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

Irene Barnes1*, Pedro W. Crous2, Brenda D. Wingfield1 and Michael J. Wingfield1

1Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 0002; 2 Centraalbureau voor Schimmelcultures (CBS), Fungal Biodiversity Centre, P.O. Box 85167, 3508 AD Utrecht, The Netherlands

*Correspondence: I. Barnes, irene.barnes@fabi.up.ac.za*

**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 1-α 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* (Pančić) Purkyne (Karadžić 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 Hedgcock (1929) believed that the red band fungus was conspecific with *L. acicola*, although Dearness referred to it as *Cryptosporium acicola* Thüm., and Hedgcock used the name *Septoria acicola* (Thüm.) Sacc. Sydow & Petrk (1942) later recognised that *A. marginatum* represented a *nomen confusum* and referred to the fungus as *L. acicola*. Independently, Hulbary (1941) described the red band fungus occurring on *Pinus nigra* Arn. var. *austriaca* Aschers. & Graebn., collected in Illinois, and erected the name *Dothistroma pini* Hulbary for it. Siggers (1944) discovered that the material previously referred to as *L. acicola*, *C. acicola*, *S. acicola* and *A. marginatum* on *P. nigra* var. *austriaca* was not conspecific with the type specimen of *L. acicola*, but rather with that of *Dothistroma pini*.

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

Three different varieties of *D. septosporum* have been described based on differences in the average conidial size. *Dothistroma septosporum* var. *septosporum* (= *D. pini* var. *pini*) and *D. septosporum* var. *lineare* (= *D. pini* var. *lineare*), proposed by Thr & Shaw (1964), are respectively the varieties with short (15.4–28.6 × 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 conidiospores 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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*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 Autosequencer (Applied Biosystems) and sequence electropherograms were analyzed using Sequence Navigator version 1.0.1 (Applied BioSystems).

Phylogenetic analysis

Sequences were aligned using Clustal X (Thompson 1997) and checked visually before analyses were run using PAUP v. 4.0 (Phylogenetic Analysis Using Parsimony) (Swofford 2002). Intron and exon positions were identified using the original sequences from which each primer set was designed. The Neurospora crassa sequence (GenBank M13630) was used for the 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 combinability of the four data sets. All sequences derived in this study have been deposited in the GenBank database with accession numbers AY808275–AY808308 (ITS), AY808170–AY808204 (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 Alu (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 type of degraded DNA associated with the fact that the sequence was recovered. Poor PCR could be the result up to exon 6 was not comparable with *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. PCR-RFLP profiles were visualized on an ethidium bromide-stained agarose gel (3 %), under UV illumination.

**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.](image-url)
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 only had 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.

![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.](image)

![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.](image)
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).

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**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 *Dothistroma septosporum* is retained, and *Dothistroma pini* is resurrected for isolates belonging to Lineage II.

### Dothistroma pini


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

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


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

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


≡ *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. Conidiogenous 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_ 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_ 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).

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

**DISCUSSION**

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

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

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

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

Recognition that two species cause the single disease known as red band needle blight has important consequences for disease control and quarantine. Our choice has been to retain the names that have been most closely associated with the red-band fungus and to amend the description of D. septosporum to exclude the genetically distinct isolates from Central U.S.A. We have consequently also restored the use of D. pini to represent this distinctly different fungus that occurs in the North Central United States, including Illinois, where the type specimen of D. pini was collected. This specimen, described by Hulbary in 1941, could not be analysed based on sequence data but is morphologically consistent with isolates in phylogenetic Lineage II/D. pini. All other isolates associated with red band needle blight, including those from Western North America and Europe, are in Lineage I. They should be referred to as D. septosporum as proposed by Morelet (1968).

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

The teleomorph Mycosphaerella pini, associated with the red band fungus, was not observed in the current study. So far, it has been reported only from Central America (Evans 1984), the western U.S.A. (Peterson 1974), western Canada (Funk & Parker 1966) and Europe (Kowalski & Jankowiak 1998). The original description of M. pini was from needles of Pinus sylvestris collected in Denmark. Scirrhia 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 sylvestris collected in Denmark. Scirrhia 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 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.

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

Jean Carlson Batzer  
Maria Mercedes Diaz Arias  
Thomas C. Harrington  
Mark L. Gleason

Department of Plant Pathology, Iowa State University, Ames, Iowa 50011

Johannes Z. Groenewald  
Pedro W. Crous

CBS Fungal Biodiversity Centre, P.O. Box 85167, 3508 AD Utrecht, the Netherlands

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, *Gloeodes pomigena* (Schwein.) Colby and *Leptothyrium pomi* A. Selby. The name *L. pomi* was synonymized with *Mycosphaerella 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. The aim of the present study was to identify and describe species of flyspeck fungi based on DNA study. The isolates and other flyspeck isolates from 10 eastern states were used for taxonomic analysis. These isolates and other flyspeck isolates were grouped 30 isolates from six eastern states in the USA based on growth patterns, colony color, numbers of sclerotium-like body produced, optimal temperature and pH ranges. 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 Zygothia 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 Prepman Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California). Primer pairs used for amplification and sequencing of the ITS region were ITS1F/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 X 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 denaturation at 94 C for 95 s followed by 35 cycles of denaturation at 94 C for 35 s, annealing at 49 C for LSU and at 52 C for ITS for 60 s, and extension at 72 C for 2 min. The PCR product was purified with a QiAquick DNA Purification Kit (QiAGEN, Valencia, California) and quantified on a Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech, San Francisco, California). Automated sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility.
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 10000. Alignable gaps were treated as a “fifth base”. All characters were given equal weight. To assess the robustness of clades and internal branches, a strict consensus of the most parsimonious trees was generated and a bootstrap analysis of 1000 replications was performed. We rooted the LSU tree to four species from the Chaetothyriales (Ceramothyrium carniolicum [Rehm] Petr., Exophiala dermatitidis [Kano] de Hoog, Rhinocladiella atrorubens Nannf. and Ramichloridium aniceps [Sacc. & Ellis] de Hoog). Outgroup for ITS phylogenetic analysis was Mycosphaerella markii Carnegie & Keane. MP analysis, treating gaps as missing data, also was conducted on the LSU alignment because of concerns that gaps could be over-weighted in the analysis where gaps were treated as a fifth character. Alignments and the representative trees (Figs. 1, 2) were deposited in TreeBASE SN3221.

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>CBS Accession No.</th>
<th>Herbarium Accession No.</th>
<th>GenBank Accession</th>
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<tr>
<td></td>
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<tr>
<td>Schizothyrium pomi</td>
<td>CUA1a, CBS 118957</td>
<td>438789, CBS-H19787</td>
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<td></td>
<td>ZJ001</td>
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<td></td>
<td>AHA2a</td>
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<td></td>
<td>GTA1a</td>
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<td>CBS 228.57</td>
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<td>CBS 486.50</td>
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<td>438791, CBS-H19785</td>
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<tr>
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<td>MWA8a</td>
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<td></td>
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<tr>
<td></td>
<td>KY1 1.2A1c</td>
<td></td>
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<td>Zygophiala tardicrescens</td>
<td>MWA1a, CBS 118946</td>
<td>438792, CBS-H19788</td>
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<tr>
<td>Zygophiala wisconsinensis</td>
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<td>438790, CBS-H19786</td>
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<td>GTA4b</td>
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</tbody>
</table>

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 cryptogama sp. nov. A well supported clade (89% bootstrap support) contained isolates obtained from Wisconsin, Ohio, Michigan and Kentucky. Isolates from the last clade (100% bootstrap support and sister of Z. wisconsinensis) were obtained from a colony description 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.

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.
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...
single Iowa orchard and are described as *Zygothiala 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 resulted in 10 equally most parsimonious trees (Fig. 2). Parsimony analysis of the LSU sequences resulted in 11 uninformative and 297 were constant. Maximum parsimony informative, 42 were variable and parsimony for the analyses. Of these characters 215 were 

\[
\text{medium brown septum to below phialide), terminating in a}
\]

\[
\text{fungoidellipsoidal, widest in the middle of the apical cell, which is acutely rounded, while the lower cell is subobtusely rounded, (10–)12–(13) × (3–)3.5–}
\]

\[
\text{Ascomata situated on a thin, hyaline,}
\]

\[
\text{basal stroma.}
\]

\[
\text{layer consisting of interwoven mycelium, forming 2–4}
\]

\[
\text{layers of thick-walled, brown, pseudoparenchymatous cells,}
\]

\[
\text{2–3 μm thick; ostiole central, but upper layer}
\]

\[
\text{splitting at maturity via irregular ruptures from the}
\]

\[
\text{elevated center; ascomata situated on a thin, hyaline,}
\]

\[
\text{basal stroma.}
\]

\[
\text{basal stroma.}
\]

\[
\text{ELEVATED CENTERS, but frequently in circles, superficial on leaves,}
\]

\[
\text{prior to oviposition.}
\]

\[
\text{specimen examined.}
\]

\[
\text{TAXONOMY.—Isolates could be grouped into four}
\]

\[
\text{species based on their morphology on cultural media,}
\]

\[
\text{growth characteristics and DNA phylogeny. Sclerotium-like}
\]

\[
\text{bodies of *Schizothyrium pomi*} \text{on apple were}
\]

\[
\text{250(155–480) μm diam and with a density of}
\]

\[
\text{of 2.4/μm². Sclerotium-like bodies of *Zygothiala crypto-
}\]

\[
\text{gama} \text{were also round but slightly smaller, 230(150–}
\]

\[
\text{364) μm diam, and were more densely arranged, averaging}
\]

\[
\text{of 3.6 sclerotium-like bodies/μm². *Zygothiala wiscon-
}\]

\[
\text{sinensis} \text{sclerotium-like bodies were ovoid, larger,}
\]

\[
\text{380}(300–450) × 500(425–600) μm and were more}
\]

\[
\text{sparserly arranged with a density of 0.8/μm². Sclerotium-
}\]

\[
\text{like bodies of *Zygothiala tardicrescens* were similar}
\]

\[
\text{to} \text{S. pomi, 260(250–270) μm diam and were arranged}
\]

\[
\text{at a density of 2.8/μm².}
\]

\[
\text{Three new species of *Zygothiala* were distinguished}
\]

\[
\text{and are described below.}
\]


**Anamorph.** *Zygothiala* 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 hamathelial 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) × (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 distortion, 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× (4–)5–6× (8) μm, distinguisha.

*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

---

**Fig. 5.** *Zygophiala cryptogama* on oatmeal agar (CBS 118949).  
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 obtuse, base subtruncate, with a darkened, thickened hilum, 1–2 μm wide. 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 verrucose, medium brown apical cell, 3–4 × 4–6 μm, that gives rise to two medium brown, finely verrucose, 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, regular; olivaceous gray throughout; developing submerged to erumpent, globose ascomatal initials.

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).

Zygophialae jamaicensi similis, sed colonii lentius crescentibus et conidiis 20 μm vel magis longinis, 6 μm vel magis latis distinguenda.

Zygophiala wisconsinensis Batzer & Crous, sp. nov.

Figs. 3, 7. MycoBank MB501245.

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

Zygophialae jamaicensi similis, sed coloniiis 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–)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.

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 *Schizothyrium* (Schizothyriaceae) in the Microthyriales, characterized by strongly flattened, crustose, rounded or elongated ascomata, opening by irregular splits, with bitunicate asci lacking an apical chamber (but see descriptions above), and some interascal tissue composed of remnants of stromatal cells, and may form ascospores that are 3-septate (*S. pomi* s. str.) (Crous et al 2003), taxa placed in *Sphaerulina* in the Dothideales: a Saccardoan myth? Sydowia 55:136–152.


**LITERATURE CITED**


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

Uwe BRAUN¹, 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 (Venturiaeaceae). Cladosporium-like anamorphs of the Venturia (conidia catenate) are referred to Fusicladium. Human-pathogenic Cladosporidium 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. Phaeoramularia kelleriana, Cladophialophora kellerianiana) as well as Cladosporium porophorum. Based on differences in conidogenesis and the structure of the conidiogenous loci, further supported by molecular data, C. malorum is allocated to Alternaria.


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 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. Erysipheae; BRAUN & TAKAMATSU 2000). In other cases, the morphological variation in the anamorphs is much greater than in the teleomorphs, e.g. in Myco-

¹ Martin-Luther-Universität, FB. Biologie, Institut für Geobotanik und Botanischer Garten, Neuwark 21, D-06099 Halle (Saale), Germany.
² Corresponding author: Pedro Crous, Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. Phone +31-31-2122643; Fax +31-31-2122601; e-mail crous@cbu.naw.nl
³ Western Regional Plant Introduction Station, USDA-ARS, Washington State University, Pullman, WA 99164-6402, USA.

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 *Pseudocladosporium*, a genus introduced for anamorphs of *Caproventuria*. Ho et al. (1999) recognized *C. malorum*, *C. porophorum* and *P. kelleriana* as con-specific. 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’-TTC CGG TAG GTG AAC CTG C-3’) and ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) (White et al. 1990) were used to amplify part of the nuclear rRNA operon spanning the 3’ end of the 18S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS (ITS2) region and the 5’ end of the 28S (large subunit) of the rRNA gene. The reaction mixture contained 5 µL of diluted sample, 1 x buffer, 8 mM MgCl2, 500 µM of each of the dNTPs, 2.5 U (Bioline) Taq polymerase and 10 pm of each primer and made up to a total volume of 200 µL. PCR conditions were the same for this region, except for the MgCl2 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 (Swoford 2000). Alignment gaps were treated as missing data and all characters were unordered and of equal weight. The robustness of the trees was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). Resulting trees were printed with TreeView Version 1.6.6 (Page 1996).

**Morphology**

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

**Results**

**Phylogenetic analysis**

For ITS, approximately 530 bases were determined for each isolate (spanning the 3’ end of 18S, ITS1, the 5.8S rRNA gene, ITS2 and the 5’ end of the 28S rRNA gene) and added to the alignment. The manually adjusted alignment of the ITS
nucleotide sequences contained 72 taxa and 575 characters including alignment gaps (data not shown). Approximately 1075 bases of the 5’ end of the SSU gene were determined for each isolate and the manually adjusted alignment of the nucleotide sequences contained 59 taxa and 1394 characters including alignment gaps (data not shown). The SSU sequence of *Mycosphaerella juvenis* (STE-U 1004) contained an insertion spanning bases 514 to 838, which was excluded from the analysis. New sequences were deposited in GenBank (Tab. 1), and the alignments in TreeBASE (S872, M1413, M1414).

The NJ tree for the ITS sequencing data (Fig. 1) contains isolates from five main groups (Herpotrichiellaceae, Amorphalesaceae, Mycosphaerellaceae, Pleosporaceae and Venturiaceae). The Herpotrichiellaceae formed a well-supported clade (100 % bootstrap support) comprising species of *Cladophialophora* and *Philophora*. The Amorphalesaceae clade was also well-supported with a bootstrap support value of 100 % and contained isolates of *Amorphales resiniae* (anamorph *Sorocybe resiniae*) and *Cladosporium* breviramulosum. The Herpotrichiellaceae and Amorphalesaceae 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. belyncii* AF222831. The clade for the Pleosporaceae was also well-supported (100 %) and contained isolates of *Alternaria malorum* and additional species of *Alternaria* and *Leuwa*. An isolate of *Mycosphaerella iridis* (CBS 281.49) grouped with 100 % bootstrap support outside the Pleosporaceae clade. The Venturiaceae clade consisted of *Phaeoramularia hachijoensis* (STE-U 3679) (60 % bootstrap support) and a well-supported (100 %) clade containing *Fusicladium convolvulorum* (STE-U 3884), *Pseudocladosporium hachijoense* (STE-U 5391) and species of *Venturia* as well as isolates of *Fusicladium effusum*. *Anungitopsis amoena* (CBS 254.95) 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 *Leuwa*. The Paraphaeosphaeriaceae clade was well supported (100 %), and was grouped inside a clade that also contained a single isolate of the Leptosphaeriaceae (100 % bootstrap support).

**Morphology**

*Cladosporium malorum* (Pleosporaceae) clade

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

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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.

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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, monotetric, determinate or polytetric, sympodial, usually with 1–2 conspicuous loci, 0.5–1.5 μm wide, unthickened, with minute central port, 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, ramoconidia 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, fruit 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

differt a var. malorum conidii latoribus, ca 3.5–6 μm latis, atrioribus, leviter crassitunicatis, interdum longitudine septatis, raro alternarioidei intermiscibus.

Etymology: Referring to its variable conidial shape.


Cladosporium s. s. (Mycosphaerellaceae) clade

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

Davidiella Crous & U. Braun, gen. nov.


Ascomata at in Mycosphaerella sect. Tassiana (asci non numerosi, sacci; ascosporeae obovatae, utrinque rotundatae). Differt a Mycosphaerella statu conidiali, i.e. Cladosporium sensu DAVID (1997).

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

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

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

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


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


**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.**


The link between the teleomorph and anamorph has not been clearly established for Davidiella ornithogali and Cladosporium ornithogali, though the discussion provided by David (1997) suggests that de Vries (1952) was correct in stating the teleomorph to be representative of ‘Mycosphaerella’. Another species that needs clarification is Didymellina intermedia, and its presumed anamorph Cladosporium allii (David 1997). Fresh collections are required to resolve this possible anamorph-teleomorph association.

When the genus Mycosphaerella was treated by von Arx (1949), he divided it into three sections, including Didymellina (with Cladosporium and Heterosporium spp.), for which he chose Mycosphaerella tassiana as type. As pointed out by David (1997), this was erroneous as Didymellina was formerly described at the generic level by von Höhnel (1918), having Dothidea iridis (syn. Didymellina iridis) as type, with Sphaera iridis (syn. Mycosphaerella iridis) as proposed synonyms. The literature is filled with erroneous links between *C. iridis* and a fungus initially identified as *Mycosphaerella iridis*, but later described as *M. macrospora*. This confusing situation is explained by David (1997). We have examined the type specimen of *Dothidea iridis* in PC, which is a species of *Mycosphaerella* and not of *Dothidea*; it is morphologically distinct from *M. iridis* (CBS 281.49, herb. CBS 4933; CBS 282.49, herb. CBS 4907). Further, no link between *Dothidea iridis* and a *Cladosporium* has ever been established. The fungus present on the two specimens from CBS represents *M. iridis*. The cultures, however, represent two different fungi, neither of which appear to be *M. iridis*. Further studies are therefore presently underway to resolve the *Mycosphaerella* spp. occurring on *Iris*. In conclusion, we were unable to find any evidence linking a *Cladosporium* state to either *Mycosphaerella iridis* or *Dothidea iridis*, and have therefore decided not to choose the name Didymellina as teleomorph genus for Cladosporium.

**Discussion**

This study has provided further evidence for the separation of *Cladosporium s. str.* anamorphs from the main *Mycosphae-rella* clade, and has provided the basis for the introduction of a new teleomorph genus, Davidiella, for this group of fungi. Furthermore, it has also shown that several *Cladosporium*-like fungi are clearly not congeneric with *Cladosporium s. str.*, and that the relatively minor differences in the scars and conidial loci, are supportive of their different phylogenetic affinities. Similarly, *C. malorum* appears to be best assigned to *Alternaria* based on its ITS and SSU phylogenetic placement, and such placement is also supported by its unique mode of conidiogenesis. As in other hyphomycetes in this complex (Crous, Kang & Braun 2001), conidial septation, and the presence of oblique septa, are of less importance at the generic level. Höller, Gloer & Wicklow (2002) identified various metabolites produced by an undetermined *Cladosporium*-like hyphomycete, which was isolated from a resupinate polypore in the USA. These metabolites, which included altersolanol and macrosporin, are commonly produced by *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* is thus further supported by these metabolite data from Höller, Gloer & Wicklow (2002).

The phylograms derived in the present study delineate several clades (families) in which *Cladosporium*-like taxa are presently accommodating. These are discussed below:

**Herpotrichiellaceae and Venturiaceae**

Of particular interest in the *Herpotrichiellaceae* are those species pathogenic to humans, which are presently placed in *Cladophialaphora* (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, Gerrits van den Ende & de Hoog 1999, Unterreiner & Naveau 1999, Unterreiner, Gerrits van den Ende & de Hoog 1999, de Hoog et al. 2000). It has been clearly demonstrated in all phylogenetic analyses that the truly human-pathogenic *Cladosporium* species are Capronia anamorphs belonging to the Herpotrichiellaceae, and all species concerned have been placed in Cladophialophora. The morphological distinction between Cladophialophora and Cladosporium s. str. has also been demonstrated by several authors (Braun & Feiler 1995, Braun 1998, de Hoog et al. 2000). Cladophialophora species are characterised by truncate, unthickened, barely darkened, often somewhat denticle-like conidiogenous loci, whereas Cladosporium loci are ‘coronate’ (David 1997), e.g. protuberant and with raised periclinal rings that surround a central convex dome. True Cladosporium species also differ from Cladophialophora physiologically in their ability to liquefy gelatine (de Hoog et al. 1995).

The morphological distinction between Cladophialophora and Pseudocladosporium is rather difficult, but the two genera are ecologically and phylogenetically clearly distinct, viz. species of *Pseudocladosporium* are saprobic fungi, usually isolated from leaf litter, and anamorphs of Caproventuria (Venturiaceae), whereas Cladophialophora spp. are true human-pathogenic fungi connected with Capronia (Herpotrichiellaceae).

Anamorphs of the Venturiaceae have recently been monographed by Ritschel (2001) and Schubert (2001), including molecular examinations (rDNA ITS) of numerous taxa in which Venturia species and their anamorphs formed a single monophyletic clade. Some Fusicladium species with catenate conidia have often been confused with Cladosporium, e.g., *C. carpophilum* (syn. Fusicladium carpophilum), *C. cerasi* (syn. *F. cerasi*) and *C. Caryigenum* (syn. *F. effusum*). As already discussed by Morgan-Jones & Jacobsen (1988), these anamorphs should rather be referred to Fusicladium (Venturia anamorphs), a conclusion supported by the present molecular data. Furthermore, the structures of the conidiogenous loci in Fusicladium species with solitary as well as catenate conidia are very uniform, and quite distinct from those of *Cladosporium* s. str. In Fusicladium the conidiogenous loci are more or less denticle-like, apically truncate to slightly convex, unthickened or almost so, and not or only slightly darkened. These loci, therefore, more closely resemble those of some saprobic genera, like Anungitea and Pseudocladosporium. The form genus Fusicladium is also associated with various other genera of the Venturiaceae, viz. Acantharia, Apiosporina and Venturia.

Several authors have dealt with *Phaeoramularia hachijoensis* (Matsushima 1975), but all reassessments of this species were based on non-type material, since type material and strains were not available and are possibly not extant any 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 brevicaenate), assigned it to the Venturiaceae and proposed the combination Venturia hanliniana. In the present study, the isolate of *P. hachijoensis* used by Dugan, Roberts & Hanlin (1995) (ATCC 96019 = STE-U 5391) also clustered in Venturia, thus supporting the conclusion by 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*, Pleurotheciospis and Polyscytalum, 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.
They considered *H. resinae* to be the mononematous form of *S. resinae*, and noted that the connection between *Amorphaecoea* (Parbery 1969) as teleomorph and *S. resinae* as anamorph, remains to be resolved. *Sorocymbium 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; Partidge & Morgan-Jones 2002). The clustering of two isolates of *Cladosporium breviramnosum* (AF393683, 393684) in the *Amorphaecoeae* is unusual, and the original strains will have to be re-examined to resolve their identity and position.

**I ncertae 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*, *Cladophilalbarum*, *Phaseoramuralaria* or *Pseudeocladosporium*, suggesting that the conidiogenesis was holoblastic. These treatments were undoubtedly influenced by the cladosporoid habit of this fungus, e.g., pigmented, 0–2–septate conidia formed in long acroelat chains (Fig. 5). However, the conidiogenous cells possess minute, but conspicuous pores, and should rather be regarded as poroconidia. Within the genus *Alternaria*, however, *A. malorum* is not totally unique in having largely aseptate, cylindrical conidia, as this is also 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 aseptaroid (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 chains (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) aseptaroid 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*, *Mycevollosiella* and *Phaeoramuralaria*). Comprehensive morphological and molecular analyses of this fungal group were recently conducted (Crous et al. 2000, 2001), in which it was shown that *Mycosphaerella* isolates form a single large monophyletic clade, with species of *Mycosphaerella* with *Cladosporium* s. str. anamorphs in a distinct subclade. These molecular data further showed that *Passalora fulva* [= *Fulvia fulva*, *Cladosporium fulvum*, *Mycevollosiella 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 cercosporid scar types (Braun 1995).

Various authors confused *Cladosporium* with *Biharia*, *Fulvia*, *Mycevollosiella* and *Stenella*. For instance, von Arx (1981) reduced these names to synonymy with *Cladosporium*. Ellis (1971) listed *Biharia* as a synonym of *Mycevollosiella*, but since the superficial hyphae of the type species, *B. van-gueriae*, are verruculose, Deighton (1979) reduced *Biharia* to synonymy with *Stenella*. Von Arx (1983) recognised *Mycevollosiella*, 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, Untereiner & Naveau 1999, Crous et al. 2000, 2001) (Figs. 1–2).

True *Cladosporium* species are easily separable from all other *Cladosporium*-like hyphomycetes by their distinctive conidiogenous loci, which were described in detail by David (1997), who pointed out that this scar type is a significant ge-
neric character. The first detailed examinations of *Cladosporium* scars were published by Roquebert (1981). The conidiogenous loci (scars) and conidial hila are usually distinctly protuberant, thickened, darkened and composed of a raised periclinal rim that surrounds a central convex part (dome or mound, David 1997). This type of scar has been called ‘coronate’ (David 1997) or it may simply be described as ‘Cladosporium-type’, since it is so characteristic and unique. *Cladosporium* s. str. should be confined to Davidiella amanmorphs with coronate conidiogenous loci. The first clear circumscripti

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 *Davi
diella* teleomorphs, cluster within the *Cladosporium* clade.

**Acknowledgements**

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RIDDLE RW (1950) Permanent stained mycological preparations obtained by slide culture. – Mycologia 42: 265-270.


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</tr>
<tr>
<td>SSU</td>
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<td>U04202</td>
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<td>Kenya</td>
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<tr>
<td>ITS</td>
<td><em>Mycosphaerella iridis</em> (syn. <em>Sphaerella iridis</em>)</td>
<td>CBS 282.49</td>
<td>AY251088</td>
<td>Leaf spot in <em>Iris pseudacorus</em></td>
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<tr>
<td>ITS</td>
<td><em>Mycosphaerella iridis</em> (syn. <em>Sphaerella iridis</em>)</td>
<td>CBS 281.49</td>
<td>AY251089</td>
<td>Leaf spot in <em>Iris pseudacorus</em></td>
<td>Switzerland, Zürich</td>
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<tr>
<td>SSU</td>
<td><em>Mycosphaerella latebrosa</em> (syn. <em>Sphaerella latebrosa</em>)</td>
<td>CBS 652.85</td>
<td>AY251114</td>
<td>Leaf spot in <em>Acer pseudoplatanus</em></td>
<td>Netherlands</td>
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<tr>
<td>SSU</td>
<td><em>Mycosphaerella nubilosa</em> (syn. <em>Sphaerella nubilosa</em>)</td>
<td>STE-U 4661</td>
<td>AY251120</td>
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<td>Ponte Areas, Spain</td>
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<td>–</td>
<td>AF271130</td>
<td>Protea sp.</td>
<td>Drakensberg, Kwazulu-Natal, South Africa</td>
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<tr>
<td>SSU</td>
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<td>STE-U 1731</td>
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<tr>
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<td><em>Passalora arachidica</em> (syn. <em>Cercospora arachidis</em>) / <em>Mycosphaerella arachidis</em></td>
<td>STE-U 3837</td>
<td>AY251116</td>
<td>–</td>
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<td><em>Paraphaeosphaeria michotii</em> (syn. <em>Sphaeria michotii</em>)</td>
<td>CBS 591.73</td>
<td>AY250817</td>
<td>Juncus squarrosus</td>
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<td>CBS 102207</td>
<td>AY250821</td>
<td>Juncus roemerianus</td>
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<tr>
<td>ITS</td>
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<td>AF22831</td>
<td>–</td>
<td>USA</td>
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<tr>
<td>ITS</td>
<td><em>Passalora dissiliens</em> (syn. <em>Phaeoramularia dissiliens</em>)</td>
<td>CBS 199.77</td>
<td>A22832</td>
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<td>Switzerland</td>
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<tr>
<td>SSU</td>
<td><em>Passalora dodonaeae</em></td>
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<td>A251110</td>
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<td>Western Cape, South Africa</td>
</tr>
<tr>
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<td><em>Passalora fulva</em> (syn. <em>Cladosporium fulvum</em>)</td>
<td>ATCC 44960</td>
<td>A293701</td>
<td>–</td>
<td>Netherlands</td>
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<tr>
<td>ITS</td>
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<td>IIMI 050487</td>
<td>L25430</td>
<td>Lycopersicon esculentum</td>
<td>Zimbabwe</td>
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<tr>
<td>ITS; SSU</td>
<td><em>Passalora fulva</em> (syn. <em>Cladosporium fulvum</em>)</td>
<td>CBS 119.46</td>
<td>A251069; A251109</td>
<td>–</td>
<td>Netherlands</td>
</tr>
<tr>
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<td>A284389</td>
<td>–</td>
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<td><em>Passalora janseana</em> (syn. <em>Napilaidium janseanum</em>)</td>
<td>CBS 145.37</td>
<td>A251103</td>
<td>–</td>
<td>Arkansas, USA</td>
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<tr>
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<td><em>Passalora vaginata</em> (syn. <em>Mycocelosia vaginata</em>)</td>
<td>CBS 140.34</td>
<td>A228332</td>
<td>Saccharum officinarum</td>
<td>Taiwan</td>
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<tr>
<td>ITS; SSU</td>
<td>“<em>Phaeoramularia hachijoiensis</em>”</td>
<td>ATCC 96545</td>
<td>A251068; A251100</td>
<td>Air</td>
<td>New York, USA</td>
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<tr>
<td>ITS</td>
<td>“<em>Phaeoramularia hachijoiensis</em>”</td>
<td>CBS 462.82</td>
<td>A251068</td>
<td>–</td>
<td>De Vuursche, Baam, Netherlands</td>
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<tr>
<td>ITS</td>
<td><em>Phialophora americana</em></td>
<td>CDC 10</td>
<td>U31838</td>
<td>Paper pulp</td>
<td>Wisconsin, USA</td>
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Tab. 1: Isolates of *Cladosporium* and allied genera studied (continued)

<table>
<thead>
<tr>
<th>Sequence data</th>
<th>Species</th>
<th>Accession no.</th>
<th>GenBank accession no.</th>
<th>Substrate</th>
<th>Origin</th>
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<td>ITS</td>
<td><em>Phialophora americana</em></td>
<td>FMC 2214</td>
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<td>Decaying timber</td>
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<td>CBS 840.69</td>
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<td>–</td>
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<td><em>Pleospora betae</em></td>
<td>IMI 156653</td>
<td>U43466</td>
<td>Seed of <em>Beta</em> sp.</td>
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<td>CBS 462.75</td>
<td>AY251105</td>
<td><em>Phaseolus</em> sp.</td>
<td>Labasa, Fiji</td>
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<tr>
<td>SSU</td>
<td><em>Pseudocercospora protearum</em> var. <em>leucadendri</em> (syn. <em>Cercospora protearum</em> var. <em>leucadendri</em>)</td>
<td>STE-U 1869</td>
<td>AY251107</td>
<td><em>Leucadendron</em> sp.</td>
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<td>ITS; SSU</td>
<td><em>Pseudocladosporium hachijoense</em></td>
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<td>AY251083;</td>
<td><em>Prunus avium</em></td>
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<td>AY251106</td>
<td>Leaf of <em>Citrus sinensis</em></td>
<td>Bié, Angola</td>
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<td><em>Sorghum</em> sp.</td>
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<td><em>Sorghum</em> sp.</td>
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<td>IMTSP 373</td>
<td>AF397133</td>
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<td>SSU</td>
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<td>Leaf of <em>Rosa</em> sp.</td>
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<td>AY251095</td>
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<td>Onion leaf</td>
<td>Colorado</td>
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<tr>
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<td><em>Sorocybe resiniae</em> (syn. <em>Hormodendrum resiniae</em>) / <em>Amorphotheca resiniae</em></td>
<td>ATCC 200942</td>
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<td>UK</td>
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<td>CBS 406.88</td>
<td>AY251067</td>
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<td>St Louis, Missouri, USA</td>
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<tr>
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<td><em>Protea</em> sp.</td>
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<td>STE-U 1004b</td>
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<td>Leaves of <em>Eucalyptus grandis</em></td>
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<td>ITS</td>
<td><em>Venturia cerasi</em></td>
<td>ATCC 12119</td>
<td>AF065847</td>
<td><em>Prunus cerasus</em></td>
<td>East Germany</td>
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*a* 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.

*b* Ex-type isolates.