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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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.

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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 ascal 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. zeae-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 (Carlier 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 Persson (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, Tomlin 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. subradians*.

**Section Tassiana**: pyriform ascus and irregularly arranged, thick-walled ascospores that are often long 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 *Heterocarium* 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 Fusiispora**: pyriform ascus 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 obvoid 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*, *Kirrhyacmes*, *Passalora*, *Phaeolehospora*, *Pseudocercospora*, *Pseudocercosporella*, *Sonterenlia*, *Stenella*, etc. Several representative species are listed by Crous (1998) on *Eucalyptus*.

**Section Cymadothea**: This section is now accepted as the genus *Cymadothea* (*Polystriicum* 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. trifoli* occurring on *Trifolium*.

Von Arx (1949) proposed separating species with separate ascomata immersed within the host tissue, and those with ascomata occurring in pseudoparenchymatous stroma. 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 Tomlin (1979). However, Müller and von Arx (1962) found that the type species of *Phaeosphaerella, P. maculosa* was identical to *Venturia macularis*. A generic new 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 *Septoria* (*Septoria* anamorphs), *Ramularisphaerella* (*Ramularia* anamorphs), *Cercosphaerella* (*Cercospora* anamorphs) and *Ovosphaerella* (*Ovularia* anamorphs).
Lesions also vary in colour at different stages of development, and can be smooth and amphiogenous, 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. Ascii are aparyphylate, bitunicate, subsessile, 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 ascii of large-spored species, or multisieriate 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 ilicis spot on Ligustrum; i. T. fimбриata 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 fijiensis 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 µm.
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. alicantai (Type C); g. T. jonkershoekensis (Type G); h. M. elaeocarpi (Type H); i. M. graminicola (Type D); j. T. suberosa (Type E); k. M. parkiiaffinis (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 µm.
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 brunneotingens 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 majorzuluensis 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 alistanii on potato-dextrose agar (note crystals); r. Septoria proteae on OA.
Anamorphic Mycosphaerella in the post-Chupp era

Asteromella is now commonly accepted as the sexual 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 or on host material (Crous & Wingfield 1996), and it is possible that occurs with species of Mycosphaerella. Anamorphic pigmentation, width, constriction at septa, etc.

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 recognizable 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, 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 Breejen 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 conidial 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 (Batzner 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, conidial structure (conidiophores solitary, fasciculate to synnematal, 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 paraplythetic 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 conidiofores 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, Boter 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 Breejen et al. 2006), Eucalyptus (Crous et al., 2004a, 2006e, 2007c, Cheewangkoon et al. 2008), Zea maydis (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. konae (Leucospermum in Hawaii, Eucalyptus in Thailand) (Crous et al. 2007c), M. markisi (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. rubidus (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 Aeglopis, Citrus, Fortuneella, 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 Cibiessia dimorphospora; l. conidiophore of Cladosporium sphaerospermum; m. conidia of Sonderhenia eucalyptica 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 Stigmina eucalypti; h. conidia of Sonderhenia eucalyptorum anamorph of M. swartii; i. conidia of Septoria eucalyptorum; j. conidiophore of Polythrincium trifoli, anamorph of Cymadothea trifoli; 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 µm, except d = 40 µm.
### Table 1 Anamorph genera linked to *Mycosphaerellaceae* (M) and *Teratosphaeriaceae* (T)¹.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Conidiomata²</th>
<th>Synanamorph</th>
<th>Proliferation³</th>
<th>Colour⁴</th>
<th>Conidial septation</th>
<th>Loci⁵</th>
<th>Arrangement⁶</th>
<th>Mycelium⁷</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Batcheloromyces</strong></td>
<td>S</td>
<td>catenulostrum-like</td>
<td>P</td>
<td>P</td>
<td>0–3</td>
<td>I</td>
<td>S,C</td>
<td>I,E</td>
<td>Taylor et al. 2003</td>
</tr>
<tr>
<td><strong>Baudoinia</strong></td>
<td>Ph</td>
<td>--</td>
<td>Ph</td>
<td>P</td>
<td>0–1</td>
<td>I</td>
<td>C</td>
<td>E</td>
<td>Scott et al. 2007</td>
</tr>
<tr>
<td><strong>Capnobotryella</strong></td>
<td>Ph</td>
<td>Endoconidia</td>
<td>Ph</td>
<td>P</td>
<td>0–1</td>
<td>I</td>
<td>C</td>
<td>E</td>
<td>Sugiyama &amp; Amano 1987</td>
</tr>
<tr>
<td><strong>Catenulostruma</strong></td>
<td>A,S,F</td>
<td>--</td>
<td>Ph</td>
<td>P</td>
<td>0-multi</td>
<td>I</td>
<td>S,C</td>
<td>I,E</td>
<td>Crous et al. 2007a</td>
</tr>
<tr>
<td><strong>Cercospora</strong></td>
<td>F</td>
<td>--</td>
<td>S</td>
<td>H (conidia)</td>
<td>0-multi</td>
<td>T,D,R</td>
<td>S</td>
<td>I</td>
<td>Crous &amp; Braun 2003</td>
</tr>
<tr>
<td><strong>Cercosporella</strong></td>
<td>F</td>
<td>--</td>
<td>S</td>
<td>P (conidioph.)</td>
<td>multi</td>
<td>T,R</td>
<td>S</td>
<td>I</td>
<td>Braun 1995</td>
</tr>
<tr>
<td><strong>Cibbiessiae</strong></td>
<td>Ph</td>
<td>readeriella-like</td>
<td>Ph</td>
<td>P</td>
<td>1–3</td>
<td>I</td>
<td>C</td>
<td>I,E</td>
<td>Crous et al. 2007c</td>
</tr>
<tr>
<td><strong>Clypeispora</strong></td>
<td>P</td>
<td>--</td>
<td>M</td>
<td>H</td>
<td>0</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>Ramaley 1991</td>
</tr>
<tr>
<td><strong>Colletogloeopsis</strong></td>
<td>A,P</td>
<td>--</td>
<td>P,S</td>
<td>P</td>
<td>0–1</td>
<td>I</td>
<td>S</td>
<td>I,E</td>
<td>Crous &amp; Wingfield 1997</td>
</tr>
<tr>
<td><strong>Devriesia</strong></td>
<td>M</td>
<td>coelomycete</td>
<td>P</td>
<td>P</td>
<td>0</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>Crous et al. 2006e</td>
</tr>
<tr>
<td><strong>Dothistroma</strong></td>
<td>A</td>
<td>Sol Chlamydospores</td>
<td>S</td>
<td>P</td>
<td>0–3</td>
<td>T,D</td>
<td>C</td>
<td>E</td>
<td>Seifert et al. 2004</td>
</tr>
<tr>
<td><strong>Hortaea</strong></td>
<td>Sol</td>
<td>--</td>
<td>P,M</td>
<td>H (conidia)</td>
<td>0–2</td>
<td>I</td>
<td>S</td>
<td>I,E</td>
<td>Bonifaz et al. 2008, Plemenitaš et al. 2008</td>
</tr>
<tr>
<td><strong>Kirramyces</strong></td>
<td>P</td>
<td>pseudocercosporale-like</td>
<td>P,S</td>
<td>P</td>
<td>0-multi</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>Andjic et al. 2007</td>
</tr>
<tr>
<td><strong>Lecanosticta</strong></td>
<td>A</td>
<td>--</td>
<td>P</td>
<td>P</td>
<td>0-multi</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>Suto &amp; Ougi 1998</td>
</tr>
<tr>
<td><strong>Miuraea</strong></td>
<td>F,Sol</td>
<td>--</td>
<td>S</td>
<td>H,P</td>
<td>muriform, multi</td>
<td>I</td>
<td>S</td>
<td>I,E</td>
<td>von Arx 1983</td>
</tr>
<tr>
<td><strong>Nothostrasseria</strong></td>
<td>P</td>
<td>--</td>
<td>M</td>
<td>P</td>
<td>0</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>Crous et al. 2007c</td>
</tr>
<tr>
<td><strong>Passalora</strong></td>
<td>F,S,Sol</td>
<td>--</td>
<td>S</td>
<td>P</td>
<td>0-multi</td>
<td>T,D,R</td>
<td>S,C</td>
<td>I,E</td>
<td>Crous &amp; Braun 2003</td>
</tr>
<tr>
<td><strong>Penidiella</strong></td>
<td>Sol,F,Syn</td>
<td>--</td>
<td>S</td>
<td>P</td>
<td>0–1</td>
<td>I or T,D</td>
<td>C</td>
<td>I,E</td>
<td>Crous et al. 2007a</td>
</tr>
</tbody>
</table>
Table 1 (continued) Anamorph genera linked to *Mycosphaerellaceae* (M) and *Teratosphaeriaceae* (T)\(^1\).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Conidia</th>
<th>Synanamorph</th>
<th>Proliferation(^2)</th>
<th>Colour(^3)</th>
<th>Conidial septation</th>
<th>Loci(^4)</th>
<th>Arrangement(^5)</th>
<th>Mycelium(^7)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Periconiella</em> (M)</td>
<td>Sol</td>
<td>–</td>
<td>S</td>
<td>P</td>
<td>0-multi</td>
<td>I,D</td>
<td>C</td>
<td>I,E</td>
<td>Arzanlou et al. 2007b</td>
</tr>
<tr>
<td><em>Phloeospora</em> (M)</td>
<td>A</td>
<td>–</td>
<td>S</td>
<td>H</td>
<td>multi</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>Sivanesan 1984</td>
</tr>
<tr>
<td><em>Pseudothecidea</em> (T)</td>
<td>En</td>
<td>–</td>
<td>En</td>
<td>P</td>
<td>0–2</td>
<td>I</td>
<td>S</td>
<td>I,E</td>
<td>Crous et al. 2007c</td>
</tr>
<tr>
<td><em>Pseudotaeniolina</em> (T)</td>
<td>Sol</td>
<td>–</td>
<td>Ph</td>
<td>P</td>
<td>0–2</td>
<td>I</td>
<td>C</td>
<td>I,E</td>
<td>Crane &amp; Schoknecht 1986</td>
</tr>
<tr>
<td><em>Ramichloridium</em> (M)</td>
<td>Sol</td>
<td>–</td>
<td>S</td>
<td>P</td>
<td>0–1</td>
<td>D</td>
<td>S</td>
<td>I,E</td>
<td>Arzanlou et al. 2007b</td>
</tr>
<tr>
<td><em>Ramulispora</em> (M)</td>
<td>S</td>
<td>Chlamydospores</td>
<td>S</td>
<td>H</td>
<td>0-multi</td>
<td>I</td>
<td>S</td>
<td>I,E</td>
<td>Crous et al. 2003</td>
</tr>
<tr>
<td><em>Readeriella</em> (T)</td>
<td>P</td>
<td>–</td>
<td>P,M</td>
<td>P</td>
<td>0</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>Crous et al. 2007a</td>
</tr>
<tr>
<td><em>Septoria</em> (M)</td>
<td>P/A</td>
<td>–</td>
<td>S</td>
<td>H</td>
<td>1-multi</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>Von Arx 1983</td>
</tr>
<tr>
<td><em>Sonderhenia</em> (M)</td>
<td>P</td>
<td>–</td>
<td>P</td>
<td>P</td>
<td>0–5</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>Swart &amp; Walker 1988</td>
</tr>
<tr>
<td><em>Staninwardia</em> (T)</td>
<td>A</td>
<td>–</td>
<td>P</td>
<td>P</td>
<td>1–2</td>
<td>I</td>
<td>C</td>
<td>I</td>
<td>Summerbell et al. 2006</td>
</tr>
<tr>
<td><em>Trochophora</em> (M)</td>
<td>F,Sol</td>
<td>–</td>
<td>S</td>
<td>P</td>
<td>3</td>
<td>I</td>
<td>S</td>
<td>I,E</td>
<td>Zhao et al. 2007</td>
</tr>
<tr>
<td><em>Zasmidium</em> (M)</td>
<td>F,Sol</td>
<td>–</td>
<td>S</td>
<td>P</td>
<td>0-multi</td>
<td>T,D,R</td>
<td>S</td>
<td>I,E</td>
<td>Arzanlou et al. 2007b</td>
</tr>
</tbody>
</table>

\(^1\)Asteronella spermatial states have also been described in Ascochyta, Asteroma, Phyllosticta and Phoma. Excluded genera are Dissoconium (= Uwebraunia), Cladosporium (Davidiellaceae), Thedgonia (Helotiales), Xenostigmina (Pleosporales), Mycovellotisella and Phaeoramularia are treated as synonyms of Passalora, Phaeosaniospiza, Paracercospora and Stigmina as synonyms of Pseudocercosporella.

\(^2\)Fasciculate (F), sporodochial (S), solitary (Sol), pycnidial (P), acervular (A), synnematous (Syn), phragmosporous (Ph), hyphae with endoconidia (En), multilocular (M).

\(^3\)Sympodial (S), percurrent (P), monoblastic, determinate (M), phragmospores (Ph), endoconidia (En).

\(^4\)Thickened (T), darkened (D), refractive (R), protruding (P), inconspicuous (I).

\(^5\)Solitary (S), chains (C).

\(^6\)Internal (I), external (E).
bread 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 crypta (syn. M. cryptica) have a broad host range and cause disease on 38 species across the Eucalyptus sub-genera Monocalyptus and Symphyomyrtus, while T. rubitosa 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 straightforward. 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-Ferraez 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 Gelatinospora 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. 2008b). Much attention was also devoted to the elucidation of the mating systems active in the Mycosphaerella spp. occurring on banana (Conde-Ferraez 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/Mycfi1/Mycfi1.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 (Mycosphaeriaceae) (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
categories 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.
References


Nauka, Leningrad, USSR.
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 various generic issues within the *Mycosphaerella* complex with sex in some prominent sexual and asexual members of the *Mycosphaerella* complex have thus also been included.


Phylogenetic and morphotaxonomic revision of *Ramichloridium* and allied genera


Key words: Capnodiales, Chaetothyriales, Mycosphaerellaceae, 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* Näff. 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 by 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 reintroduced *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-Hedaily is a serious human pathogen, causing cerebral phaeohyphomycosis (Al-Hedaily et al. 1988, Campbell & Al-Hedaily 1993), whereas *R. musae* causes tropical speckle disease on members of the *Musaceae* (Stathel 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. bastonum* de Hoog and occasionally *R. schulzeri* (Sacc.) de Hoog, while the remaining species tend to be common soil sapropoles.

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, Unterreiner & 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, Rhinocladiella* 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 gene (LSU) was amplified with primers LR0R (Rehner & Samuels 1994) and LR5 (Vigelays & 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 MgCl₂, 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 *Paullicorticium anastum* Liberta 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).

**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.

<table>
<thead>
<tr>
<th>Species</th>
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<th>Source</th>
<th>Origin</th>
<th>GenBank numbers (LSU, ITS)</th>
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</table>
RESULTS

Phylogeny
The manually adjusted alignment of the 28S rDNA data contained 373 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 516 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 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 analyses, the trees differed in the hierarchical order of the main families). The fifth clade (in the Sordariomycetes clade) includes a veronaea-like isolate from Rhinocladiella, with phylogenetic affinity to the Annulatascaceae (Sordariomycetidae). The sixth 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 Magnaporthaceae. The seventh clade (in the Sordariomycetes clade) includes R. fulvum, R. fulvum var. fulvum, and R. fulvum var. flexuosum de Hoog, the closest relatives being Thyridium and Magnaporthe. The eighth clade (in the Sordariomycetes 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 a sister taxon to the Venturiaea representing the eighth clade (Dothideomycetes). The type species of Periconiella, P. velutina, clusters within the Mycosphaerellaceae (Capnodiaceae clade), whereas P. papuana Apte and Veronaea also resides in the Herpotrichiellaceae 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 asceptate conidia. Other than conidial morphology, features of the conidiogenous apparatus that

<table>
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<th>Species</th>
<th>Accession number</th>
<th>Source</th>
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<tr>
<td>Veronaea compacta</td>
<td>South Africa</td>
<td>EU041876, EU041819</td>
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<tr>
<td>Veronaea japonica</td>
<td>On dead bamboo culm</td>
<td>Japan</td>
<td>EU041875, EU041816</td>
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<tr>
<td>Veronaeopsis simplex</td>
<td>Acacia karroo</td>
<td>South Africa</td>
<td>EU041877, EU041820</td>
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<tr>
<td>Zasmidium cellare</td>
<td>Wine cellar</td>
<td>EU041878, EU041821</td>
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</table>

1ATCC: 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 Toxicoes, Chiba University, Chiba, Japan; IMI: International Mycological Institute, CAB-I-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.

2Ex-type cultures.
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

2. Conidia 0–2(–3)-septate, conidial base truncate, retaining a marginal frill after liberation [anamorphs of Sordariomycetes] ........................................................................................................ Rhodoveronaea

3. Conidiophores composed of a well-developed erect stalk and a terminal branched head ............................................................................................................................. 4

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

6. Conidia predominantly septate ................................................................................................................................................................................................. 7

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] ................................................................................ Veronaeopsis

8. Vegetative mycelium entirely hyaline; rachis long, hyaline, with widely scattered pimple-shaped, terminally pointed, unpigmented denticles ........................................................................................................................................................................ Myrmecridium

9. Rachis distinctly raduliform, with distinct, prominent blunt denticles, 0.5–1 μm long; scars and hila unthickened, but pigmented ................................................................................................................................. Radulidium

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] ........................................................................... Rhinocladiella

11. 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 Arzaniou & 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, Streltzia 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 pontifera and Ischyrolepis subverticillata in South Africa. Zasmidium cellare (Pers.) Fr., the type species of Zasmidium (Pers.) Fr., is also shown to cluster within the Mycosphaerellaceae.

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 sejate 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 obclavate, with a slightly darkened and thickened hilum; conidial secession schizolytic.

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 conidiogenous loci.

**Fig. 1.** (Continued).
Periconiella velutina (G. Winter) Sacc., Miscell. mycol. 2: 17. 1884. Fig. 3.

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.

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

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

Ab allis speciebus *Periconiellae* conidia 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. *Conidigenous 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 μm.

RAMICHLORIDIUM AND ALLIED GENERA

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 μm.

Fig. 6. A. Pseudovirgaria hyperparasitica (CBS 121739 = CPC 10753). B. Periconiella levispora (CBS 873.73). Scale bar = 10 μm.
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* conidii levibus et maioribus, ad 23 μm longis distinguendia.

**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.


**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.


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


**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 μm with a truncate base and a darkened, slightly thickened hilum, 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.

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 μm wide; aerial hyphae pale brown, warty. Conidiophores arising vertically and clearly differentiated from creeping aerial hyphae, up to 400 μm tall, with several additional thin septa; intercalary cells, 8–40 × 2–5 μm, from the broadest part at the base tapering towards the apex, subhyaline, later becoming pale brown and warty in the lower part. Subtending hyphae thick-walled, warty. Conidiogenous cells integrated, terminal, 10–18 μm long, proliferating sympodially, giving rise to a short rachis with conspicuous conidiogenous loci; scars slightly thickened and darkened, about 1 μm diam. Conidia solitary, aseptate, thin-walled, smooth, subhyaline, subcylindrical to obclavate, (10–)12–15(–23) × 2.5–3 μm, with a truncate base and a slightly darkened and thickened hilum, 1.5–2 μm diam, rarely fusing at the basal part.

Cultural characteristics: Colonies on MEA rather slow growing, reaching 8 mm diam after 14 d at 24 °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°34′47.2″ S, 145°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. 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 μm.
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 μm.

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 μm.

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


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. Conidigenous 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 hyphae arising vertically from creeping aerial hyphae, pale brown, 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. Conidigenous 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, obvoido 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: Japan, isolated from Sasa sp., K. Tubaki, CBS 103.59, ex-type CBS H-19933.

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).


Etymology: Named after its country of origin, Brazil.
A simii Ramichloridio cerophilo conidios minoribus, ad 8 μm longis, et conidios secundarius absenbitus distinguendum.

In vitro: Submerged hyphae pale olivaceous, smooth or 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. Conidigenous 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, obvoido 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 27 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.


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

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. Conidigenous cells terminally integrated, cylindrical, variable in length, 15–50 μm long, rachis short 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, dacrystoid to pyriform, (2)–3–4(–6) × (1.5)–2(–2.5) μm, smooth, thin-walled, with an inconspicuous hilum.

Cultural characteristics: Colonies on MEA rather 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.
**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.


*In vitro:* Submerged hyphae smooth, thin-walled, hyaline, 1–2.5 μm wide, with thin septa; aerial hyphae coarsely verrucose, olivaceous-green, rather thick-walled, 2–2.5 μm 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 μm long, 2–4 μm wide, often with inflated basal cells. Conidiogenous cells terminally integrated, up to 165 μm long, smooth, dark brown, sympodially proliferating, rachis straight or flexuose, geniculate or nodose, subhyaline; scars thickened and darkened, clustered at nodes, approx. 0.5 μm 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) × (4–)6–6.5(–9) μm, with truncate base; hilum conspicuous, slightly darkened, not thickened, about 1 μm 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.


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 Ramichloridi conidiophoribus 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.


**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

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**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.
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. **Conidia** in short chains, cylindrical to fusiform, verrucose, obovate to obconical, pale brown; polyblastic, proliferating sympodially producing crowded, conspicuously pigmented scars that are thickened and refractive, about 1 μm diam.


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


**In vitro:** Submerged hyphae smooth, thin-walled, hyaline, 2–3 μm wide, with thin septa; aerial hyphae coarsely verrucose, olivaceous-green, rather thick-walled, 2–2.5 μm 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 μm long and 2–2.5 μm wide, pale brown, proliferating sympodially producing crowded, conspicuously pigmented scars that are thickened and refractive, about 1 μm diam. Conidia cylindrical to fusiform, verrucose, obovate to obconical, pale brown, with truncate base, (6–)9–14(-27) × 2–2.5 μm, with a conspicuous, slightly pigmented, refractive hilum, approx. 1 μm 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 *Rhinoiodiella* (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 aniceps*, *R. mackenziei*, *R. fasciculatum* and *R. basitonum*.
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.


*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. Conidiophores slightly differentiated from vegetative hyphae, arising from prostrate aerial hyphae, consisting of either unbranched or loosely branched stalks, golden to dark brown, up to 350 μm tall, which may have up to 15 thin, additional septa, intercalary cells 9–14 μm 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 × 1.5–2 μm; with crowded, slightly prominent, unpigmented, conidiophore-bearing denticles, about 0.5 μm diam. Conidia solitary, subhyaline, thin-walled, smooth, subglobose to ellipsoidal, 2.5–4 × 2–2.5 μm, with a less conspicuous, slightly darkened hilum, less than 0.5 μm diam.

*Cultural characteristics*: Colonies on MEA reaching 6–12 mm diam after 14 d at 24 °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 (isotype); CBS H-7716 (isotype); CBS H-7717 (isotype); CBS H-7718 (isotype); CBS H-7719 (isotype), ex-type strain, CBS 181.65 = ATCC 18655 = DAOM 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 μm long) resembles *Rh. phaeophora* Veerkamp & W. Gams (1983) (conidia 5.5–6 μm long), but has shorter conidia.

*Fig. 20. Rhinocladiella basitona* (CBS 101460). A–B. Semi-micronematous conidiophores with verticillate branching pattern. C–D. Symposidially 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 μm.
**RAMICHLORIDUM AND ALLIED GENERA**

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


*In vitro:* Submerged hyphae hyaline, smooth, thin-walled, 2 μm 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 μm wide. *Conidiogenous cells* terminal, variable in length, 10–100 μm long, pale brown, straight or geniculate, proliferating sympodially, giving rise to a long, 2–2.5 μm 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) × 1–2 μm, hilum conspicuous, slightly darkened and thickened, less than 0.5 μm diam.

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

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


*In vitro:* Submerged hyphae subhyaline, smooth, thick-walled, 2–2.5 μm 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 μm long and 2–3 μm wide, with 0–5 thin additional septa. *Conidiogenous cells* terminal, cylindrical, 30–100 μm long, thin-walled, smooth, pale brown, fertile part as wide as the basal part, up to 2 μm wide, proliferating sympodially, giving rise to a rachis with hardly prominent, slightly pigmented, not thickened scars, less than 0.5 μm diam. *Conidia* solitary, smooth, thin-walled, subhyaline, ellipsoidal, (2.5–)4–5(–6) × 2–3 μm, with truncate, slightly pigmented hilum, about 0.5 μm diam. *Synnanamorph* forming on torulose hyphae originating from giant cells; compact heads of densely branched hyphae forming thinly-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 μm long, 1–1.5 μm wide. *Conidia* smooth, thin-walled, aseptate, subhyaline, globose, 2–2.5 μm diam.

*Cultural characteristics:* Colonies on MEA reaching 8 mm diam after 14 d at 24 °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 μm.
Fig. 23. 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 μm.

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 μm.

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, unthickened, 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.


Notes: Morphologically Rhinocladiella mackenziei is somewhat similar to Pleurothecium obovodeum (Matsush.) Arzanlou & Crous, which was originally isolated from dead wood. However, P. obovodeum 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. obovodeum clusters in the Chaetosphaeriales.

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

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

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


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; micromenatous 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, 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 conidiyum-bearing denticles, about 1 μm diam, unthickened 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.


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 terminal, 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.


**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 μm wide and up to 200 μm long. **Conidiogenous cells** terminal, occasionally intercalary, cylindrical, 10–100 μm 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) × (1.5–)2–2.5(–3) μm, rounded at the apex and truncate at the base, pale brown, 1(–2)-septate, with a faintly darkened, unthickened hilum, about 0.5 μm 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 254.65 = 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 μm 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 μm wide, unbranched or branched at acute angles, with 1–3 additional septa, cells often inflated and flask-shaped, pale-brown, up to 60 μm long. **Conidiogenous cells** terminal, occasionally intercalary, variable in length, up to 10 μm long, pale brown, cylindrical to doliform or flask-shaped, with hardly prominent denticles; scars flat, slightly pigmented, not thickened, about 0.5 μm diam. **Conidia** solitary, pale brown, smooth, thin-walled, ellipsoidal to ovoid, 0–1(–2)-septate, often constricted at the septa, (4–)6–7(–9) × 2–3 μm, with a round apex and truncate base; hilum prominent, slightly darkened, unthickened, about 0.5 μm 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.
**Veronaea japonica** Arzanlou, W. Gams & Crous, sp. nov. MycoBank MB504557. Figs 17B, 27.

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

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

*In vitro:* Submerged hyphae subhyaline, smooth, thin-walled, 1.5–3 μm 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 μm wide, up to 65 μm long, unbranched or occasionally branched.
Conidiogenous cells terminal, occasionally intercalary, variable in length, up to 15 μm long, pale brown, cylindrical to clavate, with hardly prominent denticles; scars flat, slightly pigmented, not thickened, about 0.5 μm diam.

Conidia solitary, pale brown, smooth, thin-walled, ellipsoidal to ovoid, (0–)1-septate, often constricted at the septum, (6–)7–8(–10) × 2–2.5(–4) μm, with a round apex and truncate base; hilum unthickened but slightly darkened, about 1 μm diam.

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


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” (Rhinocladiella) 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: Rhinocladiella obovoidea Matsush., Icones Microfung. Mats. lect.: 123. 1975.

In vitro: Submerged hyphae smooth, hyaline, thin-walled, 1–2 μm wide; aerial hyphae hyaline to subhyaline, smooth. Conidiophores arising vertically from creeping hyphae, ascending hyphae thick-walled and dark brown; conidiophores 10–35 μm 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 μm long, pale brown, elongating sympodially; with a short rachis giving rise to denticles, 1 μm 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) × (3–)4(–5) μm, smooth, thin-walled, with a more or less rounded apex, a truncate base and a slightly darkened, unthickened hilum, 1.5 μm 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 -ridum from Chloridium.

Genus ab allis generibus Ramichloridi similibus rachide recta longa, subhyalina, denticulis distintissimis distinguendum.

In vitro: Colonies moderately fast-growing, flat, with mainly submerged mycelium, and entire margin, later becoming powdery due to superficial mycelium, and entire margin, later becoming powdery due to sporulation, which occurs in concentric zones when incubated on the laboratory bench.

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 Gomphinaria Preuss is not yet sufficiently documented. Our examination of G. amoenae Preuss (B1) 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.


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

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, 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, ellipsoidal, 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 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.


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.


In vitro: Submerged hyphae hyaline, thin-walled, 1–2 μm wide. Conidiophores unbranched, flexuose, arising from creeping aerial
hyphae, pale brown, up to 250 μm tall, 3–3.5 μm wide, thick-walled, smooth, with up to 24 thin septa, delimiting 8–12 μm long cells. Conidiogenous cells integrated, elongating sympodially, cylindrical, 20–150 μm 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 μm long denticles. Conidia solitary, subhyaline, thin-walled, finely verrucose, with a wing-like gelatinous sheath, approx. 0.5 μm wide, ellipsoid to obovoid, (5–)6–7(–9) × 3–4 μm; hilum slightly prominent, unpigmented, approx. 0.5 μm diam.

Cultural characteristics: Colonies reaching 40 mm diam after 14 d at 24 °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.


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 Myrmecidium. 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 μm.

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 μm.

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


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 subcylindric 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 schizolytica.

**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 *Neovolutaria* 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 discernible. 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.


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

Hyphae 1.5–4 μm lateae, 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 alis. Conidia saepe obovoidae, 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, plurisepitate, occasionally slightly constricted at the septa. Conidiogenous cells integrated in creeping fertile threads, terminal or intercalary, 15–50 μm long, 2–5 μm wide, subcylindric 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 round 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 *Fromonœlia sp.*, on *Dichaea chrysanthi*, 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 sympodially proliferating conidiogenous cells, resulting in a conidium-bearing rachis. C–D. Rachis with crowded, blunt conidium-bearing denticles. E. Conidia. Scale bar = 10 μm.


Radulidium distinct and a new genus is introduced below to accommodate it.

**Type species:** Referring to the radula-like denticles on the rachis.


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


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

**In vitro:** Colonies fast-growing, velvetiy, 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* 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 aciccular, 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, more or less cylindrical, with a pigmented refractive hilum, about 1 μm diam.

**Cultural characteristics:** Colonies reaching 55 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. Samšiňáková, ex-type culture CBS 405.76; Opatovický pond, from *Lasioptera arundinis* (gall midge) mycangia on *Phragmites australis*, M. Skuhravá, CBS 101010.

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

**Basionym:** *Botrytis epichloës* Ellis & Dearn., Allionia 14: 38. 1968.

**Etymology:** Latin *epichloës* = the rose, referring to the red-brown 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, 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, verruculose, 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 *Epiclädö 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 Annulatascaceae**

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


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


**In vitro:** Colonies slow-growing, velvetiy, 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-
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 conidal frill, and variably septate conidia.
Rhodoveronaea varioseptata Arzanlou, W. Gams & Crous, sp. nov. MycoBank MB504570. Figs 10D, 34.

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


**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. Conidiogenæ 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 obvoid, 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, Effel, Berndorf, on Berberis vulgaris, 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 Fusidiadium 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 Veronaea simile sed conidiophoribus brevioribus (ad 60 μm longis) et rachide dense denticulatâ 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. **Conidiogenæ** cells terminally integrated on simple or branched conidiophores, polyblastic, smooth, thin-walled, pale brown; rachis common 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 subcyllindrical, (0–)1-septate, with a slightly darkened, thickened, hilum; conidial secession schizolytic.

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


**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 Wentiomyces 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 Wentiomyces 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 Thysanorea, 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 obovodeum (Ur-Rahman et al. 1988). Data presented here reveal, however, that although morphologically similar, these species are phylogenetically separate, with P. obovodeum belonging to the Sordariales, where it clusters with sexual species of Carpophila 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 Mymercidium, Veronaeaopsis, 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 fucoginal 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 re-collected 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 Perticoniella 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


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 aapi, M. citri and M. thailandica, and also identified Mycosphaerella communis, M. lateralis and Passalora loranthis 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.

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-Willemsen 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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1 Accession numbers correspond to GenBank entries.
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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*. 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 hilum. 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).
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 recherché 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-sporo 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 26S 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 µl, which was composed of 1 × PCR Buffer (Applied Biosystems, Foster City, USA), variable MgCl2 concentrations, 60 µM dNTPs, 0.2 µM 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 DYEEnamic 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).

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 (×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) (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 = 881 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 number 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). The phylogenetic results obtained from the ITS and mtSSU data (data not shown for individual loci, variation within clades in Fig. 2). The phylogenetic results obtained from the ITS and mtSSU data (data not shown for individual loci, variation within clades in Fig. 2). The phylogenetic results obtained from the ITS and mtSSU data (data not shown for individual loci, variation within clades in Fig. 2).

**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.
Cercospora api Fren., Beitr. Mykol. 3: 91. 1863


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

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

Dissoconium communi similis, sed colonis in vitro tarde crescentibus (usque ad 10 mm diam post 30 dies ad 24 °C in agarato malato).

Etyymology. 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. 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.
Conidiophores arising orthotropically from vegetative hyphae, often reduced to conidiogenous cells and continuous with supporting hyphae, thin-walled, smooth, pale brown, unbranched, straight, (10–)19–25(–53) × (2.5–)3–5 µm.

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 forming in sympodial order in pairs on a conidiogenous cell; the primary conidium is 2-celled, while the secondary conidium is asceptate; primary conidia pale olivaceous-brown, thin-walled, smooth, ellipsoidal to obclavate, 1-septate, apex obtuse, base obconically-truncate, (11–)22–26(–35) × (3–)4–5 µm, hilum unthickened; about 1 µm diam. Secondary conidia 1-celled, pale olivaceous-brown, pyriform to turbinate, 4–5 × 3–4 µm, base truncate, flat, unthickened, about 0.5 µm diam. Both conidia slightly darkened and thickened.

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 µm.
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. Secondary conidia germinate from both ends and produce several anastomoses. 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


*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.


*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).

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

Anamorph. Ramichloridium-like.

Ascosporae rectae vel curvatae, fusdeo-ellipsoidae utrinque obtusae, ad septum medianum vix constrictae, (9–)10–11(–12) × 3–3.5(–4) µm.

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

In vivo: *Leaf spots* amphiogenous, irregular to subcircular, 1–7 mm diam, grey to pale brown on adaxial surface, grey on abaxial surface, with dark brown margins. *Ascomata* amphiogenous, intermingled among those of *M. musicola*, dark brown, subepidermal, becoming erumpent, globose, 70–90 µm diam; wall consisting of 2–3 layers of medium brown textura angularis. *Asci* apaparyphylate, fasciculate, bitunicate, subseptate, obvoid or broadly ellipsoid, straight to slightly curved, 8-spored, 28–35 × 7–9 µm. *Ascosporae* bi- to tri-seriate, overlapping, hyaline, non-guttulate, thin-walled, straight or 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 µm.

Towards both ends, but more prominently towards the lower end, (9–)10–11(–12) × 3–3.5(–4) µm; ascospores becoming distorted upon germination after 24 h on MEA, becoming constricted at the septum, 6–7 µm 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 µm 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 µm wide and up to 35 µm 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) × 2–2.5(–3) µm; hilum truncate, flat, broad, unthickened, slightly darkened, about 1 µm 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.

Fig. 6 *Mycosphaerella mozambica* (CBS 122464). a. Ascus with biseriate 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 µm.
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 Chaetothyiales. 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


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.


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


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, plurisepate, (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.](image_url)
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 × 2–6 µm; 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 agaro 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 µm 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 µm long, 2–2.5 µm 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. 8 Pseudocercospora assamensis (CBS 122467). — Scale bar = 10 µm.](image-url)
**Pseudocercospora longispora** Arzanlou & Crous, sp. nov. — MycoBank MB505976; Fig. 11, 12

*Pseudocercosporae musae similis, sed conidiis longioribus, 82–120 × 2.5–4 μm.*

*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 μm wide; aerial hyphae smooth, medium brown; hyphal cells become thick-walled, swollen, forming dark-brown monilioid, muriform cells, 5–17 × 7–12 μm. *Conidiophores* solitary, arising from superficial hyphae; conidiophores medium brown, smooth, unbranched or branched above, 0–2-septate, subcylindrical, straight, up to 30 μm long, 2–3 μm wide. *Conidiogenous cells* integrated, terminal, subcylindrical, tapering to truncate or (40–)78–95(–120) × 2–3 μm; 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 longispora* 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.

**Fig. 10** *Pseudocercospora indonesiana* (CBS 122473). — Scale bar = 10 μm.

**Fig. 11** *Pseudocercospora longispora* (CBS 122469). a–e. Conidia. — Scale bar = 10 μm.
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 × 2.5–4 µm; hila about 1 µm 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.


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 × 2–6 µm; 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 µm longa et 2–2.5 µm lata. Cellulae conidiogenae integratae, terminales, interdum intercalares, modice brunneae, verruculosa, subcylindraceae, apicem versus attenuatae, sympodialis, locis truncatis, subdenticulatis, 1–1.5 µm 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) × 1.5–3 µm, hilo inspissato obscuriore refringente, 1–1.5 µm 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 µm wide, with thin septa; aerial hyphae coarsely verrucose, olivaceous-brown to medium brown, rather

Fig. 12 Pseudocercospora longispora (CBS 122469). — Scale bar = 10 µm.

Fig. 13 Stenella musae (CBS 122477). a–d. Conidiophores with sympodially proliferating conidiogenous cells; e–f. conidia. — Scale bar = 10 µm.
thick-walled, 2–2.5 µm 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 µm long, 2–2.5 µm wide. **Conidiogenous cells** integrated, terminal, sometimes intercalary, unbranched, medium brown, finely verrucose, subcylindrical, tapering towards flat-tipped, subdenticate apical loci, 1–1.5 µm 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 subtruncatet base, straight to curved, 0–7-septate, (7–)27–40(–70) × 1.5–3 µm; hilum thickened, darkened, refractive, 1–1.5 µm diam.

**Cultural characteristics** — Colonies on MEA reaching 30 mm diam after 30 d at 24 °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 °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 × 1.5–3 µm) than ‘*Cercospora non-virulentum*’ (55–200 × 2.6–3.2 µm).

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

*Stenella 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 *Stenella 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-

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**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 verruculose, 0–2-septate, occasionally continuous with supporting hyphae, subcylindrical, straight to geniculate-sinuous, unbranched, (18–)30–36 µm wide, with thin septa. Conidiophores arising from superficial hyphae, pale brown, rather thick-walled, finely verruculose, 0–2-septate, occasionally continuous with supporting hyphae, subcylindrical, straight to geniculate-sinuous, 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 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 µm wide; 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 margins; surface olive-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.

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 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. 17

Stenellae musae similis, sed conidios longioribus, 51–83 × 2–2.5 µm. A Stenella musicola conidiophoris 1–4-septatis et conidios 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 olive-grey-brown, 2–3 µm wide, with thin septa; aerial hyphae coarsely verruculose, olive-grey-brown, rather thick-walled, 2–2.5 µm wide, with thin septa. Conidiophores arising from superficial hyphae, pale brown, thin-walled, finely verruculose, 1–4-septate, occasionally reduced to conidiogenous cells, subcylindrical, straight to geniculate-sinuous, unbranched, 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 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 × 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.

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

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**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.
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 multitudes 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), β-tubulin and elongation factor 1α 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* (Pančič) Purkyne (Karađzić 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, 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 Hedgcoc (1929) believed that the red band fungus was conspecific with *L. acicola*, although Dearness referred to it as *Cryptosporium acicola* Thüm., and Hedgcoc used the name *Septoria acicola* (Thüm.) Sacc. Sydow & Petrak (1942) later recognised that *A. marginatum* represented a nomen consufus and referred to the fungus as *L. acicola*. Independently, Hulbary (1941) described the red band fungus occurring on *P. 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 “septospore”), 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* (= *D. pini* var. *pini*) and *D. septosporum* var. *lineare* (= *D. pini* var. *lineare*), proposed by Thy & Shaw (1964), are respectively the varieties with short (15.4–28 × 2.6–4 μm) and long (23–42 × 1.8–2.9 μm) conidia. *Dothistroma septosporum* var. *keniense* (= *D. pini* var. *keniense*), proposed by Ivory (1967), accommodates collections of the fungus with conidia of intermediate (15–47.5 × 1.5–3.5 μm) 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.
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| CMW 14905 | CBS 116483 | U.S.A. | McBain, Riverside Township, Massaukee County, Michigan | var. pini | P. nigra | G. Adams | – | 2001
| CMW 14820 | ATCC MYA-609 | U.S.A. | Central, Minnesota | var. pini | P. nigra | – | 1970
| CMW 14821 | ATCC MYA-606 | U.S.A. | Lincoln, Nebraska | var. pini | P. nigra | – | 1964
| – | ILLS 27093 T | U.S.A. | DeKalb County, Illinois | var. pini | P. nigra var. austriaca | J.C. Carter | 1938
| Actinothyrium marginatum (T of D. septosporum var. lineare) | – | WSP 48361 | U.S.A. | Meadow Creek, Clearwater Ranger District, Idaho | var. lineare | P. ponderosa | F. Matzner | 1957
| Mycosphaerella dearnessii | CMW 9985 | CBS 871.95 | France | Le-Teich, Gironde prefecture (Aquitaine) | – | P. radiata | M. Morelet | 1995
| Dothistroma rhabdoclinis | CMW 12519 | CBS 102195 | Germany | Wolfenbüttel | – | Pseudotsuga menziesii | H. Butin | 1998

*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. 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 μL 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 β-tubulin gene were amplified using the primer pairs Bt2a/Bt2b and Bt1a/Bt1b (Glass & Donaldson 1995). The translation elongation factor (EF1-α) gene was amplified using the forward EF1-728F and reverse primer EF1-986R (Carbone & Kohn 1999).

PCR was performed in total volumes of 25 μL. The reaction mixtures consisted of ± 5 ng DNA template, 200 nM of the forward and reverse primers, 0.2 mM of each dNTP, 1U Taq DNA Polymerase with 10× buffer (Roche Molecular Biochemicals, Mannheim, Germany) and 1.5 mM MgCl₂. 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 Autosampler (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 β-tubulin gene regions and the Puccinia graminis sequence (GenBank X73529) for the EF1-α 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 combinatoriality 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 (β-tubulin 1), AY808205–AY808239 (β-tubulin 2) and AY808240–AY808274 (EF1-α). 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 Alul (Roche 10 U/μL) restriction enzyme in 20 μL reaction mixtures containing 2 μL 10x 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’TGCAGTTGTATCGGAACAAGCT3’ and EF1R- 5’AGCATGTTGTCGCCGTGAG3’, 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 (Actinomthyrium 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 β-tubulin 1 (5 bp differences), β-tubulin 2 (1 bp differences), and EF-1α 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.

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 β-tubulin-1 region contained 15 polymorphisms, the β-tubulin-2 showed 95 polymorphisms, and the EF-1α 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.

### Phylogeny of the red band needle blight fungi

**Fig. 3.** Phylogeny of the red band needle blight fungi based on the β-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.

**Fig. 4.** Phylogeny of the red band needle blight fungi based on the β-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.
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. 5. One of 12 most parsimonious trees inferred from the EF1-α 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.

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 Dothis- troma septosporum is retained, and Dothistroma pini is resurrected for isolates belonging to Lineage II.


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 sub-truncate 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 *Alu*I restriction site in *D. pini* isolates. Upon digestion of the PCR product, this yields distinctive fragments of 170 and 350 base pairs in length.


![Fig. 7](image1.png)  
**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. 8](image2.png)  
**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.


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


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 µm.


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 × 2–3.5 µm. *Conidigenous cells* integrated, hyaline, smooth, subcylindrical, tapering towards flattened apices, proliferating percurrently or rarely sympodially near the apex, 7–15 × 2–3 µm. *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) × 2(–2.5) µm (av. 28 × 2 µm) *in vivo*, (15–)25–30(–40) × 1.5–2(–2.5) µm (av. 28 × 2 µm) *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 *Alu*I restriction site, and thus, upon exposure of the PCR product to *Alu*I, 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.


**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 *Alu*I 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 *Alu*I at base pair position 371, giving it an RFLP profile distinguishable from those of the red band fungi (Fig. 12).

**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 Thyrv & 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. septosporum. The separation of M. pini from needles of Pinus contorta Douglas ex Loudon from British Columbia, Canada (Funk & Parker 1966), and has been linked taxonomically to 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 Zygophiala (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 Zygophiala 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 Zygophiala, 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 ascomata. These data question the value of ascomatal 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, Zygophiala 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 Zygophiala 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, Gloodes 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 and Mueller (1975), only a single anamorph species, phylogeny and phenotype. Describe species of flyspeck fungi based on DNA study. The aim of the present study was to identify and orchards in 10 eastern states were used for taxonomic collected during a survey in 2005 covering 30 apple morphology on the host and cultural growth character-istics. Although the morphology of diverse ellipsoidal to ovate (rarely obclavate), constricted at medianly or unevenly 1-septate (rarely multiseptate), produced in pairs, have a slightly granular surface, are and somewhat refractive conidial scars. Conidia are verruculose, ovate to ampulliform to elongated finely verruculose terminal cell and at its apex, two 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 l-septate (rarely multiseptate), ellipsoidal to ovate (rarely obclavate), constricted at sepa, with prominently thickened, darkened, refractive scars. Although the morphology of diverse Zygophiala 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 Zygophiala 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 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 Prepmun Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California). Primer pairs used for amplification and sequencing of the ITS region were ITS1/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 MgCl2, 5% DMSO, 1× Sigma buffer, 200 μM dNTPs, 0.5 μM 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 denatur-ation 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, 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.
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. 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 Zygophiala (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 Zygophiala tardicrescens. The second well supported clade (89% bootstrap support) contained isolates obtained from Wisconsin, Ohio, Michigan, Virginia and Missouri and is described as Zygophiala wisconsinensis. Isolates from the last clade (100% bootstrap support and sister of Z. wisconsinensis) were obtained from a

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.

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

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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*, *Rhinocladiella atrovirens* and *Zygosporium pumilum*).
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 of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (Fig. 2).

Maximum 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) μm diam and with a density of 2.4/mm². Sclerotium-like bodies of *Zygophiala cryptosinensis* were also round but slightly smaller, 230(155–364) μm diam, and more densely arranged, averaging of 3.6 sclerotium-like bodies/mm². *Zygophiala wisconsinensis* sclerotium-like bodies were ovoid, larger, 380(300–450) × 500(425–600) μm and were more sparsely arranged with a density of 0.8/mm². Sclerotium-like bodies of *Zygophiala tardicrescens* were similar to *S. pomi*, 260(250–270) μm diam and were arranged at a density of 2.8/mm².

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


**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 μm diam, 30–50 μm high, with irregular margins; upper layer consisting of interwoven mycelium, forming 2–4 layers of thick-walled, brown, pseudoparenchymatal cells, 4–8 μm 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 μm wide. *Asci* bitunicate, 8-spored, ovoid to subglobose or ellipsoid to clavate, apical chamber present but inconspicuous at maturity, 20–45 × 8–16 μm; 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) × (3–)3.5–4(–5) μm. 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 μm wide, not distorting, germinating from both ends, with 2–3 germ tubes; cultures are homothallic.

*Conidiophores* arising from superficial hyphae, 2–3 μm 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 × 7–8 μm (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, 6–7 × 6–7 μm, that gives rise to two (rarely three) medium brown, finely verruculose, doliiform to ellipsoid or subcylindrical, polyblastic conidiogenous cells, 8–12 × 6–7 μm; scars prominent, apical, darkened, thickened, somewhat refractive, with 1(–2) per conidiogenous cell, 2 μm wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, transversely 1(–7)-septate, prominently constricted at septa, (20–)22–25(–30) × 5–7(–8) μm if 1-septate but up to 110 μm long if 7-septate; apex subobtuse, base subtruncate, with a darkened, thickened hilum, 2 μm 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 CUAI = CBS 118957, GenBank: AY598895.
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 16–24 × 3–5 μm and conidia 15–18 × 3–5 μm. 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.

MycoBank MB501243.

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

*Zygophialae jamaicensi similis, sed conidiis latioribus, (12–) 14–18(–20) × (4–)5–6(–8) μm, distinguenda.

*Conidiophores* arising from superficial hyphae, 1.5–3 μm wide, erect, scattered, 3-septate, subcylindrical, irregularly flexuous, consisting of a hyaline supporting cell that gives rise to a smooth, dark brown stipe, 17–22 × 4–5 μm (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, 3–4 × 4–5 μm, that gives rise to two medium brown, finely verruculose, doliform to elongated subcylindrical, polyblastic conidiogenous

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cells with 1–10 loci, 6–15 × 5–6 μm; scars prominent, apical and lateral, darkened, thickened, somewhat refractive, 1–2 μm wide. Conidia solitary, fusiform to obclavate, hyaline, smooth and thick-walled, transversely (0–)1(–2)-septate; aseptate, 6–7(–9) × 5–6(–7) μm, 1-septate, (12–)14–18(–20) × (4–)5–6(–8) μm, 2-septate, 19–24(–30) × 5–6(–7) μm, prominently constricted at septa; apex subobtuse, base subtruncate, with a darkened, thickened hilum, 1–2 μm wide.

**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.

**Zygophialae jamaicensi** similis, sed colonis lentius crescentibus et conidiis 20 μm vel magis longis, 6 μm vel magis latis distinguenda.

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

**Zygophialae jamaicensi** similis, sed coloniis celerius
crescentibus et conidiis 20 μm vel magis longis, 6 μm vel magis latis distinguenda.

Conidiophores arising from superficial hyphae, 2–3 μm 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 × 4–7 μm (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, 3–4 × 4–5 μm, that gives rise to two medium brown, finely verruculose, doliform to ellipsoidal, polyblastic conidiogenous cells, 7–11 × 5–6 μm, with 1–2 prominent scars, apical and lateral, darkened, thickened, somewhat refractive, 2 μm wide. Conidia solitary, fusiform to obclavate, hyaline, smooth and thick-walled, granular, aseptate, 6–8 × 6–8 μm, or transversely 1-septate (rarely median), (13–)15–18(–23) × (6–)7–8 μm, prominently constricted at the septum; apex obtuse, base subtruncate, with a darkened, thickened hilum, 2–3 μm 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 × 4–5 μm (Martyn 1945). Ellis (1971) reported conidia to be 13–20 × 5–6 μm, while Williamson and Sutton (2000) cited them as 13–20 × 4–6 μm, whereas the present study found conidia of *S. pomi* to be 1(–7)-septate, prominently constricted at septa, (20–)22–25(–30) × 5–7(–8) μm if 1-septate, but up to 110 μm 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 hamathecium 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 Dothideales (von Arx and Müller 1975). The Dictio-

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<th>Key to Species of <em>Zygophiala</em></th>
<th>Description</th>
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| 1. Conidia (0–1) to multisep
tate on OA | 2           |
| 2. One-septate conidia (20–)22–25(–30) × 5–7(–8) μm | *Schizothyrium pomi* | 1 |
| 3. One-septate conidia shorter than 20 μm, and narrower than 6 μm; conidia 15–18 × 4–5 μm | *Zygophiala jamaicensis* |
| 4. Colonies fast-growing, reaching 13.5–22.5 mm diam on MEA after 2 wk at 25 C. | *Zygophiala wisconsinensis* |
| 5. Colonies slow-growing, reaching 2.5–4.5 mm diam on MEA after 2 wk at 25 C. | *Zygophiala tardicrescens* |

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LITERATURE CITED


Durbín RD, Davis LH, Snyder WC, Baker KF. 1953. The


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

**Uwe BRAUN**¹, Pedro W. CROUS²*, Frank DUGAN³, J. Z. (Ewald) GROENEWALD² and G. Sybren DE HOOG²

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 kellerianum* (syn. *P. kellerianiana*, *Cladophialophora kellerianiana*) 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*.


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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 holomorph 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 anamorph is much greater than in the teleomorphs, e.g. in *Myco-*

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 kelleriana, and Matsushima (1975) treated it as C. porophorum. Braun & Feiler (1995) excluded P. kelleriana from Phaeoramularia, and assigned it to Cladophialophora, which contains morphologically similar human-pathogenic hyphomycetes. Later Braun (1998) placed it in Pseudoacladosporium, a genus introduced for anamorphs of Caproventuria. Ho et al. (1999) recognized C. malorum, C. porophorum and P. kelleriana 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 rRNA gene (large subunit) of the rRNA gene. The reaction mixture contained 5 µL of diluted sample, 1 x buffer, 8 mM MgCl$_2$, 500 µM of each of the dNTPs, 2.5 U (Bioline) Taq polymerase and 10 pmol of each primer and made up to a total volume of 200 µL. The cycling conditions were the same for this region, except for the MgCl$_2$ 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 5' 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 (Hepatomyccaceae, Amorphothecaceae, Mycosphaerellaceae, Pleosporaceae and Venturiaceae). The Herpotrichiellaceae formed a well-supported clade (100 % bootstrap support) comprising species of Cladorhialophora and Phialophora. The Amorphothecaceae clade was also well-supported with a bootstrap support value of 100 % and contained isolates of Amorphotheca resinae (anamorph Sorocybe resiniae) and ‘Cladosporium’ breviremosum. The Herpotrichiellaceae and Amorphothecaceae 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. vaginae AF222832 and P. bellynickii 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. Anungiotopsis 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, Botryosphaeraceae, 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, ‘Cladorhialophora 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. Anungiotopsis 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. Congenital genuses 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, Cladorhialophora 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.


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 μm wide; fertile hyphae with conidiophores (Fig. 3) sometimes darker, brown, to 7 μm 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) μm, 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 μm long, monomorphic, determinate or polytretic, sympodial, usually with 1–2 conspicuous loci, 0.5–1.5 μm wide, unthickened, with minute central pore, 0.5–1 μm 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 μm, conidia 0–2(3)-septate, very rarely to 6-septate, to 35 x 7 μm, 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 μm diam, unthickened, composed of a minute central pore, 0.3–0.5 μm wide, and a narrow darker margin or margin sometimes lacking.

Substrata and distribution: Generally saprobic, isolated from soil, grass litter (Bromus inermis, Hordeum sp., Triticum aestivum), stored grains, chef 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 (SCHINELLHARDT & HEALD 1936).


Alternaria malorum var. polymorpha Dugan, var. nov.

Figs. 3–12

Differs a var. malorum conidiis latioibus, ca 3.5–6 μm latis, atriobus, levis cruciitunicatis, interdum longitudinal septatis, raro alternarioidibus intermixtis.

Etymology: Referring to its variable conidial shape.


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 corona 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 corona 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.


Ascomata in Mycosphaerella sect. Tassiana (asci non numerosi, sacculati; ascoporeae obovatae, utrinque rotundatae). Differit 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 anamorphis Cladosporium herbarum.

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

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


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


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


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


**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, Gloor & 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 *Cladosporium* 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, Gloor & 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 Venturaceae**

Of particular interest in the *Herpotrichiellaceae* are those species pathogenic to humans, which are presently placed in *Cladophialophora* (Fig. 1). Human-pathogenic cladosporoid
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, Unterreiner 1997, Gerriets van den Ende & de Hoog 1999, Unterreiner & Naveau 1999, Unterreiner, Gerriets 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 (tDNA 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 longer. 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). Unterreiner & 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*). Unterreiner (1997) reduced the latter species to synonymy with *Capronia hanliniana* (anamorph *Cladophialophora brevicatecata*), assigned it to the *Venturaceae* 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 Unterreiner (1997). Braun (1998) recognised Unterreiner’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 *Polyscytum*, is difficult and partly vague, since morphology and conidio-genesis 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*. © DGfM 2003
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. *Sorocye 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 *Amorphotheca-caceae* 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, Cladophilalophora, Phaeoramularia* or *Pseucladosporium*, suggesting that the conidiogenesis was holoblastic. These treatments were undoubtedly influenced by the cladosporial habit of this fungus, e.g., pigmented, 0–2-septate conidia formed in long acroptel chains (Fig. 5). However, the conidiogenous cells possess minute, but conspicuous pore, 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 cercosporoid 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 clade. These molecular data further showed that *Passalora fulva* [= *Fulvia falva*, *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 cercosporoid 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* (Clements & 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, Unterreiner & 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 circum-
scription 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 telemorphs, cluster within the Cladosporium clade.

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Accepted: 10.3.2003
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**Tab. 1:** Isolates of *Cladosporium* and allied genera studied
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Tab. 1: Isolates of *Cladosporium* and allied genera studied (continued)

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<td>U43466</td>
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<td>AY251105</td>
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* ATCC: American Type Culture Collection, Virginia, U.S.A.;
* IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham lane, 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 for 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.

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b Ex-type isolates.
Species of *Mycosphaerella* and related anamorphs on *Eucalyptus* leaves from Thailand

R. Cheewangkoon¹, P.W. Crous², K.D. Hyde³, J.Z. Groenewald², C. To-anan¹

Key words

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 rDNA, ITS2) and the 28S rDNA (LSU) regions. To help distinguish species of Pseudocercospora, the dataset was expanded by generating partial sequences of the translation elongation factor 1-α and actin genes. By integrating the morphological and molecular datasets, five new taxa were distinguished, namely Mycosphaerella irregularis, *M. pseudomarksii*, *M. quasiparkii*, *Penidiella eucalypti* and *Pseudocercospora chiangmaiensis*, 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). *Eucalyptus* 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 heimi*, *M. konae* and *M. thailandica* (Crous et al. 2007b). Species of this pathogen complex reported from *Eucalyptus* in Asia include *Dissoconium acciculare*, *Kirramyces destructans*, *K. eucalypti*, *M. cristallina*, *M. eucalyptorum*, *M. gracilis*, *M. heimioides*, *M. marksii*, *M. obscuris*, *M. parkii*, *M. robusta*, *M. stramenticola*, *M. sumatrensis*, *M. verrucosiafricana*, *M. vietnamensis*, *M. yunanensis*, *Ps. deglupta*, *Ps. eucalyptorum*, *Ps. fatouae*, *Ps. paraguayensis*, *Ps. robusta*, *Septoria eucalyptorum*, *S. xenoparkei*, *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 rRNA 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
Table 1 Isolates of Mycosphaerella and related anamorphs used for DNA analysis and morphological studies.

<table>
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¹ 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 (QA; 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 LR5 (Vilgalys & Hester 1990) were used to amplify part of the 28S rRNA gene (LSU). 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 µL solution containing 10–20 ng of template DNA, 1 × PCR buffer, 2.5 mM MgCl2, 15 pmol for each primer, 60 µM 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-1α 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 × 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-1α 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 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 1000 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).
54 were variable and parsimony-uninformative and 622 were constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values. A single most parsimonious tree was obtained from the heuristic search and is shown in Fig. 3 (TL = 876, CI = 0.822, RI = 0.852, RC = 0.700). The two trees differed only in their placement of *Ps. basiramifera*; in the one tree it is the closest sister of *Ps. chiangmaiensis* and in the other it is the sister of *Ps. flavomarginata*.

The manually adjusted combined (ITS, EF-1α and ACT) alignment contained 16 taxa (including the outgroup sequence) and, of the 1 012 characters used in the phylogenetic analysis, 339 were parsimony-informative, 139 were variable and parsimony-uninformative and 534 were constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values. A single most parsimonious tree was obtained from the heuristic search and is shown in Fig. 3 (TL = 876, CI = 0.822, RI = 0.852, RC = 0.700). The two trees differed only in their placement of *Ps. basiramifera*; in the one tree it is the closest sister of *Ps. chiangmaiensis* and in the other it is the sister of *Ps. flavomarginata*.

**Fig. 2** One of two 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 *Cladosporium bruheii* (GenBank accession EU019261).
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 germinalibus irregularist 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 sub-globose, 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 slightly curved; *ascospores* numerous, subglobose to ellipsoidal, smooth, brown, 15–20 × 15–20 µm, without germ tubes.

**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.*

**Fig. 4** *Mycosphaerella irregulari.* a. Asci; b. germinating ascospores; c. ascospores. — Scale bar = 10 µm.
slightly curved, 8-spored, (25–)35–40(−45) × (6–)7–8(−10) μm. 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) × 2.5–3(–3.5) μm.

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) × 4–5(−5.5) μm, slightly distorting (Type I; Crous 1998).

Cultural characteristics — Colonies reach 15 mm diam on MEA after 15 d at 25 °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) × (2.5–)3(−3.5) μm.

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 × 45–80 μm; apical ostiole 20–35 μm wide; wall consisting of 2–3 layers of medium brown textura angularis. Asci apophysate, fasciculate, bitunicate, subsessile, subcylindrical to narrowly ovoid, slightly curved, 8-spored, (40–)42–45(−48) × (6.5–)7–8(−8.5) μm. 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) × (2.5–)3(−3.5) μm.

Spermatogonia well developed, amphigenous, dark brown, subepidermal to erumpent, globose to subglobose, up to 90 μm diam. Spermatia hyaline, smooth, rod-shaped, with obtuse ends, (3.5–)4–5(−5.5) × 1.5–2 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 μm; d–f = 10 μm.
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).
**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* aparaphysate, fasciculate, bitunicate, subsessile, 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, ellipsoid to ovoid with obtuse ends, widest in the middle of the apical cell, medially 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**, Buriram, 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).

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**Fig. 8** *Mycosphaerella quasiparkii*. a. Leaf spot; b. section through an ascoma; c, d. ascii; e. ascospores; f, g. germinating ascospores; h. colony on MEA. — Scale bars: a = 1 mm; b = 20 µm; c–g = 10 µm.

**Fig. 9** *Mycosphaerella quasiparkii*. a. Asci; b. germinating ascospores; c. ascospores. — Scale bar = 10 µm.
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, septeate, smooth to slightly verruculose, pale to medium brown, (2.5–)3–4(–5) μm 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 ≤ 1 μm wide, smooth to finely verruculose, (30–)150–

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 μm; c, d = 80 μm; e = 30 μm; f, g = 20 μm; h, i = 15 μm; j, k = 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(–6) 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, Payakopoepisai, 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 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.

Pseudocercospora basiramiferae similis, sed cellulis conidiogenis terminalibus et intercalaribus 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. Stroma immersed, becoming erumpent, medium brown, 25–70 × 30–70 µm wide. Conidiophores reduced to conidiogenous
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) × 3–4(–4.5) µm, 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) × (2–)3(--5) µm, 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-subtruncatate, (2–)3–5(--10)-septate, straight to slightly curved, pale to medium brown, smooth, thin-walled, guttulate, (40–)50–60(--100) × (2–)2.5–3(--3.5) µm (up to 140 µm 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 °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 stromata. Phylogenetically it is closely related to Ps. basiramifera (Fig. 3) but differs from it with 5 nucleotide positions on ITS, 34 on EF-1α 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. marksi, 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. ellipsoides, 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, distinguisht 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. assimensis, 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 conidigenous 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.
Acknowledgements Dr G. Hunter is thanked for providing assistance with various Pseudocercospora spp. examined during this study. We thank Misses Marjan Vermaas for help in preparing the photographic plates and Arjen van Iperen for preparing all the fungal cultures for examination.

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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 (BLSD), 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 (Marín 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 BLSD 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
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 HGM 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Based on</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIKRP-F</td>
<td>5′-AAGATCAAGCGYCCAAG-3′</td>
<td>mat1-2 from M. graminicola and S. passerinii</td>
</tr>
<tr>
<td>SEKKR-R</td>
<td>5′-ATGGCRGTTTCTCTCGG-3′</td>
<td>mat1-2 from M. graminicola and S. passerinii</td>
</tr>
<tr>
<td>DNAlyde3F</td>
<td>5′-CGGCCGCTACAGCGNAGARGG-3′</td>
<td>DNA lyase from M. graminicola, N. crassa, F. graminearum, M. grisea and A. nidulans</td>
</tr>
<tr>
<td>DNAlyde3R</td>
<td>5′-CATCTCTTCGTTGCCCARYRNGTTR-3′</td>
<td>DNA lyase from M. graminicola, N. crassa, F. graminearum, M. grisea and A. nidulans</td>
</tr>
<tr>
<td>DNAlyaseMMF</td>
<td>5′-TCTCATGTCGTCGCCAGATG-3′</td>
<td>DNA lyase from M. fijiensis</td>
</tr>
<tr>
<td>DNAlyaseMRF</td>
<td>5′-TGAGTTTGGCATCCGATGG-3′</td>
<td>DNA lyase from M. fijiensis</td>
</tr>
<tr>
<td>SLA2degF1</td>
<td>5′-CATCAAGCGCCCAAYGARGGNTAYGA-3′</td>
<td>sla2 from A. nidulans, N. crassa and M. grisea</td>
</tr>
<tr>
<td>SLA2degR1</td>
<td>5′-CCCTCTGGACGAGCATTTC-3′</td>
<td>sla2 from A. nidulans, N. crassa and M. grisea</td>
</tr>
<tr>
<td>ES6</td>
<td>5′-CATCAGAAATGGATTGGAACG-3′</td>
<td>Flanking region upstream of M. fijiensis idiomorphs</td>
</tr>
<tr>
<td>ES6</td>
<td>5′-CCCTCTGGACGAGCATTTC-3′</td>
<td>Flanking region downstream of M. fijiensis idiomorphs</td>
</tr>
<tr>
<td>fla4739-F</td>
<td>5′-GCGTTTGGAGCCGCTAGG-3′</td>
<td>Flanking regions upstream of M. fijiensis idiomorphs</td>
</tr>
<tr>
<td>inver5656-R</td>
<td>5′-GAATCTCGGATTACTGACGAGG-3′</td>
<td>Inverted sequence within idiomorphs (upstream mat1-2)</td>
</tr>
<tr>
<td>inver8486-F</td>
<td>5′-GACCTCAGGGGAGCATTGG-3′</td>
<td>Inverted sequence within idiomorphs (downstream mat1-2)</td>
</tr>
<tr>
<td>fla9352-R</td>
<td>5′-TGATGCATCCTGCACAGACC-3′</td>
<td>Flanking regions downstream of M. fijiensis idiomorphs</td>
</tr>
</tbody>
</table>
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.
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 idiomorphs (Fig. 3). In *M. fijiensis* a portion of 1127 bp in the 5′ end of the *mat1-1* idiomorph shows 77% identity (in nucleotide sequence) to a portion of the 3′ end of the *mat1-2* idiomorph, 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 idiomorphs 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 idiomorph and the minus (−) strand of the other.

**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...
Isolation of Mycosphaerella fijiensis idiomorphs

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 *M. 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 (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).
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).
Isolation of Mycosphaerella fijiensis idiomorphs

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 (AJ884598) and mat1-2 is 3150 bp (AJ884599). 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 (AABX0100036) and

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).
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 identification 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>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Host</th>
<th>Mating type</th>
<th>Geographical origin</th>
<th>Reference</th>
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<td>IPO942E9</td>
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<td>86</td>
<td><em>M. fijiensis</em></td>
<td>Grand Naine</td>
<td>A*</td>
<td>Cameroon</td>
<td>Mourichon and Zapater (1990)</td>
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<td>89</td>
<td><em>M. fijiensis</em></td>
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<td>a*</td>
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<td>This work</td>
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<td>This work</td>
</tr>
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</table>

*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 mat-1-2 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 mat-1-1 idiomorph was obtained by long-range PCR with primers E56 and E66 (Table 1), which were designed based on the mat-1-2 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 SmaI (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/b2seq/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.html) 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. passerini. Conserved intron boundaries (GT/AG) and branch- ing signals (RCURAY) most commonly found in other fungi (Kupfer et al., 2004) were also identified in the predicted genes.

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REFERENCES


**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 Mycosphaerellaceae complex.


**Key words:** Ascomycetes, Batcheloromyces, Colletogloeopsis, Readeriella, Teratosphaeria, Tritomatosoma, DNA sequence comparisons, systematics.

**INTRODUCTION**

The genus *Mycosphaerella* Johansen 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 anamorphic 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 assexual 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 detected 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 comparisons, systematics.
<table>
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<th>Teleomorph</th>
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<th>Country</th>
<th>Collector</th>
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<td>CPC 11149; CPC 23</td>
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<td>Triticum aestivum</td>
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<td>Betula verrucosa</td>
<td>Netherlands</td>
<td>W.M. Loerakker</td>
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<td>Salix alba</td>
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<td>U. Braun</td>
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<td>J.E. Taylor</td>
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<td>A. Carnegie</td>
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Table 1. (continued).

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**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 FABI, Pretoria, South Africa; **IAM:** Institute of Applied Microbiology, University of Tokyo, Institute of Molecular and Cellular Biosciences, Tokyo, Japan; **IMI:** International Mycological Institute, CABI-Bioscience, Egham, Buckinghamshire, U.K.; **INIFAT:** Alexander Humboldt Institute for Basic Research in Tropical Agriculture, Ciudad de la Habana, Cuba; **ICM:** Japan Collection of Microorganisms, RIKEN BioResource Center, Japan; **VRM:** All-Russian Collection of Microorganisms, Pushchino, Russia.

*Ex-type cultures.

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 *Coccodinium bartschii* A. Massal., fresh material had to be collected from South Africa and Canada, respectively. Excised tissue pieces bearing ascomata 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).

#### 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 LR6 (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) 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* Mura (Crous et al. 2003), *Batcheloromyces* Marasas, P.S. van Wyk & Knox-Dav. (Taylor et al. 2003), *Phaeophilospora* 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 RNA (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.
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 *Capnodiaceae* (100 % bootstrap support). These are discussed in detail in the Taxonomy and Discussion sections. A new collection of *Coccodinium bartshii* 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 *Dermataceae* (*Helotiales*). The *Capnodiaceae* encompasses members of the *Capnodiales*, *Trichosphaeriaceae*, *Davidiellaceae*, *Schizothyriaceae* and taxa traditionally placed in the *Mycosphaerellaceae*, which is divided here into the *Tratosphaeriaceae* (65 % bootstrap support), and the *Mycosphaerellaceae* (76 % bootstrap support), which contains several subclades. Also included in the *Capnodiaceae* 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 *Capnodiales* and the *Tichosphaeriaceae* / *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 *Capnodiaceae*, 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 238,850 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. Ascomata thyrothecial; anamorph *Zygosphorium* .......................................................................................................................... Schizothyrium 2

2. Ascomata pseudothecial ................................................................................................................................................................. 2

3. Ascospores with irregular, angular lumens typical of *Davidiella*; anamorph *Cladosporium s. str.* .................................................. Davidiella

4. Ascospores guttulate or not, lacking angular lumens; anamorph other than *Cladosporium* ................................................................................................. 3

5. Ascomata frequently linked by superficial stroma; hamathecial tissue, ascospore sheath, multi-layered endotunica, prominent periphysoids, and ascospores turning brown in asci frequently observed ............................................................ Teratosphaeria

6. Ascomata not linked by superficial stroma; hamathecial tissue, ascospore sheath, multi-layered endotunica, prominent periphysoids, ascospores turning brown in asci not observed ............................................................ 4

7. Conidiophores solitary, pale brown, giving rise to primary and secondary, actively discharged conidia; anamorph *Dissoconium* ................. ................................................................. teleomorph *Mycosphaerella*-like

8. Conidiomata variable from solitary conidiophores to sporodochia, fascicles to pycnidia, but conidia not actively discharged ................................................................. *Mycosphaerella* *s. str.*
Fig. 1. One of 1135 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 1000 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 Paullicorticium 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 Paullicorticium ansatum AY586693).
Treatment of phylogenetic clades

Davidiellaceae clade


Description: Schubert et al. (2007 – this volume).


Notes:

Teratosphaeriaceae clade


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. Ascosporas of Teratosphaeria become brown and verrucose 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. Ascosporas 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. pseucdrypctica 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 ascosporas of M. cryptica (Cooke) Hansf. and M. rubiosa (Cooke) Hansf. to have a weakly definable sheath. Germinating ascosporas of species in this clade all exhibit a prominent mucoid sheath.

5. Ascii 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. alistairi, M. pseudosuberosa, M. fimbriata (Crous et al. 2006b, 2007b, Crous & Groenewald 2006a, b), and also M. rubiosa.

6. Finally, ascomata of T. fibrillosa and T. proteae-arboresae 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 hereafter proposed to accommodate species of Teratosphaeria:

Teratosphaeriaceae Crous & U. Braun, fam. nov. MycoBank MB504464.

Ascomata pseudothecial, superficiales vel immersa, saepe in stromate ex cellulis brunneis pseudoparenchymatibus disposita, globulares, uniloculares, papillata, apice ostiolato, periphysata, saepe cum periphysoidibus: tunica multistratata, ex cellulis brunneis angularibus composita, strato int erno ex cellulis applanatis hyaliniis; saepe cum pseudoparaphysibus subcylindricis, ramosis, septatis, anastomosibus. Ascii fasciculati, octospori, bitunicati, saepe cum endotunica multistratata. Ascospora ellipsoide-fusiformes vel obovoideae, 1-septatae, hyalinae, deinde pallide brunneae et verruculose, saepe mucosae.

Ascomata pseudothecial, superficial or immersed, frequently situated in a stroma of brown pseudoparenchymatous cells, globose, unilocular, papillate, ostiolate, canal periphysate, with periphysoid frequently present; wall consisting of several layers of brown textura angularis; inner layer of flattened, hyaline cells. Pseudoparaphyses frequently present, subcylindrical, branched, septate, anastomosing. Ascii fasciculate, 8-spored, bitunicate, frequently with multi-layered endotunica. Ascospores ellipsoid-fusoid to obovoid, 1-septate, hyaline, but becoming pale brown and verrucose, frequently covered in mucoid sheath.


Teratosphaeria africana (Crous & M.J. Wingf.) Crous & U. Braun, comb. nov. MycoBank MB504466.

**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:** Batcheloromycetes sp.
**Teratosphaeria bellula** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504469.

**Teratosphaeria cryptica** (Cooke) Crous & U. Braun, **comb. nov.** MycoBank MB504470.
Basionym: Sphaerella cryptica Cooke, Grevillea 20: 5. 1891.
Anamorph: *Readeriella nubilosa* (Ganap. & Corbin) Crous & U. Braun, **comb. nov.** MycoBank MB504471.

**Teratosphaeria dendritica** (Crous & Summerell) Crous & U. Braun, **comb. nov.** MycoBank MB504472.

**Teratosphaeria excentrica** (Crous & Carnegie) Crous & U. Braun, **comb. nov.** MycoBank MB504473.
Anamorph: *Catenulostrum excentricum* (B. Sutton & Ganap.) Crous & U. Braun, **comb. nov.** MycoBank MB504475.

Specimens examined: **South Africa**, Western Cape Province, Bains Kloof near Wellington, on living leaves of Protea grandiflora, 26 Feb. 1911; E.M. Dodge; holotype PREM; Stellenbosch, Jonkershoek valley, 53°34'59" 44°7" E; 18°58'50.6" E, 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.

**Teratosphaeria flexuosa** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504477.

**Teratosphaeria gamsii** (Crous) Crous & U. Braun, **comb. nov.** MycoBank MB504478.

**Teratosphaeria jonkershoekensis** (P.S. van Wyk, Marasas & Knox-Dav.) Crous & U. Braun, **comb. nov.** MycoBank MB504479.

**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.

Anamorph: *Catenulostroma microsporum* (Joanne E. Taylor & Crous) Crous & M.J. Wingf., **comb. nov.** MycoBank MB504482.

**Teratosphaeria molleriana** (Thüm.) Crous & U. Braun, **comb. nov.** MycoBank MB504483.
≡ Mycosphaerella molleriana (Thüm) Lindau, Nat. Pfanzenfam.: 424. 1897.
Anamorph: *Readeriella molleriana* (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504484.

**Teratosphaeria nubilosa** (Cooke) Crous & U. Braun, **comb. nov.** MycoBank MB504485.

**Teratosphaeria ohnowa** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504486.

**Teratosphaeria parkiiaffinis** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504487.

**Teratosphaeria parva** (R.F. Park & Keane) Crous & U. Braun, **comb. nov.** MycoBank MB504488.

**Teratosphaeria perpendicularis** (Crous & M.J. Wingf.) Crous & U. Braun, **comb. nov.** MycoBank MB504489.

**Teratosphaeria pluritubularis** (Crous & Mansilla) Crous & U. Braun, **comb. nov.** MycoBank MB504490.


Key to treated anamorph genera of Teratosphaeria (Teratosphaeriaceae)

1. Hyphae submerged to superficial, disarticulating into arthroconidia .......................................................................................................................... 2
2. 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 (= Friedmanniomycetes)
3. Hyphae superficial, brown to green-brown, smooth, disarticulating to form pare brown, cylindrical, 0–3-septate conidia with subtruncate ends, frequently with a Readeriella synanamorph ……………………………………… Cibiessia
4. Conidiogenous cells integrated in hyphae; well-developed conidiomata or long, solitary, macronematous, terminally penicillate conidiophores absent 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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 ................................................................................................................. *Nothostrasseria*

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 ............................................................................................... *Statinwella*

10. Conidiophores usually solitary, rarely densely fasciculate to synnematous (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 ........................................ *Catenulostroma*

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 unilocular, 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:


*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.


*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 conidogenesis on hyphal tips of the aerial mycelium, forming elongate-globose to ellipsoidal, murriformly 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. salcis*, 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).


*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 µm.

Fig. 5. Trimmatostroma salicis. A. Sporodochia on twig. B–E. Chains of disarticulating conidia. Scale bars = 10 µm.

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, pseudoparenchymatous stromata. Setae and hyphopodia absent. Conidiophores arising from hyphae or stroma, solitary, fasciculate to sporodochial, in biotrophic, plant pathogenic species emerging through stroma, little differentiated, semimacronematous, branched or not, continuous to septate, brown, smooth to verruculose. Conidigenous cells integrated, terminal or conidiophores reduced to conidiogenous cells, holoblastic-holoblastic, meristematic, unilocular, delimitation of conidium by a single septum with retrogressive delimitation of next conidium giving an unconnected chain of conidia, brown, smooth to verruculose, conidigenous scars (conidigenous 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, Koge 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, Koge 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

<table>
<thead>
<tr>
<th>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</th>
<th><em>C. excentricum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Conidia variable in shape, but without two basal cells giving rise to two lateral arms</td>
<td>2</td>
</tr>
<tr>
<td>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</td>
<td>3</td>
</tr>
<tr>
<td>Conidia distinctly verruculose to verrucose; plant pathogenic, forming leaf spots</td>
<td>5</td>
</tr>
<tr>
<td>3. Conidia (8–)20–35×(–60) μm, 1–10-septate</td>
<td><em>C. chromoblastomycosum</em></td>
</tr>
<tr>
<td>Conidia much shorter, 8–20 μm long, 0–5-septate</td>
<td>4</td>
</tr>
<tr>
<td>4. Conidia 0–5 times transversely septate, mostly two-celled; usually foliicolous on conifer needles or saxicolous</td>
<td><em>C. abietis</em></td>
</tr>
<tr>
<td>Conidia 2–4 times transversely septate and often with 1–2 oblique septa; isolated from stone</td>
<td><em>C. germanicum</em></td>
</tr>
<tr>
<td>Conidia rather broad, usually wider than 10 μm</td>
<td>6</td>
</tr>
</tbody>
</table>
Phylogenetic lineages in the Capnodiales

5. Conidia narrower, width below 10 µm

6. Conidia distoseptate, rather long, (12–)25–35(–45) × (7–)10–15(–25) µm; conidiomata large, up to 250 µm diam, on Protea anceps
   ................................................................................................................................................................................. C. protearum
6. Conidia euseptate, shorter, (9–)6–20(–36) × (0–)4–8(–27) µm; sporodochia 90–100 × 40–80 µm; on Protea grandiceps
   ................................................................................................................................................................................. C. elginense

7. Conidia 1-to multiseptate, (10–)15–17(–23) × (5–)6.5–7(–9) µm; on various Proteaceae
   ................................................................................................................................................................................. C. macowanii
7. Conidia in vivo predominantly 1-septate, (8–)13–15(–21) × (3.5–)5.5–6(–8) µm; on Protea cynaroides
   ................................................................................................................................................................................. C. microsporum (Teratosphaeria microspora)

Catenulostroma abietis (Butin & Pehl) Crous & U. Braun, comb. nov. MycoBank MB504504.

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. abieti et C. germanico conidiis longioribus, (8–)20–35(–60) × 4–5(–7) µm, 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 µm wide hyphae. Conidiomata brown, superficial,

CrouS et al.

...sporodochial, up to 350 µm diam. Conidiophores reduced to inconspicuous conidiogenous loci on hyphae, 2–4 µm wide, neither darkened nor thickened or refractive. Conidia occurring in branched chains, that tend to remain attached to each other, subcylinrdrical with subtruncate ends, straight to slightly curved, (8–)20–35(–60) × 4–5(–7) µm, 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).


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.

Fig. 7. Catenulostroma germanicum (type material). A–D. Chains of disarticulating conidia in vitro. Scale bars = 10 µm.


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. abieti conidiis –2 oblique septatis.

Mycelium consisting of branched, septate, smooth, pale to medium brown, 2–4 µm wide hyphae, giving rise to conidial chains. Conidiophores integrated, subcylinrdical, branched or not, septate, little differentiated, micronematous, 3–5 µm wide, 3- to multisepatate, 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 Taeiniola 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. 

≡ Coniothecium punctiforme G. Winter, Hedwiga 24: 33. 1885, non C. punctiforme Corda, Icones Fungorum (Prague) 1: 2. 1837. 

Catenulostroma microsorum, see Teratosphaeria microsora. 

Catenulostroma protearum (Crous & M.E. Palm) Crous & U. Braun, comb. nov. MycoBank MB504509. 


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. 


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 (= Fuscladium Bonord.: Venturiaceae), though phylogenetically Devriesia is not allied to this family. 


Description: de Hoog et al. (2000, illust. p. 721). 

Notes: The genus forms brown, septate, thick-walled hyphae, with ellipsoidal, 0–1-septate (becoming munformly 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 linea 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 conidiophores apice penicillata ex cellulis conidiogenis et ramoconidiis compositis, cellulis conidiogenis saeppe 1–3(–4) locis conidiogenis, terminalibus vel subterminalibus, subdenticulatis, non vel subincrassatis, non vel leviter fusciis-refractivis, ramoconidiis praeconstitus, saepe numerosis, conidiis ramicatenatis. 

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, fuscate 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, terminal 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 ovoidaceous 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-prodicescent, 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 penicilli apices, e.g. *P. strumelloidea* (Milko & Dunaev) Crous & U. Braun, are comparable with species of the genus *Pleurothecopsis* 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

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<tr>
<td>1. Conidiophores in <em>vivo</em> in well-developed, dense fascicles and distinct synnemata arising from a basal stroma; on fallen leaves of <em>Ficus</em> sp., Cuba</td>
<td><em>P. cubensis</em></td>
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<td>2. Conidiophores solitary, at most loosely aggregated</td>
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<td>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) µm wide, and asetate, ramoconidia sometimes heterochromous; on living leaves of <em>Nectandra coriacea</em>, Cuba</td>
<td><em>P. nectandrae</em></td>
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<tr>
<td>4. Hyphae, conidiophores and conidia frequently distinctly constricted at the septa; penicilli apex of the conidiophores sparingly developed, branches more or less divergent; isolated from leaf litter of <em>Smilax</em> sp., Cuba</td>
<td><em>P. rigidophora</em></td>
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<tr>
<td>5. Conidiophores short, up to 120 × 3–4 µm, frequently with intercalary conidiogenous cell, swollen at the conidiogenous portion just below the upper septum which render the conidiophores subnodulose to distinctly nodulose, apex ± loosely penicillate; conidia (4–)5–7(–8) µm long; occasionally with micronematous conidiophores; isolated from man with <em>tinea nigra</em>, Venezuela</td>
<td><em>P. venezuelensis</em></td>
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<tr>
<td>6. Conidiophores much longer, up to 800 µm, 7–9 µm wide at the base, not distinctly nodulose, penicilli apex loose to often more compact, tight, metula-like; conidia longer, 7–25 × 2–5 µm; micronematous conidiophores lacking; isolated from dead leaf of <em>Paepalanthus columbianus</em>, Colombia</td>
<td><em>P. columbiana</em></td>
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**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 µm latis compositum. *Penidiella* ex hyphis superficiis obiunda, penicillata, erecta, brunnea, crassitunicata, minute verruculosa, ad 800 µm longa, ad basim 7–9 µm lata, ad apicem plurimorsa, ex ramibus diversibus et cellulis conidiogenis composita, ramibus primarios (−2) subcylindraceis, 1–7-septatis, 50–120 × 4–6 µm; ramibus secundariorum (−2) subcylindraceis, 1–5-septatis, 40–60 × 4–6 µm; ramibus tertiariorum et subsequenteribus 1–4-septatis, 10–30 × 3–5 µm. Cellulæ conidiogenæ terminales vel laterales, non ramosae, 15–55 × 3–5 µm; conidia subcylindraceae, minute verruculose, apicem versus attenuatae, truncatae vel rotundatae, polyblasticae, sympodialia, cicatrices conidiales incrasatae, sed leviter fuscatæ et non refractivæ. Ramoconidia 0–1-septata, modice brunnea, levia, ellipsoidea, obclavata vel obovoidæ, sum 1–3 hili terminalibus, 10–20 × 3–5 µm; conidia subcylindrica vel ellipsoidea, 0–1-septata, pallide brunnea, catenata (−10), hila truncata, non incrassata, vix vel leviter fuscatæ. *Penidiella* consists of branched, septate, smooth, pale brown, 2–3 µm wide hyphae. Conidiophores arising from superficial mycelium, terminally penicillate, erect, brown, wall up to 1 µm wide, almost smooth to finely verruculose, up to 800 µm tall, 7–9 µm 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 × 4–6 µm; secondary branches (−2), subcylindrical, 1–5-septate, 40–60 × 4–6 µm; tertiary and additional branches 1–4-septate, 10–30 × 3–5 µm. *Conidiophores* terminal, intercalary or lateral, unbranched, 5–15 × 3–5 µm, 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-septata.
medium brown, smooth, wall ≤ 1 µm wide, ellipsoidal to obclavate or obovoid, with 1–3 apical hila, 10–25 × 3–5 µm, ramoconidia with broadly truncate base, not or barely attenuated, up to 4 µm wide, or at least somewhat attenuated at the base, hila 1.5–3 µm wide. Conidia subcylindrical to ellipsoidal, 0(–1)-septate, pale brown, in chains of up to 10, 7–15 × 2–3 µm, hila truncate, unthickened, barely to somewhat darkened, 1–2 µm 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.

**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 µm wide. *Stromata* present, 40–80 µm diam, brown, immersed. *Conidiophores* densely fasciculate or in distinct synnemata, arising from stromata, erect, synnemata up to about 1000 µm long and (10–)20–40(–50) µm wide, individual threads filiform, pluriseptate throughout, brown, thin-walled (≤ 0.5 µm), smooth or almost so to distinctly verruculose, apically penicillate. *Conidiogenous cells* integrated, terminal and intercalary, 10–30 µm 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, –2 µm 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) × 2–3 µm, aseptate, pale olivaceous, olivaceous-brown to brown, thin-walled, smooth or almost so to
faintly verruculose, ramoconidia with broadly truncate base, barely
narrowed, or ramoconidia more or less attenuated at the base, hila
1–2 µm wide, unthickened or almost so, but often slightly refractive
or darkened-refractive. Conidia in long acropetal chains, narrowly
ellipsoid-ovoid, fusiform, 5–12(–15) × (1–)1.5–3 µm, aseptate, pale
olivaceous to brownish, thin-walled, smooth to faintly rough-walled,
ends attenuated, hila 1–1.5 µm wide, unthickened, not darkened,
at most somewhat refractive.


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 synnematous conidiomata, arising from stromata.

Penidiella nectandrae Crous, U. Braun & R.F. Castañeda, nom. nov. MycoBank MB504512. Fig. 11.


In vivo: Colonies amphigenous, brown. Mycelium internal and external, superficial, composed of sparingly branched hyphae, septate, 1–3 µm wide, pale olivaceous-brown or brown, thin-walled (≤ 0.5 µm), smooth or almost so to distinctly verruculose, fertile cells giving rise to conidiophores somewhat swollen at the branching point, up to 5 µm diam, and somewhat darker. Stromata lacking. Conidiophores erect, straight, filiform, up to 350 µm long, 2.5–4 µm 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 µm 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 × 2 µm, aseptate, at the apex with 2–3(–4) subdenticulate hila, subcylindrical,
very pale olivaceous, olivaceous-brown to brown, sometimes with different shades of brown (heterochromatous), thin-walled (≤ 0.5 µm), smooth to faintly verruculose. Conidia in long acropetal chains, narrowly ellipsoid-ovoid, fusiform to cylindrical, 5–16 × (1.5–)2(–2.5) µm, 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 1–1.5 µm 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 °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 CB6745, 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*.

≡ *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, 2-6 µm wide hyphae, swollen cells up to 8 µm wide, wall up to 1(–1.5) µm wide. Conidiophores dimorphic.

**Macronematous conidiophores** separate, erect, subcylindrical, predominantly straight to slightly curved, terminally loosely penicillate, up to 120 µm long, and 4–5 µm 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(–1.5) µm wide. **Micronematous conidiophores** erect, subcylindrical, up to 40 µm tall, 3–4 µm 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, 10–20 × 5–6 µm, loci (predominantly single in micronematous conidiophores, but up to 4 in macronematous conidiophores) flat-tipped, sub-denticulate or not, 1–1.5 µm 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(–1.5) µm wide, frequently constricted at septa, which turn dark with age; ramoconidia (10–)13–17(–25) × 3–4(–5) µm, 1(–3)-septate; secondary conidia (7–)8–10(–12) × 3–4(–5); hila unthickened to very slightly thickened and darkened, not refractive, (0.5–)1(–1.5) µm.

**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 °C in the dark; colonies fertile.
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. 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.](image-url)


Mycelium consisting of branched, septate, smooth, hyaline to pale olivaceous, 1–4 µm 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 × (2–)2.5–4 µm, 0–6-septate, medium brown, smooth, wall ≤ 0.75 µm, penicillate apex formed by a terminal conidiogenous cell giving rise to a single set of ramoconidia. Conidiogenous cells terminal, integrated, subcylindrical, straight, 8–12 × 1.5–2(–2.5) µm, 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, 0.5 µm 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) × 2(–3) µm, subhyaline to olivaceous-brown, smooth, thin-walled; hila unthickened or almost so to somewhat thickened and darkened, not refractive, 1 µm 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 °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.


Etymology: Named after the geographic location of its type strain, Venezuela.

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).


Etymology: Named after the geographic location of its type strain, Venezuela.

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 strumelloidea is the oldest name. Other genera such as Readeriella are 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.


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.


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, Sonderhennia H.J. Swart & J. Walker and Lecanosticta Syd. belong to Mycosphaerellaceae.
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 *Pliidiella* Petr. & Syd. (*Diaporthales*) (Van Niekerk 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
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 brunneotingens** Crous & Summerell, **sp. nov.** MycoBank MB504517. Fig. 19. *Etymology:* Named after the diffuse brown pigment visible in agar when cultivated on MEA.

*Readeriellae* gauchensi similis, sed coloniis viridi-atris et pigmento brunneo in agar diffundente distinguenda.

Leaf spots amphiogenous, 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, asetate, doliform to ampulliform, or reduced to inconspicuous loci on hyphae (*in vitro*), proliferating percurrent 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 truncate or subtruncate 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 abundant, becoming swollen, and up to 2-septate; in older cultures becoming swollen, and up to 2-septate, 15 µm long and 5 µm wide.

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, W.P. Crous, CBS-H 9838, holotype, culture ex-type CPC 13303 = CBS 120747.

Notes: Conidial dimensions of *R. brunneotingens* 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. brunneotingens* 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).


≡ *Cercospora eucalypti* Cooke & Massee, Grevillea 18: 7. 1899.

Notes: The epithet “eucalypti” is preoccupied by *Readeriella eucalypti* (Gonz. Frag.) Crous (Summerell et al., 2006), and thus the synonym “pulcerrima” becomes the next available name for this species.

**Readeriella readeriellophora**, see *Teratosphaeria readeriellophora*. Fig. 18.


**Description:** Sutton (1971; fig. 1).

Notes: The genus *Staninwardia* presently contains two species, namely *S. brevisculea* 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. 19. Readeriella brunneotingens (type material). A. Leaf spot. B. Colony on MEA. C–D. Conidia. Scale bar = 10 µm.
**Schizothyriaceae clade**


_Description:* Batzer et al. (2007; figs 3–7).

_Notes:* Species of *Schizothyrium* (Schizothyriaceae) have zygosiphial 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**


_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**


_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**


_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 relavance 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).


_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, semiglbose, collapsed when dry, brownish, uniculate, 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 m Bunform, 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*. 
Stigmidium Trevis., Consp. Verruc.: 17. 1860. (Fig. 21).

Type species: Stigmidium schaereri (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, bitunicate, (endotunica not giving a special reaction in Congo red or toluidine blue). Ascospores are fusoid-ellipsoidal, medianly - septate, guttulate, thin-walled, lacking a sheath. Presently no culture is available, and thus the placement of Stigmidium 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 stigmina-platani F.A. Wolf to have a Cercostigmina U. Braun and Xenostigmina Crous synanamorph, and recent collections also revealed the presence of a similar species that has typical “Stigmina” (distoseptate conidia) and Pseudocercospora (euseptate conidia) synanamorphs (Crous, unpubl. data), and Crous (1998) reported Readeriella epicoccoides (coelomycete) to
have a Cercostigmata (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 Paracerospora Deighton (scars minutely thickened along the rim), was shown to be synonymous with the older genus Pseudocercospora. Similarly, Crous et al. (2001) showed that Cercostigmata (rough, irregular percurrently 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. Mycocelosmosa Rangel and Phaeoramularia Munt.-Cvetk. were subsequently reduced to synonymy with the older name, Paracerospora. Due to the unavailability of cultures, no decision was made regarding Sterella (verrucose conidia and mycelium), Stigmina (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 Verrucispora 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 Stigmina (distoseptate conidia) were also synonyms of Pseudocercospora.

The present study shows that most anamorph genera are polyphyletic within Teratosphaeriaceae, 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 *Pseudocercosporella* 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 *Pseudocercosporella* Deighton.

The eyespot fungus was originally described as *Cercosporella herpotrichoides* Fron (Fron, 1912). Deighton (1973) established the new genus *Pseudocercosporella* for anamorphs of *Mycospheraella* Johanson that were *Cercosporella*-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, *Pseudocercosporella 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 *Pseudocercosporella* 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 eye-spot 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 GCT TAT TGA TAT GC3′) (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 MgCl₂, 500 µM of each of the dNTPs, 2.5 U (Bioline) Taq polymerase and 10 µl 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.
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*).
Tapesia (Mycosphaerellales), comprising species of *Mollisia*, *Tapesia* and *Pyrenopeziza* Fuckel of the *Dermateaceae* (Helotiiales). *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. cinerea* 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* serve as 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 congeneric *Ramulispora* sensu (Oudem.) Davis formed a subclade with 88% bootstrap support.

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 been linked to a *Cystodendron* or *Cadophora*-like anamorphs (Hütter, 1958). *T. fusca* (Pers.) Fockel, 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 *Mycosphaerellales*. 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.

*Oculimacula* sesilis, gregaria, 0.5–2.5 mm diam., circularis vel lobata, subiculo hypharum plus minusve brunearum persistentium insidentia, texto superficiali hypharum pallide brunneorum, angustarum substrato affixa. Discus levis, griseus, marginem versus pallide griseus, maturus emarginatus, applanatus ad convexus. Receptaculum pallide brunneum ad griseo-brunneum, crateriforme. Ascii 8-spori, unitunicati, clavati vel subcylindrici vel fusoidii, breviter stipitati, poro apicali iodi ope caeruleante. Ascosporae biseriatae ad multisieriatae, hyalinae, leves, unicellulares, fusoidae vel subcylindricae-fusidiae vel clavatae, utrinque rotundatae, plerumque rectae. Paraphyses filiformes, sursum obtusatae, ascis longitudinalis similis. Excipulum medullare ex hyphis multisieriatae, hyalinis compositis, excipulum ectale e cellulis tenuitunicatis, fusci, 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, gregarios, 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 integrae, 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*). 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-sinuous, 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


Oculimacula acuformis (Boerema, R. Pieters & Hamers) Crous & W. Gams, comb. nov. Figure 7


*Helgardia anguioides* (Nirenberg) Crous & W. Gams, comb. nov. Figure 8

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 ascal layer. (5) Ascospores of *O. yallundae*. (6) Conidia and conidiogenous cells of *H. herpotrichoides*. (7) Conidial hila and conidiogenous cell of *H. acuformis*. (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 μm in (a)–(e), and 1 μm in (f)–(h).
Helgardia aestiva (Nirenberg) Crous & W. Gams, comb. nov. Figure 9


Discussion

A recent reclassification of the eyespot pathogens in Ramulispora seemed to correct the inadequacy of their placement in Pseudocercosporella, 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. anguioides 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 Philalophora 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 ascomata 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 Pseudocercosporella to accommodate taxa with unthickened, not darkened or refractive conidial hila that were formerly placed in Cercosporella Sacc. He did not, however, consider the morphological similarity of Pseudocercosporella with Ramulispora, and hence placed C. herpotrichoides in Pseudocercosporella. 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 Pseudocercosporella. 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 Pseudocercosporella and Helgardia.

A further issue not addressed in the present paper concerns the distinction between and priority of the genera Pseudocercosporella (1973) and Ramulispora (1920). Although Deighton (1973) did not compare these two genera when he introduced Pseudocercosporella, 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 Pseudocercosporella actually would have to
be transferred to *Ramulispora*. To reach a final conclusion, however, more species of *Pseudocercosporella* 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 *Pseudocercosporella* would deserve conservation over the lesser-known *Ramulispora*. A further 13 species of *Ramulispora* are known (www.speciesfungorum.org), but without cultures and molecular analyses, their correct phylogenetic affinities remain unclear. *Pseudocercosporella* 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 *Pseudocercosporella* nor *Ramulispora* is available for the anamorphs, nor are *Tapesia* or *Mollisia* for the teleomorphs.

Acknowledgements

We are grateful to Dr. B. Robbertse, who provided the SEM photomicrographs used in this study.

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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-α, 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 conidiotheca, 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 Lattrell & 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-α, 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-α 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.

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1 CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.

*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. zeae-maydis, C. zeina sp. nov. and an undescribed Cercospora species, respectively:

- CzeaeHIST (5'-TCGACTCGTCTTTCATCTTG-3'), CzeinaHIST (5'-TCGAGTGCCCTACCGT-3') and CmaizeHIST (5'-TCGAGTCATTCGACTTCC-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 MgCl₂, 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.
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 × 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
isolates of the new species (C. zeina, formerly C. zeae-maydis Group II). The isolates of C. sorghi var. sorghi and C. canescens had ITS sequences similar to those of C. zeae-maydis Group II (≡ C. zeina), but there was no bootstrap support for this branch. The third clade (78 % bootstrap support) contained isolates of C. zeae-maydis (formerly C. zeae-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 parsimony-uninformative, and 1227 were constant.

Development of a species-specific diagnostic test

Easy and rapid identification of C. zeae-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. zeae-maydis, C. zeina and the Cercospora sp., respectively, and can be used for their identification and detection.

Taxonomy

Cercospora zeae-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 amphiogenous, mostly hypophyllous, punctiform to subeffuse, brown. Mycelium internal. Stromata lacking or small, with a few swollen substomal 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; conidigenous cells integrated, terminal, occasionally intercalary, 10–40 µm long, conidigenous 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).


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,
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 µm 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 µm high and wide. *Mycelium* internal, consisting of pale brown, septate, branched, smooth hyphae, 3–4 µm wide. *Stromata* lacking or small, a few swollen substomatal cells, brown, up to 30 µm 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 genculate–sinuous, unbranched or branched above, 40–100 × 5–7 µm, 1–5-septate, uniformly pale olivaceous to medium brown, thin-walled, smooth; conidiogenous cells integrated, terminal, 40–60 × 5–6 µm, with several conidiogenous loci that are conspicuously thickened, darkened and refractive, 2–3 µm wide. *Conidia* solitary, broadly fusiform, (40–)60–75(–100) × (6–)7–8(–9) µm, (1–)3–5(–10)-septate, hyaline, thin-walled, smooth, apex subobtuse, base subtruncate, hila somewhat thickened, darkened and refractive, 2–3 µm wide (based on type specimen).


*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*.


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 µm) and more broadly fusiform conidia, versus longer conidiophores (up to 180 µm) 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
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. zeae-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. zeae-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. zeae-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. zeae-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. sorghi var. sorghi than to that of the presumably American species C. zeae-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. zeae-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 api (C. api 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. zeae-maydis and C. zeina have the ability to form male 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 zeae-maydis, and our current mating studies with isolates of C. zeae-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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REFERENCES


CERCOSPORA SPP. ON ZEA MAYS


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-α and the β-tubulin gene regions were used to resolve species in the Mycosphaerella genus. A total of 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 madeirensis Crous & Denman sp. nov., M. toledana Crous & G. Bills sp. nov. (anamorph Phaeophysospora 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. ohnova Crous & M.J. Wingf. sp. nov., Passalora zambiae Crous & T. Coutinho sp. nov., Pseudocercospora pseudoeucalyptorum Crous sp. nov., Readeriella novaazelandiae Crous sp. nov.

Key words: Ascomycetes, Dissoconium, DNA sequence comparisons, Mycosphaerella, Passalora, Phaeophysospora, Pseudocercospora, Readeriella, systematics.

INTRODUCTION

Species of Eucalyptus L’Hér., primarily native to Australia, are widely planted as exotics in the tropics, Mediterranean region and Southern Hemisphere. These plantations 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, Phaeophysospora 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. 2004)
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 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-α and the β-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 inside 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 examined 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 (Bio-line, London, U.K.), 1× PCR buffer, 1.5 mM MgCl2, 0.2 µM 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 µL 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-1α) gene was amplified with primers EF1-728F and EF1-986R (Carbone & Kohn 1999) and part of the β-tubulin gene was amplified with primers T1 (O’Donnell & Cigelnik 1997) and Bt-2b (Glass & Donaldson 1995). PCR conditions for EF-1α and β-tubulin genes were the same as those for ITS, except for the MgCl2 concentration, which was increased to 2.0 mM for β-tubulin. PCR products were separated by electrophoresis at 80 V for 1 h in a 0.8 % (w/v) agarose gel in 0.5x 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.

<table>
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<th>Teleomorph</th>
<th>Anamorph</th>
<th>Accession no.</th>
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<th>Country</th>
<th>Collector</th>
<th>GenBank accession</th>
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1CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS. 2Ex-type cultures. *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 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 (×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. ellipsioidea 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 Spec. 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. irregularivamosa 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. heimitii 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).
0.1 substitutions per site
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 β-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 *Phaeophileospora* 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 β-tubulin gene and 300 bases of the EF-1α 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-1α and β-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, β-tubulin and EF-1α 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.

**Mycosphaerella nubilosa** similis, sed coloniis avellaneis distinguendus.

**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, subsessile, 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.

*Dissoconium dekkeri* simile, sed coloniis avellaneis distinguendus.

**Mycelium** internal and external, consisting of smooth, branched, septate, pale 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, Areeiro, 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*).


Etymology: Named after the location from which it was collected.

*Mycosphaerellae heimioide similis*, sed ascii spori germinantis 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 µm diam; apical ostiole 10–15 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* ap paraphysate, fasciculate, bitunicate, sessile, obvoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 30–50 × 8–12 µm. *Ascospores* 3- to multiseptate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid-ellipsoid with subobtuse apex, 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) × 2.5–3(–3.5) µm in vivo.

*Mycelium* internal and external, consisting of smooth, branched, septate, pale to medium brown, 3–6 µm wide hyphae; external mycelium extensive on abaxial leaf surface. *Conidiomata* fasciculate, hypophyllous, medium brown, up to 90 µm wide and 150 µm high. *Conidiophores* arising from superficial mycelium, or aggregated in loose fascicles arising from the upper cells of a brown stroma up to 80 µm wide and 90 µm high; conidiophores pale to medium brown, smooth, unbranched or branched, 1–5-septate, subcylindrical, straight to variously curved, 15–45 × 2.5–4 µm. *Conidiogenous cells* terminal or lateral, unbranched, subcylindrical, pale brown, smooth, proliferating sympodially, or 1–4 times percurrently near apex, 7–15 × 2.5–3 µm; conidial scars inconspicuous. *Conidia* solitary, pale brown, smooth, subcylindrical, but tapering from a subobtuse apex, 3–6- or multiseptate, 35–85 × 2.5–4 µm; 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 µm diam.


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 °C in the dark; teleomorph but no conidia formed in culture.
*Host:* *E. globulus.*

*Distribution:* Madeira.

*Notes:* *Mycosphaerella madeirensis* 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. madeirensis*, 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.


≡ *Sphaerella nubilosa* Cooke, Grevillea 19: 61. 1892.


*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 ellipsoidal, 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 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.

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. cypellocarpa*, *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).


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.

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 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown textura angularis. Ascii aparaphysate, fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoid, straight or slightly incurved, 8-spored, 40–60 × 8–11 µm. 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) × (3–)3–4 µm in vivo.


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.


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.


Anamorph: Readeriella readeriellophora Crous & J.P. Mansilla, sp. nov.

Etymology: Named after the anamorph genus Readeriella.

Mycosphaerellae nubilose similis, sed coloniis olivaceis distinguenda.
brown, aseptate, finely verruculose, (5–)6–7(–9) × (3–)4(–4.5) µm; inconspicuous marginal frill present.

**Holotypes**: Spain, Pontevedra, Lourizán, Areeiro, 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 *Uvebraunia* anamorph. Furthermore, ascospores of *M. readeriellophora* do not distort on MEA (type C), while those of *M. nubilosa* and *M. ohnowa* do (type F).

**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.

*Phaeophleospora toledana* Crous & G. Bills, sp. nov. MycoBank MB500057.

**Colletogloeopsis nubilosa** (Ganap. & Corbin) Crous & M.J. Wingf. similis sed pycnidii 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** amphotigenous, pyenidial, substomatal; wall consisting of 3–4 layers of textura angularis. **Conidiophores** reduced to conidiogenous cells.
Conidiogenous cells ampulliform to subcylindrical, pale brown, smooth to finely verruculose, proliferating 1–3 times percurrently near apex, 6–10 × 3–4 µm; occurring intermixed between hyaline, smooth, subcylindrical, aseptate paraphyses, 10–20 × 2–2.5 µm. Conidia fusoid with acutely rounded apices and truncate bases, medium brown, verruculose, aseptate, (8–)10–12(–14) × (2.5–)3–3.5(–4) µm; 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 11313.

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 5–6 µm 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.


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 conidinis minoribus, 0(–2)-septatis, anguste ellipsoideis, 10–20 × 2–3 µm, 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 µm wide hyphae; frequently with hyphal swellings that develop into thick-walled, dark brown chlamydospore-like structures, up to 15 µm diam. Conidiophores arising from the mycelium, medium brown, smooth, branched or unbranched, 0–2-septate, subcylindrical, straight to variously curved, 10–30 × 2–4 µm. Conidiogenous cells terminal and intercalary, subcylindrical, tapering to truncate apices, pale to medium brown, smooth, proliferating sympodially, 10–30 × 2–4 µm; 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, 10–20 × 2–3 µm in vitro.


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 µm diam. Lateral branches also commonly observed 24–48 h after germination.

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 pseudoeucalyptorum** Crous, *sp. nov.* MycoBank MB500059. Figs 34, 35.

*Etymology:* Morphologically similar to *P. eucalyptorum*.

*Pseudocercosporae eucalyptorum* similis, sed conidiomata 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 genulate-sinuous, 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.
**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 *vivo* 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, textura angularis. Conidia holoblastic, solitary, asperate, pale to medium brown, finely verrucose, base subtruncate, apex flattened with three lateral, obulate 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°text 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 *Kirriymyces* J. Walker, B. Sutton & Pascoe (Walker et al. 1992), and subsequently transferred to *Phaeotheospora* 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* (Cook) 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 nova zealandiniae* 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 (Dodige 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* fide 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...
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, Ponteareas, 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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REFERENCES


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 Southeast 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 1α 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. colombiensis*, 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 phyllodenous *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* Freesen. 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 phyllodenous species of *Acacia*, *A. mangium* and *A. crassicarpa*, 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-α, histone, actin and calmodulin gene regions.

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**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 subcultured 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 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. Sequences and unique character positions were calculated using the sequences of the two isolates (94% bootstrap support). The Mycosphaerella species (CPC 10520 and 10521) cluster with 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. konae.

RESULTS

DNA Phylogeny
For each of the five loci sequenced, approximately 500, 320, 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 outgroups) 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 api and C. beticola (63% bootstrap support) and four isolates of C. accaciae-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. konae.
Table 1. Isolates of *Mycosphaerella* spp. and their anamorphs included for sequence analysis.

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<th>Species</th>
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<th>Host</th>
<th>Country</th>
<th>Collector</th>
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<td>Tonga</td>
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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.

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.
Several well-supported clades are seen in the tree, one of which (100 % bootstrap support) contains sequences of *C. api* 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*.

**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-sinuosa. Cellulae conidiogenae integratae, terminales vel intercalares, ad 100 µm longae, sympodiales; cicatrices conidiales incrassatae, pluriseptatae, ad 30 µm latae. Conidiae aciculares, 50–350 × 3.5–5 µm, pluriseptatae, basi (in hilo) incassata, fuscata, refractiva. 

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. api*/*C. beticola* isolates (87 %) and *C. acaciae-mangii* (92 %). The *M. citri* clade (100 %) contains three isolates, two of which once more 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.

**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.
MYCOSPHAERELLA SPP. OCCURRING ON ACACIA

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 multiserial, overlapping, hyaline, non-guttulate, thin-walled, curved, fusoid-ellipsoïdal with obtuse ends, medianly 1-septate, widest at the median, unconstricted 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, subseissile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 25–40 × 8–11 µm. *Ascospores* tri- to multiserial, 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, \( (8–)9–10(–11) \times (2.5–)3 \, \mu m \) in vivo. Spermatogonia intermixed with and similar to the ascomata in general morphology. Spermatia rod-shaped, hyaline, \( 3–6 \times 1 \, \mu m \) in vivo.


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 bruneis, 2–2.5(–3) \, \mu m latis.

Conidiomata amphigenous, pale brown, up to 80 \, \mu m diam; stromata well developed, brown, up to 60 \, \mu m wide and 30 \, \mu m high. Mycelium predominantly internal, consisting of smooth, branched, septate, pale brown, 3–4 \, \mu m 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-sinuous, 15–30 \times 3–5 \, \mu m.

Conidiogenous cells terminal, pale brown, smooth, subcylindrical, tapering to flat tipped apical loci, proliferating sympodially, or several times percurrently, 15–20 \times 3–4 \, \mu m; conidial scars inconspicuous. Conidia solitary, pale brown, smooth, guttulate, narrowly obclavate, apex subobtuse, base long obconically subtruncate, straight to curved, 3–15-septate, \( (40–)50–75(–80) \times 2–2.5(–3) \, \mu m \) in vivo; hila inconspicuous.


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 smokery-grey (19′′′′′d) and olivaceous-grey (21′′′′′i); reverse olivaceous-grey (21′′′′′). 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.


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 culture, conidia of CBS 116366 closely resembled the morphology of isolates described from *Citrus* (Fisher 1961, Sivanesan 1984).  

**Culture examined:** Thailand, Chachoengsao Province, Sanamechaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, CBS 116366 = CPC 10522 (single-ascospore isolate).


**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, subsessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 30–40 × 6–8 µm. *Ascospores* trito multiseriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid-ellipsoid with obtuse ends, medially 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. Spermatia rod-shaped, hyaline, 3–5 × 1 µm in vivo.

*Pseudocercospora thailandica* Crous, Himaman & M.J. Wingf., sp. nov. MycoBank MB500122.

Differt a *P. acaciigena* conidios brevioribus, ad 6-septatis; a *P. acaciae-confusae*, *P. hyaloconidiophora* et *P. acaciae* conidios obclavatisubcylindraceis, 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, 10–20 × 5–6 µm. Conidiogenous cells terminal, pale brown, smooth, subcylindrical, tapering to flat tipped apical loci, proliferating sympodially, 10–15 × 3–5 µm; 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–)30–45(–60) × 2–2.5(–3) µm in vivo; hila inconspicuous.


**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 2.5–3 µm wide, developing several lateral branches.

**Cultures:** Colonies slightly crumpled, 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 2003, 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-confusiae (Sa-wada) 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 conidio-phores 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 conidio- phores, 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. konae 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. crasscarpa which clusters with Cercospora loraanti 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-1α, 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 unre- lated 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 speci- ation 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. colombien- sis/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 asco- spores, 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 de- scribed in this study is where they might have origi- nated. 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.

REFERENCES


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. Verna & 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 synnematous 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 synnematous 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 synnematous 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).
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 Stigmina 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.
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¹ATCC: 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, CAB-Bioscience, Egham, U.K.

²ITS: 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

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**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; Cl = 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.

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. Mushii, CBS H-19683, epitype designated here. CBS 119096 = 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 ≥ 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.


Illustrations (selection): Saccardo, Fungi italicci, Pl. 838, Padova 1881; Brioso & Cavara, Funghi parasiti 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, subglobe, depressed to lacrimoid, up to 70 µm diam, brown. Conidiophores numerous, up to approx. 40, in dense fascicles, often forming synnematous conidiomata, erumpent, 100–500 × 20–70 µm, rarely longer, olivaceous-brown, composed of a more or less firm stipe of closely appressed conidiophores and a terminal, loose capitulum, i.e. conidiophores spaying out at the end of the conidiomata, free ends usually up to 100 µm long, individual conidiophores filiform, appressed threads 2–5 µm wide, up to 7 µm wide towards the apex, pluriseptate, subhyaline to olivaceous-brown, thin-walled, occasionally becoming rough-walled with age. Conidiogenous cells integrated, terminal, 20–100 µm 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 µm diam, usually flat, non-protruding. Conidia solitary, oblate-cylindrical, broadly subfuscous, short conidia sometimes ellipsoid-ovoid to short cylindrical, straight to curved, 20–75(–85) × 4–9 µm, (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) µm 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.

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),
**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 recognize 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 conidial 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 *Phaeophleospora*-like species with asperate 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 cladograms. 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 synnematosous 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-synnematosous species, e.g., *Spiroopes* 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 synnematosous 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 (= *Fuscidialum 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 & Lin 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 *P. griseola* in detail, based on a wide range of samples in *vivo* and *in vitro*, including type material of *Isariopsis griseola*, *Cylindrosporum 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. 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 *Paracerascospora* Deighton and *Pseudophaeoramularia* U. Braun had to be reduced to synonymy with *Pseudocercospora* (Crous et al. 2000; 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 *Stigmata* Sacc. [*S. platani* (Fockel) 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) synnematous 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*.

There is no specific combination to apply: 

"predicament arises as to what name should be phylogenetically reside in the same clade, the next extent has been prepared for Taxon (Braun & Crous 2006). The latter type species of *Stigmina* (1910; 1171 names), or *Stigmata* (1880, 161 names). Although *Stigmata* is the oldest name, *Pseudocercospora* is the most commonly used, and many species of *Stigmata* 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*. *Stigmata* predates *Phaeoisariopsis*. If the Code of Botanical Nomenclature were to be strictly applied, all species in this complex should be transferred to *Stigmata*. 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 *Stigmata*. 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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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.

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.
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; Unterreiner 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 starophorum 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 Centraalbureau voor Schimmelcultures (CBS; Utrecht), and CABI Bioscience (IM; 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 IT51/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 natrophila 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, heuristics 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 cm²) 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
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 musica (91 % bootstrap support) and Metulocladosporiella musae (99 % bootstrap support). The two

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession No.</th>
<th>Source</th>
<th>Origin</th>
<th>GenBank accession no. (ITS, LSU)</th>
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<td>USA</td>
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<td>Mycophilic</td>
<td>USSR</td>
<td>—, DQ008145</td>
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<td>CBS 109501</td>
<td>Deep mycosis of human patient</td>
<td>Turkey</td>
<td>—, DQ008146</td>
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<td>Puccinia allii</td>
<td>UK</td>
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<td>Páramo soil</td>
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<td>—, DQ008150, DQ008151</td>
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</tr>
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<td>Musa sp.</td>
<td>France</td>
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<td>M. paradiisaca</td>
<td>Uganda</td>
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<td>Cameroon</td>
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<td>Passalora fulva</td>
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<td>Australia</td>
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<td>Apple juice drink</td>
<td>Australia</td>
<td>DQ008141, —</td>
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</tr>
</tbody>
</table>

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).

Metulocladosporiella species are joined with a bootstrap support value of 100 %. Sequences of ‘Ramichloridium’ mackenziei and ‘Ramichloridium’ ances 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
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 musae (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 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.
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 subdenticulate, 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 hyphomycete genera (see below) revealed that C. musae does not fit into the concepts of any of the genera concerned. The present
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.


Mycobank MB500224.

Hyphae ramosae, septatae, hyalinae, subhyalinae vel pallide olivaceae, tenutunicae. Conidiophora solitaria vel laxe aggregata, erecta, subcilindrica, septata, brunneo, lev; ramuli terminales ex cellsulis conoidigenis et ramoconidii compositi; cellulae conoidigenae integratae, terminales, polyblasticae; cicatrices conidiales subconspicuae vel conspicuae. Conidia et ramoconidia catenata vel rami-catenata, ellipsoidea, ovoidea, subcilindrica vel fusiformia septata, subhyalina vel pallide olivacea, hila non-incrassata, leviter fuscata-refractiva, secessio schizolytica.


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; branch 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, subcilindrical, 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


**(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) μm wide, secession slightly constricted at the septa, with small swellings, hyaline, subhyaline to very pale olivaceous, thinned-tailed, 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) μm long, composed of a subcylindrical stipe, 3–8 μm wide, 2–12-septate, swollen or lobed at the base, 10–17 μm 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 subcilindrical branchlets; primary branches aseptate, 15–30 × 3.5–5 μm, giving rise to 1–2 secondary branches, or to conidigenous cells; secondary branches 0(–1)-septate, 30–50 × 3.4–5 μm, giving rise to 1–3 conidigenous cells; conidigenous cells subcylindrical, 10–45 × 3.4–4 μm, terminal or occasionally intercalary, sympodial, polyblastic, conidiogenous loci subconspicuous to conspicuous, subdenticulate, somewhat protuberant, truncate, wall unthickened, but somewhat darkened-refractive, 1–2 μm wide. *Conidia* in simple and branched acropetal chains, ellipsoid-ovoid, fusiform, subcilindrical, (6–)8–11(–16) × (3.5–)4(–5) μm [ramoconidia (10–)15–19(–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 wide (up to 3 µm 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 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. × paradisiaca (incl. var. sapientum) and M. schweinfurthii (syn. Ensete gillesii); Africa (Burundi, Cameroon, Côte d’Ivoire, Cameroun, Gabon, Kamerun).

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 µm, (B) = 20 µm, (C–D), (H) = 10 µm, (E–G) = 8 µm, (I–K) = 4 µm.
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).


**Notes**: Mason (in Martyn 1945) described long conidiophores, 60–500 x 3.5–6 μm, and aseptate conidia, 6–22 x 2.5–4 μm. In the type material from IMI (slide only), the conidiophores are much shorter, 45–150 x 3–6 μm, but the conidia agree well with the original description [5–16 x 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)

Differs a M. musae conidiophoros ad apicem valde ramosis, conidia (9–)11–13(–16) μm longis, locis conidiogenis latoribus, (1–)2–(–4) μm, saepe distinctioribus.


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, medium to dark brown in the lower half, paler towards the apex, tips pale olivaceous or even subhyaline, thin-walled below, thin-walled towards the apex, smooth; apex persistently branched, branched part composed of usually fairly compact, closely arranged subcylindrical branches; primary branches 0(–2)-septate, 15–85 x 3.5–6 μm, giving rise to 1–3 secondary branches, or to conidiogenous cells; secondary branches 0(–1)-septate, 20–40 x 3–4 μm, giving rise to (1–)2–3 conidiogenous cells; conidiogenous cells subcylindrical, 20–30 x 3–5 μm, terminal or occasionally intercalary, sympodial, polyblastic, conidiogenous loci subconspicuous to conspicuous, subdenticulate, somewhat protuberant, truncate, wail 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) x (3.5–)4 (–5) μm [ramoconidioid (12–)15–20(–25) x (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 ramosconidia), 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 NW 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 μm, (B–D) = 10 μm, (E–I) = 4 μm.
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 μm; and (3) wider loci, (1–)2(–4) vs 1–2(–3) μm. M. musae is also more prone to form micronematous conidiphores 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, micromematous or semi-micromematous to semi-macronematous conidiophores: Bispora (saprobiic), Cladophialophora (human pathogenic), Deuvriesia (heat-resistant soil fungi), Dimorphospora (saprobiic on submerged leaves), Pseudocladosporium (saprobiic), Torula (mostly saprobiic), Xylohypha, Xylohyphopsis (saprobiic, 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-micromematous to semi-macronematous conidiophores. Furthermore, the lignonicolous 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. Mycocellosiella, Phaeomuraria, and Stenella (all anamorphs of either Davidiella or Mycosphaeriella, 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 Venturicaceae. 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 Ramulia. 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 in Castanedaea 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 Parapleurotheciospis are pale, subhyaline as in Metulocladosporiella, but the conidiophores arise from lobed basal cells. Species of Pleurotheciospis 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, Beltriopsis, Beltriella, Hembeltrania and Pseudobeltania, 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 (‘Rhombospires’ sensu Kiffer & Morelet 1999; obovoid in Hembeltrania). Cordana, Parappycularia and Sterigmatabotrys 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 Heteronemium 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.

Haplotrichium (syn. Acladium, Alysidium; Partridge et al. 2001a), Sorocybe resinae (Partridge & Morgan-Jones 2002), Parahaplotrichum (Partridge et al. 2001b), Pheabolastophora and Subramaniomyces are some morphologically comparable genera with branched conidiophores. Haplotrichium, comprising wood-inhabiting lyphomycetes, differs in having quite distinct, denticulate conidiogenous cells and pigmented unicellular conidia. Parahaplotrichum species are also lignonicolous, denticulate, amerosporous and pigmented throughout. Sorocybe resinae is morphologically very close to Metulocladosporiella, but ecologically, genetically and also morphologically distinguishable. S. resinae 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 Pheabolastophora 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 saprobiic and possess conidiophores with lobed bases as well as aseptate conidia (amosporous).

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.

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**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 asceptate to septate .........................................................4
Conidiophores without branched head, irregularly branched, sometimes deeply cleft; conidia consistently asceptate .........................................................5

4(3) Branched head of the conidiophores loose to dense, but not typically metuloid; conidiogenous loci conspicuous, thickened and darkened, nondenticulate ........................................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, doliform 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 yellow ........................................................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

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**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 Kifer & 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 Cladosporium (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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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*, *Phaeotheca*, *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. konae* 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
connections (Crous and Braun, 2003). The *Mycosphaerellaceae* (*Capnodiales*) (Schoch *et al.*, 2006) includes species that are plant pathogens, saprobes, endophytes (saprobic 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 Kleing, 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.MycoBank.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 (× 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>.

**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 *Dissocconium 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.

<table>
<thead>
<tr>
<th>Teleomorph</th>
<th>Anamorph / Synanamorph</th>
<th>Strain no.</th>
<th>Substrate</th>
<th>Country</th>
<th>Collector</th>
<th>GenBank number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycosphaerella acaciigena</em></td>
<td></td>
<td>CPC 13290 = CBS 120740</td>
<td>Eucalyptus sp.</td>
<td>Australia</td>
<td>B. Summerell</td>
<td>EF394822</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPC 13350</td>
<td>Eucalyptus camaldulensis × E. urophylla</td>
<td>Venezuela</td>
<td>M.J. Wingfield</td>
<td>EF394823</td>
</tr>
<tr>
<td><em>Mycosphaerella associata</em></td>
<td></td>
<td>CPC 13108 = CBS 120732</td>
<td>Eucalyptus dunnii</td>
<td>Australia</td>
<td>A.J. Carnegie</td>
<td>EF394824</td>
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Table 1. Isolates of *Mycosphaerella* spp. and its anamorphs included for sequence analysis and morphological comparison.

<table>
<thead>
<tr>
<th>Teleomorph</th>
<th>Anamorph / Synanamorph</th>
<th>Strain no.¹</th>
<th>Substrate</th>
<th>Country</th>
<th>Collector</th>
<th>GenBank number</th>
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<td><em>Cibiessia minutispora</em></td>
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<td>CPC 12968 = CBS 120749</td>
<td>Leaf litter of <em>Cussonia</em> sp.</td>
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<td><em>Colletogleopsis</em> sp.</td>
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¹CBS: 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 100 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 hyphomycetum ad Mycosphaerellaceas pertinens. Hyphae pallide brunneae, leves, 3–5 μm latae, in conidia dilute brunnea, cylindrica, 0–3-septata, utrinque subtruncata disarticulatae, synanamorphe Readeriella.

Hyphomycetous, Mycosphaerellaceae. Hyphae pale brown, smooth, 3–5 μm 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 × 2–3 μm, 1(–3)-septata, synanamorphe Readeriellae in vitro formata, conidia ellipsoidea vel subcylindrica, dilute brunnea, 4.5–7 × 2–2.5 μm.

Arthroconidia occurring on brown lesions associated with a Pseudocercospora sp. Hyphae pale brown, smooth, 3–5 μm wide, disarticulating at septa to form short, pale brown, cylindrical conidia with obtusely rounded to subtruncate ends; aseptate conidia 5–7 × 2–3 μm, 1(–3)-septate conidia 5–9 × 2–3 μm; conidia developing further, becoming medium brown, predominantly aseptate, verruculose, ellipsoidal to subglobose or globose, 5–7 μm 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 × 3–4 μm, monop- or polyphialide with visible periclinal thickening, or phialide proliferating percurrently near apex; frequently intermingled with cylindrical paraphyses that can extend 5–10 μm above the conidiophores. Conidia narrowly ellipsoid to subcylindrical with rounded ends, pale brown, smooth to finely verruculose, 4.5–7 × 2–2.5 μm.

*Cultural characteristics*: Colonies on PDA slow growing, reaching 30 mm diam after 2 months at 25°C. Surface appearing grey-oliveaceous to green-oliveaceous 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 4–6 × 2–3 μm.

*Hyphae* pale brown, smooth, 2–3 μm wide, disarticulating at septa to form short, pale brown, cylindrical conidia with obtusely rounded to subtruncate ends; aseptate conidia 4–6 × 2–3 μm, 1(–2)-septate conidia 6–10 × 2–3 μm; 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.


*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.  
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 µm.

**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 pigimento 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 µm wide; apical ostiole 5–10 µm wide; wall consisting of 2–3 layers of medium brown textura angularis. Asci aparaphysate, fasciculate, bitunicate, subsessile, obovoid to narrowly ellipsoidal to subcylindrical, straight to slightly curved, 8-spored, 35–45 × 8–11 µm. 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
more prominently towards the lower end, (9–)10–11 × 2–3(–3.5) µm; 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 µm wide, disarticulating at septa to form short, pale brown, cylindrical conidia with obtusely rounded to subtruncate ends; aseptate conidia 4–10 × 3–5 µm, 1(–3)-septate conidia 7–12 × 3–5 µm; 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 × 3–4 µm,
mono- or polyphialidic. *Conidia* narrowly ellipsoid to subcylindrical with rounded ends, pale brown, smooth, 4–6 × 2–3 µm.

*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 µm long and 5 µm wide... ................................................................. *C. nontingens*

2. *Readeriella* state produced in culture; aseptate *Cibiessia* conidia up to 7 µm long and 2.5 µm wide................................................................................................................................. *C. dimorphospora*
2. *Readeriella* state not produced in culture; aseptate *Cibiessia* conidia up to 6 µm long and 3 µm wide ................................................................. *C. minutispora*

**Dissoconium australiensis** Crous & Summerell, *sp. nov.* (Fig. 6)

*MycoBank* 501260.

*Etymology:* Named for Australia, the country of origin.

*Dissoconio communis* similis, sed conidios minoribus, (20–)23–25(–27) × (3–)4(–5) µm, distinguendum.

*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 blunted rounded or truncate apex, straight to curved, smooth, medium brown, aseptate, 20–27 × 4–5 µm; loci terminal and lateral, indistinct. *Conidia* (20–)23–25(–27) × (3–)4(–5) µm, solitary, pale olivaceous-brown, smooth, ellipsoidal to obclavate, 1-septate, apex obtuse, base obconic-truncate, hilum unthickened, 1–1.5 µm 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.
Fungal Diversity

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 primariss minoribus, (8–)10–12(–14) × (4.5–)5–6 µm, secondariis 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
thickened, darkened scars, 0.5–1 µm wide. Conidia (8–)10–12(–14) × (4.5–)5–6 µm, solitary, pale olivaceous-brown, smooth, ellipsoid to obclavate, 1-septate, apex obtuse, base obconic-truncate, hilum thickened, somewhat darkened, 1–1.5 µm wide. Secondary conidia developing adjacent to primary

Fig. 7. Dissoconium eucalypti (CBS H-19770). Conidia and conidiogenous cells in vitro. Scale bar = 10 µm.
conidia, pale olivaceous to subhyaline, aseptate, pyriform, with a truncate base, 4–7 × 2.5–3 μm; 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, 152º 36’ 47” E, 28º 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 (12–25 × 3.5–6 μm), and secondary (7.5–12 × 3.5–6 μm) 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.

Ascosporae fusoideae-ellipsoideae, (12–)13–16(–17) × (3.5–)4–5(–6) μm.

*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 μm wide; apical ostiole 10–15 μm wide; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, but with remains of hamathecium visible, fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoidal, straight to slightly curved, 8-spored, 30–38 × 9–12 μm. *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–)13–16(–17) × (3.5–)4–5(–6) μm; 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 μm wide, at times developing 1–2 additional spore septa and additional germ tubes (germination Type H sensu Crous, 1998).

*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.


*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.


*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* aparáphysate, fuscilate, bitunicate, subessile, 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.


**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. dendritic*a. Phylogenetically *Nothostrasseria* clusters with species of *Readeriella*, but is different from *R. novaezelandiae* 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)

*Etymology:* Named after its characteristic long ascospores.

*Ascospores* fusoid-ellipsoidal, (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 tp 30 µm wide; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* ap paraphysate, fasciculate, bitunicate, subsessile, ovoid 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.


*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 µm.

*Mycosphaerella excentrica* Crous & Carnegie, *sp. nov.* (Fig. 11) MycoBank 501263.


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 µm wide; apical ostiole up to 20 µm 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 × 8–10 µm. Ascospores

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) × (3–)4 µm. *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 × 3–4 µm, secondary cells 2.5–4.5 µm wide, 4–6 µm long, but with additional septa these arms can become up to 15 µm 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.

Ascosporae 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* aparaphysate, fasciculate, bitunicate, subsessile, 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
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.


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.

MycoBank 501265.

Etymology: Name refers to its morphology which is similar to that of Mycosphaerella parkii.

Ascospores fusoid-e-ellipsoidal, (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 aparaphysate, 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. parkii affinis 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

*Stenella* anamorph, and having smaller ascospores than those of *M. parkii*, which are up to 15 µm 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 liliæ* similis, sed conidiis minoribus, (25–)30–33(−35) × (3.5)4(−5) µm, 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 µm diam; wall of 3–4 layers of dark brown *textura angularis*. *Conidiogenous cells* pale brown,
smooth, ampulliform to doliiform, 3–7 × 3–5 μm, 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–)30–33(–35) × (3.5)4(–5) μm.

*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.

*Phaeotheca* 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 triangualria, 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 collarette 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 µm.

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) × (5–)6(–7) µm.
Fig. 16. Pseudocercospora norchiensis (CBS H-19841). A. Conidiophores. B–F. Conidia. Scale bar = 10 µm.

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 µm wide; superficial mycelium developing once incubated in moist chambers. Caespituli fasciculate, epiphyllous, pale brown on leaves, up to 160 µm wide and 150 µm high. Conidiophores aggregated in highly dense fascicles arising from the upper cells of a brown stroma up to 160 µm wide and 90 µm high; conidiophores medium brown, smooth, 3–5-septate, subcylindrical, straight to variously curved, unbranched, 40–70 × 4–7 µm. Conidiogenous cells terminal, unbranched, medium brown, smooth, tapering to flat-tipped apical loci, proliferating sympodially, rarely percurrently near apex, 12–45 × 4–6 µm. 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) × (5–)6(–7) µm; hila inconspicuous, 2–3 µm 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.


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. nogalesii* (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 fusideo-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-sinuous, 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.


**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.,
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.**

Leaf spots absent, conidiomata associated with leaf litter. Mycelium internal, consisting of smooth, branched, septate, pale brown, 1.5–2 µm wide hyphae. Conidiomata pycnidal, immersed, brown, globose in OA, up to 100 µm 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 µm.

Fig. 19. Conidia and conidiogenous cells of *Septoria* sp. (CBS H-19831). Scale bar = 10 µm.
hyaline, smooth, proliferating sympodially near the apex, 6–12 × 3–8 µm. 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–)17–20(–24) × 1.5(–2) µm; hila inconspicuous, 0.5–1 µm 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.


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 µm wide; terminal hyphal ends characteristically ending in clusters of globose, multi-celled chlamydospore-like structures, frequently surrounded by a mucus sheath; clusters 10–30 µm diam. Conidiophores arising singly from superficial mycelium, dark brown, finely verruculose, multi-septate, subcylindrical, straight to geniculate-sinuous, mostly unbranched, or branched below, 50–200 × 5–8 µm. Conidiogenous cells terminal, mostly unbranched, medium brown, smooth to finely verruculose, tapering to flat-tipped apical loci, proliferating sympodially, 10–15 × 4–5 µm; scars thickened, darkened, refractive. Conidia solitary, pale brown, finely verruculose, guttulate, subcylindrical to narrowly obclavate, apex subobtusae, base long obconically subtruncate to obconically subtruncate, straight to slightly curved, (0–)1–3(–5)-septate, (10–)20–35(–60) × (2–)3–4(–6) µm; hila thickened, darkened, refractive, 1.5–2 µm wide.

**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**


*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).


*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.


*Anamorph:* *Pseudocercospora* sp.


*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.


*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*.


*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.


*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.


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)

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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References


Fungal Diversity


(Received 25 February 2007; accepted 28 March 2007)
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 leucadendron, B. proteae, Catenulostroma macowanii, Mycosphaerella markisii, Teratosphaeria bellula, T. jonkershoekensis, T. parva, and T. proteae-arboeae. Several species were also newly described, namely Batcheloromyces edgeweldii, Catenulostroma wingfieldii, Dissocionis proteae, Teratosphaeria personii, T. knoxdaviesii, 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. konaee (Leucospermum, Eucalyptus), M. markisii (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-arboeae. 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 Cylindrocladium root rot (Schoch et al. 1999, 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 ascii, 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...
### 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.

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1 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, CABI-Bioscience, Egham, Bakeham Lane, 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 Petri dish lids, with the top half of the dish containing 2 % malt extract agar (MEA; Oxoid, Hampshire, England). Ascospor 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 (cont.)
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.

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.
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 (× 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 the latter two models). The neighbour-joining trees support the results obtained are discussed where applicable

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.


Batcheloromyces proteae Marasas, P. S. van Wyk, & Knox-Dav., J. S. African Bot. 41: 43. 1975 — Fig. 2


Description — 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.


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 agaro (MEA) colonios tardes 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.
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.


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 macowanii (Sacc.) Crous & U. Braun, Stud. Mycol. 58: 17. 2007 — Fig. 4


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.


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. Conidiomata formed in simple and branched chains, dry, 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.
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.


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

Dissoconium aciculari simile, sed conidiis primaris minoribus, (9–)10–11(–12) × (3–)3.5(–4) µm, conidiis secondaris 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. Conidiophores 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.
**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 \(\times\) 2–3 \(\mu\)m, darkening and distorting upon germination), *M. buckinghamiae* has larger ascospores (9–13 \(\times\) 2.5–3.5 \(\mu\)m), 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. 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 \(\mu\)m.

*Specimen examined.* **CANYON 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 \(\times\) 4.5–6 \(\mu\)m, and secondary conidia, 4–7 \(\times\) 2.5–3 \(\mu\)m), and *D. aciculare* (primary conidia, 12–25 \(\times\) 3.5–6 \(\mu\)m, and secondary conidia, 7.5–12 \(\times\) 3.5–6 \(\mu\)m), but has smaller and narrower primary and secondary conidia (9–12 \(\times\) 3–4 \(\mu\)m, and 7–10 \(\times\) 3–4 \(\mu\)m, respectively), than both.
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 μm.
**Mycosphaerella communis** Crous & Mansilla, Stud. Mycol. 50: 203. 2004


Description — Crous et al. (2004b).


**Mycosphaerella holualoana** Crous, Joanne E. Taylor & M.E. Palm, Mycotaxon 78: 458. 2001

Descriptions — Taylor et al. (2001b), Crous et al. (2004a).


**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. **South Africa**, Western Cape Province, Stellenbosch, Elsenburg Farm, on leaves of Protea repens, 23 July 1999, S. Denman, holotype CBS H-20092, cultures ex-type CPC 2828–2830, 2831 = CBS 110942.

**Mycosphaerella marksii** Carnegie & Keane, Mycol. Res. 98: 414. 1994


**Mycosphaerella stromatosa** 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.

**Phaeotheoidea proteae** Crous, sp. nov. — MycoBank MB506594; Fig. 7

Phaeotheoidea eucalypti similis, sed conidias 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 oblivate, (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, Elsenburg Farm, on leaves of Protea repens, 23 July 1999, S. Denman, holotype CBS H-20092, cultures ex-type CPC 2828–2830, 2831 = CBS 114219.

Notes — The present species clusters close to the type of the genus Phaeotheoidea, *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.


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.

Description — Crous et al. (2000b).


**Septoria protearum** Viljoen & Crous, S. Afr. J. Bot. 64: 144. 1998

Description — Crous et al. (2004a).


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. jonkheerensis* 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).


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 henryi*, 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, Bungawaltbin, Robertson Plantation, 

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**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.
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 µm.

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. australis 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


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
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


**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. nida, 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


**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. asiatica* and *T. maxii* (Fig. 1).

**Teratosphaeria knoxdavesii** Crous, sp. nov. — MycoBank MB506595; Fig. 11

**Teratosphaeriaceae** bellulae similis, sed ascosporis cum tubis germinalibus parallelis ad axem longum sporisae.

**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 apaphysate, fasciculate, bitunicate, subsessile, obvoid to broadly ellipsoid, straight to slightly curved, 8–(35–)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.


**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


**Cultural characteristics** — Colonies black and erumpent, devoid of aerial mycelium, slow-growing; 3 mm diam after 6 mo
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. Extrae. 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 marasasi (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-artaboeae. 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 recognizable 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 obtained 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

*Teratosphaeria 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** amphiogenous, circular, 2–3 mm diam, pale brown to grey-brown, with a thin, raised, dark brown border. **Ascomata** amphiogenous, black, immersed, substomatia, up to 120 µm diam; wall consisting of 2–3 layers of medium brown **textura angularis**. **Asci** ap 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 ascii; germinating ascospores on MEA become brown and verruculose, 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 *Colernea 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.


*Basionym. Mycosphaerella maxii* Crous in *Crous & Groenewold, Fungal Planet No. 6*. 2006.

**Description** — Crous & Groenewold (2006b).


**Notes** — **Teratosphaeria maxii** is associated with leaf spot symptoms reminiscent of those of **T. alicantii**, **T. bellula** and **T. jonkershoekensis**. It is distinct from **T. alicantii** 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 & Groenewold 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**.


**Notes** — **Teratosphaeria microspora** is presently the only known teleomorph connection for species of *Catenulostroma* (Crous et al. 2007a). *Catenulostroma microsporum* 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.
**Teratosphaeria parva** (R.F. Park & Keane) Crous & U. Braun, Stud. Mycol. 58: 10. 2007 — Fig. 14


**Teratosphaeria persoonii** Crous & L. Mostert, sp. nov. — Myco-Bank 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. *Ascospora* amphigenous, black, immersed, substomatal, up to 120 µm diam; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* ap paraphysate, fasciculate, bitunicate, subsessile, obvoid 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.

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**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.

**Fig. 15** Teratosphaeria persoonii Crous & L. Mostert, sp. nov. — Myco-Bank MB506597, Fig. 15
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. persoonii 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 persoonii 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


Leaf spots initially indistinct, chlorotic, raised, circular, with catenulate margins, with substomatal ascomata appearing as black spots in lesions; spots not extending through lamina; 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-arboresae (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 µm, all others = 10 µm.
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 fissional 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 ascii, (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 mainly straight, fasciculate, bitunicate with fissitunicate dehiscence, with a rounded apex with a distinct ocular chamber, 2–4 µm diam, to obovoid, tapering abruptly to a small pedicel, narrowing to 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; 25–30(–35) × (5–)6–7(–9) 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; 25–30(–35) × (5–)6–7(–9) 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.

Notes — *Teratosphaeria proteae-arboareae* 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 undeveloped pseudoascospores. The reason for the smaller ascospores occurring in ascii along with normally developed ascospores is unknown. It is thus tempting to speculate that microascospores (which frequently aggregate at the ascal apex in *T. proteae-arboareae* 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.

Notes — This species, which is closely related to *T. proteae-arboareae*, 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); 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.

Notes — This species, which has ascospores that distort and turn brown upon germination, occurred on spots in association with *T. fibrillosa* and *T. proteae-arboareae*. It could not be described, however, due to paucity of material.

**Teratosphaeria sp.**

Cultural characteristics — Colonies on PDA 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.

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.

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 marksi, Teratosphaeria bellula, T. jonkershoekensis, T. parva, and T. proteae-arbovae. Several species are also newly described, namely Batcheloromyces sedgefieldii, Catenulostroma wingfieldii, Dissoconium proteae, Teratosphaeria knoxdavesii, T. marasasi 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 magna (in Australia; Mycosphaerella konae (Leucospermum, Hawaii), which also occurs on Eucalyptus in Thailand (Crous et al. 2007d), M. marksi (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 hypothesis 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.

Acknowledgements The University of Stellenbosch is thanked for financial support to P.W.C. during a recent collecting trip to the fynbos region in South Africa. Prof. dr U. Braun (Martin-Luther-Univ., Halle, Germany) is thanked for providing the Latin diagnoses. Profs W.F.O. Marasas and M.J. Wingfield, who have been honoured in the present paper, have both been awarded the Hendrik Persoon Gold Medal by the Southern African Society for Plant Pathology. The medal was designed by Prof. P.S. Knox-Davies (deceased), also remembered here. They all love(d) fungi.

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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 *Sordothrix*-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), *Cyphonectria* 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 such as 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 following the protocol of Lee & Taylor (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, hyphomyceticum. 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, 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, hilo consipuco, inspissato, fuscato, refringente, 1.5–2 µm diam, praeditis distinguendae; porus hili centralis 0.5 µm latus; coloniae in agaro mali pigmentum rubrum formantes; chlamydosporeae 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, producing abundant amounts of diffusing red pigment. Chlamydospores absent.

Cladoriella eucalypti Crous, sp. nov. MycoBank MB500800. Figs 1–2.

Deurviesiae thermodorantii similis, sed conidia 0–1-septata, (11–)13–15(–22) × (2.5–)3–3.5(–4) µm, hilo consipuco, inspissato, fuscato, refringente, 1.5–2 µm diam, praeditis distinguendae; porus hili centralis 0.5 µm latus; coloniae in agaro mali pigmentum rubrum formantes; chlamydosporeae absentes.

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).


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 (Venturaceae). 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession number</th>
<th>Host</th>
<th>Country</th>
<th>Collector</th>
<th>GenBank numbers (ITS, LSU, SSU)</th>
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<td>P.W. Crous</td>
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<td>Eucalyptus sp.</td>
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<td>M.J. Wingfield</td>
<td>DQ195779, DQ195791, DQ195802</td>
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<td>Colombia</td>
<td>M.J. Wingfield</td>
<td>DQ195780, DQ195792, DQ195803</td>
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<tr>
<td><strong>Macrohilum eucalypti</strong></td>
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<td>Eucalyptus sp.</td>
<td>New Zealand</td>
<td>J.A. Stalpers</td>
<td>DQ195781, DQ195793, DQ195804</td>
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<td><strong>Pestalotiopsis disseminata</strong></td>
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<td>Eucalyptus botryoides</td>
<td>New Zealand</td>
<td>M.A. Dick</td>
<td>DQ195782, DQ195794, DQ195805</td>
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<td>Colombia</td>
<td>M.J. Wingfield</td>
<td>DQ195783, DQ195795, DQ195806</td>
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<tr>
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<td>CBS 420.93</td>
<td>Pimenta dioica</td>
<td>Cuba</td>
<td>R.F. Cañáeda</td>
<td>DQ195784, DQ195796, DQ195807</td>
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<td>CPC 10972</td>
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<td>M.J. Wingfield</td>
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<tr>
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<td>DQ195787, DQ195799, DQ195810</td>
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<td>Indonesia</td>
<td>M.J. Wingfield</td>
<td>DQ195789, —, —</td>
</tr>
</tbody>
</table>

1CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.

Claviceps Tul., Calonectria De Not. (Hypocreales) and Gaemannomyces 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 clavispora* (Peck) Seaver (Hysteriales), *Candelariella vitellina* (Hoffm.) Müll. Arg. (Lecanorales), *Polysporina simplex* (Davies) Vězda (Lecanorales), *Botryosphaeria ribis* Grossenb. & Duggar (Dothidea, 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), *Fusicladium convolvulorum* Ondřej (Pleosporales) and others.

**Fulvoflamma eucalypti** Crous, sp. 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 sympodialiter cellularum conidigenorum et setis marginalibus hyalinis tenunticaxis et conidis 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. *Conidioforma* 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 conidigenous cells. Conidiogenous cells hyaline, smooth, subcylindrical, proliferating basally and sympodially. Conidia subcylindrical, straight or slightly curved with obtuse ends, septate, hyaline, smooth, guttulate.

**Fulvoflamma eucalypti** Crous, sp. nov. MycoBank MB500802. Fig. 3.

Conidioforma sporodochialia, erecta, aurantiaca, flammam candelae fingentia. Cellulae conidigenae hyalinae, leves, subcylindraceae, 7–15 × 1.5–2.5 μm, sympodialiter proliferantes. Conidia subcylindraceae, 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. *Conidioforma* 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-

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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-18046, 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 Potentiamyces pyri (Berk. & Broome) Dennis (7e-123; Rhytismatales), Phacidioypynn.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 caliciformis Fr. (Helotiales), Leuconeouspora pulcherima (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.


Ascomata indistinct on host, intermingled with those of Mycosphaeraella 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 subobtuse ends, multisepate (septa at approx. 10 µm intervals), 130–165 × 1–1.5 µm.

Cultural characteristics: Colonies spreading on MEA, slightly erumpent with moderate aerial mycelium; surface pale 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.


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
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.


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 µm diam. *Conidiogenous cells* pale brown, cylindrical, proliferating percurrently near the apex, 10–15 × 3–5 µm. *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 µm wide, (15–)17–19(–20) × (8–)10–12(–13) µm.

*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., Mihaljč. & M. Petrov (1e-96; *Diaporthales*), *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.


*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 µm wide, 11–20 µm long, 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, 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).


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(–5) 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 concentrical 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.


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 *Libertia* 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 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 collarate. *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.


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 calciciformis* Fr. (*Helotiales*), *Leucoeurospora pulcherrima* (G. Winter) Malloch & Cain (*Helotiales*), *Vibrissa albofusca* 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.

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 µm long, smooth, with dark brown walls thickened up to 1.5 µm, septate, paler at the blunt top, attenuated and bent at the base. Asci cylindrical-clavate, apex conical-rounded, the apical apparatus bluing in Melzer’s reagent, croziers present, 8-spored, 75–100 × 7–9 µm; ascospores fusoid, 0–septate, narrowly rounded at both ends, contents guttulate, hyaline, each end provided with a central, everted (umbrella-shaped) mucilaginous appendage, 17–25 × 3–4 µm; sometimes producing ellipsoid microspores 3–5.5 × 1.5–2 µm directly from apertures at one or both ends. Paraphyses simple or branched near the base, obtuse, hyaline, somewhat inflated and up to 3.5 µm 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 × 2–4 (–5) µm 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) × (1.5–)1.8–2 µm.
Substrate and distribution: Eucalyptus sp., Indonesia.


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 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). A number of similarities with an E-value of 0.0 were obtained from the LSU data: Hymenoscyphus fructigenus (Bull.) Fr. (8e-135) and Pezizella amanti (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 different media, morphology of the anamorph and teleomorph structures as well as ascospore germination patterns were used to describe 21 new species.

**Taxonomic novelties:** Colletogleopsis stellensboschiana Crous sp. nov., Mycosphaerella davisoniellae Crous sp. nov. (anamorph Davisoniella eucalypti H.J. Swart), Mycosphaerella eucalyptorum Crous & M.J. Wingf. sp. nov., Mycosphaerella gamssii Crous sp. nov., Mycosphaerella perpendicularis Crous & M.J. Wingf. sp. nov., Mycosphaerella pluritubulata Crous & J.P. Mansilla sp. nov., Mycosphaerella pseudaficanica Crous & T. Coutinho sp. nov., Mycosphaerella pseudocyptica Crous sp. nov. (anamorph Colletogleopsis sp.), Mycosphaerella pseudosedophytica Crous & G. Hunter sp. nov. (anamorph Pseudocercospora sp.), Mycosphaerella pseudusberosa Crous & M.J. Wingf. sp. nov. (anamorph Trimmatostruma sp.), Mycosphaerella quasicercospora Crous & T. Coutinho sp. nov., Mycosphaerella scytalidi Crous & M.J. Wingf. sp. nov. (anamorph Stenella sp., synanamorph, Scytalidium-like.), Mycosphaerella secundaria Crous & A.C. Alfenas sp. nov., Mycosphaerella stramenti 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 provencias 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, Colletogleopsis, Davisoniella, DNA sequence comparisons, Mycosphaerella, Pseudocercospora, Pseudocercosporella, Scytalidium, Septoria, Stenella, systematics, Trimmatostruma.

**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* in many parts of the world (Wingfield & Purnell 1987). MLB is known to occur 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 telemorphs (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.

<table>
<thead>
<tr>
<th>Teleomorph</th>
<th>Anamorph</th>
<th>Strain no.</th>
<th>Substrate</th>
<th>Country</th>
<th>Collector</th>
<th>ITS GenBank number</th>
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<td><em>Mycosphaerella communis</em></td>
<td><em>Dissoconium commune</em></td>
<td>CPC 11700</td>
<td><em>Eucalyptus globulus</em></td>
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<td><em>Colletogloeopsis nubilosum</em></td>
<td>CBS 111679; CPC 1576</td>
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\(^1\)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.

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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 (Swoford 2002). Phylogenetic analysis of the aligned ITS sequence data consisted of neighbour-joining analysis with the incorrected (“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 (× 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>.

**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. nibulosa* (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 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 second 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.

Coniothyrium ovato similis sed conidiis minoribus, (6.5–)7–9(–10) × (33.5(–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
**Mycosphaerella davisoniellae** Crous, sp. nov. MycoBank MB500834. Fig. 6.


Asci subcylindrici, subsessiles, 50–70 × 9–12 µm. Ascosporae bi- vel triseriatae, textura angularis, 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 subcylindric, subsessile, straight or slightly incurved, 8-spored, 50–70 × 9–12 µm. Ascosporae bi- to triseriate, overlapping, hyaline, thin-walled, straight, obvoid 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. **Conidigenous 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 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*. **Conidigenous 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 unicellular 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 conidigenous 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 unicellular 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*.

*Mycosphaerella eucalyptorum* Crous & M.J. Wingf., sp. nov. MycoBank MB500835. Fig. 7.

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, subsessile, 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.

**Host:** *Eucalyptus* sp.

**Distribution:** Indonesia.

Notes: Conidia of a *Stenella* anamorph were found on some lesions. This link is, however, unconfirmed, and...
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 × 3.5–4.5 µm) germinate with a Type B germination pattern as observed in *M. gracilis* (10–20 × 2–3 µm) (Fig. 8) and *M. marksii* (11–22.5 × 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 × (2–)3 µm, 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 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, sub sessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spired, 25–35 × 7–9 µm. *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 × (2–)3 µm *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 µm 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 × 2–3 µm, 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) × (2.5–)3 µm, modo M germinantibus, distinguenda.*

Leaf spots amphigenous, irregular to sub-circular, 5–15 mm diam, medium brown, frequently with a orange-red discolouration in the central part; border raised, dark brown. *Ascomata* pseudothecial, epiphyllous, single and black, subepidermal, globose, up to 90 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 broadly ellipsoid, slightly incurved, 8-spored, 25–35 × 7–8 μm. 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) × (2.5–)3 μm in vivo.


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 μm 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) × 3(–4) μm, saepe plures quam 2 tubos germinationis proferentibus, 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 μm diam; apical ostiole 10–15 μm diam; wall of 2–3 layers of medium brown textura angularis. Asci apariaphysate, fasciculate, bitunicate, subsessile, obovoid to subcylindrical, straight to slightly incurved, 8-spored, 30–45 × 7–10 μm. 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) × 3(–4) μm in vivo.

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 µm 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 pseudafricana 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 × 2.5–3 µm, distinguenda.
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. pseudafricana* (8–11 × 2.5–3 µm) germinate with a Type G pattern similar to that observed in *M. africana* (7–11 × 2–3 µm). Ascospores of *M. pseudafricana* 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. pseudafricana* 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. pseudafricana* 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.


Anamorph: *Colletogloeopsis* sp.

Etymology: Morphologically similar to *M. cryptica*.

*Mycosphaerellae crypticae* similis, sed ascosporis minoribus, (11–)12–14(–15) × (3–)3.5(–4) µm, 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 µm diam; apical ostiole 10–15 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* apparaphysate, fasciculate, bitunicate, subsessile, narrowly ellipsoid to subcylindrical, straight or slightly incurved, 8-spored, 35–45 × 9–11 µm. *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) × (3–)3.5(–4) µm, *in vivo*; frequently encased in an irregular mucous sheath. *Mycelium* internal, consisting of branched, septate, medium brown, smooth, 3–4 µm wide hyphae. *Conidiomata* intermixed among ascomata or separate, predominantly on the lower

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**Fig. 13. Mycosphaerella pseudafricana** (CBS 114782). A. Leaf spot. B–E. Asci and ascospores. F–H. Germinating ascospores. Scale bars: B = 3 µm, F = 7 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.

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*.
Isolates also form a \textit{Colletogloeopsis} anamorph in culture, which is similar to \textit{M. cryptica}. Ascospores of \textit{M. pseudocryptica} are 11–15 × 3–4 µm, and conidia 10–17 × 3.5–6 µm, while ascospores of \textit{M. cryptica} are 9–17.5 × 2–5.5 µm, and conidia are 8.5–18 × 4–6 µm. Phylogenetically \textit{M. pseudocryptica} is closely related to the \textit{M. molleriana} complex (Fig. 1), and distinct from \textit{M. cryptica}.


\textbf{Anamorph:} \textit{Pseudocercosporella} sp.

\textbf{Etymology:} Named after its morphological similarity to \textit{M. endophytica}.

\textit{Mycosphaerellae endophyticae} similis, sed ascosporis modo \textit{C} germinantibus distinguenda.

\textbf{Leaf spots} amphigenous, irregular to subcircular or angular, 2–5 mm diam, brown, with a raised, dark brown margin. \textit{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 \textit{textura angularis}. \textit{Asci} aparaphysate, fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoid, straight or slightly incurved, 8-spored, 30–40 × 8–10 µm. \textit{Ascospores} multiseriate, overlapping, hyaline, sparsely guttulate, thin-walled, straight to slightly curved, fusoid–ellipsoidal with obtuse ends, medially 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, \textit{in vivo}. \textit{Mycelium} internal, consisting of branched, septate, pale to medium brown, smooth, 3–4 µm wide hyphae. \textit{Conidiomata in vitro} sporodochial, hyaline. \textit{Conidiogenous cells} aggregated, unbranched or branched, hyaline, smooth, tapering to flat-tipped apical and lateral loci, proliferating sympodially, 8–15 × 2–3.5 µm. \textit{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.


\textbf{Ascospore germination on MEA after 24 h:} Type C. \textit{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.

\textbf{Cultures:} Similar to those of \textit{M. endophytica} (Crous 1998).

\textbf{Host:} \textit{E. nitens}.

\textbf{Distribution:} South Africa.

\textbf{Notes:} \textit{Mycosphaerella pseudoendophytica} has been known to us for some time, but its formal description required a molecular comparison with ex-type strains of \textit{M. endophytica} (which it resembles in anamorph morphology), and \textit{M. ellipsoidea} (which it resembles in ascospore germination pattern). As can be seen here, \textit{M. pseudoendophytica} (Fig. 4) is clearly a distinct species, sharing features of both of these taxa.

\textbf{Mycosphaerella pseudosuberosa} Crous \& M.J. Wingf., sp. nov. MycoBank MB500842. Fig. 18.

\textbf{Anamorph:} \textit{Trimmatostroma} sp.

\textbf{Etymology:} Morphologically similar to \textit{M. suberosa}.

\textit{Mycosphaerellae suberosae} similis, sed ascosporis minoribus, (11–)12–14(–15) × (3–)3.5(–4) µm, distinguenda.

\textbf{Leaf spots} amphigenous, associated with brown, corky spots on leaf petioles. \textit{Ascomata} pseudothecial, single
to aggregated, black, immersed becoming erumpent, globose, up to 120 µm diam; apical ostiole 10–20 µm diam; wall of 3–6 layers of brown textura angularis.

Ascii aparaphysate, fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoid, straight or slightly incurved, 8-spored, 35–45 × 12–16 µm. 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) × (3–)3.5(–4) µm in vivo; frequently surrounded by an irregular mucous sheath.


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 µm 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 µm vs. 3–6 µm), 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 quasicercospora 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 × 3–4 µm, distinguenda.
Ascospores tri- to multisieriate, 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) × (3–)3.5(–4) µm in vivo; apical cell 4–6 µm long, basal cell 6–8 µm 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. quasicercospora (10–14 × 3–4 µm) germinate with a Type F germination pattern, similar to that observed in M. nubilosa (11–16 × 3–4.5 µm), 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 × (2.5–)3 µm, 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 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown textura angularis. Asci aparyphysate, fasciculate, bitunicate, sessile, obovoid to ellipsoid, straight or slightly incurved, 8-spored, 25–30 × 7–9 µm. Ascospores tri- to multisieriate, 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 × (2.5–)3 µm in vivo. Mycelium internal and external, consisting of septate, branched, verruculose hyphae, 2–3 µm wide. Conidiophores aggregated in loose fascicles arising from the upper cells of a brown stroma up to 50 µm wide and 60 µm high. Conidiophores medium brown, finely verruculose, 1–4-septate, subcylindrical, straight to geniculate–sinuous, unbranched, 20–40 × 2–4 µm. Conidiogenous cells terminal, unbranched, medium brown, smooth to verruculose, tapering to the flat-tipped apical loci, proliferating sympodially, 7–15 × 2–3 µm, 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 × 3–3.5 µm; hila thickened, darkened, refractive. Aerial mycelium disarticulating into hyaline, smooth arthroconidia that are Scytalidium-like, 12–35 × 3–5 µm.

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 µm wide.
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...
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 × 2.5–3 µm, 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 µm diam. Asci paraphysate, fasciculate, bitunicate, subsessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 20–30 × 7–9 µm. 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 × 2.5–3 µm *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
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 × 2.5–3 µm) was originally treated as *M. parkii* (ascospores 8–15 × 2–3.5 µm) (Crous 1998).

Additional culture examined: Brazil, Picadão, Conceição da Barra, on leaves of *E. grandis*, 27 Apr. 1992, A.C. Alfenas, CBS 115608 = CPC 504.

*Mycosphaerella stramenti* 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) × 3(–3.5) µm, modo I germinantibus, distinguenda.

**Leaf spots** absent, ascomata associated with leaf litter. *Ascomata* pseudothecial, amphigenous, but predominantly hypophysoidal, single, black, immersed becoming erumpent, globose, up to 120 µm diam. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, narrowly ellipsoid to subcylindrical, straight or slightly incurved, 8-spored, 25–40 × 7–8 µm. *Ascospores* tritio multiseriatae, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, 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–)10–12(–13) × 3(–3.5) µm, *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 µm 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. stramenti* germinate with a Type I pattern. Several taxa are known to have this pattern of ascospore germination (Crous 1998), from which *M. stramenti* 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 × 3–3.5 µm, distinguenda.

**Leaf spots** absent, associated with leaf litter. *Ascomata* pseudothecial, amphigenous, but predominantly hypophysoidal, 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, subsessile, narrowly ellipsoid to subcylindrical, straight or slightly incurved, 8-spored, 30–35 × 7–9 µm. *Ascospores* tri- to multiseriatae, 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) × 3(–3.5) µm, *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.

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 µm 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 × 3–4 µm), *M. ellipsoidea* (8–11 × 2–3 µm), *M. endophytica* (8–11 × 2–3 µm), *M. lateralis* (7–16 × 2–3 µm), *M. irregulariramosa* (7–10 × 1.5–2.5 µm) and *M. tasmaniensis* (10–13 × 2.5–4 µm). Ascospores of *M. stramenticola* are 8–11 × 3–3.5 µm, 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.

*Mycosphaerella* keniensi similis, sed ascosporis maioribus, 12–16 × 3–4 µm, 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 µm diam; apical ostiole 15–20 µm diam; wall of 2–3 layers of medium brown *textura angularis*. Asci aparaphysate, fasciculate, bitunicate, subsessile, obovoid, straight or slightly incurved, 8-spored, 30–40 × 9–11 µm. 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) × (3–)4 µm, *in vivo*.


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 µm 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...
distinct from other species occurring on Eucalyptus (Fig. 2). Ascospores (12–16 × 3–4 µm) germinate with Type J germination patterns, as do M. colombiensis (11–15 × 3–4 µm) and M. keniensis (7–11 × 2.5–3 µm). However, ascospores of M. sumatrensis are larger than those of M. keniensis, and it has no anamorph, while M. colombiensis 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 lariotibus, verruculosis, (7–)8–9(–10) × 3(–3.5) µm, 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. **Ascomata** pseudothecial, amphigenous but chiefly hypophyllous, single, black, immersed becoming erumpent, globose, up to 60 µm diam; apical ostiole 10–15 µm diam; wall of 2–3 layers of medium brown **textura angularis**. **Asci** aparaphysate, fasciculate, bitunicate, subssesile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 18–27 × 7–8 µm. **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) × 3(–3.5) µm *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 µm 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.
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.  

*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*.  


Fig. 27. *Pseudocercospora subulata* (CBS 118489). A. Leaf spot. B. Conidiophores. C–E. Conidia. Scale bars = 5 µm.
Phylogeny of Mycosphaerella spp. on Eucalyptus

S. linicolae similis, sed conidiis brevioribus, 8–22 × 2–2.5 µm, distinguenda.

Leaf spots absent, conidiomata associated with leaf litter. Mycelium internal, consisting of smooth, branched, septate, pale brown, 2–2.5 µm wide hyphae. Conidiomata pycnidial, immersed, brown, globose on leaves, up to 160 µm 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, 5–10 × 2–2.5 µm. 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–)12–16(–22) × 2(–2.5) µm; hila inconspicuous, 0.5–1 µm diam.


 Cultures: Colonies after 3 wk on MEA 30–40 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 redisposed to other genera. The exceptions are S. eucalypti G. winter & Roum. (conidia filiform–acicular,
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.


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,
proliferating sympodially, 15–25 × 2–3 µm. 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 × 2.5–3 µm; 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 µm 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 × 2.5–3 µm) than Stenella parkii (25–200 × 2–2.5 µm) (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 × 3–5 µm, 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 × 3–4 µm. Conidiogenous cells terminal, unbranched, medium brown, verruculose, tapering to flat-tipped apical loci that are darkened and refractive, proliferating sympodially, 10–25 × 3–4 µm. 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 × 3–5 µm; hila thickened, darkened and refractive.


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. pseudafriaca. 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 dekeri (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. dekeri 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. form 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.

**ACKNOWLEDGEMENTS**

We are grateful to a large number of colleagues in different countries who have collected or enabled us to collect the material studied here, without which this work would not have been possible. In this regard, we thank particularly Mr. Carlos Rodas, Mr. Paul Clegg, Prof. Alan J.L. Phillips, and Prof. Teresa Coutinho. We thank Miss Marjan Vermaas for preparing the photographic plates, and Miss Arien van Liperen for doing the growth studies in pure culture.

**REFERENCES**


Foliicolous microfungi occurring on *Encephalartos*

P.W. Crous¹,², A.R. Wood³, G. Okada⁴, J.Z. Groenewald¹

**Key words**

Catenulostroma  
Cladophialophora  
Dactylaria  
ITS nrDNA  
LSU nrDNA  
Ochroconis  
Phaeomoniella  
Saccharatra  
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. Novelties include *Phaeomoniella capensis*, *Saccharatra kirstenboschensis*, *Teratosphaeria altensteinii* and *T. encephalarti*. New host records of species previously only known to occur on Proteaceae include *Cladophialophora proteae* and *Catenulostroma microsporum*, as well as a hyperparasite, *Dactylaria leptosphaerii–cola*, 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/). 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/).

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).

**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
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 rDNA gene (LSU). The primers ITS4 (White et al. 1990) and LROR (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). 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 (∼ 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 *Phaeomonella 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 *Fusidium* (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 *Phaeomonella*, *Saccharata* and *Teratosphaeria*. New records for *Encephalartos* include *Catenulostroma microsporum*, *Cladophialophora pro­teae*, *Dactylaria leptosphaericola*, and undescribed species of *Teratosphaeria*, *Lophiostoma* and *Ochroconis*. 

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain no.</th>
<th>Substrate</th>
<th>Collector(s)</th>
<th>GenBank Accession number</th>
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<td><em>Catenulostroma abietis</em></td>
<td>CPC 1496</td>
<td>Dead leaf tissue of <em>E. altersteinii</em></td>
<td>P.W. Crous</td>
<td>FJ372387</td>
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<td><em>Cladophialophora proteae</em></td>
<td>CPC 14902</td>
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<td><em>Lophiostoma</em></td>
<td>CPC 15000; CBS 123543</td>
<td>Living leaves of <em>E. altersteinii</em></td>
<td>P.W. Crous et al.</td>
<td>FJ372389</td>
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<td><em>Ochroconis</em> sp.</td>
<td>CPC 15461; CBS 123536</td>
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<td>A.R. Wood</td>
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<td><em>Saccharata kirstenboschensis</em></td>
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<td><em>Teratosphaeria altersteinii</em></td>
<td>CPC 15133; CBS 123539</td>
<td>Living leaves of <em>E. altersteinii</em></td>
<td>P.W. Crous et al.</td>
<td>FJ372394</td>
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<td>CPC 14886; CBS 123540</td>
<td>Living leaves of <em>E. altersteinii</em></td>
<td>P.W. Crous et al.</td>
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1. CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.
2. ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; LSU: 28S nrDNA.
**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.
with patches of apricot and flesh, apricot in reverse, reaching 15 mm diam after 1 mo.


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, 3.5–5 × 1.5–2 µm, thus larger than observed in P. capensis (Sydow & Sydow 1912). The second species, Phoma encephalarti, is distinct in having larger, bigutulate conidia, 6.3–7.2 × 2.7–3.6 µm (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–)18–22(–24) × 3.5–4(–5) µm.

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 µm 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, 15–45 × 2–3.5 µm. Conidiogenous cells terminal, subcylindrical, hyaline, 15–20 × 2–3 µm; 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 µm wide, extending above conidiophores. Conidia hyaline, smooth, fusiform to narrowly ellipsoid, apex subobtuse, base truncate with minute marginal frill, guttulate, thin-walled, (16–)18–22(–24) × 3.5–4(–5) µm, base 2–3 µm wide.
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 20–30 × 4.5–6 µm; Deman et al. 1999, Crous et al. 2006b) and *S. capensis* (conidia 13–18 × 3.5–5.5 µm; Marincowitz et al. 2008). *Saccharata kirstenboschensis* represents an intermediate species, having conidia 16–24 × 3.5–5 µm. 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

*Teratosphaeria bellulae* similis, sed ascosporis minoribus, 7–8(–9) × 2.5–3(–3.5) µm.

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 µm diam; ostiole lined with periphyses; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, obovoid, straight to slightly curved, 8-spored, 35–37 × 8–9 µm. *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, 7–8(–9) × 2.5–3(–3.5) µm; 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 µm wide.
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.


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 × 2.5–3.5 µm) than those of T. bellula (8–11 × 2–3.5 µm; 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) × (3–)3.5–4 µm.

Etymology. Name refers to its host genus, Encephalartos.

Leaves with tip-blight symptoms; nectrotic tissue grey-brown. Ascomata hypophyllous, black, immersed, substomatal, up to 90 µm diam; ostiole lined with periphyses; wall consisting of 2–3 layers of medium brown textura angularis. Asci ap paraphysate, fasciculate, bitunicate, subsessile, obvoid to broadly ellipsoid, straight to curved, 8-spored, 30–40 × 10–13 µm. Pseudoparaphyses intermingled among asci, branched, septate, hyaline, 2–3 µm wide. Ascospores bi- to triseriate, overlapping, hyaline, gutulate, 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) × (3–)3.5–4 µm; turning brown and verruculose in older asci; germinating ascospores on MEA.
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.
MEAs become brown and verruculose, generating 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. 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.


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 distant relatives (88 % based on ITS) to T. associaata, which occurs on Eucalyptus and Protea spp. (Crous et al. 2007a, 2008). The ITS sequences of the ex-type strain 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; centre olivaceous-grey; reverse iron-grey; reaching 25 mm diam after 1 mo.


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, dense, 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.


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 eruptent, 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.


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...
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 *Phaeomoniella*, *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 leptosphaericola* (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. leptosphaericola* 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 µm.

Fig. 8  *Cladophialophora proteae* in vitro (CPC 14902). a–c. Colony on MEA, OA and PDA, respectively; d, e. conidial chains. — Scale bar = 10 µm.
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 Fusiclidium (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 altsteinii* (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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**REFERENCES**


Characterization and Distribution of Mating Type Genes in the Dothistroma Needle Blight Pathogens

Marizeth Groenewald, Irene Barnes, Rosie E. Bradshaw, Anna V. Brown, Angie Dale, Johannes Z. Groenewald, Kathy J. Lewis, Brenda D. Wingfield, Michael J. Wingfield, and Pedro W. Crous

Abstract


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 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 Zealand 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 (1. 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 (*MAT1-1*) of the MAT locus (40,43,44,55). These include the *MAT1-1-1* encoding an α domain protein, the *MAT1-1-2* encoding an amphipathic α helix protein, the *MAT1-1-3* gene encoding a high mobility group (HMG) domain protein, and the *MAT1-1-4* gene encoding a metallothionein protein. Only one gene has been characterized for the mating type 2 idiomorph (*MAT1-2*) 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 *MAT1-1* and, because only a single *MAT1-2* gene has been identified for filamentous ascomycetes, this gene is referred to as *MAT1-2* (49).

DNA and amino acid sequences of the *MAT1-1-1* and *MAT1-2* genes in fungi show no obvious similarities, although the mating type locus has common flanking regions (48). Except for the HMG and α 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 *MAT1-1-1* and *MAT1-2* 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 *MAT1-1-1* and *MAT1-2* in isolates of the pathogens. A global collection of isolates subsequently was screened to determine which mating types are present in these collections.

**MATERIALS AND METHODS**

**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 Tsyuryupinsk, 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 *MAT1-1-1* of *Dothistroma* spp.** The *MAT1-1-1*-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 *MAT1-1-1* 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 *MAT1-1* 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 µg/ml in 1× 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. Amplifications 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 *MAT1-1-1* 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 *MAT1-1-1* 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 gencode 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 *MAT1-1-1* gene sequences for the *Dothistroma* spp. was calculated using the alignment tool of ALIGN (37).

**Isolation and characterization of *MAT1-2* of *Dothistroma* spp.** The *MAT1-2*-specific degenerate primers (MgMfSpMat1-2f1 and MgMfSpMat1-2r1) (Table 2), designed by Groenewald et al. (25), were used to screen isolates of *D. septosporum* and *D. pini*.
<table>
<thead>
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<th>Country, area, site</th>
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**TABLE 1. Origins of the Dothistroma septosporum and D. pini strains used during this study and the distribution of their mating types**
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 Dothistroma 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 (DseptoMat1f) or D. pini (DpiniMat1f) 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. DseptoMat2f 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 MgCl₂, 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) v4b.3.10 (Swofford D. L. 2003. Sinauer Associates, Sunderland, MA). Maximum parsimony analyses were conducted as described by Groenenwald 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 × 10⁻⁵⁰) and 37% identity to a 78-aa region (E = 2 × 10⁻⁵⁰) 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 × 10⁻⁵⁰) of the S. passerinii MAT1-1 protein (GenBank accession no. AAQ49357). 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 77.1% 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 Cerco- spora 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 MgMfSpMAT1-2f1 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 190-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

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<th>Primer</th>
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<td>DotMat2r</td>
<td>Dothistroma MAT1-2-specific primer</td>
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</table>

* Nucleotides specific to the given Dothistroma spp. 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.
spp. studied. The translated sequence showed 55% identity to a 65-aa (E = 1 × 10^{-19}) and 70% identity to a 27-aa region (E = 1 × 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 × 10^{-17}) of the S. passerinii mating type 1-2 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 × 10^{-19}) and 68% identity to a 29-aa region (E = 1 × 10^{-19}) of the M. graminicola mating type 1-2 protein (GenBank accession no. AAL30836) as well as a 47% (E = 7 × 10^{-17}) and 68% identity (E = 7 × 10^{-17}) to the same amino acid regions of the S. passerinii MAT2 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 corresponded with the number of predicted introns. None of the isolates amplified both fragments.

The alignment of partial MAT1-1-1 nucleotide sequences (α 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 the 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 α domain (Fig. 4A) and HMG domain (Fig. 4B) sequences were similar.

Phylogenetic analyses. The alignment of partial MAT1-1-1 nucleotide sequences (α 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 the 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 α 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).](image-url)
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 α 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 α domain and an HMG domain also described for the MAT1-1-1 and MAT1-2 of other ascomycetes. The two putative introns in the α 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 α 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...
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 *Alul* (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 stage 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 a 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 enable the quality of quarantine regulations in the future.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


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 Valleeau (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 Valleeau 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 morphological, 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 FastDNA 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-α 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 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 viewed under UV-light.

Amplicons were sequenced in both directions with the PCR primers and a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Roosendal, 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×). 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-
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C. beticola Sacc.

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*a CBS strain numbers, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
b CPC strain numbers, Collection of Pedro Crous, housed at CBS, The Netherlands.
c 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 Maluccella, 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. apiicola 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 apiicola 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, conidios obclavatus-cylindraceis, nonacicularibus, tantum 1–6-septatis.*

*Specimen examined.* VENEZUELA. La Guanota, Caripe, 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. *Conidiophores* arising in fascicles of 4–10, moderately dense, arising from stromata, emerging through stomata or erumpent through the cuticle, subcylindrical, upper part geniculate-sinuous, unbranched, 1–
CPC 10547
CPC 10548 Mycosphaerella thailandica
CPC 10549
CBS 117.47 Beta vulgaris Czechia
CPC 5062 Beta vulgaris Rumania
CPC 10166 Beta vulgaris New Zealand
CPC 10195 Beta vulgaris New Zealand
CPC 12027 Beta vulgaris Germany
CPC 12022 Beta vulgaris Germany
CPC 12031 Beta vulgaris Germany
CPC 5057 Helianthemum sp. Rumania
CPC 5064 Beta vulgaris Germany
CPC 5070 Beta vulgaris Rumania
CPC 5072 Beta vulgaris Germany
CPC 10204 Beta vulgaris New Zealand
CPC 11557 Beta vulgaris Italy
CPC 10197 Beta vulgaris New Zealand
CPC 12030 Beta vulgaris Egypt
CPC 12028 Beta vulgaris Egypt
CPC 10171 Beta vulgaris New Zealand
CPC 5074 Beta vulgaris Netherlands
CPC 5123 Apium graveolens New Zealand
CBS 116501 Beta vulgaris Iran
CBS 116502 Beta vulgaris Germany
CBS 116503 Beta vulgaris Italy
CBS 116505 Beta vulgaris France
CBS 116506 Beta vulgaris The Netherlands
CPC 12029 Beta vulgaris Egypt
CPC 5069 Beta vulgaris Japan
CPC 5071 Beta vulgaris Spain
CPC 5065 Malva pusilla Rumania
CPC 5128 Beta vulgaris New Zealand
CPC 5113 Limonium sinuatum New Zealand
CPC 5125 Beta vulgaris New Zealand
CPC 5073 Beta vulgaris Austria
CPC 10923 Apium graveolens Italy
CPC 10924 Apium graveolens Italy
CPC 5087 Apium graveolens Rumania
CPC 5063 Beta vulgaris Netherlands
CPC 5083 Plumbago europaea Rumania
CPC 5119 Beta vulgaris Hungary
CPC 5084 Plantago lanceolata Rumania
CPC 5112 Moluccella laevis New Zealand
CPC 5111 Moluccella laevis USA
CPC 5110 Moluccella laevis USA
CPC 5369 Spinacia sp. Botswana
CPC 5370 Spinacia sp. Botswana
CPC 11558 Beta vulgaris Germany
CPC 10168 Beta vulgaris New Zealand
CBS 116504 Apium graveolens Germany
CBS 116507 Apium graveolens Germany
CPC 5086 Apium graveolens
CPC 10925 Apium sp. Austria
CPC 11556 Apium graveolens Germany
CPC 11341 Chrysanthemum coronarium L. var. spathiosum Korea
CPC 11344 Chrysanthemum coronarium L. var. spathiosum Korea
CPC 10220 Apium sp. Venezuela
CPC 10248 Apium sp. Venezuela
CPC 10265 Apium sp. Venezuela
CPC 10266 Apium sp. Venezuela
CPC 10267 Apium sp. Venezuela
CPC 10279 Apium sp. Venezuela
CPC 10657 Apium graveolens Korea
CPC 10666 Apium sp. Korea
CPC 10759 Apium graveolens Korea
CPC 11641 Apium graveolens Greece
CPC 11642 Apium graveolens Greece

Cercospora apii
Cercospora beticola

1 change
3-septate, 25–70 × 4–6 μm, medium brown, becoming pale brown toward the apex, smooth, wall somewhat thickened. Conidiogenous cells integrated, terminal, 15–30 × 4–5 μm, occasionally unilocal, usually multilocular, sympodial; loci subcircular, planate, thickened, darkened, refractive, 2.5–3 μm wide. Conidia solitary, cylindrical when small, obclavate-cylindrical when mature, not acicular, (50–)80–120 (–150) × (3–)4–5 μm, 1–6-septate; apex subobtuse, base obconically subtruncate; hila 2–2.5 μm 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
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* 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. helianthemi*), *Moluccella* (*C. molucellae*), *Plantago* (*C. panteleuca*, *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. apicola* (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

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**Fig. 4.** Line drawing of conidiophores and conidia of the *C. apicola* holotype (CBS 116457). Bar = 10 μm.
using only HIS sequences. The first analysis separated the C. apiicola 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. apiicola s.s./ C. beticola s.s. isolates, it was unable to distinguish between C. apiicola s.s. and C. beticola s.s. isolates. Using HIS data a small clade representing seven C. beticola s.s. and one C. apiicola s.s. isolate grouped separately from other C. apiicola 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. apiicola 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. apiicola 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.

ACKNOWLEDGMENTS

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Gams W, Hoekstra ES, Aptroot A. 1998. CBS Course of Mycology. 4th ed. Baarn, the Netherlands: Centraal-
bureau voor Schimmelcultures.
Distinct Species Exist Within the Cercospora apii Morphotype

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ABSTRACT


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 other’s 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.

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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 ng 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-α 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 CyH3F and CyH3R (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 MgCl2 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 µl and contained 1 µl of diluted gDNA, 1× PCR buffer, 48 µM 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 µg/ml in 1× 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 consisted 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 reconnection 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 replicates (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× T4 DNA ligase buffer, 50 mM NaCl, 2 U of MseI, 2 units of EcoRI, 40 U of T4 DNA ligase, 10 µg of bovine serum albumin, 50 pmol of MseI adaptor, and 5 pmol EcoRI adaptor made up to a final volume of 11 µl (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 dH2O and 4 µl was used in the preselective PCR. The preselective PCR was performed in a 20-µl volume containing 25 pmol of primer EcoRI-0 (39), 25 pmol of primer MseI-0 (39), 1.5 mM MgCl2, 1× 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 dH2O 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 MgCl2, 0.5 units of Bioline Taq polymerase, 1× Bioline Taq polymerase buffer, 0.1 mM each dNTP, 0.5 µl of EcoRI primer, and 0.5 µl of MseI primer made up to a final volume of 10 µl. Selective PCR products (2 µl), amplified with the different primer combinations for each of the isolates, were mixed together with 0.5 µl of GeneScan 500 (labeled with 6-carboxy-X-rhodamine) (Applied Biosystems) and made up to a final volume of 25 µl 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

<table>
<thead>
<tr>
<th>Strain, accession no.(^a)</th>
<th>Host</th>
<th>Origin</th>
<th>Collector</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Korea</td>
<td>H. D. Shin</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
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<td>Meutri</td>
</tr>
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<td>K. Schrameyer</td>
</tr>
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<td>Venezuela</td>
<td>N. Pons</td>
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</table>

\(^a\) 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.

\(^b\) 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, 1x PCR buffer, 1.5 mM MgCl₂, 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. api* 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. api* 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. api* and *C. beticola*. For *C. api*, 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"). Isoneotypes are duplicate specimens of the neotype and ex-epitype cultures (to facilitate molecular studies) are derived from the epitype material.


Lectotype (proposed here): on *Apium graveolens*, Germany, Oestrich, garden, Fuckel, Fungi rhen. 117, in HAL, Fresenius (15) cited material of *C. api* 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.


Colonies of *C. beticola* and *C. api* 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. api* 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. api* (12°C), whereas *C. beticola* and *C. api* 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. api* 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. api* grew 3.5 and 2.7 mm/day at their respective optimal temperatures. Differences in growth rate between *C. api* and *C. beticola* were observed for most of the

### Table 2. Primers designed from calmodulin sequences for the species identification amplifications

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Nucleotide position</th>
<th>Description</th>
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</thead>
<tbody>
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<td>CGCGAGGCGAGAGTACTACGA</td>
<td>61–79</td>
<td>Positive control forward primer</td>
</tr>
<tr>
<td>CercoCal-beta</td>
<td>GCCACCCCTCTGGAATGTA</td>
<td>117–137</td>
<td><em>Cercospora beticola</em>-specific primer</td>
</tr>
<tr>
<td>CercoCal-apii</td>
<td>GACACCCCTCTGCAACTGCG</td>
<td>117–137</td>
<td><em>C. api</em>-specific primer</td>
</tr>
<tr>
<td>CercoCal-sp</td>
<td>GACACCTTCTGACTGCA</td>
<td>117–137</td>
<td>*Cercospora sp.-specific primer</td>
</tr>
<tr>
<td>CercoCal-R</td>
<td>GTCGAGAATTGCGGAATC</td>
<td>275–294</td>
<td>Reverse primer</td>
</tr>
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</table>

* The calmodulin sequence of *C. api* 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. apiit* (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 combined (*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. apiit* 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. apiit* 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. apiit*, 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-apiit, and CercoCal-sp are specific for *C. beticola*, *C. apiit*, 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. apiit* (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. apiit* sensu lato complex. Our data, however, refute the hypothesis that all morphologically indistinguishable *Cercospora* forms represent one species (7,13).
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-α, 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.
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.](image-url) Visualization of the amplified fragment length polymorphism (AFLP) band patterns were done using Bionumrics software. A, AFLP fingerprints of different isolates of the *Cercospora* sp., *Cercospora beticola*, and *C. apii* using primer combination *Eco*RI-A [FAM]/MseI-CT. B, AFLP fingerprints of *C. beticola* and *C. apii* isolates using primer combination *Eco*RI-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” host genera. For instance, the 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.

ACKNOWLEDGMENTS

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LITERATURE CITED


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. zeae-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. zeae-maydis* and *C. zeina* were found to be dissimilar. The presence of both mating types in approximately even proportions in *C. beticola*, *C. zeae-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.

Keywords: Allele frequency; *Cercospora* leaf spot; Geographic distribution; Mating types; *Mycosphaerella*; PCR amplification

1. Introduction

The genus *Cercospora* was described by Fresenius (Fuckel, 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* senso 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*, *Moluccella*, *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. zeae-maydis*, *C. zeina*, and an
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. zeae-maydis* and *C. zeina*. The description of *C. zeina* (Crous et al., 2006) has resolved some of the taxonomic uncertainty surrounding groups in *C. zeae-maydis*. The previously described *C. zeae-maydis* group II is now *C. zeina*, whereas group I is *C. zeae-maydis sensu stricto* (Crous et al., 2006; Dunkle and Levy, 2000; Goodwin et al., 2001).

No telemorphs are known for the *Cercospora* species causing leaf spot on celery, sugar beet or maize, although there was an unconfirmed report of a telemorph for *C. zeae-maydis* (Latterell and Rossi, 1977). Wang et al. (1998) were unable to find evidence of the MAT-2 idiomorph in isolates of *C. zeae-maydis*, and *in vitro* pairing studies with isolates of *C. zeae-maydis* and *C. zeina* have thus far proven unsuccessful in producing a telemorph (Crous et al., 2006). Wang et al. (1998) reported that there is little genotypic variation in populations of Group I and Group II (*C. zeae-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-Herrenthay, 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 has 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 (*MAT-I-1* and *MATI-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 *MATI-2* gene (Arie et al., 1997).

Four *MATI-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 *MATI-1-1* gene contains an alpha domain, the *MATI-1-2* gene has a MAT A-2 domain, and the *MATI-1-3* gene has a HMG domain, whereas the *MATI-1-4* encodes for a metallothionein protein (Kronstad and Staben, 1997; Turgeon, 1998). Only a single gene, *MATI-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 *MATI-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 *MATI-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 coerulescens* 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 telemorphs 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. apicola*, *C. zee-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. apicola*) 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. zee-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. fijensis* (Abeln, unpublished data) and the *MAT1-2* sequences of *M. graminicola* (GenBank Accession No. AF440398), *S. passerinii* (GenBank Accession No. AF483194) and *M. fijensis* (Abeln, unpublished data) were aligned using MegAlign from the Lasergene package (DNASTAR, 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’-CATTNGCNATCCCTTG-3’ and MgMfSpMat1-1r1 5’-GGGTNGANACCATGTTGAG-3’) and the other in a conserved region of the *MAT1-2* (MgMfSpMat1-2f1 5’-CAAGAANGCTTCTNTGATCT-3’ and MgMfSpMat1-2r1 5’-TTCCTTCCNGATGGCTTG-3’) genes. 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 MgCl2, 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 DYEEnamic 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. apicola*, *C. zee-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. Blasts (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)
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<td>C. polygonaceae</td>
<td>Persicaria</td>
<td>Korea</td>
<td>H.D. Shin</td>
<td>DQ264745; —</td>
</tr>
<tr>
<td>C. violae</td>
<td>Viola</td>
<td>Romania</td>
<td>O. Constantinescu</td>
<td>DQ264746; —</td>
</tr>
<tr>
<td>C. zeae-maydis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bCBS 117758</td>
<td>Zea</td>
<td>Iowa, U.S.A.</td>
<td>B. Fleener</td>
<td>DQ264747; —</td>
</tr>
<tr>
<td>bCBS 117760</td>
<td>Zea</td>
<td>Pennsylvania, U.S.A.</td>
<td>B. Fleener</td>
<td>— ;DQ264761</td>
</tr>
<tr>
<td>C. zeina</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bCPC 11995</td>
<td>Zea</td>
<td>South Africa</td>
<td>P. Caldwell</td>
<td>— ;DQ264762</td>
</tr>
<tr>
<td>bCPC 11998</td>
<td>Zea</td>
<td>South Africa</td>
<td>P. Caldwell</td>
<td>DQ264748; —</td>
</tr>
</tbody>
</table>
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 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. apiicola*, *C. beticola*, *C. zeae-maydis*, and *C. zeina*. The aligned MAT1-1-1 sequences included *C. beticola*, *C. apiicola*, *C. zeae-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. zeae-maydis*, and *C. zeina* (GenBank Accession Nos. DQ192582, DQ264752, DQ264761 and DQ264762, respectively). The sequences of each gene were aligned using MegaAlign from the Lasergene package (DNASTAR). To robustly amplify partial *Cercospora* mating type genes, the primers CercosporaMat1f (5′-GAGGCCATGGTGAGTGAG-3′) and CercosporaMat1r (5′-GAGGCCCATGGTGAGTGAG-3′) were designed from the conserved regions of the MAT1-1 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 *S. passerinii* and *S. pseudopasserinii* 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, CercosporaMat1f and CercosporaMat2r, were used in a multiplex PCR to screen for the presence of the two mating type genes in the *C. apiicola*, *C. apiicola*, *C. beticola*, *C. zeae-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, MgMISpMAT1-1f1 and MgMISpMAT1-1r2, 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.
and the translated sequence showed 77% identity to a 57 amino acid region of the \textit{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 \textit{M. graminicola} MAT1 protein using Blastx on the GenBank database. This confirmed that the 922 bp fragment is part of the \textit{MAT1-1-1} gene of \textit{C. beticola}. A homologous fragment was also obtained from \textit{C. apiicola}, \textit{C. zeae-maydis} and \textit{C. zeina} isolates during the first round of amplification using the MgMfSpMAT11f1 and MgMfSpMAT11r2 degenerate primers. The \textit{C. apiicola} population of 47 isolates, as well as 11 additional \textit{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 \textit{MAT1-2} gene.

The full-length \textit{MAT1-1-1} gene sequences for all four \textit{Cercospora} species were obtained by chromosome walking. The geneid software predicted that the \textit{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 \textit{MAT1-1-1} of the two maize pathogens and that of \textit{C. apiii} and \textit{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 \textit{Cercospora} species are listed in Table 2. Perfect

### Table 2

<table>
<thead>
<tr>
<th>C. zeae-maydis</th>
<th>C. zeina</th>
<th>C. apiicola</th>
<th>C. apiii</th>
<th>C. beticola</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. zeae-maydis</td>
<td>—</td>
<td>92.6</td>
<td>NA</td>
<td>87.4</td>
</tr>
<tr>
<td>C. zeina</td>
<td>74.5</td>
<td>—</td>
<td>NA</td>
<td>87.3</td>
</tr>
<tr>
<td>C. apiicola</td>
<td>70.3</td>
<td>90.8</td>
<td>—</td>
<td>NA</td>
</tr>
<tr>
<td>C. apiii</td>
<td>NA</td>
<td>NA</td>
<td>—</td>
<td>99.9</td>
</tr>
<tr>
<td>C. beticola</td>
<td>90.2</td>
<td>70.6</td>
<td>76.4</td>
<td>—</td>
</tr>
</tbody>
</table>

NA = not available due to the absence of the specific gene in the isolates tested.

![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.](image1)

![Fig. 2. Diagrammatic representation of the full-length mating type genes of Cercospora zeae-maydis, C. zeina, C. apiicola, C. apiii 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).](image2)
lariat sequences (RCTRAC) (Brunchez et al., 1993) were present in the introns of all four Cercospora species, except in the first intron of C. beticola and C. apiicola, that contained a GCTGAT sequence starting at 16nt 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 (MgMfSpMAT1-2f1 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. zeae-maydis and C. zeina) using the degenerate primers. A C. apiicola population of 32 isolates as well as 17 additional C. apiicola 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. zeae-maydis 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. zeae-maydis 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. zeae-maydis 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. zeae-maydis 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,
were constant, 139 were variable and parsimony-uninformative, and 273 characters were parsimony-informative. The MAT1-2 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.

Fig. 4. (A) One of five most parsimonious trees obtained from the MAT1-1-1 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 MAT1-2 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.

Similar trees were obtained irrespective of whether neighbor joining or parsimony was used. Five most parsimonious trees were obtained from the MAT1-1-1 sequences, and three most parsimonious trees were obtained from the MAT1-2 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% (MAT1-1-1) and 96% (MAT1-2). The trees obtained for both the MAT1-1-1 and MAT1-2 datasets showed that the two isolates that do not belong to the C. apii complex, namely C. zeae-maydis and C. zeina, group together with a 100% bootstrap support for MAT1-1-1 and 94% bootstrap support for MAT1-2. 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).
The *MAT1-1* 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 *MAT1-2* 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 *MAT1-1* 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 *MAT1-2* 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. apiicola* 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 MAT1-1-1 and MAT1-2 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 *Cercospora*MAT1 (805 bp fragment) and *Cercospora*MAT2 (442 bp fragment). Each tested isolate showed either the 442 bp fragment or the 805 bp fragment of the respective MAT1-1-1 or MAT1-2 genes, and no isolate showed both fragments. The MAT1-1-1 and MAT1-2 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 MAT1-1-1:MAT1-2 ratio calculated for each of the populations tested.

A total of 43 *C. zeae-maydis*, 49 *C. zeina*, 32 *C. apiicola* and 47 *C. apiicola* isolates were screened for the presence of the mating type genes, and no isolate showed both fragments. The MAT1-1-1 and MAT1-2 genes were distributed in the *C. zeae-maydis* and *C. zeina* populations at observed MAT1-1-1:MAT1-2 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 MAT1-2, whereas all the *C. apiicola* isolates obtained from Germany were found to be MAT1-1-1.

### Table 3

<table>
<thead>
<tr>
<th>Populations (country; region)</th>
<th>MAT1-1-1</th>
<th>MAT1-2</th>
<th>Ratio</th>
<th>χ²</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. beticola</em> (France; Longvic)</td>
<td>46</td>
<td>25 (0.54)</td>
<td>21 (0.46)</td>
<td>1.19</td>
<td>0.35</td>
</tr>
<tr>
<td><em>C. beticola</em> (Germany; Niedersachsen)</td>
<td>41</td>
<td>22 (0.54)</td>
<td>19 (0.46)</td>
<td>1.16</td>
<td>0.22</td>
</tr>
<tr>
<td><em>C. beticola</em> (Italy; Ravenna)</td>
<td>33</td>
<td>11 (0.33)</td>
<td>22 (0.67)</td>
<td>0.50</td>
<td>3.77</td>
</tr>
<tr>
<td><em>C. beticola</em> (Netherlands; Bergen op Zoom)</td>
<td>48</td>
<td>22 (0.46)</td>
<td>26 (0.54)</td>
<td>0.85</td>
<td>0.33</td>
</tr>
<tr>
<td><em>C. beticola</em> (Iran; Pakajik)</td>
<td>50</td>
<td>26 (0.52)</td>
<td>24 (0.48)</td>
<td>1.08</td>
<td>0.08</td>
</tr>
<tr>
<td><em>C. beticola</em> (New Zealand; Unknown)</td>
<td>37</td>
<td>19 (0.51)</td>
<td>18 (0.49)</td>
<td>1.06</td>
<td>0.03</td>
</tr>
<tr>
<td><em>C. zeae-maydis</em> (USA; Iowa)</td>
<td>43</td>
<td>21 (0.49)</td>
<td>22 (0.51)</td>
<td>0.95</td>
<td>0.02</td>
</tr>
<tr>
<td><em>C. zeina</em> (South Africa; KwaZulu-Natal)</td>
<td>49</td>
<td>30 (0.61)</td>
<td>19 (0.39)</td>
<td>1.58</td>
<td>2.5</td>
</tr>
<tr>
<td><em>C. apiicola</em> (Venezuela; Caripe)</td>
<td>47</td>
<td>0 (0)</td>
<td>47 (1)</td>
<td></td>
<td>62.67</td>
</tr>
<tr>
<td><em>C. apiicola</em> (Germany; Baden Württemberg)</td>
<td>32</td>
<td>32 (1)</td>
<td>0 (0)</td>
<td></td>
<td>58.33</td>
</tr>
</tbody>
</table>

The numbers in brackets represent the frequency of the gene.

* a Number of isolates analyzed.
* b MAT1-1-1:MAT1-2 ratio.
* c χ² value for the deviation from the expected 1:1 ratio.
* d Probability of a greater χ² value under the null hypothesis of 1:1 ratio (1 degree of freedom).
* e MAT1-1-1 was not detected in *C. apiicola*.
* f MAT1-2 was not detected in *C. apiicola*.

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. apiicola* 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 MAT1-1-1 and/or MAT1-2 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*), cucurbits (*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 MAT1-1-1 gene characterized during this study contains an area that corresponds to a putative alpha domain of MAT1-1-1, and DNA sequences in the MAT1-2 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 MAT1-1-1 and MAT1-2 genes of other ascomycetes. Species containing only one
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), Gibberella 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 (Paolletti 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. apiicola 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. apiicola complex and it is clear that the MAT1-1-1 sequences cannot separate C. apiicola 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. apiicola 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. 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 MAT1-1-1 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 MAT1-1-1 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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Singh, G., Ashby, A.M., 1998. Cloning of the mating-type loci from Pyrenopeziza brassicae reveals the presence of a novel mating type gene within a discomycete MAT-1 locus encoding a putative metallothio


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; Holtschulte, 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
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. sorgii (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. zeae-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 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 ≤ 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. apis 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

<table>
<thead>
<tr>
<th>Country of origin</th>
<th>Sample size</th>
<th>Location</th>
<th>Collector</th>
</tr>
</thead>
<tbody>
<tr>
<td>France (Fr)</td>
<td>46</td>
<td>Longvic</td>
<td>S. Garressus</td>
</tr>
<tr>
<td>Germany (Ger)</td>
<td>39</td>
<td>Niedersachsen</td>
<td>S. Mittler</td>
</tr>
<tr>
<td>Italy (It)</td>
<td>32</td>
<td>Ravenna</td>
<td>V. Rossi</td>
</tr>
<tr>
<td>Netherlands (Neth)</td>
<td>48</td>
<td>Bergen op Zoom</td>
<td>Unknown</td>
</tr>
<tr>
<td>New Zealand (NZ)</td>
<td>35</td>
<td>Unknown</td>
<td>C.F. Hill</td>
</tr>
<tr>
<td>Iran (Ir)</td>
<td>50</td>
<td>Pakajik</td>
<td>A.A. Ravanlou</td>
</tr>
</tbody>
</table>
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 ($r_d$) were used. $I_A$ and $r_d$ 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.

TFFPGA (Miller, 1997) and POPGENE v1·32 (Yeh et al., 1997) were used to analyse the 0/1 matrix. The population genetic analyses program TFFPGA 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 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 ($N_m$) 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

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>No. of bands</th>
<th>NZ</th>
<th>Fr</th>
<th>Ger</th>
<th>Ir</th>
<th>It</th>
<th>Neth</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI-A/MseI-CT</td>
<td>54</td>
<td>14</td>
<td>14</td>
<td>16</td>
<td>14</td>
<td>16</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>EcoRI-AG/MseI-C</td>
<td>52</td>
<td>11</td>
<td>16</td>
<td>17</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>EcoRI-G/MseI-CG</td>
<td>52</td>
<td>8</td>
<td>11</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>EcoRI-AT/MseI-C</td>
<td>48</td>
<td>10</td>
<td>11</td>
<td>17</td>
<td>9</td>
<td>14</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>206</td>
<td>43</td>
<td>52</td>
<td>63</td>
<td>52</td>
<td>58</td>
<td>59</td>
<td>79</td>
</tr>
<tr>
<td>% Polymorphic loci</td>
<td>20·9%</td>
<td>25·2</td>
<td>30·6</td>
<td>25·2</td>
<td>28·6</td>
<td>28·6</td>
<td>28·6</td>
<td>38·3</td>
</tr>
</tbody>
</table>

*NZ = New Zealand, Fr = France, Ger = Germany, Ir = Iran, It = Italy, Neth = Netherlands, All = total of all six populations.*
The high gene flow (\(N_m\)) values of 6.8 and 5.8 across the four European and five Eurasian populations, respectively, indicate high genetic exchange between these populations, but \(N_m\) was low when the New Zealand population was included in the calculation (\(N_m = 2.2\)) (Table 4). Low \(N_m\) values (1.3–1.8) were observed between the New Zealand population and every other population analysed. The highest \(N_m\) values were obtained in pairwise comparisons between Italy and the Netherlands (\(N_m = 19.1\)), followed by Netherlands/France (\(N_m = 11.4\)) (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 suggesting frequency-dependent selection, except in the population from Italy where MAT1-2 isolates were more predominant (Table 3). Multilocus measures of association (\(I_a\) and \(\Phi_{st}\) values at \(P < 0.01\)) were significant for all populations (Table 3). All four loci showed significant \(I_a\) and \(\Phi_{st}\) 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 \(I_a\) and \(\Phi_{st}\) values at \(P < 0.01\) 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. apicola (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(S) and I(A)) showed that all six populations were in gametic disequilibrium. This suggests that asexual reproduction is predominant and that random mating occurs only rarely, if at all. However, although significant, the values of I(S) and I(A) were low for populations from Italy, France and the Netherlands. Furthermore, r(S) 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 a separate gene. 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. Second, 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 electrophoresis. 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 *C. beticola* because it can 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-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.

**Acknowledgements**

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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-1α) 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-1α 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-apressorium 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 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-1α) 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 0.1 M CaCl2. DNA isolation was cleaned by washing with 70 % Ethanol (70 % EtOH) and dried under vacuum. SABAX water was used to resuspend the isolated DNA. RNaseA (10 µg/µL) 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 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 µL containing 10× PCR Buffer (5 mM Tris-HCl, 0.75 mM MgCl2, 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 µM of forward and reverse primers (Inqaba Biotech, South Africa) and 1.25 U Taq DNA Polymerase (Roche Diagnostics, South Africa) and DNA (20 ng/µL). Sterilised distilled water was added to obtain a final volume of 25 µL.

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′−GTT TGG TTT CTT TTC CT−3′) (Vilgalys & Hester 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-1α was amplified using the primers EF1-728F (5′−CAT CGA GAA GTT CGA GAA G−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-738R (5′−TAC TAC CAC CAA GAT CT−3′) (Carbone & Kohn 1999). 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 (Katoh 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-1α 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-1α and ACT have been deposited in GenBank (White were in the case of the ITS region where two internal primers used for PCR reactions. Exceptions

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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, Modelfit 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. ambiphilla* A. Maxwell, *M. suttoniae* Crous & M.J. Wingf.), *Colletogloeopsis* Crous & M.J. Wingf. (*M. molleriana* (Thüm.) Lindau, *M. vespa* Carnegie & Keane, *M. cryptica* (Cook) Hansf.), *Uwebraunia* Crous & M.J. Wingf. (*M. nubilosa* (Cook) Hansf., *M. ohnowa*, *Readierea* 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. walkeri R.F. Park & Keane.), *Pseudocercospora* Speg. (*M. heimii* Crous & M.J. Wingf., *M. heimii* Crous, *M. crystallina* Crous & M.J. Wingf., *M. irregulararamosa* 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. basistruncata* 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.

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Table 1. Isolates of Mycosphaerella used in this study for DNA sequencing and phylogenetic analysis.

<table>
<thead>
<tr>
<th>Teleomorph</th>
<th>Anamorph</th>
<th>Isolate No.</th>
<th>Host</th>
<th>Country</th>
<th>Collector</th>
<th>GenBank Accession No.</th>
<th>LSU</th>
<th>ITS</th>
<th>ACT</th>
<th>EF-1a</th>
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</thead>
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<td>E. viminallis</td>
<td>South Africa</td>
<td>P.W. Crous</td>
<td>DQ246258 DQ267577 DQ147608 DQ235098</td>
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<td>Phaeophleospora sp.</td>
<td>4945</td>
<td>E. viminallis</td>
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<td>P.W. Crous</td>
<td>DQ246257 AF309602 DQ147609 DQ235099</td>
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</tr>
<tr>
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<td>Unknown</td>
<td>14180</td>
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*a* 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-1α) phylogeny**: The EF-1α 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.](image-url)
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 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. psuedoeucalyptorum, Ps. robusta, Ps. basiruncta, 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. ambiphylla, M. molleriana and M. vespa also clustered together with 100% bootstrap support. Isolates of M. intermedia M.A. Dick & Dobbie, M. marksi Carnegie & Keane and Pseudocercospora epispermogonia Crous & M. J. Wingf. grouped together in a clade that was supported with a bootstrap value of 80% included isolates of M. lateralis and M. communis and was basal to Clades 1 and 2.
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-1α alignment conducted in PAUP resulted in a P-value of 0.001 for all possible combinations of the LSU, ITS and EF-1α 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-1α 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-1α 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

![Phylogram obtained from the Elongation factor 1-alpha (EF-1α) 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.](image-url)
(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 data.
data from the LSU, ITS, EF-1α 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-1α 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

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### Table: Phylogram obtained from the combined LSU, ITS and EF-1α DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus*

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<td><strong>Phaeophleospora</strong></td>
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<td>Colletogloeopsis</td>
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<td>Uwebraunia</td>
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<td><strong>Pseudocercospora / Uwebraunia</strong></td>
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<td><strong>Dissoconium</strong></td>
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**Figure 5.** Phylogram obtained from the combined LSU, ITS and EF-1α 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.

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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 *Phaeolephospora* 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 constrict at the median septum, typical of a type C ascospore germination and ascospores with a slight constriction at the median tubes that grow parallel to the long axis of the spore (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) x (2.5−)3.5−4(−4.5) μm, those of *M. ambiphylla* are (12−)14−15(−22) x(3.5−)4.5−5(−6) μm and those of *M. vespa* 9.5−16.5 x 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.


*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-1α 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 of these species are required to determine whether they 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. 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. african*. However, the ascospores of *M. aurantia* are not constricted at the median septum whereas those of *M. african* had such constrictions, and ascospores of *M. aurantia* are longer (7−)8−10(−11) µm than those of *M. african* (7−)8−10(−11) µm (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. african* 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.
Eucalyptus Mycosphaerella relationships based on the phylogenetic position within larger part the evolution of the anamorph genera. It is evident that for the cryptic speciation. Studies of the deeper branches for will aid in the resolution of species complexes and increased resolution at the species level. This in turn of greater numbers of data sets should allow for – this volume). To study species complexes, variable

\[ \text{REFERENCES} \]


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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 ap 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

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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 hygiosgenously 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 for details). The ha-
Phoma have sympodially proliferating conidiogenous cells. Many of the hyphomycetes are anamorphic species. These include both hyphomycetes and coelomycetes. Many of the hyphomycetes (e.g. Pleosporaceae, Mycosphaerellaceae, Tubeufiaceae) include a high proportion of families in this class (e.g. Pleosporaceae, Mycosphaerellaceae); 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 (Winke 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.
within the framework of a multiple gene analysis showing intraordinal relationships in the Dothideomyces.

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 Dothideomyces. 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 cucurbitaecearum*) 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 MULTEES in effect. Maximum likelihood was performed with PHYML (Guindon and Gascuel 2005) using a GTR+I+F 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 20917 steps (CI = 0.204, RI = 0.535) and three MPTs with 54319.54 steps, respectively. In model-based methods, ML heuristic search analysis resulted in a tree of $-94457.67$ 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 $-93955$. 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 “*Epipolaeolum longisetosum*” = *Raciborskiomyces longisetosus*” 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 pines* (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 periphsate ostioles (Verrucariales, Chaetothyriales, Pyrenulales). The phylogeny (Fig. 2) confirms the separation of the Chaetothyriales and Verrucariales from the Dothideomycetes.

**Dothideomycetes-Arthoniomycetes clade.** The relationships 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, Teehler 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 ascohymenial 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 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 ap paraphyate 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 Sordariomycetiidae (syn. Sordariomycetes s. str.) and Arthoniomycetiidae (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 Dothidea, Capsmodiales and Myriangiales, which lack paraphyses, pseudoparaphyses and paraphysoids. This emended subclass overlaps with the Loculopar enchymatomycetiidae (Barr 1983) but differs by including the Myriangiales and excluding the Asterinales, now listed under its constituent families as Dothideomycetes et Chaetothyriomycetiidae incertae sedis by Eriksson (2006).


Ascomata immersa vel erumpentia vel superficialia, minuta vel magnitudine media, separata vel in stromate basali, aggregata, unilocularia vel plurilocularia, ostiolata, nonnumquam peripherysata. Pseudo-paraphyses absentia, peripherysiadeae nonnumquam praesentia. Asci globosi vel ellipoidei vel clavati vel
subcyllindrical. 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 multiloculate apical pore mostly present, when present ostiolar canal at times periphrystate, stromatic tissues may contain pseudoparenchymatous cells. Pseudo-paraphyses lacking, periphysoids may be present; Asci globose, subglobose, ovoid to ellipsoid, saccate, oblong, clavate or subcyllindrical, 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.


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 Dothisa sambuci, the type of the genus Dothisa (Shoemaker et al 2003). The family Dothideaceae includes biotrophs, necrotrophs and saprobes on plant tissue. Stylodothis puccinoides was redescribed as a separate species from Dothisa but remains closely associated with the genus in our phylogeny.

Three members of the Dothioraceae are polythetic 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 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 citrinum (Lumbsch and Lindemuth 2001). Synapomorphies are limited in this expanded order and these taxa have not been grouped together before. The presence of short, periphyses-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 Pidiaceae. The constituent families are discussed below.

Capnodiales. An ascostromatal family without pseudoparaphyses, the Capnodiales are leaf epi- phytes 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 pleonan- morphic. The order includes many anamorphic species, all dematiaceous, including several conidio- matal, 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-
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* 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 *Mycosphaerellaceae* similia, sed lumen ascosporum 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. Ascii bitunicate, 8-spored, obovoid 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*.


The position of a single representative of the Piedraeiaceae, *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 (13) 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 *Sphaeloma* 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 perithecioid, hysterothecioid or cleistotheioid, conchate or dolabriform, immersed, erumpent or superficial; globose, sphaeroid, turbinate, ovoid, obpyriform, conoid, doliiform, dimidiate. Hamatheccium of wide to narrow cellular or trabecular pseudoparaphyses, deliquescent at maturity in some. Ascii 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
the ostiolate marine species, cioid ascostromata. This clade unexpectedly contains
by Eriksson (2006). Members of this family are mainly
 provisionally included among Ascomycota incertae sedis
 supports two members of the Testudinaceae, pro-
 (FIG. 2).

There was also strong support for the monophyly of
the strain used (results not shown). Members of this
family are hypersaprotrophic on old dung and
exposed wood.

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 sup-
ported (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, pro-
visionally included among Ascomycota incertae sedis
by Eriksson (2006). Members of this family are mainly
isolated from soil and produce reduced, cleistotho-
ceiid 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 (Lophio-
stromataceae 2) includes members of the Lophiosto-
mataceae and Pleomassariaceae as well as Melanom-
mataceae. 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 impor-
tant plant pathogens, saprobes and animal pathogens
with numerous anamorphs. Didymella cucurbita-
ceanum forms a clade with the anamorphs Ascochyta
pisi and Phoma herbarum (D8), parasites on agricul-
tural 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 pseudotrichia with
narrow asci and a characteristic thin apex. Many
species are associated with coelomycetous ana-
morphs. Phoma anamorphs are particularly common
(Camara et al 2001, Verkley et al 2004). The Phaeo-
sphaeriaceae (D9) are distinguished from the Lepto-
sphaeriaceae by ascosomal wall morphology and all
have pycnidial coelomycetes, mostly classified in
Stagonospora, characterized by holoblastic or some-
times annellidic conidio genesis and the production of
phragmoconidia. Unnamed pycnidial microconidi-
dial anamorphs also are reported in some species
(Leuchtmann 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 telemorphs Leptosphaeria and
Herpotrichia.

The next well supported node (D10) contains the
Pleosporaceae, which have ascostromata that are
mainly flask-shaped pseudotrichia 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 Leuca, 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), Stempylidium, 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 nucc 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 apothecid 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 Tyrrannosorus pnicola (Fig. 2). Phaeotrichum is characterized by dark-brown, septate spores and cleistothecial ascostromata. T. pnicola 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. pnicola 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 nucc 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, multisepate ascospores (Rossman 1987). The best-known anamorphs of the Tubeufiaceae are helicosporous hyphomycetes and well known genera include Helicodendron, Helicomyces 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.


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 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. *Ascosphaera* hyaline to pigmented, septate or not, ellipsoid to ovoid, with or without mucoid appendages or sheath. *Anamorphs* have unito 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 Botryosphaeriales, and redefine the Capnodiales to include the currently defined Mycosphaerellaceae and Pie-draiacae. 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 *Trypethe-lium* species was included, but numerous lichenized ascosporic 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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**LITERATURE CITED**


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. fassiana), C. macrocarpum (D. macrocarpa) and C. brunnei (D. alticina) are distinguishable by having conidia of different width, and by teleomorphic 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. brunnei. Several species are newly described from hypersaline water, namely C. ramotenellum, C. tenuellum, C. subtilissimum, and C. herbaroides. Cladosporium pseudiditis 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 hereafter proposed to facilitate future morphological examination of Cladosporium spp. in culture, and neotypes or epitypes are proposed for all species treated.


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 carophores 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, Prasid & de Hoog 1988), and there are literally thousands of records of this species in the literature. McKerny & 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-α, 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).

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).

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 λ 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 FST (index: θ) was calculated with 1–6 runs using the same software. The null hypothesis for this analysis is no population differentiation. When observed theta (θ) 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 (iST; 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 iST 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 LAN v. 3.5 (Haubold & Hudson 2000). Degrees of recombination or horizontal gene transfer were also visualised using SPLITSTREE 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. Conidigenous cells with more than one conidigenous 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 µm. K. Schubert del.
**Morphology of the teleomorph: Telemorphs 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 (∗ 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 × 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. bruheii* 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.,...
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**Cladosporium iridis**

- **Davidiella macrospora**
  - CPC 107.20: Iris sp. (epitype)
  - CPC 138.40*: Iris sp. (epitype)

**Cladosporium macrocarpum**

- **Davidiella macrocarpa**
  - CPC 175.82: Water from Romania
  - CPC 223.31: ATCC 11287
  - CPC 299.67: Triticum aestivum
  - CPC 12181*: CPC 12755 (neotype)
  - CPC 11817: Corylus sp.

**Cladosporium oasifragi**

- CPC 842.91*: Iris sp. (epitype)
  - CPC 843.91: Narthecium oasifragum

**Cladosporium pseudinidis**

- CPC 110663*: LYN 1065 = ICMP 15579 (ex-type)
- CPC 121628*: CPC 12043 = EXF-454 (ex-type)
- CPC 12047: EXF-967
- CPC 121629*: CPC 11839 = ICMP 15819 (ex-type)

**Cladosporium spinulosum**

- CPC 102044: Hypersaline water from salterns
- CPC 110907*: CPC 12040 = EXF-334 (ex-type)
- CPC 121630*: CPC 12041 = EXF-343 (ex-type)
- CBS 172.52: ATCC 11320
- CPC 113741: Grape berries
- CPC 113742: Grape berries
- CPC 113744: Grape buds
- CPC 12484: Pinus ponderosa
- CPC 12485: Pinus ponderosa
- CPC 12153*: CPC 12053 = EXF-1735 (ex-type)

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**Cladosporium subinflatum**

- CPC 113753: Bing cherry fruits
- CPC 113754*: Grape berries
- CPC 12044: EXF-462
- CPC 121634*: CPC 12053 = EXF-1735 (ex-type)
The intraspecific variation in the *C. brunheii* clade is due to genetic variation present in the sequence data of all loci except 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 FST varied between 0.1362 and 0.3381. Linkage disequilibrium calculated using the standardised index of association (I\textsuperscript{S}A) for the entire dataset (observed variance V\textsubscript{o} = 0.5602, expected variance V\textsubscript{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).
with correlated allele frequencies. Group indications (18) are taken from a tree based on EF sequences with AIC under the HKYG model.

Fig. 4. Distance tree of the Cladosporium herbarum complex based on ACT sequence data, generated with UPGA Neighbor-joining structure analysis at K = 6, under admixture model.
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)
2. Conidia with different surface ornamentation, minutely to distinctly verruculose, verrucose to echinulate or spiny .......................................................... 2

2. Conidiophores uniform, macronematous; conidia solitary, sometimes formed in short unbranched chains .......................................................... 3
3. Conidiophores both macronematous and micronematous; conidia always catenate, usually formed in branched chains .......................................................... 5

Fig. 5. Split decomposition of the Cladosporium herbarum complex using SplitTree 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
3. Conidiophores due to geniculations often growing zigzag-like, (4–)5–7 μm wide; conidia 9–21 × (5–)6–8 μm, 0–1-septate; conidiogenous loci and conidial hila 1.2–2(–2.2) μm diam ................................................................. C. sinuosum

3. Conidiophores not growing zigzag-like, wider, 6–11 μm; conidia very large and wide, 15–75(–87) × (7–)10–19(–21) μm, often with more septa; conidiogenous loci and conidial hila wider, (2–)2.5–4 μm diam .................................................. C. variabile

4. Conidia (18–)30–75(–87) × (7–)10–16(–18) μm, (0–)2–6(–7)-septate, walls thickened, especially in older conidia, up to 1 μm thick ................................................................................................................ C. pseudiridis

4. Conidia shorter and wider, 15–55 × (9–)11–19(–21) μm, 0–3-septate, walls distinctly thickened, up to 2 μm, usually appearing zonate ........................................................................................................ C. herbaroides

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 μm wide, swellings 5–11 μm wide ........................................................................................................ 7

6. Macronematous conidiophores somewhat narrower, (1.5–)2.5–5 μm wide, swellings 3–8 μm 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. variabile

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) μm wide, swellings 3–6.5 μm wide; conidia 4–17(–22) μm long, ornamentation variable, but usually densely echinulate, spines up to 0.8 μm long ................................................................................ C. subinflatum

8. Macronematous conidiophores slightly wider, 3–5 μm, swellings (4–)5–8(–9) μm wide; conidia longer, up to 25(–35) μm, ornamentation minutely verruculose to verrucose, but not echinulate or spiny ........................................................................................................ C. iridis

9. Conidia formed by macronematous conidiophores 3–33 × (2–)3–6(–7) μm, with age becoming wider, (3.5–)5–9(–11) μm, darker and more thick-walled ................................................................................ C. herbaroides

9. Conidia formed by macronematous conidiophores not becoming wider and darker with age, usually up to 7 μm wide ................................................................................................................ C. herbarum

10. Conidiophores usually with small head-like swellings, sometimes also with a second intercalary nodule; small terminal conidia 4–9 × 2.5–3.5 μm, secondary ramoconidia and occasionally formed ramoconidia 10–24(–31) × 3–5(–7) μm .......................................................... C. bruhaei

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 × 3–5(–6) μm, intercalary conidia 6–16 × 4–6 μm, secondary ramoconidia 12–25(–35) × (3–)5–7(–9) μm ............................................................... C. herbarum

11(5) Small terminal and intercalary conidia 4–15 × 3–5 μm, secondary ramoconidia 16–36(–40) × (4–)5–8 μm, 0–3(–4)-septate, ramoconidia absent .................................................................................................................. C. ossifragi

11. Small terminal conidia, ramoconidia and secondary ramoconidia distinctly narrower, 2–5(–6) μm wide, 0–2(–3)-septate ................................................................. 12

12. Mycelium dimorphic, narrow hyphae 1–3 μm wide, hyaline to subhyaline, thin-walled, hyphae of the second type wider, 3.5–8(–9) μm, pale to dark greyish olivaceous or olivaceous-brown, thick-walled, sometimes even two-layered, 1(–1.5) μm 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 .................................................................................................................. C. spinulosum

13. Conidial ornamentation distinctly echinulate, spiny (baculate, digitate or capitulate under SEM), spines 0.5–1.3 μm long, loose to moderately dense, conidial hila usually situated on small peg-like prolongations or denticles .................................................................................. C. variabile

13. Conidial ornamentation different, minutely verruculose to verrucose, 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) μm 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) μm diam ................................................................. 15
Key to the Davidiella species treated

1. Ascospores not wider than 7 µm when mounted in Shear's solution or lactic acid, apical cell acutely rounded .......... 1
2. Pseudoparaphyses prominent; ascii frequently >95 µm; ascospores (22–)23–26(–28) × (6–)6.5–7(–8) µm .................. D. macrocarpa
3. Pseudoparaphyses mostly absent in older ascostroma; ascii <95 µm .................. D. variabile

2. 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

3. 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

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:


Ascomata pseudothecial, black to red-brown, globose, inconspicuous and immersed beneath stomata to superficial, situated on a reduced stroma, with 1(–3) short, periphysal ostiolar necks; periphysoids frequently growing down into cavity; wall consisting of 3–6 layers of textura angularis. Asci fasciculate, short-stalked or not, bitunicate, subseusalis, 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 luminal 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.


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 conoid conidiogenous loci and conidial hila consisting of a central convex dome and a raised periclinal rim.


Etymology: Refers to Antarctica, where the fungus was collected.

Differt a Cladosporio licheniphilho 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, vellerosus vel verrucosis.

Mycelium immersus und superficialis, dimorphus, branchus, 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 micromatous and macromatous, solitary or in loose groups, arising from plagiotorous 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. Conidigenous 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
geniculate with several conidiogenous loci at the apex, 2–22 × 2–3 μm, pale greyish olivaceous, loci denticulate. Ramoconidia occasionally occurring, cylindrical, up to 30 μm long, 4–5 μm 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 μm wide. Conidia catenate, in branched chains, straight, small terminal conidia obvoid, limoniform or narrowly ellipsoidal, 4–14 × 2.5–4 μm [av. ± SD, 8.5 (± 3.3) × 3.5 (± 0.6)], 0(–1)-septate, secondary ramoconidia ellipsoidal to cylindrical, often with several or numerous conidial hila crowded at the distal end, up to 12, 13–30 × 4–5 μm [av. ± SD, 20.1 (± 5.8) × 4.3 (± 0.5) μm], 0–3-septate, sometimes slightly constricted at the median septum, pale olivaceous-brown or greyish brown, minutely verrucose 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) μm diam, thickened and darkened-refractive; microcyclic conidiogenesis occurring.

Cultural characteristics: Colonies on PDA attaining 9 mm diam after 14 d at 25 ºC, 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 prominent exudates not formed, sporulation dense, covering almost the whole colony. Colonies on MEA attaining 12 mm diam after 14 d at 25 ºC, 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 ºC, 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 μm, with numerous characteristic terminal branches and much shorter, 0–1-septate, smooth conidia, 3.5–13 × 3–7 μm (Heuchert & Braun 2006). Cladosporium lichenicolum 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 foliicolous 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 × 2–6.5(–8) μm, and wider conidiogenous loci and conidial hila, 0.5–3
Cladosporium herbarum species complex

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 μm, C–D = 5 μm, E = 2 μm.


μm (Heuchert et al. 2005); C. alneum, which causes leaf spots on Alnus glutinosa, possesses longer and wider conidiophores, 25–260 × (2–)3–7(–8.5) μm, 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 μm, and wider conidiogenous loci and conidial hila, 1–3 μm diam (Ellis 1972, Schubert 2005).
Cladosporium bruhnei  
≡ Cladosporium herbarum (Pers.: Fr.) Link var. (δ) cerealium Sacc. f.  


Ascomata pseudothecial, black, superficial, situated on a small stroma, globose, up to 250 µm diam; ostioles periphysate, with apical periphysoids present; wall consisting of 3–6 layers of reddish brown textura angularis. Asci fasciculate, bitunicate, subsessile, ovoid to broadly ellipsoid, straight to slightly curved, 8-spored, 65–90 × 16–25 µm; with pseudoparenchymatal cells of the hamathecium persistent. Ascospores tri- to multiserial, 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) × (5.5–)6–7 µm.  

Mycelium superficial, hyphae branched, 1.5–8 µm wide, pluriseptate, broader hyphae usually slightly constricted at the septa and somewhat swollen, hyaline to subhyaline, almost to somewhat verrucose or irregularly rough-walled, sometimes appearing to have a slime coat, walls unthickened. Conidiophores macroconidial, sometimes also microconidial, 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 µm, sometimes even longer, (2–)3–5 µm wide, swellings (4–)5–8 µm wide, pluriseptate, not constricted at the septa, septa sometimes not very conspicuous, subhyaline to pale brown or pale olivaceous, smooth or somewhat verrucose, 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 µm long, proliferation sympodial, with few conidiogenous loci confined to swellings, up to five per swelling, loci protuberant, conspicuous, 1–2 µm 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 × 2.5–3.5 µm [av. ± SD, 6.5 (± 1.5) × 3.1 (± 0.5) µm], aseptate; secondary ramoconidia and occasionally formed ramoconidia ellipsoid to subcylindrical or cylindrical, 10–24(–31) × 3–5(–7) µm [av. ± SD, 16.1 (± 4.1) × 4.1 (± 0.8) µm], rarely up to 40 µm long, 0–1–3-septate, very rarely 5-septate, subhyaline to pale brown or pale olivaceous, minutely verrucose to verrucose (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 µm wide, up to 1 µm high, thickened and darkened-refractive; microcyclic conidiogenesis occurring.  

Fig. 9. Cladosporium bruhnei (CPC 12211). Macro- and micronematous conidiophores and conidia. Scale bar = 10 µm. K. Schubert del.
Cladosporium herbarum SpecieS coMplex

Cultural characteristics: Colonies on PDA reaching 22–32 mm diam after 14 d at 25 ºC, 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 ºC, 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 wrinkles in the colony centre, without prominent exudates, sporulating. Colonies on OA reaching 20–32 mm diam after 14 d at 25 ºC, 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 118.54 = 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 12041.


Substrate and distribution: Living and decaying plant material, man, air, hypersaline and industrial water; widespread.

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 µm 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. villosum, 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 × 6–8.5 µm, RO) was treated as synonymous to D. allicina (ascospores 20–27 × 6–7 µm, 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.


Etymology: Refers to its morphological similarity to Cladosporium herbarum.

Differt a Cladosporio herbaro conidiis polymorphis, 3–33 × (2–)3–6(–7) µm, postremo latioribus, (3.5–)5–9(–11) µm, fuscis et crassitunicatis; et a Cladosporio macrocarpo conidiophoris leniter angustioribus, 3–5 µm latis, nodulis angustioribus, 5–8 µm latis.

Mycelium branched, (1–)2–8 µm 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 multitateral swellings, often numerous swellings in short succession giving them a gnarled appearance, often forming somewhat protruding or prolonged lateral swellings or a branch-
like prolongation below the terminal swelling (due to sympodial proliferation), unbranched or sometimes branched, 30–230 µm long or even longer, 3–5 µm wide, swellings 5–8 µm 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 µm 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 µm 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 × 2–3 µm, 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 µm 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 × (2–)3–6(–7) µm [av. ± SD, 14.5 (± 7.9) × 5.2 (± 1.2) µm], 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 × (3.5–)5–9(–11) µm [av. ± SD, 14.4 (± 6.9) × 7.2 (± 1.9) µm], walls up to 1 µm thick, hila protuberant, 0.8–2(–2.5) µm 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) × 2–3.5 µm [av. ± SD, 15.6 (± 6.2) × 2.9 (± 0.5) µm], 0–1(–3)-septate, subhyaline to pale brown, almost smooth to minutely verruculose, walls unthickened, hila protuberant, 1–1.5 µm diam, thickened and somewhat darkened-refractive.

Fig. 13. Cladosporium herbaroides (CPC 12052). Macro- and micronematous conidiophores and conidia. Scale bar = 10 µm. K. Schubert del.
**Cultural characteristics:** Colonies on PDA attaining 23 mm diam after 14 d at 25 ºC, 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 sparse. Colonies on MEA attaining 24 mm diam after 14 d at 25 ºC, 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 ºC, 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.


**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.


For additional synonyms see Dugan et al. (2004), Schubert (2005).


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

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 µm 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 µm, B–C, F (inset) = 10 µm, D–E = 5 µm, F = 2 µm.

Ascomata pseudothecial, black, globose, erumpent to superficial, up to 200 µm diam, with 1(–3) short, periphysate ostiolar necks; wall consisting of 3–6 layers of medium red-brown textura angularis. Asci fasciculate, bitunicate, subsessile, obvoid to broadly ellipsoid, straight to slightly curved, 8-spored, 65–85 × 13–17 µm. Pseudoparaphyses absent in host material, but remnants observed when studied in culture, hyaline, septate, subcylindrical, anastomosing, 3–4 µm wide. Ascospores tri- to multiseriate, overlapping, hyaline, with irregular luminar 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) × (6–)7(–8) µm; 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 µm wide, hyaline to pale brown and appearing somewhat verruculose, enclosed in a mucoid sheath, with germ tubes being irregular, somewhat nodular.
**Mycelium** superficial, loosely branched, (0.5–)1–5 µm 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 genculate-sinusous, 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 × 3.5–5 µm, swellings 5–8(–9) µm 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 µm 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 µm 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 genculate, unbranched, 5–120 × 1.5–3(–4) µm, pluriseptate, not constricted at septa, cells usually very short, 5–15 µm long, subhyaline to pale brown, almost smooth to minutely verruculose or irregularly rough-walled, sometimes forming clavate conidia, up to 33 µm 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 obvoid, 4–10 × 3–5(–6) µm [av. ± SD, 7.8 (± 1.9) × 4.7 (± 0.9) µm], asperate, intercalary conidia with a single or sometimes up to three distal hila limoniform, ellipsoid to subcylindrical, 6–16 × 4–6 µm [av. ± SD, 12.4 (± 1.6) × 5.3 (± 0.6) µm], 0–1-septate, secondary **ramoconidia** with up to four distal hila, ellipsoid to cylindrical-oblong, 12–25(–35) × (3–)5–7(–9) µm [av. ± SD, 18.8 (± 4.5) × 6.2 (± 0.9) µm], 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 to brown to medium brown or greyish brown, minutely verruculose to verrucose, 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) µm wide, 0.5–1 µm 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 ºC, 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, felyt, 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 ºC, 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 ºC, 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.


**Substrate and distribution:** On fading and decaying plant material, on living leaves (phyllolane fungus), as secondary invader, as an endophyte, isolated from air, soil, foodstuffs, paints, textiles and numerous other materials; cosmopolitan.
Cladosporium herbarum

**SpecieS complex**


**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) × (5–)6–7(–8) µm. 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) × (6–)7–8(–8.5) µm, 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.


For additional synonyms see Dugan *et al.* (2004).


**Fig. 17.** Cladosporium herbarum (CPC 11600). Macro- and micronematous conidiophores and conidia. Scale bar = 10 µm. K. Schubert del.


Mycelium branched, 2–8 µm 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 µm long, 6–11 µm wide, swellings 8–11(−14) µm 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 µm 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 µm 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, soleform 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) × (7–)10–16(−18) µm [av. ± SD, 53.3 (± 17.8) × 12.6 (± 2.2) µm], (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 µm thick, hila protuberant, often stalk-like or conically prolonged, up to 2 µm long, (2–)2.5–3.5(–4) µm 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 ºC, 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 ºC, pale olivaceous-grey to olivaceous-grey, olivaceous-grey reverse, felty, margin slightly 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.

Fig. 20. Cladosporium iridis (CBS 138.40). Conidiophores and conidia. Scale bar = 10 µm. K. Schubert del.

Fig. 21. Cladosporium iridis (teleomorph Davidiella macrospora) (CBS 138.40). A–C. Conidiophores with conidia. D. Conidium. Scale bar = 10 µm.
Cladosporium herbarum SpecieS coMplex

Substrates and distribution: Leaf spot and blotch of Iris spp. including I. crocea, I. florentina, I. foetidissima, I. germanica, I. gueldenstaedtiana, 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).


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) µm wide, conidia (11–)15–23(–28) µm].

For additional synonyms see Dugan et al. (2004), Schubert (2005).

Davidiellae tassianae similis, sed pseudoparaphysibus prominentibus et ascosporis macrocarpibus, (22–)23–26(–28) × (6–)6.5–7(–8) µm.

Ascomata superficial on a small stroma, black, up to 200 µm diam, globose, separate, but developing with 1–3 necks with age; ostioles consisting of pale brown to subhyaline cells, periphysate, with paraphyses growing into the cavity; wall consisting of 3–6 layers of medium brown textura angularis. Pseudoparaphyses present, hyaline, subcylindrical, septate, anastomosing, 3–4 µm diam; hamathelial cells persistent in cavity. Asci fasciculate, bitunicate, subsessile, broadly ellipsoid with a long tapered stalk, straight to curved, 8-spored, 70–110 × 15–20 µm. Ascospores tri- to multiseriate, overlapping, hyaline, guttulate, irregular lumina.
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) × (6–)6.5–7(–8) µm; mucoid sheath rarely observed, mostly absent.

Mycelium unbranched or loosely branched, 1–4.5(–5) µm 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 plagiotropic 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 × (3–)4–6 µm, swellings 5–10 µm wide, pluriseptate, sometimes slightly constricted at septa, pale to medium brown or olivaceous-brown, somewhat paler at apices,

Cladosporium herbarum Species Complex

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 µm long, with up to 12 loci per cell, usually with up to six, loci conspicuous, protuberant, (1–)1.5–2 µm 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. 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 µm, D, F = 5 µm, E = 2 µm.
Conidia formed by micronematous conidiophores, obovoid to ellipsoid, 1.5–3 µm 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 µm diam, thickened and somewhat darkened-refractive. Conidia catenate in branched chains, small terminal conidia subglobose, obovoid, oval, limoniform, 4–11 × (3–)4–6 µm [av. ± SD, 7.6 (± 1.9) × 5.0 (± 0.8) µm], aseptate, intercalary conidia broadly ovoid-ellipsoid, 10–17 × (4.5–)5–9 µm [av. ± SD, 12.7 (± 2.1) × 6.8 (± 0.8) µm], 0–1-septate; secondary ramoconidia broadly ellipsoid to subcylindrical, 14–25(–30) × (5–)6–9(–10) µm [av. ± SD, 19.4 (± 3.5) × 7.6 (± 1.0) µm], 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), dark brown, verruculose to echinulate (muricate under SEM), brown to medium olivaceous-brown or brown, sometimes even 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), dark brown, verruculose to echinulate (muricate under SEM), or branched chains, subglobose, obovoid to limoniform, ellipsoid or fusiform, 2.5–16 × 1.5–5 µm, 0(–1)-septate, few longer conidia subcylindrical to clavate, up to 37(–43) µm 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 µm diam, thickened and darkened-refractive.

Cultural characteristics: Colonies on PDA reaching 30–43 mm in diam after 14 d at 25 ºC, 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 ºC, 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 ºC, 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, flat, white to smoke-grey or grey-olivaceous, felty, growth flat, regular, without exudates, sporulating.


Substrate and distribution: Decaying plant material, human, hypersaline water, water, widespread.


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 isonome material,
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 × (4–)5.5–8(–9) µm, 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 µm, wider and more frequently septate conidia [small terminal conidia 4–11 × (3–)4–6 µm versus 4–10 × 3–5(–6) µm in *C. herbarum*, intercalary conidia 10–17 × (4.5–)5–9 µm versus 6–16 × 4–6 µm in *C. herbarum*, secondary ramoconidia 14–25(–30) × (5–)6–9(–10) µm versus 12–25(–35) × (3–)5–7(–9) µm 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) µm wide, and also the conidia can be somewhat wider, sometimes up to 13(–15) µm.

*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 luminar ascospore inclusions.


≡ *Heterosporium ossifragi* (Rostr.) Lind, Dan. fung.: 531. 1913.
Fig. 27. Cladosporium ossifragi (CBS 842.91). A. Macronematous conidiophore. B. Micronematous conidiophore. C–D. Conidia. E. Conidia and microcyclic conidiogenesis. Scale bars = 10 µm.

Fig. 28. Cladosporium ossifragi (CBS 842.91). A. Survey on different secondary ramoconidia and conidia. B. Details of conidia and hila. 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 hila. D. Formation of a new conidium. Note the broad scar behind it (> 1 µm). E. Formation of a new conidium from a smooth-walled stalk. F. Hila 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 µm, B–D, F = 2 µm, E = 5 µm.
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. Conidiothecium macroseta and microseta, 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, plurisepate, 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, obvoid to ellipsoid, 4–15 × 3–5 µm [av. ± SD, 9.3 (± 3.7) x 4.0 (± 0.7) µm], 0–1-septate, not constricted at the septa, pale brown, hila 0.8–1 µm diam, secondary ramosconidia cylindrical, sometimes ellipsoid or subbisporous, 16–36 (–40) × (4)–5–8 µm [av. ± SD, 26.6 (± 7.4) x 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, septate sometimes slightly sinuous, pale brown to pale medium brown, densely verruculose, verrucose to echinulate walls usually distinctly thickened, sometimes even two-layered, up to 1 (–2) µm thick, protoplasm granular, often clearly contrasting in vivo.

Cultural characteristics: Colonies on PDA reaching 53 mm diam after 14 d at 25 ºC, greenish olive-brown, grey-olive-brown 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 ºC, olive-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 ºC, 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.

Substrate and distribution: Causing leaf spots on Narthecium ossifragum; Europe (Austria, Denmark, Germany, Great Britain, Ireland, Norway).


Notes: Type material of Naplicadium ossifragi is not preserved in Rostrup’s herbarium (on Narthecium ossifragum, Faeroe Islands, Viderø, Viderejde and Østerø, Svina, sine dato, leg. O. Lind & 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 Narthecium ossifragum, Denmark, Tänder, Rämé, peat bog by Twismark, Jul.–Aug. 1901, Jaap), but not designated as type, is not preserved (David 1997). It is neither deposited at BHG 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.


Etymology: Epithet derived from its similar morphology to Cladosporium iridis.

Differs a Cladosporio iridis conidiis 0–3-septatis, brevioribus et latioribus, 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, gulletate or protoplasma appearing granular, sometimes enveloped by a slime coat. Conidiothecium 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 olive-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, protoplasma 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.
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 × (9–)11–16(–18) µm [av. ± SD, 19.2 (± 4.3) × 14.2 (± 3) µm], 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 × 12–19(–21) µm [av. ± SD, 41.5 (± 6.8) × 17.1 (± 2.1) µm], 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 µm 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 µm 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 µm diam, thickened and darkened-refractive; microcyclic conidiogenesis not observed.

Cultural characteristics: Colonies on PDA attaining 6 mm diam after 14 d at 25 ºC, 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
Cladosporium herbarum
SpecieS coMplex
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 ºC, 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 umbontate, sometimes with elevated colony centre, prominent exudates lacking, sporulation sparse. Colonies on OA attaining 8 mm diam after 14 d at 25 ºC, 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.


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–)30–75x(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.


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 verruculosis; 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 micromematous, arising as lateral branches of plagiotrephous 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

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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.
branched, branches often only as short lateral prolongations, mainly formed below a septum, 14–110 × 2–4 µm, 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) µm long, proliferation sympodial, with few conidiogenous loci, mostly 1–3, loci sometimes situated on small lateral prolongations, protuberant, 0.5–1.5(–2) µm diam, thickened and somewhat darkened-refractive. Ramoconidia formed, cylindrical-oblong, up to 47 µm long, 2–4 µm 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 µm 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 × 2–4(–4.5) µm [av. ± SD, 5.1 (± 1.3) × 3.1 (± 0.6) µm], aseptate, without distal hilum or with a single apical scar, intercalary conidia ellipsoid to subcylindrical, 8–15 × 3–4(–4.5) µm [av. ± SD, 11.5 (± 2.4) × 3.6 (± 0.5) µm], 0–1-septate; secondary ramoconidia subcylindrical to cylindrical-oblong, 17–35 × 3–4(–5) µm [av. ± SD, 22.5 (± 5.6) × 3.7 (± 0.5) µm], 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) µm diam, somewhat thickened and darkened-refractive; microcyclic conidiogenesis occurring.

Cultural characteristics: Colonies on PDA reaching 46–49 mm diam after 14 d at 25 ºC, 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 ºC, grey-olivaceous to olivaceous-grey, velvety, olivaceous-grey to

Fig. 31. Cladosporium ramotenellum (CPC 12043). Conidiophores and conidia. Scale bar = 10 µm. K. Schubert del.
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.
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 ºC, 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; Sečovlje, 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) µm 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) × (2.5–)3–5(–6) µm. 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 × 2–6.5 µm) which become larger and more frequently septate with age (up to 32 µm 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) × 2–5 µm, becoming distinctly swollen, darker, longer and wider with age, 5–7 µm, with the septa often being constricted (Schubert 2005).

**Etymology:** Refers to the usually distinctly sinuous conidiophores.

*Differs from Cladosporium herbarum* conidiophores distincte sinuosis, conidiis solitariis vel breve catenatis, catenis non ramosis, echinulatis.

**Mycelium** sparingly branched, 1–7 µm 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 plagiotropic hyphae or terminally from ascending hyphae, erect, more or less straight to flexuous, often once or several times slightly to distinctly geniculate-sinuous, 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 × 5–7 µm, shoulders up to 10 µm 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 µm thick. **Conidiogenous cells** integrated, terminal or intercalary, often slightly to distinctly geniculate-sinuous, nodulose with small to large laterally swollen shoulders, 8–30 µm 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) µm diam, mainly 2 µm, 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 × (5–)6–8 µm [av. ± SD, 14.5 (± 2.5) × 6.6 (± 0.7) µm], 0–1-septate, not constricted at the septa, septum more or less median, pale greyish brown, densely echinulate, spines up to 1 µm long, walls thickened, apex mostly broadly rounded or sometimes attenuated, towards the base mostly distinctly attenuated forming a peg-like prolongation, up to 2 µm long, hilum protuberant, 1.2–2 µm diam, mainly 2 µm, somewhat thickened and darkened-refractive; microcyclic conidiogenesis not observed.

**Cultural characteristics:** Colonies on PDA attaining 20 mm diam after 14 d at 25 ºC, pale olivaceous-grey due to abundant aerial mycelium, olivaceous-grey to iron-grey towards margins, 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 ºC, 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
prominent exudates. Colonies on OA attaining 18 mm diam after 14 d at 25 ºC, 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.


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. arthropodi 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).

**Etymology:** Refers to its nodulose conidiophores.

Differt a Cladosporio bruhnei conidiophoris cum nodulis angustioribus, 3–6.5 µm latis, conidiis brevioribus, 4–17(–22) µm longis, spinulosis, cum spinulis ad 0.8 µm longis; et a Cladosporio spinuloso conidiophoris nodulosis, conidiis spinulosis, cum spinulis brevioribus, ad 0.8 longis, locis conidiogenis et hilis latoribus, (0.5–)1–2 µm latis.

**Mycelium** unbranched or occasionally branched, 1.5–3 µm wide, later more frequently branched and wider, up to 7 µm 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 × (1.5–)2.5–4.5(–5.5) µm, swellings 3–6.5 µm 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 µm 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 µm long, proliferation sympodial, with several loci, up to four situated at nodules or on lateral swellings, protuberant, conspicuous, denticulate, (0.8–)1–2 µm diam, thickened and darkened-refractive. **Conidia** catenate, in branched chains, more or less straight, numerous globose and subglobose conidia, ovoid, obvoid, broadly ellipsoid to cylindrical, 4–17(–22) × (2.5–)3.5–5.5(–7) µm [av. ± SD, 11.7 (± 4.6) × 4.5 (± 0.8) µm], 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 µm high, sometimes irregularly verrucose with few scattered tubercles.

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*Fig. 37. Cladosporium subinflatum (CPC 12041). Macro- and micronematous conidiophores and conidia. Scale bar = 10 µm. K. Schubert del.*
Schubert et al.

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 µm diam, thickened and darkened-refractive; microcyclic conidiogenesis observed.

**Cultural characteristics:** Colonies on PDA attaining 29 mm diam after 14 d at 25 ºC, 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 ºC, 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 diffusely grown, 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 ºC, 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) µm], nodulose macronematous conidiophores with conidiogenous loci confined to swellings, wider conidiogenous loci and hila, (0.8–)1–2 µm, and spiny conidia with shorter spines than in *C. spinulosum* (up to 0.8 µm versus 0.5–1.3 µm 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 µm, and longer conidia 4–24(–31) µm, rarely up to 40 µm long which are minutely verruculose to verrucose but not spiny.

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**Fig. 40.** *Cladosporium subtilissimum* (CBS 113754). Macro- and micronematous conidiophores and conidia. Scale bar = 10 µm. K. Schubert del.

Etymology: Refers to its narrow conidiophores and conidia.

Differt a Cladosporio cladosporioide conidiophoris et conidiis semper asperulatis ad verruculosis, conidiis 0–1(–2)-septatis.

Mycelium unbranched or sparingly branched, 1–5 µm wide, septate, smooth to minutely verruculose, walls unthickened or almost so, protoplasm somewhat guttulate or granular. Conidiophores macronematous and micronematous, arising laterally from plagiotropic 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 × 2–4 µm, 0–4-septate, not constricted at the septa, smooth to minutely verruculose to verruculose, walls unthickened or slightly thickened, protoplasm some guttulate or granular. Conidiogenous cells integrated, terminal or pleurogenous, sometimes also intercalary, filiform to narrowly cylindrical, non-nodulose, 14–57 µm long, with usually sympodial clusters of pronounced conidiogenous loci at the apex or on a lower level, intercalary conidiogenous cells usually with a short denticle-like lateral outgrowth below a septum, protuberant, denticulate, 1.2–2 µm 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) µm long, 1.5–4 µm 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 µm 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 ovoid, limoniform or fusiform, 4–9 × 2–3.5 µm [av. ± SD, 6.4 (± 1.5) × 2.8 (± 0.4) µm], with up to three distal scars, aseptate, hila (0.5–)0.8–1 µm diam, intercalary conidia narrowly ellipsoid, fusiform to subcylindrical, 9–18 × 3–4(–6) µm [av. ± SD, 13.0 (± 2.5) × 3.8 (± 0.3) µm], 0(–1)-septate, hila 1–1.2(–1.8) µm diam, with up to four distal scars, secondary ramoconidia ellipsoid, fusiform or subcylindrical, (13)–17–32(–37) × 3–5(–6) µm [av. ± SD, 21.4 (± 4.4) × 4.1 (± 0.5) µm], 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) µm 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 verruculose or granular, hila protuberant, denticulate, 0.5(–)0.8–2(–2.2) µm diam, thickened and darkened-refractive; microcyclic conidiogenesis occasionally observed.

Cultural characteristics: Colonies on PDA attaining 24 mm diam after 14 d at 25 °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

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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.


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 teneillum (CPC 12053). Macro- and micronematous conidiophores and conidia. Scale bar = 10 µm. K. Schubert del.

Fig. 44. Cladosporium teneillum (CPC 12053). A–C. Macronematous conidiophore. D. Micronematous conidiophore. F. Ramoconidium and conidia. Scale bars = 10 µm.
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.

**Cultural characteristics:** Colonies on PDA reaching 27–34 mm diam after 14 d at 25 ºC, 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, feltly, 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 ºC, 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 ºC, grey-olivaceous, olivaceous-

**MycoBank MB504581.**

**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 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 µm, B = 10 µm, C, E = 2 µm, D = 5 µm.
Cladosporium tenellum, which morphologically also resembles C. ramotenellum, possesses longer and narrower, 0–3-septate conidia, 2.5–35 × 2–4(–5) µm, but forms only few conidiogenous loci and conidial hila at the apices of conidiophores and conidia.


≡ Cladosporium subnodosum Cooke, Grevillea 17(83): 67. 1889.
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 µm, D = 2 µm, E = 5 µm.


Davidiellae tassianae similis, sed ascosporis maioribus, (22–)26–30(–35) × (7–)7.5–8(–9) µm, et ascis latioribus, plus quam 18 µm.

Ascomata pseudothecial, black, superficial, situated on a small stroma, globose, up to 250 µm 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 × 18–28 µm; 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, mediately 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) × (7–)7.5–8(–9) µm.

Mycelium immersed and superficial, irregularly branched, aerial mycelium twisted and spirally coiled, 1–3 µm 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 µm, 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-sinuous 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 × (2.5–)3–6 µm, swellings (3–)6–11 µm wide, septate, not constricted at the septa, pale to medium olivaceous-brown or brown, usually verruculose, walls somewhat thickened, about 1 µm 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 plurisepaete, 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 conidigenous 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; micrcyclic conidiosgenesis occurring.

Cultural characteristics: Colonies on PDA attaining 29 mm diam after 14 d at 25 ºC, olivaceous to olivaceous-grey or iron-grey, iron-grey or olivaceous-grey reverse, velvety to powdery, margin regular, entire edge to fimbriate, 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 ºC, 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 ºC, 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.


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.).


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 ornamentated, 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 conidiosgenesis 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 herbaroides* 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. bruheii* and *C. herbaroides* are rather long, and can appear as aerial hyphae.

An important feature of the conidiophore is the location were 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 nodulous, 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. bruheii*, *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-corneate 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 (aculate, 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. bruheii* (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 muriculate. 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 (Gronenwald 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^S) was high (0.3914), indicating an overabundance of clonality and/or inbreeding, the latter possibly matching with observed homothallism of Davidiella telemorphs. 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 (θ) around 0.14, but in most cases there was considerable overlap in representation of motifs, with θ 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 MAT1-1-1 and MAT1-2-1, 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.
(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 creates 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°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...
in glasshouses, while a few were collected from outdoor
grown tomatoes. Unfortunately, records on the year of isola-
tion 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 manu-
facturer’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-I and MAT1-2-I, respec-
tively, of different Mycosphaerella species (Groenewald et al.,
2006), were used to screen nine C. fulvum strains for the pres-
ence 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-I and
MAT1-2-I sequences obtained. These primers in combination with
primers from the DNA walking kit (Seegene Inc., Rock-
ville, Maryland), were used to amplify the full length
sequences of MAT1-1-I and MAT1-2-I as well as for
sequencing of the complete MAT idiomorphs from
strains #22 (MAT1-1) and #54 (MAT1-2). In all cases,

<table>
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matting-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.softerry.com/berry.phpml). 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 (Biologeo bv, Niijmegen, 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 H2O. 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 GFXTM 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 399 bp (MAT1-1_P3F) and 881 bp (MAT1-1_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 (Biologeo 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 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 Cockermans’s (1984) theta (θ), the equivalent of Wright’s Fst. We interpreted the resultant θ(Fst) values based on Wright’s (1978) suggested qualitative guidelines of θ(Fst) values. In that respect, θ(Fst) = 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 θ(Fst) was generated using 10,000 bootstrap replicates. Confidence limits around θ 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: dememorization number = 1000, number of batches = 1000, number of iterations = 10,000. The null hypothesis for genetic differentiation was H0: “the allele distribution of AFLP loci is identical across different geographic collections.}

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Primers used for the AFLP analysis</th>
</tr>
</thead>
</table>
| Primers | Sequence (5’-3’)<br />
| E00     | GACTGGCTACAAATTC                  |
| E15     | E00 + CA                         |
| E18     | E00 + CT                         |
| E20     | E00 + GC                         |
| E23     | E00 + TA                         |
| M00     | GATGGAATCTGAGTAA                  |
| M00     | M00 + C                          |
| M00     | M00 + G                          |
| M04     | M00 + T                          |

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. fumago* strains examined. This fragment showed highest similarity to the alpha-domain of MAT1-1-1 from *M. graminicola* and other filamentous ascomycetes 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. fumago* 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. fumago* 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* sequences of several *Cercospora* species (Greenwald et al., 2006). The positioning of the third putative intron present in MAT1-1-1 of *C. fumago* is in perfect synteny with the third intron suggested for the *Cercospora* species (Fig. 2). Blastp analysis showed that MAT1-1-1 from *C. fumago* 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 ascomyceteous 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. fumago*. This ORF is located on the opposite DNA strand 350 bp upstream of the MAT1-1 gene and it has been designated as ORF1-1-1 (Fig. 1A). ORF1-1-1 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 ORF1-1-1 and any other proteins currently present in the NCBI GenBank database.

Using the degenerate primers MgMSpMAT1-2f2 and MgMSpMAT1-2r1 a 236 bp fragment was amplified from all strains screened as well as a 333 bp fragment, which was found only in the *C. fumago* strain of the test panel that did not generate a PCR product using the degenerate MAT1-1 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](image-url)

**Fig. 1.** In scale physical map of the mating-type idiomorphs (A) and the mating-type genes (B) of *C. fumago*. (A) Idiomorphs are presented as boxes and their flanking regions as solid black lines. The positioning and transcriptional direction of the mating-type genes in each idiomorph is indicated by an arrow. “Islands” of high homology between the two idiomorphs are shown as shaded-grey boxes (I, Ia, Ib). The additional putative ORFs (ORF1-1-2 and ORF1-2-2) identified in each idiomorph are indicated by arrows and putative introns are shown in black. Segments of these ORFs that are only partially present in the opposite idiomorphs 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.
both upstream and downstream directions of this 333 bp fragment, generated a 6.344 kb fragment that contained the full MAT1-2 idiomorph along with 881 and 1910 bp of 5′/H11032- and 3′/H11032-anking sequences, respectively. The MAT1-2 idiomorph is 3.553 kb long and contains at least a putative MAT1-2-1 ORF flanked by 1.259 kb of 5′-sequences and 985 bp of 3′-idiomorph-sequences (Fig. 1A). The predicted 1.155 kb MAT1-2-1 ORF is interrupted by three putative introns of 51, 52, and 52 bp, respectively, and encodes a protein of 384 amino acids (Fig. 1B). Two of the predicted introns disrupt the HMG-domain of MAT1-2-1 and to our knowledge this is the first time that more than one intron is identified in this domain of MAT1-2-1. The predicted position of the first intron (Ile138) disrupting the HMG-domain, corresponds to the position of the intron present in the same region of other fungal species (Fig. 2). No expression data are available that could confirm the existence of the additional predicted intron. However, when this intron is not spliced this would result in a premature stop codon for the protein (Fig. 2). Blastp analysis showed that the MAT1-2-1 protein of C. fulvum shows highest similarity to MAT1-2-1 proteins from C. beticola (46% identity, 55% similarity), M. graminicola (55% identity, 65% similarity), S. passerinii (51% identical, 61% similarity), A. fumigatus (26% identity, 43% similarity), and other ascomycetous fungi. Sequence similarity was particularly high within the HMG-domains of these proteins. Sequence analysis revealed the presence of an additional putative ORF within the MAT1-2 idiomorph of C. fulvum (Cercospora zeae-maydis (DQ264747, DQ264761), Cercospora beticola (DQ192581, DQ192582), Mycosphaerella graminicola (AAL30838, AAL30836), and Septoria passerinii (AAO49357, AAO49358).
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 \((P < 10^{-6})\). 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. MAT1-1-1 and MAT1-2-1 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, MAT1-1-1 was observed at a higher frequency than MAT1-2-1 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

<table>
<thead>
<tr>
<th>Collection</th>
<th>(N_{strains}) ((N_{genot})^a)</th>
<th>MAT-type Frequency</th>
<th>(\chi^2) values(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>86 (75)</td>
<td>MAT1-1-1 0.61 (0.63)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Europe</td>
<td>50 (41)</td>
<td>MAT1-1-1 0.64 (0.70)</td>
<td>2.98 (4.81)</td>
</tr>
<tr>
<td>Americas</td>
<td>15 (15)</td>
<td>MAT1-1-1 0.40 (0.40)</td>
<td>3.92 (7.05)</td>
</tr>
<tr>
<td>Japan</td>
<td>12 (11)</td>
<td>MAT1-1-1 0.83 (0.82)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Turkey</td>
<td>4 (4)</td>
<td>MAT1-1-1 0.5 (0.5)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Former USSR</td>
<td>2 (0)</td>
<td>MAT1-1-1 0.0 (0.0)</td>
<td>n.d.</td>
</tr>
<tr>
<td>New Zealand</td>
<td>2 (2)</td>
<td>MAT1-1-1 0.5 (0.5)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Africa</td>
<td>1 (1)</td>
<td>MAT1-1-1 0.0 (0.0)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\(^a\) Numbers refer to the actual number of strains. Numbers inside the parenthesis refer to the data as clone-corrected for haplotypes only.

\(^b\) \(\chi^2\) “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.

### Table 5

<table>
<thead>
<tr>
<th>Nucleotide substitutions</th>
<th>Amino acid substitutions</th>
<th>Strains containing the substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT1-1-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G &gt; A 159 bp</td>
<td>Gly52(^c) &gt; Lys</td>
<td>#31, #41, #42, #51, #74, #78, #85</td>
</tr>
<tr>
<td>G &gt; C 435 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C &gt; T 1856 bp</td>
<td>Ser334 &gt; Ser (silent)</td>
<td>#31, #41, #42</td>
</tr>
<tr>
<td>MAT1-2-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T &gt; C 1067 bp</td>
<td>Pro304 &gt; Pro (silent)</td>
<td>#30</td>
</tr>
<tr>
<td>C &gt; T 1270 bp</td>
<td>Pro372 &gt; Leu</td>
<td>#30</td>
</tr>
<tr>
<td>C &gt; A 1271 bp</td>
<td>Pro372 &gt; Leu</td>
<td>#30</td>
</tr>
</tbody>
</table>

\(^a\) Indicates position of the substitution relative to the A nucleotide (+1 bp) of the ATG start codon.

\(^b\) 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), 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

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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). Pairwise 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

![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.](image)
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.](image)

Table 7

<table>
<thead>
<tr>
<th></th>
<th>Europe</th>
<th>Americas</th>
<th>Japan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td>***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Americas</td>
<td>(P &lt; 0.0001)</td>
<td>(\theta = 0.213^a (0.293-0.139^b))</td>
<td>(\theta = 0.235^a (0.347-0.130))</td>
</tr>
<tr>
<td>Japan</td>
<td>(P &lt; 0.0001)</td>
<td>***</td>
<td>(\theta = 0.133 (0.194-0.071))</td>
</tr>
</tbody>
</table>

\(^a\) Significant at the \(P < 0.05\) level.

\(^b\) 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 Dothideomycetes 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; Pogge, 2001; Schoch et al., 2006). However, although the individual MAT genes of C. fulvum are highly similar to the MAT genes from the Dothiomyce-tes, 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 MAT1-2-2 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-spe-cific 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 patho-gen has been attributed to the lack or failure of some other important components of the mating signal-transduction pathway, and not to disfunctionality 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 Mycosphae-rella 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− mutants of a closely related and sexually highly active species, such as M. grami-nicola, 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 equilib-rium 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 geno-type-corrected collections analyzed, therefore suggesting that asexual propagation is predominant in the epidemi-ology 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 mainte-nance of cell wall integrity, virulence and other cellular pro cesses (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 partic-u-larly 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. ful-vum 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 (Arnaud et al., 1994; Arnaud 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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**References**


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 Cladosporium anamorphs was recently segregated under the name Davidiella (Braun et al. 2003); it is a close sister group of other Mycosphaerella.
be the only sporulation observed in most ecological and endophyte studies, we consider it useful to also formally name this conidial state. Fresh ascocarps of *M. punctiformis* were collected on dead fallen leaves of the host species *Quercus robur*, checked for discharges on to 2% malt extract agar (MEA). We also obtained ecological data from a biodiversity study of foliar ascomycetous endophytes of *Quercus* in the ephysite 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 ascocarps were collected in March to May of 2002 and 2003 in the forested area ‘De Stompet’ in The Netherlands, from three mature trees of *Quercus robur*. Leaves were incubated in a moist chamber for several hours in the laboratory at 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 cm² 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 °C 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 °C, 30 cycles (1 min 95 °C, 1 min 55 °C, 2 min 72 °C) followed by a final extension of 5 min at 72 °C. 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 GFX 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 10000. 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 sequences were matched using SeqMan (DNASTAR, Madison, WI).
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-frigidae, 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**

*Ascomata* developing on fallen dead leaves, predominantly hypophyllous, black, subepidermal, erumpent to superficial, globose, 70–110 μm diam; apical ostiole 5–10 μm wide; wall consisting of 2–3 layers of medium brown textura angularis. **Asci** apophysate, fasciculate, bitunicate, subsessile, cylindrical, straight, 8-spored, 30–45 × 5–7(–9) μm. **Ascospores** multisieriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obfuse ends, widest just above the septum, medially 1-septate, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (6–)8–9(–10) × (2–)3 μm (av. 9 × 3 μm). Germinating ascospores hyaline, distorting, forming germ tubes 4–6 μm 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 °C): **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...
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*Note: CBS, Centraalbureau voor Schimmelcultures; MPPD, MycoBank; ATCC, American Type Culture Collection; IMI, International Mycological Institute; STE, State Type Ectomycorrhizal; UC, University of California; UB, University of British Columbia.*
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 m, recta vel geniculata-sinuosa, hyalina, levia; cicatrices conidiales leniter incrassatae et fuscae, circa 1 m latae; conidia formed holoblastically, hyaline, walls smooth to minutely roughened, hila conspicuous, thickened, darkened, refractive, 0.5–1(–1.5) m lata; conidia primaria solitaria, ovoidea, ellipsoidea vel subcylindrica, aseptata, rounded at the top and somewhat attenuated towards the base, 6–15 × 2–4 μm; primary conidia solitary, ovoid, or ellipsoid to subcylindrical, aspetate, rounded at the top and somewhat attenuated towards the base, 6–15 × 2–4 μm; secondary conidia catenate, often in branched, acropetal chains, straight to curved, 0–1-septata, 6–15 μm long.


Conidiophores simple, subcylindrical or cylindrical, (6–)10–30 × 2.5–4(–5) μm, straight or genticulate-sinuous, 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, aspetate, 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 ovoid to ellipsoid-fusiform, 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; conidial 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).
Mycosphaerella punctiformis revisited

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.

Fig. 3–4. Mycosphaerella punctiformis, epitype (CBS herb. 7949). Fig. 3. Ascospores and asci in planta. Fig. 4. Germinating ascospores on MEA. Bars = 10 μm.


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 spermatiophores with acropleurogenous openings; spermatia ellipsoid to subcylindrical, with rounded ends, hyaline, smooth-walled, aseptate, 3–4(–5) × 1–1.5 μm, whitish in mass.


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. Verkley
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 μm.

Fig. 16. *Mycosphaerella punctiformis* (CBS 113265 – ex-epitype). Conidiogenous cells and conidia on MEA. Bar = 10 μm.
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 8–10 × 2–3 μm.


**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 (11–13 × 3.5–5 μm 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 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 asymptptomatically 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 ascii (35–50 × 9–12 μm) and longer ascospores (13–20 × 3.5–5 μm, av. 17 × 4.5 μm), 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.

**REFERENCES**


Corresponding Editor: H. T. Lambsch
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, 1951; Rasmusson and Rogers, 1958; Rasmusson and Rogers, 1961; Rasmusson and Rogers, 1963*).
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; Rasmussen 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., 1996c).

Table 1
Summary information about the isolates of Septoria passerinii and Mycosphaerella graminicola used in this study

<table>
<thead>
<tr>
<th>Species Isolate</th>
<th>Collection location</th>
<th>Mating type</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. passerinii⁶ P62</td>
<td>North Dakota, USA</td>
<td>mat 1-1</td>
</tr>
<tr>
<td>P63</td>
<td>North Dakota, USA</td>
<td>mat 1-1</td>
</tr>
<tr>
<td>P64</td>
<td>North Dakota, USA</td>
<td>mat 1-1</td>
</tr>
<tr>
<td>P65</td>
<td>North Dakota, USA</td>
<td>mat 1-1</td>
</tr>
<tr>
<td>P66</td>
<td>North Dakota, USA</td>
<td>mat 1-2</td>
</tr>
<tr>
<td>P67</td>
<td>North Dakota, USA</td>
<td>mat 1-2</td>
</tr>
<tr>
<td>P68</td>
<td>North Dakota, USA</td>
<td>mat 1-1</td>
</tr>
<tr>
<td>P71b</td>
<td>North Dakota, USA</td>
<td>mat 1-1</td>
</tr>
<tr>
<td>P75</td>
<td>North Dakota, USA</td>
<td>mat 1-1</td>
</tr>
<tr>
<td>P78</td>
<td>Minnesao, USA</td>
<td>mat 1-2</td>
</tr>
<tr>
<td>P81</td>
<td>Minnesao, USA</td>
<td>mat 1-2</td>
</tr>
<tr>
<td>P83b</td>
<td>North Dakota, USA</td>
<td>mat 1-2</td>
</tr>
<tr>
<td>M. graminicola IPO323</td>
<td>The Netherlands</td>
<td>mat 1-1</td>
</tr>
<tr>
<td>IPO4269</td>
<td>The Netherlands</td>
<td>mat 1-2</td>
</tr>
</tbody>
</table>

a The isolates of S. passerinii were as reported previously by Goodwin et al. (2003).
b 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

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Cultivars for crossing</th>
<th>Locations for crossing</th>
</tr>
</thead>
<tbody>
<tr>
<td>P71 × P83</td>
<td>Topper 33 and Kindred</td>
<td>Wageningen and West Lafayette</td>
</tr>
<tr>
<td>P71 × P83</td>
<td>Topper 33 and Kindred</td>
<td>Wageningen</td>
</tr>
<tr>
<td>P71 × P83</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P62 × P81</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P62 × P83</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P62 × P81</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P63 × P78</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P64 × P81</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P65 × P66</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P68 × P67</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P71 × P81</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P71 × P83</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P75 × P78</td>
<td>Kindred</td>
<td>West Lafayette</td>
</tr>
<tr>
<td>P63 × P67</td>
<td>Topper 33 and Kindred</td>
<td>Wageningen and West Lafayette</td>
</tr>
<tr>
<td>IPO323 × IPO94269</td>
<td>Taichung 29</td>
<td>Wageningen</td>
</tr>
<tr>
<td>P71 × IPO94269</td>
<td>Topper 33 and Taichung 29</td>
<td>Wageningen</td>
</tr>
<tr>
<td>IPO323 × P83</td>
<td>Topper 33 and Taichung 29</td>
<td>Wageningen</td>
</tr>
</tbody>
</table>

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 (Genentra 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 MgCl₂, 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-F</td>
<td>CTTCTTGCTCGCCACACGGG</td>
<td>mat 1-1 and mat 1-2 PCR</td>
</tr>
<tr>
<td>Alpha(584)(R)</td>
<td>CGGTATGAGGATGGGAAGAAGG</td>
<td>mat 1-1 PCR</td>
</tr>
<tr>
<td>HMG(849)(R)</td>
<td>TAGTCGGGACCTGAAGGCTG</td>
<td>mat 1-2 PCR</td>
</tr>
<tr>
<td>OPA-9</td>
<td>GGTTAACGCC</td>
<td>RAPD</td>
</tr>
<tr>
<td>EcoRI adapter</td>
<td>CTCTGATAGTCGGTAC</td>
<td>AFLP</td>
</tr>
<tr>
<td>MseI adapter</td>
<td>AATTTGAATCCAGGTCGAT</td>
<td>AFLP</td>
</tr>
<tr>
<td>TACTCAGGACTCAT</td>
<td>GACGATTGATCCGCTGAG</td>
<td>AFLP</td>
</tr>
<tr>
<td>E00</td>
<td>GCTGCGTACCAATTC</td>
<td>AFLP</td>
</tr>
<tr>
<td>M00</td>
<td>GATGAGTCCTGAGTAA</td>
<td>AFLP</td>
</tr>
<tr>
<td>E19</td>
<td>GATTCGGAATCATCTC</td>
<td>AFLP</td>
</tr>
<tr>
<td>M16</td>
<td>GATTCGGAATCATCTC</td>
<td>AFLP</td>
</tr>
<tr>
<td>ITS4</td>
<td>TCCCTCCGTATTGATGC</td>
<td>ITS sequencing</td>
</tr>
<tr>
<td>ITS5</td>
<td>GGAAGTAAAGTGTCAACAGG</td>
<td>ITS sequencing</td>
</tr>
</tbody>
</table>
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 buffer, 0.25 μl of 50 mM MgCl₂, 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 AFLWin Evaluation software (Amersham Pharmacia Biotech, Roesendaal, 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 MgCl₂, 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 Sau3A1 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 Leptosphaeria, and four species of Para-phaeoasphaeria (including P. michotii) were isolated commonly. In addition, two-celled ascospores of Didymella tassiana, the teleomorph of Cladosporium herbarum, also were encountered regularly on older leaf material. Single-sporo 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 (*mat*1-1:*mat*1-2 = 10:7; $\chi^2 = 0.53$; $P = 0.05$) typical for an organism with a heterothallic, bipolar mating system (data not shown).
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 *Eco*RI-GA and *Mse*I-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 *Sau*3AI. 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 multicelled 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
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 telemorph for this fungus that was hitherto considered to be asexual (Cunfer and Ueng, 1999). Therefore, we proceeded to test the hypothesis of a functional telemorph by crossing isolates of *S. passerinii* with opposite mating types using the *in planta* protocol developed for the closely related sexual species *Mycosphaerella graminicola* (Kema et al., 1996c). This led to the generation of the telemorph 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 telemorph 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 telemorph 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 telemorph. 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* telemorph 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 telemorph 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 (pynnidiospores) of the closely related *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 telemorph 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 (Buayen 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.

Acknowledgments

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References


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 β-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.

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 (Ein Gedi, 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 μm), 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 Schimmelculturen (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.

<table>
<thead>
<tr>
<th>Strain Nr.*</th>
<th>Source</th>
<th>Geography</th>
<th>GenBank accession Nr.*</th>
<th>ITS rDNA / 18S rDNA</th>
<th>actin</th>
<th>β-tubulin</th>
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<td><strong>Cladosporium bruhnei</strong></td>
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<td></td>
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<td>EF101453</td>
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<td>UAMH 7686</td>
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<td>U.S.A., Alta, Clyde Comer</td>
<td>AY625063 / –</td>
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<td>AJ300332 / DQ780941</td>
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<td>Dominican Republic, salt lake Enriquillo</td>
<td>DQ780392 / –</td>
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<td>EXF-699</td>
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<td>DQ780394 / –</td>
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<td>Hypersaline water</td>
<td>Dominican Republic, saltern</td>
<td>DQ780393 / –</td>
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<td>Mycosis Brazil</td>
<td>DQ780379 / DQ780932</td>
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<tr>
<td>CBS 701.84</td>
<td>Picea abies, wood</td>
<td>Germany, Göttingen</td>
<td>DQ780382 / –</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CBS 101880</td>
<td>Moist aluminium school window frame</td>
<td>Belgium, Lichtenvoorde</td>
<td>DQ780380 / –</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CBS 109868</td>
<td>Mortar of Muro Farnesiano</td>
<td>Italy, Parma</td>
<td>DQ780377 / –</td>
<td>–</td>
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<tr>
<td>EXF-604</td>
<td>Hypersaline water</td>
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<td>DQ780376 / DQ780931</td>
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<td>Cladosporium langeronii</td>
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<td>Mycosis Brazil</td>
<td>DQ780379 / DQ780932</td>
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<td>CBS 101880</td>
<td>Moist aluminium school window frame</td>
<td>Belgium, Lichtenvoorde</td>
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<tr>
<td>CBS 109868</td>
<td>Mortar of Muro Farnesiano</td>
<td>Italy, Parma</td>
<td>DQ780377 / –</td>
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<td>Spain, Barcelona, Salines de la Trinitat</td>
<td>DQ780351 / –</td>
<td>–</td>
<td>EF101378</td>
<td>EF101411</td>
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<td>Gardening peat substrate</td>
<td>Russia, Kaliningrad</td>
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<td>EXF-385</td>
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<td>EXF-464</td>
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<td>– / DQ780927</td>
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<td>Spain, Santa Pola</td>
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<td>EXF-715</td>
<td>Hypersaline water</td>
<td>Dominican Republic, saltern</td>
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<td>EXF-738</td>
<td>Bathroom</td>
<td>Slovenia</td>
<td>DQ780348 / –</td>
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<td>Bathroom</td>
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<td>DQ780344 / –</td>
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<td>EF101381</td>
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<td>DQ780347 / –</td>
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<td>Bathroom</td>
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<td>–</td>
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<td>EXF-1069</td>
<td>Hypersaline water</td>
<td>Israel, Eilat saltern</td>
<td>– / –</td>
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<td>EF101376</td>
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<td>Hypersaline water</td>
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<td>DQ780346 / –</td>
<td>–</td>
<td>EF101379</td>
<td>EF101408</td>
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<td>EXF-1726</td>
<td>Hypersaline water</td>
<td>Israel, Dead Sea</td>
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<td>EXF-1732</td>
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<td>– / DQ780928</td>
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<td>AF455481* / –</td>
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<td>Cladosporium spinulosum</td>
<td>EXF-333</td>
<td>Hypersaline water</td>
<td>Slovenia, Sečovlje saltern</td>
<td>DQ780404 / –</td>
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<td>Cladosporium subinflatum</td>
<td>EXF-334 T</td>
<td>Hypersaline water</td>
<td>Slovenia, Sečovlje saltern</td>
<td>DQ780406 / –</td>
<td>–</td>
<td>EF101355</td>
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<td>EXF-382</td>
<td>Hypersaline water</td>
<td>Slovenia, Sečovlje saltern</td>
<td>DQ780407 / DQ780936</td>
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<td>New Caledonia</td>
<td>AF393724 / –</td>
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<td>Cladosporium velox</td>
<td>EXF-324</td>
<td>Hypersaline water</td>
<td>Slovenia, Sečovlje saltern</td>
<td>– / DQ780926</td>
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<td>EXF-371</td>
<td>Hypersaline water</td>
<td>Slovenia, Sečovlje saltern</td>
<td>DQ780396 / –</td>
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<td>EXF-452</td>
<td>Hypersaline water</td>
<td>Slovenia, Sečovlje saltern</td>
<td>DQ780397 / –</td>
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<td>DQ780398 / –</td>
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<td>DQ780361 / DQ780937</td>
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<td>–</td>
<td>EF101386</td>
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<td>EXF-471</td>
<td>Hypersaline water</td>
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<td>DQ780360 / –</td>
<td>–</td>
<td>EF101387</td>
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Table 1. (Continued).
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 unilatral. 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) \( n_1 \ldots n_3 \) or (ii) \( (n_1 \ldots n_3)(-n_2) \), 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 cm³ 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 (DNASTar; 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 Fusarium 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); their accession numbers are listed in Table 1. Alignments and trees were deposited in TreeBASE (www.treebase.org).

### Table 1. (Page 158–160)

<table>
<thead>
<tr>
<th>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.</th>
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Table 2. Statistical parameters describing phylogenetic analyses performed on sequence alignments of four different loci.

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<th>Parameter</th>
<th>SSU rDNA</th>
<th>ITS rDNA</th>
<th>β-tubulin</th>
<th>Actin</th>
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<td>Number of parsimony informative characters (PIC)</td>
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<td>68</td>
<td>220</td>
<td>103</td>
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<td>Length of tree / number of steps</td>
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<td>102</td>
<td>714</td>
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<td>Consistency Index (CI)</td>
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<td>0.804</td>
<td>0.538</td>
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<td>Retention Index (RI)</td>
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<td>0.975</td>
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<td>Rescaled Consistency Index (RC)</td>
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<td>0.784</td>
<td>0.475</td>
<td>0.518</td>
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<tr>
<td>Homoplasy index (HI)</td>
<td>0.369</td>
<td>0.196</td>
<td>0.462</td>
<td>0.414</td>
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<tr>
<td>Number of equally parsimonious trees retained</td>
<td>30</td>
<td>600</td>
<td>90</td>
<td>32</td>
</tr>
</tbody>
</table>

1Including the internal transcribed spacer region 1 and 2 and the 5.8S rDNA.
2Including partial sequences of 4 exons and complete sequences of 3 introns.
3Including partial sequences of 3 exons and 2 introns.

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).
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* J ohanson teleomorphs, containing various anamorphic genera such as *Septoria* Sacc.,
Ramularia Unger, Cercospora Fresen., Pseudocercospora Speg., “Trimmatostrona” Corda (now Catenulostrona 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. cladosporioideis (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 β-tubulin and actin genes.

Analyses of the more variable ITS rDNA and partial β-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 β-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. 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 β-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).
Cladosporium sphaerospermum SpecieS coMplex
growing colonies at all tested temperatures and relatively large,
oblong conidia. However, phylogenetic analyses of the β-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 β-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 β-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 β-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 β-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 β-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. 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).
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 verrucose (*C. halotolerans*) (Fig. 5). The verrucose 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 µm and a width of the main conidiophore stipe of 3.5–4 µm, (ii) spherical to ellipsoidal, acrogenously formed conidia of 3.4–4 µm diam, and (iii) ramoconidia of 6–14 × 3.5–4 µm. 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 substrate 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 salters. 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 Alhara 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 salters 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 loboi Taborda, V.A. Taborda & McGinnis (GenBank AF0335674) 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 loboi 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 loboi 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 (Treujs 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 may 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 intrabranchial 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 nodule infilations (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 (Wirsel 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 lecotypified based on CBS 170.54 (de Vries 1952), which Bisby considered a standard culture of C. herbarum. The Cladosporium sp. complexes require 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, Wirsel et al. 2002) and ATCC 66670 (Braun et al. 2003, as Davidiella tassiana) have been re-identified as C. brunneifolium (Linder) 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 Morica 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 Wirsel 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 sallers 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–)2.5–3(–4) 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.

<table>
<thead>
<tr>
<th>Key to species treated in this study</th>
<th>C. culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Conidial ornamentation conspicuously echinulate / digitate because of up to 1.3 μm long projections</td>
<td>C. spinulosum</td>
</tr>
<tr>
<td>1. Conidial ornamentation verruculose to verrucose or smooth, not conspicuously echinulate or digitate</td>
<td></td>
</tr>
<tr>
<td>2. Conidiophores micronematous, poorly differentiated, once or several times geniculate-sinuous,</td>
<td>C. salinae</td>
</tr>
<tr>
<td>2. Conidiophores micro- or macronematous, not geniculate or only slightly so, usually up to 100</td>
<td></td>
</tr>
<tr>
<td>μm long; terminal conidia obovoid</td>
<td></td>
</tr>
<tr>
<td>3. Terminal conidia 0–3(–4)–septate; septa of conidiophores and conidia darkened and thickened</td>
<td></td>
</tr>
<tr>
<td>3. Secondary ramoconidia 0–1(–2)–septate; septa neither darkened nor thickened</td>
<td></td>
</tr>
<tr>
<td>4. Conidiophores (5–)10–50(–300) × (2–)2.5–3(–5.5) μm; terminal conidia (2–)3–4(–6) × (2–)2.5–3(–5)</td>
<td>C. halotolerans</td>
</tr>
<tr>
<td>μm; secondary ramoconidia (5–)7–12(–37.5) × (2–)2.5–3(–6.5) μm; ramoconidia sporadically formed</td>
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<tr>
<td>4. Conidiophores mostly longer and somewhat wider, (10–)45–130(–300) × (2.5–)3–4(–6) μm; terminal</td>
<td></td>
</tr>
<tr>
<td>conidia mostly wider, (2.5–)3–4(–7) × (2–)3–3.5(–4.5) μm; secondary ramoconidia (4–)8.5–16(–37.5)</td>
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<tr>
<td>× (2–)3–3.5(–5) μm; ramoconidia often formed, up to 40 μm long, with up to 5 septa</td>
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<tr>
<td>5. Terminal conidia usually fusiform</td>
<td>C. fusiforme</td>
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<tr>
<td>5. Terminal conidia globose, subglobose or ovoid</td>
<td></td>
</tr>
<tr>
<td>6. Conidia and secondary ramoconidia irregularly verruculose to sometimes loosely verrucose; radial</td>
<td>C. langeronii</td>
</tr>
<tr>
<td>growth on PDA at 25 °C after 14 d typically less than 5 mm</td>
<td></td>
</tr>
<tr>
<td>6. Conidia and secondary ramoconidia smooth to minutely verruculose; radial growth on PDA at 25 °C</td>
<td></td>
</tr>
<tr>
<td>after 14 d typically more than 10 mm</td>
<td></td>
</tr>
<tr>
<td>7. Conidiophores (3–)3.5–4(–7.5) μm wide, thick-walled; conidiogenous loci and conidial hila 0.5–2</td>
<td>C. psychrotolerans</td>
</tr>
<tr>
<td>μm diam; ramoconidia sometimes formed with a broadly truncate, up to 2 μm wide non-cladosporioid</td>
<td></td>
</tr>
<tr>
<td>base; no growth observed after 14 d at 30 °C on MEA</td>
<td></td>
</tr>
<tr>
<td>7. Conidiophores mostly narrower, 2–4 μm wide, only with slightly thickened walls;</td>
<td></td>
</tr>
<tr>
<td>conidiogenous loci and conidial hila narrower, 0.5–1.5 μm diam; ramoconidia rarely formed;</td>
<td></td>
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<tr>
<td>colony showing at least weak growth after 14 d at 30 °C on MEA</td>
<td></td>
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<tr>
<td>8. Secondary ramoconidia (4–)6.5–13(–24.5) μm long; no visible colony growth after 14 d at 10 °C</td>
<td>C. dominicanum</td>
</tr>
<tr>
<td>on MEA</td>
<td></td>
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<tr>
<td>8. Secondary ramoconidia mostly longer, (3.5–)5.5–19(–42) μm; radial growth of colonies after 14</td>
<td></td>
</tr>
<tr>
<td>d at 10 °C on MEA more than 5 mm</td>
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Zalar et al.
Description of *Cladosporium* species

*Cladosporium dominicanum* Zalar, de Hoog & Gunde-Cimerman, sp. nov. MycoBank MB510995. Fig. 6.

Etyymology: Refers to the the Dominican Republic, where most strains were encountered.

Conidiophora lateralia vel terminalia ex hyphis rectis oriunda; stipes longitundine variabili, (5–)10–100(–200) × (1.5–)2.5–(5.3–) μm, olivaceo-brunneus, levis vel leniter verruculosus, tenuitunicatus, plurumque unicellularis, simplex vel ramosum. Conidiorum catenae undique divergentes, ad 8 conidia in parte continua continentes. Cellulare conidiogeneae indistinctae. Conidia levia vel leniter verruculosa, dilute brunnea, unicellularia, plurumque breviter ovoidea, utrinque angustata, (2.5–)3.5–(5.5) × (2–2.5–(2.5) μm, long. : lat. 1.4–1.6; ramoconidica secundaria cylindrical 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 polysaccharidicae carentes.

Myecelium 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–)2.5–(5.3–) μm, olivaceo-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. *Conidigenous 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) × (2–2.5–2.5(–2.5) μm [av. (± SD) 3.4 (± 0.6) × 2.2 (± 0.2)]; *secondary* ramoconidica 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 (2B6), hairly granular, flat or slightly furrowed, with flat margin. Druplets 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.


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.


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.

Etyymology: Refers to its usually fusiform conidia.

Conidiophora erecta, lateralia vel terminalia ex hyphis rectis oriunda; stipes longitundine variabili, (10–)25–50(–100) × (2–2.5–3.5(–4) μm, olivaceo-brunneus, levis, crassitunicatus, complices septatus (cellulis 9–23 μm longis), plurumque simplex. Conidiorum catenae undique divergentes, in parte continua ad 5 conidia continentes. Cellulare conidiogeneae indistinctae. Conidia leniter verruculosa, dilute brunnea, unicellularia, plurumque fusiformia, utrinque angustata, (2.5–)3.5–5.5–(6.5) × (2–)2.5–(2.5–5.3) μm, long. : lat. 1.8–2.0; ramoconidica secundaria cylindrical, 0(–1)-septata, (5–6)–11(–22) × (2.5–)2.5–3(–3) μm, ad 4 cicatrices terminales ferentia; cicatrices inspissatae, conspiciueae, 0.7–1.0 μm diam. Hyphae vagina polysaccharidicae carentes.

Myecelium without extracellular polysaccharide-like material. *Conidiophores* erect, arising laterally and terminally from straight hyphae, stipes of variable length, (10–)25–50(–100) × (2–2.5–3.5(–4) μm, olivaceo-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. *Conidigenous cells* undifferentiated. *Ramoconidia* rarely formed. *Conidia* minutely verruculose, light brown, asperate, usually fusiform and narrower at both ends, length : width ratio = 1.8–2.0; (2.5–)3.5–5.5(–6.5) × (2–2.5–2.5(–3) μm [av. (± SD) 4.4 (± 0.8) × 2.2 (± 0.2)]; *secondary* ramoconidica cylindrical, 0(–1)-septata, (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.


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 ºC 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-ZS19; 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 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 ºC 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 ºC 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, lateralia vel terminalia ex hyphis rectis oriunda; stipes longitudine variabilis, (5–)10–50(–300) × (2–)2.5–3(–5.5) μm, pallide olivaceo-brunneus, levis vel leniter verruculosus, tenuitunicatus, 0–3-septatus, interdum plusseptatus, simplex, denticulatus. Conidiorum catenae undique 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. *Ramoconiaida* rarely formed. *Conidia* verrucose, brown to dark brown, non-septate, usually subglobose to globose, less often short-ovoid, narrowing 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 ramoconiaida** 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.


**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:** Verrucose 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-648, 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 (Kantarcioglu 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* (Auerw.) Starbäck on *Hypericum perforatum* in Switzerland. According to Aapro (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.


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 ramoconiaida. *Ramoconiaida* 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 verrucose, dark brown, non-septate, usually ovoid, length : width ratio = 1.3–1.5; conidial size (3–)4–5(–8) × (2–)3–4(–5) μm [av. (± SD) 4.8 (± 1.0) × 3.5 (± 0.6)]; **secondary ramoconiaida** cylindrical to almost spherical, mostly 0–1(–2)-septate, (5.5–)7.5–12.5(–35.5) × (2.5–)3.5–4.5(–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 ulceroid-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 biomat; conifer wood and window frame in Europe; humans; strains originating from nasal mucus (Buzina et al. 2003) have 100 % sequence homology with...
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.

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**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 µm, F = 30 µm, G–I = 10 µm.
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. Conidiophores erect or ascending, macronematous, stipes (10–)50–100(–150) × (3.5–)4–(–7.5) μm, olivaceous-brown, levis, crassitunicatus, complutes regulariter septatis (cellulis 10–40 μm longis), idemident dichotome ramosus. Conidionum catenae undique divergentes, terminales partes simplices ad 4 conidia continentes. Cellulae conidiogenae indistinctae. Ramoconidia primaria 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) × (3.5–)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(–24) × (2.5)3–3.5(–4) μm, aspere, 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 (± 0.5) × 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).


**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 involute. Conidiophora vix distincta, lateralia vel terminalia ex hyphis aeriis orundae; stipes longitudine variabilis, [5–]25–50(–60) × (2–)2.5–3(–4) μm, olivaceous-brown, levis vel linter verruculosus, crassitunicatus, irregulariter dense septatis (cellulis 6–29 μm longis), simplex, interdum ruamosus. Conidionum catenae undique divergentes, terminales ad 6 conidia continentes. Cellulae conidiogenae nonnumquam integratae, in summo sequentiam sympodialen denticulorum formantes. Conidia levia, interdum linter verruculosa, dilute brunnea, unicellulata, 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–)15.5–13.5(–19) × (2.5–)2.5–3(–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, microconematous, stipes (5–)25–50(–60) × (2–)2.5–3(–5) μm, olivaceous-brown, smooth to often minutely verruculose or irregularly rough-walled, thick-walled, irregularly densely septate (lengths 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, aspere, usually oblong elliptoidal to fusiform, length : width ratio = 1.9–2.4; (4.5–)5.5–7.5(–10) × (2–)2.5–3(–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–)2.5–3(–3.5) 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 granular 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.


**Habitats and distribution:** Hypersaline water in the Mediterranean basin.

**Differential parameters:** Sympodial conidiogenous cells with pronounced denticles, narrow temperature amplitude.
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. 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 µm, F = 50 µm, G–I = 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. 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.
SD) 3.8 (± 0.8) × 3.1 (± 0.4)]; secondary ramoconidia cylindrical to almost spherical, 0–3(–4) septate, (4–)8.5–16(–37.5) × (2–)3–3.5(–5) μm [av. (± SD) 13.1 (± 6.3) × 3.2 (± 0.5)], with up to 4, rarely up to 6 distal scars. Conidiogenous scars thickened and conspicuous, protuberant, 0.9–1.1(–1.4) μm 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.
Cladosporium sphaerospermum

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.


Diagnostic parameters: Thick-walled, melanised, densely septate mycelium, almost spherical, verruculose to verrucose terminal conidia, growth on 20% NaCl after 7 d.


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.
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. Conidiorum catenae undique ramosae, ad 4 conidiis in partibus linearibus continuis cohaerentibus. Cellulae conidiogenae integratae vel discretae, acervos distales denticulorum conspicuum 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, asceptate, usually subpherical 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, velvet, 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 with 5 % NaCl reaching 12–18 mm diam, consisting of submerged mycelium only. Reverse pale to dark green forming a crater-like structure; margin furrowed, paler in colour, (29F6), either flat or radially furrowed. Colony centre wrinkled, Margin arachnoid. Reverse pale brown to dark brown. Colonies on MEA reaching 12–18 mm diam, 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.


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 globose 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 polysaccaricida carentes. Conidiophora erecta, lateralia vel terminalia ex hyphis aeris orundae; stipes (10–)25–150(–250) × (2.5–)3–4(–5) μm, olivaceous-bruneus, leiva, crassilaticatus, ad 7-septatus (cellula 10–60 μm longis), identidem dichotome ramosus. Conidiorum catenae unidique divergentes, terminales partes similes ad 5 conidia continentes. Cellulae conidioinae indistinctae. Conidia levia vel leniter verruculosa, dilute brunea, unicellularia, ovoidvia, (2–)3–4(–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 terminalis ferenti: cicatrices inspissatæ, protuberantes, conspueæ, 0.5–1.5 μm diam.

Mycelium partly superficial partly submerged; hyphae without extracellular polysaccaricida-like material. Conidiophores erect, stipes (10–)25–150(–250) × (2.5–)3–4(–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 verrucose, pale brown, non-septate, ovoid, length : width ratio = 1.4–1.7; (2–)3–4(–5) × (1.5–)2–2.5(–3) μm [av. (± SD) 3.6 (± 0.8) × 2.3 (± 0.2)]; secondary ramoconidia cylindrica, 0–1-septata, (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, velvet, 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, velvet, 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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