-brown or brown, usually echinulate, sometimes coarsely verrucose, walls distinctly thickened, up to 2

$\mu\text{m}$  thick, often appearing layered with a large lumen in the centre of the cell, broadly rounded to flattened at apex and base, hila often very prominent, often peg-like elongated, up to 3  $\mu\text{m}$  long, with age becoming less prominent, visible as a thickened flat plate just below the outer echinulate wall layer, slightly raised towards the middle, 2–3.5  $\mu\text{m}$  diam, thickened and darkened-refractive; microcyclic conidiogenesis not observed.

*Cultural characteristics:* Colonies on PDA attaining 6 mm diam after 14 d at 25  $^{\circ}\text{C}$ , whitish, smoke-grey to pale olivaceous-grey due to abundant aerial mycelium, olivaceous-black reverse, margin narrow, white, more or less crenate, aerial mycelium zonate, fluffy, covering most of the colony, mainly in the colony centre, growth

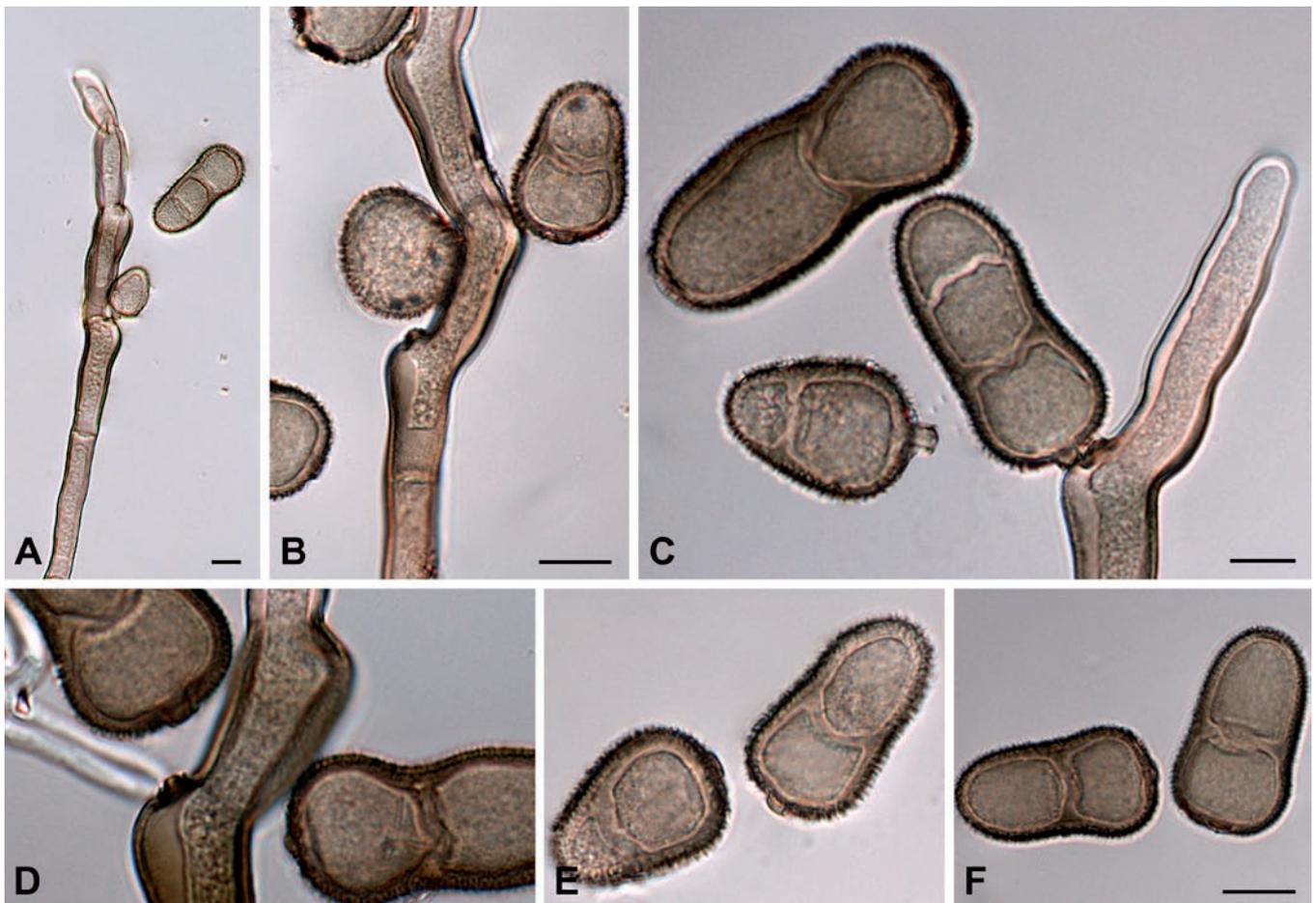


Fig. 30. *Cladosporium pseudiridis* (CBS 116463). A–C. Conidiophores and conidia. D. Part of a conidiogenous cell showing a protuberant cladosporioid conidiogenous locus. E–F. Conidia. Scale bars = 10 µm.

convex to raised, deep into the agar, with age few large prominent exudates formed, sparingly sporulating. Colonies on MEA attaining 7 mm diam after 14 d at 25 °C, olivaceous-grey, pale olivaceous-grey to pale rosy-buff due to abundant aerial mycelium covering almost the whole colony, iron-grey reverse, margin colourless or white, broad, regular, more or less glabrous, aerial mycelium fluffy, dense, high, growth convex to umbonate, sometimes with elevated colony centre, prominent exudates lacking, sporulation sparse. Colonies on OA attaining 8 mm diam after 14 d at 25 °C, white, pale buff to pale olivaceous-grey in the centre, margin grey-olivaceous, olivaceous- to iron-grey reverse, margin entire edge or somewhat undulate, somewhat feathery, growth raised with a somewhat depressed centre forming an elevated outer rim, without prominent exudates, sporulation more abundant.

*Specimen examined:* New Zealand, Auckland, Mt. Albert, Carrington Road, Unitec Campus, isolated from large leaf lesions on *Iris* sp. (*Iridaceae*), 15 Aug. 2004, C.F. Hill, CBS-H 19861, **holotype**, culture ex-type CBS 116463 = LYN 1065 = ICMP 15579.

*Substrate and distribution:* On living leaves of *Iris* sp.; New Zealand.

*Notes:* *Cladosporium pseudiridis* closely resembles *C. iridis*, a common and widespread species causing leaf spots on numerous *Iris* spp. and a few additional hosts of the host family *Iridaceae*, but the latter species is easily distinguishable by having longer and narrower, more frequently septate conidia, (18–)30–75(–87) × (7–)10–16(–18) µm, (0–)2–6(–7)-septate.

It is unlikely that *C. pseudiridis* is of New Zealand origin since the genus *Iris* is not indigenous to New Zealand. All *Iris* species that are found in this country have been introduced, mainly for horticultural purposes. The species is, therefore, probably more common than indicated above. However, within the course of the recent monographic studies in the genus *Cladosporium* numerous herbarium specimens, mainly of European origin, have been examined and proved to be correctly identified agreeing with the species concept of *C. iridis*. Additional collections and cultures are necessary to determine its distribution.

***Cladosporium ramotenellum*** K. Schub., Zalar, Crous & U. Braun, **sp. nov.** MycoBank MB504577. Figs 31–33.

*Etymology:* Refers to the morphological similarity with *Cladosporium tenellum*.

Differt a *Cladosporio* cladosporioide conidiophoris et conidiis leniter angustioribus, 2–4(–5) µm latis, conidiis 0–2(–3)-septatis, semper verruculosus; et a *Cladosporio* tenello locis conidiogenis non numerosis et non aggregatos ad apicem, conidiis longioribus et angustioribus, 2.5–35 × 2–4(–5) µm, 0–3-septatis.

*Mycelium* unbranched or only sparingly branched, 1.5–4 µm wide, septate, without swellings and constrictions, hyaline or subhyaline, smooth, sometimes irregularly rough-walled, walls unthickened. *Conidiophores* solitary, macronematous and micronematous, arising as lateral branches of plagiotropous hyphae or terminally from ascending hyphae, erect, straight or slightly flexuous, cylindrical, neither geniculate nor nodulose, without head-like swollen apices or intercalary swellings, unbranched, sometimes

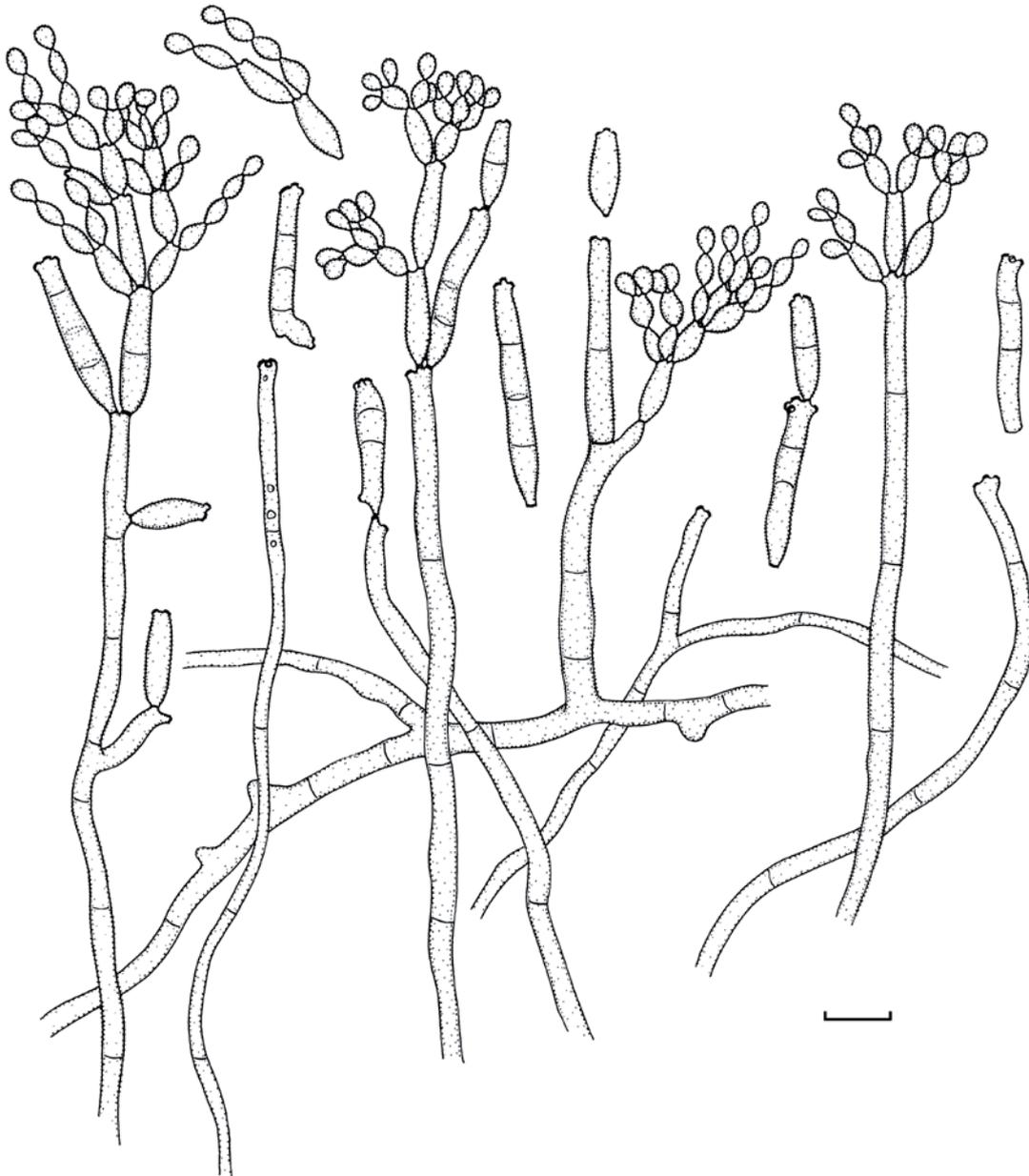


Fig. 31. *Cladosporium ramotenellum* (CPC 12043). Conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert del.

branched, branches often only as short lateral prolongations, mainly formed below a septum, 14–110  $\times$  2–4  $\mu\text{m}$ , septate, not constricted at the septa, subhyaline to pale olivaceous or brown, smooth to minutely verruculose, walls unthickened, sometimes guttulate. *Conidiogenous cells* integrated, terminal, sometimes also intercalary, cylindrical, not geniculate, non-nodulose, 10–28(–50)  $\mu\text{m}$  long, proliferation sympodial, with few conidiogenous loci, mostly 1–3, loci sometimes situated on small lateral prolongations, protuberant, 0.5–1.5(–2)  $\mu\text{m}$  diam, thickened and somewhat darkened-refractive. *Ramoconidia* formed, cylindrical-oblong, up to 47  $\mu\text{m}$  long, 2–4  $\mu\text{m}$  wide, 0–1-septate, rarely up to 4-septate, subhyaline to very pale olivaceous, smooth or almost so, with a broadly truncate base, without any dome and raised rim, 2–3  $\mu\text{m}$  wide, not thickened but somewhat refractive. *Conidia* numerous, polymorphous, catenate, in branched chains, straight, sometimes slightly curved, small terminal conidia numerous, globose, subglobose or ovoid, obovoid or limoniform, 2.5–7  $\times$  2–4(–4.5)  $\mu\text{m}$  [av.  $\pm$  SD, 5.1 ( $\pm$  1.3)  $\times$  3.1 ( $\pm$  0.6)  $\mu\text{m}$ ], aseptate, without distal hilum or with a single apical scar, intercalary conidia ellipsoid to

subcylindrical, 8–15  $\times$  3–4(–4.5)  $\mu\text{m}$  [av.  $\pm$  SD, 11.5 ( $\pm$  2.4)  $\times$  3.6 ( $\pm$  0.5)  $\mu\text{m}$ ], 0–1-septate; secondary ramoconidia subcylindrical to cylindrical-oblong, 17–35  $\times$  3–4(–5)  $\mu\text{m}$  [av.  $\pm$  SD, 22.5 ( $\pm$  5.6)  $\times$  3.7 ( $\pm$  0.5)  $\mu\text{m}$ ], 0–3-septate, not constricted at the septa, subhyaline to very pale olivaceous, minutely verruculose (granulate under SEM), walls unthickened or almost so, apex broadly rounded or slightly attenuated towards apex and base, sometimes guttulate, hila protuberant, conspicuous, 0.8–1.5(–2)  $\mu\text{m}$  diam, somewhat thickened and darkened-refractive; microcyclic conidiogenesis occurring.

*Cultural characteristics:* Colonies on PDA reaching 46–49 mm diam after 14 d at 25  $^{\circ}\text{C}$ , olivaceous to grey-olivaceous due to abundant sporulation, appearing zonate in forming concentric zones, margin entire edge to slightly undulate, white, glabrous, aerial mycelium absent or sparse, growth flat with a somewhat folded and wrinkled colony centre, without prominent exudates, sporulation profuse. Colonies on MEA reaching 48–49 mm diam after 14 d at 25  $^{\circ}\text{C}$ , grey-olivaceous to olivaceous-grey, velvety, olivaceous-grey to

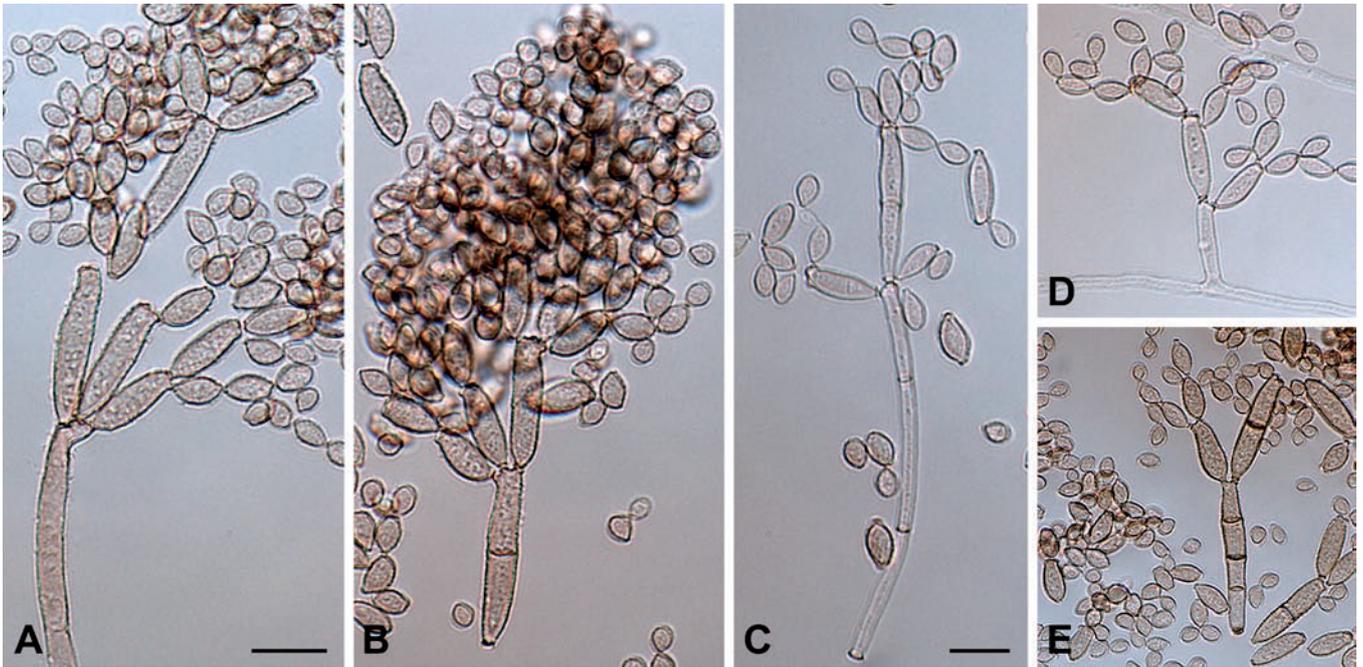


Fig. 32. *Cladosporium ramotenellum* (CPC 12043). A, C. Macronematous conidiophore. B. Conidial chain. D. Micronematous conidiophore. E. Ramoconidia and conidia. Scale bars = 10 µm.

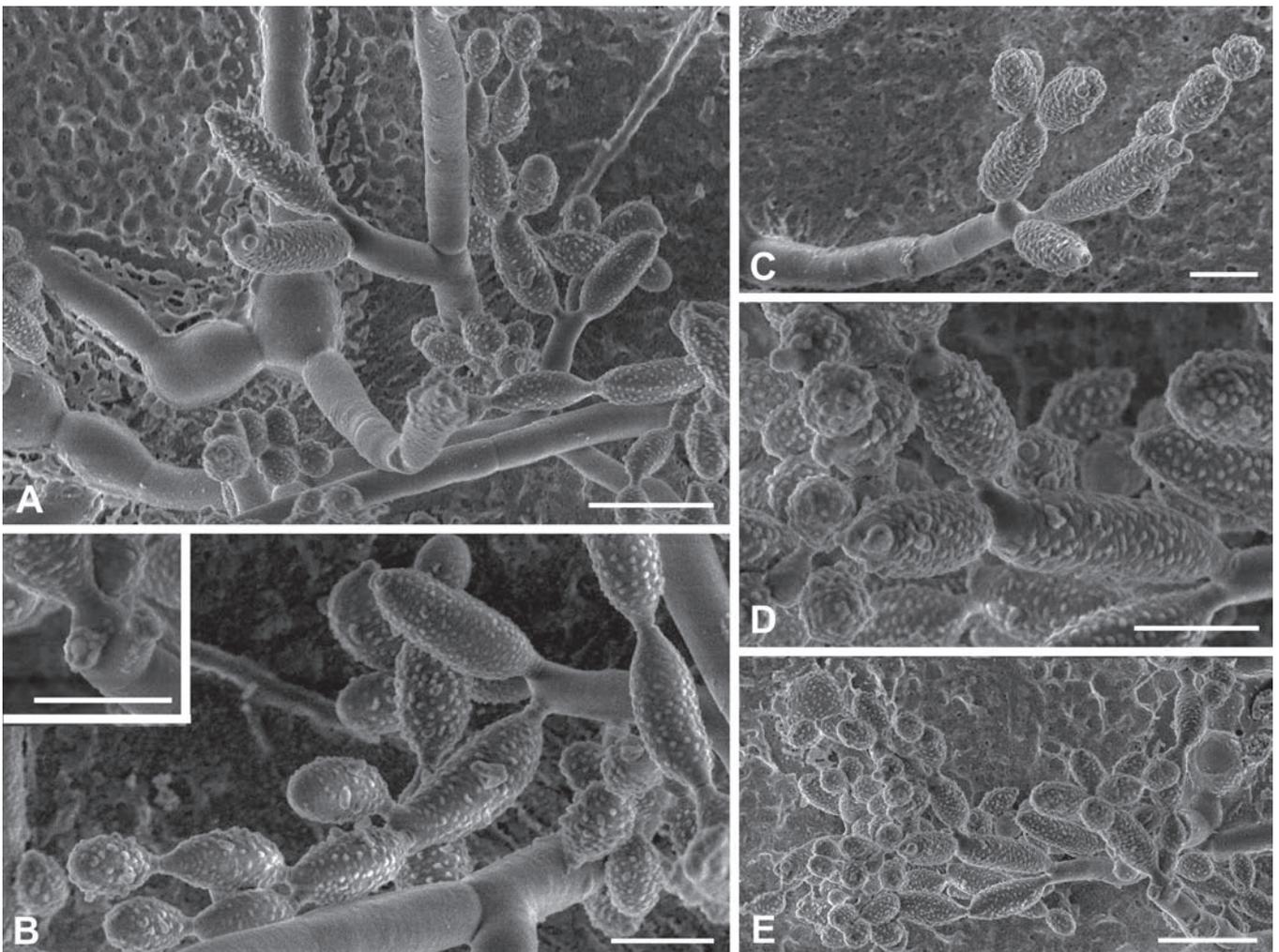


Fig. 33. *Cladosporium ramotenellum* (CPC 12043). A. Survey of colony development showing a large bulbous "foot cell" that gives rise to conidiophores, which can be branched. B. Details of conidiophores showing secondary ramoconidia and conidia. The inset shows scar formation on a conidiophore. C. Conidiophore and several conidia. D. Details of ornamentation on conidia. Note the wide, but relatively low ornamentation units. E. A micrograph illustrating the organisation within a conidiophore. Scale bars A–D = 5 µm, E = 10 µm.



Fig. 34. *Cladosporium sinuosum* (CPC 11839). Conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert del.

iron-grey reverse, margin entire edge to undulate, radially furrowed, colourless, glabrous to feathery, aerial mycelium sparse, diffuse, growth flat with slightly elevated colony centre, distinctly wrinkled, prominent exudates not formed, abundantly sporulating. Colonies on OA attaining 40 mm diam after 14 d at 25 °C, grey-olivaceous, margin entire edge, colourless or white, glabrous, aerial mycelium absent or sparse, growth flat, without exudates, sporulation profuse.

*Specimens examined:* **Slovenia**, Ljubljana, isolated from an air conditioning system (bathroom), 2004, M. Butala, CBS 121627 = CPC 12047 = EXF-967; Sečovelje, isolated from hypersaline water from reverse ponds, salterns, 2005, P. Zalar, CBS-H 19862, **holotype**, isotype HAL 2026 F, culture ex-type CBS 121628 = CPC 12043 = EXF-454.

*Substrate and distribution:* Hypersaline water, air; Slovenia.

*Notes:* *Cladosporium ramotenellum*, which appears to be a saprobe in air and hypersaline water, morphologically resembles *C. cladosporioides* and *C. tenellum* K. Schub., Zalar, Crous & U. Braun, but is quite distinct from *C. cladosporioides* by having somewhat narrower conidiophores and conidia, 2–4(–5)  $\mu\text{m}$

wide, and 0–3-septate, always minutely verruculose conidia. *Cladosporium tenellum*, a newly introduced species (see below) isolated from hypersaline water and plant material, possesses conidiophores with numerous conidiogenous loci, usually crowded towards the apex forming sympodial clusters of pronounced scars, and shorter and somewhat wider, 0–1(–2)-septate conidia, 3–20(–28)  $\times$  (2.5–)3–5(–6)  $\mu\text{m}$ . Besides these morphological differences, *C. ramotenellum* is faster growing in culture than *C. tenellum*.

*Cladosporium arthrinioides* Thüm. & Beltr. and *C. hypophyllum* Fuckel are also close to *C. ramotenellum*, but *C. arthrinioides*, known from Italy on leaves of *Bougainvillea spectabilis*, deviates in having shorter and wider, 0–1(–2)-septate, mostly smooth conidia (2–18  $\times$  2–6.5  $\mu\text{m}$ ) which become larger and more frequently septate with age (up to 32  $\mu\text{m}$  long and with up to four septa); and *C. hypophyllum* occurring in Europe on leaves of *Ulmus minor* differs in having often mildly to distinctly geniculate-sinuous, sometimes subnodulose conidiophores and shorter and somewhat wider, 0–1(–3)-septate conidia, 4–17(–19)  $\times$  2–5  $\mu\text{m}$ , becoming distinctly swollen, darker, longer and wider with age, 5–7  $\mu\text{m}$ , with the septa often being constricted (Schubert 2005).

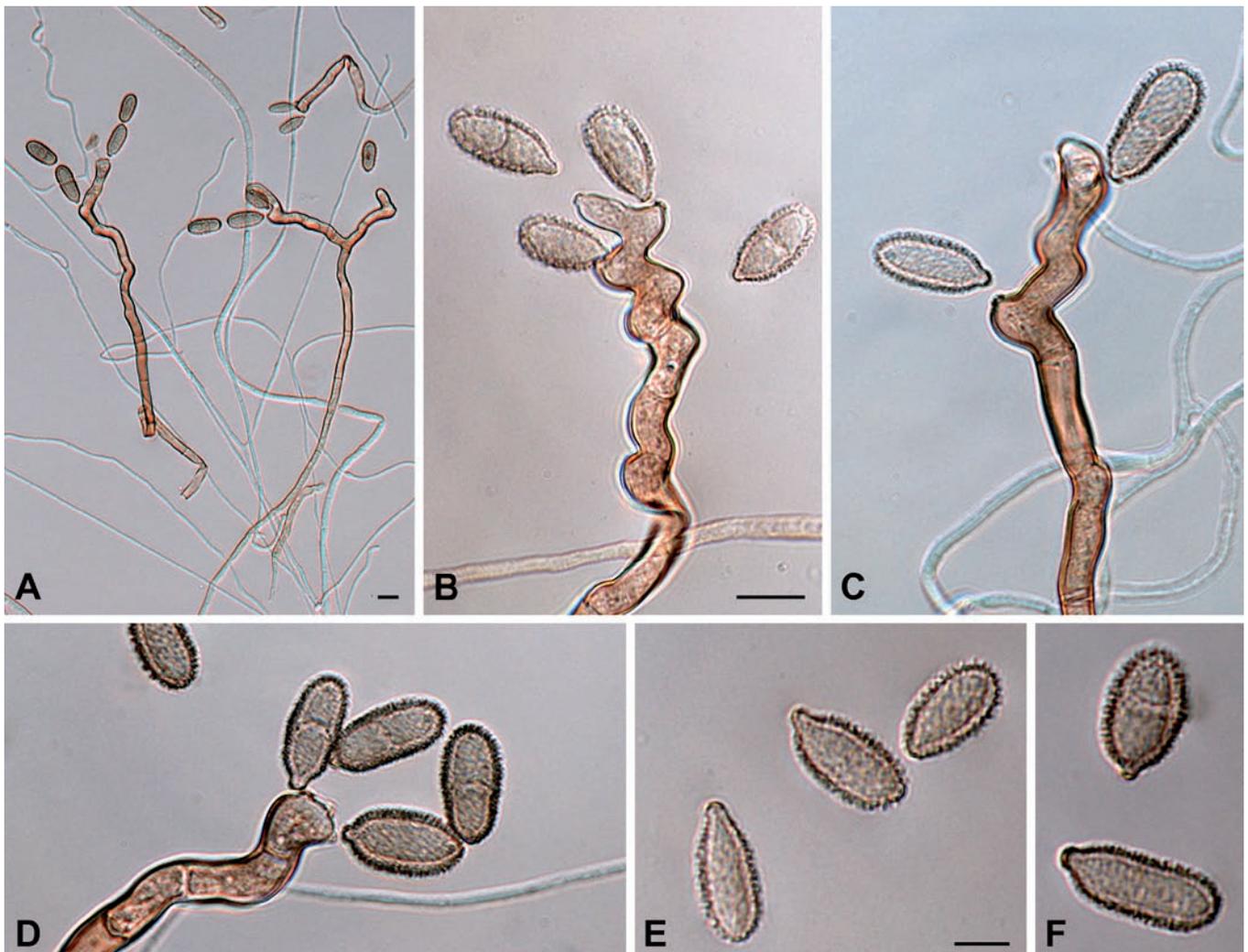


Fig. 35. *Cladosporium sinuosum* (CPC 11839). A–D. Conidiophores. E–F. Conidia. Scale bars = 10  $\mu$ m.

***Cladosporium sinuosum*** K. Schub., C.F. Hill, Crous & U. Braun, sp. nov. MycoBank MB504578. Figs 34–35.

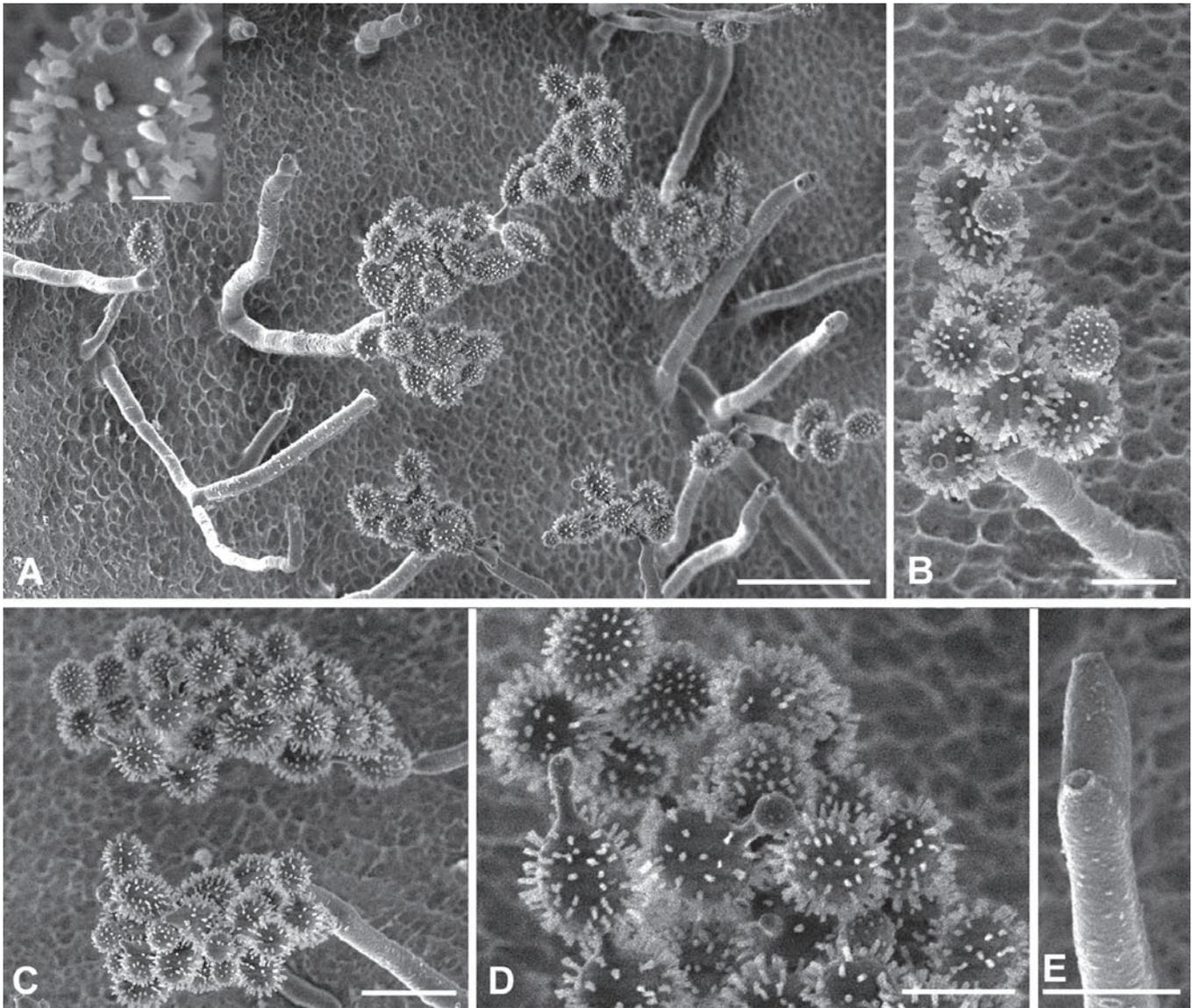
**Etymology:** Refers to the usually distinctly sinuous conidiophores.

Differt a *Cladosporio herbaro* conidiophoris distincte sinuosis, conidiis solitariis vel breve catenatis, catenis non ramosis, echinulatis.

**Mycelium** sparingly branched, 1–7  $\mu$ m wide, septate, not constricted at the septa, subhyaline to pale brown, smooth to minutely verruculose, walls unthickened or slightly thickened, sometimes with small swellings. **Conidiophores** arising laterally from plagiotropous hyphae or terminally from ascending hyphae, erect, more or less straight to flexuous, often once or several times slightly to distinctly geniculate-sinuuous, sometimes even zigzag-like, nodulose with small to large lateral shoulders, shoulders somewhat distant from each other or in close succession giving them a knotty/gnarled appearance, unbranched or once branched, 25–260  $\times$  5–7  $\mu$ m, shoulders up to 10  $\mu$ m wide, pluriseptate, septa sometimes in short succession, not constricted at the septa, pale brown to medium brown, smooth to minutely verruculose, walls thickened, often distinctly two-layered, up to 1  $\mu$ m thick. **Conidiogenous cells** integrated, terminal or intercalary, often slightly to distinctly geniculate-sinuuous, nodulose with small to large laterally swollen shoulders, 8–30  $\mu$ m long, proliferation sympodial, with a single or up to three conidiogenous loci, usually confined to lateral

shoulders, protuberant, often denticle-like or on the top of short cylindrical stalk-like prolongations, 1.2–2(–2.2)  $\mu$ m diam, mainly 2  $\mu$ m, somewhat thickened and darkened-refractive, dome often slightly higher than the surrounding rim. **Conidia** solitary or in short unbranched chains with up to three conidia, straight, obovoid, oval, broadly ellipsoid to subcylindrical or sometimes clavate (broader at the apex), 9–21  $\times$  (5–)6–8  $\mu$ m [av.  $\pm$  SD, 14.5 ( $\pm$  2.5)  $\times$  6.6 ( $\pm$  0.7)  $\mu$ m], 0–1-septate, not constricted at the septa, septum more or less median, pale greyish brown, densely echinulate, spines up to 1  $\mu$ m long, walls thickened, apex mostly broadly rounded or sometimes attenuated, towards the base mostly distinctly attenuated forming a peg-like prolongation, up to 2  $\mu$ m long, hila protuberant, 1.2–2  $\mu$ m diam, mainly 2  $\mu$ m, somewhat thickened and darkened-refractive; microcyclic conidiogenesis not observed.

**Cultural characteristics:** Colonies on PDA attaining 20 mm diam after 14 d at 25  $^{\circ}$ C, pale olivaceous-grey due to abundant aerial mycelium, olivaceous-grey towards margins, iron-grey to olivaceous-black reverse, margin regular, entire edge, aerial mycelium abundant, cottony, dense, high, growth regular, low convex, radially furrowed in the centre, growing deep into the agar, with age numerous small to large prominent exudates, sporulation sparse. Colonies on MEA attaining 16 mm diam after 14 d at 25  $^{\circ}$ C, white to pale smoke-grey, fawn reverse, velvety, margin undulate, glabrous, aerial mycelium abundant, dense, high, fluffy, growth raised with elevated colony centre, laterally furrowed, without



**Fig. 36.** *Cladosporium spinulosum* (CPC 12040). A. Overview on agar surface with conidiophores arising from the surface. The spore clusters on the conidiophore are very compact. Note several simple, tubular conidiophore ends. The inset shows details of a conidium showing two pronounced hila and a unique, very distinct ornamentation on the cell wall. B. Conidiophore with globose or subsphaerical secondary ramoconidia and conidia. Note the newly forming cells and hila. C. Two conidiophores. D. Details of spores and spore formation. E. The end of a conidiophore and two scars. Scale bars: A = 20 µm, A (inset) = 1 µm, B, D–E = 5 µm, C = 10 µm.

prominent exudates. Colonies on OA attaining 18 mm diam after 14 d at 25 °C, olivaceous, white to pale olivaceous-grey in the centre due to abundant aerial mycelium, olivaceous-grey reverse, margin white, entire edge, glabrous, aerial mycelium loose to dense, high, fluffy to felty, growth flat to low convex, regular, without prominent exudates, sporulating.

*Specimen examined:* **New Zealand**, Te Anau, isolated from leaves of *Fuchsia excorticata* (Onagraceae), 31 Jan. 2005, A. Blouin, Hill 1134A, CBS-H 19863, **holotype**, culture ex-type CBS 121629 = CPC 11839 = ICMP 15819.

*Substrate and distribution:* On living leaves of *Fuchsia excorticata*; New Zealand.

*Notes:* This new species is well characterised by its slightly to distinctly geniculate-sinuous, often zigzag-like conidiophores and its conidia formed solitary or rarely in short unbranched chains and is therefore morphologically not comparable with any of the species described until now. Most *Cladosporium* species with conidia usually formed solitary or in short unbranched chains have previously been treated as species of the genus *Heterosporium* Klotzsch ex

Cooke, now considered to be synonymous with *Cladosporium*. All of them, including the newly introduced *C. arthropodii* K. Schub. & C.F. Hill from New Zealand, which also belongs to this species complex (Braun *et al.* 2006), possess very large and wide, often pluriseptate conidia quite distinct from those of *C. sinuosum* (David 1997). *Cladosporium alopecuri* (Ellis & Everh.) U. Braun, known from the U.S.A. on *Alopecurus geniculatus* is also quite different by having larger and wider conidia, 20–40 × 7–13(–15) µm, and wider conidiogenous loci and conidial hila, 3.5–5 µm diam (Braun 2000).

*Cladosporium herbarum* is superficially similar but the conidiophores of the latter species are sometimes only slightly geniculate-sinuous but never zigzag-like and the verruculose to verrucose conidia are frequently formed in unbranched or branched chains.

***Cladosporium spinulosum*** Zalar, de Hoog & Gunde-Cimerman, *Studies in Mycology* 58: 180. 2007 – this volume. Fig. 36.

*Note:* This new species is described and illustrated in Zalar *et al.* (2007 – this volume).

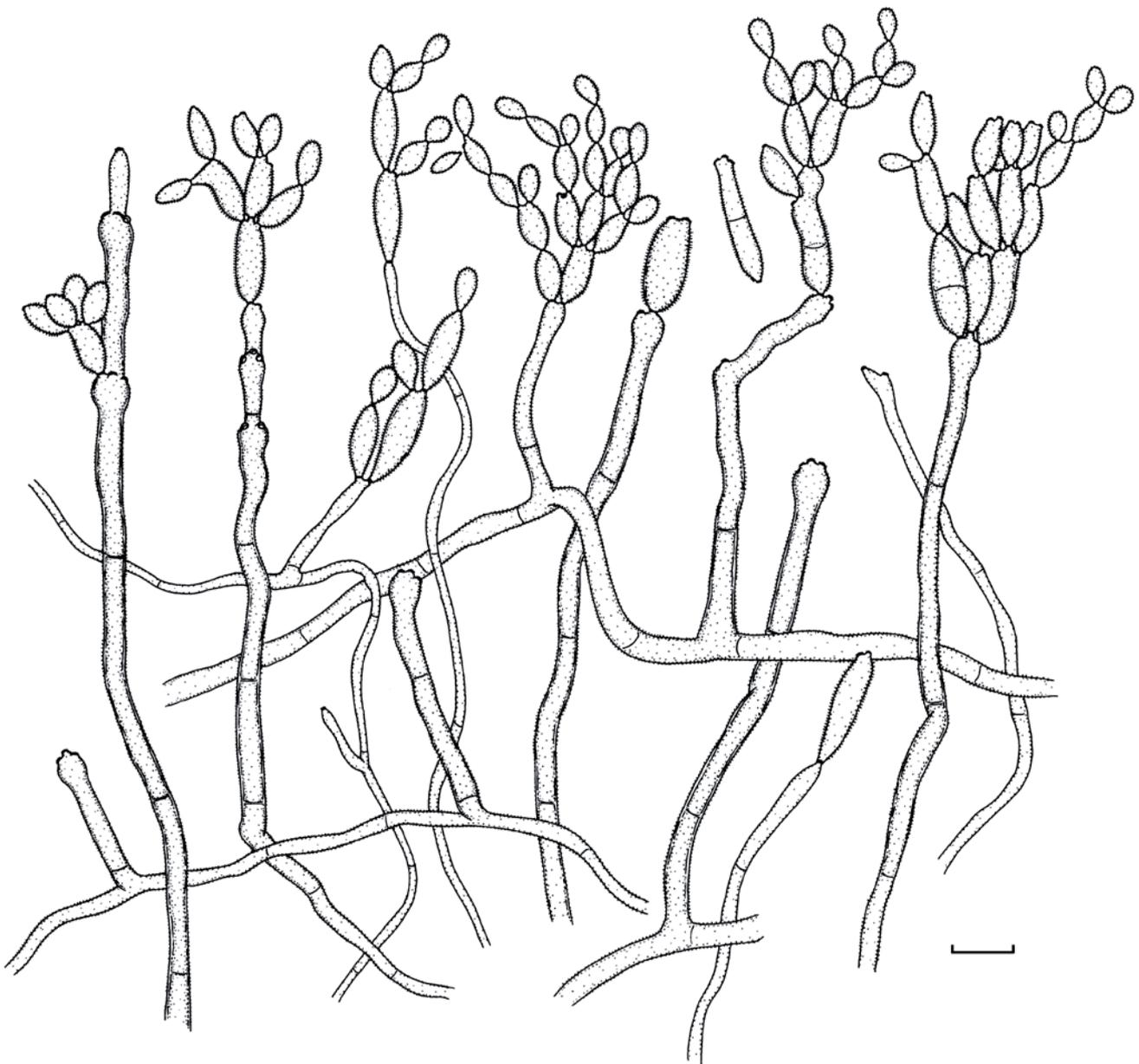


Fig. 37. *Cladosporium subinflatum* (CPC 12041). Macro- and micronematous conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert del.

***Cladosporium subinflatum*** K. Schub., Zalar, Crous & U. Braun, sp. nov. MycoBank MB504579. Figs 37–39.

**Etymology:** Refers to its nodulose conidiophores.

Differt a *Cladosporio bruhnei* conidiophoris cum nodulis angustioribus, 3–6.5  $\mu\text{m}$  latis, conidiis brevioribus, 4–17(–22)  $\mu\text{m}$  longis, spinulosis, cum spinulis ad 0.8  $\mu\text{m}$  longis; et a *Cladosporio spinuloso* conidiophoris nodulosis, conidiis spinulosis, cum spinulis brevioribus, ad 0.8 longis, locis conidiogenis et hilis latioribus, (0.5–)1–2  $\mu\text{m}$  latis.

**Mycelium** unbranched or occasionally branched, 1.5–3  $\mu\text{m}$  wide, later more frequently branched and wider, up to 7  $\mu\text{m}$  wide, septate, not constricted at the septa, hyaline or subhyaline, almost smooth to somewhat verruculose or irregularly rough-walled, walls unthickened. **Conidiophores** mainly macronematous, sometimes also micronematous, arising terminally from ascending hyphae or laterally from plagiotropous hyphae, erect or subdecumbent, straight or flexuous, sometimes bent, cylindrical, nodulose, usually with small head-like swellings, sometimes swellings also on a lower level or intercalary, occasionally geniculate, unbranched, occasionally branched, (5–)10–270  $\times$  (1.5–)2.5–4.5(–5.5)  $\mu\text{m}$ ,

swellings 3–6.5  $\mu\text{m}$  wide, aseptate or with few septa, not constricted at the septa, pale brown, pale olivaceous-brown or somewhat reddish brown, smooth, usually verruculose or irregularly rough-walled and paler, subhyaline towards the base, walls thickened, sometimes appearing even two-layered, up to 1  $\mu\text{m}$  thick. **Conidiogenous cells** integrated, usually terminal or conidiophores reduced to conidiogenous cells, cylindrical, nodulose, usually with small head-like swellings with loci confined to swellings, sometimes geniculate, 5–42  $\mu\text{m}$  long, proliferation sympodial, with several loci, up to four situated at nodules or on lateral swellings, protuberant, conspicuous, denticulate, (0.8–)1–2  $\mu\text{m}$  diam, thickened and darkened-refractive. **Conidia** catenate, in branched chains, more or less straight, numerous globose and subglobose conidia, ovoid, obovoid, broadly ellipsoid to cylindrical, 4–17(–22)  $\times$  (2.5–)3.5–5.5(–7)  $\mu\text{m}$  [av.  $\pm$  SD, 11.7 ( $\pm$  4.6)  $\times$  4.5 ( $\pm$  0.8)  $\mu\text{m}$ ], 0–1(–2)-septate, not constricted at septa, pale brown or pale olivaceous-brown, ornamentation variable, mainly densely verruculose to echinulate (loosely muricate under SEM), spines up to 0.8  $\mu\text{m}$  high, sometimes irregularly verruculose with few scattered tubercles



Fig. 38. *Cladosporium subinflatum* (CPC 12041). A–C. Macronematous conidiophores. D–E. Conidia. Scale bar = 10 µm.

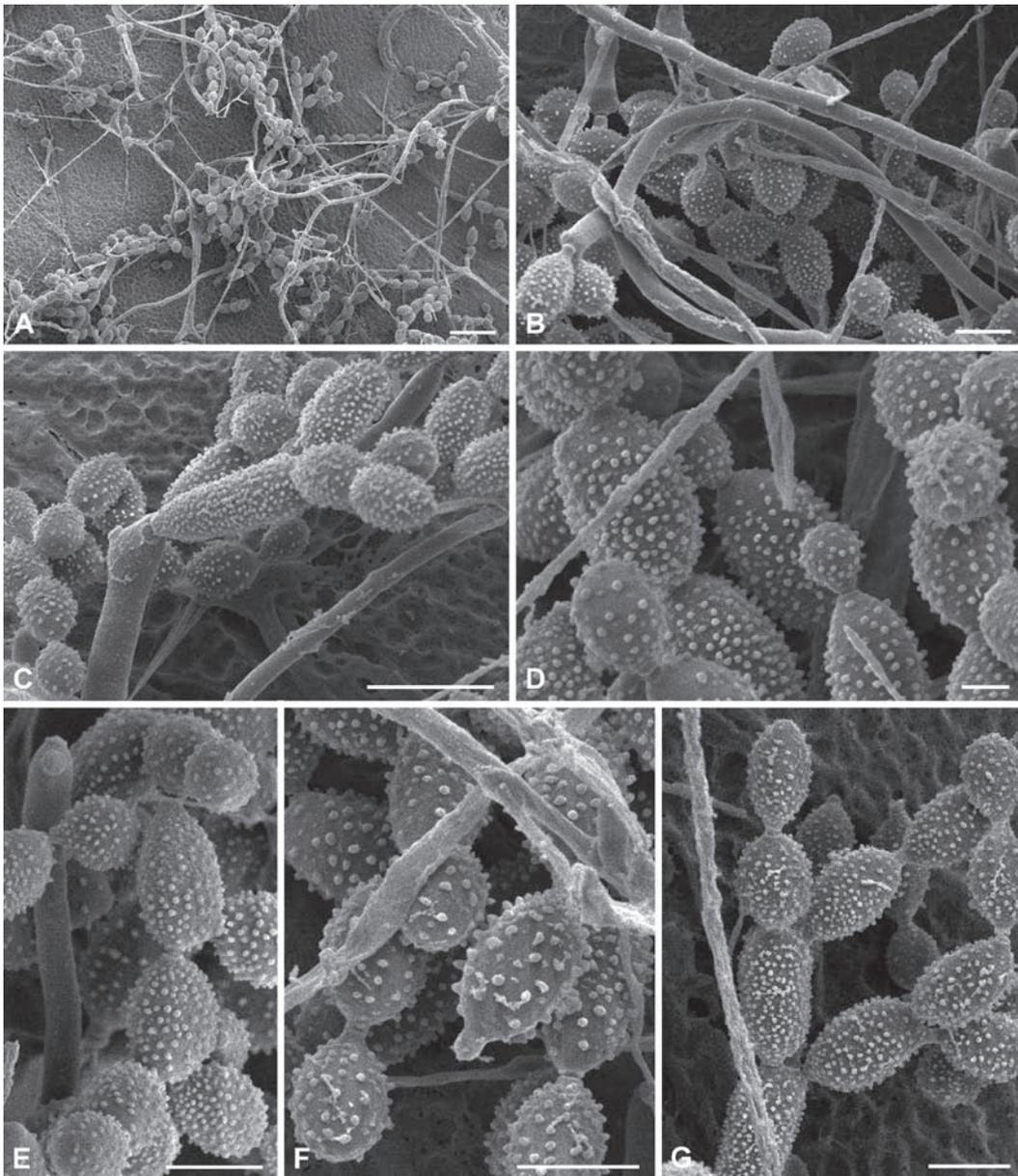


Fig. 39. *Cladosporium subinflatum* (CPC 12041). A–G. Images of an 11-d-old culture on SNA. A. Overview of colony with clusters of conidia and aerial hyphae. Many of the hyphae have a collapsed appearance. B. Detail of colony with conidiophores, conidia and aerial hyphae that are partly collapsed. C. Detail of a conidiophore end and a secondary ramoconidium. Note the scars at the end of the conidiophore. D. Details of conidia and ornamentation. The ornamentation consists out of markedly defined units, which have a relatively large distance from each other. Note the hilum on the right conidium. E. Conidiophore with large scars and conidia. F. Different blastoconidia with very early stages of new spore formation in the middle of the picture. G. Pattern of spore development. Scale bars: A = 20 µm, B, E–G = 5 µm, C = 10 µm, D = 2 µm.

or irregularly echinulate, walls unthickened or slightly thickened, apex rounded or slightly attenuated towards apex and base, hila conspicuous, protuberant, denticulate, 0.5–2  $\mu\text{m}$  diam, thickened and darkened-refractive; microcyclic conidiogenesis observed.

**Cultural characteristics:** Colonies on PDA attaining 29 mm diam after 14 d at 25 °C, olivaceous-black to olivaceous-grey towards margin, margin regular, entire edge, narrow, colourless to white, glabrous to feathery, aerial mycelium formed, fluffy, mainly near margins, growth flat, somewhat folded in the colony centre, deep into the agar, few prominent exudates formed with age, sporulation profuse. Colonies on MEA attaining 25 mm diam after 14 d at 25 °C, olivaceous-grey to olivaceous due to abundant sporulation in the colony centre, pale greenish grey towards margin, iron-grey reverse, velvety to powdery, margin crenate, narrow, white, glabrous, radially furrowed, aerial mycelium diffuse, growth convex with papillate surface, wrinkled colony centre, without prominent exudates, sporulation profuse. Colonies on OA attaining 26 mm diam after 14 d at 25 °C, olivaceous, iron-grey to greenish black reverse, growth flat, deep into the agar, with a single exudate, abundantly sporulating.

**Specimen examined:** Slovenia, Sečovlje, isolated from hypersaline water from crystallization ponds, salterns, 2005, S. Sonjak, CBS-H 19864, **holotype**, isotype HAL 2027 F, culture ex-type CBS 121630 = CPC 12041 = EXF-343.

**Substrate and distribution:** Hypersaline water; Slovenia.

**Notes:** *Cladosporium subinflatum*, an additional saprobic species isolated from hypersaline water, was at first identified as *C. spinulosum*, but proved to be both morphologically as well as phylogenetically distinct from the latter species in having somewhat wider [(1.5–)2.5–4.5(–5.5)  $\mu\text{m}$ ], nodulose macronematous conidiophores with conidiogenous loci confined to swellings, wider conidiogenous loci and hila, (0.8–)1–2  $\mu\text{m}$ , and spiny conidia with shorter spines than in *C. spinulosum* (up to 0.8  $\mu\text{m}$  versus 0.5–1.3  $\mu\text{m}$  long) (Zalar *et al.* 2007). With its narrow, nodulose macronematous conidiophores and catenate conidia, *C. bruhnei* is morphologically also similar but differs by having conidiophores with wider swellings, (4–)5–8  $\mu\text{m}$ , and longer conidia 4–24(–31)  $\mu\text{m}$ , rarely up to 40  $\mu\text{m}$  long which are minutely verruculose to verrucose but not spiny.

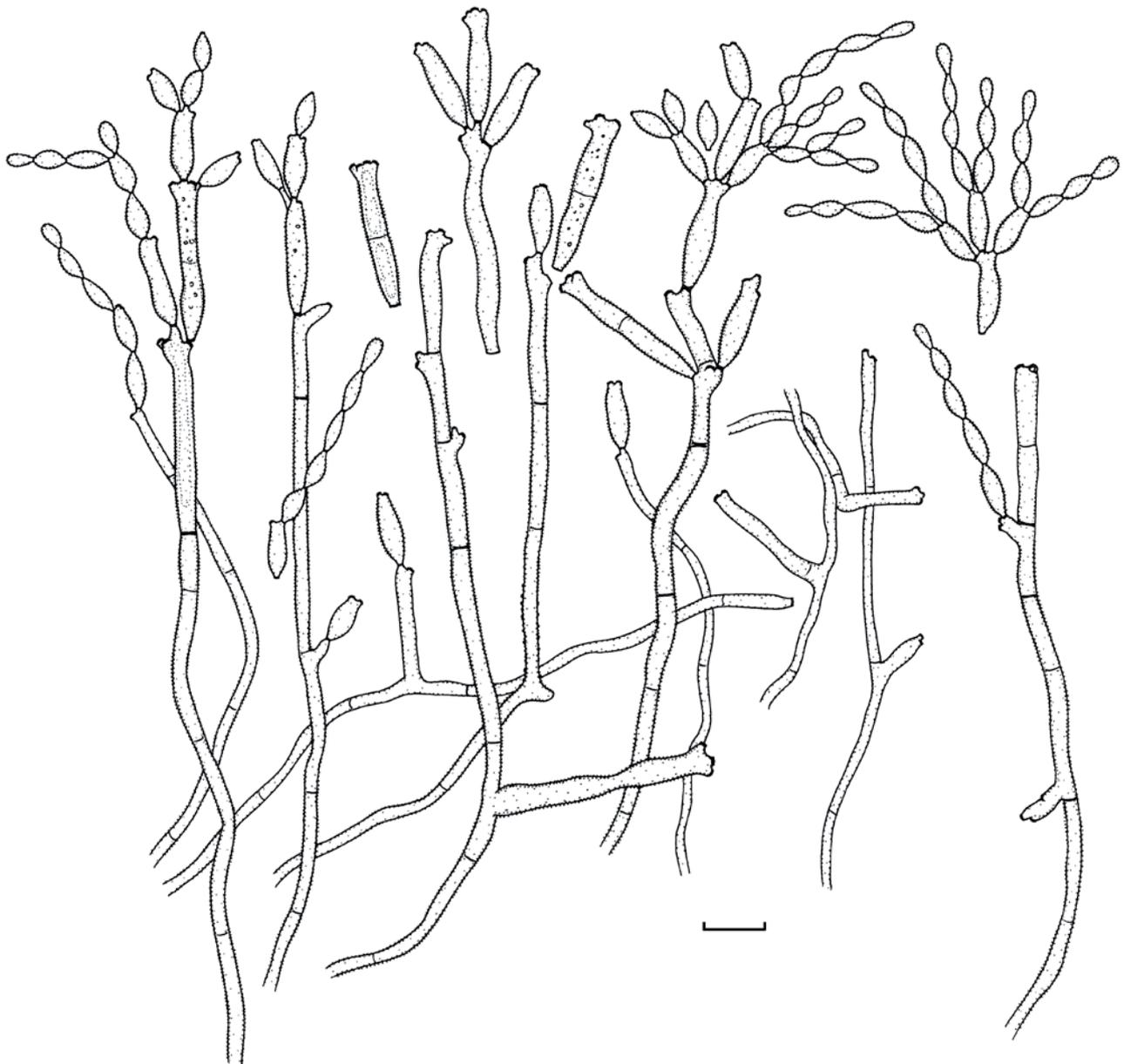


Fig. 40. *Cladosporium subtilissimum* (CBS 113754). Macro- and micronematous conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert *del.*

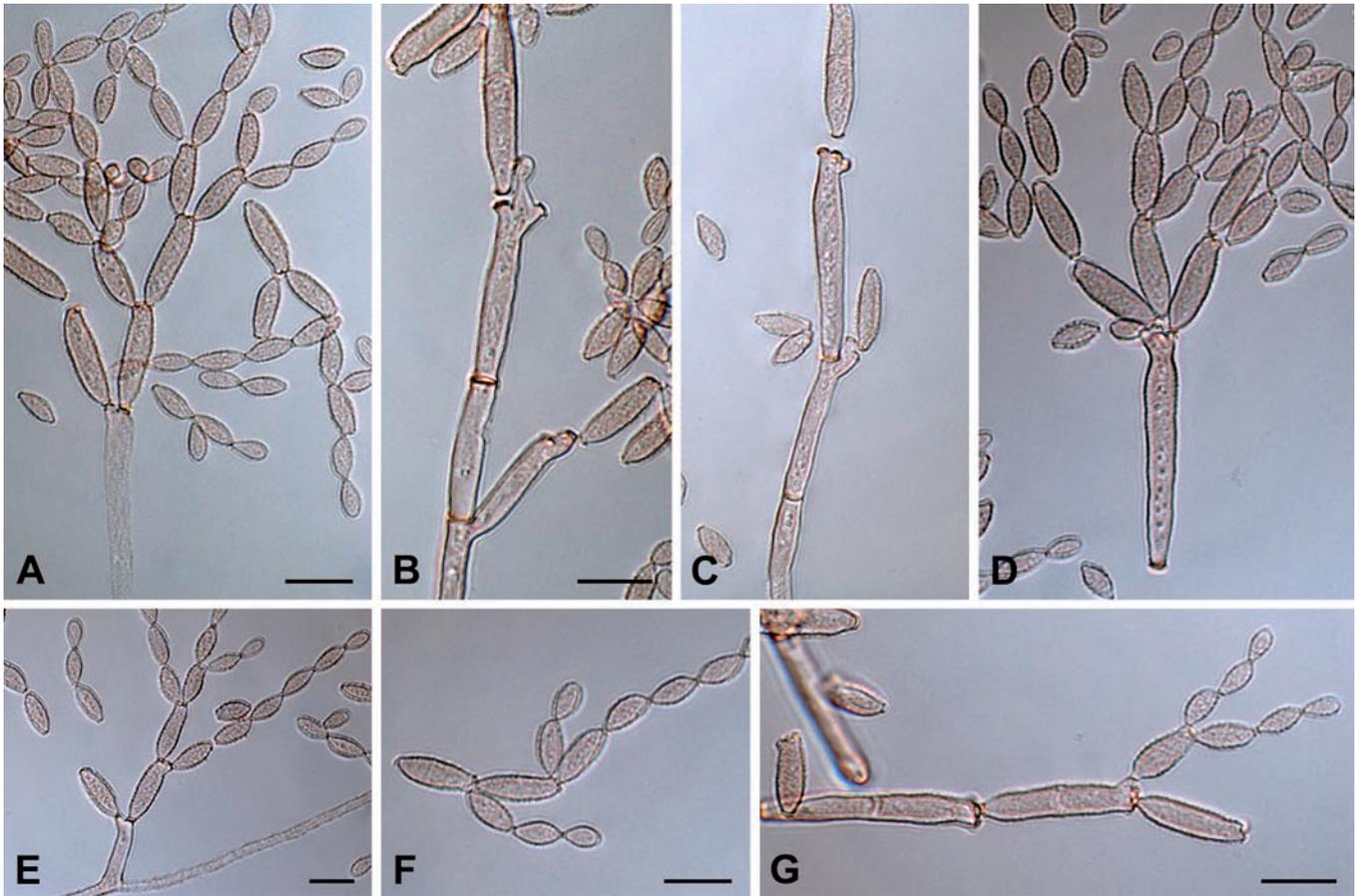


Fig. 41. *Cladosporium subtilissimum* (CBS 113754). A–C. Macronematous conidiophores. D. Conidial chain. E. Micronematous conidiophore. F–G. Conidia. Scale bars = 10  $\mu\text{m}$ .

***Cladosporium subtilissimum*** K. Schub., Dugan, Crous & U. Braun, **sp. nov.** MycoBank MB504580. Figs 40–42.

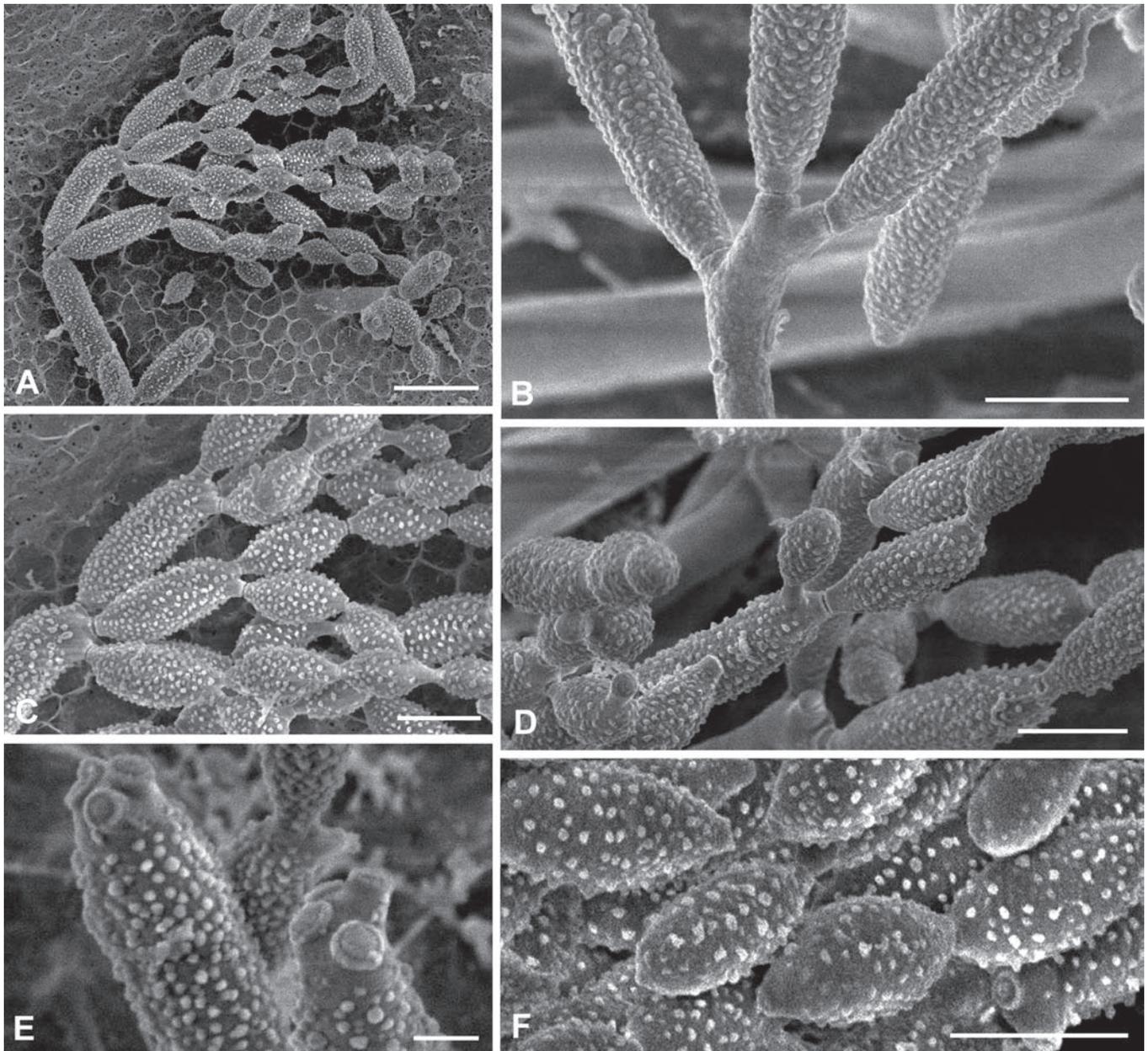
**Etymology:** Refers to its narrow conidiophores and conidia.

Differt a *Cladosporio cladosporioides* conidiophoris et conidiis semper asperulatis ad verruculosus, conidiis 0–1(–2)-septatis.

**Mycelium** unbranched or sparingly branched, 1–5  $\mu\text{m}$  wide, septate, without swellings and constrictions, hyaline to subhyaline or pale brown, smooth to minutely verruculose, walls unthickened or almost so, protoplasm somewhat guttulate or granular. **Conidiophores** macronematous and micronematous, arising laterally from plagiotropous hyphae or terminally from ascending hyphae, erect, straight to slightly flexuous, filiform to cylindrical-oblong, non-nodulose, sometimes geniculate towards the apex, unbranched or once branched, branches short to somewhat longer, usually formed below a septum, sometimes only short, denticle-like or conical, 25–140  $\times$  2–4  $\mu\text{m}$ , 0–4-septate, not constricted at the septa, subhyaline to pale brown, almost smooth, minutely verruculose to verruculose, sometimes irregularly rough-walled in the lower part, walls unthickened or slightly thickened, protoplasm guttulate or somewhat granular. **Conidiogenous cells** integrated, terminal or pleurogenous, sometimes also intercalary, filiform to narrowly cylindrical, non-nodulose, sometimes geniculate, 14–57  $\mu\text{m}$  long, with usually sympodial clusters of pronounced conidiogenous loci at the apex or on a lower level, denticle-like or situated on short lateral prolongations, up to five loci, intercalary conidiogenous cells usually with a short denticle-like lateral outgrowth below a septum, protuberant, denticulate, somewhat truncate, 1.2–2  $\mu\text{m}$  diam, thickened and darkened-refractive. **Ramoconidia** sometimes occurring, conidiogenous cells seceding at one of the upper septa

of the conidiophore and behaving like conidia, filiform or cylindrical, 20–40(–55)  $\mu\text{m}$  long, 1.5–4  $\mu\text{m}$  wide, 0–1-septate, concolorous with conidiophores, not attenuated towards apex and base, base broadly truncate, non-cladosporioid, without any dome and raised rim, 2–3.5  $\mu\text{m}$  wide, neither thickened nor darkened, sometimes slightly refractive. **Conidia** catenate, in branched chains, up to 12 or even more in a chain, straight, small terminal conidia numerous, subglobose, narrowly obovoid, limoniform or fusiform, 4–9  $\times$  2–3.5  $\mu\text{m}$  [av.  $\pm$  SD, 6.4 ( $\pm$  1.5)  $\times$  2.8 ( $\pm$  0.4)  $\mu\text{m}$ ], with up to three distal scars, aseptate, hila (0.5–)0.8–1  $\mu\text{m}$  diam, intercalary conidia narrowly ellipsoid, fusiform to subcylindrical, 9–18  $\times$  3–4(–6)  $\mu\text{m}$  [av.  $\pm$  SD, 13.0 ( $\pm$  2.5)  $\times$  3.8 ( $\pm$  0.3)  $\mu\text{m}$ ], 0(–1)-septate, hila 1–1.2(–1.8)  $\mu\text{m}$  diam, with up to four distal scars, secondary ramoconidia ellipsoid, fusiform or subcylindrical, (13–)17–32(–37)  $\times$  3–5(–6)  $\mu\text{m}$  [av.  $\pm$  SD, 21.4 ( $\pm$  4.4)  $\times$  4.1 ( $\pm$  0.5)  $\mu\text{m}$ ], 0–1(–2)-septate, septum median or somewhat in the lower half, usually not constricted at the septa, with up to six distal hila crowded at the apex, hila (1.2–)1.5–2(–2.5)  $\mu\text{m}$  diam, apex often somewhat laterally enlarged or prolonged with hila crowded there, very pale or pale brown or olivaceous-brown, minutely verruculose to verruculose (granulate under SEM), walls unthickened or only slightly thickened, often slightly attenuated towards apex and base, protoplasm often guttulate or granular, hila protuberant, denticulate, (0.5–)0.8–2(–2.2)  $\mu\text{m}$  diam, thickened and darkened-refractive; microcyclic conidiogenesis occasionally observed.

**Cultural characteristics:** Colonies on PDA attaining 24 mm diam after 14 d at 25  $^{\circ}\text{C}$ , grey-olivaceous to olivaceous, olivaceous-grey, iron-grey or olivaceous-black reverse, velvety, margin regular, entire edge, white or pale greenish olivaceous, glabrous to feathery, aerial mycelium sparse, only few areas with abundant



**Fig. 42.** *Cladosporium subtilissimum* (CBS 113754). A. Overview on the organisation of spore formation. The micrograph shows a large basal secondary ramoconidium which has chains of secondary ramoconidia, intercalary and small terminal conidia. The conidia are formed in rows of often three cells. Note the size difference in the different cells. B. Conidiophore showing very pronounced scars that almost appear as branches. C. Detail of (A), illustrating the scar formation between the cells. D. Conidia during different stages of formation. E. Details of pronounced hila, and prominent ornamentation on secondary ramoconidia with the central dome-formed area. F. Different conidia and hila. Scale bars: A = 10  $\mu\text{m}$ , B–D, F = 5  $\mu\text{m}$ , E = 2  $\mu\text{m}$ .

mycelium, diffuse, growth regular, flat or with a raised and wrinkled colony centre, radially furrowed, effuse, usually without prominent exudates, with age several exudates formed, sporulation profuse, colonies consisting of two kinds of conidiophores, short and a few longer ones. Colonies on MEA reaching 25 mm diam after 14 d at 25 °C, greenish olivaceous to grey-olivaceous in the centre, olivaceous-grey to iron-grey reverse, velvety, margin entire edge, crenate or umbonate, narrow, pale greenish olivaceous, sometimes radially furrowed, aerial mycelium absent or sparse, growth low convex with distinctly wrinkled colony centre, without prominent exudates, abundantly sporulating. Colonies on OA attaining 25 mm diam after 14 d at 25 °C, dark grey-olivaceous to olivaceous due to profuse sporulation, iron-grey reverse, sometimes releasing some olivaceous-buff pigments into the agar, velvety, margin regular, entire edge or crenate, narrow, colourless or white, glabrous or feathery, aerial mycelium sparse, growth flat with slightly raised colony centre, prominent exudates lacking, sporulation profuse.

*Specimens examined:* **Slovenia**, Sečovlje, isolated from hypersaline water from salterns (reserve pond), 2005, P. Zalar, CPC 12044 = EXF-462. **U.S.A.**, isolated from bing cherry fruits, F. Dugan, CBS 113753; isolated from a grape berry, F. Dugan, wf 99-2-9 sci 1, CBS-H 19865, **holotype**, isotype HAL 2028 F, culture ex-type CBS 113754.

*Excluded strains within the subtilissimum complex:* **Argentina**, isolated from *Pinus ponderosa* (*Pinaceae*), 2005, A. Greslebin, CPC 12484, CPC 12485. **U.S.A.**, isolated from grape berry, F. Dugan, CBS 113741, CBS 113742; isolated from grape bud, F. Dugan, CBS 113744.

*Substrate and distribution:* Plant material and hypersaline water; Slovenia, U.S.A.

*Notes:* *Cladosporium cladosporioides* is morphologically comparable with the new species but deviates in having usually smooth conidiophores and conidia, with the conidia being mainly aseptate. *C. subtilissimum* is represented by three isolates of different origins

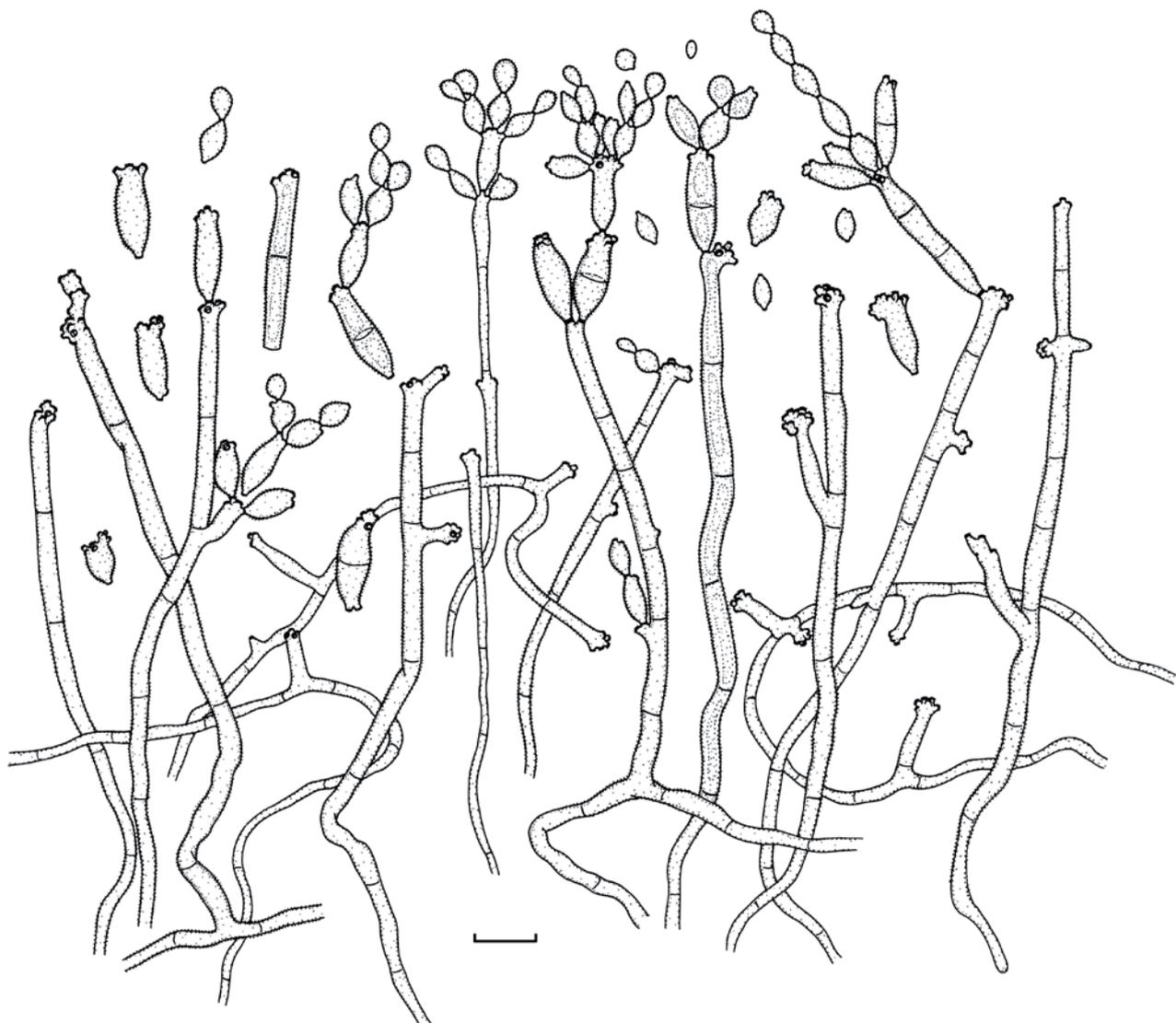


Fig. 43. *Cladosporium tenellum* (CPC 12053). Macro- and micronematous conidiophores and conidia. Scale bar = 10  $\mu$ m. K. Schubert del.

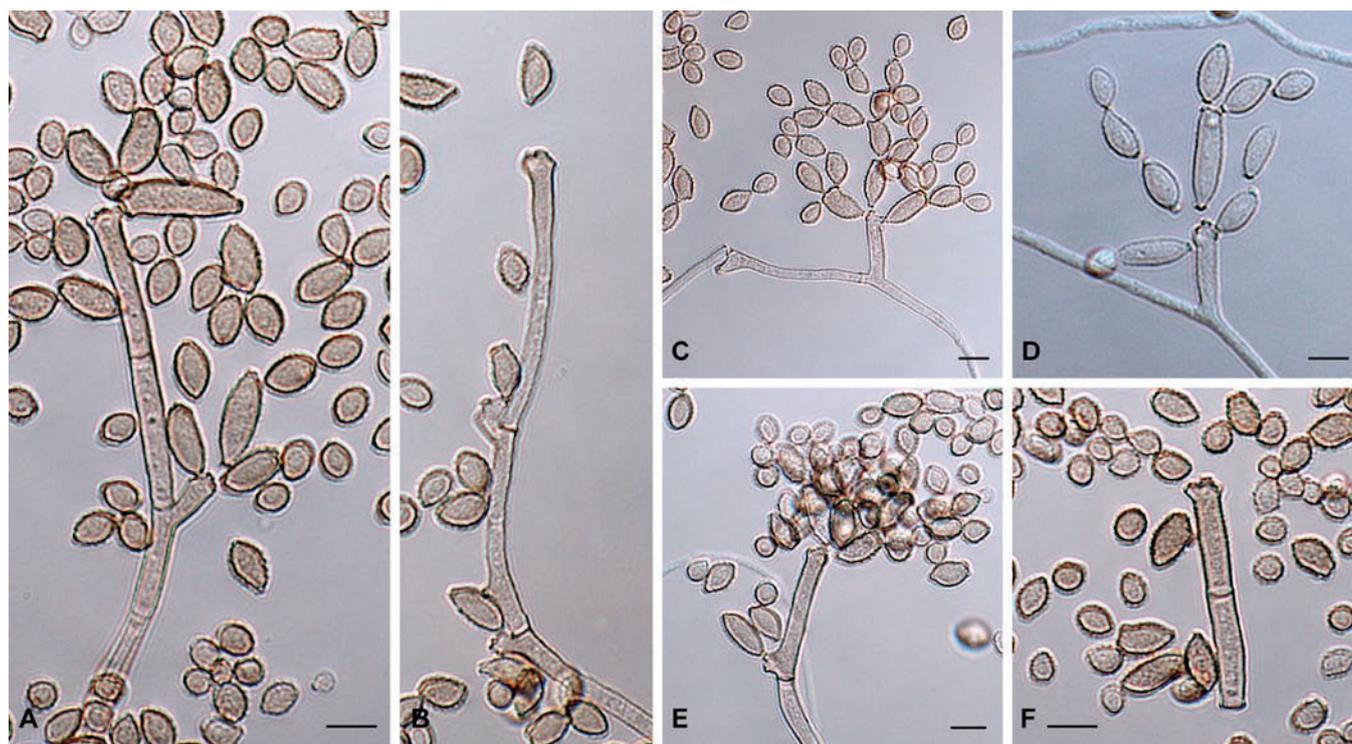
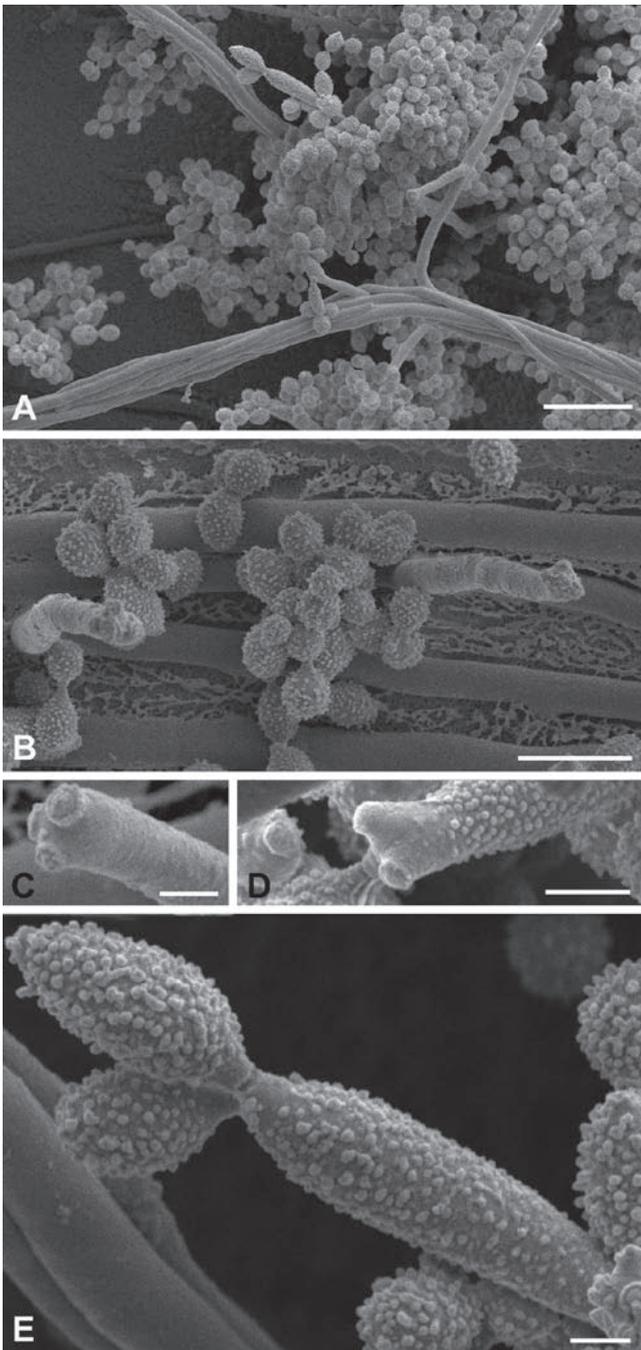


Fig. 44. *Cladosporium tenellum* (CPC 12053). A–C, E. Macro-nematous conidiophore. D. Micronematous conidiophore. F. Ramoconidium and conidia. Scale bars = 10  $\mu$ m.



**Fig. 45.** *Cladosporium tenellum* (CPC 12053). A. A bird's eye view of a colony of *C. tenellum* with its very characteristic bundles of aerial hyphae. Numerous conidia are visible, formed on simple conidiophores. B. Hyphae that run on the agar surface give rise to conidiophores and numerous conidia, that are relatively rounded. C. Conidiophore ends are rather simple and have large scars. D. Hila on a secondary ramoconidium with non-ornamented area. E. Detail of the prominent ornamentation on a secondary ramoconidium. Scale bars: A = 20  $\mu\text{m}$ , B = 10  $\mu\text{m}$ , C, E = 2  $\mu\text{m}$ , D = 5  $\mu\text{m}$ .

and substrates. Besides these strains, several additional isolates listed under excluded strains are morphologically indistinguishable from *C. subtilissimum* in culture, but genetically different, clustering in various subclades. They are indicated as *Cladosporium* sp. in the tree (Fig. 3).

***Cladosporium tenellum*** K. Schub., Zalar, Crous & U. Braun, **sp. nov.** MycoBank MB504581. Figs 43–45.

**Etymology:** Refers to its narrow conidiophores and conidia.

Differt a *Cladosporio cladosporioide* conidiophoris et conidiis semper asperulatis, locis conidiogenis apicalibus, numerosis, hilis quoque numerosis, conidiophoris angustioribus, (1–)1.5–3.5(–4)  $\mu\text{m}$  latis; et a *Cladosporio subtilissimo* loci conidiogenis et hilis apicalibus, numerosis, angustioribus, saepe 1–1.5  $\mu\text{m}$  latis, conidiis minutis numerosis, saepe globosis.

*Mycelium* sparingly branched, 1–3  $\mu\text{m}$  wide, septate, septa often not very conspicuous, not constricted at the septa, sometimes slightly swollen, subhyaline, smooth, walls unthickened. *Conidiophores* macronematous and micronematous, solitary, arising terminally or laterally from plagiotropous or ascending hyphae, erect or subdecumbent, almost straight to more or less flexuous, cylindrical, sometimes geniculate towards the apex, but not nodulose, sometimes with short lateral prolongations at the apex, unbranched to once or twice branched (angle usually 30–45° degree, sometimes up to 90°), branches usually below a septum, 6–200  $\times$  (1–)2–4(–5)  $\mu\text{m}$ , septate, septa not very conspicuous, not constricted at the septa, subhyaline to pale brown, almost smooth to usually asperulate, walls unthickened or almost so. *Conidiogenous cells* integrated, terminal or intercalary, sometimes conidiophores reduced to conidiogenous cells, cylindrical, sometimes geniculate, non-nodulose, 6–40  $\mu\text{m}$  long, proliferation sympodial, with several conidiogenous loci often crowded at the apex and sometimes also at a lower level, situated on small lateral shoulders, unilateral swellings or prolongations, with up to 6(–10) denticulate loci, forming sympodial clusters of pronounced scars, intercalary conidiogenous cells with short or somewhat long lateral outgrowths, short denticle-like or long branches with several scars at the apex, usually below a septum, loci protuberant, 1–1.5(–2)  $\mu\text{m}$  diam, thickened and darkened-refractive. *Ramoconidia* sometimes occurring, cylindrical, up to 32  $\mu\text{m}$  long, 2.5–4  $\mu\text{m}$  wide, with a broadly truncate, unthickened base, about 2  $\mu\text{m}$  wide. *Conidia* catenate, formed in branched chains, straight, small terminal conidia globose, subglobose, ovoid, oval, 3–6  $\times$  2.5–3.5  $\mu\text{m}$  [av.  $\pm$  SD, 4.5 ( $\pm$  1.3)  $\times$  2.8 ( $\pm$  0.4)  $\mu\text{m}$ ], aseptate, asperulate, with 0–2 distal hila, intercalary conidia and secondary ramoconidia ellipsoid-ovoid, ellipsoid to subcylindrical, 3.5–20(–28)  $\times$  (2.5–)3–5(–6)  $\mu\text{m}$  [av.  $\pm$  SD, 12.4 ( $\pm$  5.4)  $\times$  4.1 ( $\pm$  0.7)  $\mu\text{m}$ ], 0–1-septate, rarely with up to three septa, sometimes slightly constricted at the septa, subhyaline, pale brown to medium olivaceous-brown, asperulate or verruculose (muricate, granulate or colliculate under SEM), walls unthickened or slightly thickened, apex rounded or slightly to distinctly attenuated towards apex and base, often forming several apical hila, up to 7(–9), crowded, situated on small lateral outgrowths giving them a somewhat irregular appearance, hila protuberant, 0.5–1.5  $\mu\text{m}$  diam, thickened and darkened-refractive; microcyclic conidiogenesis sometimes occurring.

**Cultural characteristics:** Colonies on PDA reaching 27–34 mm diam after 14 d at 25 °C, smoke-grey, grey-olivaceous to olivaceous-grey, olivaceous-grey to iron-grey reverse, velvety to powdery, margin regular, entire edge, narrow, colourless to white, aerial mycelium absent or sparingly formed, felty, whitish, growth regular, flat, radially furrowed, with folded and elevated colony centre, deep into the agar, with age forming few to numerous prominent exudates, sporulation profuse, few high conidiophores formed. Colonies on MEA reaching 25–44 mm diam after 14 d at 25 °C, olivaceous-grey to olivaceous- or iron-grey due to abundant sporulation in the colony centre, velvety, margin regular, entire edge, narrow, colourless, white to pale olivaceous-grey, aerial mycelium loose, diffuse, growth convex with papillate surface, radially furrowed, wrinkled, without prominent exudates, sporulating. Colonies on OA reaching 23–32 mm diam after 14 d at 25 °C, grey-olivaceous, olivaceous-

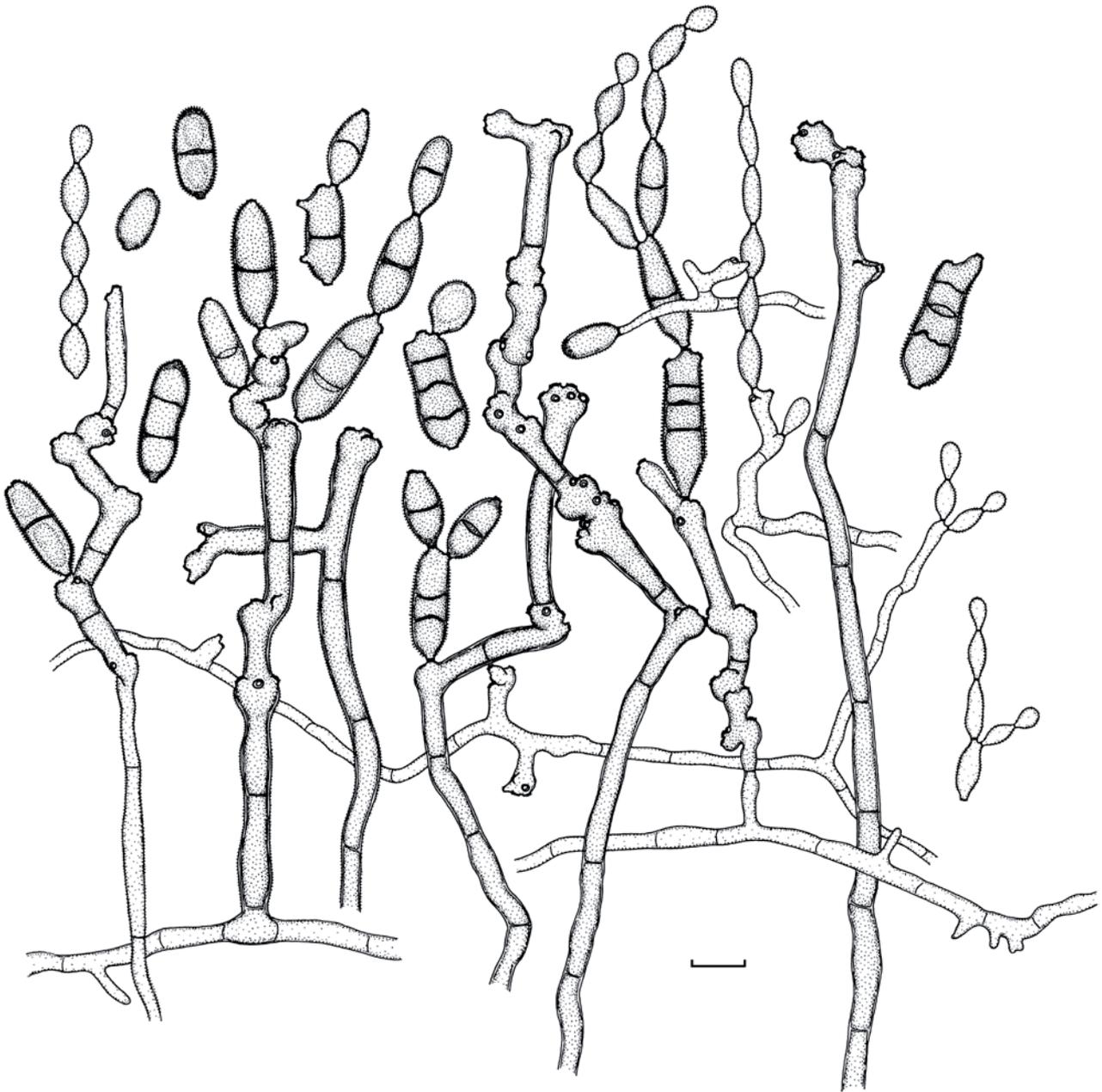


Fig. 46. *Cladosporium variabile* (CPC 12751). Macro- and micronematous conidiophores and conidia. Scale bar = 10  $\mu\text{m}$ . K. Schubert del.

grey to olivaceous due to abundant sporulation in the colony centre, olivaceous- or iron-grey reverse, velvety, margin regular, entire edge, narrow, colourless or white, aerial mycelium sparse, diffuse, floccose, growth flat to low convex, radially furrowed, wrinkled, without prominent exudates, sporulation profuse.

*Specimens examined:* Israel, Eilat, isolated from hypersaline water from salterns, 2004, N. Gunde-Cimerman, CBS 121633 = CPC 12051 = EXF-1083; Ein Bokek, isolated from hypersaline water of the Dead Sea, 2004, M. Ota, CBS-H 19866, **holotype**, isotype HAL 2029 F, culture ex-type CBS 121634 = CPC 12053 = EXF-1735. **U.S.A.**, Seattle, University of Washington campus, isolated from *Phyllactinia* sp. (*Erysiphaceae*) on leaves of *Corylus* sp. (*Corylaceae*), 16 Sep. 2004, D. Glawe, CPC 11813.

*Substrates and distribution:* Hypersaline water and plant material; Israel, U.S.A.

*Notes:* *Cladosporium subtilissimum* and *C. cladosporioides* are morphologically comparable with the new species *C. tenellum*, but *C. cladosporioides* deviates in having usually smooth conidiophores

and conidia with only few conidiogenous loci and conidial hila crowded at the apex and somewhat wider conidiophores, 3–5(–6)  $\mu\text{m}$ ; and in *C. subtilissimum* the small terminal conidia are not globose but rather narrowly obovoid to limoniform, the conidiogenous loci and conidial hila are somewhat wider, (0.5–)0.8–2(–2.2)  $\mu\text{m}$ , and at the apices of conidiophores and conidia only few scars are formed.

*Cladosporium ramotenellum*, which morphologically also resembles *C. tenellum*, possesses longer and narrower, 0–3-septate conidia, 2.5–35  $\times$  2–4(–5)  $\mu\text{m}$ , but forms only few conidiogenous loci and conidial hila at the apices of conidiophores and conidia.

***Cladosporium variabile*** (Cooke) G.A. de Vries, *Contr. Knowl. Genus Cladosporium*: 85. 1952. Figs 46–48.

*Basionym:* *Heterosporium variabile* Cooke, *Grevillea* 5(35): 123. 1877.

= *Helminthosporium variabile* Cooke, *Fungi Brit. Exs. Ser. 1*, No. 360. 1870, *nom. inval.*

= *Cladosporium subnodosum* Cooke, *Grevillea* 17(83): 67. 1889.

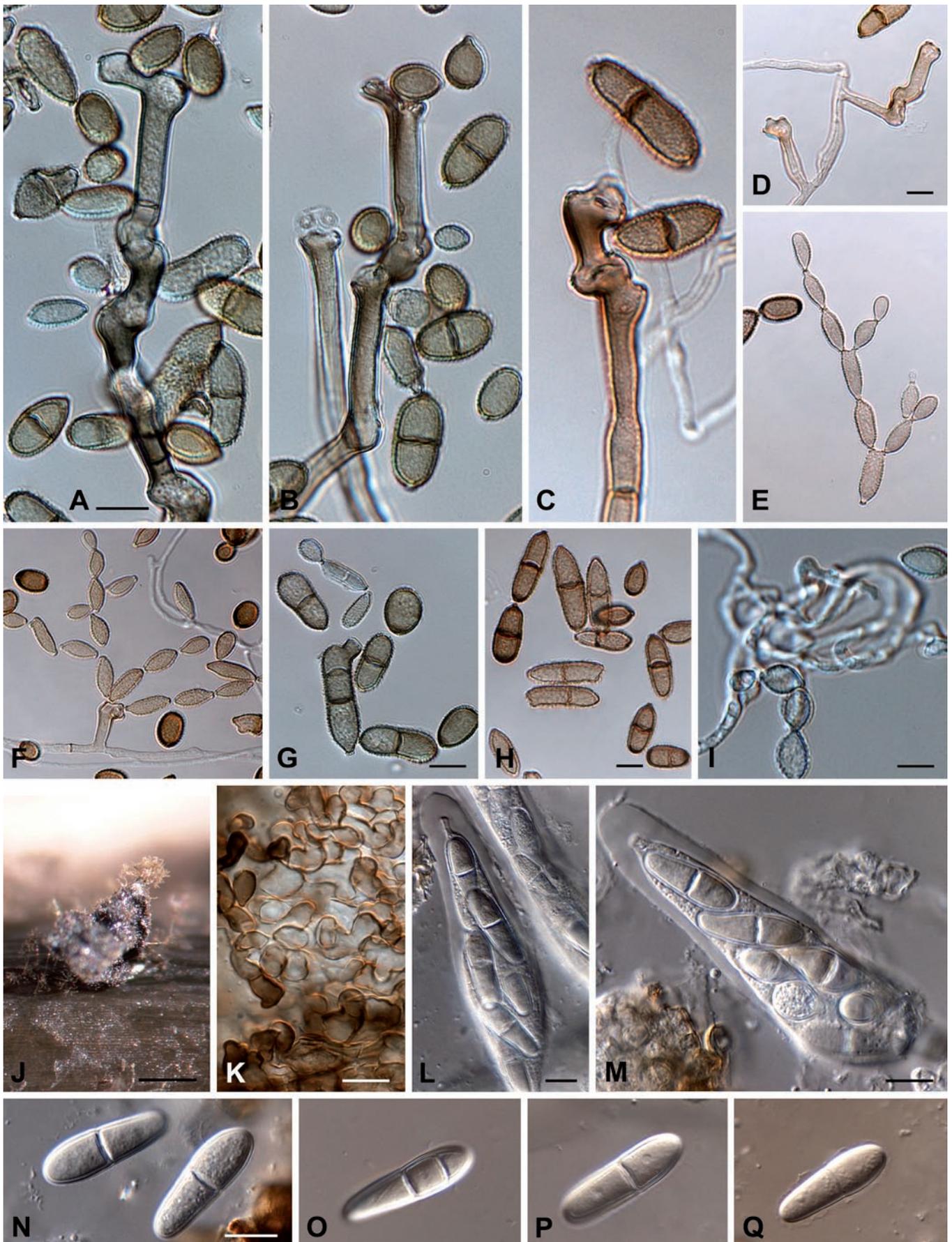
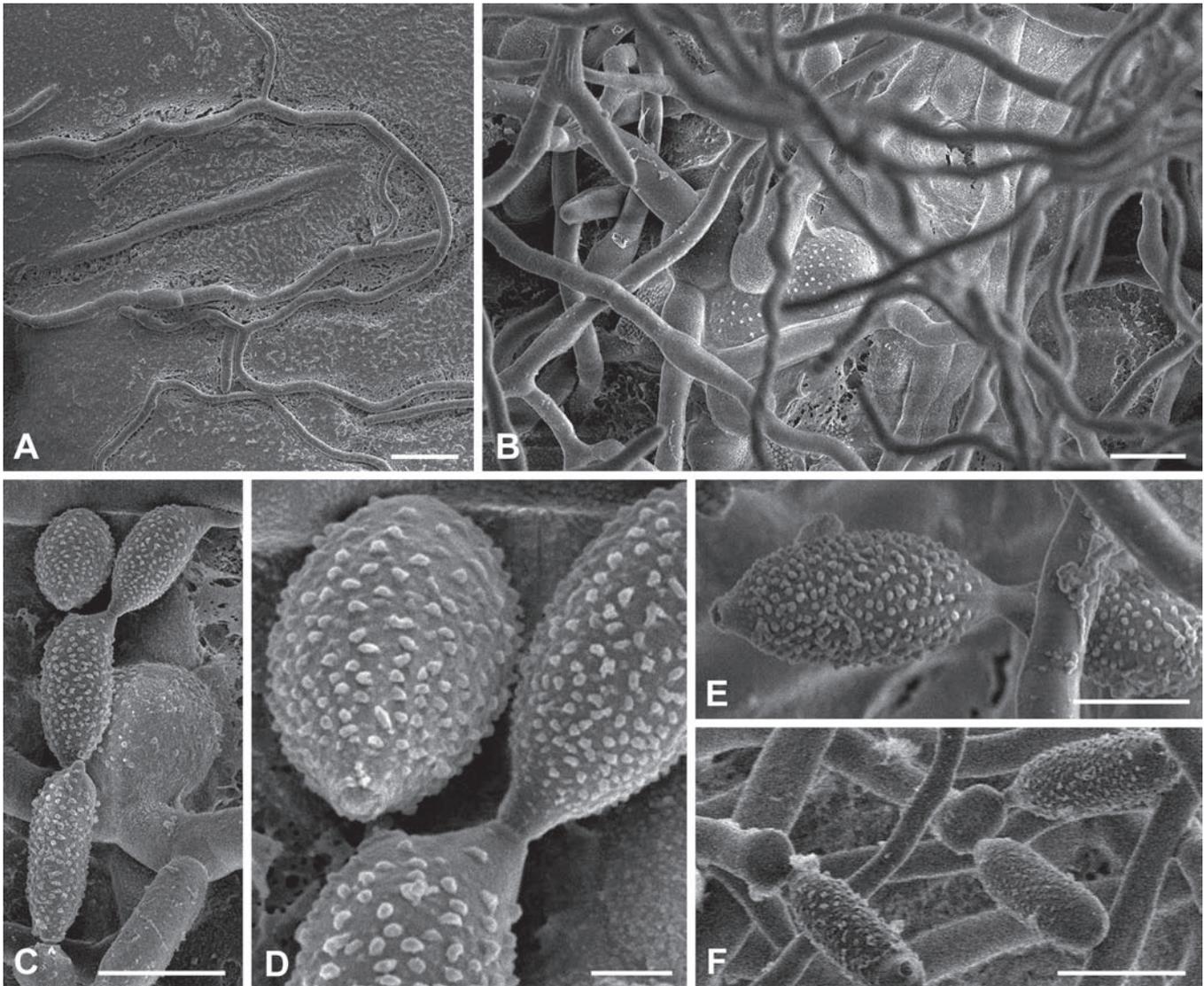


Fig. 47. *Cladosporium variabile* and its teleomorph *Davidiella variabile* (CPC 12751). A–C. Macronematous conidiophores. D, F. Micronematous conidiophores. E, G–H. Conidia. I. Twisted aerial mycelium. J. Ascomata formed on nettle stem in culture. K. Surface view of ascomal wall of *textura epidermoidea*. L–M. Asci. N–P. Ascospores. Q. Ascus with a sheath. Scale bars A, D, G–J, K–N = 10  $\mu$ m, J = 250  $\mu$ m.



**Fig. 48.** *Cladosporium variabile* (CPC 12753). A. Survey of hyphae that grow on the agar surface. Some of the fungal cells have a swollen appearance and could develop into a "foot cell" that gives rise to a conidiophore. B. A number of aerial hyphae obstruct the swollen, large structures on the agar surface, which give rise to conidiophores. Some of them appear ornamented. C. A series of conidia formed on a conidiophore (bottom of the micrograph). D. Detail of the ornamented conidia. The ornamentations are isolated and dispersed. Note also the ornamentation-free scar zone and the hilum of the left cell. E. Two conidia behind an aerial hypha. F. Two conidiophores forming secondary ramoconidia. Note the bulbous shape of the spore-forming apparatus. This micrograph is from an uncoated sample. Scale bars: A–C, F = 10  $\mu\text{m}$ , D = 2  $\mu\text{m}$ , E = 5  $\mu\text{m}$ .

**Teleomorph: *Davidiella variabile*** Crous, K. Schub. & U. Braun, **sp. nov.** MycoBank MB504583.

*Davidiellae tassianae* similis, sed ascosporis maioribus, (22–)26–30(–35)  $\times$  (7–)7.5–8(–9)  $\mu\text{m}$ , et ascis latoribus, plus quam 18  $\mu\text{m}$ .

*Ascomata* pseudothecial, black, superficial, situated on a small stroma, globose, up to 250  $\mu\text{m}$  diam, with 1–3 ostiolate necks; ostioles periphysate, with apical periphysoids present; wall consisting of 3–6 layers of dark brown *textura angularis*, *textura epidermoidea* in surface view. *Asci* fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoid, straight to slightly curved, 8-spored, 70–95  $\times$  18–28  $\mu\text{m}$ ; with pseudoparenchymatal cells of the hamathecium persistent. *Ascospores* tri- to multiseriate, overlapping, hyaline, with irregular lumina, thick-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, widest near the middle of the apical cell, medianly 1-septate, not to slightly constricted at the septum, at times developing a second septum in each cell, several ascospores with persistent, irregular mucoid sheath, (22–)26–30(–35)  $\times$  (7–)7.5–8(–9)  $\mu\text{m}$ .

*Mycelium* immersed and superficial, irregularly branched, aerial mycelium twisted and spirally coiled, 1–3  $\mu\text{m}$  wide, septate, sometimes with swellings or small lateral outgrowths, hyaline to subhyaline, smooth, walls unthickened, hyphae which give rise to conidiophores somewhat wider, 3–4.5  $\mu\text{m}$ , subhyaline to pale brown, almost smooth to minutely verruculose, sometimes enveloped by a polysaccharide-like cover. *Conidiophores* usually macronematous, but also micronematous, arising terminally from ascending hyphae or laterally from plagiotropous hyphae. *Macronematous conidiophores* erect, more or less straight to flexuous, often distinctly geniculate-sinuuous forming lateral shoulders or unilateral swellings, sometimes zigzag-like or somewhat coralloid, nodulose, swellings at first terminal, then becoming lateral due to sympodial proliferation, often as distinct lateral shoulders, unbranched, sometimes once branched, 6–180  $\times$  (2.5–)3–6  $\mu\text{m}$ , swellings (3–)6–11  $\mu\text{m}$  wide, septate, not constricted at the septa, pale to medium olivaceous-brown or brown, usually verruculose, walls somewhat thickened, about 1  $\mu\text{m}$  thick, sometimes appearing to be two-layered. *Conidiogenous cells* integrated, terminal and intercalary, cylindrical, nodulose to nodose,

with a single or two swellings per cell, swellings apart from each other or formed in short succession, loci confined to swellings, up to six per node, protuberant, 1–2 µm diam, thickened and darkened-refractive. *Micronematous conidiophores* erect, straight to slightly flexuous, unbranched, usually without swellings, filiform to narrowly cylindrical, sometimes only as short lateral outgrowths of hyphae, often almost indistinguishable from hyphae, up to 50 µm long, 1.5–2.5(–3) µm wide, longer ones pluriseptate, septa appear to be somewhat more darkened, with very short cells, 4–12 µm long, subhyaline to pale brown, smooth, walls unthickened or almost so. *Conidiogenous cells* integrated, usually terminal, rarely intercalary, cylindrical, non-nodulose, with a single, two or few conidiogenous loci at the distal end, protuberant, up to 2 µm diam, thickened and darkened-refractive. *Conidia* catenate, in branched chains, straight, subglobose, obovoid, oval, broadly ellipsoid to cylindrical, sometimes clavate, 4–26(–30) × (3.5–)5–9(–10) µm [av. ± SD, 16.8 (± 6.9) × 6.5 (± 1.4) µm], 0–3-septate, usually not constricted at the septa, septa becoming sinuous with age, often appearing to be darkened, pale to medium or even dark brown or olivaceous-brown, verruculose to densely verrucose or echinulate (granulate under SEM), walls slightly to distinctly thickened in larger conidia, apex and base broadly rounded, sometimes broadly truncate or somewhat attenuated, apex and base often appear to be darkened or at least refractive, hila protuberant to somewhat sessile (within the outer wall ornamentation), (0.8–)1–2 µm diam, thickened and darkened-refractive; microcyclic conidiogenesis occurring.

**Cultural characteristics:** Colonies on PDA attaining 29 mm diam after 14 d at 25 °C, olivaceous to olivaceous-grey or iron-grey, iron-grey or olivaceous-grey reverse, velvety to powdery, margin regular, entire edge to fimbriate, almost colourless, aerial mycelium whitish turning olivaceous-grey, sometimes reddish, greyish rose, woolly-felty, growth flat with elevated colony centre, somewhat folded or radially furrowed, with age forming several very small but prominent exudates, sporulation profuse. Colonies on MEA attaining 27 mm diam after 14 d at 25 °C, olivaceous-grey to iron-grey, white to pale olivaceous-grey in the centre due to abundant aerial mycelium, velvety, margin very narrow, colourless, more or less entire edge, radially furrowed, aerial mycelium fluffy to floccose, dense, growth low convex with wrinkled and folded centre, without exudates, sporulation profuse. Colonies on OA attaining 25 mm diam after 14 d at 25 °C, iron-grey or olivaceous, margin regular, entire edge, narrow, white, glabrous, aerial mycelium whitish, at first mainly in the colony centre, high, dense, floccose, growth flat, abundantly sporulating, no exudates.

**Specimens examined:** Great Britain, Wales, Montgomeryshire, Welshpool, Forden Vicarage, on *Spinacia oleracea* (*Chenopodiaceae*), J.E. Vize, Cooke, Fungi Brit. Exs. Ser. I, No. 360, K, holotype. U.S.A., Washington, isolated from *Spinacia oleracea*, 1 Jan. 2003, L. DuToit, CBS-H 19867, epitype designated here of *C. variabile* and *D. variabile*, cultures ex-epitype CBS 121635 = CPC 12753, CPC 12751.

**Substrate and distribution:** Leaf-spotting fungus on *Spinacia oleracea*; Asia (China, India, Iraq, Pakistan), Europe (Austria, Belgium, Cyprus, Denmark, France, Germany, Great Britain, Hungary, Italy, Montenegro, Netherlands, Norway, Romania, Spain, Turkey), North America (U.S.A.).

**Literature:** de Vries (1952: 85–88), Ellis (1971: 315), Ellis & Ellis (1985: 429), David (1995b; 1997: 94, 96–98), Ho *et al.* (1999: 144).

**Notes:** *In vivo* the conidia are usually longer, somewhat wider and more frequently septate, (6.5–)10–45(–55) × (4.5–)6–14(–17) µm,

0–4(–5)-septate (Schubert 2005). In culture the dimensions tend to be smaller, which was already mentioned by de Vries (1952).

This leaf-spotting fungus superficially resembles *C. macrocarpum*, but besides its pathogenicity to *Spinacia*, *C. variabile* differs from the latter species in having distinctly larger and more frequently septate conidia on the natural host, forming twisted and spirally coiled aerial mycelium in culture and in having lower growth rates in culture (29 mm after 14 d on PDA versus 38 mm on average in *C. macrocarpum*). Furthermore, the conidial septa of *C. variabile* are often distinctly darkened, become sinuous with age and the apex and base of the conidia often appear to be distinctly darkened. A *Davidiella* teleomorph has not previously been reported for this species.

### The cladosporioides complex

This species complex will be treated in an additional paper in this series, dealing with the epitypification of this common and widespread species, and with numerous isolates identified and deposited as *C. cladosporioides*.

## DISCUSSION

In the present study, a multilocus genealogy supported by light and SEM microscopy, and cultural characteristics was used to redefine species borders within *Cladosporium*, especially within the *C. herbarum* complex. Most of the diagnostic features used for species delimitation on host material (Heuchert *et al.* 2005, Schubert 2005), proved to be applicable in culture. However, morphological features were often more pronounced *in vivo* than *in vitro*. For instance, conidiophore arrangement is not applicable to cultures, conidiophore and conidium widths were often narrower in culture than on the natural host, and macro- as well as microconidiophores were often observed in culture, but not on host material. All species belonging to the *C. herbarum* complex are characterised by possessing conidia which are ornamented, the ornamentation ranging from minutely verruculose to verrucose, echinulate or spiny whereas in the *C. sphaerospermum* complex species with both smooth-walled as well as ornamented conidia are included (Zalar *et al.* 2007). The surface ornamentation varies based on the length of surface protuberances and in the density of ornamentation. Furthermore, the conidia are mainly catenate, formed in unbranched or branched chains. However, species previously referred to the genus *Heterosporium*, which usually produce solitary conidia or unbranched chains of two or three conidia at the most on the natural host, also belong to this species complex (e.g., *C. iridis*). *In vitro* these chains can become longer and may even be branched. The conidiophores formed in culture are mostly macro- but may also be micronematous, sometimes forming different types of conidia that vary in shape and size from each other. Most of the species possess nodulose conidiophores with the conidiogenesis confined to the usually lateral swellings. However, this phenetic trend is not consistently expressed in all of the species belonging to the *C. herbarum* complex. The various *Cladosporium* species within the *C. herbarum* complex were observed to have subtle differences in their phenotype which were visible via cryo-electron microscopy (cryoSEM), and are discussed below.

**Fungal colonies:** CryoSEM provides the opportunity to study the organisation of the fungal colony at relatively low magnifications. *Cladosporium tenellum* proved to be the only fungus able to form

aerial hyphal strands under the conditions studied. *Cladosporium variabile* formed abundant aerial hyphae, but in *C. spinulosum* these were sparse, and only conidiophores were observed on the agar surface. Three-day-old colonies of *C. subinflatum* formed numerous, long aerial hyphae, and no conidiophores could be discerned under the binocular. After 11 d the aerial hyphae seemed to have disappeared, giving rise to conidiophores. *Cladosporium antarcticum*, *C. variabile* and *C. ramotenellum* showed very large, swollen (> 10 µm) cells which gave rise to conidiophores. With *C. variabile* possible earlier stages of these cells were visible (Fig. 48), which gave rise to conidiophores. More than one conidiophore could be formed on such a structure (*C. variabile* and *C. ramotenellum*). *Cladosporium herbarum* has very wide hyphae on the agar surface, which gave rise to conidiophores as lateral branches. These wide hyphae were observed to anastomose, which may provide a firm interconnected supporting mycelium for these conidiophores. In *C. herbaroides* these wide hyphae could also be discerned, but conidiophore formation was less obvious. Similarly, *C. tenellum* has wide, parallel hyphae that gave rise to conidiophores.

These observations reveal fungal structures in *Cladosporium* that have not previously been reported on, and that raise intriguing biological questions. For instance, why are hyphal strands observed in some species (*C. tenellum*), and not in others, and what happens to the aerial hyphae during incubation in some species such as *C. subinflatum*? Furthermore, these preliminary results suggest that CryoSEM provide additional features that can be used to distinguish the different species in the *C. herbarum* complex.

*Fine details of morphological structures:* CryoSEM provides the opportunity to study fine details of the conidiophore, (ramo)conidia and scars. Samples can be studied at magnification up to × 8 000, revealing details at a refinement far above what is possible under the light microscope (LM) (Fig. 2). However, the LM micrographs provide information about the different compartments of ramoconidia, as well as the thickness and pigmentation of the cell wall of different structures. With other words, the different techniques are complementary, and both reveal fungal details that build up the picture that defines a fungal species.

Conidiophores can vary with respect to their width and the length. *Cladosporium ramotenellum*, *C. antarcticum* and *C. variabile* have tapered conidiophores formed on large globoid “foot cells”. The conidiophore itself can be branched. *Cladosporium spinulosum* has conidiophores that rise from the agar surface, but can have a common point of origin. These conidiophores are not tapered, but parallel and slender. The conidiophores of *C. bruhnei* and *C. herbaroides* are rather long, and can appear as aerial hyphae.

An important feature of the conidiophore is the location where the conidia are formed. Conidiophore ends can be simple and tubular, or rounded to more complex, several times geniculate, with several scars. Conidiophore ends become more elaborate over time. *Cladosporium spinulosum* and *C. tenellum* have nearly tubular conidiophore ends, with often very closely aggregated scars. The conidiophore ends of *C. subinflatum* are also near tubular with a hint of bulbousness. *Cladosporium subtilissimum* is similar, but with somewhat more elevated scars that look denticulate. *Cladosporium variabile* has nodulose, somewhat swollen apices with often sessile, almost inconspicuous scars. In the case of *C. macrocarpum*, these structures are also nodulose to nodose and somewhat bent, with only slightly protuberant loci. *Cladosporium ramotenellum* has tubular conidiophore ends with pronounced scars. *Cladosporium antarcticum* has very characteristic, tapered ends, and widely dispersed (5 µm) scars. More complex conidiophore ends are more irregular in shape, and have scars dispersed over a longer

distance, such as observed in *C. bruhnei*, *C. herbaroides*, and *C. herbarum*.

Secondary ramoconidia are usually the first conidia formed on a conidiophore. They are often multicellular, and have one basal cladosporioid hilum, and more at the apex. Few *Cladosporium* species additionally form true ramoconidia representing apical parts of the conidiophore which secede at a septum resulting in an undifferentiated non-coronate base and function as conidia. Ramification of conidial chains is realised through these conidia. They can occur in up to three stages, which results in elaborated spore structures. The basal secondary ramoconidium is invariably the largest, and cell size decreases through a series of additional secondary ramoconidia, intercalary conidia, and small, terminal conidia. The elongation of secondary ramoconidia varies among the different species. *Cladosporium macrocarpum* has broadly ellipsoid to cylindrical secondary ramoconidia usually with broadly rounded ends, like *C. variabile*, while *C. spinulosum* has secondary ramoconidia that can often hardly be discerned from the conidia that are formed at later stages. The conidia of the other species roughly fall between these species. The most notable structures on these conidia are their ornamentation, scar pattern and morphology. *Cladosporium spinulosum* forms numerous globose to subsphaerical spores with digitate, non-tapered surface ornamentation, which is unique for all the species discussed here. In his study on *Cladosporium* wall ornamentation, David (1997) recognised three classes of echinulate surfaces (aculeate, spinulose, digitate), and five classes of verrucose surfaces (muricate, granulate, colliculate, pustulate and pedicellate) (Fig. 2). The ornamentation particles vary in shape, width, height and density. The most strongly ornamented conidia of the species examined by SEM are formed by *C. ossifragi*, with the ornamentation both large (up to 0.5 µm wide) and high, and can be regarded as densely muricately ornamented. Strong ornamentation is also seen in *C. herbaroides*, which is mostly granulate. *Cladosporium tenellum* (with muricate, granulate and colliculate tendencies) and *C. bruhnei* (mostly granulate with some muricate projections) have relatively large ornamentation structures with slightly more space between the units than the other two species. *Cladosporium antarcticum*, *C. ramotenellum*, *C. variabile* and *C. subtilissimum* exhibit rather large granulate ornamentations that have a more irregular and variable shape. *Cladosporium subinflatum* shows the widest dispersed structures of the series, being muricate. In contrast, *C. macrocarpum* has a very neat and regular pattern of muricate ornamentation. The area of formation of new spores on conidia is invariably not ornamented, and hila all have the typical *Cladosporium* morphology with a central dome and a ring-like structure around it.

*Branching patterns:* Spores usually show a “line of weakness” between them where the coronate scars form. It seems that scars at both sides of the line of weakness have the central dome structure, which appears to play a major role in the effective mechanism *Cladosporium* employs for spore dispersal, with the dome actively pushing the conidia apart. This mechanism is also illustrated in David (1997, fig. 2E). Indeed, conidia of *Cladosporium* are very easily dislodged; even snap freezing or the electrical forces inside the SEM often result in dislodgement of the spores in a powdery “wave”. It is no surprise, therefore, that *Cladosporium* conidia are to be found in most air samples. In *Cladosporium*, conidia are mostly formed in chains, with the size invariably decreasing from the base to the apex of the row. Upon formation each conidium is separated from the conidiophore, or previously formed conidium, and hence from its nutrients. The basal ramoconidium or secondary ramoconidia have the nutrients and metabolic power to produce

a number of additional secondary ramoconidia that in turn could produce a chain of intercalary conidia, and finally, some small, single-celled, terminal conidia. Further research is still necessary to determine if specific branching patterns can be linked to different species.

A surprising finding from the present study is the huge diversity in species and genotypes that exist in nature, be it in the indoor environment, on fruit surfaces, or in extreme ecological niches such as salterns, etc. It is clear that detailed studies would be required to find and characterise other species of *Cladosporium* and obtain a better understanding of their host ranges and ecology. A further surprise lay in the fact that several of these species are capable of sexual reproduction, and readily form *Davidiella* teleomorphs in culture. The *Davidiella* states induced here were all from homothallic species. Further attention now needs to be given to elucidating teleomorphs from other species which, as in *Mycosphaerella* (Groenewald *et al.* 2006, Ware *et al.* 2007) could be heterothallic, and experiencing clandestine sex.

Despite the occurrence of many different genotypes in variable genes, the degree of diversity in the entire data set was low. For the majority of the species ITS was almost invariant, with only six genotypes in the entire dataset. This suggests a very recent evolution. The standardised index of association ( $I_A^S$ ) was high (0.3914), indicating an overabundance of clonality and / or inbreeding, the latter possibly matching with observed homothallism of *Davidiella* teleomorphs. Clonality was visualised with SPLITSTREE software, where star-shaped representations without any sign of reticulation were obtained for all genes, though at different branch lengths (Fig. 5). With STRUCTURE software an optimal subdivision was achieved at six putative groups. Some of them were distinctly separated, yielding a theta ( $\theta$ ) around 0.14, but in most cases there was considerable overlap in representation of motifs, with  $\theta$  at significantly higher values. Results are difficult to interpret due to the small size of the data set compared to the number of predicted groups, and due to unknown but probably large sampling effects. With optimal subdivision of the 79 strains at a hypothesised value of  $K = 6$  (Fig. 4), still a large degree of inter-group similarity was noted, as was the case at any other level of  $K$ . This was particularly obvious when data from the most variable genes (EF and ACT) are superimposed (Fig. 4). The ACT groups are further subdivided by EF data, but in many cases the same EF motif (indicated with arrows) was encountered in different (multilocus) species, for example in *C. antarcticum*, *C. spinulosum*, *Davidiella* sp., and the various clusters comprising *Cladosporium* strains which are phenotypically almost indistinguishable but genetically distinct from *C. subtilissimum*. A similar situation was found with the distribution of EF genotypes (indicated with doughnuts) in *C. herbarum* and *C. macrocarpum*. Nevertheless, the data set showed significant structuring, partly correlating with geography, e.g. the EF-determined cluster of *C. bruhnei* that contained isolates from different sources in The Netherlands. Differences may be over-accentuated by known sampling effects, particularly in *C. herbarum* and *C. macrocarpum*, where single-spore isolates from a single collection are included.

Taken together the data suggest a recent, preponderantly clonal evolution, combined with limited natural selection at a low level of evolutionary pressure. As a result, many genotypes produced by hot spots in the genes analysed have survived, leading to nearly random variation in the data set. Many combinations of motifs that possibly could emerge have maintained in the course of time due to the absence of recombination. This indicates that the observed structure is that of populations within a single species, and consequently a distinction of clonal "species" could be redundant.

This conclusion is underlined by the fact that a single source in a single location can be colonised by various genotypes, such as grapes in the U.S.A. containing three different, closely related genotypes. However, the phenomenon of co-inhabitation by different *Mycosphaerella* species on the same lesion of *Eucalyptus* has been described before (Crous 1998, Crous *et al.* 2004) and it is therefore not surprising that different genotypes occurring close together are also observed for the related genus *Cladosporium*. There is no obvious ecological difference between genotypes, and hence isolates seem to have equal fitness.

However, in general we noticed a remarkable concordance of genetic and phenetic characters. The morphological study was done prior to sequencing, and nearly all morphotypes clustered in separate molecular entities. There are some exceptions, such as with *C. antarcticum* with striking morphology that was almost identical on the molecular level to *Cladosporium* spp. that resemble *C. subtilissimum* and would normally have been interpreted to be a mutant. Conversely, nearly all genetically distinguishable groups proved to be morphologically different, with the exception of members of the *C. subtilissimum* s. lat. complex (indicated as *Cladosporium* sp. in Fig. 3 and Table 1). The possibility remains that the found genetic parameters correlate with phenetic markers other than morphology, such as virulence, toxins or antifungal susceptibilities. For this reason we introduce the established entities here as formal species. They can be diagnosed by ACT sequencing or by phenetic characters provided in the key. For simple routine purposes, however, they can be seen and treated as the "*C. herbarum* complex", based on their close phylogenetic relationships.

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# Mating-type genes and the genetic structure of a world-wide collection of the tomato pathogen *Cladosporium fulvum*

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## Abstract

Two mating-type genes, designated *MATI-1-1* and *MATI-2-1*, were cloned and sequenced from the presumed asexual ascomycete *Cladosporium fulvum* (syn. *Passalora fulva*). The encoded products are highly homologous to mating-type proteins from members of the Mycosphaerellaceae, such as *Mycosphaerella graminicola* and *Cercospora beticola*. In addition, the two MAT idiomorphs of *C. fulvum* showed regions of homology and each contained one additional putative ORF without significant similarity to known sequences. The distribution of the two mating-type genes in a world-wide collection of 86 *C. fulvum* strains showed a departure from a 1:1 ratio ( $\chi^2 = 4.81$ ,  $df = 1$ ). AFLP analysis revealed a high level of genotypic diversity, while strains of the fungus were identified with similar virulence spectra but distinct AFLP patterns and opposite mating-types. These features could suggest the occurrence of recombination in *C. fulvum*.

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**Keywords:** AFLP; Ascomycete; Asexual; Genotypic diversity; Mating-type genes; Polymorphism; Population differentiation; Race; Recombination

## 1. Introduction

*Cladosporium fulvum* [syn *Passalora fulva* (Braun et al., 2003)] is a non-obligate biotrophic fungus that causes leaf mold on tomato plants (*Lycopersicon esculentum*). It is an asexual hyphomycetous member of the Mycosphaerellaceae (*Capnodiales*), suggesting that if a teleomorph state were to be found for this fungus, it would be a species of *Mycosphaerella* (Braun et al., 2003; Goodwin et al., 2001). Typical disease symptoms on tomato plants are patches of white mold on the abaxial leaf surface that turn brown when the fungus starts to sporulate (Thomma et al., 2005). The disease is thought to have originated from South America, the centre of origin of tomato and other wild

*Lycopersicon* species (Cooke, 1906), but to date it has an almost world-wide distribution as tomatoes are globally produced outdoors and in glasshouses, under cultivation practices that are often conducive to *C. fulvum* infections.

*Cladosporium fulvum* used to be an economically important disease that caused considerable yield losses. However, the introduction during the last 50 years of *Cf*-resistance (for *C. fulvum*) genes into cultivated tomato from wild *Lycopersicon* species, successfully contained the disease in most cultivation areas (Joosten and De Wit, 1999; Rivas and Thomas, 2005). Over the last few decades, the pathosystem *C. fulvum*-tomato has been intensively studied, and has become a model for the study of gene-for-gene interactions (De Wit et al., 2002). In that respect, *C. fulvum* was the first pathogen from which fungal avirulence (*Avr*) genes were cloned and were shown to induce *Cf*-mediated resistance responses in tomato. In a similar way, many cognate *Cf*-resistance genes have been cloned from wild *Lycopersicon* species that are resistant to this pathogen

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(Rivas and Thomas, 2005). However, in many cases resistance based on *Cf* genes has been rapidly overcome after their deployment into commercial tomato lines by the appearance of new races of the fungus (Westerink et al., 2004b). Such “boom-and-bust” cycles (Stakman, 1957) have been described for many gene-for-gene-based pathosystems, and are thought to have major impacts on pathogen evolution and population structure (McDonald and Linde, 2002). To date many races of *C. fulvum* exist that are able to evade recognition from several combinations of *Cf*-resistance genes present in cultivated tomato lines. It is believed that such specific race configurations of the fungus have arisen from a few clonal lineages by the consecutive accumulation of mutations in the different *Avr* genes (Joosten and De Wit, 1999; Westerink et al., 2004a).

Sexual reproduction in fungi is controlled by mating-type genes, which have been characterized for several species of ascomycetes (Arie et al., 2000; Coppin et al., 1997; Kronstad and Staben, 1997; Poggeler, 2001). These include species of Mycosphaerellaceae, such as *Mycosphaerella graminicola* (Waalwijk et al., 2002), *Septoria passerinii* (Goodwin et al., 2003), and several *Cercospora* species (Groenewald et al., 2006). Heterothallic fungi can only reproduce sexually when two individuals of opposite mating-type are present. In most heterothallic filamentous ascomycetous fungi mating is controlled by a single mating-type (MAT) locus, which is represented by two idiomorphs known as MAT1-1 and MAT1-2. Although the two idiomorphs are surrounded by identical flanking regions, they are otherwise completely dissimilar in their structural organization, as they encode proteins that differ in number and function (Metzenberg and Glass, 1990; Turgeon, 1998). Members of Loculoascomycetes (Mycosphaerellaceae) exhibit a similar organizational structure in their mating-type locus; each MAT idiomorph contains a single gene encoding a protein with an alpha-domain (MAT1-1-1) or a protein with a DNA-binding domain of the high-mobility group (HMG) (MAT1-2-1) (Poggeler, 2001; Turgeon and Yoder, 2000). Regions with high similarities can be found in the alpha-domain as well as HMG-domain of different species (Turgeon, 1998), and such homologous regions have been extensively explored in PCR-based approaches for cloning of mating-type genes from various fungi (Arie et al., 1997; Poggeler, 2001).

*Cladosporium fulvum* is thought to be a strictly asexual fungus, since its teleomorph has never been found. However, failure to detect sexual structures, does not necessarily exclude that genetic recombination occurs in fungal populations. With the availability of novel molecular genetic tools in recent years and significant advances in molecular markers technology, it is now possible to test for evidence of recombination in the absence of a known sexual stage (Milgroom, 1996; Tibayrenc et al., 1991). As a result, several studies have revealed an ever growing number of fungi that were previously thought to reproduce strictly asexually, but which in fact undergo cryptic sex in nature (Dodgson et al., 2005; Litvintseva et al., 2003; Taylor et al., 1999). The presence of regular out-crossing in a fungal population constantly cre-

ates new genotypes that result in higher levels of genotypic diversity. This type of genetic structure is seen for example in most populations of *M. graminicola* and *Phaeosphaeria nodorum* as well as for other pathogens that appear to be randomly mating (Keller et al., 1997; Linde et al., 2002; Zhan and McDonald, 2004; Zhan et al., 2003). The occurrence and frequency distribution of *MAT* genes in a population may also be indicative of the reproductive behavior of a pathogen. Thus, in populations of sexually reproducing pathogens the two *MAT* genes occur in approximately equal frequencies, whereas skewed ratios are indicative for asexual populations (Milgroom, 1996). However, the presence of the mating-type idiomorphs in a given species alone is insufficient to prove the existence of a sexual stage, as has been demonstrated for the filamentous ascomycetes *Alternaria alternata* and *Fusarium oxysporum* (Arie et al., 2000).

In this study, we describe the cloning, characterization and population distribution of the mating-type idiomorphs from *C. fulvum*. It is presently accepted that this pathogen only reproduces asexually, but here we show that strains of the fungus contain *MAT1-1-1* or *MAT1-2-1* genes that show high similarity to homologous genes from other filamentous ascomycetous fungi. In addition, by using amplified fragment length polymorphism (AFLP) multilocus fingerprints (Vos et al., 1995), we explored the genetic variation of a worldwide collection of strains of this fungus. AFLP analysis revealed a level of genotypic diversity that is too high for a fungus that is expected to reproduce solely asexually, and which contrasts the idea of the dispersal of a few clonal lineages of the pathogen around the world. Therefore, we suggest that sexual recombination might occur in *C. fulvum*.

## 2. Materials and methods

### 2.1. Fungal material and culture conditions

Eighty-six *C. fulvum* strains were isolated over a period of 50 years from commercially cultivated tomato lines in different parts of the world (Table 1) and stored at  $-80^{\circ}\text{C}$  at the laboratory of Phytopathology, Wageningen University, The Netherlands. Strains were collected from different geographical regions and were grouped according to the continent from which they were collected. As the strains used in this study were collected over long distances and over a period of several decades and from often previously resistant tomato plants, they represent a collection of strains that could be biased rather than a random population. The collection from Europe contained 50 strains originating from The Netherlands ( $n=22$ ), France ( $n=13$ ), Belgium ( $n=4$ ), Bulgaria ( $n=2$ ), UK ( $n=5$ ), Italy ( $n=1$ ), and Poland ( $n=3$ ). The collection from the Americas contained 15 strains originating from Canada ( $n=9$ ), USA ( $n=2$ ), Argentina ( $n=1$ ), Brazil ( $n=1$ ), and other South American regions ( $n=2$ ). Additional but substantially smaller collections originated from Japan ( $n=12$ ), former USSR ( $n=2$ ), Turkey ( $n=4$ ), New Zealand ( $n=2$ ), and one from the African continent, namely Zimbabwe ( $n=1$ ). Most strains were isolated from tomato plants grown



Table 1  
Strains of *C. fulvum* used in this study

Strain (#)	Code	Origin and year of isolation	MAT-type	Strain (#)	Code	Origin and year of isolation	MAT-type
1	0	Netherlands	MAT1-1	51	IMI Day2 054978	UK	MAT1-1
2	2	Netherlands	MAT1-2	52	IMI Day5 054977	UK	MAT1-2
3	4E	Netherlands, 1968	MAT1-2	53	IMI Day8 054979	UK	MAT1-2
4	2 4	Netherlands, 1971	MAT1-1	54	IMI Day9 054980	UK	MAT1-2
5	2 4 11	Poland	MAT1-1	55	IMI Zimba 050487	Zimbabwe	MAT1-2
6	2 4 5	Netherlands, 1977	MAT1-1	57	IPO 2459 (30787)	Netherlands, 1981	MAT1-2
7	2 4 5 11	Netherlands	MAT1-2	58	IPO 2459 (50381)	Netherlands, 1987	MAT1-1
8	2 4 5 7	Netherlands	MAT1-1	59	IPO 2459 (60787)	Netherlands, 1987	MAT1-2
9	2 4 5 9	Netherlands, 1980	MAT1-1	60	IPO 248911 Polen	Poland, 1987	MAT1-1
10	2 4 5 9 11 IPO	Netherlands	MAT1-2	61	IPO 249 France	France	MAT1-1
11	2 4 8 11	Netherlands	MAT1-1	62	IPO 2679 SECRET	New Zealand	MAT1-2
12	2 4 9 11	Poland	MAT1-1	63	IPO 5 (104)	Netherlands	MAT1-2
13	2 5	Bulgaria	MAT1-1	64	IPO 5 (116)	Netherlands	MAT1-2
15	2 5 9	France, 1987	MAT1-1	65	IPO 5 (15104)	Netherlands	MAT1-2
16	4	Netherlands, 1971	MAT1-1	66	IPO 80379	Netherlands	MAT1-1
17	4 (2)	Netherlands	MAT1-1	67	Jap 12	Japan	MAT1-1
18	5	France, 1979	MAT1-2	68	Jap 14	Japan	MAT1-1
19	5 Kim	France, 1979	MAT1-2	69	Jap 15	Japan	MAT1-1
20	5 Marmeisee	France, 1979	MAT1-2	70	Jap 16	Japan	MAT1-1
21	5.1	France, 1979	MAT1-2	71	Jap Cf32	Japan	MAT1-1
22	Alenya B	Netherlands, 1979	MAT1-1	72	Jap Cf44	Japan	MAT1-1
23	AP 0	Netherlands	MAT1-1	73	Jap Cf5	Japan	MAT1-1
24	Brest 84	France	MAT1-1	74	Jap Cf56	Japan	MAT1-1
25	Brest Rianto 85	France, 1986	MAT1-1	75	Jap Cf9	Japan	MAT1-2
26	Bul 20	Bulgaria	MAT1-1	76	La Maxe 2	France, 1978	MAT1-1
30	Can Brasil	Brazil, 1989	MAT1-2	78	MUCL723	Belgium, 1959	MAT1-1
31	Can USA Amherst	USA	MAT1-1	79	MUCL724	Belgium, 1959	MAT1-1
32	Can 35	Canada, 1980-1983	MAT1-2	80	MUCL725	Belgium, 1959	MAT1-1
34	Can 38	USA, 1962	MAT1-1	81	MUCL726	Belgium, 1959	MAT1-1
35	Can 43	Canada, 1969	MAT1-2	82	Nantes 89	France	MAT1-1
36	Can 47	Canada, pre-1965	MAT1-2	83	NZ	New Zealand	MAT1-1
37	Can 48	Canada, 1980-1983	MAT1-1	84	Pons 89	Netherlands	MAT1-1
38	Can 56	Canada, 1980-1983	MAT1-2	85	Sarrians 86	France, 1978	MAT1-1
39	Can 57	Canada, 1980	MAT1-1	86	Sicile 93	Italy	MAT1-1
40	Can 62	Canada, 1980	MAT1-2	87	T Hijwegen	Netherlands	MAT1-2
41	Can 69	Canada, 1980-1983	MAT1-1	110	VKM 1392	Former USSR	MAT1-2
42	Can 84	Canada, 1982	MAT1-1	111	VKM 1437	Former USSR	MAT1-2
44	Gattieres Furon 90	France	MAT1-2	112	Z. Am 1	South America	MAT1-2
46	IPO 31254	Japan	MAT1-1	113	Zuid Amerika 1336	South America	MAT1-2
47	IPO 8419	Japan	MAT1-1	117	Turk 1a	Turkey, 2005	MAT1-2
48	IPO 9759	Japan	MAT1-2	119	Turk 3a	Turkey, 2005	MAT1-1
49	IMI Argent 358077	Argentina, 1991	MAT1-2	121	Turk 3b	Turkey, 2005	MAT1-2
50	IMI Day? 054976	UK	MAT1-1	122	Turk 3c	Turkey, 2005	MAT1-1

in glasshouses, while a few were collected from outdoor grown tomatoes. Unfortunately, records on the year of isolation of many of these strains were not available. Strains were cultured on half potato-dextrose agar (PDA 19.5 g/L, agar technical 15 g/L; Oxoid Ltd., Hampshire, England) at 22 °C. Conidia were harvested from 15-day-old cultures and freeze-dried prior to DNA extraction. Genomic DNA isolations were performed using the DNeasy® Plant Mini Kit (Qiagen Benelux bv, Venlo, The Netherlands) according to the manufacturer's instructions.

## 2.2. Cloning and characterization of the mating-type genes and idiomorphs

Two degenerate primer sets, MgMfSpMAT1-1 and MgMfSpMAT1-2 (Table 2) that were previously designed to

amplify a region within *MAT1-1-1* and *MAT1-2-1*, respectively, of different *Mycosphaerella* species (Groenewald et al., 2006), were used to screen nine *C. fulvum* strains for the presence of mating-type genes. These included strains from The Netherlands (#23, #66), France (#22, #82), Belgium (#79, #80), the UK (#54), Japan (#46), and New Zealand (#83). PCR conditions as described by Groenewald et al. (2006) were used for the amplification of both gene regions.

Internal walking primers (Table 2) were designed based on the partial *C. fulvum* *MAT1-1-1* and *MAT1-2-1* sequences obtained. These primers in combination with primers from the DNA walking kit (Seegene Inc., Rockville, Maryland), were used to amplify the full length sequences of *MAT1-1-1* and *MAT1-2-1* as well as for sequencing of the complete MAT idiomorphs from strains #22 (MAT1-1) and #54 (MAT1-2). In all cases,

Table 2  
MAT1-1- and MAT1-2-related primers used in this study

MAT1-1		MAT1-2	
Primer	Sequence 5'–3'	Primer	Sequence 5'–3'
<i>Degenerate<sup>a</sup></i>			
MgMfSpMAT1-1f1	Groenewald et al. (2006)	MgMfSpMAT1-2f2	Groenewald et al. (2006)
MgMfSpMAT1-1r2	Groenewald et al. (2006)	MgMfSpMAT1-2r1	Groenewald et al. (2006)
<i>Gene-walking</i>			
CfMat1-CW1	CATTCATCCTCATGTGCTAAC	CfMat2-CW1	CTGTCAAAGACGAGTACAAGC
CfMat1-CW2	CTTCACCTCAAACCTCGACAC	CfMat2-CW2	TGAGGTCGGTCTTCATCTTCC
CfMat1-CW3	GACCTGGTCAACCACTGCTAC	CfMat2-CW3	GTGACTGACATCTCGCAGGAC
CfMat1-CW4	GACACGATGTGTCTTCCAG	CfMat2-CW4	CATGAGTGTGAGTGGATG
CfMat1-CW5	GAAGGTTCCGAAATCGTCTG	CfMat2-CW5	TGAGGATGCTCAGTAGCATGG
CfMat1-CW6	AAATCGTCTGCCATTGTGTG	CfMat2-CW6	TGTTATGCATTCCAGGGTACG
CfMat1-CW7	GTTGATGGCACAGAATGAGG	CfMat2-CW7	CAACATAGCCTTGATGATCG
CfMat1-CW8	TGGCACAGAATGAGGAAGG	CfMat2-CW8	AGCCCTCCTCCAACCTTCTCC
CfMat1-CW9	CTGGGAGGACTTCATCAACG	CfMat2-CW9	TCATTGATGACGATGCTTGC
CfMat1-CW10	TATGTGATGATCGAACTTGC	CfMat2-CW10	CACTCGTGTGGTCTTGTGC
CfMat1-CW11	TAGTGCAGTGCACGATGAC	CfMat2-CW11	GGTCTTGTGCTTGCAGTGG
CfMat1-CW12	AAGTTTCGCAACGGCCTATC	CfMat2-CW12	AAAGCAGAAGTGGCAGAAGG
CfMat1-CW13	TGACTTTCTTGATGTAGATGC	CfMat2-CW13	CAGTGTCTCAGACGATAGACC
CfMat1-CW14	GGACTCATCTTCGTCTGTGTCC	CfMat2-CW14	TTGTCTGAACCGCTGTCTAATG
CfMat1-CW15	CAGCTTGAGGTCGAGTGAGG	CfMat2-CW15	TACCAACGGAAGGATTTAGCC
CfMat1-CW16	GAGTCTCAGCGTGAGAGG	CfMat2-CW16	GAGTCTCAGCGTGAGAGG
CfMat1-CW17	GAGAGTGGAACAAGGCTTCG	CfMat2-CW17	GAGAGTGGAACAAGGCTTCG
CfMat1-CW18	TGATGTTTCTGTTGTGATGTGC	CfMat2-CW18	TGATGTTTCTGTTGTGATGTGC
<i>PCR amplifications</i>			
MAT1-1_P1F	CTTCACCACACCCAAAC	MAT1-2_P1F	CTGCCAGTTCTGCTTTG
MAT1-1_P4R	TGTTCCGGTGTCTGTGATG	MAT1-2_P4R	TCCACGTCGAAGTAGAG
<i>Sequencing</i>			
MAT1-1_P3F	AATGCTCAGAGGACACAC	MAT1-2_P3F	ATCTACCGTCTCAACCAC
MAT1-1_P6F	ACACACATGACATCTTTC	MAT1-2_P6F	CCTTACCAGAACAACAC

<sup>a</sup> Groenewald et al. (2006).

mating-type genes and idiomorphs were specified according to the nomenclature proposed by Turgeon and Yoder (2000). Primer design and amplification conditions were according to the manufacturer's instructions. The amplified products were sequenced and analyzed as described above. Blastx and Blastp (Altschul et al., 1997) were used to compare the sequences obtained from *C. fulvum* with nucleotide or protein sequences present in the NCBI non-redundant database. Open reading frames (ORFs) were predicted by comparing the *C. fulvum* mating-type sequences to known *MAT* sequences of other filamentous fungi as well as by predictions using the "geneid v1.2 web server" software package (<http://www1.imim.es/geneid.html>; © Genome Bioinformatics Research laboratory, Barcelona, Spain) and the FEX (Solovyev et al., 1994) and FGENESH (Salamov and Solovyev, 2000) programs from the MOLQUEST software package (Softberry Inc. NY, USA) available at (<http://sun1.softberry.com/berry.phtml>). In all cases intron/exon boundaries were predicted using the genetic code of *Fusarium graminearum* as a model. FGENESH has been described as the most accurate gene finding program (Yu et al., 2002). However, the validity of these programs in identifying potential intron/exon boundaries was examined by analyzing first *MAT* sequences from other fungal species.

### 2.3. Mating-type determination and characterization of polymorphisms

The presence of *MAT1-1-1* and/or *MAT1-2-1* in the collection of 86 fungal strains was examined by PCR amplification using gene-specific primers (Table 2). *MAT1-1-1*-specific primers were MAT1-1\_P1F (forward), located 39 bp before the predicted translation start of *MAT1-1-1* and MAT1-1\_P4R (reverse), located 31 bp after the predicted stop-codon of this gene. *MAT1-2-1* specific primers were MAT1-2\_P1F (forward), located 113 bp before the predicted translation start of *MAT1-2-1* and MAT1-2\_P4R (reverse), located 148 bp after the predicted stop-codon of this gene. PCR-reaction mixes included 5.0 µl of 10× SuperTaq PCR-reaction buffer, 10 mM of each dNTP (Promega Benelux bv, Leiden, The Netherlands), 20 µM of each primer (Biologio bv, Nijmegen, The Netherlands), 1 U of SuperTaq DNA polymerase (HT Biotechnology Ltd., Cambridge, England) and approximately 100 ng genomic DNA. The final reaction volume was adjusted to 50 µl with sterile H<sub>2</sub>O. The PCR program consisted of an initial 5 min denaturation step at 94 °C, followed by 35 cycles of denaturation at 94 °C (30 s), annealing at 54 °C (90 s) and extension at 68 °C (30 s). A final extension step at 68 °C (7 min) concluded the reaction.

Following amplifications, the full-length *MAT1-1-1* and *MAT1-2-1* genes were sequenced from 21 and 19 *C. fulvum* strains, respectively, in order to determine possible sequence variation among the genes. PCR products were excised from 0.8% agarose gels and purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences UK limited, Buckinghamshire, England). *MAT1-1-1*-specific fragments were sequenced using primers MAT1-1\_P1F and MAT1-1\_P4R as well as two internal forward primers located 308 bp (MAT1-1\_P3F) and 881 bp (MAT1-1\_P6F) after the predicted translation–initiation codon, respectively (Table 2). In a similar way, *MAT1-2-1*-specific fragments were sequenced using primers MAT1-2\_P1F and MAT1-2\_P4R as well as two internal forward primers located 399 bp (MAT1-2\_P3F) and 839 bp (MAT1-2\_P6F) after the predicted translation–initiation codon, respectively. Sequencing was performed at Macrogen Inc. (Seoul, South Korea) directly on the purified PCR products and generated chromatographs were analyzed using the Vector NTI Suite 8 (InforMax Inc., Europe Headquarters, Oxford, UK).

#### 2.4. AFLP analysis

The intra-specific diversity among 67 *C. fulvum* strains from the world-wide collection was analyzed by AFLP fingerprinting. These included strains from Europe ( $n = 39$ ), the Americas ( $n = 13$ ), Japan ( $n = 10$ ), the former USSR ( $n = 2$ ), New Zealand ( $n = 2$ ), and Africa ( $n = 1$ ). AFLP analysis was performed according to Vos et al. (1995) with minor modifications as described by Zhao et al. (2005). Genomic DNA (350 ng) from 67 the *C. fulvum* strains was digested with the restriction enzymes *EcoRI* (E) and *MseI* (M) (New England Biolabs Inc., Ipswich, Massachusetts) and ligated to the corresponding adaptors. Pre-amplifications were performed using the non-selective primers E00 and M00. Selective amplifications were carried out with primers that contained two selective nucleotides for *EcoRI* primers and one selective nucleotide for *MseI* primers. In preliminary experiments, 104 E + 2/M + 2 and E + 2/M + 1 primer-pairs were tested on 10 *C. fulvum* strains and the produced AFLP fingerprints were evaluated for overall quality, and the number of polymorphic fragments generated (data not shown). From the set of 104 tested primer-pairs, five E + 2/M + 1 primer combinations, namely E15/M02, E18/M02, E18/M03, E20/M04, and E23/M02 were selected and used for the final analysis (Table 3). The *EcoRI* primers were fluorescently labeled with either IRD700 (E15, E23) or IRD800 (E18, E20) at their 5'-end (Biolegio bv, Nijmegen, The Netherlands). AFLP fingerprints were analyzed using the AFLP-QUANTAR™ 1.0 fingerprint analysis software package (KeyGene Products bv, Wageningen, The Netherlands).

AFLP bands were scored manually as binary characters and bands at the same migration height were treated as putative unique AFLP loci with absence or presence of amplification products as putative alleles. A binary matrix was constructed containing all AFLP amplified fragments and all

Table 3  
Primers used for the AFLP analysis

Primers	Sequence (5'–3')
E00	GACTGCGTACCAATTC
E15	E00 + CA
E18	E00 + CT
E20	E00 + GC
E23	E00 + TA
M00	GATGAGTCCTGAGTAA
M02	M00 + C
M03	M00 + G
M04	M00 + T

strains. In subsequent analyses, marker data were combined to haplotype data. Genetic similarities were calculated with Jaccard's similarity coefficient by NTSYS-pc version 2.02j (Rohlf, 1997). Jaccard's similarity coefficient only takes the presence of bands into account, while absence of bands is not interpreted as a similar character between strains. The similarity matrix was used to construct a dendrogram by the UPGMA cluster method. Bootstrap values were calculated for 1000 replicates with SplitsTree version 4 (Huson, 1998). Branches with at least 70% bootstrap support were considered as informative. The indices of genotypic diversity were calculated using Nei's (1987) diversity index corrected for sample size using GENODIVE (Meirmans and Van Tienderen, 2004). TFPGA version 1.3 (Miller, 1997) was used to calculate Nei's unbiased measure of genetic identity between geographically diverse collections (Nei, 1978) as well as Wright's geometric average modification on Rogers' genetic distance (Rogers, 1972; Wright, 1978). TFPGA was also used to quantify collection subdivision using hierarchical *F*-statistics by calculating Weir and Cockerham's (1984) theta ( $\theta$ ), the equivalent of Wright's  $F_{st}$ . We interpreted the resultant  $\theta(F_{st})$  values based on Wright's (1978) suggested qualitative guidelines of  $\theta(F_{st})$  values. In that respect,  $\theta(F_{st}) = 0–0.05$  indicates no or little collection differentiation,  $0.05–0.15$  indicates moderate differentiation,  $0.15–0.25$  indicates great differentiation, and  $>0.25$  indicates very great differentiation. The 95% confidence level of  $\theta(F_{st})$  was generated using 10,000 bootstrap replicates. Confidence limits around  $\theta$  that did not overlap with 0 were taken as evidence for significant genetic differentiation of collections. The multi-loci statistic of Fisher's combined probability test of genetic differentiation was estimated using Genepop DOS version 3.4 (Raymond and Rousset, 1995). The following settings were used: dememorisation number = 1000, number of batches = 1000, number of iterations = 10,000. The null hypothesis for genetic differentiation was  $H_0$ : 'the allele distribution of AFLP loci is identical across different geographic collections.'

### 3. Results

#### 3.1. Cloning and characterization of the mating-type idiomorphs of *C. fulvum*

Using the degenerate primers MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 a 912 bp PCR fragment was amplified

from eight of the *C. fulvum* strains examined. This fragment showed highest similarity to the alpha-domain of MAT1-1-1 from *M. graminicola* and other filamentous ascomycetous fungi. Subsequent chromosome-walking steps, in both upstream and downstream directions, generated a 5.433 kb fragment that contained the entire MAT1-1 idiomorph along with 661 and 611 bp of 5'- and 3'-flanking sequences, respectively. The MAT1-1 idiomorph is 4.161 kb long and contains at least a putative *MAT1-1-1* ORF flanked by 1.509 and 1.349 kb of 5'-3' idiomorph-sequences, respectively (Fig. 1A). The predicted *MAT1-1-1* ORF from *C. fulvum* is 1.170 kb and encodes a protein of 389 amino acids. The ORF is interrupted by three putative introns of 48, 48, and 53 bp, respectively (Fig. 1B). Perfect lariat sequences (RCTRAC) could be found within the nucleotide sequences of all three introns. Alignment of the *C. fulvum* MAT1-1-1 protein with similar proteins from other fungal species showed that the first two putative introns are located in the alpha-domain of MAT1-1-1 at the same positions (S83 and W114, respectively) as introns found in related fungal species, such as *M. graminicola* (Waalwijk et al., 2002), *S. passerinii* (Goodwin et al., 2003), and others (Fig. 2). Recently, the presence of an additional third intron downstream of the alpha-domain region was suggested to be present in *MAT1-1-1* sequences of several *Cercospora* species (Groenewald et al., 2006). The positioning of the third putative intron present in *MAT1-1-1* of *C. fulvum* is in perfect synteny with the third intron suggested for the *Cercospora* species (Fig. 2). Blast analysis showed that MAT1-1-1 from

*C. fulvum* exhibits highest similarity to the MAT1-1-1 proteins from *Cercospora beticola* (47% identity, 60% similarity), *M. graminicola* (49% identity, 62% similarity), *S. passerinii* (40% identity, 52% similarity), *Aspergillus fumigatus* (40% identity, 55% similarity), *Rhynchosporium secalis* (39% identity, 55% similarity), and several other ascomycetous fungi. High similarity was found within the alpha-box domains but only limited similarity was present outside this domain. Sequence analysis revealed the presence of an additional putative ORF within the MAT1-1 idiomorph of *C. fulvum*. This ORF is located on the opposite DNA strand 350 bp upstream of the *MAT1-1-1* gene and it has been designated as *ORF1-1-2* (Fig. 1A). *ORF1-1-2* is 1.074 kb long and is interrupted by a putative intron of 50 bp, encoding a putative protein of 357 amino acids. Blast analysis showed no significant similarity between the predicted protein product of *ORF1-1-2* and any other proteins currently present in the NCBI GenBank database.

Using the degenerate primers MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1 a 236 bp fragment was amplified from all strains screened as well as a 333 bp fragment, which was found only in the *C. fulvum* strain of the test panel that did not generate a PCR product using the degenerate *MAT1-1-1* primers. Sequencing revealed that the 236 bp fragment did not show similarity to any protein sequence present in the database. However, the translated product of the 333 bp fragment showed highest similarity to the HMG-domain present in the MAT1-2-1 proteins of *S. passerinii* and *M. graminicola*, respectively. Subsequent chromosome-walking steps, in

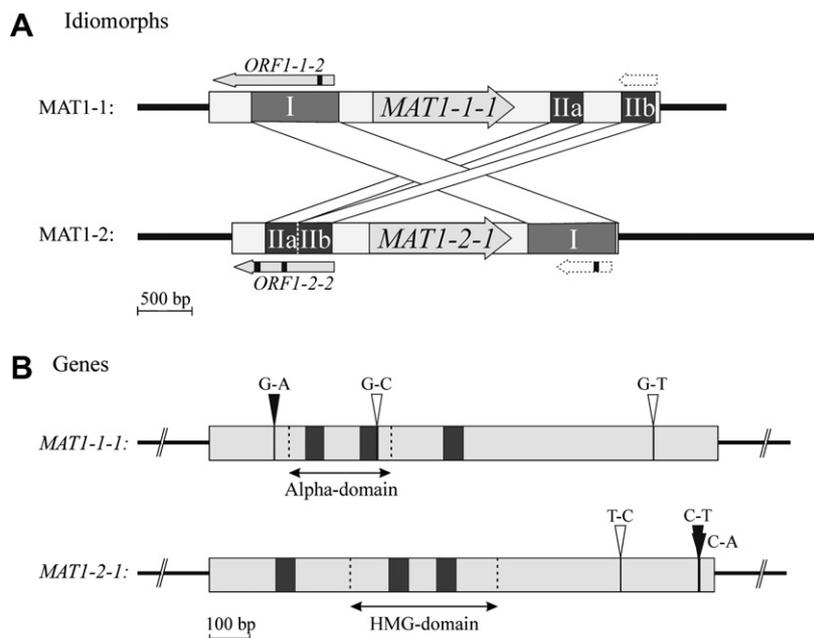


Fig. 1. In scale physical map of the mating-type idiormorphs (A) and the mating-type genes (B) of *C. fulvum*. (A) Idiormorphs are presented as boxes and their flanking regions as solid black lines. The positioning and transcriptional direction of the mating-type genes in each idiormorph is indicated by an arrow. “Islands” of high homology between the two idiormorphs are shown as shaded-grey boxes (I, IIa, IIb). The additional putative ORFs (*ORF1-1-2* and *ORF1-2-2*) identified in each idiormorph are indicated by arrows and putative introns are shown in black. Segments of these ORFs that are only partially present in the opposite idiormorphs are indicated as transparent arrows. (B) Open reading frames (ORFs) are indicated as grey-filled boxes. Introns are presented as dark-grey boxes. The relative position of the alpha-domain of MAT1-1-1 and the HMG-domain of MAT1-2-1 is indicated below the ORFs by double-headed arrows. Identified polymorphisms within *MAT1-1-1* and *MAT1-2-1* are shown as black arrow-heads whenever they are predicted to cause a mutation in the produced protein, or as white arrow-heads when there is no predicted effect on the protein.



putative *ORF1-2-2* is 816 bp long and contains two predicted introns of 54 and 64 bp, respectively, encoding a putative protein of 271 amino acids. However, Blast search showed no significant similarity between the putative ORF1-2-2 protein and other proteins present in the NCBI database.

Pairwise sequence alignment showed that the 616 and 600 bp of 5'- and 3'-sequences flanking the two MAT idiomorphs of *C. fulvum* share 97 and 99% identity, respectively. No significant similarities were found between the flanking sequences of the two idiomorphs and other sequences currently present at the NCBI database ( $P < 10^{-5}$ ). Besides the identity in the flanking regions, regions of high homology between MAT1-1 and MAT1-2 were identified that are not part of their flanking regions. The first of these regions is 806 bp long and exhibits 90% identity between the two idiomorphs (Fig. 1A). This region encompasses almost the entire sequence of *ORF1-1-2* in MAT1-1, whereas a similar ORF is only partially present in MAT1-2 as it is interrupted by several stop codons. The second DNA region of high homology between the two idiomorphs is 613 bp long and in MAT1-2 is included entirely within *ORF1-2-2*. However, in MAT1-1 this region is split into a segment of 301 bp with 75% identity to its homologous MAT1-2 counterpart and a segment of 312 bp with 88% identity to its MAT1-2 counterpart, separated by an insertion of 349 bp (Fig. 1A).

The genomic sequences of the two MAT idiomorphs have been deposited in the NCBI GenBank under the Accession Nos. DQ659350 (MAT1-1) and DQ659351 (MAT1-2).

### 3.2. Continental distribution of the mating-type genes

The geographic distribution of both mating-type genes of *C. fulvum* was examined in a world-wide collection of 86 strains (Table 1). None of the 86 strains contained both *MAT* genes or lacked one of these two genes. *MATI-1-1* and *MATI-2-1* were identified in strains collected from all continents that were examined, except the ones that were represented by too small sample-sizes. In that respect, binomial  $\chi^2$  “goodness-of-fit” tests were performed only for the overall collection ( $n=87$ ) and the European collection ( $n=50$ ) of strains. The sample-sizes of the other collections were too small for reliable statistical analyses (Table 4). In both collections, the frequency distribution of *MAT* genes deviated significantly from a 1:1 ratio, thus suggesting a potential unbalanced distribution of the two mating-type genes. Indeed, *MATI-1-1* was observed at a higher frequency than *MATI-2-1* in most of the collections examined, except for the American collection of strains. Similar results were also obtained when the different collections were corrected to include haplotypes only (Section 3.5).

Table 4  
Frequency distribution of *MATI-1-1* and *MATI-2-1* of *C. fulvum* in a geographically diverse collection of 86 strains

Collection	$N_{\text{strains}}$ ( $N_{\text{genot.}}$ ) <sup>a</sup>	MAT-type		Frequencies		$\chi^2$ values <sup>b</sup>
		<i>MATI-1-1</i>	<i>MATI-2-1</i>	<i>MATI-1-1</i>	<i>MATI-2-1</i>	
Overall	86 (75)	51 (47)	35 (28)	0.61 (0.63)	0.39 (0.37)	2.98 ( <b>4.81</b> ) <sup>c</sup>
Europe	50 (41)	32 (29)	18 (12)	0.64 (0.70)	0.36 (0.30)	<b>3.92 (7.05)</b>
Americas	15 (15)	6 (6)	9 (9)	0.40 (0.40)	0.60 (0.60)	n.d. <sup>d</sup>
Japan	12 (11)	10 (9)	2 (2)	0.83 (0.82)	0.17 (0.18)	n.d.
Turkey	4 (4)	2 (2)	2 (2)	0.5 (0.5)	0.5 (0.5)	n.d.
Former USSR	2 (0)	0 (0)	2 (0)	0.0 (0.0)	1.0 (1.0)	n.d.
New Zealand	2 (2)	1 (1)	1 (1)	0.5 (0.5)	0.5 (0.5)	n.d.
Africa	1 (1)	0 (0)	1 (1)	0.0 (0.0)	1.0 (1.0)	n.d.

<sup>a</sup> Numbers refer to the actual number of strains. Numbers inside the parenthesis refer to the data as clone-corrected for haplotypes only.

<sup>b</sup>  $\chi^2$  “goodness-of-fit” tests.  $\chi^2$  values calculated for a 1:1 ratio with one degree of freedom. Tests were performed only for the Overall and European collection of strains.

<sup>c</sup> Values in bold indicate frequencies that are statistically significantly different at the  $P < 0.05$  level.

<sup>d</sup> Frequencies were not determined (n.d.) due to small sample-sizes.

Table 5  
Sequence variation in the *MATI-1-1* and *MATI-2-1* genes of *C. fulvum* at the nucleotide and amino acid level

Nucleotide substitutions	Amino acid substitutions	Strains containing the substitutions
<i>MATI-1-1</i>		
G > A 159 bp <sup>a</sup>	Gly52 <sup>b</sup> > Lys	#31, #41, #42, #51, #74, #78, #85
G > C 435 bp	—	#31, #41, #42
C > T 1856 bp	Ser334 > Ser (silent)	#31, #41, #42
<i>MATI-2-1</i>		
T > C 1067 bp	Pro304 > Pro (silent)	#30
C > T 1270 bp	Pro372 > Leu	#30
C > A 1271 bp	Pro372 > Leu	#30

<sup>a</sup> Indicates position of the substitution relative to the A nucleotide (+1 bp) of the ATG start codon.

<sup>b</sup> Indicates the amino acid affected relatively to the start of the protein (Met is +1 amino acid).

### 3.3. Sequence variation in the MAT genes

The full length *MAT1-1-1* sequence was determined from 21 *C. fulvum* strains originating from Europe (#1, #4, #11, #12, #15, #16, #25, #26, #51, #58, #60, #78, #85), the Americas (#31, #41, #42), Japan (#46, #67, #69, #74), and Turkey (#119). Sequence variation within the *MAT1-1-1* gene was very limited (Table 5 and Fig. 1B). One nucleotide substitution (G>A at 159 bp), predicted to result in an amino acid substitution (Gly52>Lys) was detected in seven strains originating from Europe (#85, #51, #78), the Americas (#31, #41, #42), and Japan (#74). Furthermore, the strains originating from the Americas (#31, #41, #42) showed the presence of two additional nucleotide substitutions (G>C and C>T at 435 and 1856 bp, respectively) but these substitutions are not predicted to affect the amino acid composition of the produced protein as the G>C substitution is located inside the second putative intron of *MAT1-1-1* and the C>T substitution is silent.

Among the 19 strains of *C. fulvum* analyzed, only three nucleotide substitutions were observed, all present in the *MAT1-2-1* gene of the Brazilian strain (#30). These were a T>C at 1067 bp, C>T at 1270 bp, and C>A at 1271 bp nucleotide substitutions, predicted to cause a silent (T>C) or a Pro372>Leu amino acid substitution (C>T and C>A combined). All other strains originating from Europe (#2, #7, #18, #44, #53, #57, #63, #87, #117), the Americas (#32, #49, #35, #36), Japan (#75), Turkey (#117, #121), former USSR (#111), New Zealand (#62), and Africa (#55) showed no nucleotide substitutions.

### 3.4. AFLP analysis

Each of the five primer-pairs used for the AFLP analysis, produced evenly distributed AFLP fragments between 100 and 700 bp. However, the number of AFLP fragments produced by each primer-pair differed significantly. For example primer-pair E17/M25 generated 21 clearly visible fragments, while primer-pair E19/M25 resulted in 38 clear fragments (data not shown). In general, good results were obtained with E+2/M+1 primer-pairs, which produced between 50 and 60 clearly distinguishable fragments per primer-pair and of which almost one third were polymorphic. Therefore, five E+2/M+1 primer-pairs, i.e. E15/M02,

E18/M02, E18/M03, E23/M02, and E20/M04, were selected in order to determine the intra-specific diversity in the collection of 67 *C. fulvum* strains (Table 3).

In total 255 AFLP fragments between 100 and 700 bp were generated using the five selected primer combinations, of which 72 (28.2%) were polymorphic among the overall collection of *C. fulvum* strains analyzed. Of the 72 polymorphic fragments, 55 (76.4%) showed different alleles in more than 5% of the strains, while the remaining 17 AFLP loci (23.6%) showed different alleles at a frequency of 5% or less, indicating possible rare alleles (Hartl and Clark, 1997). No considerable differences were observed among the different primer-pairs with respect to the number of polymorphic fragments generated within each geographic collection of strains. However, when data from all five primer-pairs were combined, then higher levels of polymorphic fragments were observed within the American (24.3%) as compared to the European (18.4%) and Japanese (18.8%) collection of strains.

### 3.5. Haplotypic diversity

Among the 67 strains of *C. fulvum*, 55 different multilocus AFLP haplotypes were identified (Table 6). Six haplotypes were detected more than once. The most frequent haplotype was detected five times and included four Dutch strains (#3, #23, #58, #66) and a French strain (#61),

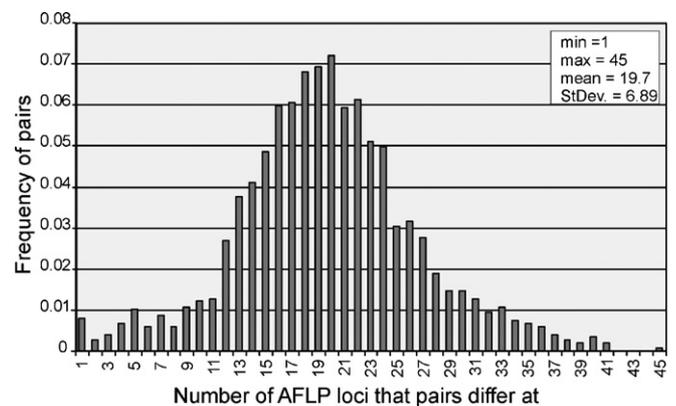


Fig. 3. Pair-wise comparison of 55 unique AFLP haplotypes showing the frequency of haplotype-pairs (*y*-axis) differing in one or more AFLP fragments (*x*-axis).

Table 6  
Haplotypic diversity within the geographical collections of *C. fulvum* strains based on 255 AFLP fragments

Collection	Number of strains	Distinct haplotypes	Maximum frequency <sup>a</sup>	% Different haplotypes <sup>b</sup>	Haplotypic diversity <sup>c</sup>
Overall	67	55	5	82.1%	0.990
Europe	39	30	5	76.9%	0.977
Americas	13	13	1	100%	1.000
Japan	10	9	2	90%	0.978
New Zealand	2	2	1	—	—
Former USSR	2	2	1	—	—
Africa	1	1	1	—	—

<sup>a</sup> Frequency of the most frequent haplotype.

<sup>b</sup>  $G_{max}$  (100 × number of distinct haplotypes/number of strains).

<sup>c</sup> Nei's (1987) diversity index corrected for sample size.

whereas another haplotype was detected four times and included three French strains (#19, #20, #21) and one strain from the former USSR (#111). One haplotype was identified three times and included only Dutch strains (#10, #57, #59), while three additional haplotypes were detected twice and included pairs of Dutch (#64, #65) and Japanese strains (#68, #71), and a pair of a French (#18) strain together with a strain from the former USSR (#110). Pair-

wise comparisons of the 55 unique haplotypes showed that they differ between one and 45 AFLP fragments, following a normal distribution within this range of fragments. On average haplotypes differed in 20 AFLP fragments out of the 255 scored on the fingerprints (Fig. 3). In total, 97% of the haplotypes varied in more than five fragments, while only 3% of the haplotypes differed in five or less fragments. Nei's (1987) genotypic diversity corrected for sample size

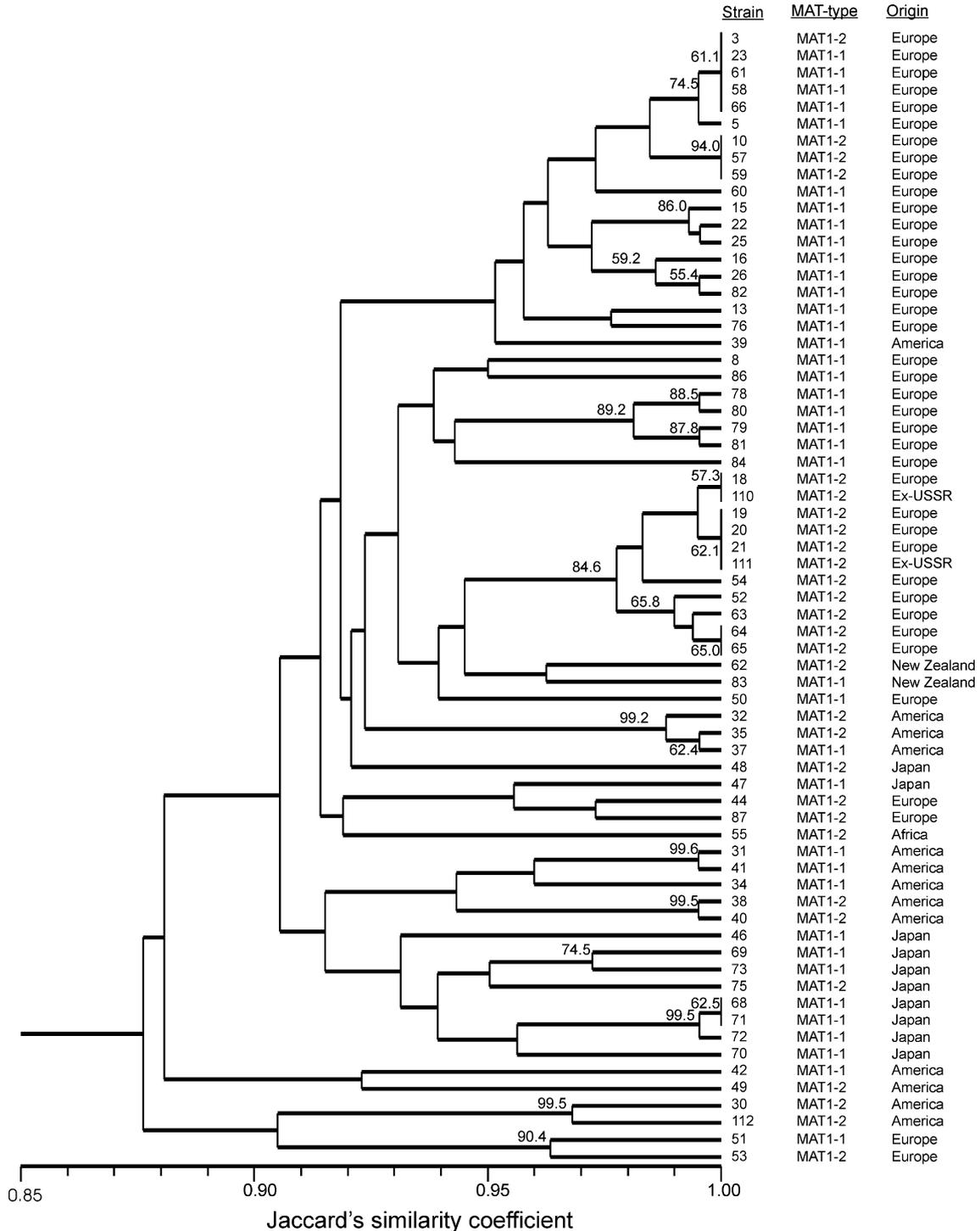


Fig. 4. UPGMA clustering of 67 strains of *C. fulvum* based on Jaccard's correlation coefficient, calculated from 255 AFLP fragments. Bootstrap values higher than 50% are shown above each branch. Mating-type as well as macro-geographic origin of the strains are also indicated.

was 0.99 for the overall collection, thus indicating that almost every strain represented a unique haplotype. Indices of haplotypic diversity were also almost maximal for the different continental collections. Nei's genotypic diversity corrected for sample size was 0.97, 0.98, and 1.0 for the European, American, and Japanese collections, respectively. Estimates of haplotypic evenness were quite high and were estimated at 0.69 and 0.93 for the European and Japanese collections, respectively. Collections from the former USSR, New Zealand, and Africa, are too small to draw any reliable conclusions.

### 3.6. Genetic distances and clustering of the strains

Jaccard's similarity coefficient was used to evaluate the genetic relatedness among the different strains of *C. fulvum*, using the combined AFLP data of all five primer combinations. These data were subsequently used to construct a UPGMA dendrogram of the 67 *C. fulvum* strains analyzed. Significant clustering was detected by bootstrap analysis (Fig. 4). Similarity coefficient values ranged between 0.87 and 1.0. Bootstrap analyses showed 14 nodes with support values higher than 70%, of which seven supported clusters with more than two strains. Most of the supported nodes contained strains of the same mating-type. One cluster with 84.6% of bootstrap support contained eleven strains that originated from Europe and the former USSR and were all of MAT1-2-type. A second cluster with 89.2% of bootstrap support consisted of four Belgian strains that were collected in 1959 and were MAT1-1-type. However, since the deepest nodes of the cladogram were not highly supported by bootstrap no reliable grouping of the strains could be made based on their geographic origin or mating-type.

### 3.7. Genetic differentiation

Genetic differentiation among the different geographical collections of strains was evaluated using Wright's  $F$ -statistics as estimated by theta ( $\theta$ ) (Cockerham and Weir, 1993) and Fisher's combined probability tests (Fisher, 1954) (Table 7). Collections from New Zealand, former USSR, and Africa were excluded from the analysis due to their small sample-sizes. Pair-wise comparisons at the 95% confidence interval level showed that the European collection was significantly differentiated from the American ( $\theta = 0.213$ ) and Japanese ( $\theta = 0.235$ ) collections, whereas the latter two collections were only moderately differentiated

( $\theta = 0.133$ ) (Weir and Cockerham, 1984). Fisher's combined probability test further provided additional support for these results. Nei's (1972) genetic distance as well as Wright's (1978) geometric average modification on Rogers' distance (1972) was lowest for the pair of America and Japan (0.0207 and 0.138, respectively) as compared to pairs of Europe and America (0.024 and 0.150, respectively) and Europe and Japan (0.026 and 0.156, respectively). Bootstrap analysis and a UPGMA dendrogram produced based on Nei's (1972) genetic distances supported (83.9%) the clustering between the American and Japanese collections (Fig. 5).

## 4. Discussion

Here, we report on the cloning of mating-type idiomorphs from *C. fulvum*, a pathogen that until now was considered to be strictly asexual. However, the presence of opposite mating-type genes and the high levels of genotypic diversity observed in this pathogen suggest the occurrence of recombination or other sources of genetic variation.

The cloning and characterization of the mating-type genes from the tomato pathogen *C. fulvum* was performed using an approach that has been successfully applied in the past for the cloning of mating-type genes from other ascomycetous fungi (Arie et al., 1997; Groenewald et al., 2006). All *C. fulvum* strains analyzed thus far have either the MAT1-1-1 or MAT1-2-1 gene present in their genome, thus indicating that if a sexual cycle were to be found for *C. fulvum* then the fungus would be heterothallic. The mating-type genes of *C. fulvum* showed highest similarity to the MAT genes of two phylogenetically closely related species, namely *M. graminicola* and *S. passerinii* (Crous et al., 2001; Goodwin et al., 2001). Both fungal species exhibit a similar

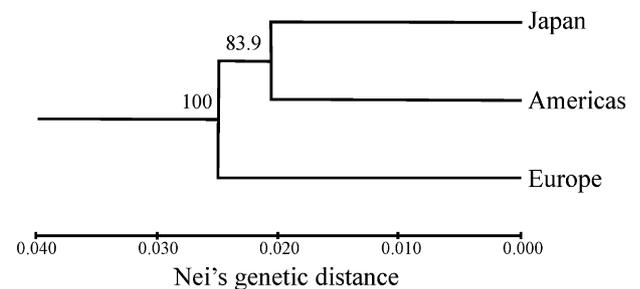


Fig. 5. UPGMA dendrogram generated based on Nei's (1972) genetic distances among the different *C. fulvum* geographic collections. Bootstrap support was obtained after 10,000 permutations over 255 AFLP fragments.

Table 7

Genetic differentiation among three *C. fulvum* collections, inferred from Wright's  $F_{st}$  statistics as estimated by theta ( $\theta$ ) (above diagonals) and Fisher's combined probability test (below diagonals)

	Europe	Americas	Japan
Europe	***	$\theta = 0.213^a$ (0.293–0.139 <sup>b</sup> )	$\theta = 0.235^a$ (0.347–0.130)
Americas	$P < 0.0001$	***	$\theta = 0.133$ (0.194–0.071)
Japan	$P < 0.0001$	$P = 0.056$	***

<sup>a</sup> Significant at the  $P < 0.05$  level.

<sup>b</sup> Confidence intervals (CI) derived after bootstrapping with 10,000 permutations.



organizational structure in their mating-type locus; each *MAT* idiomorph contains a single gene encoding a protein with an alpha-domain (*MAT1-1-1*) or an HMG-domain (*MAT1-2-1*). This organization is commonly present in heterothallic members of the *Dothiomyces* to which *M. graminicola*, *S. passerinii* and *C. fulvum* belong (Braun et al., 2003; Crous et al., 2001; Goodwin et al., 2001; Groenewald et al., 2006; Poggeler, 2001; Schoch et al., 2006). However, although the individual *MAT* genes of *C. fulvum* are highly similar to the *MAT* genes from the *Dothiomyces*, the overall organization of the *MAT* idiomorphs in *C. fulvum* seems to be deviating. In this study the presence of an additional ORF in each of the two idiomorphs of *C. fulvum* was observed. It is currently unknown whether the ORFs are transcribed into functional proteins and it is difficult to speculate on their putative function since they do not exhibit significant similarity to other sequences currently present in the database. Moreover, two highly homologous “islands” of identity between the otherwise largely dissimilar idiomorphs were identified. Although these homologous regions are part either of *ORF1-1-2* or *ORF1-2-2* in *MAT1-1* and *MAT1-2*, respectively, *ORF1-1-2* and *ORF1-2-2* are only partially present in the opposite idiomorphs. Similar “islands” of identity, but only containing eight to nine bps, have been reported to be present in heterothallic *Cochliobolus* species where they might function as putative sites for rare homologous recombination events, and may in this way be involved in the evolution of homothallic fungi from heterothallic progenitors (Yun et al., 1999).

The number of introns predicted to be present in the *MAT1-1-1* and *MAT1-2-1* gene of *C. fulvum* is higher than observed in other fungal species. Three introns were predicted for *MAT1-1-1* and they display a positional conservation to introns predicted in this gene of several *Cercospora* species, suggesting a close phylogenetic relation among these species (Goodwin et al., 2001; Groenewald et al., 2006). For *MAT1-2-1* an additional intron is predicted to be located in the HMG-box domain, which has not been observed in other fungal species. Although a growing number of studies indicate introns that are present at specific positions in one species but are absent in closely related taxa, the biological significance and mechanisms of intron gain are not yet clear (Logsdon, 1998; Logsdon et al., 1998; Lynch and Richardson, 2002). It has been postulated that introns can be gained and lost in different genomes in response to strong selective forces (Belshaw and Bensasson, 2006) and as such could constitute a significant driving force in the evolution of fungal genes (Nielsen et al., 2004). Introns of orthologous genes aligning at the same position are thought to have been inherited from a common ancestor, whereas lineage-specific introns mostly reflect gain events at later stages of evolution (Fedorov et al., 2002; Rogozin et al., 2003; Sverdlov et al., 2005). Therefore, the presence of the additional predicted introns in the *MAT* genes of *C. fulvum* might suggest recent evolutionary divergence of these

genes from similar genes present in closely related species, such as *M. graminicola* and *S. passerinii*.

It is tempting to speculate on the functionality of the mating-type genes in the absence of a known sexual stage, as in the case of *C. fulvum*. However, heterologous expression and functionality of mating-type genes from supposedly asexual fungi in the genetic background of close sexual relatives has been demonstrated for *A. alternata* (Arie et al., 2000). In this case, absence of a sexual stage in this pathogen has been attributed to the lack or failure of some other important components of the mating signal-transduction pathway, and not to dysfunctionality of *MAT* genes (Arie et al., 2000; Yun et al., 2000). Despite the fact that the functionality of *MAT1-1-1* and *MAT1-2-1* of *C. fulvum* has not been investigated yet, their high similarity to mating-type genes from other sexually active members of *Mycosphaerella* and the presence in their coding regions of only limited polymorphisms, suggests that they are still functional. Therefore, heterologous expression of *MAT1-1-1* and *MAT1-2-1* from *C. fulvum* in *MAT*<sup>-</sup> mutants of a closely related and sexually highly active species, such as *M. graminicola*, could confirm the functionality of the mating-type genes of *C. fulvum* as well.

Mating-type genes are frequently used in population studies as their presence, relative frequency and distribution within a population could be indicative of the reproductive mode of a fungus (Groenewald et al., 2006; Tredway et al., 2003; Zhan et al., 2002). In a sexual population, negative frequency-dependent selection is expected to retain an equilibrium in the two mating-type idiomorphs over several spatial scales, whereas in asexual populations this ratio would be skewed (Goodwin et al., 2003; Richman, 2000). A deviation from 1:1 ratios was observed for all of the *C. fulvum* genotype-corrected collections analyzed, therefore suggesting that asexual propagation is predominant in the epidemiology of this pathogen. However, both mating-types were present in almost all collections and none of them seemed to be in fixation in a particular collection, suggesting that the potential for sexual reproduction is at least present in the collections. Skewed mating-type ratios may also be caused by factors that are unrelated to the reproduction mode of a fungus (Milgroom, 1996). For instance, it has been reported that mating-type genes may also function in the maintenance of cell wall integrity, virulence and other cellular processes (Kunz and Haynes, 1981; Kwon-Chung et al., 1992; Verna and Ballester, 1999). In these cases, selection pressure acting on a mating-type or a closely linked locus due to for example fungicide applications or a resistant cultivar, might favor the propagation of one of the two mating-type idiomorphs in a population. Gene-for-gene systems can be particularly influenced by epistatic selection of particular avirulence genes, based on the resistance genes employed in host crop plants (Kolmer, 1992; Wolfe and McDermott, 1994). Such selection has also been imposed on the *C. fulvum* avirulence (*Avr*) genes following the introduction of the *Cf*-resistance genes into commercially grown tomato plants (Westerink et al., 2004b). It is possible that the major part of



the collection of *C. fulvum* strains used in this study has been sampled from resistant plants that had become susceptible to newly arisen virulent races of the fungus. This means that the collection of strains is not a random, but a skewed sample, as it might have been strongly affected by the employment of *Cf*-genes, which could have influenced the spatial distribution of the two mating-type genes. Unfortunately, for the major part of the collection it is not known from which commercial cultivars the strains were collected, while conclusions drawn from small sample-sizes are only indicative. Therefore, the presence of the two mating-type genes alone does not allow us to draw any firm conclusion on frequency and occurrence of recombination in *C. fulvum*, unless supported by additional genetic data.

In *C. fulvum*, AFLP analysis distinguished 55 haplotypes among the 67 strains analyzed in our collection, thereby revealing the overall high genotypic diversity present in this collection. On average, most haplotypes differed from each other at 20 AFLP loci out of the 255 amplified fragments, indicating that haplotypes were unambiguously identified. The high levels of genotypic diversity and the large number of loci in which *C. fulvum* strains differ are not typical for a strictly asexual fungus, but suggest the occurrence of recombination in this pathogen. This could also explain the fact that strains of the fungus were identified that shared the same virulence spectrum but were of opposite mating-type. Several mutations have been identified in *C. fulvum* *Avr* genes that determine the virulence spectrum of the different races of the fungus (Westerink et al., 2004b), while specific complex virulence spectra were thought to have arisen in a few clonal lineages by successive accumulation of mutations in the different *Avr* genes (Joosten and De Wit, 1999; Westerink et al., 2004a). We found evidence to partially reject this hypothesis as strains of the fungus with the same complex virulence spectrum but with opposite mating-types were identified. For example, the Dutch strains IPO2459 (50381) and IPO2459 (30787) are both races 2.4.5.9 and overcome the resistance genes *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* (Boukema, 1981; Lindhout et al., 1989), but have opposite mating-types and share distinct AFLP patterns. Therefore, clonal propagation and the dispersal of a clonal lineage around the world can not account for the occurrence of strains with an identical virulence spectrum, but opposite mating-types. In this case, the virulence spectrum of such strains would either have to be defined by different mutations in the respective *Avr* genes, or otherwise a chromosomal exchange containing the mating-type locus must have taken place.

Despite the fact that recombination could explain a number of features revealed in the collection of *C. fulvum* strains analyzed, it should also be taken into account that events, such as high mutation rates, highly active transposons, mitotic recombination, or the occurrence of a parasexual cycle (Fierro and Martin, 1999) could also act as a source of genetic variation. As mentioned above, the successive introduction of *Cf*-resistance genes into commercial

tomato cultivars since the early 1940s has imposed an enormous selection pressure on *C. fulvum*, which has generated races with complex virulence spectra, some of which can overcome as many as five different *Cf* genes (Lindhout et al., 1989). This transition from avirulence to virulence is generally associated with DNA modifications in the *Avr* genes of the fungus that code for race-specific elicitors. Such modifications include point and frameshift mutations, insertions of transposon-like elements, or even deletion of an entire ORF (Westerink et al., 2004b). Moreover, pulse-field gel electrophoresis revealed chromosome length polymorphism including large deletions in different races of *C. fulvum* (Talbot et al., 1991). Chromosome polymorphisms have been frequently observed in natural strains of many fungal species and this phenomenon seems to occur more frequently in asexual than sexual pathogens (Fierro and Martin, 1999). In addition, a high content of repetitive DNA sequences and retro-transposons has been identified in many chromosomes of *C. fulvum* (Talbot et al., 1991), which can trigger extensive chromosome rearrangements through various molecular processes (McHale et al., 1992). Such phenomena have been reported to occur frequently in the rice pathogen *Magnaporthe grisea* (Skinner et al., 1993). Lastly, it has been shown that during an induced parasexual cycle in *C. fulvum*, mitotic recombination can lead to a high degree of sequence rearrangement in this pathogen (Arnau et al., 1994; Arnau and Oliver, 1993). In conclusion, in addition to sexual recombination the later phenomena could also contribute to the genetic variability observed in *C. fulvum*.

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# *Mycosphaerella punctiformis* revisited: morphology, phylogeny, and epitypification of the type species of the genus *Mycosphaerella* (Dothideales, Ascomycota)

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*Mycosphaerella punctiformis*, the type species of the genus *Mycosphaerella*, is epitypified by material collected on *Quercus robur* in The Netherlands. The teleomorph is described *in planta*, and the *Ramularia* anamorph, for which the new name *R. endophylla* is proposed, and the *Asteromella* spermatial state are characterized *in vitro*. Sequence data of the nuclear ribosomal DNA are presented and analyzed together with other *Mycosphaerella* spp. with *Ramularia* and several other anamorphs. Several strains originating from *Quercus*, *Acer* and *Tilia* showed diverging ITS sequences, indicating that the *M. punctiformis* complex may comprise more than a single phylogenetic species, but this could not be confirmed by the analysis of our dataset. An endophytic phase is established for the first time in the life-cycle of *M. punctiformis*, as the species was repeatedly isolated from surface sterilized green healthy leaves of *Quercus robur* in summer at the epitype locality.

## INTRODUCTION

The genus *Mycosphaerella* is one of the largest genera of ascomycetes, comprising many plant pathogens of economically important crops, but also saprobic species. Teleomorph morphology is relatively simple and uniform in *Mycosphaerella*, but the genus is unequalled in the diversity of the associated anamorphs. Indeed, 27 anamorphic genera have been associated with *Mycosphaerella* (von Arx 1983, Sutton & Hennebert 1994), 23 of which were accepted by Crous *et al.* (2000). Klebahn (1918) and Laibach (1922) suggested segregating groups of species from *Mycosphaerella* based on their association with a particular anamorph, but genera they proposed did not become widely used. Recent molecular studies indicate that characters used to define the anamorph genera, such as conidiomatal structure, and conidial shape, size, and septation, are not always phylogenetically informative, and that some generic concepts for the anamorphs need to be revised (Crous *et al.* 2000, Crous, Kang & Braun 2001, Verkley *et al.* 2004). However, a group of species with *Cladospodium* anamorphs was recently segregated under the name *Davidiella* (Braun *et al.* 2003); it is a close sister group of other *Mycosphaerella*.

*Mycosphaerella punctiformis*, the type species of *Mycosphaerella*, was originally described as *Sphaeria punctiformis* from fallen leaves of *Quercus robur*. Microscopical examination of the lectotype material of *M. punctiformis* deposited in L, confirmed the identity. However, the over 200 yr old herbarium specimen does not provide an unambiguous application of the name, because recent molecular work has shown that *M. punctiformis* as currently circumscribed comprises cryptic species that are morphologically indistinguishable. Several strains in the CBS collection that had been morphologically identified as *M. punctiformis* from *Quercus*, *Acer* and *Tilia*, were found to be heterogeneous in their sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene array. As no ex-type strain is available, we tried to obtain ribosomal DNA from the type material of *M. punctiformis*, but failed. In accordance with Art. 9.7 of the *Code*, we sought to settle the application of the name by selecting an epitype for *M. punctiformis*. The main purpose of this paper is to epitypify *M. punctiformis* with material recently collected from the type host *Quercus robur* in The Netherlands, and to give a full phenotypic characterization of the teleomorph, and (syn)anamorphs in culture. Because the anamorph will



be the only sporulation observed in most ecological and endophyte studies, we consider it useful to also formally name this conidial state. Fresh ascomata of *M. punctiformis* were collected on dead fallen leaves of the type host *Quercus robur*, checked for agreement with the lectotype material, and ascospore isolates were made. We also obtained ecological data from a biodiversity study of foliar ascomycetous endophytes of *Quercus* in the epitype locality. We sequenced the ITS region of rDNA of the available strains of *M. punctiformis*, and also included a number of additional taxa in the sequence analyses to investigate the phylogenetic relationships of *M. punctiformis* with other *Mycosphaerella* species with *Ramularia* and several other anamorphs. Furthermore, partial small subunit (SSU) sequences of the ex-epitype strain of *M. punctiformis* were analysed with other data available in order to obtain further support for the phylogenetic position within the *Mycosphaerella* clade.

## MATERIALS AND METHODS

### *Isolation from fruit bodies on decaying leaves and endophytic mycelia from green leaves*

Strains used in this study are listed in Table 1. Dead fallen leaves with ascomata were collected in March to May of 2002 and 2003 in the forested area 'De Stompert' in The Netherlands, from three mature trees of *Quercus robur*. Leaves were incubated in a moist chamber for several hours in the laboratory at ca 20 °C. They were then cut into square pieces and glued to the inside of Petri dish lids to allow ascospores to be discharged on to 2% malt extract agar (MEA). Germinating ascospores were examined after 24 h, illustrated and transferred to MEA. Fresh green leaves from the same trees were collected monthly between May and November, put in plastic bags and transported to the lab. On the same day, leaves were sterilized in domestic bleach water (5% chlorine) for 5 min, followed by three rinses in sterile water. Small squares of about 0.5 cm<sup>2</sup> were placed onto MEA with 50 ppm streptomycin, aureomycin and penicillin to inhibit bacterial growth, placed on the laboratory bench in diffuse daylight, and regularly checked for fungal growth. Mycelia growing out of the margin were transferred to 2% MEA and oatmeal agar (OA; Centraalbureau voor Schimmelcultures 2001) and preliminarily identified morphologically.

### *Phenotypic characterization*

For microscopic examination, fruiting structures were mounted in tap water. Line drawings were made with a drawing tube, and photographic images with a Nikon Coolpix 995 digital camera. For the description of colony features and sporulating structures, isolates were transferred onto OA and 3% MEA plates and placed in an incubator at 15 ° under n-uv (12 h

rhythm). Colours are described according to Rayner (1970).

### *DNA extraction and sequencing*

Strains were transferred from agar cultures to 2 ml liquid medium (2% malt extract) and incubated on a rotary shaker (300 rpm) for 3 wk at room temperature. Liquid cultures were transferred to 2-ml tubes, centrifuged and washed twice with sterile water. DNA was extracted using the FastDNAkit (Omnilabo 6050073, BIO 101, CA) according to the manufacturer's instructions. For ITS sequence analysis a part of the ribosomal RNA gene cluster was amplified by PCR using primer pairs V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990). Part of the 18S rRNA gene (SSU) was amplified using primers NS1 and NS4 (White *et al.* 1990). PCR was performed in 50 µl reaction volumes, each reaction containing 10–100 ng of genomic DNA, 25 pM of each primer, 40 µM dNTP, 1.0 unit Supertaq DNA polymerase and 5 µl 10 × PCR buffer (SphaeroQ, Leiden). PCR was performed in an Applied Biosystems (Foster City, CA) thermocycler with the following program: 1 min at 95 °, 30 cycles (1 min 95 °, 1 min 55 °, 2 min 72 °) followed by a final extension of 5 min at 72 °. PCR products were cleaned with GFX columns (Amersham Pharmacia, NJ) and analyzed on a 2% agarose gel to estimate concentrations. ITS1 and ITS4 (White *et al.* 1990) were used as internal sequencing primers for the ITS region. The SSU region was sequenced using the PCR primers. Sequencing was performed with the BigDye terminator chemistry (Part no. 403049, Applied Biosystems) following the manufacturer's instructions. The sequencing products were cleaned with G50 Superfine Sephadex columns (Amersham Pharmacia 17-0041-01), and separated and analyzed in an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Forward and reverse sequences were matched using SeqMan (DNASTar, Madison, WI).

### *Phylogenetic analyses*

Pairwise and global alignment of consensus sequences were performed in Bionumerics 3.0 (Applied Maths, Kortrijk, Belgium), and manually adjusted where necessary. Parsimony analysis was done using PAUP v. 4.0b10 (Swofford 2003). The heuristic search was performed with the following parameters: characters unordered with equal weight, random taxon addition, branch swapping with tree bisection-reconnection (TBR) algorithm, branches collapsing if the maximum branch length was zero. Maxtrees was set at 10 000. Alignment gaps were treated as missing characters. Parsimony bootstrap analyses were performed using the full heuristic search option, random stepwise addition, and 1000 replicates, with maxtrees set at 100.

Neighbour-joining analyses was performed using PAUP, with GTR (Gamma = 0.5, and rates for variable

sites equal), and 1000 neighbour-joining bootstrap replications to test the stability of clades. BLAST searches in GenBank revealed highest similarity to species of *Mycosphaerella*. GenBank accession numbers, taxon names and other information about the sequences from GenBank used in this study are given in Table 1. GenBank accession numbers (marked with \*) of sequences generated in this study are also given in Table 1. A strain of *Davidiella tassiana* (sub *Mycosphaerella tassiana*) was defined as outgroup for the ITS dataset and sequences of *Botryosphaeria* species were used as outgroup for the SSU dataset. The alignments and trees were lodged in TreeBASE (study accession S1126).

## RESULTS

### Phylogenetic analyses

The alignment of the ITS sequences comprised 513 characters, of which 168 (36%) were parsimony-informative. 23 of these characters were excluded from the analysis because they were positioned in small insertions/deletions or regions with ambiguous position homology. Furthermore, 322 uninformative characters were also excluded, so that 145 characters were used in the parsimony analysis. In the neighbour joining analysis in total 213 characters were included, as constant characters were excluded, but autapomorphic characters were included to obtain accurate branch lengths in the phylogram. The heuristic search yielded 580 most parsimonious trees (MPT) of 535 steps ( $C.I.=0.505$ ,  $R.I.=0.878$ ,  $R.C.I.=0.443$ , and homoplasy index = 0.495). The strict consensus tree is shown in Fig. 1. Several highly supported multi-taxon clades were the same in the parsimony and neighbour joining analyses (neighbour joining trees not shown). Among these was a clade comprising all included strains with *Ramularia* anamorphs (parsimony 99%/neighbour joining minimum 100%), which in the parsimony analysis formed a sister group to the clade with the cereal pathogens *Mycosphaerella graminicola* and *Septoria passerini* (100/92). The support for the two clades together was, however, lower (61/<50). Further highly supported clades were the one with *Cercospora* spp. (90/97), a clade with *M. crystallina*, *M. heimii*, *M. heimioides* and *M. colombiensis* (99/95), and a clade with *M. africana*, *M. keniensis*, *M. aurantia*, *M. hedericola*, *Mycosphaerella* sp. (from *Coprosma* sp.), *M. confusa*, and *Passalora fulva* (91/81). The *Ramularia* clade was rather unresolved in parsimony and neighbour joining analyses. In the parsimony analysis, only a clade comprising four strains identified as *M. punctiformis* from *Quercus*, *Acer* and *Tilia* was well-supported (100/95). With their closest sister *M. phacae-frigidiae*, these strains also obtained good support in both analyses (91/77).

BLAST results of the SSU sequence of *M. punctiformis* (AY490775) supported the close association of *M. punctiformis* with other *Mycosphaerella* species. The

alignment of the SSU sequences included 1067 characters, of which 1006 were constant, 21 were parsimony uninformative and 40 were parsimony informative. The heuristic search yielded eleven most parsimonious trees of 81 steps ( $C.I. 0.852$ ,  $R.I. 0.919$ ,  $R.C. index 0.783$ , and  $H.I. 0.148$ ). The strict consensus tree is shown in Fig. 2. The topology of the eleven trees only differed in the internal ordering of groups in the *Mycosphaerella* clade. Two main clades are delimited in the SSU tree, the first clade contains isolates of *Mycosphaerella* (98% bootstrap support) and the other isolates of *Davidiella* (100% bootstrap support). The sequence of *M. punctiformis* groups closest to the sequences of a *Mycosphaerella* sp. isolated from *Acacia* (AY251116) and a sequence of *Septoria tritici* (AY251117). However, this association does not have significant bootstrap support.

### Phenotypic characterization

(Figs 3–10)

A description of the teleomorph *in planta*: *Leaf spots* not observed. *Ascomata* developing on fallen dead leaves, predominantly hypophyllous, black, subepidermal, erumpent to superficial, globose, 70–110  $\mu\text{m}$  diam; apical ostiole 5–10  $\mu\text{m}$  wide; wall consisting of 2–3 layers of medium brown textura angularis. *Asci* paraphysate, fasciculate, bitunicate, subsessile, cylindrical, straight, 8-spored, 30–45  $\times$  5–7(–9)  $\mu\text{m}$ . *Ascospores* multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest just above the septum, medianly 1-septate, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (6–)8–9(–10)  $\times$  (2–)3  $\mu\text{m}$  (av. 9  $\times$  3  $\mu\text{m}$ ). Germinating ascospores hyaline, distorting, forming germ tubes 4–6  $\mu\text{m}$  diam apically, parallel to the long axis from both ascospore cells, and simultaneously also laterally, from one or both ascospore cells, at an angle of 90° or less to the long axis (Germination pattern D; Crous 1998).

Free conidia possibly belonging to *M. punctiformis* were occasionally observed in late summer on older leaf lesions caused by pathogens such as *Septoria quercicola* and *Discula* sp.

Colony description (diffuse daylight, 15°): *Colonies* on OA reaching 28–31 mm diam in 27 d, spreading (low), sometimes in the centre with some elevated mycelium, margin even or slightly lobed, glabrous, pale Honey to Olivaceous Buff or Rosy Vinaceous to Rosy Buff, colony surface glabrous or with appressed pure white aerial hyphae or conidiophores; in the centre submerged and superficial mycelium Rosy Buff to Salmon and concolourous on reverse, or becoming Dark Violet to dark Purple due to the deposition of violet pigment on the outer surface of vegetative hyphae, surrounding medium then often becoming Coral to red by diffusing pigments, and Coral to Flesh on reverse. In a few isolates, the colony was dominated by olivaceous colours (underneath a white covering of

**Table 1.** Fungal isolates included for ITS and SSU sequence analyses (in alphabetical order of the teleomorph names).

GenBank accession no.				
ITS	SSU	Teleomorph	Anamorph	Origin
	U42476	<i>Botryosphaeria rhodina</i>	<i>Lasiodiplodia theobromae</i>	No data available
	U42477	<i>B. ribis</i>	<i>Fusicoccum</i> sp.	No data available
AY251078	AY251096	<i>Davidiella tassiana</i>	<i>Cladosporium herbarum</i>	ATCC 66670 (=‘STE-U 5101’); CCA-treated Douglas-fir pole, New York, USA
	AY251094	<i>Davidiella</i> state unknown	<i>Cl. cladosporioides</i>	ATCC 66669 (=‘STE-U 5100’); Creosote-treated southern pine pole, New York, USA
	AY251092	<i>Davidiella</i> state unknown	<i>Cl. colocasiae</i>	STE-U 4323; <i>Colocasia esculenta</i> , Fiji Islands
	AY251098	<i>Davidiella</i> state unknown	<i>Cl. sphaerospermum</i>	CBS 188.54 (=‘STE-U 3686’, ATCC 11290)
	AY251097	<i>Davidiella</i> state unknown	<i>Cl. uredinicola</i>	ATCC 46649 (=‘STE-U 5390’); Fungicolous on <i>Cronartium fusiforme</i> f. sp. <i>quercum</i> on <i>Quercina nigra</i> leaves, Alabama, USA
AF173314		<i>Mycosphaerella africana</i>	Unknown	STE-U 794 (ex-type); <i>Eucalyptus viminalis</i> , South Africa
AY490773		<i>M. africana</i>	Unknown	CBS 680.95 (=STE-U 796; ex type); <i>Eucalyptus viminalis</i> , South Africa
AY150331		<i>M. aurantia</i>	Unknown	CBS 110500; <i>Eucalyptus globulus</i> , Australia
AF222838		<i>M. colombiensis</i>	<i>Pseudocercospora colombiensis</i>	STE-U 1106; <i>Eucalyptus</i> , Colombia
AF362058		? <i>M. confusa</i>	<i>Ps. rubi</i>	CBS 256.35
AF222839		<i>M. crystallina</i>	<i>Ps. crystallina</i>	STE-U 801 (ex type); <i>Eucalyptus bicostata</i> , South Africa
AY490757		<i>M. crystallina</i>	<i>Ps. crystallina</i>	CBS 681.95, STE-U 802 (ex type); <i>Eucalyptus bicostata</i> , South Africa
AY266152		<i>M. fijiensis</i>	<i>Ps. fijiensis</i>	ATCC 22116, PF7; <i>Musa</i> sp., Philippines
AY266150		<i>M. fijiensis</i>	<i>Ps. fijiensis</i>	ATCC 36054, PFD9; <i>Musa</i> sp., Honduras
AF181706		<i>M. musicola</i>	<i>Ps. musae</i>	ATCC 22115; <i>Musa</i> sp., Philippines
AY288148		<i>M. musicola</i>	<i>Ps. musae</i>	PM11, ATCC 36143; <i>Musa</i> , Honduras
AY152590		<i>M. laricina</i>	<i>Pseudocercospora</i> sp.	CBS 326.52; <i>Larix decidua</i> , Switzerland
AY152595		<i>M. fragariae</i>	<i>Ramularia grevilleana</i>	CBS 259.36; <i>Fragaria</i> sp., The Netherlands
AY152597		<i>M. fragariae</i>	<i>R. grevilleana</i>	CBS 719.84; <i>Fragaria</i> sp., The Netherlands
AY152596		<i>M. fragariae</i>	<i>R. grevilleana</i>	CBS 298.34; <i>Fragaria</i> sp., The Netherlands
AF297235		<i>M. fragariae</i>	<i>R. grevilleana</i>	ATCC 24113; <i>Fragaria</i> sp., Illinois, USA
AF173312		<i>M. fragariae</i>	<i>R. grevilleana</i>	STE-U 656; <i>Fragaria</i> sp., South Africa
AY152601		<i>M. graminicola</i>	<i>Septoria tritici</i>	CBS 100330 (=IPO 6566.1); <i>Triticum aestivum</i> , The Netherlands
AY152602		<i>M. graminicola</i>	<i>S. tritici</i>	CBS 100335; <i>Triticum aestivum</i> , The Netherlands
AY152603		<i>M. graminicola</i>	<i>S. tritici</i>	CBS 392.59; <i>Triticum aestivum</i>
AF181692		<i>M. graminicola</i>	<i>S. tritici</i>	IPO 323; <i>Triticum aestivum</i> , The Netherlands
AF181693		<i>M. graminicola</i>	<i>S. tritici</i>	T1; <i>Triticum aestivum</i> , Minnesota, USA
AF362068	AY251117	<i>M. graminicola</i>	<i>S. tritici</i>	STE-U 658; <i>Triticum</i> sp., South Africa
AY152581		<i>M. grossulariae</i>	<i>S. ribis</i>	CBS 235.37; <i>Ribes nigrum</i> , The Netherlands
AY490772		<i>M. hedericola</i>	Unknown	CBS 441.86; <i>Hedera helix</i> , France
AF452508		<i>M. heimii</i>	<i>Pseudocercospora heimii</i>	CMW5705
AF452509		<i>M. heimii</i>	<i>Ps. heimii</i>	CMW5707
AF222841		<i>M. heimii</i>	<i>Ps. heimii</i>	No data available
AF452512		<i>M. heimii</i>	<i>Ps. heimii</i>	CMW5713
AF222842		<i>M. heimioides</i>	<i>Ps. heimioides</i>	STE-U 1312; <i>Eucalyptus</i> , Indonesia
AF173300		<i>M. keniensis</i>	Unknown	STE-U 1084; <i>Eucalyptus grandis</i> , Kenya
AY490768		<i>M. latebrosa</i>	<i>Septoria aceris</i>	CBS 183.97; <i>Acer pseudoplatanus</i> , The Netherlands
AY152553		<i>M. latebrosa</i>	<i>S. aceris</i>	CBS 687.94; <i>Acer pseudoplatanus</i> , The Netherlands
AY490769	AY251114	<i>M. latebrosa</i>	<i>S. aceris</i>	CBS 652.85; <i>Acer pseudoplatanus</i> , The Netherlands
AY152600		<i>M. marksii</i>	Unknown	CBS 682.95 (=‘STE-U 842’); <i>Eucalyptus grandis</i> , South Africa



AY152599		<i>M. parkii</i>	<i>Stenella parkii</i>	CBS 387.92 (= 'STE-U 353; ex type); <i>Eucalyptus grandis</i> , Brazil
AY490758		<i>M. phacae-frigidiae</i>	<i>Ramularia</i> sp.?	CBS 234.55; <i>Phaca frigida</i> , Switzerland
AY152583		<i>M. populicola</i>	<i>Septoria populicola</i>	CBS 100045; <i>Populus trichocarpa</i> , Washington, USA
AY152584		<i>M. populicola</i>	<i>S. populicola</i>	CBS 100052; <i>Populus trichocarpa</i> , Washington, USA
AY152585		<i>M. populicola</i>	<i>S. populicola</i>	CBS 100044; <i>Populus trichocarpa</i> , Washington, USA
AY152586		<i>M. populicola</i>	<i>S. populicola</i>	CBS 100051; <i>Populus trichocarpa</i> , Washington, USA
AY152587		<i>M. populicola</i>	<i>S. populicola</i>	CBS 100047; <i>Populus trichocarpa</i> , Washington, USA
AY490759		' <i>M. punctiformis</i> '	<i>Ramularia</i> sp.	CBS 515.69; <i>Acer pseudoplatanus</i> , The Netherlands
AY490760		' <i>M. punctiformis</i> '	<i>Ramularia</i> sp.	CBS 742.79; <i>Tilia</i> sp., Germany
AY152593		' <i>M. punctiformis</i> '	<i>Ramularia</i> sp.	CBS 943.97; <i>Quercus</i> sp., The Netherlands
AY152594		' <i>M. punctiformis</i> '	<i>Ramularia</i> sp.	CBS 184.97; <i>Acer pseudoplatanus</i> , The Netherlands
AY490761		<i>M. punctiformis</i>	<i>R. endophylla</i>	CBS 942.97; <i>Quercus</i> sp., Belgium
AY490762		<i>M. punctiformis</i>	<i>R. endophylla</i>	CBS 113871 (SS); <i>Quercus robur</i> , The Netherlands
AY490763	AY490775*	<i>M. punctiformis</i>	<i>R. endophylla</i>	CBS 113265 (SS; ex-epitype); <i>Quercus robur</i> , The Netherlands
AY490764		<i>M. punctiformis</i>	<i>R. endophylla</i>	CBS 113868; leaf endophyte <i>Quercus robur</i> , The Netherlands
AY490765		<i>M. punctiformis</i>	<i>R. endophylla</i>	CBS 113869; leaf endophyte <i>Quercus robur</i> , The Netherlands
AY490766		<i>M. punctiformis</i>	<i>R. endophylla</i>	CBS 113870; leaf endophyte <i>Quercus robur</i> , The Netherlands
AF222848		<i>M. punctiformis</i>	<i>R. endophylla</i>	KC1
AY152591		<i>M. pyri</i>	<i>Septoria pyricola</i>	CBS 222.31; <i>Pyrus communis</i>
AY152592		<i>M. pyri</i>	<i>S. pyricola</i>	CBS 640.72; <i>Pyrus communis</i> , The Netherlands
AY490767		<i>M. rubella</i>	<i>Ramularia</i> sp.?	CBS 288.49; <i>Angelica sylvestris</i>
AY152575		<i>M. ulmi</i>	<i>Phloeospora ulmi</i>	CBS 344.97; <i>Ulmus glabra</i> , Austria
AY490774		<i>Mycosphaerella</i> sp.	<i>Septoria</i> sp. (in culture)	CBS 113113; <i>Coprosma</i> sp., New Zealand
AY490771		<i>Mycosphaerella</i> sp.	<i>S. quercicola</i>	CBS 663.94; <i>Quercus robur</i> , The Netherlands
	AY251115	<i>Mycosphaerella stromatosa</i>	<i>Pseudocercospora stromatosa</i>	STE-U 1731; <i>Protea</i> sp., South Africa
	AY251116	<i>Mycosphaerella</i> sp.		STE-U 3837; <i>Acacia</i> sp., Venezuela
AF173310		<i>Mycosphaerella</i> state unknown	<i>Ramularia collo-cygni</i>	STE-U 2045; <i>Hordeum</i> sp., Germany
AJ417496		<i>Mycosphaerella</i> state unknown	<i>Ramularia</i> sp.	'ascomycete 2'; <i>Quercus robur</i> , Germany
AY259131	AY251110	<i>Mycosphaerella</i> state unknown	<i>Ramulispora sorghi</i>	STE-U 905; <i>Sorghum</i> sp., South Africa
AY259132	AY251111	<i>Mycosphaerella</i> state unknown	<i>R. sorghi</i>	STE-U 906; <i>Sorghum</i> sp., South Africa
AY166268		<i>Mycosphaerella</i> state unknown	<i>Cercospora apii</i>	CA1, ATCC 12246
AY152576		<i>Mycosphaerella</i> state unknown	<i>C. beticola</i>	CBS 539.71; <i>Beta vulgaris</i> , Romania
AY266165		<i>Mycosphaerella</i> state unknown	<i>C. beticola</i>	MPPD12120, CB4; <i>Beta vulgaris</i> , Minnesota, USA
AY152577		<i>Mycosphaerella</i> state unknown	<i>C. kikuchii</i>	CBS 128.27 (ex type); <i>Glycine max</i> , Japan
AY166260		<i>Mycosphaerella</i> state unknown	<i>C. kikuchii</i>	CK 39; <i>Glycine max</i> , Illinois, USA
AY266161		<i>Mycosphaerella</i> state unknown	<i>C. kikuchii</i>	CK 35; <i>Glycine max</i> , Illinois, USA
AY260078	AY251104	<i>Mycosphaerella</i> state unknown	<i>C. zebrina</i>	STE-U 3955; <i>Trifolium pratense</i> , Canada
AY152572		<i>Mycosphaerella</i> state unknown	<i>Septoria apiicola</i>	CBS 395.52 (= IMI 092627); <i>Apium</i> sp., The Netherlands
AY152573		<i>Mycosphaerella</i> state unknown	<i>S. apiicola</i>	CBS 389.59; <i>Apium graveolens</i> , Italy
AY152574		<i>Mycosphaerella</i> state unknown	<i>S. apiicola</i>	CBS 400.54 (= IMI 092628); <i>Apium graveolens</i> , The Netherlands
AY152588		<i>Mycosphaerella</i> state unknown	<i>S. castaneicola</i>	CBS 102377; <i>Castanea sativa</i> , The Netherlands
AF279582	AF279583	<i>Mycosphaerella</i> state unknown	<i>S. epambrosiae</i>	<i>Ambrosia artemisiifolia</i>
AY490770		<i>Mycosphaerella</i> state unknown	<i>S. hippocastani</i>	CBS 411.61; <i>Aesculus hippocastanum</i> , Germany
AY152563		<i>Mycosphaerella</i> state unknown	<i>S. lamiicola</i>	CBS 109113; <i>Lamium album</i> , Austria
AY152564		<i>Mycosphaerella</i> state unknown	<i>S. lamiicola</i>	CBS 102328; <i>Lamium album</i> , The Netherlands
AF181697		<i>Mycosphaerella</i> state unknown	<i>S. passerinii</i>	ATCC 26516; <i>Hordeum vulgare</i> , Minnesota, USA
AF181699		<i>Mycosphaerella</i> state unknown	<i>S. passerinii</i>	P78; <i>Hordeum vulgare</i> , Minnesota, USA
	AY251108	<i>Mycosphaerella</i> state unknown	<i>Passalora dodonaeae</i>	STE-U 1223; <i>Dodonaea</i> sp., South Africa



Table 1. (Cont.)

GenBank accession no.		Teleomorph	Anamorph	Origin
ITS	SSU			
AY251069	AY251109	<i>Mycosphaerella</i> state unknown	<i>Pas. fulva</i>	CBS 119.46 (= 'STE-U 3688'); <i>Lycopersicon esculentum</i> , The Netherlands
AY152559	AY251103	<i>Mycosphaerella</i> state unknown	<i>Pas. jansseana</i>	CBS 145.37 ('STE-U 4303'); <i>Oryza sativa</i> , Arkansas, USA
AY152561		<i>Mycosphaerella</i> state unknown	<i>Septoria scabiosicola</i>	CBS 102336; <i>Knaulia arvensis</i> , The Netherlands
	AY251095	<i>Mycosphaerella</i> state unknown	<i>S. scabiosicola</i>	CBS 182.93; <i>Succisa pratensis</i> , France
	AY251113	<i>Sphaerulina polyspora</i>	Unknown	CBS 354.29 (= 'STE-U 4301')
	AY251106	? <i>Sphaerulina rhemiana</i>	<i>S. rosae</i>	CBS 355.58 (= 'STE-U 4302'); leaf of <i>Rosa</i> sp.
	AY251107	Unknown	<i>Pseudocercospora angolensis</i>	CBS 149.53 (= 'ATCC 11669'); leaf of <i>Citrus sinensis</i> , Angola
		Unknown	<i>Ps. protearum</i> var. <i>leucadendri</i>	STE-U 1869; <i>Leucadendron</i> sp., South Africa

\* GenBank accession no. of LSU sequence = AY490776.

conidiophores) and greyish Sepia to Hazel or Olivaceous on reverse.

*Colonies* on MEA reaching 21–30 mm diam in 27 d, restricted and up to 5 mm high in the centre, margin weakly to distinctly lobed, glabrous or finely felty of pure white aerial hyphae, Buff, pale Olivaceous or Rosy Buff, colony surface Pale Vinaceous or Pale Violet, and then often the surrounding medium becoming Coral to red by diffusing pigments, or greyish, but largely covered by pure white aerial hyphae or conidiophores; reverse Dark Purple to Blood Colour, or Fawn to Vinaceous Buff with Dark Brick, Brick and Cinnamon areas.

## TAXONOMY

**Ramularia endophylla** Verkley & U. Braun, **sp. nov.**  
(Figs 11–16)

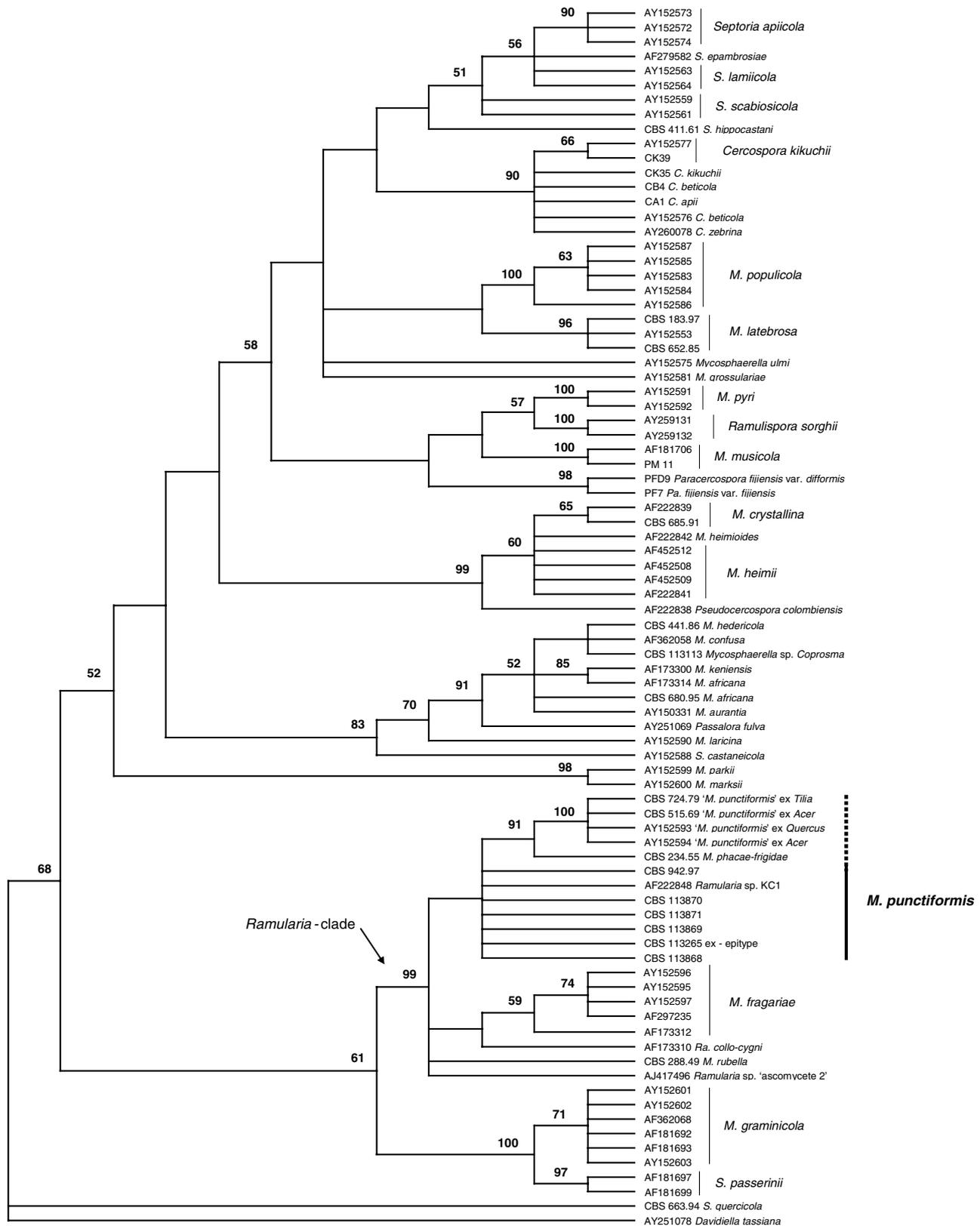
*Conidiophora* unicellulares (=cellulae conidiogenae), simplicia, subcylindrica vel cylindrica, (6–)10–30 × 2.5–4(–5) µm, recta vel geniculata-sinuosa, hyalina, levia; cicatrices conidiales leniter incrassatae et fuscae, circa 1 µm latae; *conidia* hyalina, levia vel sublevia, hila incrassata, fusca, refractiva, 0.5–1(–1.5) µm lata; *conidia* primaria solitaria, ovoidea, ellipsoidea vel subcylindrica, continua, apice rotundato, basin versus leniter attenuata, 6–15 × 2–4 µm; *conidia* secundaria catenata, saepe ramificata, in OA praecipue ellipsoidea vel cylindrica, in MEA ovoidea vel ellipsoidea-fusififormia, recta vel curvata, 0–1-septata, in OA 7–29 × 3–4(–5) µm, in MEA (4–)7–10(–15) × (3–)4–5 µm.

*Typus*: **The Netherlands**: Utrecht: Soesterberg, 'De Stompert', on dead leaf of *Quercus robur* ('B3'), April 2003, G. Verkley s.n. [ex-epitypus *Mycosphaerella punctiformis*] (CBS 113265–holotypus; culture kept metabolically inactive, in liquid nitrogen and lyophilized).

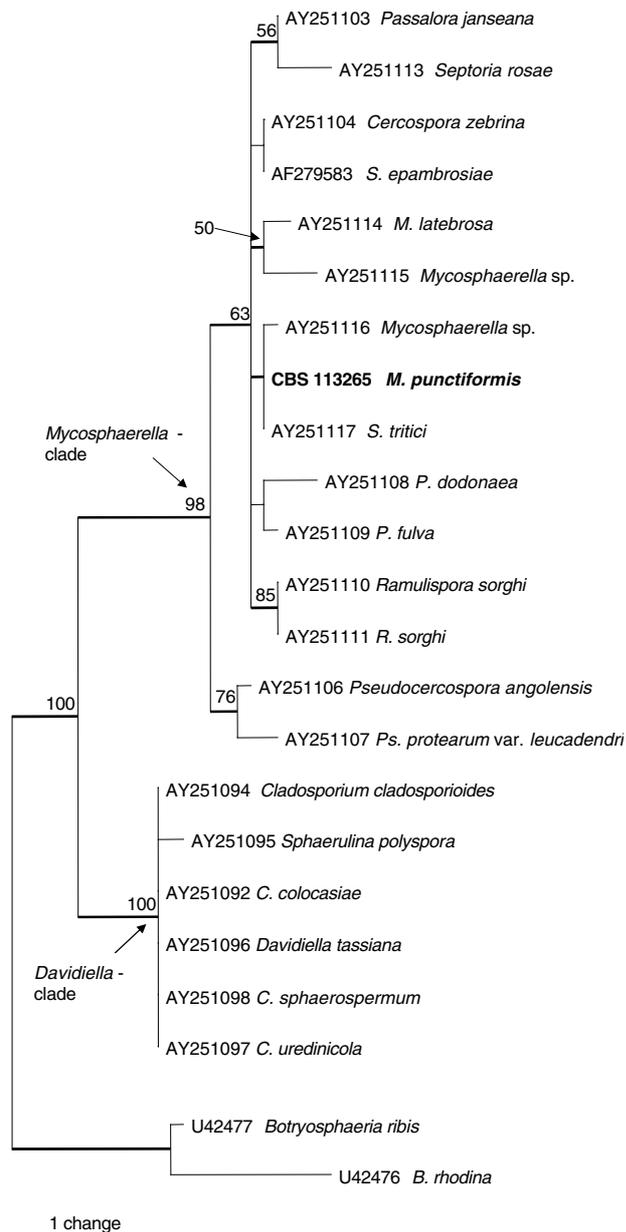
*Conidiophores* simple, subcylindrical or cylindrical, (6–)10–30 × 2.5–4(–5) µm, straight or geniculate-sinuuous, hyaline, smooth-walled, arising from terminal or intermediary hyphal cells at the colony surface, often without a basal septum; conidial scars somewhat thickened and darkened, about 1 µm wide; *conidia* formed holoblastically, hyaline, walls smooth to minutely roughened, hila conspicuous, thickened, darkened, refractive, 0.5–1(–1.5) µm wide; *primary conidia* solitary, ovoid, or ellipsoid to subcylindrical, aseptate, rounded at the top and somewhat attenuated towards the base, 6–15 × 2–4 µm; *secondary conidia* catenate, often in branched, acropetal chains, on OA predominantly ellipsoid to cylindrical, on MEA ovoid to ellipsoid-fusifiform, straight to curved, 0–1-septate, ends with a single hilum rounded to attenuated, branching ends often with hila on short projections, on OA 7–29 × 3–4(–5) µm, on MEA (4–)7–10(–15) × (3–)4–5 µm.

### *Asteromella spermatial* state

Description *in vitro*: *Spermogonia* submerged or on the agar surface, pycnidial, globose, mostly aggregated in larger complexes containing several merging cavities and one or several rather undifferentiated ostioles, black to dark brown; *conidiomatal walls* composed of



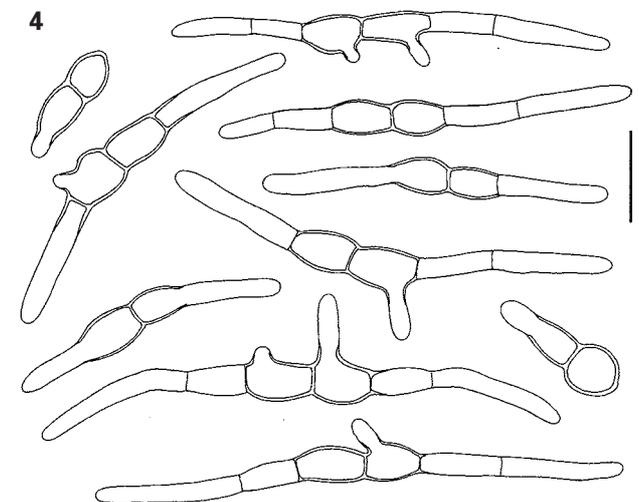
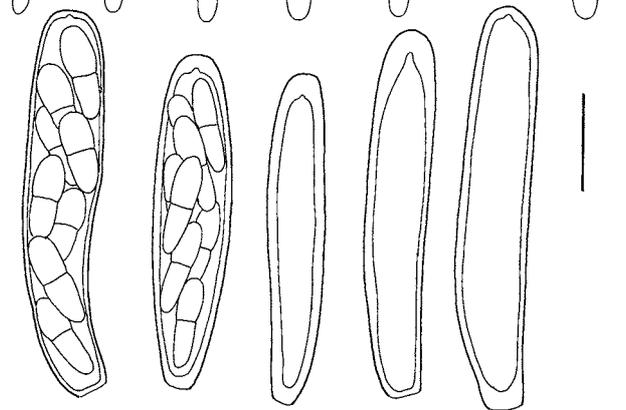
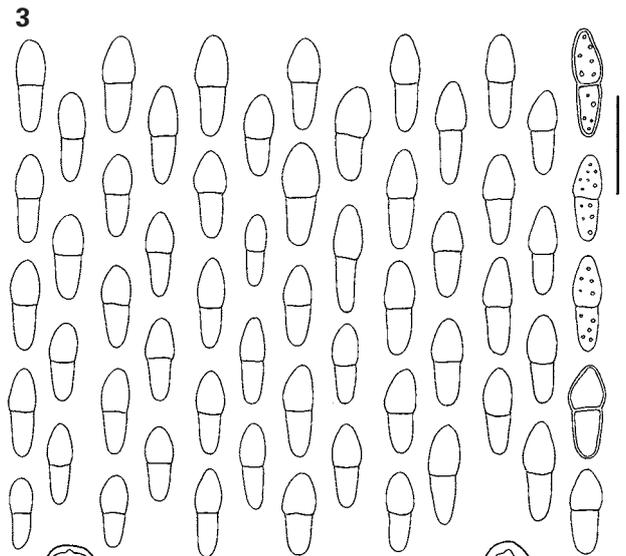
**Fig. 1.** Strict consensus tree of 580 most parsimonious trees of 535 steps obtained in a heuristic search of 168 parsimony-informative characters of the ITS1-5.8SrDNA-ITS2 region calculated in PAUP. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Species are labelled by teleomorph name, if known (anamorph names are given in Table 1).



**Fig. 2.** One of eleven most parsimonious trees obtained from a heuristic search of the SSU sequence alignment. Bootstrap support values from 1000 replicates are shown at the nodes and the scale bar represents a single change. Branches that were maintained in the Strict consensus tree are thickened and the tree is rooted to *Botryosphaeria ribis* and *Botryosphaeria rhodina*.

an outer layer of thick-walled, brown textura angularis, and an inner layer of hyaline, irregular to isodiametric cells; *spermatogenous cells* phialidic, determinate, hyaline, discrete or integrated in simple, septate, more rarely branched, hyaline spermatophores with acropleurogenous openings; *spermatia* ellipsoid to sub-cylindrical, with rounded ends, hyaline, smooth-walled, aseptate, 3–4(–5) × 1–1.5 µm, whitish in mass.

***Mycosphaerella punctiformis*** (Pers.: Fr.) Starbäck, *Bih. Kongl. Svenska Vetensk.-Akad. Handl.* **15**(3, 2): 9 (1889).

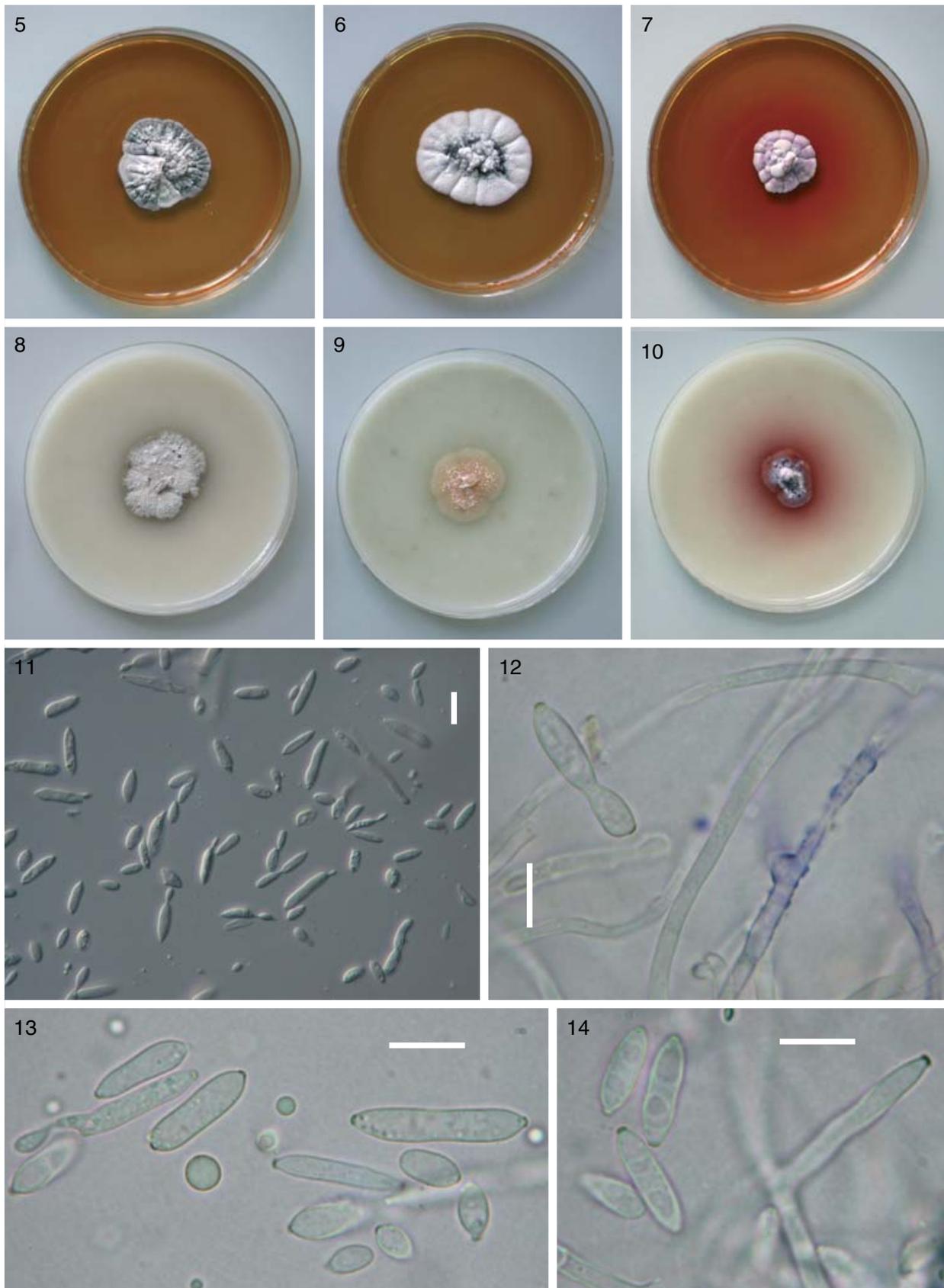


**Figs 3–4.** *Mycosphaerella punctiformis*, epitype (CBS herb. 7949). **Fig. 3.** Ascospores and asci *in planta*. **Fig. 4.** Germinating ascospores on MEA. Bars = 10 µm.

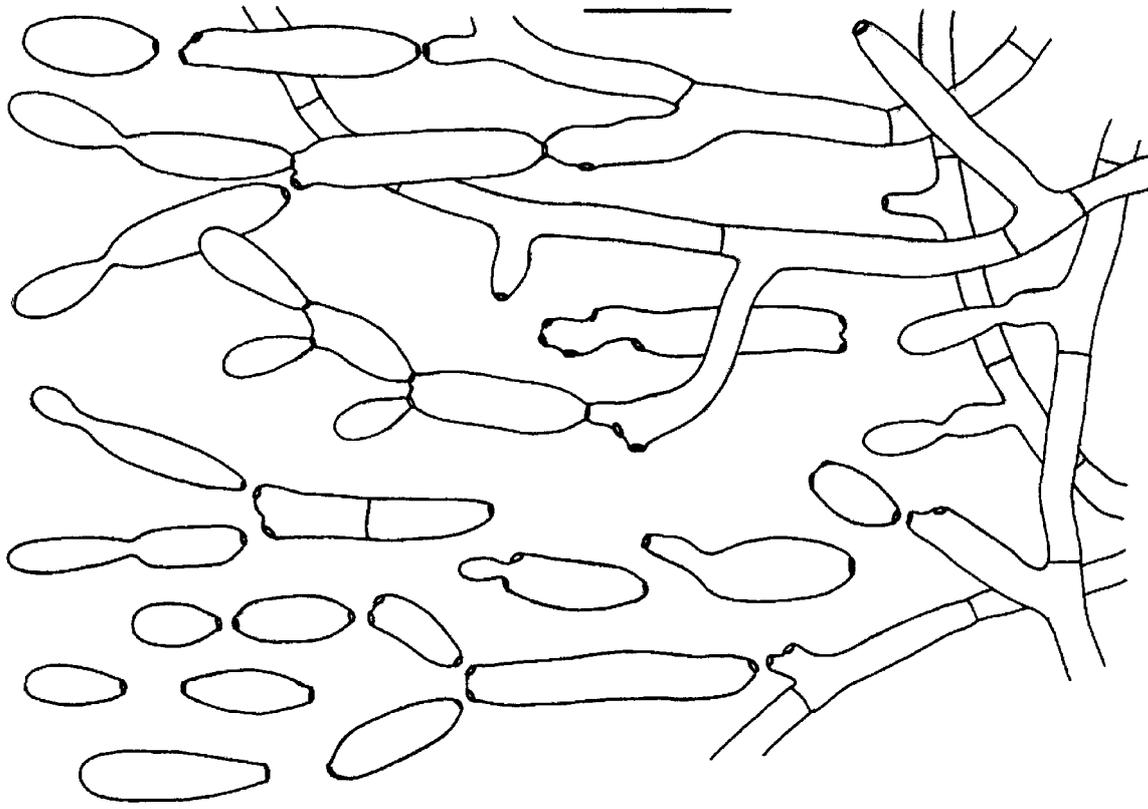
*Sphaeria punctiformis* Pers., *Ann. Bot. (Usteri)* **11**: 26. 1794: Fr., *Syst. Mycol.* **2**: 525 (1823).

*Sphaerella punctiformis* (Pers.: Fr.) Rabenh., *Herb. Vivum Mycol., ed. nov., cent. 3, no. 264* (1856).

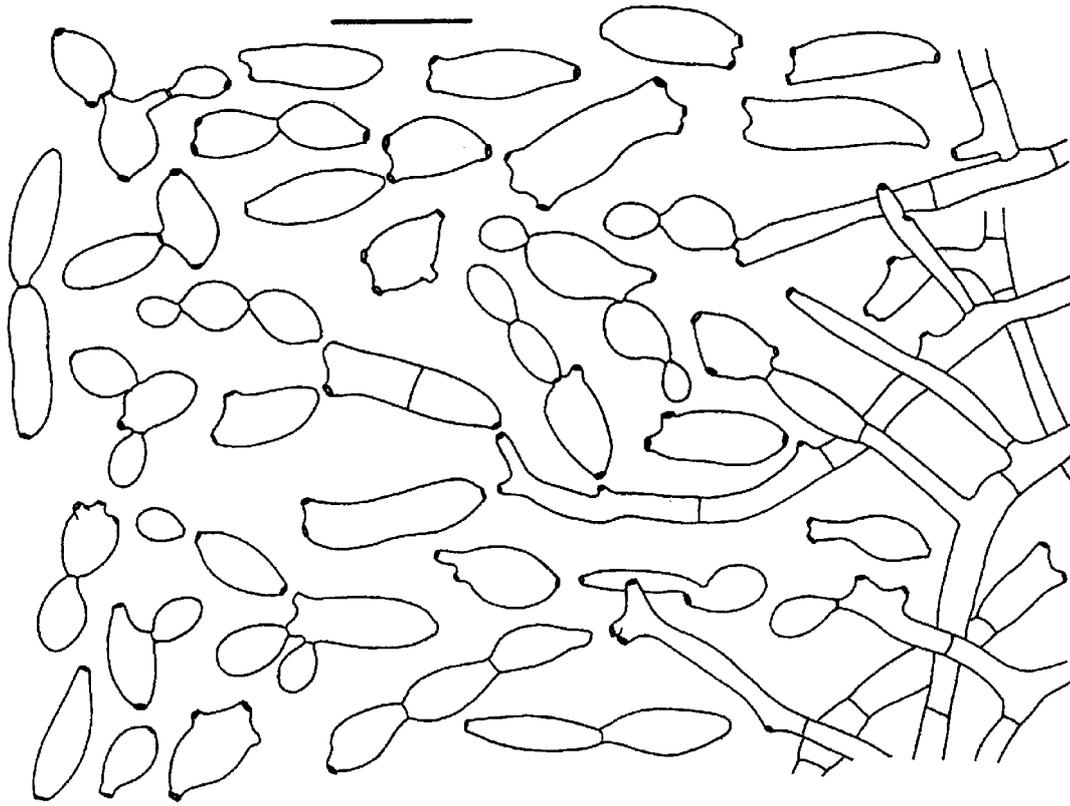
*Typus*: **The Netherlands**: On lower surface of dead leaves of *Quercus* (Fagaceae), Persoon s.n. (L-Persoon – *lectotypus hic designatus*); **Utrecht**: Soesterberg, 'De Stompert', G. Verkleij



**Figs 5–14.** *Mycosphaerella punctiformis* *in vitro* (diffuse daylight, 18 °C). **Figs 5–7.** Isolates on MEA, after 27 d. **Fig. 5.** CBS 113870. **Fig. 6.** CBS 113868. **Fig. 7.** CBS 113869. **Figs 8–10.** Isolates on OA, after 27 d. **Fig. 8.** CBS 113870. **Fig. 9.** CBS 113869. **Fig. 10.** CBS 113868. **Figs 11–14.** Conidia and conidiogenous cells on OA. Bars = 10 µm.



**Fig. 15.** *Mycosphaerella punctiformis* (CBS 113265 – ex-epitype). Conidiogenous cells and conidia on OA. Bar = 10  $\mu$ m.



**Fig. 16.** *Mycosphaerella punctiformis* (CBS 113265 – ex-epitype). Conidiogenous cells and conidia on MEA. Bar = 10  $\mu$ m.

*s.n.*, on dead leaf of *Quercus robur* ('B3'), April 2003 (CBS herb. Nr 7949 – *epitypus hic designatus*); living single ascospore (SS) culture CBS 113265 – (ex-epitype; also with the holotype of *Ramularia endophylla*).

The lectotype is the only material under this name in the Persoon herbarium that was not classified in another (often invalid) variety by himself. It is typical for the species, with cylindrical asci, and ascospores  $8\text{--}10 \times 2\text{--}3 \mu\text{m}$ .

*Endophytic isolates examined: The Netherlands: Utrecht:* Soesterberg, 'De Stompert', ex living leaf of *Quercus robur*, 'AugB3H8', Aug. 2002 (CBS 113868); *loc. cit.*, substr., 'AugB2L12', Aug. 2002 (CBS 113869), and 'AugB3H7' (CBS 113870).

## DISCUSSION

Previous work showed that ITS sequences are fairly constant within most species of *Mycosphaerella*, and that some species may not even be discriminated by ITS sequences (Verkley *et al.* 2004). ITS sequence divergences among *Mycosphaerella* states which are identified as *M. punctiformis* found on dead leaves of *Quercus*, *Tilia*, and *Acer*, indicate that this morphospecies could in fact represent a species complex. *M. phacae-frigidae*, which grouped with four *M. punctiformis* strains, can be distinguished morphologically from *M. punctiformis* by the larger ascospores ( $11\text{--}13 \times 3\text{--}3.5 \mu\text{m}$  in the holotype of *M. phacae-frigidae* in ZT; A. Aptroot, unpubl.). *M. punctiformis*, as we epitypify it here, has been fully characterized phenotypically on the basis of isolates from *Quercus*. Future work including morphological analysis of strains from other hosts, and also sequence analysis of additional genes, may provide evidence to delimit *M. punctiformis s. str.* from other cryptic species. The host range of *M. punctiformis* in this restricted sense is therefore still unknown. The characters of the teleomorph from which CBS 113265 was isolated comply with the original material of *M. punctiformis* in Persoon's herbarium in L. The main aim of the work presented here, is to link the name *M. punctiformis* to this material, and in accordance with Art. 9 of the Code, to epitypify *M. punctiformis* with herbarium specimen CBS 7949 (teleomorph on leaves), and an ex-epitype strain CBS 113265. Other *M. punctiformis* strains which originated from *Tilia*, *Acer*, and *Quercus* differ in ITS sequence by more than 20 positions from the epitype strain and other strains of *M. punctiformis s. str.* However, the ITS data proved insufficient to resolve possible cryptic species within the *M. punctiformis* complex. Therefore, all isolates studied here are for the moment considered as *M. punctiformis s. lat.*

We repeatedly isolated endophytic *Ramularia* strains from surface-sterilized, fresh, green leaves of *Quercus robur* trees collected between June and September. Because they were morphologically and genetically identical to the epitype strain, we were able to prove that *M. punctiformis* can asymptotically colonize

living *Quercus* leaves. Its presence becomes evident by the spermogonia, which develop in large numbers when oak leaves or parts hereof go into senescence naturally or due to activities of fungi or other invaders. Although *R. endophylla* conidia were occasionally seen near leaf lesions, we were unable to confirm that conidial sporulation of *M. punctiformis* does occur *in planta* or on dead leaves in nature. This is in accordance with Braun (1998), who listed the *Ramularia* anamorph of *M. punctiformis* as an insufficiently known taxon, formed in culture only. The life-cycle of *M. punctiformis* seems to be similar to that described in *M. buna*, a fungus with a *Pseudocercospora* anamorph which endophytically colonizes *Fagus crenata* foliage in Japan (Kaneko & Kakishima 2001, Kaneko, Kakishima & Tokumasu 2003).

On oaks in The Netherlands, *M. punctiformis* is commonly accompanied by the weakly pathogenic *Septoria quercicola*, which forms pycnidia within small leaf spots. We recently also discovered its teleomorph in small numbers on dead leaves, including those of the epitype specimen. The teleomorph of *S. quercicola* differs from *M. punctiformis* in the wider asci ( $35\text{--}50 \times 9\text{--}12 \mu\text{m}$ ) and longer ascospores ( $13\text{--}20 \times 3.5\text{--}5 \mu\text{m}$ , av.  $17 \times 4.5 \mu\text{m}$ ), which are not constricted at the septum and taper about equally towards both ends. Our ITS sequence analyses indicate that this *Mycosphaerella* species, which is probably different from all published species on oaks (Gilman & Wadley 1952) and for which an applicable name has not yet been found, is relatively distant from taxa of the *Ramularia* clade, as well as other taxa with *Septoria* anamorphs.

Host specificity in the *M. punctiformis* complex is still insufficiently known. Brefeld & Tavel (1891) regarded *M. punctiformis* as a plurivorous species. They noted that it was less abundant on oak than *M. maculiformis*, a species originally described from *Corylus*. According to Brefeld & Tavel, *M. maculiformis* can be distinguished from *M. punctiformis* by the more densely arranged ascomata, cylindrical asci and larger ascospores. However, they have been seen as synonymous for a long time, and the type specimens of both species were recently re-examined and found to contain (at least) morphologically indistinguishable fungi. Klebahn (1918) studied the ascomata of *M. punctiformis* on *Tilia*, *Corylus*, and *Quercus* and briefly described and illustrated the *Ramularia* anamorphs in culture. Klebahn noted that there were only minor differences between the teleomorphs from the various tree species, and that the isolates showed only some differences in pigmentation but were otherwise indistinguishable. He tentatively classified these fungi as host-specific forms of *M. punctiformis*. Von Arx (1949, Müller & von Arx 1962) considered *M. maculiformis* as a synonym of *M. punctiformis*, which he regarded as plurivorous. Later authors followed this concept (Barr 1972, Sivanesan 1984), but as is shown here, the situation is more complex and may involve more than one species.



All *Mycosphaerella* species with *Ramularia* anamorphs grouped in a single, monophyletic group which obtained high bootstrap support particularly in the parsimony analysis. This was also the case in earlier molecular studies, in which fewer taxa had been included (Crous *et al.* 2001, Goodwin, Dunkle & Zismann 2001, Verkley *et al.* 2004). As in those studies, *M. graminicola* and *Septoria passerinii* form the closest sister group, but support for the joined clades remains limited. The epitypification of the type species of *Mycosphaerella* will enable the unambiguous application of the name *M. punctiformis*, and facilitate the naming of possible future segregates from *Mycosphaerella*.

## ACKNOWLEDGEMENTS

Irma van Kempen is kindly thanked for isolating and sequencing the oak endophytes, and Mieke Starink-Willemse for sequencing additional strains.

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## Discovery of a functional *Mycosphaerella* teleomorph in the presumed asexual barley pathogen *Septoria passerinii*

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### Abstract

We studied the possibility of a teleomorph associated with the genotypically diverse septoria speckled leaf blotch (SSLB) pathogen of barley, *Septoria passerinii*. A teleomorph in the genus *Mycosphaerella* had been predicted previously based on phylogenetic analyses. This prediction was tested with experiments in the Netherlands and the United States by co-inoculating isolates with opposite mating types onto susceptible barley cultivars and monitoring leaves for sexual structures and for the discharge of ascospores. Characterization of putative hybrid progeny by both molecular (AFLP, RAPD, mating type, and ITS sequencing) and phenotypic analyses confirmed that a *Mycosphaerella* teleomorph of *S. passerinii* has been discovered approximately 125 years after the description of the anamorph. Progeny had recombinant genotypes of the molecular alleles present in the parents, and the identities of representative progeny isolates as *S. passerinii* were confirmed by ITS sequencing. A previously unknown sexual cycle explains the high degree of genetic variation among isolates found in nature. The experimental identification of a predicted teleomorph for *S. passerinii* indicates that cryptic sexual cycles may be common for many other “asexual” fungi with high levels of genotypic diversity.

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**Keywords:** *Septoria passerinii*; Septoria speckled leaf blotch (SSLB); *Hordeum vulgare*; Barley; Teleomorph; Sexual cycle; *Mycosphaerella*; Crossing protocol

### 1. Introduction

*Septoria passerinii* Sacc. causes septoria speckled leaf blotch (SSLB) on barley (*Hordeum vulgare*) and was first discovered in Italy in 1879 (Passerini). Since then, SSLB has been reported around the globe in such areas as the Upper Midwest region of the United States, the Prairie Provinces of Canada, Northern Europe, Northern Africa, Western Asia, and Australia (Cunfer and Ueng, 1999; Mathre, 1997). Over the past decade, SSLB epidemics have

increased in frequency, and SSLB has become one of the most important, albeit sporadic, foliar diseases of barley in the United States and in Canada (Mathre, 1997; Steffenson, 2003; Toubia-Rahme et al., 2003). Yield losses of up to 38% have been reported in Minnesota and North Dakota, with similar reports of losses up to 20% in Canada (Green and Bendelow, 1961; Toubia-Rahme and Steffenson, 1999). In addition to reductions in yield, SSLB can render the remaining barley grain unacceptable for malting due to reductions in both kernel size and amount of malt extract (Green and Bendelow, 1961).

Many barley cultivars are resistant to *S. passerinii* (Banttari et al., 1975; Buchannon, 1961; Green and Dickson, 1957; Koble et al., 1959; Rasmusson and Rogers,

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1963; Toubia-Rahme and Steffenson, 2004). Green and Dickson (1957) reported that 50 of 126 *H. vulgare* cultivars tested were resistant to this pathogen, but these resistant cultivars were of low malting quality. Extensive breeding programs exist for barley, but there has been little attempt to incorporate resistance to *S. passerinii* into new cultivars (Toubia-Rahme and Steffenson, 2004). This is because breeding programs mainly focus on developing cultivars with high yields and high malting qualities and thus have used parents with little or no resistance to SSLB. Consequently, all of the commercially important cultivars for malt and feed in the Upper Midwest region of the United States grown over the past 25 years have been and still are highly susceptible to this pathogen, even though the major cultivars have changed throughout the years (Helm et al., 2001; Toubia-Rahme and Steffenson, 1999, 2004). Toubia-Rahme and Steffenson (2004) argued that because of the increasing importance of SSLB, there should be more invested in the development of cultivars that incorporate resistance with high yield and malting quality characteristics. They reported that resistance could be found in cultivars from diverse geographical origins, such as North America, South America, Europe, North Africa, and East Asia.

Presently there is evidence of up to six genes controlling resistance to SSLB in barley (Buchannon, 1961; Metcalfe et al., 1970; Rasmusson and Rogers, 1963). These specific resistance genes in the host suggest the presence of avirulence genes in the pathogen. However, such avirulence genes have not yet been identified in *S. passerinii*. Furthermore, formal genetic analysis of the pathogen is not possible due to the fact that only the imperfect stage has been reported (Cunfer and Ueng, 1999). Our previous work, however, provided lines of evidence suggesting the possibility of sexual recombination in this fungus. Despite the fact that *S. passerinii* was generally considered to be an asexual fungus (Cunfer and Ueng, 1999), we used heterologous mating-type probes from the wheat pathogen *Mycosphaerella graminicola* (Waalwijk et al., 2002) to clone the mating-type genes of *S. passerinii* (Goodwin et al., 2003), based on a previously identified close phylogenetic relationship between these two species (Goodwin et al., 2001; Goodwin and Zismann, 2001). In addition, it was shown that both mating-type idiomorphs of *S. passerinii* were found commonly in natural populations on the same leaf among 22 isolates tested, suggesting that sexual recombination under field conditions was possible. This was further substantiated by combined isozyme and RAPD genotyping of these 22 isolates, which yielded 22 unique haplotypes, as expected for sexual, but not asexual, populations (Goodwin et al., 2003).

The purpose of this paper was to test the hypothesis that *S. passerinii* has a cryptic teleomorph in the genus *Mycosphaerella*. The relative ease of generating the predicted teleomorph of *S. passerinii*, which has not been noticed in nature over the past 120-plus years, has broad implications

for mycology and indicates that many other fungi may be incorrectly classified as asexual.

## 2. Materials and methods

### 2.1. Isolates, crossing, and phenotyping procedures

Twelve isolates of *S. passerinii* and two isolates of *M. graminicola* were used in this study (Table 1). Crosses were made both in Wageningen, The Netherlands, and in West Lafayette, IN, USA. Inoculum preparation, inoculations, and crossing procedures were as described previously for *M. graminicola* by Kema et al. (1996c), except that spore suspensions were sprayed onto seedlings instead of being applied with cotton. Environmental conditions for growing seedlings both before and after inoculation were as described previously (Kema et al., 1996a). Isolate combinations for crosses are listed in Table 2. *S. passerinii* crosses were made on 10-day-old seedlings of the barley cvs. Topper 33 and/or Kindred. A cross between *S. passerinii* isolates with the same mating type was included as a negative control to differentiate ascospores generated from environmental contaminants on barley from those generated by *S. passerinii*. *M. graminicola* test crosses were made on the wheat cv. Taichung 29 and served as a positive control for the crossing procedure, as a negative control to differentiate ascospores generated from environmental contaminants on wheat, and as a reference for diagnostic comparison of *M. graminicola* ascospores with those potentially produced by the *S. passerinii* teleomorph, since we speculated earlier that ascospores from these species were likely to be similar morphologically (Goodwin et al.,

Table 1  
Summary information about the isolates of *Septoria passerinii* and *Mycosphaerella graminicola* used in this study

Species	Isolate	Collection location	Mating type
<i>S. passerinii</i> <sup>a</sup>	P62	North Dakota, USA	<i>mat 1-1</i>
	P63	North Dakota, USA	<i>mat 1-1</i>
	P64	North Dakota, USA	<i>mat 1-1</i>
	P65	North Dakota, USA	<i>mat 1-1</i>
	P66	North Dakota, USA	<i>mat 1-2</i>
	P67	North Dakota, USA	<i>mat 1-2</i>
	P68	North Dakota, USA	<i>mat 1-1</i>
	P71 <sup>b</sup>	North Dakota, USA	<i>mat 1-1</i>
	P75	North Dakota, USA	<i>mat 1-1</i>
	P78	Minnesota, USA	<i>mat 1-2</i>
	P81	Minnesota, USA	<i>mat 1-2</i>
	P83 <sup>b</sup>	North Dakota, USA	<i>mat 1-2</i>
	<i>M. graminicola</i>	IPO323	The Netherlands
IPO94269		The Netherlands	<i>mat 1-2</i>

<sup>a</sup> The isolates of *S. passerinii* were as reported previously by Goodwin et al. (2003).

<sup>b</sup> Cultures of these isolates have been deposited into the collection of the Fungal Biodiversity Center (Centraalbureau voor Schimmelcultures) in Utrecht, the Netherlands, under accession numbers: P71 = CBS 120383 and P83 = CBS 120382. Progeny isolates P71 × P83 A = CBS 120384 and P71 × P83 B = CBS 120385 also were deposited.

Table 2

*In planta* crosses between and among isolates of *Septoria passerinii* and *Mycosphaerella graminicola*

Isolates	Cultivars for crossing	Locations for crossing
P71 × P83	Topper 33 and Kindred	Wageningen and West Lafayette
P78 × P83	Topper 33	Wageningen
P62 × P81	Kindred	West Lafayette
P62 × P83	Kindred	West Lafayette
P62 × P81	Kindred	West Lafayette
P63 × P78	Kindred	West Lafayette
P64 × P81	Kindred	West Lafayette
P65 × P66	Kindred	West Lafayette
P68 × P67	Kindred	West Lafayette
P71 × P81	Kindred	West Lafayette
P71 × P83	Kindred	West Lafayette
P75 × P78	Kindred	West Lafayette
P63 × P67	Topper 33 and Kindred	Wageningen and West Lafayette
IPO323 × IPO94269	Taichung 29	Wageningen
P71 × IPO94269	Topper 33 and Taichung 29	Wageningen
IPO323 × P83	Topper 33 and Taichung 29	Wageningen

2003). Finally, we also performed interspecific crosses between *S. passerinii* and *M. graminicola* because of the suggested close phylogenetic relationship between these species (Goodwin and Zismann, 2001). Plants were placed on a rotating table in an inoculation cabinet, and spore suspensions (at concentrations of  $10^7$  per ml in a total of 30, 15 ml per parental isolate) were sprayed until run-off. Incubations in Wageningen and West Lafayette were conducted as described by Kema et al. (1996c) and Adhikari et al. (2003), respectively. After symptoms developed during incubation in the greenhouse (22 °C, >85% RH), seedlings were placed outside in large plastic pots covered with 1.5-mm-mesh plastic screens. Crosses were attempted seven times between September 2002 and May 2005. Leaf samples were collected once per week from 7 to 12 weeks after inoculation in the Netherlands and from 4 to 10 weeks in the U.S. for discharge of ascospores onto 2% water agar and for microscopical identification of the sexual structure. Proposed parental isolates and the resulting progeny were inoculated onto the susceptible barley cv. Topper 33 for phenotypic comparisons.

## 2.2. Comparative taxonomical analyses of discharged ascospores and of sexual structures

Because plants were outside for up to 10 weeks, they were exposed to sexual and asexual spores of naturally occurring contaminant fungal species. In addition, it was impossible to know for certain what type of ascospores to expect for *S. passerinii* because they had not been described previously. Therefore, all discharged ascospores were meticulously categorized for size, shape, number of cells, pigmentation, and germination pattern on 2% water agar. All non-*M. graminicola* ascospore types discharged

from leaves inoculated with *M. graminicola*, as well as all ascospore types discharged from leaves that were co-inoculated with isolates of *S. passerinii* with the same mating type, were considered to be environmental contaminants. Examples of the different types of discharged ascospores were transferred as single spores to yeast-glucose broth (YGB) and then onto potato dextrose agar (PDA) for comparisons of growth with that of *S. passerinii*. Infected leaf samples that were co-inoculated with isolates of *S. passerinii* with opposite mating types were also screened microscopically to find the associated sexual structure.

## 2.3. DNA extraction and analyses

In preparation for DNA extraction, isolates were grown in YGB for 10 days, at which time spores were pelleted and subsequently lyophilized. Total genomic DNA was extracted from 10 mg of lyophilized spores using the Puregene DNA isolation kit (Gentra System Inc., Minneapolis, MN, USA) and eluted with 50 µl of TE buffer (pH 8.0). All PCRs were performed in either an MJ PTC-200 Peltier (MJ Research, Watertown, MA, USA) or a Perkin-Elmer 9600 (Perkin-Elmer, Foster City, CA, USA) thermal cycler. Primers and adapters used in this study are listed in Table 3.

To confirm ascospores as progeny from *S. passerinii* crosses and to determine allelic segregation ratios, parental isolates and presumed progeny were screened using mating-type PCR, Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Internal Transcribed Spacer (ITS) analysis. For the mating-type analysis, primers developed by Goodwin et al. (2003) were used. PCRs were done in 25-µl reactions, each containing 2.5 µl of 10 mM dNTPs, 2.5 µl of 10× PCR buffer, 1.5 µl of 25 mM MgCl<sub>2</sub>, 0.1 µl of 5 U/µl AmpliTaq DNA polymerase (Applied Biosystems), 2.5 µl of 0.01% G-2500 Gelatin (Sigma), 1.33 µl each of 4 µM

Table 3

Primers and adapters for *Septoria passerinii* used in this study

Name	Sequence (5' to 3')	Purpose
MT-F	CTTCTTGCTGCGCCACAGG	<i>mat 1-1</i> and <i>mat 1-2</i> PCR
Alpha(1594)R	CGGTATGTGGATGGAAGAAAGG	<i>mat 1-1</i> PCR
HMG(849)R	TAGTCGGGACCTGAAGGAGTG	<i>mat 1-2</i> PCR
OPA-9	GGGTAACGCC	RAPD
EcoRI adapter	CTCGTAGACTGCGTACC	AFLP
MseI adapter	AATTGGTACGCGAGTC GACGATGAGTCCTGAG TACTCAGGACTCAT	AFLP
E00	GACTGCGTACCAATTC	AFLP
M00	GATGAGTCCTGAGTAA	AFLP
E19	GACTGCGTACCAATTCGA	AFLP
M16	GATGAGTCCTGAGTAACC	AFLP
ITS4	TCCTCCGCTTATTGATATGC	ITS sequencing
ITS5	GGAAGTAAAGTCGTAACAAGG	ITS sequencing

MT-F, Alpha(1594)R, and HMG(849)R primers, 3 µl of 1 ng/µl target DNA, and 8.9 µl of sterile double-distilled (sdd) water. Thermal cycler conditions were as described previously (Goodwin et al., 2003), and the annealing temperature was 55 °C. For the RAPD analysis, PCRs were done in 25-µl reactions, each containing 2.5 µl of 2 mM dNTPs, 2.5 µl of 10× PCR + MgCl<sub>2</sub> buffer, 0.25 µl of 50 mM MgCl<sub>2</sub>, 0.06 µl of 5 U/µl Taq DNA polymerase (Roche), 2.5 µl of 10 ng/µl OPA9 primer (Operon Technologies), 1.5 µl of 0.5 ng/µl DNA, and 15.69 µl of sdd water. Cycling parameters were as described previously by Kema et al. (1996c). Amplicons from both RAPD and mating-type PCRs were run on 1.2% agarose gels for visualization. Fluorescent AFLP analysis was done according to the protocol described previously by Flier et al. (2003). DNA was digested using enzymes *EcoRI* and *MseI* with primers E00 and M00 and then ligated with *EcoRI* and *MseI* adapters. Primary amplification was with primers E00 and M00, while secondary amplification was with primers E19 (fluorescent, Cy5-labeled) and M16, each with two selective nucleotides. Amplified bands were viewed using ALFwin Evaluation software (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). For ITS sequencing, PCRs were done in 25-µl reactions, each containing 2.5 µl of 10 mM dNTPs, 2.5 µl of 10× Mango PCR buffer, 1.5 µl of 25 mM MgCl<sub>2</sub>, 0.5 µl of 1U/µl Mango Taq DNA polymerase (Bioline), 2.5 µl each of 2 µM primers ITS4 and ITS5, 1 µl of 10 ng/µl target DNA, and 12 µl of sdd water. Cycling parameters were as described previously by Goodwin and Zismann (2001). Sequencing was done with the ThermoSequenase fluorescence-labeled primer cycle sequencing kit on an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described previously (Goodwin and Zismann, 2001). Digestions of ITS regions were done with the enzyme *Sau3AI* as described previously (Goodwin and Zismann, 2001).

### 3. Results

#### 3.1. Comparative taxonomical analyses of discharged ascospores and of sexual structures

Routine test crosses between *M. graminicola* isolates IPO323 and IPO94269 that were used as positive controls for the crossing procedure discharged ascospores from eight through 12 weeks after inoculation. During weeks 11 and 12 (November 2002), we also identified a substantial number of two-celled ascospores (~80) from plants that were co-inoculated with *S. passerinii* isolates P71 and P83 that closely resembled those from *M. graminicola* in morphology and early growth development. Ascospores of the two species were similar in their germination patterns. Initially, two germ tubes arose from the polar ends and grew parallel to the long axis of the spore. Additional secondary germ tubes (1–2) arose at the ascospore septum and grew perpendicular to the long axis of the ascospore.

Ascospores remained hyaline and did not develop additional septa during the initial phase of germination. We were able to isolate 17 of those as single-ascospore cultures for further analyses. Repeated attempts to cross *S. passerinii* resulted in a positive discharge of eight ascospores of the same type as mentioned above during May 2005 in West Lafayette, this time from cv. Kindred that was co-inoculated with *S. passerinii* isolates P63 and P67. One of these was isolated as a single-ascospore culture. The colonies developing from all 18 proposed progeny on PDA plates, as well as their morphology and growth rate in YGB cultures (not shown), were identical to those of the parental isolates.

In addition, numerous different types of ascospores were discharged from barley leaves that were co-inoculated with two *S. passerinii* isolates, including the control crosses between isolates of the same mating type, during this same time period. We monitored thousands of ascospore contaminants on barley, some of which could be identified. One species of *Didymella* with an *Ascochyta* anamorph, one species of *Leptosphaerulina*, and four species of *Paraphaeosphaeria* (including *P. michotii*) were isolated commonly. In addition, two-celled ascospores of *Davidiella tassiana*, the teleomorph of *Cladosporium herbarum*, also were encountered regularly on older leaf material. Single-spore isolates from a sampling of these contaminants did not show any similarity to *S. passerinii* in *in vitro* growth tests on PDA or in YGB (not shown).

The interspecies crosses between *S. passerinii* and *M. graminicola* resulted in numerous ascospores (two to four celled), but their growth on PDA and in YGB did not resemble that of either *S. passerinii* or *M. graminicola*. Subsequent RAPD characterization (data not shown) excluded them as interspecies hybrids, so they were considered to be contaminants.

Infected leaf samples inoculated with isolates of *S. passerinii* with opposite mating types from which *Mycosphaerella* ascospores were successfully harvested were examined microscopically to locate ripe ascomata. Despite numerous attempts over several years, only a single, partly decayed ascoma was found. Ascospores were observed to be hyaline, thin-walled, obovoid, and 10–15 × 3–4 µm. Due to the poor state of the material, the sexual stage could not be officially named, although it clearly resembled *M. graminicola* in general morphology. We therefore propose that the *S. passerinii* teleomorph belongs to the genus *Mycosphaerella*, as is indicated by its DNA phylogeny (Goodwin et al., 2001).

#### 3.2. Genotyping

The 17 proposed progeny from the cross between *S. passerinii* isolates P71 and P83 were genotyped based on mating-type PCR, RAPD and AFLP markers, and by ITS analyses. The mating-type PCRs were positive and matched the expected 1:1 segregation ratio (*mat1-1:mat1-2* = 10:7;  $\chi^2 = 0.53$ ;  $P = 0.05$ ) typical for an organism with a heterothallic, bipolar mating system (data not shown).

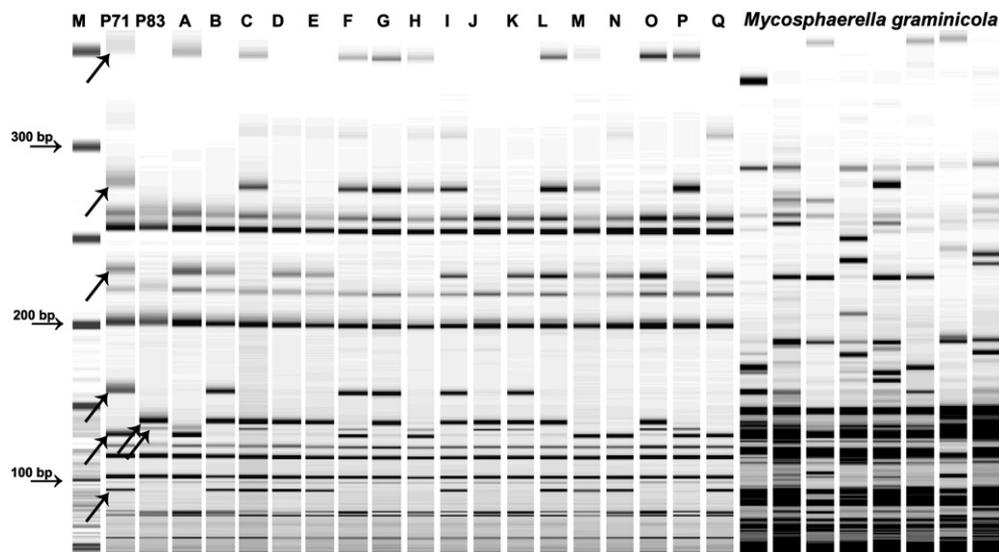


Fig. 1. Genotypes of the parental *Septoria passerinii* isolates P71 and P83 compared to 17 ascospore progeny (isolates A through Q) and seven single-ascospore isolates of *Mycosphaerella graminicola* using AFLP markers. Primers *EcoRI*-GA and *MseI*-CC were used for all isolates. Diagonal arrows indicate polymorphic bands between the *S. passerinii* parental isolates (six from P71 and two from P83). Horizontal arrows indicate reference size markers.

Furthermore, the RAPD analysis showed that the majority of these progeny had recombinant genotypes based on just three markers (not shown), indicating that these *S. passerinii* isolates were the parents of the collected offspring. Genotyping of the ascospore set using AFLP confirmed this conclusion (Fig. 1). Results of the AFLP analysis with the primers *EcoRI*-GA and *MseI*-CC showed that the parental isolates P71 and P83 had six and two unique bands, respectively, and had an additional 10 bands in common. All putative progeny isolates possessed these 10 shared bands and additionally displayed at least two of the eight unique bands observed for the parental isolates P71 and/or P83. All 17 proposed progeny had recombinant genotypes except for one that had the same genotype as P83, but this one had a recombinant genotype in the RAPD analysis. None of the progeny had bands that were not present in the parents. For comparison, seven isolates of *M. graminicola* were included on the same polyacrylamide gel using the same AFLP enzymes and primers. There was at least one (at ~230 bp) and possibly more shared bands between *M. graminicola* and *S. passerinii*, which can be expected since these species are closely related, but bands having the same size do not necessarily have the same sequences. However, the vast majority of bands were not shared between the two species, and the AFLP patterns clearly distinguish *S. passerinii* from *M. graminicola*.

To further distinguish the *S. passerinii* progeny from *M. graminicola*, the ITS region was digested with the enzyme *Sau3AI*. All *S. passerinii* progeny showed the same pattern as both of the parental isolates, P71 and P83 (not shown). This pattern was different from the pattern of *M. graminicola* isolates IPO323, IPO94269, and T48. In addition, the ITS regions of parental isolates P71 and P83 and progeny A, E, K, and M were cloned and sequenced. The ITS sequences of all isolates were identical to one another

and to archived sequences of several isolates of *S. passerinii* in a blastn search of GenBank. Isolates P71, P83, A, and B have been deposited into the culture collection of the Fungal Biodiversity Center (Centraalbureau voor Schimmelcultures) in Utrecht, the Netherlands. The one proposed progeny isolate from the cross between *S. passerinii* isolates P63 and P67 was characterized as *mat1-1*, and its ITS sequence also was identical to that of *S. passerinii*. This isolate must have been a progeny derived from isolates P63 and P67, because barley is not grown in central Indiana and *S. passerinii* has not been found on wild barley, so no source of natural inoculum exists.

### 3.3. Phenotyping

Plant inoculations confirmed the ability of the progeny isolates to infect barley. Inoculation of barley seedlings with spores from offspring from the cross between P71 and P83 caused the typical SSLB symptoms on barley (Fig. 2) that began as small chlorotic flecks at 10 days after inoculation. These slowly developed into larger chlorotic blotches that eventually turned necrotic at ~17 days after inoculation. These lesions contained numerous pycnidia, the asexual fructifications that produce the slender multi-celled pycnidiospores typical for *S. passerinii*. In contrast, inoculations using *M. graminicola* on the barley cv. Topper 33 or *S. passerinii* on the wheat cv. Taichung 29 did not develop symptoms, even after extended incubation periods (data not shown).

## 4. Discussion

High genotypic diversity in natural populations, the identification of apparently intact mating-type genes, and the occurrence of both mating types within single leaves

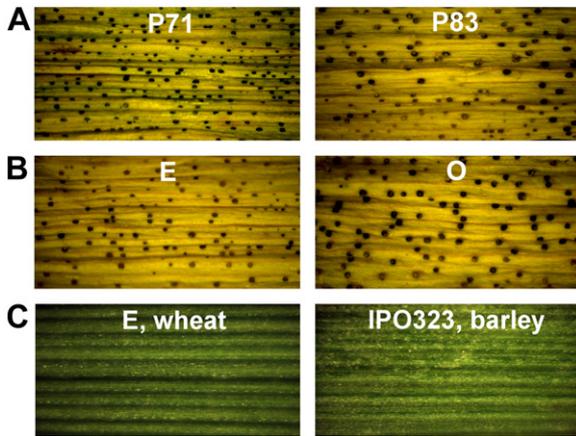


Fig. 2. Symptom development of isolates of *Septoria passerinii* and *Mycosphaerella graminicola* on the barley cultivar Topper 33 or the wheat cultivar Obelisk at 21 days post inoculation. (A) *S. passerinii* parental isolates P71 and P83 on cv. Topper 33. (B) *S. passerinii* progeny isolates E and O on cv. Topper 33. (C) Negative controls. (Left) *S. passerinii* progeny isolate E on cv. Obelisk. (Right) *M. graminicola* isolate IPO323 on cv. Topper 33.

all led to the speculation that *S. passerinii* had the capacity for sexual recombination (Goodwin et al., 2003). However, there was no concrete proof of a functional teleomorph for this fungus that was hitherto considered to be asexual (Cunfer and Ueng, 1999). Therefore, we proceeded to test the hypothesis of a functional teleomorph by crossing isolates of *S. passerinii* with opposite mating types using the *in planta* protocol developed for the closely related sexual species *M. graminicola* (Kema et al., 1996c). This led to the generation of the teleomorph for *S. passerinii* both in Europe and in the United States.

Even though we have generated and characterized sexual progeny from two crosses of *S. passerinii* isolates, we cannot formally describe the sexual stage as required by the International Code of Botanical Nomenclature due to the lack of well-preserved teleomorph material. The identification of the sexual structure has been hampered by the necessity to place inoculated plants outside for approximately two months. Due in part to this, the vast majority of the ascospores discharged from the inoculated barley leaves did not originate from crosses of *S. passerinii* isolates but instead were contaminants from fungi in the environment. Likewise, the vast majority of sexual structures observed on leaves were not produced by crosses of *S. passerinii* isolates but rather by naturally occurring contaminant species. This complicated the localization of the very few ascomata generated by the teleomorph of *S. passerinii*. Furthermore, our observations suggest that the inconspicuous, thin-walled, medium-brown ascomata degenerate quickly once ascospores are discharged, which could explain our difficulty in locating ripe ascomata on leaf sections known to harbor the teleomorph. Three species of *Mycosphaerella* have been described on *Hordeum* (barley), two on *Secale* (rye), and three on *Triticum* (wheat) (Corlett, 1991),

but the dimensions of their ascospores as well as their associated anamorphs indicate that they are distinct from the *Mycosphaerella* teleomorph of *S. passerinii*.

It is noteworthy that the success rate of crosses and the number of ascospores obtained from successful crosses are much lower for *S. passerinii* than for *M. graminicola*. Two explanations for the observed sporadic recombination are that either the sexual cycle is much less active in *S. passerinii* on barley than in *M. graminicola* on wheat, or that conditions of the crossing procedure for *M. graminicola* on wheat need to be adapted to meet the environmental requirements for formation of the teleomorph of *S. passerinii* on barley. Thus far, we do not have an indication of what these environmental requirements are, especially since ascospores were harvested from the two successful crosses during cold and wet conditions in Europe (November 2002) and during warm and dry conditions in the United States (May 2005). Other crossing procedures have been attempted for both *S. passerinii* and *M. graminicola*, including leaving the inoculated plants in the greenhouse instead of placing them outside, following the *in vitro* crossing method used for *Mycosphaerella citri* (Mondal et al., 2004), and others. However, only the protocol developed by Kema et al. (1996c) resulted in ascospore production in both species.

The need to place inoculated plants outside to complete the sexual cycle makes them vulnerable to infection by environmental inoculum of *S. passerinii* and other fungi. Contamination by unrelated fungi can be identified and eliminated easily, as described above. However, we also must be certain that environmental inoculum of *S. passerinii* can be identified and excluded. The possibility of contamination by environmental inoculum in Indiana is essentially zero. Barley is not grown commercially in Indiana so there is no nearby source of inoculum. The only wild barley that occurs commonly is *Hordeum jubatum*, and speckled leaf blotch has never been reported on this host in Indiana. Furthermore, an isolate from *H. jubatum* in Minnesota had a different-sized amplicon with the mating-type PCR assay and a different ITS sequence compared to typical *S. passerinii*, so was considered to represent a new, unnamed species of *Septoria* (Goodwin and Zismann, 2001). Thus, there is essentially a zero probability that the progeny isolate in Indiana could have arisen from contamination by environmental inoculum of *S. passerinii*.

It also is extremely unlikely, if not impossible, that we have isolated ascospores from environmental inoculum of *S. passerinii* in the Netherlands. Despite the fact that *S. passerinii* is endemic in the Netherlands, it is not a major pathogen of barley. Moreover, the size of the barley crop in the Netherlands is very small (~50,000 ha) and concentrated at least 150 km from the experimental site. This reduces the chance for splash-borne inoculum to zero, as conidia (pycnidiospores) of the closely related (Goodwin and Zismann, 2001) *S. tritici* are dispersed only over very short distances (on the order of meters) (Bannon and Cooke, 1998; Shaw, 1999) with half distances of about 10 cm (Shaw, 1999). Dispersal ranges of conidia of

*S. passerinii* have not been estimated but presumably will be similar to those for *S. tritici*. Furthermore, none of the negative controls (those inoculated with isolates of the same mating type) discharged ascospores that could be tied to *S. passerinii*, and all of the segregating markers from the AFLP and RAPD analyses came from the two inoculated isolates with no evidence of migrant alleles. An abundance of genetic data in *M. graminicola* using the same mating protocol also showed no evidence of migrant alleles (Kema et al., 1996c, 2000). We therefore conclude that there is essentially no chance that any of the progeny isolates in Indiana or the Netherlands arose from environmental inoculum of *S. passerinii*.

Recently, many presumably asexual fungi have been found to be sexual, such as: *Colletotrichum acutatum* (teleomorph *Glomerella acutata*), a pathogen of flowering plants (Guerber and Correll, 2001); *Phaeoacremonium aleophilum* (teleomorph *Togninia minima*), associated with Petri disease in grapevines (Mostert et al., 2006); and *Beauveria bassiana* (teleomorph *Cordyceps bassiana*), a widely used biological control agent against insects (Huang et al., 2002). Similarly, the identification of mating-type genes in *S. passerinii* has led to the current discovery of a cryptically active sexual cycle. However, mating-type genes have been identified in many other fungal species in which a sexual cycle has not yet been confirmed. One such example is the barley pathogen *Rhynchosporium secalis*. After a phylogenetic analysis showed that this pathogen probably has a teleomorph in the genus *Tapesia* (Goodwin, 2002), two groups cloned its mating-type genes using degenerate primers designed from sequences of *T. yallundae* and *Pyrenopeziza brassicae* (Foster and Fitt, 2003; Linde et al., 2003). Screening of natural populations of *R. secalis* revealed high genetic diversity and a 1:1 ratio for *mat1-1*:*mat1-2* in most populations sampled (Linde et al., 2003). Another example is *Fusarium oxysporum*, a well-studied plant pathogen with a wide host range (Armstrong and Armstrong, 1981). Mating-type genes from *F. oxysporum* have been cloned by Arie et al. (2000). However, attempts to cross isolates of *F. oxysporum* with opposite mating types have not yielded sexual spores (S. Ware, unpublished), nor have these spores been found in nature, although high genotypic diversity in natural populations of *F. oxysporum* also suggests the possibility of a sexual cycle (Baayen et al., 2000; Bao et al., 2002). More recently, Paoletti et al. (2005) found evidence for sexuality in the opportunistic human pathogen *Aspergillus fumigatus*.

Almost certainly, many presumably asexual fungi are sexually recombining (see review by Taylor et al., 1999, for a parallel opinion with expanded arguments). In addition to the examples given already, a brief review of findings for the human pathogen *Cryptococcus neoformans* represents an excellent example of why the reproductive capabilities of fungi should not be underestimated. The anamorph *C. neoformans* was first described by Busse (1894) and was presumed to be asexual until the discovery of a bipolar heterothallic mating system in 1976, which led

to the naming of the teleomorph *Filobasidiella neoformans* (Kwon-Chung, 1976). Twenty years later, monokaryotic fruiting between isolates with the same mating type was reported in *C. neoformans*, but this type of reproduction was considered to be strictly mitotic and asexual based on descriptions in other fungi (Wickes et al., 1996). However, in 2005 this monokaryotic fruiting was proven to be a second sexual form of mating for this pathogen (Lin et al., 2005). Thus, major ideas on mating for *C. neoformans* have changed three times since the description of the anamorph, and even a completely new type of sexual reproduction in fungi has been discovered. Therefore, the possibility and even probability of sexual recombination for presumably asexual fungi cannot be excluded, as has been demonstrated in our study.

The discovery of a functional sexual cycle for *S. passerinii* has potentially important consequences for future study of this pathogen as well as for resistance breeding efforts in the host. In a comparison between *S. passerinii* and *M. graminicola*, the time lapse between the description of the anamorph and the discovery of the corresponding teleomorph is similar (123 and 130 years, respectively). *S. tritici*, the anamorph of *M. graminicola*, was first reported in 1842. The teleomorph was discovered in 1894, but it was not linked to *S. tritici* until 1972 (Sanderson, 1972). Once this link was made, the emphasis of research efforts extended from epidemiological studies (Royle and Shaw, 1986; Shaw and Royle, 1993) to studies on population genetics (McDonald et al., 1995, 1999) and host-pathogen interactions (Kema et al., 1996a,b, 2000). The development of fungal genetics in *M. graminicola* (Kema et al., 1996c) had an important impact on the identification of resistance genes in wheat (Brading et al., 2002). To date, at least 12 resistance genes have been identified that are currently being used in practical breeding programs (Chartrain et al., 2005). In this study, we have identified the existence of the sexual stage of *S. passerinii* and report a crossing protocol that potentially can, with some adaptation, be used to generate a mapping population of *S. passerinii* progeny to study the genetics of avirulence on barley. We hypothesize that this will substantially benefit resistance breeding in barley to this economically important pathogen.

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# Phylogeny and ecology of the ubiquitous saprobe *Cladosporium sphaerospermum*, with descriptions of seven new species from hypersaline environments

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**Abstract:** Saprobic *Cladosporium* isolates morphologically similar to *C. sphaerospermum* are phylogenetically analysed on the basis of DNA sequences of the ribosomal RNA gene cluster, including the internal transcribed spacer regions ITS1 and ITS2, the 5.8S rDNA (ITS) and the small subunit (SSU) rDNA as well as  $\beta$ -tubulin and actin gene introns and exons. Most of the *C. sphaerospermum*-like species show halotolerance as a recurrent feature. *Cladosporium sphaerospermum*, which is characterised by almost globose conidia, is redefined on the basis of its ex-neotype culture. *Cladosporium dominicanum*, *C. psychrotolerans*, *C. velox*, *C. spinulosum* and *C. halotolerans*, all with globose conidia, are newly described on the basis of phylogenetic analyses and cryptic morphological and physiological characters. *Cladosporium halotolerans* was isolated from hypersaline water and bathrooms and detected once on dolphin skin. *Cladosporium dominicanum* and *C. velox* were isolated from plant material and hypersaline water. *Cladosporium psychrotolerans*, which grows well at 4 °C but not at 30 °C, and *C. spinulosum*, having conspicuously ornamented conidia with long digitate projections, are currently only known from hypersaline water. We also newly describe *C. salinae* from hypersaline water and *C. fusiforme* from hypersaline water and animal feed. Both species have ovoid to ellipsoid conidia and are therefore reminiscent of *C. herbarum*. *Cladosporium langeronii* (= *Hormodendrum langeronii*) previously described as a pathogen on human skin, is halotolerant but has not yet been recorded from hypersaline environments.

**Taxonomic novelties:** *Cladosporium dominicanum* Zalar, de Hoog & Gunde-Cimerman, sp. nov., *C. fusiforme* Zalar, de Hoog & Gunde-Cimerman, sp. nov., *C. halotolerans* Zalar, de Hoog & Gunde-Cimerman, sp. nov., *C. psychrotolerans* Zalar, de Hoog & Gunde-Cimerman, sp. nov., *C. salinae* Zalar, de Hoog & Gunde-Cimerman, sp. nov., *C. spinulosum* Zalar, de Hoog & Gunde-Cimerman, sp. nov., *C. velox* Zalar, de Hoog & Gunde-Cimerman, sp. nov.

**Key words:** Actin,  $\beta$ -tubulin, halotolerance, ITS rDNA, phylogeny, SSU rDNA, taxonomy.

## INTRODUCTION

The halophilic and halotolerant mycobiota from hypersaline aqueous habitats worldwide frequently contain *Cladosporium* Link isolates (Gunde-Cimerman *et al.* 2000, Butinar *et al.* 2005). Initially, they were considered as airborne contaminants, but surprisingly, many of these *Cladosporium* isolates were identified as *C. sphaerospermum* Penz. because they formed globose conidia (data unpublished). *Cladosporium sphaerospermum*, known as one of the most common air-borne, cosmopolitan *Cladosporium* species, was frequently isolated from indoor and outdoor air (Park *et al.* 2004), dwellings (Aihara *et al.* 2001), and occasionally from humans (Badillet *et al.* 1982) and plants (Pereira *et al.* 2002). Strains morphologically identified as *C. sphaerospermum* were able to grow at a very low water activity ( $a_w$  0.816), while other cladosporia clearly preferred a higher, less extreme water activity (Hocking *et al.* 1994). This pronounced osmotolerance suggests a predilection for osmotically stressed environments although *C. sphaerospermum* is reported from a wide range of habitats including osmotically non-stressed niches.

We therefore hypothesised that *C. sphaerospermum* represents a complex of species having either narrow or wide ecological amplitudes. The molecular diversity of strains identified as *C. sphaerospermum* has not yet been determined and isolates from humans have not yet been critically compared with those from environmental samples. Therefore, a taxonomic study was initiated with the aim to define phylogenetically and morphologically distinct entities and to describe their *in vitro* osmotolerance and their natural ecological preferences.

## MATERIALS AND METHODS

### Sampling

Samples of hypersaline water were collected from salterns located at different sites of the Mediterranean basin (Slovenia, Bosnia and Herzegovina, Spain), different coastal areas along the Atlantic Ocean (Monte Cristy, Dominican Republic; Swakopmund, Namibia), the Red Sea (Eilat, Israel), the Dead Sea (Ein Gedi, Israel), and the salt Lake Enriquillo (Dominican Republic). Samples from the Sečovlje salterns (Slovenia) were collected once per month in 1999. Samples from the Santa Pola salterns and Ebre delta river saltern (Spain) were taken twice (July and November) in 2000. A saltern in Namibia and one in the Dominican Republic were sampled twice (August and October) in 2002. Various salinities, ranging from 15 to 32 % NaCl were encountered in these ponds.

### Isolation and maintenance of fungi

Strains were isolated from salterns using filtration of hypersaline water through membrane filters (pore diam 0.45  $\mu$ m), followed by incubation of the membrane filters on different culture media with lowered water activity (Gunde-Cimerman *et al.* 2000). Only colonies of different morphology on one particular selective medium per sample were analysed further. Strains were carefully selected from different evaporation ponds, collected at different times, in order to avoid sampling of identical clones. Subcultures were maintained at the Culture Collection of Extremophilic Fungi (EXF, Biotechnical Faculty, Ljubljana, Slovenia), while a selection was deposited at the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) and the Culture Collection of the National Institute of Chemistry (MZKI, Ljubljana, Slovenia). Reference strains were obtained from CBS, and were selected either on the basis of the strain history, name, or on the basis of their ITS rDNA sequence. Strains were maintained on oatmeal agar (OA; diluted OA, Difco: 15 g of Difco 255210 OA medium, 12 g of agar, dissolved in 1 L of distilled water) with or without 5 % additional NaCl. They were preserved in liquid nitrogen or by lyophilisation. Strains studied are listed in Table 1.

**Table 1.** List of *Cladosporium* strains, with their current and original names, geography, GenBank accession numbers and references to earlier published sequences.

Strain Nr. <sup>a</sup>	Source	Geography	GenBank accession Nr. <sup>b</sup>		
			ITS rDNA / 18S rDNA	actin	β-tubulin
<b><i>Cladosporium bruhnei</i></b>					
CBS 177.71	Thuja tincture	The Netherlands, Amsterdam	DQ780399 / DQ780938	EF101354	EF101451
CBS 812.71	<i>Polygonatum odoratum</i> , leaf	Czech Republic, Lisen	DQ780401 / –	–	–
<b><i>Cladosporium cladosporioides</i></b>					
CBS 170.54 NT	<i>Arundo</i> , leaf	U.K., England, Kew	AY213640 / DQ780940	EF101352	EF101453
EXF-321	Hypersaline water	Slovenia, Sečovlje saltern	DQ780408 / –	–	–
EXF-780			DQ780409 / –	–	–
EXF-946	Hypersaline water	Bosnia and Herzegovina, Ston saltern	DQ780410 / –	–	–
<b><i>Cladosporium dominicanum</i></b>					
CPC 11683	Citrus fruit (orange)	Iran	DQ780357 / –	EF101369	EF101419
EXF-696	Hypersaline water	Dominican Republic, saltern	DQ780358 / –	EF101367	EF101420
EXF-718	Hypersaline water	Dominican Republic, salt lake Enriquillo	DQ780356 / –	EF101370	EF101418
EXF-720	Hypersaline water	Dominican Republic, saltern	DQ780355 / –	–	EF101417
EXF-727	Hypersaline water	Dominican Republic, saltern	DQ780354 / –	–	EF101416
EXF-732 T; CBS 119415	Hypersaline water	Dominican Republic, salt lake Enriquillo	DQ780353 / –	EF101368	EF101415
<b><i>Cladosporium fusiforme</i></b>					
CBS 452.71	Chicken food	Canada	DQ780390 / –	EF101371	EF101447
EXF-397	Hypersaline water	Slovenia, Sečovlje saltern	DQ780389 / –	EF101373	EF101445
EXF-449 T; CBS 119414	Hypersaline water	Slovenia, Sečovlje saltern	DQ780388 / DQ780935	EF101372	EF101446
<b><i>Cladosporium herbarum</i></b>					
ATCC 66670, as <i>Davidiella tassiana</i>	CCA-treated Douglas-fir pole	U.S.A., New York, Geneva	AY361959 <sup>2</sup> & DQ780400 / DQ780939	AY752193 <sup>11</sup>	EF101452
<b><i>Cladosporium halotolerans</i></b>					
ATCC 26362	Liver and intestine of diseased frog	U.S.A., New Jersey	AY361982 <sup>2</sup> / –	–	–
ATCC 64726	Peanut cell suspension tissue culture	U.S.A., Georgia	AY361968 <sup>2</sup> / –	–	–
CBS 280.49	Stem of <i>Hypericum perforatum</i> identified as <i>Mycosphaerella hyperici</i>	Switzerland, Glarus, Mühlehorn	DQ780369 / –	EF101402	EF101432
CBS 191.54	Laboratory air	Great Britain	– / –	–	–
CBS 573.78	<i>Aureobasidium caulivorum</i>	Russia, Moscow region	– / –	–	–
CBS 626.82	–	Sweden, Stockholm	– / –	–	–
dH 12862; EXF-2533	Culture contaminant	Brazil	DQ780371 / –	EF101400	EF101422
dH 12941; EXF-2534	Culture contaminant	Turkey	– / –		EF101421
dH 12991; EXF-2535	Brain	Turkey	DQ780372 / –		EF101423
dH 13911; EXF-2422	Ice	Arctics	DQ780370 / –	EF101401	EF101430
EXF-228; MZKI B-840	Hypersaline water	Slovenia, Sečovlje saltern	DQ780365 / DQ780930	EF101393	EF101425
EXF-380	Hypersaline water	Slovenia, Sečovlje saltern	DQ780368 / –	EF101394	EF101427
EXF-564	Hypersaline water	Namibia, saltern	DQ780363 / –	EF101395	EF101433
EXF-565	Hypersaline water	Namibia, saltern	– / –	–	–
EXF-567	Hypersaline water	Namibia, saltern	– / –	–	–
EXF-571	Hypersaline water	Namibia, saltern	– / –	–	–
EXF-572 T; CBS 119416	Hypersaline water	Namibia, saltern	DQ780364 / –	EF101397	EF101424
EXF-646	Hypersaline water	Spain, Santa Pola saltern	DQ780366 / –	EF101398	EF101428
EXF-698	Hypersaline water	Dominican Republic, saltern	– / –	–	–
EXF-703	Hypersaline water	Dominican Republic, salt lake Enriquillo	DQ780367 / –	EF101392	EF101426
EXF-944	Hypersaline water	Bosnia and Herzegovina, Ston saltern	– / –	–	–
EXF-972	Bathroom	Slovenia	– / –	–	–
EXF-977	Bathroom	Slovenia	DQ780362 / –	EF101396	EF101431

Strain Nr. <sup>a</sup>	Source	Geography	GenBank accession Nr. <sup>b</sup>		
			ITS rDNA / 18S rDNA	actin	β-tubulin
EXF-1072	Hypersaline water	Israel, Dead Sea	DQ780373 / –	EF101399	EF101428
EXF-2372	Hypersaline water	Slovenia, Sečovlje saltern	– / –	–	–
UAMH 7686	Indoor air ex RCS strip, from <i>Apis mellifera</i> overwintering facility	U.S.A., Alta, Clyde Corner	AY625063 <sup>5</sup> / –	–	–
–	rDNA from bottlenose dolphin skin infected with <i>Loboa lobo</i>	U.S.A., Texas	AF035674 <sup>6</sup> / –	–	–
–	Microcolony, on rock	Turkey, Antalya	AJ971409 <sup>7</sup> / –	–	–
–	Microcolony, on rock	Turkey, Antalya	AJ971408 <sup>7</sup> / –	–	–
–	Tomato leaves	–	L25433 <sup>8</sup> / –	–	–
<b><i>Cladosporium langeronii</i></b>					
CBS 189.54 NT	Mycosis	Brazil	DQ780379 / DQ780932	EF101357	EF101435
CBS 601.84	<i>Picea abies</i> , wood	Germany, Göttingen	DQ780382 / –	EF101360	EF101438
CBS 101880	Moist aluminium school window frame	Belgium, Lichtervoorde	DQ780380 / –	EF101359	EF101440
CBS 109868	Mortar of Muro Farnesiano	Italy, Parma	DQ780377 / –	EF101362	EF101434
dH 11736	Biomat in a lake	Antarctics	DQ780381 / –	EF101363	EF101436
dH 12459	Orig. face lesion	Brazil	DQ780378 / –	EF101358	EF101439
dH 13833	Ice	Arctics	DQ780383 / –	EF101361	EF101437
–	Nasal mucus	–	AF455525 <sup>4</sup> / –	–	–
–	Nasal mucus	–	AY345352 <sup>4</sup> / –	–	–
–	Mycorrhizal roots	–	DQ068982 <sup>9</sup> / –	–	–
<b><i>Cladosporium oxysporum</i></b>					
ATCC 66669	Creosote-treated southern pine pole	U.S.A., New York, Binghamton	AF393689 <sup>10</sup> / DQ780395	AY752192 <sup>11</sup>	EF101454
ATCC 76499	Decayed leaf, <i>Lespedeza bicolor</i>	–	AF393720	–	–
CBS 125.80	<i>Cirsium vulgare</i> , seedcoat	The Netherlands	AJ300332 <sup>12</sup> / DQ780941	EF101351	EF101455
EXF-697	Hypersaline water	Dominican Republic, salt lake Enriquillo	DQ780392 / –	–	–
EXF-699	Hypersaline water	Dominican Republic, saltern	DQ780394 / –	–	–
EXF-710	Hypersaline water	Dominican Republic, saltern	DQ780393 / –	–	–
EXF-711	Hypersaline water	Dominican Republic, saltern	DQ780391 / –	–	–
<b><i>Cladosporium psychrotolerans</i></b>					
EXF-326	Hypersaline water	Slovenia, Sečovlje saltern	DQ780387 / DQ780934	–	EF101444
EXF-332	Hypersaline water	Slovenia, Sečovlje saltern	DQ780385 / DQ780933	EF101364	EF101441
EXF-391 T; CBS 119412	Hypersaline water	Slovenia, Sečovlje saltern	DQ780386 / –	EF101365	EF101442
EXF-714	Hypersaline water	Dominican Republic	DQ780384 / –	EF101366	EF101443
<b><i>Cladosporium ramotenellum</i></b>					
EXF-454 T; CPC 12043	Hypersaline water	Slovenia, Sečovlje saltern	DQ780403 / –	–	–
<b><i>Cladosporium salinae</i></b>					
EXF-322	Hypersaline water	Slovenia, Sečovlje	DQ780375 / –	EF101391	EF101403
EXF-335 T; CBS 119413	Hypersaline water	Slovenia, Sečovlje	DQ780374 / DQ780931	EF101390	EF101405
EXF-604	Hypersaline water	Spain, Santa Pola	DQ780376 / –	EF101389	EF101404
<b><i>Cladosporium</i> sp.</b>					
CBS 300.96	Soil along coral reef coast	Papua New Guinea, Madang, Jais Aben	DQ780352 / –	EF101385	–
EXF-595	Hypersaline water	Spain, Santa Pola saltern	DQ780402 / –	–	–
<b><i>Cladosporium sphaerospermum</i></b>					
ATCC 12092	Soil	Canada	AY361988 <sup>2</sup> / –	–	–
ATCC 200384	Compost biofilter	The Netherlands	AY361991 <sup>2</sup> / –	–	–
CBS 109.14; ATCC 36950	<i>Carya illinoensis</i> leaf scale	U.S.A.	DQ780350 / –	EF101384	EF101410
CBS 122.47; IFO 6377; IMI 49640; VKM F-772; ATCC 11292	Decaying stem of <i>Begonia</i> sp., with <i>Thielaviopsis basicola</i>	The Netherlands, Aalsmeer	AJ244228 <sup>1</sup> / –	–	–
CBS 188.54; ATCC 11290; IMI 049638	de Vries (Engelhardt strain)	–	AY361990 <sup>2</sup> & AY251077 <sup>3</sup> / –	–	–
CBS 190.54; ATCC 11293; IFO 6380; – IMI 49641	–	–	AY361992 <sup>2</sup> / –	–	–
CBS 192.54; ATCC 11288; IMI 49636	Nail of man	–	AY361989 <sup>2</sup> / –	–	–

Table 1. (Continued).

Strain Nr. <sup>a</sup>	Source	Geography	GenBank accession Nr. <sup>b</sup>		
			ITS rDNA / 18S rDNA	actin	β-tubulin
CBS 193.54 NT; ATCC 11289; IMI 49637	Human nails	–	DQ780343 & AY361958 <sup>2</sup> / DQ780925	EF101380	EF101406
CBS 122.63	Plywood of <i>Betula</i> sp.	Finland, Helsinki	– / –	–	–
CBS 102045; EXF-2524; MZKI B-1066	Hypersaline water	Spain, Barcelona, Salines de la Trinitat	DQ780351 / –	EF101378	EF101411
CBS 114065	Outdoor air	Germany, Stuttgart	– / –	–	–
CPC 10944	Gardening peat substrate	Russia, Kaliningrad	DQ780350 / –	–	–
EXF-131; MZKI B-1005	Hypersaline water	Slovenia, Sečovlje saltern	AJ238670 <sup>1</sup> / –	–	–
EXF-328	Hypersaline water	Slovenia, Sečovlje saltern	– / –	–	–
EXF-385	Hypersaline water	Slovenia, Sečovlje saltern	– / –	–	–
EXF-446	Hypersaline water	Slovenia, Sečovlje saltern	– / –	–	–
EXF-455	Hypersaline water	Slovenia, Sečovlje saltern	DQ780349 / –	EF101375	EF101412
EXF-458	Hypersaline water	Slovenia, Sečovlje saltern	DQ780345 / –	EF101374	EF101409
EXF-461	Hypersaline water	Slovenia, Sečovlje saltern	– / –	–	–
EXF-464	Hypersaline water	Slovenia, Sečovlje saltern	– / DQ780927	–	–
EXF-465	Hypersaline water	Slovenia, Sečovlje saltern	– / –	–	–
EXF-598	Hypersaline water	Spain, Santa Pola	– / –	EF101377	–
EXF-644	Hypersaline water	Spain, Santa Pola	– / –	–	–
EXF-645	Hypersaline water	Spain, Santa Pola	– / –	–	–
EXF-649	Hypersaline water	Spain, Santa Pola	– / –	–	–
EXF-715	Hypersaline water	Dominican Republic, saltern	– / –	–	–
EXF-738	Bathroom	Slovenia	DQ780348 / –	EF101383	EF101414
EXF-739	Bathroom	Slovenia	DQ780344 / –	EF101381	EF101407
EXF-781; MZKI B-899	Hypersaline water	Slovenia, Sečovlje	– / –	–	–
EXF-788	Hypersaline water	Slovenia, Sečovlje	– / –	–	–
EXF-962	Bathroom	Slovenia	DQ780347 / –	EF101382	EF101413
EXF-965	Bathroom	Slovenia	– / –	–	–
EXF-1069	Hypersaline water	Israel, Eilat saltern	– / –	EF101376	–
EXF-1061	Hypersaline water	Israel, Dead Sea	DQ780346 / –	EF101379	EF101408
EXF-1726	Hypersaline water	Israel, Dead Sea	– / –	–	–
EXF-1732	Hypersaline water	Israel, Eilat saltern	– / DQ780928	–	–
–	<i>Bryozoa</i> sp.	–	AJ557744 / –	–	–
–	Nasal mucus	–	AF455481 <sup>4</sup> / –	–	–
<b><i>Cladosporium spinulosum</i></b>					
EXF-333	Hypersaline water	Slovenia, Sečovlje saltern	DQ780404 / –	–	–
EXF-334 T	Hypersaline water	Slovenia, Sečovlje saltern	DQ780406 / –	EF101355	EF101450
EXF-382	Hypersaline water	Slovenia, Sečovlje saltern	DQ780407 / DQ780936	EF101356	EF101449
<b><i>Cladosporium subinflatum</i></b>					
EXF-343 T; CPC 12041	Hypersaline water	Slovenia, Sečovlje saltern	DQ780405 / –	EF101353	EF101448
<b><i>Cladosporium tenuissimum</i></b>					
ATCC 38027	Soil	New Caledonia	AF393724 / –	–	–
EXF-324	Hypersaline water	Slovenia, Sečovlje saltern	– / DQ780926	–	–
EXF-371	Hypersaline water	Slovenia, Sečovlje saltern	DQ780396 / –	–	–
EXF-452	Hypersaline water	Slovenia, Sečovlje saltern	DQ780397 / –	–	–
EXF-563	Hypersaline water	Namibia, saltern	DQ780398 / –	–	–
<b><i>Cladosporium velox</i></b>					
CBS 119417 T; CPC 11224	<i>Bamboo</i> sp.	India, Charidij	DQ780361 / DQ780937	EF101388	EF101456
EXF-466	Hypersaline water	Slovenia, Sečovlje saltern	DQ780359 / –	EF101386	–
EXF-471	Hypersaline water	Slovenia, Sečovlje saltern	DQ780360 / –	EF101387	–

## Cultivation and microscopy

For growth rate determination and phenetic description of colonies, strains were point inoculated on potato-dextrose agar (PDA, Difco), OA and Blakeslee malt extract agar (MEA, Samson *et al.* 2002) and incubated at 25 °C for 14 d in darkness. Surface colours were rated using the colour charts of Komerup & Wanscher (1967). For studies of microscopic morphology, strains were grown on synthetic nutrient agar (SNA, Gams *et al.* 2007) in slide cultures. SNA blocks of approximately 1 × 1 cm were cut out aseptically, placed upon sterile microscope slides, and inoculated at the upper four edges by means of a conidial suspension (Pitt 1979). Inoculated agar blocks were covered with sterile cover slips and incubated in moist chambers for 7 d at 25 °C in darkness. The structure and branching pattern of conidiophores were observed at magnifications × 100, × 200 and × 400 in intact slide cultures under the microscope without removing the cover slips from the agar blocks. For higher magnifications (× 400, × 1 000) cover slips were carefully removed and mounted in lactic acid with aniline blue.

## Morphological parameters

Morphological terms follow David (1997), Kirk *et al.* (2001) and Schubert *et al.* (2007 – this volume). Conidiophores in *Cladosporium* are usually ascending and sometimes poorly differentiated. Though the initiation point of conidiophore stipes could sometimes be determined only approximately, their lengths were in some cases useful for distinguishing morphologically similar species when observed in slide cultures. The branching patterns can be rotationally symmetric or unilateral. Characters of conidial scars were studied by light and scanning electron microscopy (SEM). Conidial chains show different branching patterns, determined by the numbers of conidia in unbranched parts, the nature of ramoconidia as well as their distribution in conidial chains. Measurements are given as (i)  $n_1$ – $n_2$  or (ii)  $(n_1$ –) $n_3$ – $n_4$ (– $n_2$ ), with  $n_1$  = minimum value observed;  $n_2$  = maximum value observed;  $n_3/n_4$  = first/third quartile. For conidia and ramoconidia also average values and standard deviations are listed. The values provided are based on at least 25 measurements for the conidiophores of each strain, and at least 50 measurements for conidia.

## Ecophysiology

To determine the degree of halotolerance, strains were point-inoculated on MEA without and with additional NaCl at concentrations of 5, 10, 17 and 20 % NaCl (w/v) and incubated at 25 °C for 14 d. To determine cardinal temperature requirements for growth, plates were incubated at 4, 10, 25, 30 and 37 °C, and colony diameters measured after 14 d of incubation.

## DNA extraction, sequencing and analysis

For DNA isolation strains were grown on MEA for 7 d. DNA was extracted according to Gerrits van den Ende & de Hoog (1999) by mechanical lysis of approx. 1 cm<sup>2</sup> of mycelium. A fragment of the rDNA including the Internal Transcribed Spacer region 1, 5.8S rDNA and the ITS 2 (ITS) was amplified using the primers V9G (de Hoog & Gerrits van den Ende 1998) and LS266 (Masclaux *et al.* 1995). Sequence reactions were done using primers ITS1 and ITS4 (White *et al.* 1990). For amplification and sequencing of the partial actin gene, primers ACT-512F and ACT-783R were applied according to Carbone & Kohn (1999). For amplification and sequencing of the β-tubulin gene primers T1 and T22 were used according to O'Donnell & Cigelnik (1997). A BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) was used in sequence reactions. Sequences were obtained with an ABI Prism 3700 DNA Analyzer (Applied Biosystems). They were assembled and edited using SeqMan v. 3.61 (DNASar, Inc., Madison, U.S.A.). Sequences downloaded from GenBank are indicated in the trees by their GenBank accession numbers; newly generated sequences are indicated by strain numbers (see also Table 1). Sequences were automatically aligned using ClustalX v. 1.81 (Jeanmougin *et al.* 1998). The alignments were adjusted manually using MEGA3 (Kumar *et al.* 2004). Phylogenetic relationships of the taxa were estimated from aligned sequences by the maximum parsimony criterion as implemented in PAUP v. 4.0b10 (Swofford 2003). Data sets of the SSU rDNA, ITS rDNA and the β-tubulin and actin genes are analysed separately. Species of *Cladosporium s. str.* were compared with various taxa of the *Mycosphaerellaceae* using SSU rDNA sequences and *Fusicladium effusum* G. Winter (*Venturiaceae*) as outgroup. The other data sets focus on *Cladosporium s. str.*, using *Cladosporium salinae* Zalar, de Hoog & Gunde-Cimerman as an outgroup, because this species was most deviant within *Cladosporium* in the SSU rDNA analysis (see below). Heuristic searches were performed on all characters, which were unordered and equally weighted. Gaps were treated as missing characters. Starting tree(s) were obtained via stepwise, random, 100 times repeated sequence addition. Other parameters included a “MaxTrees” setting to 9 000, the tree-bisection-reconnection as branch-swapping algorithm, and the “MulTrees” option set to active. Branch robustness was tested in the parsimony analysis by 10 000 search replications, each on bootstrapped data sets using a fast step-wise addition bootstrap analysis. Bootstrap values larger than 60 are noted near their respective branches. Newly generated sequences were deposited in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)); their accession numbers are listed in Table 1. Alignments and trees were deposited in TreeBASE ([www.treebase.org](http://www.treebase.org)).

Table 1. (Page 158–160).

<sup>a</sup> Abbreviations used: ATCC = American Type Culture Collection, Virginia, U.S.A.; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC = Culture Collection of Pedro Crous, housed at CBS, Utrecht, The Netherlands; dH = de Hoog Culture Collection, housed at CBS, Utrecht, The Netherlands; EXF = Culture Collection of Extremophilic Fungi, Ljubljana, Slovenia; IFO = Institute for Fermentation, Culture Collection of Microorganisms, Osaka, Japan; IMI = The International Mycological Institute, Egham, Surrey, U.K.; MZKI = Microbiological Culture Collection of the National Institute of Chemistry, Ljubljana, Slovenia; UAMH = University of Alberta Microfungus Collection, Alberta, Canada; VKM = All-Russian Collection of Microorganisms, Russian Academy of Sciences, Institute of Biochemistry and Physiology of Microorganisms, Pushchino, Russia; NT = ex-neotype strain; T = ex-type strain.

<sup>b</sup> Reference: <sup>1</sup>de Hoog *et al.* 1999; <sup>2</sup>Park *et al.* 2004; <sup>3</sup>Braun *et al.* 2003; <sup>4</sup>Buzina *et al.* 2003; <sup>5</sup>Meklin *et al.* 2004; <sup>6</sup>Haubold *et al.* 1998; <sup>7</sup>Sert & Sterflinger, unpubl.; <sup>8</sup>Curtis *et al.* 1994; <sup>9</sup>Menkis *et al.* 2005; <sup>10</sup>Managbanag *et al.* unpubl.; <sup>11</sup>Crous *et al.* 2004; <sup>12</sup>Wirsel *et al.* 2002. All others are newly reported here.

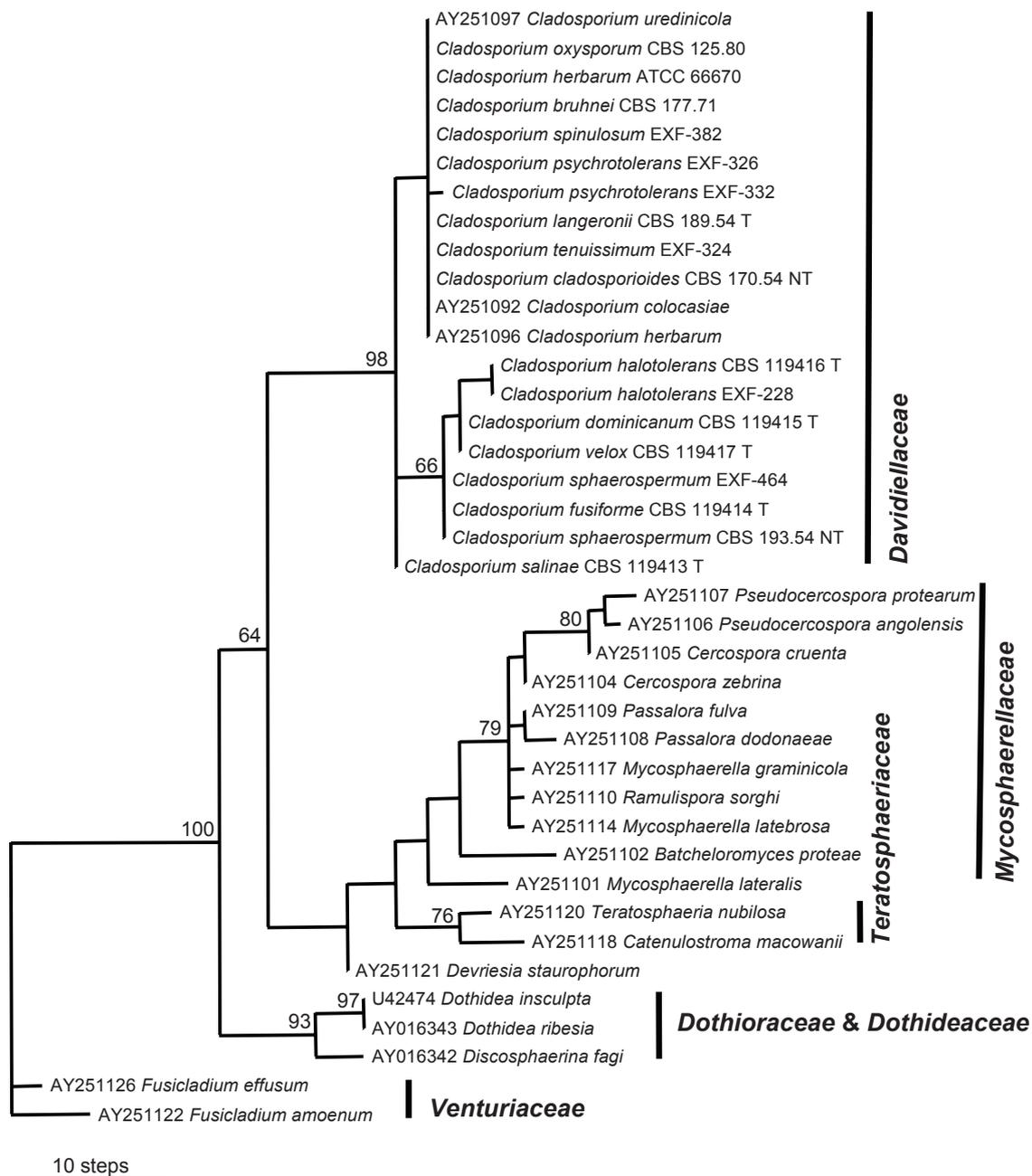


Fig. 1. One of 30 equally most parsimonious and equally looking phylogenetic trees based on a heuristic tree search using aligned small subunit ribosomal DNA sequences. The tree was randomly selected. Support based on 10 000 replicates of a fast step-wise addition bootstrap analysis is indicated near the branches. Species of *Cladosporium s. str.*, including the seven newly described species, form a strongly supported monophyletic group among other taxa of the *Mycosphaerellaceae* (*Dothideomycetes*) (CI = 0.631, RI = 0.895, PIC = 50).

Table 2. Statistical parameters describing phylogenetic analyses performed on sequence alignments of four different loci.

Parameter	SSU rDNA	ITS rDNA <sup>1</sup>	$\beta$ -tubulin <sup>2</sup>	Actin <sup>3</sup>
Number of alignment positions	1031	498	654	210
Number of parsimony informative characters (PIC)	50	68	220	103
Length of tree / number of steps	103	102	714	338
Consistency Index (CI)	0.631	0.804	0.538	0.586
Retention Index (RI)	0.895	0.975	0.883	0.885
Rescaled Consistency Index (RC)	0.565	0.784	0.475	0.518
Homoplasy index (HI)	0.369	0.196	0.462	0.414
Number of equally parsimonious trees retained	30	600	90	32

<sup>1</sup>Including the internal transcribed spacer region 1 and 2 and the 5.8S rDNA.

<sup>2</sup>Including partial sequences of 4 exons and complete sequences of 3 introns.

<sup>3</sup>Including partial sequences of 3 exons and 2 introns.

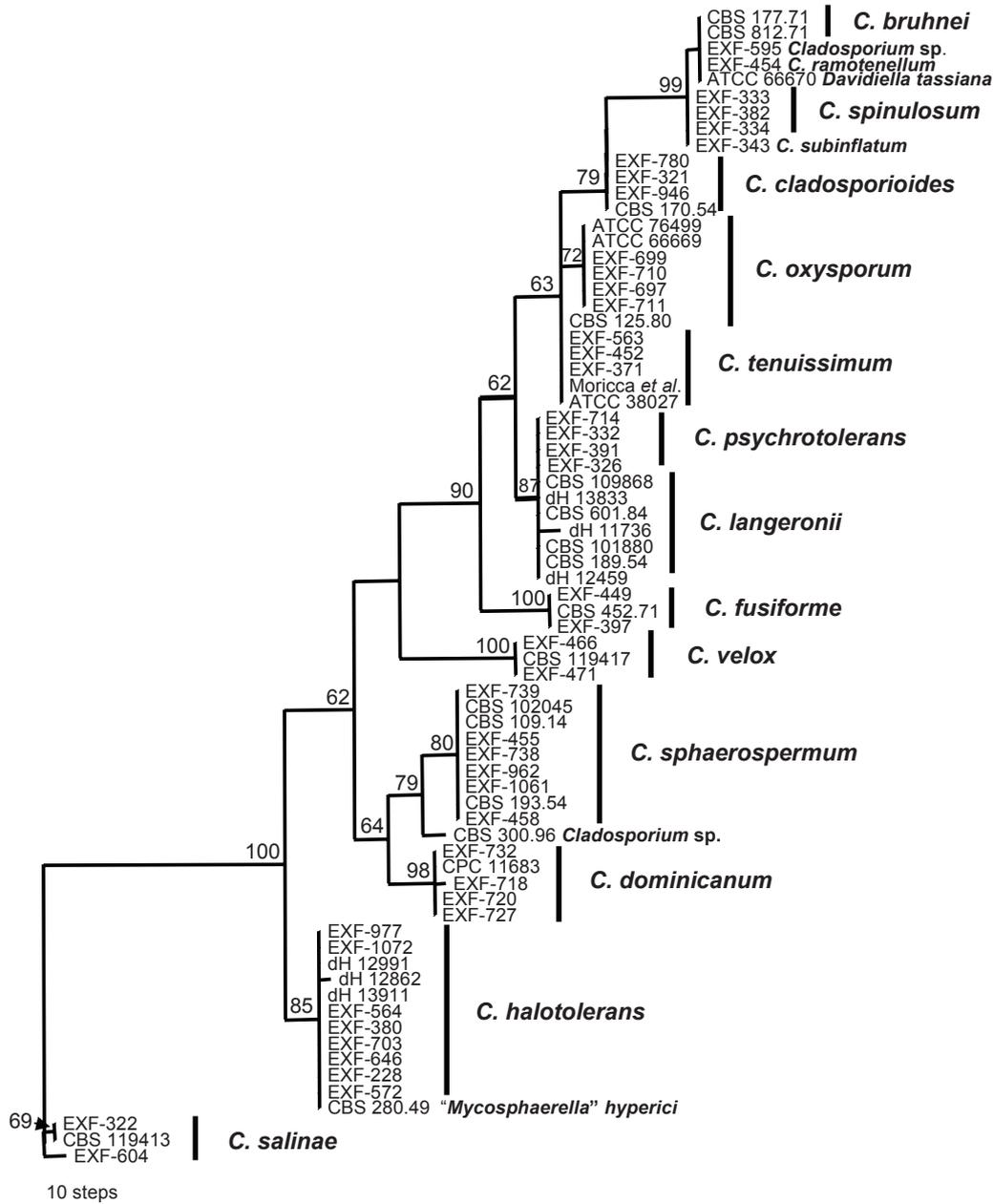


Fig. 2. One of 600 equally most parsimonious and equally looking phylogenetic trees based on a heuristic tree search using aligned sequences of the internal transcribed spacer regions 1 and 2 and the 5.8S rDNA. The tree was randomly selected. Support based on 10 000 replicates of a fast step-wise addition bootstrap analysis is indicated near the branches. Trees were rooted with the strains of *Cladosporium salinae*. Most monophyletic species clades received high, but some deeper branches moderate, bootstrap support (CI = 0.804, RI = 0.975, PIC = 68).

## RESULTS

Descriptive statistical parameters of phylogenetic analyses and calculated tree scores for each analysed sequence locus are summarised in Table 2. Mainly reference material such as ex-type or ex-neotype strains was analysed on the level of SSU rDNA sequences. Downloaded and newly generated SSU rDNA sequences of members of *Cladosporium s. str.* were compared with related taxa of the *Mycosphaerellaceae*, *Dothioraceae* and *Dothideaceae*. The somewhat more distantly related *Fusicladium effusum* (*Venturiaceae*) (Braun *et al.* 2003: Fig. 2) was selected as outgroup. *Anungitopsis amoena* R.F. Castañeda & Dugan (now placed in *Fusicladium* Bonord., see Crous *et al.* 2007b), also a member of the *Venturiaceae*, was included in the analyses. All taxa included in the SSU rDNA analysis belong to the *Dothideomycetes*

(Schoch *et al.* 2006), within which the ingroup is represented by the orders *Capnodiales* (*Davidiellaceae*, *Mycosphaerellaceae*, *Teratosphaeriaceae*) and *Dothideales* (*Dothioraceae*, *Dothideaceae*) (see also Schoch *et al.* 2006). The genus *Cladosporium*, of which some species are linked to *Davidiella* Crous & U. Braun teleomorphs (Braun *et al.* 2003), forms a statistically strongly supported monophyletic group (*Davidiellaceae*). It also accommodates species newly described in this paper, namely, *C. halotolerans* Zalar, de Hoog & Gunde-Cimerman, *C. fusiforme* Zalar, de Hoog & Gunde-Cimerman, *C. dominicanum* Zalar, de Hoog & Gunde-Cimerman, *C. salinae*, *C. psychrotolerans* Zalar, de Hoog & Gunde-Cimerman, *C. velox* Zalar, de Hoog & Gunde-Cimerman and *C. spinulosum* Zalar, de Hoog & Gunde-Cimerman (Fig. 1). A sister group relationship of *Cladosporium s. str.* with a clade of taxa characterised, among others, by *Mycosphaerella* Johanson teleomorphs, containing various anamorphic genera such as *Septoria* Sacc.,

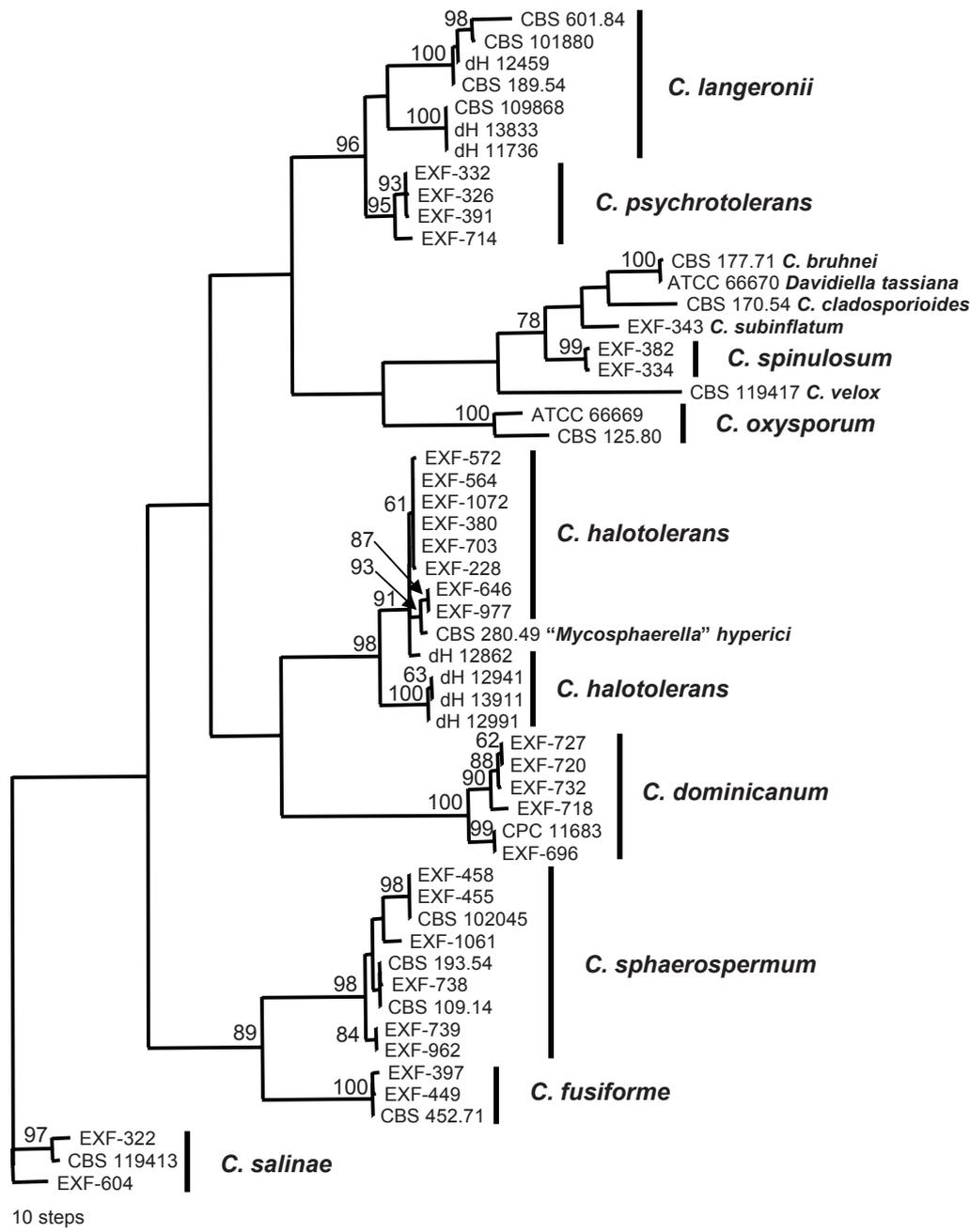


Fig. 3. One of 90 equally most parsimonious and equally looking phylogenetic trees based on a heuristic tree search using aligned exons and introns of a part of the  $\beta$ -tubulin gene. The tree was randomly selected. Support based on 10 000 replicates of a fast step-wise addition bootstrap analysis is indicated near the branches. Trees were rooted with the strains of *Cladosporium salinae*. Most monophyletic species clades received high, but deeper branches weak or no, bootstrap support (CI = 0.538, RI = 0.883, PIC = 220).

*Ramularia* Unger, *Cercospora* Fresen., *Pseudocercospora* Speg., “*Trimmatostroma*” Corda (now *Catenulostroma* Crous & U. Braun) (see Crous *et al.* 2004, 2007a – this volume) and the somewhat cladosporium-like genus *Devriesia* Seifert & N.L. Nick. (Seifert *et al.* 2004), was statistically only moderately supported (Fig. 1), whereas in an analogous analysis by Braun *et al.* (2003: Fig. 2) it was highly supported. These data also support the conclusion by Braun *et al.* (2003) and Crous *et al.* (2006) that *Cladosporium* is not a member of the distantly related *Herpotrichiellaceae* (*Chaetothyriomycetes*), which is also rich in cladosporium-like taxa (Crous *et al.* 2006). None of the fungi isolated from hypersaline environments belonged to the *Herpotrichiellaceae*. The SSU rDNA sequences do not resolve a phylogenetic structure within *Cladosporium s. str.* Only a moderately supported clade comprising *C. halotolerans*, *C. dominicanum*, *C. velox*, *C. sphaerospermum* and *C. fusiforme* is somewhat distinguished from a statistically unsupported clade with

*C. herbarum* (Pers. : Fr.) Link, *C. cladosporioides* (Fresen.) G.A. de Vries, *C. oxysporum* Berk. & Broome, *C. spinulosum*, and *C. psychrotolerans*, etc. Because *C. salinae* appeared most distinct within the genus *Cladosporium* in analyses of the SSU rDNA (Fig. 1), it was used as outgroup in analyses of the ITS rDNA and the  $\beta$ -tubulin and actin genes.

Analyses of the more variable ITS rDNA and partial  $\beta$ -tubulin and actin gene introns and exons supported the species clades of *C. halotolerans*, *C. dominicanum*, *C. sphaerospermum*, *C. fusiforme* and *C. velox* (Figs 2–4), of which *C. velox* was distinguished in the  $\beta$ -tubulin tree by a particular long terminal branch of the only sequenced strain (Fig. 3). *Cladosporium salinae* also clustered as a well-supported species clade in preliminary analyses using various *Mycosphaerella* species as outgroup (not shown). All strains of *C. langeronii* (Fonseca, Leão & Nogueira) Vuill. are particularly well distinguishable from other *Cladosporium* species by strikingly slow-

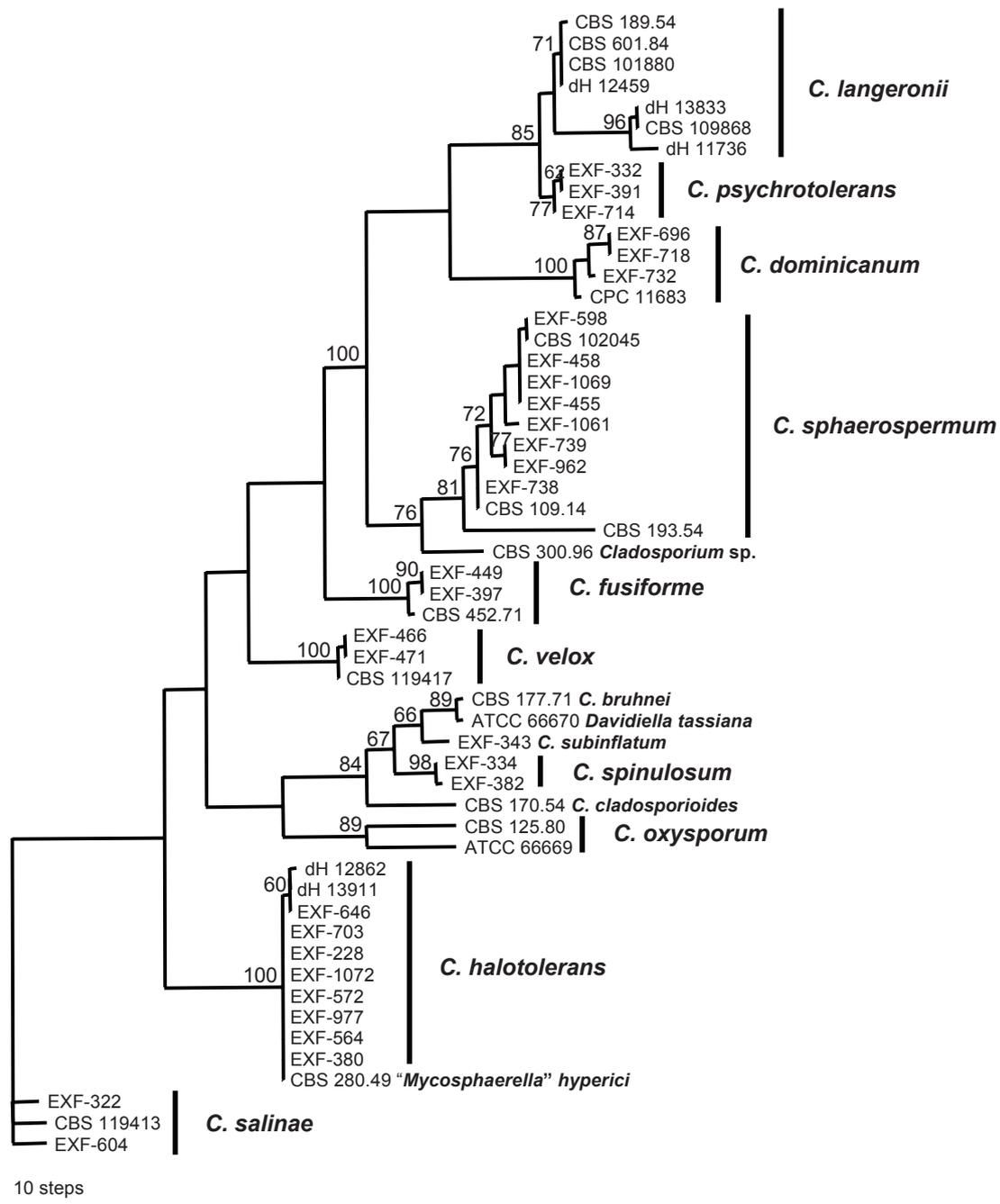
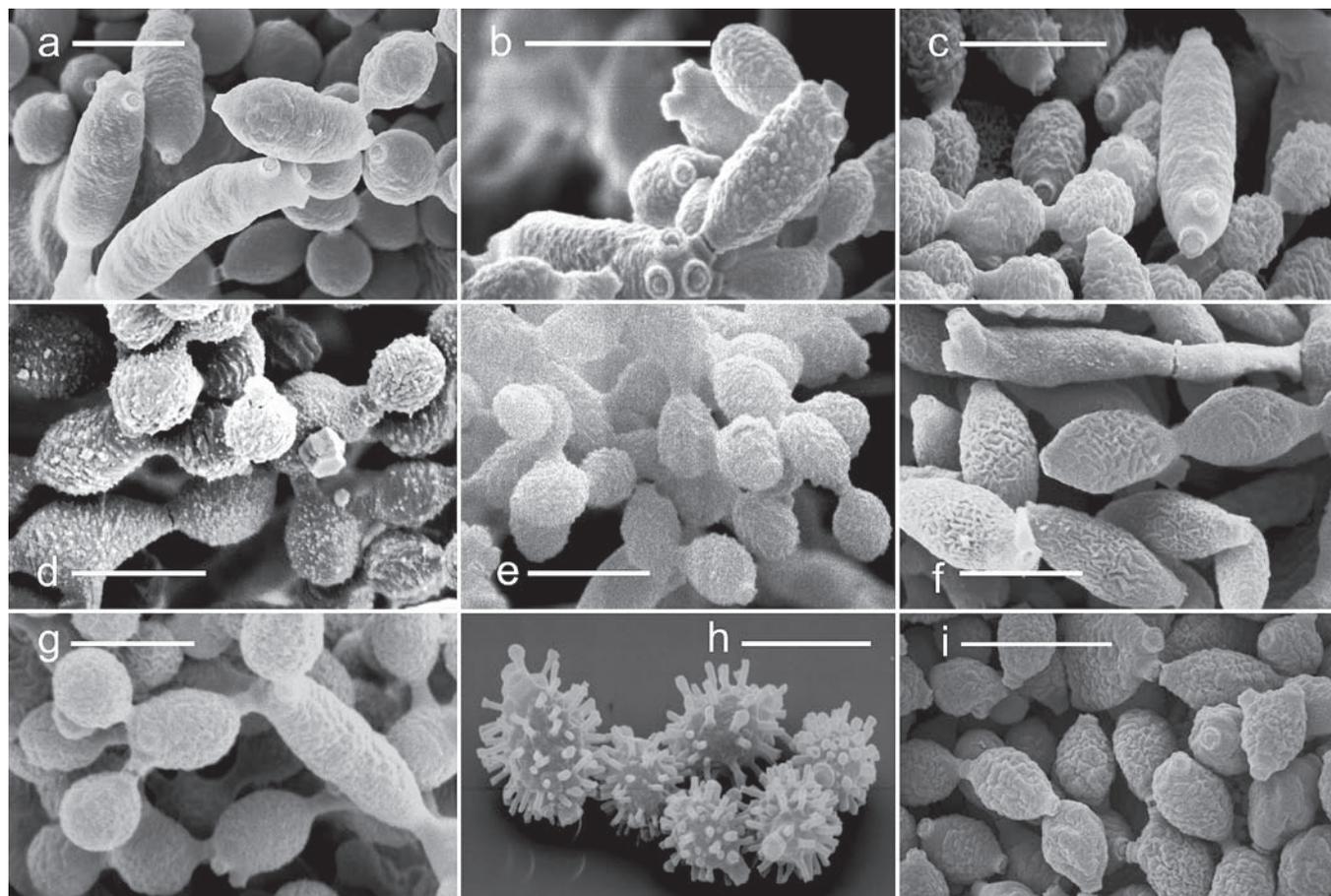


Fig. 4. One of 32 equally most parsimonious and equally looking phylogenetic trees based on a heuristic tree search using aligned exons and introns of the partial actin gene. The tree was randomly selected. Support based on 10 000 replicates of a fast step-wise addition bootstrap analysis is indicated near the branches. Trees were rooted with the strains of *Cladosporium salinae*. Most monophyletic species clades received high, but deeper branches weak or no, bootstrap support (CI = 0.586, RI = 0.885, PIC = 103).

growing colonies at all tested temperatures and relatively large, oblong conidia. However, phylogenetic analyses of the  $\beta$ -tubulin and actin gene indicate that *C. langeronii* presents two cryptic species (Figs 3–4). The species clade of *C. psychrotolerans* is moderately supported in analyses of the actin gene but highly by means of the  $\beta$ -tubulin gene. It is evident from all three analyses (Figs 2–4) that *C. langeronii* and *C. psychrotolerans* are closely related species. The species node of *Cladosporium spinulosum*, which is morphologically clearly distinguished from all other species by its conspicuous ornamentation consisting of digitate projections (Fig. 5), is supported by  $\beta$ -tubulin (Fig. 3) and actin (Fig. 4) sequence data but not by those of the ITS rDNA (Fig. 2). Analyses of all loci, however, indicate that it is a member of the *C. herbarum* complex.

The analyses of sequences of the ITS and the  $\beta$ -tubulin and actin gene introns and exons (Figs 2–4) do not allow the full elucidation of phylogenetic relationships among these *Cladosporium* species. Statistical support of the interior tree branches resulting from analyses of the  $\beta$ -tubulin and actin genes is low (bootstrap values mostly < 50 %). While the sister group relationship of *C. sphaerospermum* and *C. fusiforme* is highly supported in the analysis based on the  $\beta$ -tubulin gene, analysis of the ITS rDNA indicate that these two species are unrelated, and that *C. sphaerospermum* is closely related to *C. dominicanum*. It is clear from the data that the species morphologically resembling *C. sphaerospermum* are not phylogenetically closely related and that the data we present here do not allow their classification in natural subgroups of the genus *Cladosporium*. Only *C. spinulosum* was placed in all analyses among species of the *C. herbarum* complex.



**Fig. 5.** Conidial scars and surface ornamentation of ramoconidia and conidia (SEM). A. *C. dominicanum* (strain EXF-732). B. *C. fusiforme* (strain EXF-449). C. *C. halotolerans* (strain EXF-572). D. *C. langeronii* (strain CBS 189.54). E. *C. psychrotolerans* (strain EXF-391). F. *C. salinae* (strain EXF-335 = CBS 119413). G. *C. sphaerospermum* (strain CBS 193.54). H. *C. spinulosum* (strain EXF-334). I. *C. velox* (strain CBS 119417). Scale bars = 5  $\mu$ m. (Photos: K. Drašlar).

and all analyses supported close relatedness of *C. langeronii* and *C. psychrotolerans*.

The majority of species described here have slightly ornamented conidia ranging from minutely verruculose (*C. fusiforme*, *C. langeronii*, *C. psychrotolerans*, *C. sphaerospermum*, *C. velox*) to verruculose (*C. halotolerans*) (Fig. 5). The verruculose conidia of *C. halotolerans* can be recognised also under the light microscope and used as a distinguishing character. Almost smooth to minutely verruculose conidia are encountered in *C. dominicanum* and *C. salinae* (Fig. 5). *Cladosporium spinulosum*, a member of the *C. herbarum* species complex, has conidia with a digitate ornamentation that can appear spinulose under the light microscope; however, when using the SEM it became clear that its projections have parallel sides and a blunt end (Fig. 5).

## DISCUSSION

The genus *Cladosporium* was established by Link (1816) who originally included four species, of which *C. herbarum* is the type species of the genus (Clements & Shear 1931). In 1950, von Arx reported a teleomorph connection for this species with *Mycosphaerella tassiana* (De Not.) Johanson. Based on SSU rDNA data the majority of *Mycosphaerella* species, including the type species of the genus, *M. punctiformis* (Pers.) Starbäck, clustered within the *Mycosphaerellaceae*, a family separated from *M. tassiana* (Braun *et al.* 2003). Therefore, *Mycosphaerella tassiana* was reclassified as *Davidiella tassiana* (De Not.) Crous

& U. Braun, the type of the new genus *Davidiella*. All anamorphs with a cladosporium- and heterosporium-like appearance and with a supposed *Dothideomycetes* relationship were maintained under the anamorph name *Cladosporium*, morphologically characterised by scars with a protuberant hilum consisting of a central dome surrounded by a raised rim (David 1997).

The concept of distinguishing ramoconidia from secondary ramoconidia has been adopted from Schubert *et al.* (2007). In the species described here, ramoconidia have been observed often in *C. sphaerospermum*, sometimes in *C. psychrotolerans*, *C. langeronii* and *C. spinulosum*, and only sporadically in all other species. Therefore, ramoconidia can be seen as important for distinguishing species although sometimes, they can be observed only with difficulty. When using ramoconidia as a diagnostic criterion, colonies only from SNA and not older than 7 d should be taken into account.

*Cladosporium sphaerospermum* was described by Penzig (1882) from decaying *Citrus* leaves and branches in Italy. He described *C. sphaerospermum* as a species with (i) branched, septate and dark conidiophores having a length of 150–300  $\mu$ m and a width of the main conidiophore stipe of 3.5–4  $\mu$ m, (ii) spherical to ellipsoid, acrogenously formed conidia of 3.4–4  $\mu$ m diam, and (iii) ramoconidia of 6–14  $\times$  3.5–4  $\mu$ m. Penzig's original material is not known to be preserved. Later, a culture derived from CBS 193.54, originating from a human nail, was accepted as typical of *C. sphaerospermum*. However, de Vries (1952), incorrectly cited it as "lectotype", and thus the same specimen is designated as neotype in this study (see below), with the derived culture (CBS 193.54)

used as ex-neotype strain. Numerous strains with identical or very similar ITS rDNA sequences as CBS 193.54 were isolated from hypersaline water or organic substrata including plants or walls of bathrooms. It is not clear yet whether surfaces in bathrooms and of plants, colonised by *C. sphaerospermum*, can have a similar low water activity as salterns. In our experiments, the strains of this species, however, grew under *in vitro* conditions at a water activity of up to 0.860, while Hocking *et al.* (1994) and Aihara *et al.* (2002) reported that it can grow even at 0.815. Therefore, we consider *C. sphaerospermum* as halo- or osmotolerant. Hardly any reports are available unambiguously proving that *C. sphaerospermum* is a human pathogen. It is therefore possible that CBS 193.54 was not involved in any disease process but rather occurred as a contaminant on dry nail material. *Cladosporium sphaerospermum* is a phylogenetically well-delineated species (Figs 2–4).

Strains of *C. halotolerans* were isolated sporadically from substrata such as peanut cell suspension, tissue culture, bathroom walls and as culture contaminants. This surprising heterogeneity of substrata suggests that *C. halotolerans* is distributed by air and that it can colonise whatever substrata available, although it may have its natural niche elsewhere. We have recurrently isolated it from hypersaline water of salterns and other saline environments and it was also detected with molecular methods (but not isolated) from skin of a salt water dolphin. There are only few reports of this species from plants (Table 1). It is therefore possible that *C. halotolerans* is a species closely linked to salty or hypersaline environments although additional sampling is necessary to prove that. *Cladosporium halotolerans* is morphologically recognisable by relatively oblong to spherical, coarsely rough-walled conidia. The ITS rDNA sequence of a fungus in the skin of a bottlenose dolphin, suffering from lobomycosis, is identical to the sequences of *C. halotolerans*. This sequence was deposited as *Lacazia lobo*i Taborda, V.A. Taborda & McGinnis (GenBank AF035674) by Haubold *et al.* (1998), who apparently concluded wrongly that a fungus with a cladosporium-like ITS rDNA sequence similar to that of *C. halotolerans* can be the agent of lobomycosis. Later, Herr *et al.* (2001) showed that *Lacazia lobo*i phylogenetically belongs to the *Onygenales* on the basis of amplified SSU rDNA and chitin synthase-2 gene sequences generated from tissue lesions. By this, they confirmed an earlier supposition by Lacaz (1996) who reclassified the organism as *Paracoccidioides lobo*i O.M. Fonseca & Silva Lacaz (*Onygenales*). It is therefore possible that *C. halotolerans* was not the main etiologic agent for the lobomycosis and it was colonising the affected dolphin skin secondarily while inhabiting other seawater habitats.

*Cladosporium langeronii* and *C. psychrotolerans* are closely related but *C. langeronii* is particularly well distinguishable from all other *Cladosporium* species by its slow growing colonies (1–7 mm diam / 14 d) and relatively large conidia (4–5.5 × 3–4 µm). *Cladosporium psychrotolerans* has smaller conidia (3–4 × 2.5–3 µm) but a similar length : width ratio and faster expanding colonies (8–18 mm diam / 14 d). *Cladosporium langeronii* is most likely a complex of at least two species. Strains isolated from the Arctic and the Antarctic may need to be distinguished from *C. langeronii* s. str. on species level. This inference is particularly supported by analyses of the β-tubulin and actin genes (Figs 3–4). *Cladosporium langeronii* s. str., represented by an authentic strain of *Hormodendrum langeronii* Fonseca, Leão & Nogueira, CBS 189.54 (Trejos 1954), has been isolated from a variety of substrata but is tolerating only up to 10 % NaCl. It was originally described by da Fonseca *et al.* (1927a, b) and subsequently reclassified as *Cladosporium langeronii* by Vuillemin (1931). The authentic

strain derived from an ulcerating nodular lesion on the arm of a human patient. Because other strains of this species are ubiquitous saprobes originating from various substrata, we suspect that *C. langeronii* is not an important human pathogen. *Cladosporium psychrotolerans* has been isolated from hypersaline environments only, and tolerates up to 20 % NaCl in culture media.

In general, the human- or animal-pathogenic role of the *C. sphaerospermum*-like species described here seems to be limited. It is possible that pathogenic species of *Cladophialophora* Sacc. have been misidentified as *C. sphaerospermum* or as other species of *Cladosporium* (de Hoog *et al.* 2000). Alternatively, true *Cladosporium* species isolated as clinical strains could have been secondary colonisers since they are able to dwell on surfaces poor in nutrients, possibly in an inconspicuous dormant phase and may then be practically invisible. More likely, they could be air-borne contaminations of lesions, affected nails etc. (Summerbell *et al.* 2005) or are perhaps disseminated by insufficiently sterilised medical devices, as melanised fungi can be quite resistant to disinfectants (Phillips *et al.* 1992). They can easily be isolated and rapidly become preponderant at isolation and thus difficult to exclude as etiologic agents of a disease. For example, in 2002, a case report on an intrabronchial lesion by *C. sphaerospermum* in a healthy, non-asthmatic woman was described (Yano *et al.* 2002), but we judge the identification of the causal agent to remain uncertain, as it was based on morphology alone and no culture is available. The present authors have the opinion that all clinical cases ascribed to *Cladosporium* species need careful re-examination.

### General characteristics and description of *Cladosporium sphaerospermum*-like species

The present paper focuses on *Cladosporium* strains isolated from hypersaline environments. Comparison of data from deliberate sampling and analysis of reference strains from culture collections inevitably leads to statistical bias, and therefore a balanced interpretation of ecological preferences of the species presented is impossible. Nevertheless, some species appeared to be consistent in their choice of habitat, and for this reason we summarise isolation data for all species described. Strains belonging to a single molecular clade proved to have similar cultural characteristics and microscopic morphology. Although within most of the species there was some molecular variation noted (particularly when intron-rich genes were analysed), some consistent phenetic trends could be observed.

Conidiophores of all *C. sphaerospermum*-like species lack nodose inflations (McKemy & Morgan-Jones 1991). They are usually ascending and can sometimes be poorly differentiated from their supporting hyphae. Though the initiation point of conidiophore stipes could sometimes be determined only approximately, their lengths were in some cases useful for distinguishing morphologically similar species when observed in slide cultures. Generally, the branched part of a conidiophore forms a complex tree-like structure. The number and orientation of early formed secondary ramoconidia, however, determines whether it is rotationally symmetric or unilateral.

The variability in ITS rDNA sequences observed in all *C. sphaerospermum*-like species (about 10 %) spans the variation observed in all members of the genus *Cladosporium* sequenced to date. Thus, the *C. sphaerospermum*-like species described here may not present a single monophyletic group but may belong to various species complexes within *Cladosporium*. Verifying existing literature with sequence data of these species (Wirsal *et al.* 2002, Park *et al.* 2004), we noticed that names of the common saprobes seem to be distributed nearly at random over phylogenetic trees.

For most commonly used names, no type material is available for sequencing. Also verification of published reports is difficult without available voucher strains.

*Cladosporium cladosporioides* was incorrectly lectotypified based on CBS 170.54 (de Vries 1952), which Bisby considered a standard culture of *C. herbarum*. The *C. cladosporioides* species complex requires revision, and will form the basis of a future study. *Cladosporium herbarum* is maintained as a dried specimen in the Leiden herbarium; Prasil & de Hoog (1988) selected CBS 177.71 as a representative living strain. Strains, earlier accepted as living representatives of *C. herbarum*, CBS 177.71 and CBS 812.71 (Prasil & de Hoog 1988, Wirsal *et al.* 2002) and ATCC 66670 (Braun *et al.* 2003, as *Davidiella tassiana*) have been re-identified as *C. bruhnei* Linder by Schubert *et al.* (2007 – this volume). Ho *et al.* (1999) used strain ATCC 38027 as a representative of *C. tenuissimum* Cooke and this strain has identical ITS sequences as the non-deposited *C. tenuissimum* material used by Moricca *et al.* (1999). We tentatively accept this concept although we could not

include ATCC 38027 in our analyses. The ITS sequence of strain CBS 125.80, identified by Wirsal *et al.* (2002) as *C. oxysporum*, is identical to the sequence of ATCC 38027. Strain ATCC 76499, published by Ho *et al.* (1999) as *C. oxysporum*, appears to be identical to a number of currently unidentified *Cladosporium* strains from Slovenian salters that compose a cluster separate from all remaining species. Strains of this cluster, represented in Fig. 2 by strain ATCC 76499, morphologically resemble *C. oxysporum*.

Strain CBS 300.96 has not been identified to species level in the present study. It clusters outside the species clade of *C. sphaerospermum*, with the latter being its nearest relative. CBS 300.96 differs from *C. sphaerospermum* by having smaller structures: conidiophore stipes [(5–)20–80(–150) × (2–)2.5–3(–4) µm], 0–1 septate ramoconidia [(13–)19–27(–32) × 2–2.5 µm], conidia [(2.5–)3–3.5(–4) × (2–)2–2.5(–3) µm] and secondary ramoconidia [(5–)9–18(–30) × (2–)2.5–2.5(–3) µm]. However, based on a single isolate, we currently refrain from describing it as a new species.

## Key to species treated in this study

Macro-morphological characters used in the key are from colonies grown on PDA and MEA 14 d at 25 °C, if not stated otherwise; microscopical characters are from SNA slide cultures grown for 7 d at 25 °C.

1. Conidial ornamentation conspicuously echinulate / digitate because of up to 1.3 µm long projections that have more or less parallel sides ..... ***C. spinulosum***
1. Conidial ornamentation verruculose to verrucose or smooth, not conspicuously echinulate or digitate ..... 2
2. Conidiophores micronematous, poorly differentiated, once or several times geniculate-sinuous, short, up to 60 µm long; terminal conidia obovoid ..... ***C. salinae***
2. Conidiophores micro- or macronematous, not geniculate or only slightly so, usually up to 100 µm or 220 µm long or even longer; terminal conidia globose, subglobose to ovoid or fusiform ..... 3
3. Secondary ramoconidia 0–3(–4)-septate; septa of conidiophores and conidia darkened and thickened ..... 4
3. Secondary ramoconidia 0–1(–2)-septate; septa neither darkened nor thickened ..... 5
4. Conidiophores (5–)10–50(–300) × (2–)2.5–3(–5.5) µm; terminal conidia (2–)3–4(–6) × (2–)2.5–3(–5) µm; secondary ramoconidia (5–)7–12(–37.5) × (2–)2.5–3(–6.5) µm; ramoconidia sporadically formed ..... ***C. halotolerans***
4. Conidiophores mostly longer and somewhat wider, (10–)45–130(–300) × (2.5–)3–4(–6) µm; terminal conidia mostly wider, (2.5–)3–4(–7) × (2–)3–3.5(–4.5) µm; secondary ramoconidia (4–)8.5–16(–37.5) × (2–)3–3.5(–5) µm; ramoconidia often formed, up to 40 µm long, with up to 5 septa ..... ***C. sphaerospermum***
5. Terminal conidia usually fusiform ..... ***C. fusiforme***
5. Terminal conidia globose, subglobose or ovoid ..... 6
6. Conidia and secondary ramoconidia irregularly verruculose to sometimes loosely verrucose; radial growth on PDA at 25 °C after 14 d typically less than 5 mm ..... ***C. langeronii***
6. Conidia and secondary ramoconidia smooth to minutely verruculose; radial growth on PDA at 25 °C after 14 d typically more than 10 mm ..... 7
7. Conidiophores (3–)3.5–4(–7.5) µm wide, thick-walled; conidiogenous loci and conidial hila 0.5–2 µm diam; ramoconidia sometimes formed with a broadly truncate, up to 2 µm wide non-cladosporioid base; no growth observed after 14 d at 30 °C on MEA ..... ***C. psychrotolerans***
7. Conidiophores mostly narrower, 2–4 µm wide, only with slightly thickened walls; conidiogenous loci and conidial hila narrower, 0.5–1.5 µm diam; ramoconidia rarely formed; colony showing at least weak growth after 14 d at 30 °C on MEA ..... 8
8. Secondary ramoconidia (4–)6.5–13(–24.5) µm long; no visible colony growth after 14 d at 10 °C on MEA ..... ***C. dominicanum***
8. Secondary ramoconidia mostly longer, (3.5–)5.5–19(–42) µm; radial growth of colonies after 14 d at 10 °C on MEA more than 5 mm ..... ***C. velox***

## Description of *Cladosporium* species

***Cladosporium dominicanum*** Zalar, de Hoog & Gunde-Cimerman, sp. nov. MycoBank MB510995. Fig. 6.

**Etymology:** Refers to the Dominican Republic, where most strains were encountered.

Conidiophora lateralia vel terminalia ex hyphis rectis oriunda; stipes longitudine variabili, (5–)10–100(–200) × (1.5–)2–2.5(–3.5) µm, olivaceo-brunneus, levis vel leniter verruculosus, tenuitunicatus, plerumque unicellularis, simplex vel ramosus. Conidiorum catenae undique divergentes, ad 8 conidia in parte continua continentes. Cellulae conidiogenae indistinctae. Conidia levia vel leniter verruculosa, dilute brunnea, unicellularia, plerumque breviter ovoidea, utrinque angustata, (2.5–)3–3.5(–5.5) × (2–)2–2.5(–2.5) µm, long.: lat. 1.4–1.6; ramoconidia secundaria cylindrica vel quasi globosa, 0–1-septata, (4–)6.5–13(–24.5) × (2–)2.5–3(–4.5) µm, ad 4 cicatrices terminales ferentia; cicatrices inspissatae, protuberantes, 0.5–1.2 µm diam. Hyphae vagina polysaccharidica carentes.

**Mycelium** without extracellular polysaccharide-like material. **Conidiophores** arising laterally and terminally on erect hyphae, micronematous and semimacronematous, stipes of variable length, (5–)10–100(–200) × (1.5–)2–2.5(–3.5) µm, olivaceous-brown, smooth to minutely verruculose, thin-walled, almost non-septate, unbranched or branched. **Conidial chains** branching in all directions, up to eight conidia in the unbranched parts. **Conidiogenous cells** undifferentiated. **Ramoconidia** rarely formed. **Conidia** smooth to minutely verruculose, subhyaline to light brown, non-septate, usually short-ovoid, narrower at both ends, length : width ratio = 1.4–1.6; (2.5–)3–3.5(–5.5) × (2–)2–2.5(–2.5) µm [av. (± SD) 3.4 (± 0.6) × 2.2 (± 0.2)]; **secondary ramoconidia** cylindrical to almost spherical, 0–1-septate, (4–)6.5–13(–24.5) × (2–)2.5–3(–4.5) µm [av. (± SD) 10.3 (± 5.2) × 2.7 (± 0.6)], with up to four distal scars. **Conidiogenous scars** thickened and conspicuous, protuberant, 0.5–1.2 µm diam.

**Cultural characteristics:** Colonies on PDA reaching 18–36 mm diam, olive-yellow (2D6), hairy granular, flat or slightly furrowed, with flat margin. Droplets of light reseda-green (2E6) exudate sometimes present. Reverse dark green to black. Colonies on OA reaching 19–34 mm diam, olive (2F5), loosely powdery with raised central part due to fasciculate bundles of conidiophores. Reverse dark green. Colonies on MEA reaching 30–32 mm diam, reseda green (2E6), velvety, furrowed, with undulate margin. Reverse dark green-brown. Colonies on MEA + 5 % NaCl reaching 37–41 mm diam, reseda-green (2E6), radially furrowed, velvety, sporulating in the central part or all over the colony, margin white and regular. Reverse brownish green.

**Maximum tolerated salt concentration:** 75 % of tested strains develop colonies at 20 % NaCl after 7 d, while after 14 d all strains grow and sporulate.

**Cardinal temperatures:** No growth at 4 and 10 °C, optimum 25 °C (30–32 mm diam), maximum 30 °C (2–15 mm diam), no growth at 37 °C.

**Specimen examined:** Dominican Republic, from hypersaline water of salt lake Enriquillo, coll. Nina Gunde-Cimerman, Jan. 2001, isol. P. Zalar 25 Feb. 2001, CBS H-19733, **holotype**, culture ex-type EXF-732 = CBS 119415.

**Habitats and distribution:** Fruit surfaces; hypersaline waters in (sub)tropical climates.

**Differential parameters:** No growth at 10 °C, ovoid conidia, large amounts of sterile mycelium.

**Strains examined:** CPC 11683, EXF-696, EXF-718, EXF-720, EXF-727, EXF-732 (= CBS 119415; ex-type strain).

**Note:** Cultures of *C. dominicanum* sporulate less abundantly than *C. sphaerospermum* and *C. halotolerans* and tend to lose their ability to sporulate with subculturing.

***Cladosporium fusiforme*** Zalar, de Hoog & Gunde-Cimerman, sp. nov. MycoBank MB510997. Fig. 7.

**Etymology:** Refers to its usually fusiform conidia.

Conidiophora erecta, lateralia vel terminalia ex hyphis rectis oriunda; stipes longitudine variabili, (10–)25–50(–100) × (2–)2–3.5(–4) µm, olivaceo-brunneus, levis, crassitunicatus, compluribus septatus (cellulis 9–23 µm longis), plerumque simplex. Conidiorum catenae undique divergentes, in parte continua ad 5 conidia continentes. Cellulae conidiogenae indistinctae. Conidia leniter verruculosus, dilute brunnea, unicellularia, plerumque fusiformia, utrinque angustata, (2.5–)3.5–5(–6.5) × (2–)2–2.5(–3) µm, long. : lat. 1.8–2.0; ramoconidia secundaria cylindrica, 0(–1)-septata, (5–)6–11(–22) × (2.5–)2.5–3(–3) µm, ad 4 cicatrices terminales ferentia; cicatrices inspissatae, conspicuae, 0.7–1.0 µm diam. Hyphae vagina polysaccharidica carentes.

**Mycelium** without extracellular polysaccharide-like material. **Conidiophores** erect, arising laterally and terminally from straight hyphae, stipes of variable length, (10–)25–50(–100) × (2–)2–3.5(–4) µm, olivaceous-brown, smooth- and thick-walled, regularly-septate (cell length 9–23 µm), mostly unbranched. **Conidial chains** branching in all directions, up to 5 conidia in the unbranched parts. **Conidiogenous cells** undifferentiated. **Ramoconidia** rarely formed. **Conidia** minutely verruculose, light brown, aseptate, usually fusiform and narrower at both ends, length : width ratio = 1.8–2.0; (2.5–)3.5–5(–6.5) × (2–)2–2.5(–3) µm [av. (± SD) 4.4 (± 0.8) × 2.2 (± 0.2)]; **secondary ramoconidia** cylindrical, 0(–1)-septate, (5–)6–11(–22) × (2.5–)2.5–3(–3) µm [av. (± SD) 9.0 (± 4.7) × 2.6 (± 0.3)], with up to 4 distal scars. **Conidiogenous scars** thickened and conspicuous, protuberant, 0.7–1.0 µm diam.

**Cultural characteristics:** Colonies on PDA reaching 20–26 mm diam, dull green (30E3), granular due to profuse sporulation, flat, with flat margin. Sterile mycelium absent. Reverse blackish green. Colonies on OA reaching 24–28 mm diam, olive (3F3), granular in concentric circles, consisting of two kinds of conidiophores (low and high), flat, with flat margin. Reverse black. Colonies on MEA reaching 23–28 mm diam, olive (3E5), deeply furrowed, velvety (sporulating all over) with undulate, white margin. Reverse brownish green. Colonies on MEA + 5 % NaCl reaching 28–43 mm diam, olive (3E6), granular due to profuse sporulation, slightly furrowed with flat, olive-grey (3F2) margin. Reverse dark green.

**Maximum tolerated salt concentration:** Only one of three strains tested (CBS 452.71) developed colonies at 17 % NaCl after 14 d, the other two strains grew until 10 % NaCl.

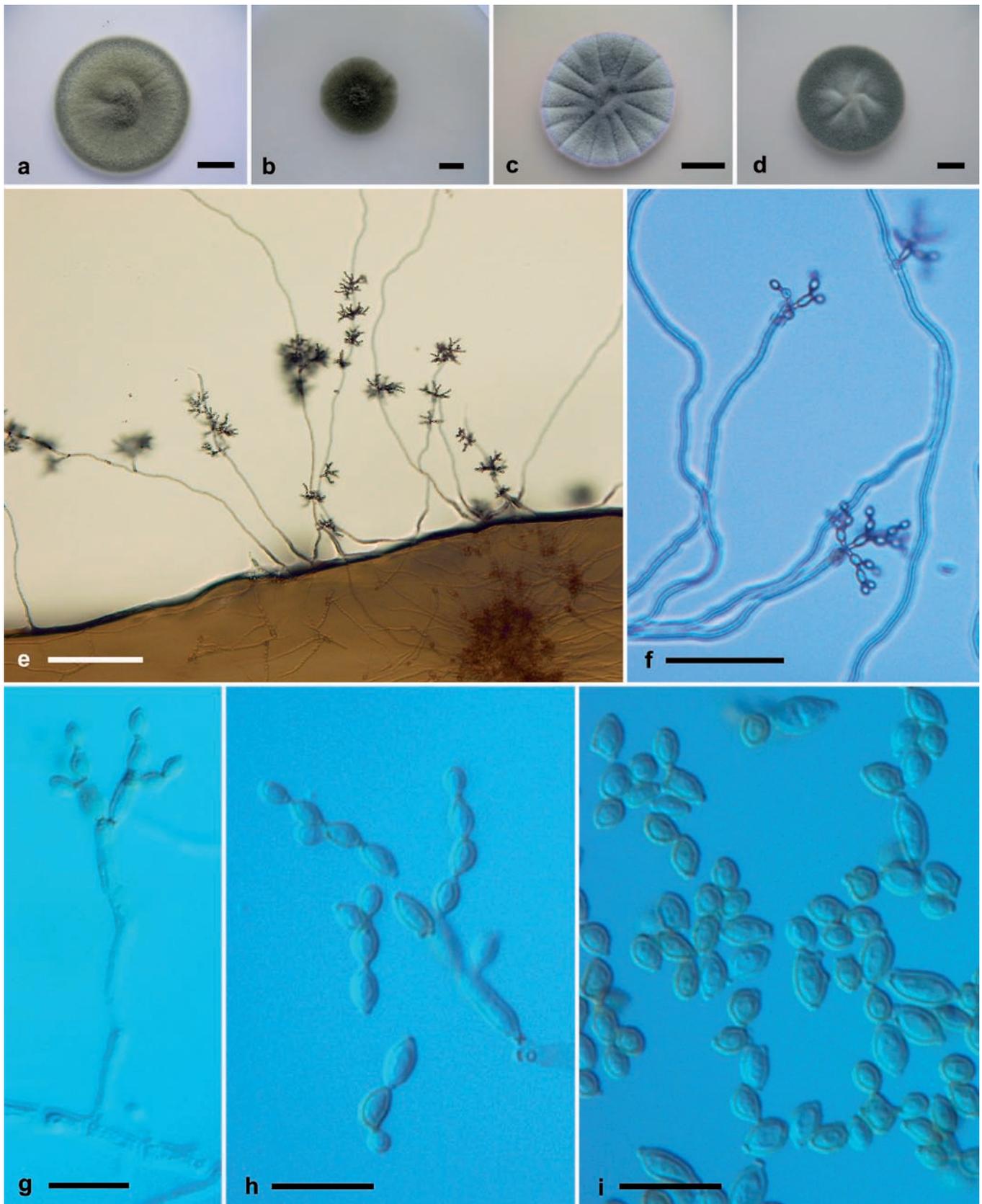
**Cardinal temperatures:** For one of three strains (CBS 452.71) the minimum temperature of growth was 4 °C (6 mm diam), for the other two 10 °C (8–9 mm diam); optimum 25 °C (23–28 mm diam), maximum 30 °C (only strain CBS 452.71 grew 5 mm diam), no growth at 37 °C.

**Specimen examined:** Slovenia, from hypersaline water of Sečovlje salterns, coll. and isol. L. Butinar, Dec. 1999, CBS H-19732, **holotype**, culture ex-type EXF-449 = CBS 119414.

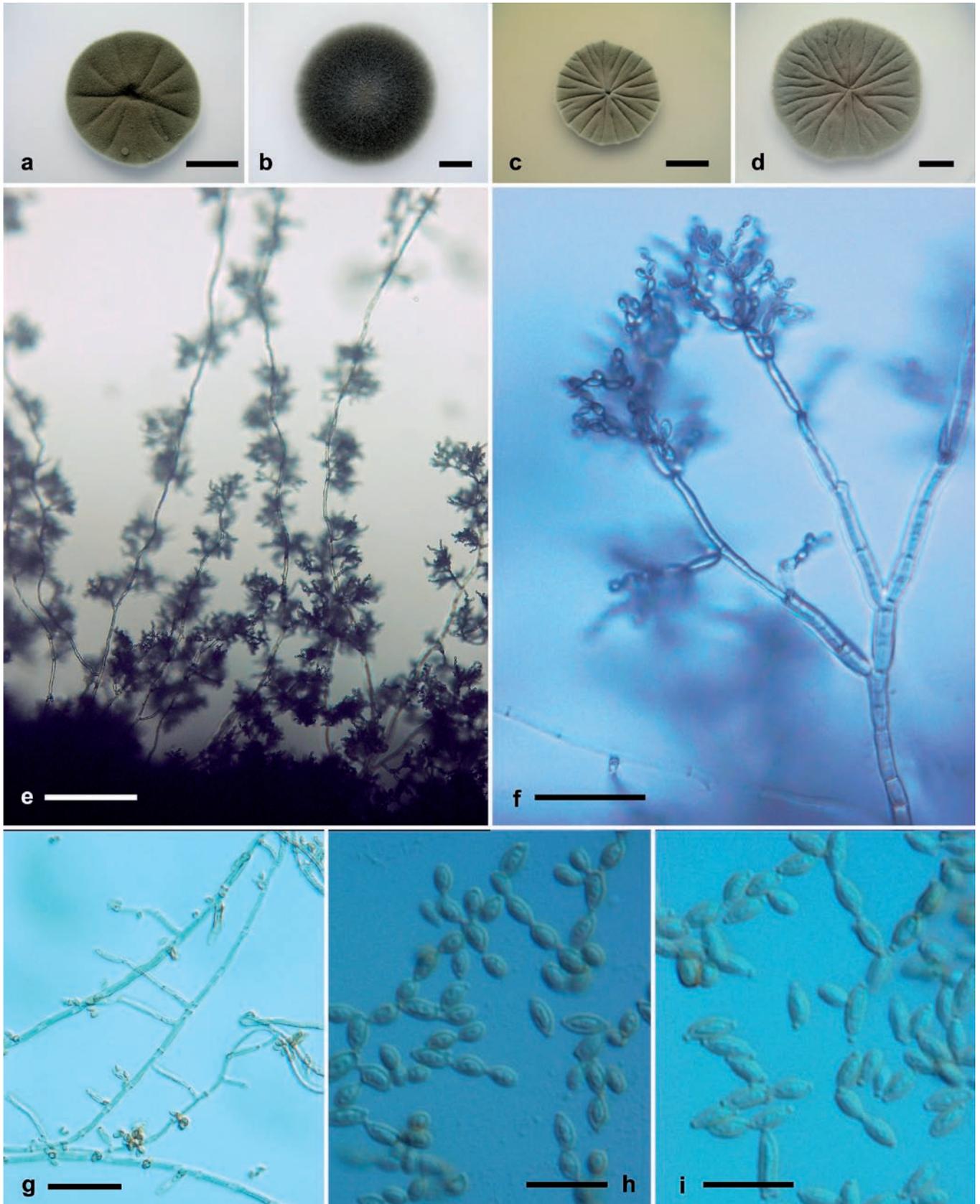
**Habitats and distribution:** Osmotic environments worldwide.

**Differential parameters:** Oblong conidia, relatively low degree of halotolerance.

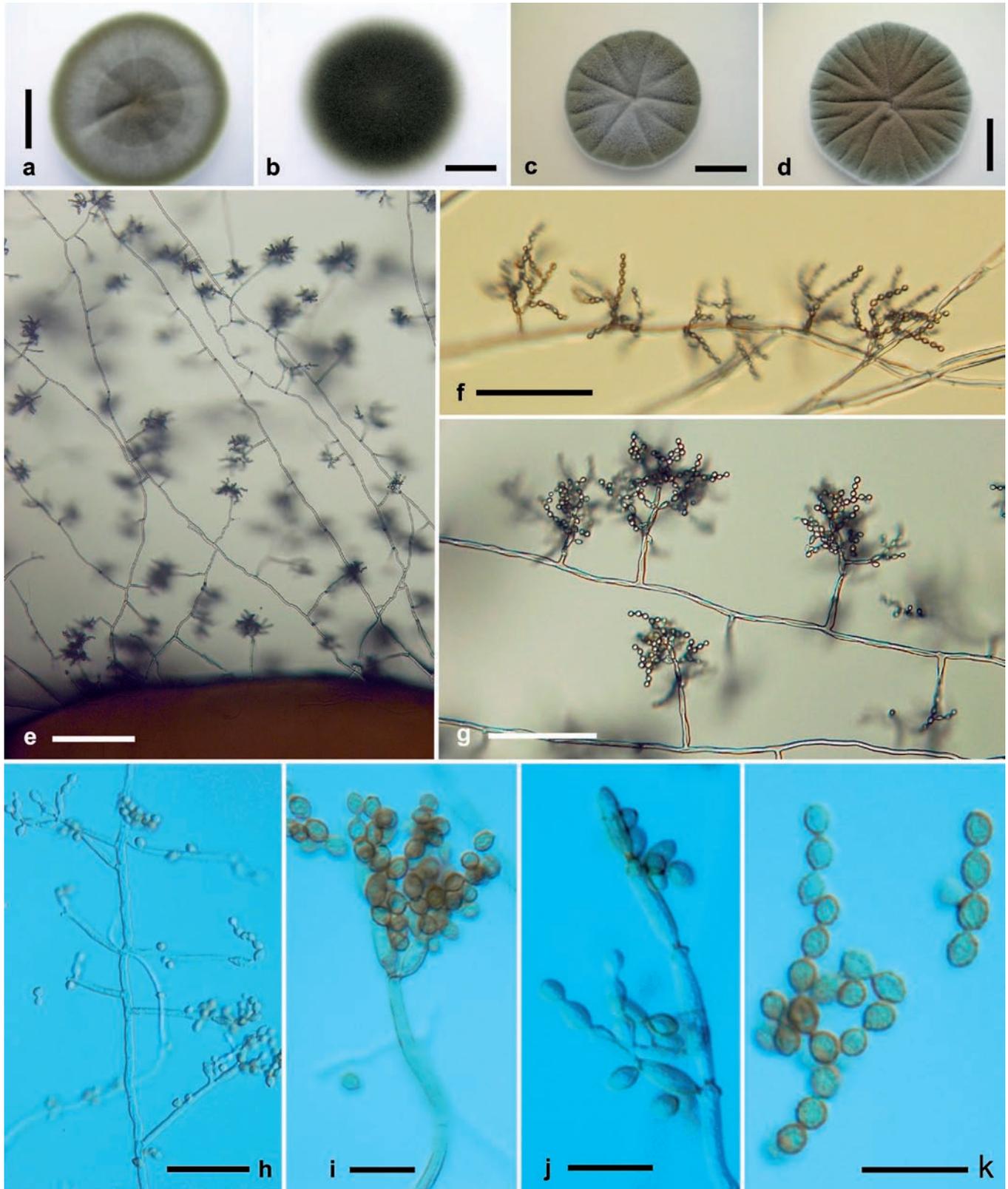
**Strains examined:** CBS 452.71, EXF-397, EXF-449 (= CBS 119414; ex-type strain).



**Fig. 6.** *Cladosporium dominicanum*. Macro- and micromorphological characters. A–D. Colony surface grown on PDA (A), OA (B), MEA (C) and MEA plus 5 % NaCl (D) of strains incubated for 14 d at 25 °C in darkness. E–F. Habit of conidiophores. G. Conidiophore. H–I. Secondary ramoconidia and conidia. E–I. All from 7-d-old SNA slide cultures. A, D, F–H, from EXF-2519; B, C, E from EXF-727; I, EXF-732 (ex-type strain). Scale bars A–D = 10 mm, E = 100 μm, F = 30 μm, G–I = 10 μm.



**Fig. 7.** *Cladosporium fusiforme*. Macro- and micromorphological characters. A–D. Colony surface grown on PDA (A), OA (B), MEA (C) and MEA plus 5 % NaCl (D) of strains incubated for 14 d at 25 °C in darkness. E–G. Habit of conidiophores. H–I. Ramoconidia and conidia. E–I. All from 7-d-old SNA slide cultures. A–H, from EXF-449 (ex-type strain); I, from CBS 452.71. Scale bars A–D = 10 mm, E = 100 µm, F–G = 30 µm, H–I = 10 µm.



**Fig. 8.** *Cladosporium halotolerans*. Macro- and micromorphological characters. A–D. Colony surface grown on PDA (A), OA (B), MEA (C) and MEA plus 5 % NaCl (D) of strains incubated for 14 d at 25 °C in darkness. E–H. Habit of conidiophores. I. Conidiophore. J. Succession of secondary ramoconidia. K. Conidia. E–K. All from 7-d-old SNA slide cultures. A–B, from EXF-572 (ex-type strain); C–D, from EXF-977; E, G, from EXF-972; F, from EXF-564; H, I, K, from EXF-1072; J, from dH 12862. Scale bars A–D = 10 mm, E = 100 μm, F–G = 50 μm, H = 30 μm, I–K = 10 μm.

***Cladosporium halotolerans*** Zalar, de Hoog & Gunde-Cimerman **sp. nov.** MycoBank MB492439. Fig. 8.

**Etymology:** Refers to its halotolerant habit.

Conidiophora erecta, lateralialia vel terminalia ex hyphis rectis oriunda; stipes longitudine variabili, (5–)10–50(–300) × (2–)2.5–3(–5.5) μm, pallide olivaceo-brunneus, levis vel leniter verrucosus, tenuitunicatus, 0–3-septatus, interdum

pluriseptatus, simplex, denticulatus. Conidiorum catenae unidique divergentes, terminales ad 9 conidia continentes. Cellulae conidiogenae indistinctae. Conidia verrucosa, brunnea vel fusca, unicellularia, plerumque subglobosa vel globosa, raro breviter ovoidea, utrinque angustata, (2–)3–4(–6) × (2–)2.5–3(–5) μm, long. : lat. 1.2–1.5; ramoconidia secundaria cylindrica vel quasi globosa, 0(–1)-septata, (5–)7–12(–37.5) × (2–)2.5–3(–6.5) μm, ad 4 cicatrices terminales ferentia; cicatrices inspissatae, conspicuae, protuberantes, 0.7–1.0(–1.5) μm diam. Hyphae vagina polysaccharidica carentes.

*Mycelium* partly submerged, partly superficial; hyphae without extracellular polysaccharide-like material. *Conidiophores* erect, arising laterally and terminally from straight hyphae, stipes of variable length, (5–)10–50(–300) × (2–)2.5–3(–5.5) µm, pale olivaceous-brown, smooth to minutely verruculose, thin-walled, 0–3-septate, unbranched, with pronounced denticles. *Conidial chains* branching in all directions, terminal chains with up to 9 conidia. *Conidiogenous cells* undifferentiated. *Ramoconidia* rarely formed. *Conidia* verruculose, brown to dark brown, non-septate, usually subglobose to globose, less often short-ovoid, narrower at both ends, length : width ratio = 1.2–1.5; (2–)3–4(–6) × (2–)2.5–3(–5) µm [av. (± SD) 3.5 (± 0.7) × 2.7 (± 0.5)]; *secondary ramoconidia* cylindrical to almost spherical, 0–1-septate, (5–)7–12(–37.5) × (2–)2.5–3(–6.5) µm [av. (± SD) 10.3 (± 4.8) × 2.9 (± 0.6)], with up to 4 distal scars. *Conidiogenous scars* thickened and conspicuous, protuberant, 0.7–1.0(–1.5) µm diam.

**Cultural characteristics:** Colonies on PDA reaching 27–43 mm diam, olive (2F5), slightly furrowed, often covered with grey secondary mycelium, except at the marginal area where only sporulating structures can be observed. Margin white and regular, with submerged hyphae. Reverse pale green to black. Colonies on OA reaching 29–40 mm diam, olive (2F6), flat, uniform, granular due to profuse sporulation and fasciculate bundles of conidiophores, without sterile mycelium. Reverse dark green to black. Colonies on MEA reaching 18–44 mm diam, highly variable in colour, but mainly olive (2E5), and from flat with regular margin to deeply furrowed with undulate margin. Colony centre wrinkled with crater-shaped appearance. Reverse pale to dark green. Colonies on MEA + 5 % NaCl reaching 24–48 mm diam, olive (3E8), furrowed, velvety, with more pale, undulate margins. Reverse dark green to black.

**Maximum tolerated salt concentration:** Only 15 % of tested strains develop colonies at 20 % NaCl after 7 d, whereas after 14 d all cultures grow and sporulate.

**Cardinal temperatures:** No growth at 4 °C, optimum 25 °C (18–44 mm diam), maximum 30 °C (6–23 mm diam). No growth at 37 °C.

**Specimen examined:** Namibia, from hypersaline water of salterns, coll. Nina Gunde-Cimerman, 1 Sep. 2000, isol. P. Zalar, 1 Oct. 2000, CBS H-19734, **holotype**, culture ex-type EXF-572 = CBS 119416.

**Habitats and distribution:** Hypersaline water in subtropical climates; indoor environments; Arctic ice; contaminant in lesions of humans and animals; plant phyllosphere; rock.

**Literature:** Haubold *et al.* (1998), Meklin *et al.* (2004).

**Differential parameters:** Verruculose conidia, short unbranched and non-septate conidiophores which arise laterally alongside erect hyphae.

**Strains examined:** CBS 191.54, CBS 573.78, CBS 626.82, dH 12862, dH 12991, dH 13911, EXF-228, EXF-380, EXF-565, EXF-567, EXF-571, EXF-572 (= CBS 119416; ex-type strain), EXF-646, EXF-698, EXF-703, EXF-944, EXF-972, EXF-977, EXF-1072, EXF-2372.

**Notes:** *Cladosporium halotolerans* strongly resembles *C. sphaerospermum*. Several strains of this species such as dH 12862, dH 12941, CBS 191.54 and UAMH 7686 have been isolated sporadically from various indoor habitats in Europe, Brazil and the U.S.A. and repeatedly from bathrooms in Slovenia (Table 1). Probably sometimes as uncertain culture contaminations, it has been isolated from plants (GenBank accession no. L25433),

inner organs of a diseased frog (AY361982) and human brain (Kantarcioğlu *et al.* 2002). The presence of *C. halotolerans* species in gypsum sediments entrapped in Arctic ice, the fact that it was repeatedly isolated from hypersaline water and possibly its presence in dolphin skin (see Discussion) suggest that it has a clear preference for (hyper)osmotic habitats. This is supported by its ability to grow at 20 % NaCl.

The teleomorph of *C. halotolerans* is predicted to be a *Davidiella* species. Strain CBS 280.49 was isolated by J.A. von Arx from teleomorphic material of a fungus labelled as *Mycosphaerella hyperici* (Auersw.) Starbäck on *Hypericum perforatum* in Switzerland. According to Aptroot (2006) this species may belong in *Davidiella* and produces a *Septoria* anamorph. In the original herbarium specimen, CBS H-4867, a *Mycosphaerella* teleomorph was present, but no sign of a *Cladosporium* anamorph. We assume that CBS 280.49 was a culture contaminant.

***Cladosporium langeronii*** (Fonseca, Leão & Nogueira) Vuill., Champ. Paras.: 78. 1931. Fig. 9.

**Basionym:** *Hormodendrum langeronii* Fonseca, Leão & Nogueira, Sci. Med. 5: 563. 1927.

= *Cladosporium langeronii* (Fonseca, Leão & Nogueira) Cif., Manuale di Micologia Medica, ed. 2: 488 (1960), comb. superfl.

*Mycelium* partly submerged, partly superficial; hyphae sometimes enveloped in polysaccharide-like material. *Conidiophores* erect or ascending, micronematous and macronematous, stipes of variable length, (20–)50–130(–200) × (3–)3.5–4.5(–6.5) µm, dark brown, rough- and thick-walled, regularly septate (cell length 9–22 µm), arising laterally and terminally from submerged or aerial hyphae, branched. *Conidial chains* dichotomously branched, up to 6 conidia in the unbranched parts. *Conidiogenous cells* undifferentiated, sometimes seceding and forming ramoconidia. *Ramoconidia* cylindrical, 0–1 septate, (10–)11–22(–42) × (3–)3.5–4.5(–5) µm, base broadly truncate, 2–3.5 µm wide, slightly thickened and somewhat darkened. *Conidia* irregularly verruculose to sometimes loosely verruculose, dark brown, non-septate, usually ovoid, length : width ratio = 1.3–1.5; conidial size (3–)4–5.5(–8) × (2–)3–4(–5) µm [av. (± SD) 4.8 (± 1.0) × 3.5 (± 0.6)]; *secondary ramoconidia* cylindrical to almost spherical, mostly 0–1(–2)-septate, (5.5–)7.5–12.5(–35.5) × (2.5–)3–4.5(–5.5) µm [av. (± SD) 10.7 (± 4.7) × 3.6 (± 0.8)], with 2, rarely 3 distal scars. *Conidiogenous scars* thickened and conspicuous, protuberant, 0.9–1.5(–2.3) µm diam.

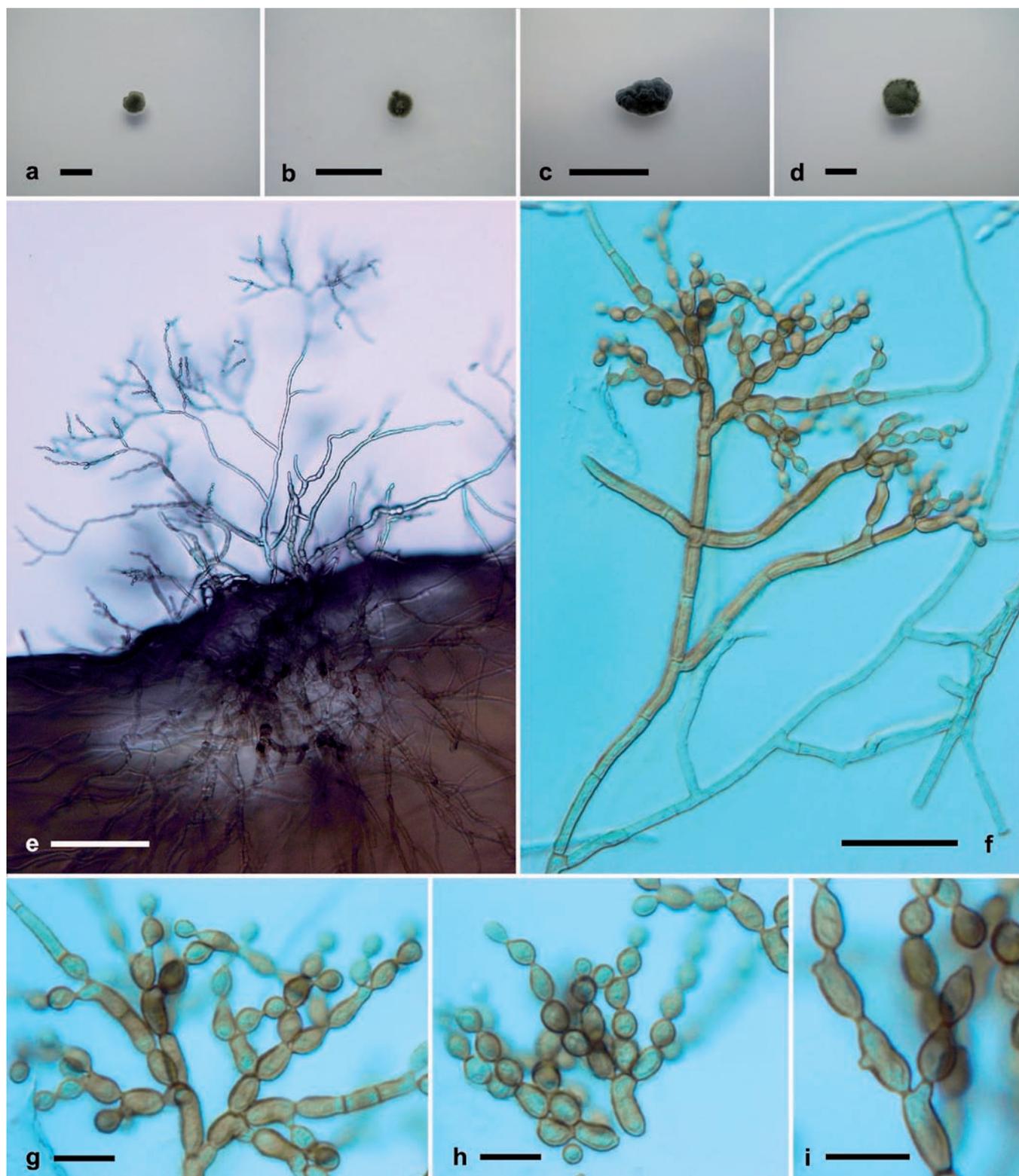
**Cultural characteristics:** Colonies on PDA, OA and MEA with restricted growth, attaining 2.5–4.5, 1.5–7.0 and 1.0–5.5 mm diam, respectively. Colonies flat or heaped (up to 3 mm), dark green (30F4), with black reverse and slightly undulate margin with immersed mycelium. Sporulating on all media. On MEA + 5 % NaCl growth is faster, colonies attaining 8.5–12.0 mm diam, sporulating and growing deeply into the agar.

**Maximum tolerated salt concentration:** All strains develop colonies at 17 % NaCl after 14 d.

**Cardinal temperatures:** No growth at 4 °C, optimum / maximum 25 °C (1.0–5.5 mm diam), no growth at 30 °C.

**Specimen examined:** Brazil, from man ulcero-nodular mycosis of hand and arm, 1927, coll. and isol. da Fonseca, CBS H-19737, **holotype**, culture ex-type CBS 189.54.

**Habitats and distribution:** Polar ice and biotams; conifer wood and window frame in Europe; humans; strains originating from nasal mucus (Buzina *et al.* 2003) have 100 % sequence homology with



**Fig. 9.** *Cladosporium langeronii*. Macro- and micromorphological characters. A–D. Colony surface grown on PDA (A), OA (B), MEA (C) and MEA plus 5 % NaCl (D) of strains incubated for 14 d at 25 °C in darkness. E–F. Habit of conidiophores. G–I. Ramoconidia and conidia. E–I. All from 7-d-old SNA slide cultures. A–D, from CBS 189.54 (ex-type strain); E, from CBS 109868; F–I, from EXF-999. Scale bars A, C–D = 10 mm, B = 5 mm, E = 100  $\mu$ m, F = 30  $\mu$ m, G–I = 10  $\mu$ m.

the strains studied, as well as with a clone from mycorrhizal roots (Menkis *et al.* 2005). The species is distributed worldwide, without any apparent predilection for a particular habitat. The strains from clinical cases probably were culture contaminants.

*Literature:* da Fonseca *et al.* (1927a, b).

*Differential parameters:* Restricted growth; lowest salt halotolerance taxon of all *C. sphaerospermum*-like species.

*Strains examined:* CBS 189.54 (ex-type strain), CBS 601.84, CBS 101880, CBS 109868, dH 11736, dH 12459 = EXF-999, dH 13833 = EXF-1933.

*Notes:* De Vries (1952) synonymised the isolate identified as *Hormodendrum langeronii* with *C. sphaerospermum*. Strains of this species have often been identified as *C. cladosporioides* (Buzina *et al.* 2003, Menkis *et al.* 2005) although it has slightly longer conidia.

***Cladosporium psychrotolerans*** Zalar, de Hoog & Gunde-Cimerman, **sp. nov.** MycoBank MB492428. Fig. 10.

**Etymology:** Refers to its ability to grow at low temperatures.

Mycelium partim submersum; hyphae vagina polysaccharidica carentes. Conidiophora erecta vel adscendentia; stipes (10–)50–100(–150) × (3–)3.5–4(–7.5) µm, olivaceo-brunneus, levis, crassitunicatus, compluribus regulariter septatus (cellulis 10–40 µm longis), identidem dichotome ramosus. Conidiorum catenae undique divergentes, terminales partes simplices ad 4 conidia continentes. Cellulae conidiogenae indistinctae. Ramoconidia primaria cylindrica, (18–)19–22(–43) × (2.5)3–3.5(–4.5) µm, 0(–1)-septata. Conidia leves vel leniter verruculosa, dilute brunnea, unicellularia, globosa vel ovoidea, (2.5–)3–4(–4.5) × (2–)2.5–3(–3) µm, long.: lat. 1.3–1.4; ramoconidia secundaria cylindrica, 0–1(–2)-septata, (5–)8–16(–36) × (2–)2.5–3(–5) µm, ad 4 cicatrices terminales ferentia; cicatrices inspissatae, conspicuae, 0.5–2 µm diam.

*Mycelium* partly superficial partly submerged; hyphae without extracellular polysaccharide-like material. *Conidiophores* erect or ascending, macronematous, stipes (10–)50–100(–150) × (3–)3.5–4(–7.5) µm, olivaceous-brown, smooth or almost so, thick-walled, regularly septate (cell length 10–40 µm), arising laterally from aerial hyphae, repeatedly dichotomously branched. *Conidial chains* branching in all directions, up to 4 conidia in the unbranched parts. *Ramoconidia* sometimes formed, cylindrical, (18–)19–22(–43) × (2.5)3–3.5(–4.5) µm, aseptate, rarely 1-septate, with a broadly truncate base, up to 2 µm wide, unthickened or slightly thickened, somewhat darkened-refractive. *Conidia* smooth to minutely verruculose, light brown, non-septate, spherical to ovoid, length : width ratio = 1.3–1.4; conidial size (2.5–)3–4(–4.5) × (2–)2.5–3(–3) µm [av. (± SD) 3.4 (± 0.5) × 2.5 (± 0.2)]; *secondary ramoconidia* cylindrical, 0–1(–2)-septate, (5–)8–16(–36) × (2–)2.5–3(–5) µm [av. (± SD) 12.7 (± 6.5) × 3.0 (± 0.5)], with up to 4 distal scars. *Conidiogenous scars* thickened and conspicuous, protuberant, 0.5–2 µm diam.

**Cultural characteristics:** Colonies on PDA reaching 13–18 mm diam, velvety, olive (3F4) due to profuse sporulation, flat with straight margin. Reverse dark green. Colonies on OA reaching 13–15 mm diam, olive (2F8), of granular appearance due to profuse sporulation; aerial mycelium sparse. Margin regular. Reverse black. Colonies on MEA reaching 8–15 mm diam, olive (2F4), velvety, radially furrowed with undulate white margin. Colonies on MEA with 5 % NaCl growing faster than on other media, reaching 25–27 mm diam, olive (3E6) and granular due to profuse sporulation, either slightly furrowed or heavily wrinkled with regular or undulate margin. Reverse dark green.

**Maximum tolerated salt concentration:** 17 % NaCl after 14 d.

**Cardinal temperatures:** Minimum at 4 °C (5 mm diam), optimum and maximum at 25 °C (8–15 mm diam).

**Specimen examined:** Slovenia, from hypersaline water of Sečovlje salterns, coll. and isol. S. Sonjak, May 1999, CBS H-19730, **holotype**, culture ex-type EXF-391 = CBS 119412.

**Habitats and distribution:** Hypersaline water in the Mediterranean basin.

**Differential parameters:** Growth at 4 °C; maximal NaCl concentration 17 % NaCl, which differentiates it from other species with similar conidia, like *C. sphaerospermum*, *C. halotolerans* and *C. dominicanum*.

**Strains examined:** EXF-326, EXF-332, EXF-391 (= CBS 119412; ex-type strain), EXF-714.

***Cladosporium salinae*** Zalar, de Hoog & Gunde-Cimerman, **sp. nov.** MycoBank MB492438. Fig. 11.

**Etymology:** Refers to salterns (= Latin *salinae*) as the habitat of this species.

Mycelium partim submersum; hyphae multa rostra lateralia ferentes, hyphae vagina polysaccharidica involutae. Conidiophora vix distincta, lateralia vel terminalia ex hyphis aeris oriunda; stipes longitudine variabili, (5–)25–50(–60) × (2–)2.5–3(–4) µm, olivaceo-brunneus, levis vel leniter verruculosus, crassitunicatus, irregulariter dense septatus (cellulis 6–29 µm longis), simplex, interdum ramosus. Conidiorum catenae undique divergentes, terminales ad 6 conidia continentes. Cellulae conidiogenae nonnumquam integratae, in summo sequentiam sympodiale denticulorum formantes. Conidia levia, interdum leniter verruculosa, dilute brunnea, unicellularia, plerumque fusiformia, (4.5–)5.5–7.5(–10) × (2–)2.5–3(–3.5) µm, long.: lat. 1.9–2.4; ramoconidia secundaria cylindrica, 0–1(–2)-septata, (7.5–)9.5–13.5(–19) × (2.5–)2.5–3.5(–4.5) µm, ad 5 cicatrices terminales ferentia; cicatrices inspissatae, conspicuae, protuberantes, 0.7–1.8 µm diam.

*Mycelium* partly superficial partly submerged, with numerous lateral pegs, consistently enveloped in polysaccharide-like material. *Conidiophores* poorly differentiated, micronematous, stipes (5–)25–50(–60) × (2–)2.5–3(–4) µm, olivaceous-brown, smooth to often minutely verruculose or irregularly rough-walled, thick-walled, irregularly densely septate (length of cells 6–29 µm), arising laterally and terminally from aerial hyphae, unbranched, occasionally branched. *Conidial chains* branching in all directions, terminal chains with up to 6 conidia. *Conidiogenous cells* sometimes integrated, producing sympodial clusters of pronounced denticles at their distal ends. *Conidia* usually smooth, occasionally minutely verruculose, light brown, aseptate, usually oblong ellipsoidal to fusiform, length : width ratio = 1.9–2.4; (4.5–)5.5–7.5(–10) × (2–)2.5–3(–3.5) µm [av. (± SD) 6.7 (± 1.3) × 2.9 (± 0.4)]; *secondary ramoconidia* cylindrical, 0–1(–2)-septate, (7.5–)9.5–13.5(–19) × (2.5–)2.5–3.5(–4.5) µm [av. (± SD) 12.1 (± 3.3) × 3.2 (± 0.6)], with up to 5 distal scars. *Conidiogenous scars* thickened and conspicuous, protuberant, 0.7–1.8 µm diam.

**Cultural characteristics:** Colonies on PDA reaching 10–27 mm diam, granular, olive (2E4) due to profuse sporulation, with white undulate margin. Aerial mycelium absent. Colonies either heaped or radially furrowed, in the marginal area growing deeply into the agar. Reverse dark brown to dark green. Colonies on OA reaching 7–20 mm diam, olive (3E6), of granular appearance due to profuse sporulation, aerial mycelium present. Margin either undulate or arachnoid, deeply furrowed. Reverse pale brown to dark green. Colonies on MEA reaching 8–19 mm diam, velvety, reseda-green (2E6), heaped. Margin furrowed, growing deeply into the agar. Colonies on MEA with 5 % NaCl growing much faster than on other media, reaching 25–38 mm diam, of different colours, mostly reseda-green (2E6) and granulate due to profuse sporulation, margin olive-yellow (2D6). Reverse yellow to dark green.

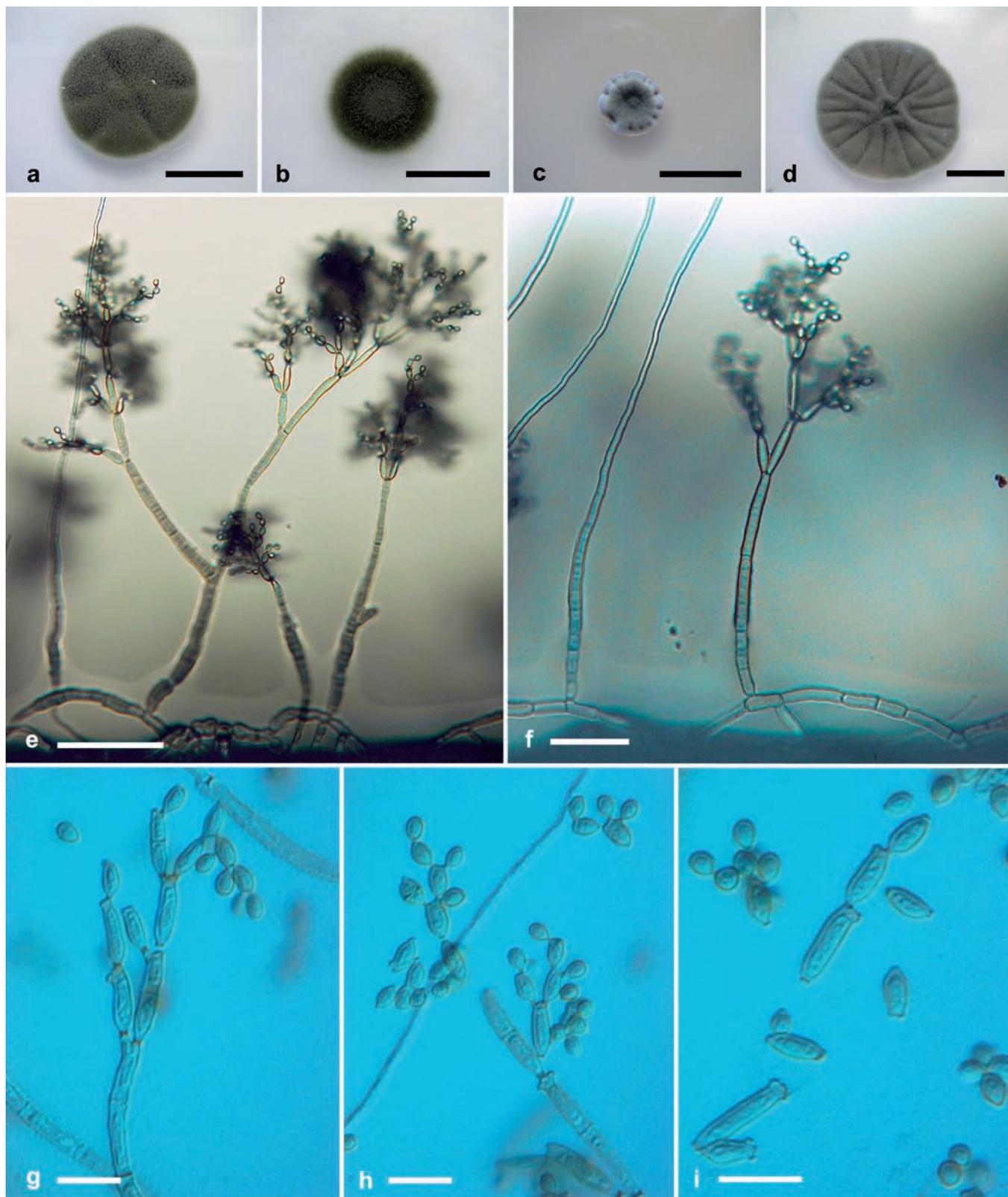
**Maximum tolerated salt concentration:** MEA + 17 % NaCl after 14 d.

**Cardinal temperatures:** No growth at 4 °C, optimum and maximum temperature at 25 °C (8–19 mm diam), no growth at 30 °C.

**Specimen examined:** Slovenia, from hypersaline water of Sečovlje salterns, coll. and isol. S. Sonjak, Feb. 1999, CBS H-19731, **holotype**, culture ex-type EXF-335 = CBS 119413.

**Habitats and distribution:** Hypersaline water in the Mediterranean basin.

**Differential parameters:** Sympodial conidiogenous cells with pronounced denticles, narrow temperature amplitude.

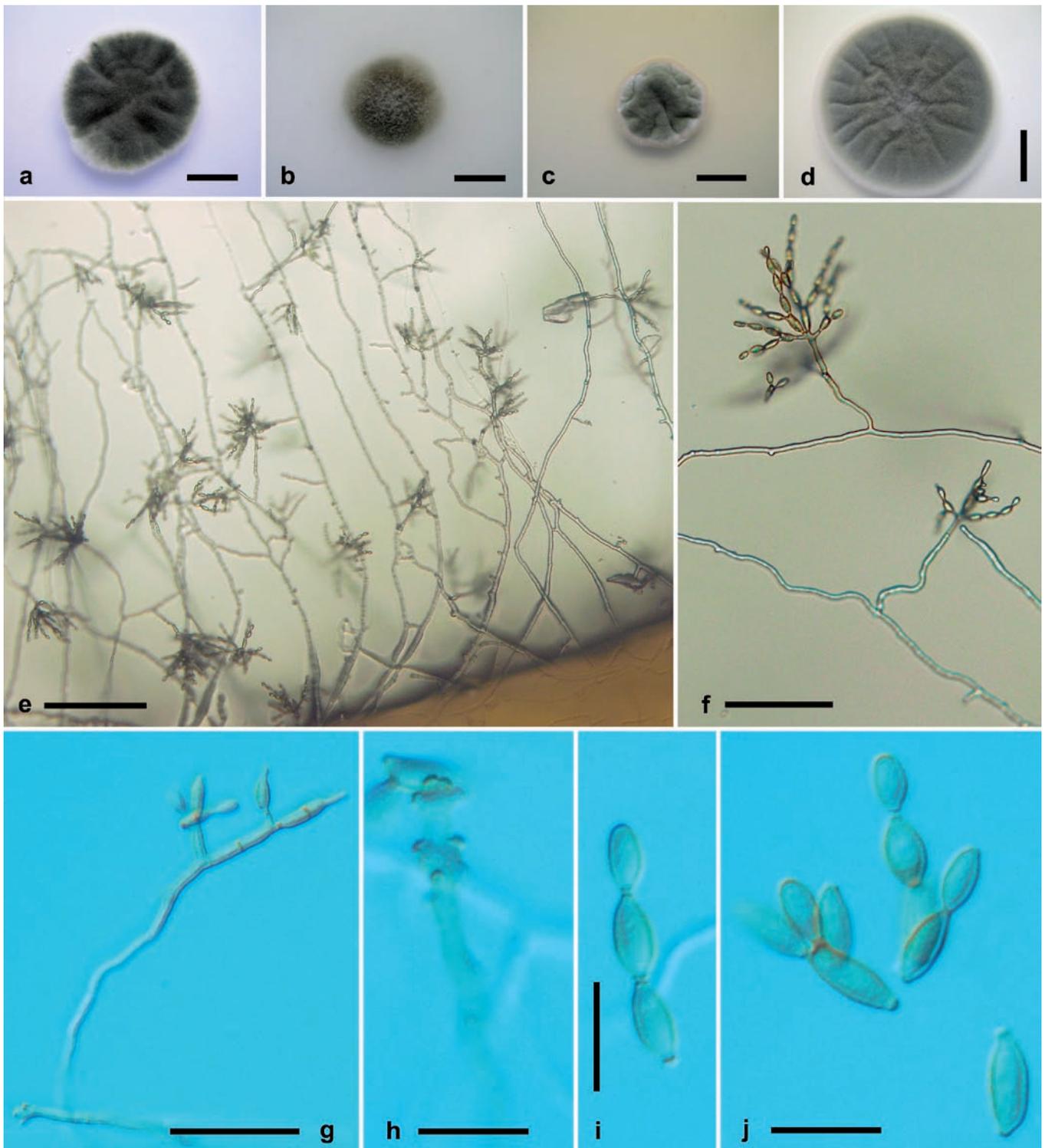


**Fig. 10.** *Cladosporium psychrotolerans*. Macro- and micromorphological characters. A–D. Colony surface grown on PDA (A), OA (B), MEA (C) and MEA plus 5 % NaCl (D) of strains incubated for 14 d at 25 °C in darkness. E–F. Conidiophores. G. Apical part of a conidiophore. H–I. Secondary ramoconidia and conidia. E–I. All from 7-d-old SNA slide cultures. All but C, from EXF-391 (ex-type strain); C, from EXF-714. Scale bars A–D = 10 mm, E = 100  $\mu$ m, F = 50  $\mu$ m, G–I = 10  $\mu$ m.

*Strains examined:* EXF-322, EXF-335 (= CBS 119413; ex-type strain), EXF-604.

*Notes:* *Cladosporium salinae* morphologically resembles species of the genus *Fusicladium* because its conidia are oblong ellipsoidal to fusiform and conidiogenous loci of ramoconidia are placed

closely together. As any other *Cladosporium* species, its conidia show typical cladosporioid scar structures, however. *Cladosporium salinae* seems to have a separate position within the genus *Cladosporium* since it seems to be distantly related to any other described *Cladosporium* species or currently known species complex within *Cladosporium*.

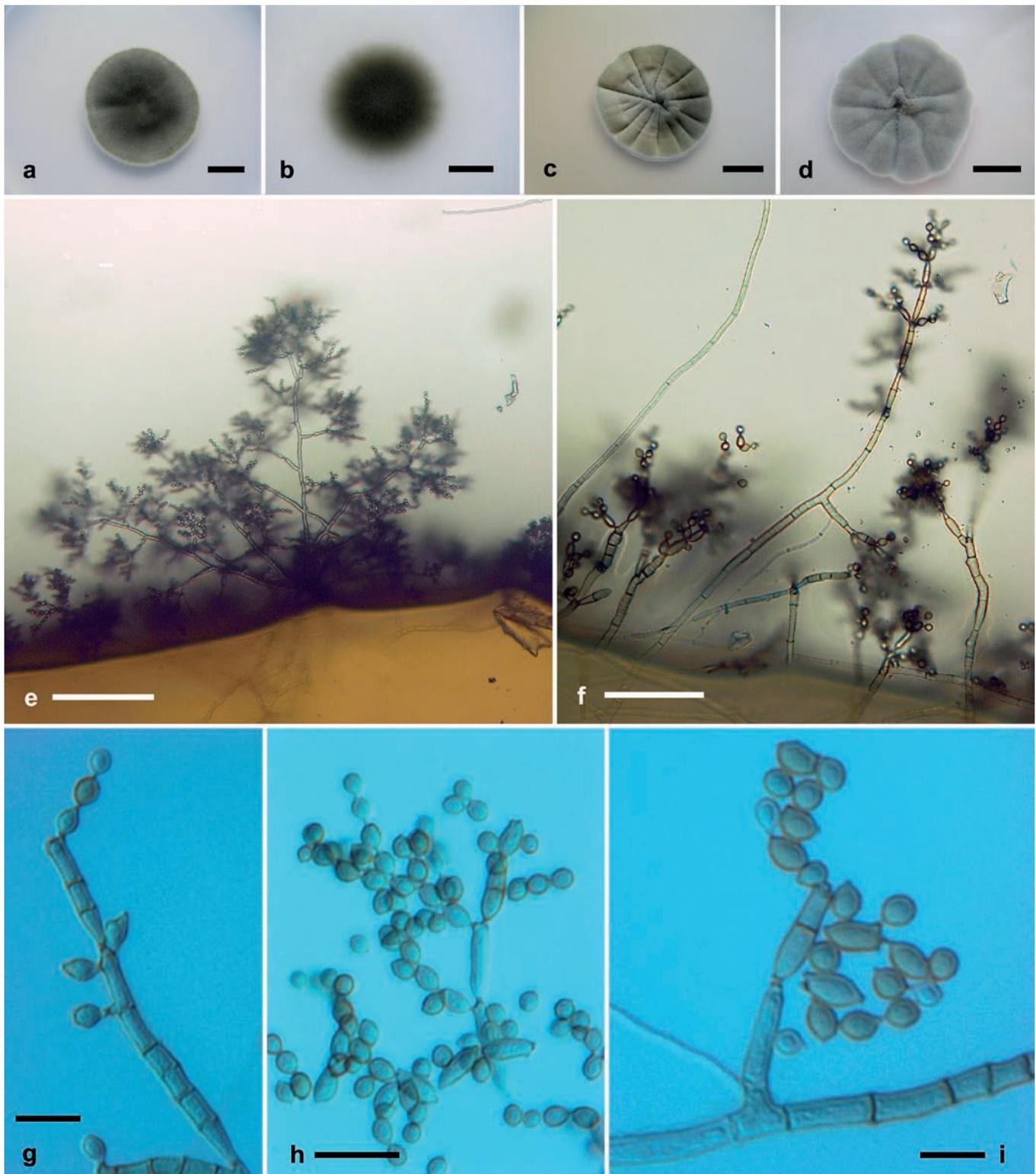


**Fig. 11.** *Cladosporium salinae*. Macro- and micromorphological characters. A–D. Colony surface grown on PDA (A), OA (B), MEA (C) and MEA plus 5 % NaCl (D) of strains incubated for 14 d at 25 °C in darkness. E–F. Habit of conidiophores. G. Conidiophore. H. Detail of apical part of conidiophore. I. Conidia. J. Secondary ramoconidia and conidia. E–J. All from 7-d-old SNA slide cultures. A–D, from EXF-604; E–J, from EXF-335 (ex-type strain). Scale bars A–C = 5 mm, D = 10 mm, E = 100 µm, F = 50 µm, G = 30 µm, H–J = 10 µm.

***Cladosporium sphaerospermum*** Penzig, *Michelia* 2(8): 473. 1882. Fig. 12.

*Mycelium* partly submerged, partly superficial; hyphae thick, darkly pigmented and densely septate in submerged mycelium, not enveloped in polysaccharide-like material. *Conidiophores* erect or ascending, micronematous and macronematous, stipes of variable length, (10–)45–130(–300) × (2.5–)3–4(–6) µm, olivaceous-brown, smooth to minutely verruculose, thick-walled, with relatively dense septation (cells mostly 4.5–23 long), septa darkened and

somewhat thickened, arising laterally and terminally from immersed or aerial hyphae, either unbranched or branched. *Conidial chains* branching in all directions, up to 6 conidia in the unbranched parts. *Conidiogenous cells* not differentiated. *Ramoconidia* often formed, cylindrical, (11.5–)20.5–40(–48) × (2.5–)3(–3.5) µm, with up to 5 septa, base broadly truncate, 2 µm wide, slightly thickened and somewhat darkened-refractive. *Conidia* verruculose, brown to dark brown, non-septate, usually subspherical to spherical, less often short-ovoid, narrower at both ends, with length : width ratio = 1.1–1.5; conidial size (2.5–)3–4(–7) × (2–)3–3.5(–4.5) µm [av. (±

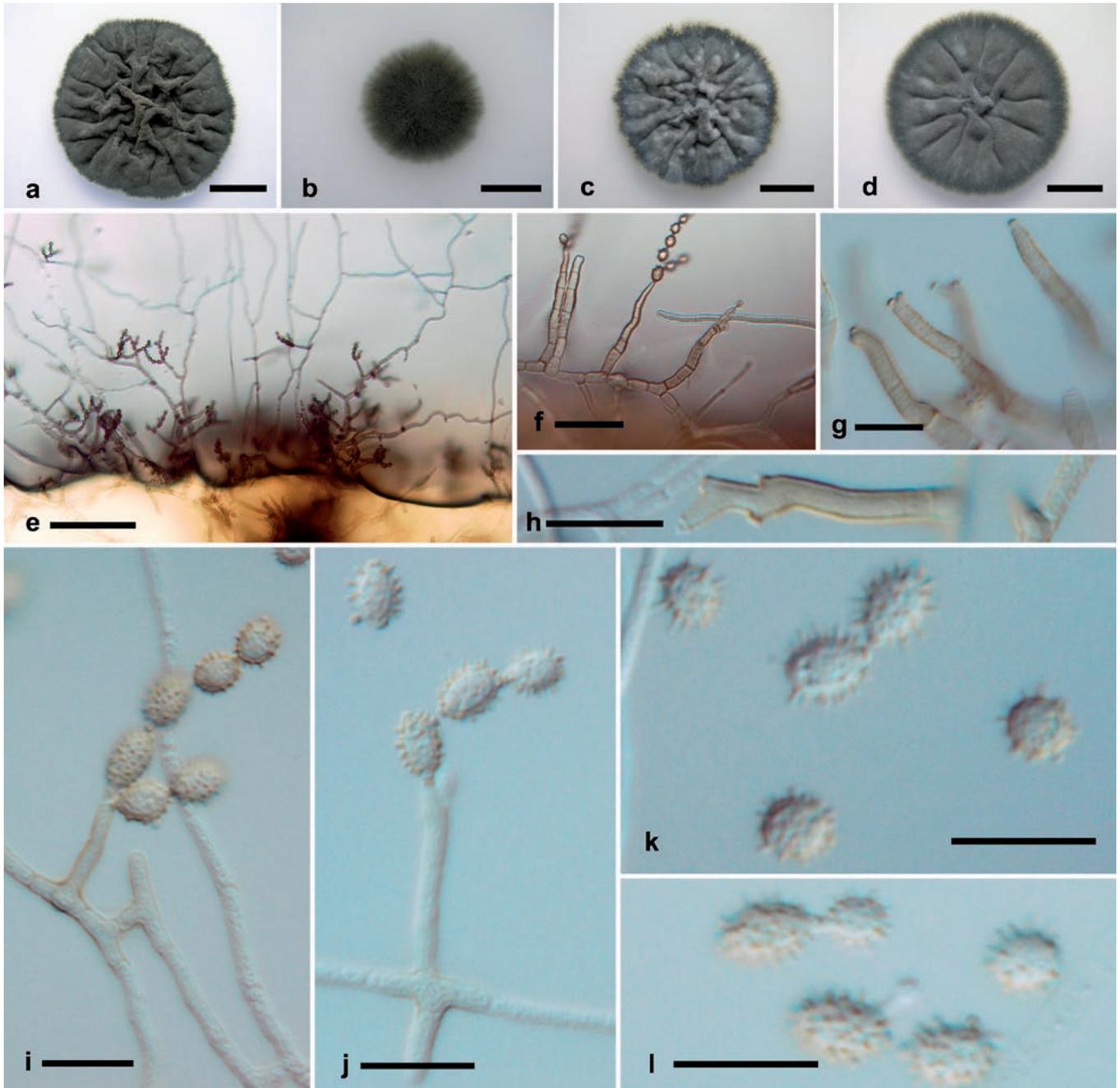


**Fig. 12.** *Cladosporium sphaerospermum*. Macro- and micromorphological characters. A–D. Colony surface grown on PDA (A), OA (B), MEA (C) and MEA plus 5 % NaCl (D) of strains incubated for 14 d at 25 °C in darkness. E–F. Habit of conidiophores. G–I. Ramoconidia and conidia. E–I. All from 7-d-old SNA slide cultures. A, C–D, F–H, from CBS 193.54 (ex-neotype strain); B, from EXF-738; E, EXF-455; I, EXF-458. Scale bars A–D = 10 mm; E = 100 µm; F = 50 µm; G–I = 10 µm.

SD)  $3.8 (\pm 0.8) \times 3.1 (\pm 0.4)$ ; *secondary ramoconidia* cylindrical to almost spherical, 0–3(–4) septate,  $(4\text{--})8.5\text{--}16(\text{--}37.5) \times (2\text{--})3\text{--}3.5(\text{--}5) \mu\text{m}$  [av. ( $\pm$  SD)  $13.1 (\pm 6.3) \times 3.2 (\pm 0.5)$ ], with up to 4, rarely up to 6 distal scars. *Conidiogenous scars* thickened and conspicuous, protuberant,  $0.9\text{--}1.1(\text{--}1.4) \mu\text{m}$  diam.

**Cultural characteristics:** Colonies on PDA reaching 21–44 mm diam, velvety, olive (2F5) due to profuse sporulation, either with white and regular, or exceptionally undulate margin. Aerial mycelium sparse. Colonies flat or rarely radially furrowed with elevated

colony centre. Exudates not prominent, some strains release green soluble pigments into the agar. Reverse blackish blue to pale green. Growth deep into the agar. Colonies on OA reaching 21–38 mm diam, olive (2F8), of granular appearance due to profuse and uniform sporulation, almost no aerial mycelium. Margin either regular or arachnoid, deeply radially furrowed. Reverse black. Colonies on MEA reaching 15–35 mm diam, velvety, linden-green (2C5), radially furrowed. Colony centre wrinkled, forming a crater-like structure; margin furrowed, lighter in colour, consisting of



**Fig. 13.** *Cladosporium spinulosum*. Macro- and micromorphological characters. A–D. Colony surface grown on PDA (A), OA (B), MEA (C) and MEA plus 5 % NaCl (D) of strains incubated for 14 d at 25 °C in darkness. E. Habit of conidiophores. F–J. Conidiophores. K–L. Conidia (also visible in I–J). E–L. All from 7-d-old SNA slide cultures. A–L, from EXF-334 (ex-type strain). Scale bars A–D = 10 mm, E = 100 µm, F = 30 µm, G–L = 10 µm.

submerged mycelium. Reverse pale to dark brown. Colonies on MEA with 5 % NaCl growing faster than on other media, reaching 31–60 mm diam, mainly olive (2D4), either being almost flat or radially furrowed, with margin of superficial mycelium; sporulation dense. Reverse ochraceous or dark green.

**Maximum tolerated salt concentration:** On MEA + 20 % NaCl 89 % of all strains tested develops colonies after 7 d, 96 % after 14 d.

**Cardinal temperatures:** No growth at 4 °C, optimum 25 °C (15–35 mm diam), maximum 30 °C (2–15 mm diam). No growth at 37 °C.

**Specimen examined:** **Netherlands**, from nail of man, 1949, coll. and isol. R.W. Zappey, CBS H-19738, **neotype designated here**, incorrectly selected by de Vries (1952) as "lectotype", culture ex-neotype CBS 193.54 = ATCC 11289 = IMI 049637.

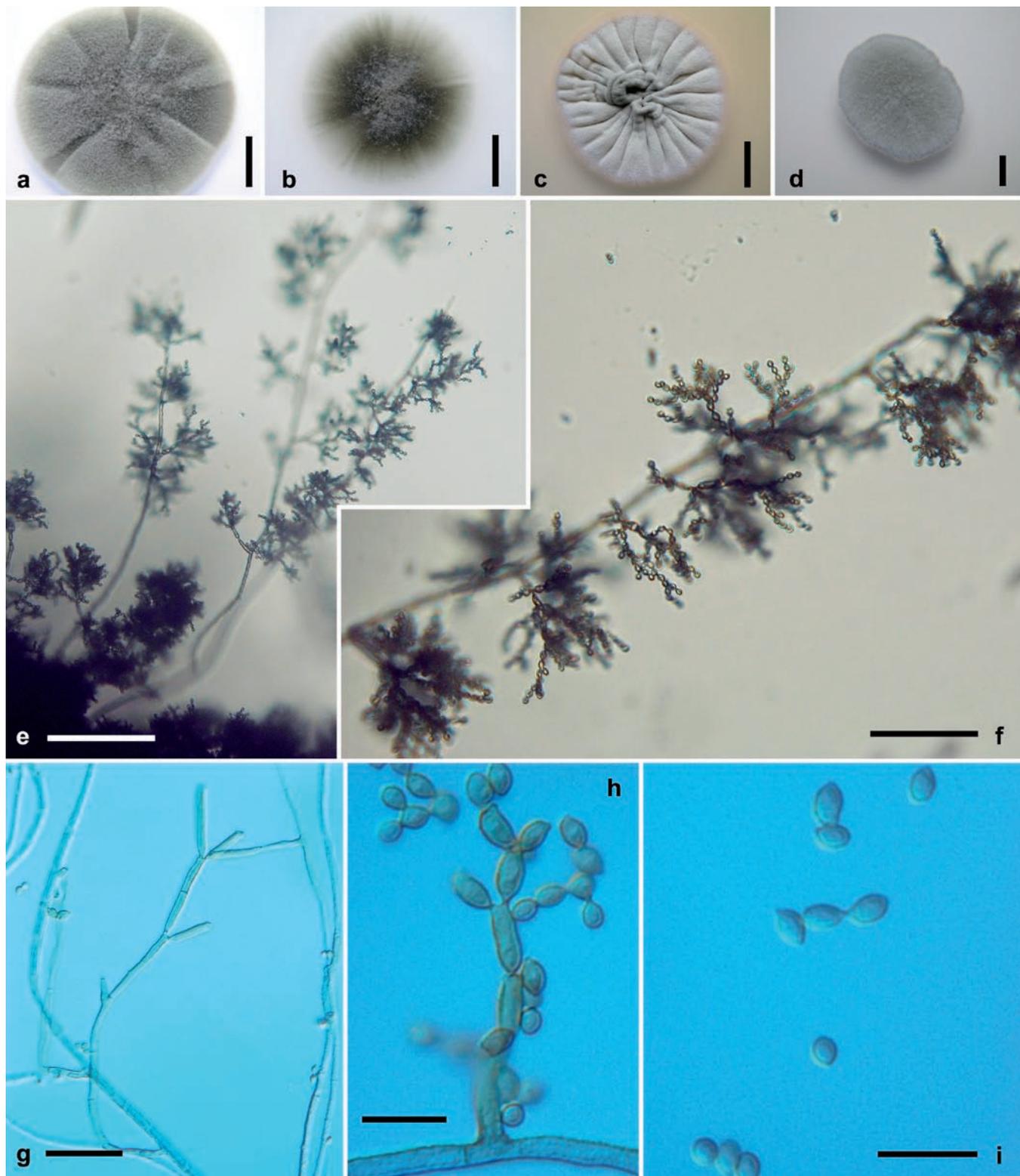
**Habitats and distribution:** Hypersaline water in mediterranean and tropics; soil and plants in temperate climates; indoor wet

cells; humans. The species does not seem to have any particular preference. Human isolates were probably culture contaminants.

**Literature:** Penzig (1882), de Vries (1952), Ellis (1971), de Hoog *et al.* (2000), Samson *et al.* (2002).

**Diagnostic parameters:** Thick-walled, melanised, densely septate mycelium, almost spherical, verruculose to verrucose terminal conidia, growth on 20 % NaCl after 7 d.

**Strains examined:** CBS 109.14, CBS 122.63, CBS 190.54, CBS 192.54, CBS 193.54 (ex-neotype strain), CBS 102045, CPC 10944, EXF-131, EXF-328, EXF-385, EXF-446, EXF-455, EXF-458, EXF-461, EXF-464, EXF-465, EXF-598, EXF-644, EXF-645, EXF-649, EXF-715, EXF-738, EXF-739, EXF-781, EXF-962, EXF-965, EXF-1061, EXF-1726, EXF-1732.



**Fig. 14.** *Cladosporium velox*. Macro- and micromorphological characters. A–D. Colony surface grown on PDA (A), OA (B), MEA (C) and MEA plus 5 % NaCl (D) of strains incubated for 14 d at 25 °C in darkness. E–F. Habit of conidiophores. G. Conidiophore. H–I. Secondary ramoconidia and conidia. E–I. All from 7-d-old SNA slide cultures. A–D, G, from CBS 119417 (ex-type strain); E–F, H–I, from EXF-466. Scale bars A–D = 10 mm, E = 100 µm, F = 50 µm, G = 30 µm, H–I = 10 µm.

***Cladosporium spinulosum*** Zalar, de Hoog & Gunde-Cimerman, sp. nov. MycoBank MB501099. Fig. 13.

**Etymology:** Refers to its conspicuously digitate conidia.

Conidiophora erecta vel adscendentia; stipites longitudine variabili, (15–)25–50(–155) × (2.5–)3–4(–5) µm, olivaceo-brunneus, levis, crassitunicatus, 0–6(–9)-septatus (cellulis 6–20 µm longis), ex hyphis submersis vel aeriis lateraliter vel terminaliter oriundus, simplex vel ramosus. Conidorum catenae undique ramosae, ad 4 conidiis in partibus linearibus continuis cohaerentibus. Cellulae conidiogenae integratae vel

discretae, acervos distales denticulorum conspicuorum sympodialium proferentes. Conidia echinulata vel digitata, brunnea vel fusca, continua, vulgo subglobosa vel globosa, (4.5–)5.5–7(–8) × (3–)4–4.5(–5) µm, long.: lat. = 1.1–1.6, digiti 0.6–1.3 µm longi; ramoconidia secundaria etiam digitata, cylindrica vel subglobosa, 0(–1)-septata, (6–)6.5–8(–18) × (4–)4.5–5(–5.5) µm, 1–3 cicatrices distales ferentia. Cicatrices inspissatae, conspicuae, protuberantes, 0.8–1.2 µm diam. Hyphae nonnumquam polysaccharido circumdatae.

**Hyphae** sometimes enveloped in polysaccharide-like material. **Conidiophores** erect or ascending, stipes of variable length, (15–)

25–50(–155) × (2.5–)3–4(–5) µm, olivaceous-brown, smooth, sometimes irregularly rough-walled to verrucose near the base, thick-walled, 0–6(–9)-septate (cells mostly 6–20 µm long), arising laterally and terminally from immersed or aerial hyphae, either unbranched or branched, somewhat tapering towards the apex. *Conidial chains* branching in all directions, up to 4 conidia in the unbranched parts. *Conidiogenous cells* sometimes integrated, producing sympodial clusters of pronounced denticles at their distal ends. *Ramoconidia* rarely formed. *Conidial wall ornamentation* conspicuously digitate, with up to 1.3 µm long projections having parallel sides and blunt ends. *Conidia* brown to dark brown, aseptate, usually subspherical to spherical, length : width ratio = 1.1–1.6; conidial size (4.5–)5.5–7(–8) × (3–)4–4.5(–5) µm [av. (± SD) 6.2 (± 1.0) × 4.2 (± 0.5)]; *secondary ramoconidia* ornamented as conidia, cylindrical to almost spherical, 0(–1)-septate, (6–)6.5–8(–18) × (4–)4.5–5(–5.5) µm [av. (± SD) 8.6 (± 4.0) × 4.8 (± 0.4)], with up to 3 distal scars. *Conidiogenous scars* thickened and conspicuous, protuberant, 0.8–1.2 µm diam.

**Cultural characteristics:** Colonies on PDA reaching 20–30 mm diam, velvety, dull green (29E4) to dark green (29F6) due to profuse sporulation, either with white and regular, or undulate margin. Aerial mycelium sparse. Colonies flat or radially furrowed with elevated colony centre. Growth deep into the agar. Exudates not prominent. Colonies on OA reaching 20–25 mm diam, dull green (29E4) to dark green (29F6), sometimes olive (3D4), of granular appearance due to profuse and uniform sporulation; almost without aerial mycelium. Margin arachnoid. Reverse pale brown to black. Colonies on MEA reaching 17–28 mm diam, velvety, dull green (29E4) to dark green (29F6), either flat or radially furrowed. Colony centre wrinkled, forming a crater-like structure; margin furrowed, paler in colour, consisting of submerged mycelium only. Reverse pale to dark green. Colonies on MEA with 5 % NaCl reaching 12–18 mm diam, of different colours, greenish grey (29D2), greyish green (29D5) to dark green (29F6); colony appearance variable, mostly either being almost flat with immersed colony centre or radially furrowed, with white to dark green margin consisting of superficial mycelium; sporulation dense. Reverse pale to dark green.

**Maximum tolerated salt concentration:** On MEA + 17 % NaCl, two of three strains tested developed colonies after 14 d.

**Cardinal temperatures:** Growth at 4 °C, optimum and maximum at 25 °C (17–28 mm). No growth at 30 °C.

**Specimen examined:** Slovenia, from hypersaline water of Sečovlje saltens, coll. and isol. S. Sonjak, Feb. 1999, CBS H-19796, **holotype**, culture ex-type EXF-334 = CBS 119907.

**Habitats and distribution:** Hypersaline water in temperate climate.

**Diagnostic parameters:** Conidia and ramoconidia with a digitate ornamentation.

**Strains examined:** EXF-334 (= CBS 119907; ex-type strain), EXF-382.

**Notes:** *Cladosporium spinulosum* is a member of the *C. herbarum* species complex (Figs 2–4) although its globoid conidia are reminiscent of *C. sphaerospermum*. Within *Cladosporium*, the species is unique in having conspicuously digitate conidia and ramoconidia. The two strains are differing in the size of conidia. The average size of conidia in EXF-334 is 6.2 (± 0.9) × 4.2 (± 0.5) µm, and in EXF-382 it is 3.9 (± 0.6) × 3.3 (± 0.4) µm.

***Cladosporium velox*** Zalar, de Hoog & Gunde-Cimerman, **sp. nov.**  
Mycobank MB492435. Fig. 14.

**Etymology:** Refers to the quick growth of strains of this species.

Mycelium partim submersum; hyphae vagina polysaccharidica carentes. Conidiophora erecta, lateralia vel terminalia ex hyphis aeriis oriunda; stipes (10–)25–150(–250) × (2.5–)3–4(–4.5) µm, olivaceo-brunneus, levis, crassitunicatus, ad 7–septatus (cellulis 10–60 µm longis), identidem dichotome ramosus. Conidiorum catenae undique divergentes, terminales partes simplices ad 5 conidia continentes. Cellulae conidiogenae indistinctae. Conidia levia vel leniter verruculosa, dilute brunnea, unicellularia, ovoidea, (2–)3–4(–5.5) × (1.5–)2–2.5(–3) µm, long. : lat. 1.4–1.7; ramoconidia secundaria cylindrica, 0–1-septata, (3.5–)5.5–19(–42) × (2–)2.5–3(–4.5) µm, ad 4(–5) cicatrices terminales ferentia; cicatrices inspissatae, protuberantes, conspicuae, 0.5–1.5 µm diam.

**Mycelium** partly superficial partly submerged; hyphae without extracellular polysaccharide-like material. *Conidiophores* erect, stipes (10–)25–150(–250) × (2.5–)3–4(–4.5) µm, slightly attenuated towards the apex, olivaceous-brown, smooth- and thick-walled, arising terminally and laterally from aerial hyphae, dichotomously branched [up to 5(–7)-septate, cell length 10–60 µm]. *Ramoconidia* rarely formed. *Conidial chains* branching in all directions, terminal chains with up to 5 conidia. *Conidia* smooth to very finely verruculose, pale brown, non-septate, ovoid, length : width ratio = 1.4–1.7; (2–)3–4(–5.5) × (1.5–)2–2.5(–3) µm [av. (± SD) 3.6 (± 0.6) × 2.3 (± 0.2)]; *secondary ramoconidia* cylindrical, 0–1-septate, (3.5–)5.5–19(–42) × (2–)2.5–3(–4.5) µm [av. (± SD) 13.4 (± 10.2) × 2.8 (± 0.5)], with up to 4(–5) distal scars. *Conidiogenous scars* thickened and conspicuous, protuberant, 0.5–1.5 µm diam.

**Cultural characteristics:** Colonies on PDA reaching 35–45 mm diam, velvety, dark green due to profuse sporulation, on some parts covered with white sterile mycelium, flat with straight white margin. Reverse dark green to black. Colonies on OA reaching 30–43 mm diam, dark green, mycelium submerged, aerial mycelium sparse. Margin regular. Reverse black. Colonies on MEA reaching 30–42 mm diam, pale green, radially furrowed, with raised, crater-shaped central part, with white, undulate, submerged margin. Sporulation poor. Colonies on MEA with 5 % NaCl reaching 35–45 mm diam, pale green, velvety, flat with regular margin. Reverse pale green. Sporulation poor.

**Maximum tolerated salt concentration:** 20 % NaCl after 14 d.

**Cardinal temperatures:** Minimum at 10 °C (9 mm diam), optimum at 25 °C (30–42 mm diam) and maximum at 30 °C (5–18 mm diam).

**Specimen examined:** India, Charidij, isolated from *Bambusa* sp., W. Gams, CBS H-19735, **holotype**, culture ex-type CBS 119417.

**Habitats and distribution:** Hypersaline water in Slovenia; bamboo, India.

**Strains examined:** CBS 119417 (ex-type strain), EXF-466, EXF-471.

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