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

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Abstract: The red band needle blight fungus, *Dothistroma septosporum* is a widely distributed pathogen of many pine species. Three morphological varieties of this pathogen have been described based on differences in conidial length. However, controversy exists as to whether spore size represents an adequate characteristic to distinguish between forms of *D. septosporum*. The aim of this investigation was to consider the phylogenetic relationships between *D. septosporum* isolates from different countries. An additional objective was to determine whether comparisons of DNA sequence data support the morphological varieties recognized for this species. DNA from portions of the nuclear ribosomal internal transcribed spacer (ITS), β-tubulin and elongation factor 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* (Pani) Purkyne (Karadži 1994), though no data exist to confirm that these incidents were caused by *D. septosporum*.

After the fungus infects via the stomata, initial symptoms appear as water-soaked lesions on the needles. Black conidiomata develop at these infection sites, which are characteristically surrounded by a red band, hence the common name of the fungus. Infected needles become necrotic and are cast (Fig. 1). In severe cases, complete defoliation occurs, leading to growth retardation and tree death (Gibson *et al*. 1964). Red band needle blight is one of the most important diseases of pines, which has seriously damaged plantation forestry in many countries.

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

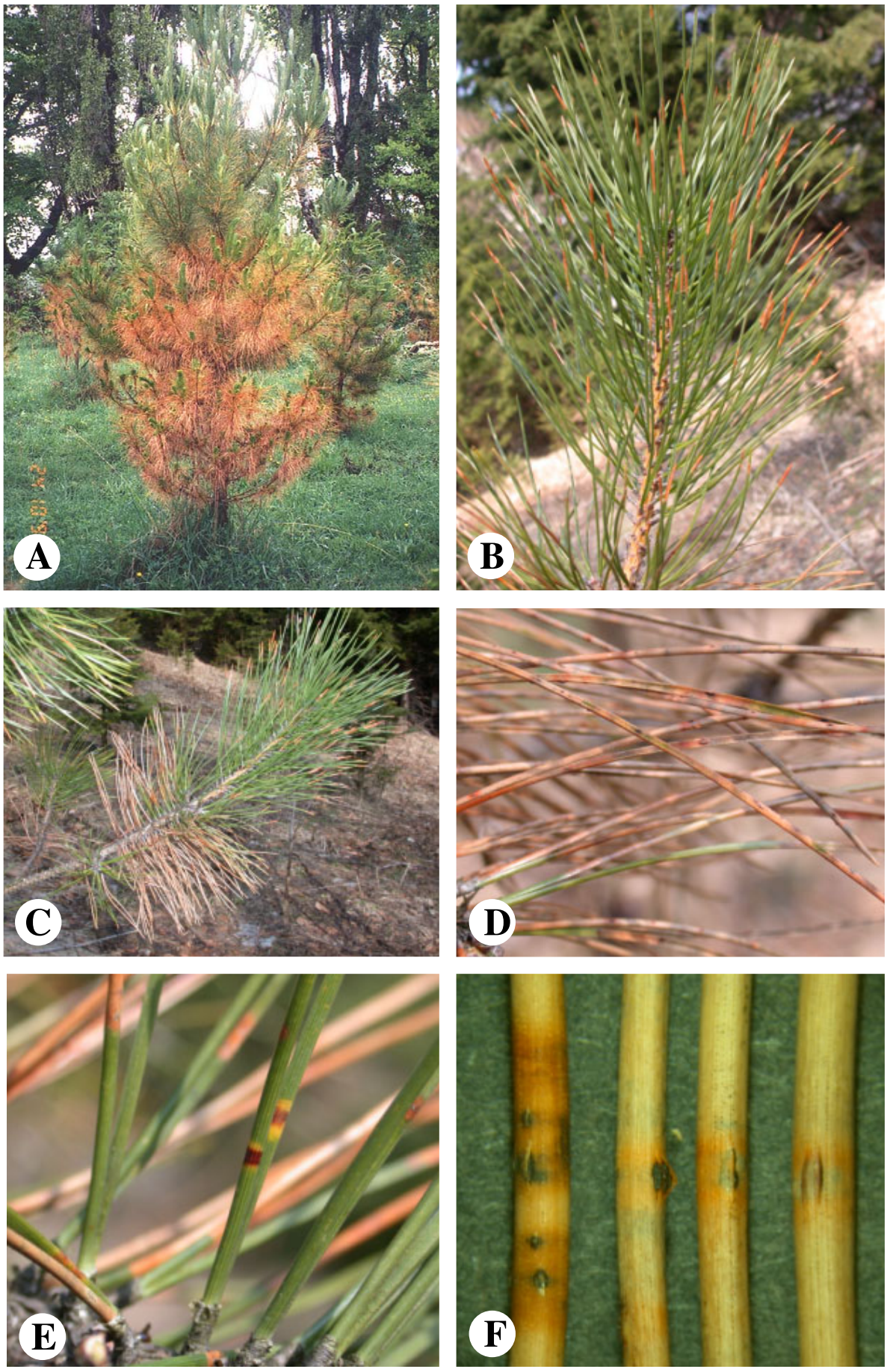

Fig. 1. Symptoms of *Dothistroma septosporum* infection on *Pinus* spp. A. 50–75 % infection on *P. radiata* in Chile. B. Tip die-back of infected *P. nigra* needles. C. Characteristically, needles from the lower branches show the first signs of disease. D. Severely infected needles showing complete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

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

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

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

Three different varieties of *D. septosporum* have been described based on differences in the average conidial length. *Dothistroma septosporum* var. *septosporum* (≡ *D. pini* var. *pini*) and *D. septosporum* var. *lineare* (≡ *D. pini* var. *lineare*), proposed by Thyr & Shaw (1964), are respectively the varieties with short (15.4–28 × 2.6–4 µm) and long (23–42 × 1.8–2.9 µm) conidia. *Dothistroma septosporum* var. *keniense* (≡ *D. pini* var. *keniense*), proposed by Ivory (1967), accommodates collections of the fungus with conidia of intermediate (15–47.5 × 1.5–3.5 µm) size. There has, however, been considerable debate as to whether conidial size represents an appropriate character by which to distinguish among forms or varieties of *D. septosporum* (Gadgil 1967, Funk & Parker 1966, Sutton 1980). Evans (1984) studied a large number of collections of these fungi from many parts of the world and found considerable differences in both anamorph and teleomorph morphology. He contested the validity of varieties in *Dothistroma*, but acknowledged that morphotypes or ecotypes probably exist.

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

MATERIALS AND METHODS

Isolates

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

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

DNA extraction, amplification and sequencing

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

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

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

	CMW 6400	–	U.S.A.	Stanton, Michigan	var. *pini*	*P. nigra*	G. Adams	–
	CMW 14905	CBS 116483	U.S.A.	McBain, Riverside Township, Massaukee County, Michigan	var. *pini*	*P. nigra*	G. Adams	2001
	CMW 14820	ATCC MYA-609	U.S.A.	Central, Minnesota	var. *pini*	*P. nigra*	–	1970
	CMW 14821	ATCC MYA-606	U.S.A.	Lincoln, Nebraska	var. *pini*	*P. nigra*	–	1964
	–	ILLS 27093 **T**	U.S.A.	DeKalb County, Illinois	*var. pini*	*P. nigra* var. *austriaca*	J.C. Carter	1938
Actinothyrium marginatum (T of *D. septosporum* var. *lineare*)	–	WSP 48361	U.S.A.	Meadow Creek, Clearwater Ranger District, Idaho	*var. lineare*	*P. ponderosa*	F. Matzner	1957
Mycosphaerella dearnessii	CMW 9985	CBS 871.95	France	Le-Teich, Gironde prefecture (Aquitaine)	–	*P. radiata*	M. Morelet	1995
	CMW 13119	ATCC 200602	China	Fujie	–	*P. elliottii*	Z.Y. Huang	–
Dothistroma rhabdoclinis	CMW 12519	CBS 102195	Germany	Wolfenböttel	–	*Pseudotsuga menziesii*	H. Butin	1998
Botryosphaeria ribis	CMW 7773	–	U.S.A.	New York	–	*Ribes* sp.	G. Hudler	2000

[a]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. [b]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 specimen.

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 $MgCl_2$. 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*I (Roche 10 U/µL) restriction enzyme in 20 µL reaction mixtures containing 2 µL 10× SuRE/Cut Buffer A and 7.5 µL water. CMW14822 was left undigested as a control. Reaction mixtures were incubated overnight at 37 °C followed by heat inactivation of the enzyme at 65 °C for 20 min. PCR-RFLP profiles were visualized on an ethidium bromide-stained agarose gel (3 %), under UV illumination.

RESULTS

Isolates

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

DNA extraction, amplification and sequencing

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

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

Phylogenetic analysis

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

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

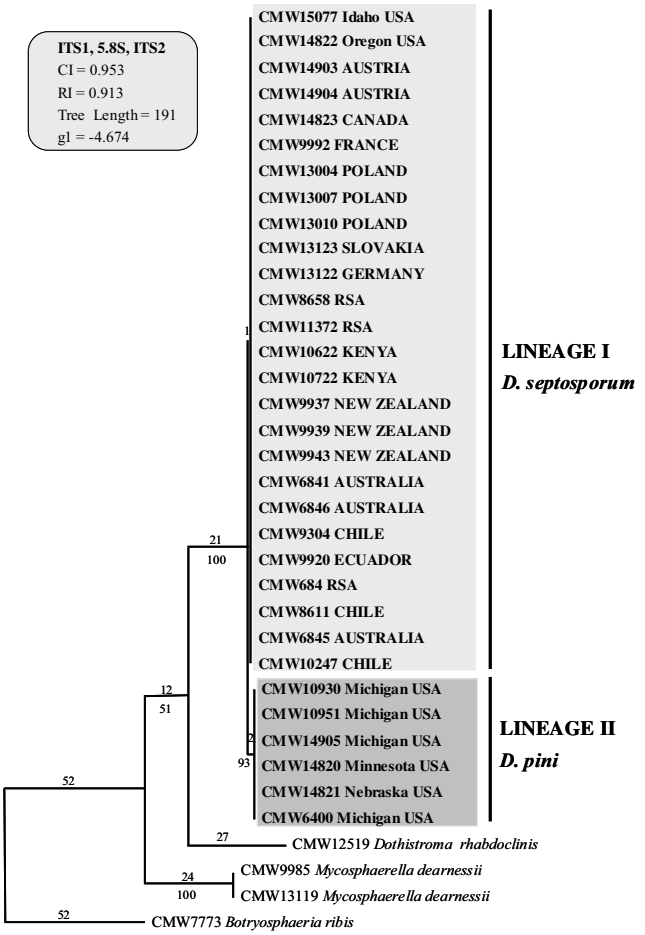

Fig. 2. One of 9 most parsimonious trees inferred from nuclear ribosomal internal transcribed spacer (ITS)1, 5.8S and ITS2 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 473 characters, 90 variable characters were parsimony-uninformative and 57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

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

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

The sequence obtained from the type material of *D. septosporum* var. *lineare* (WSP 48361), was also included in this clade (Fig. 2). The ITS sequences in this lineage were identical while slight variation was observed randomly in the 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.

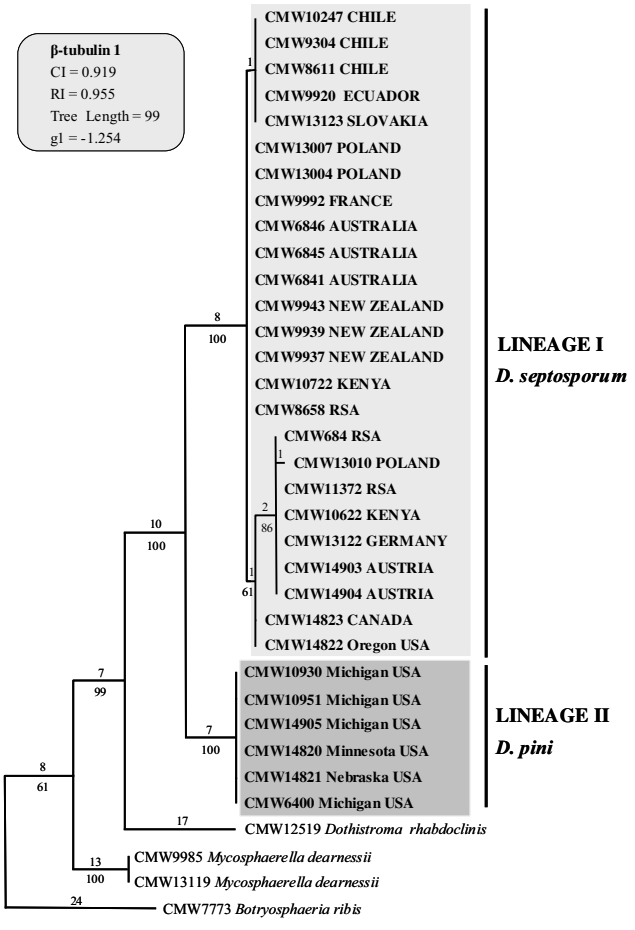

Fig. 3. Phylogeny of the red band needle blight fungi based on the β-tubulin-1 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 367 characters, 28 variable characters were parsimony-uninformative and 45 were parsimony-informative. Within-species variation 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.

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

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

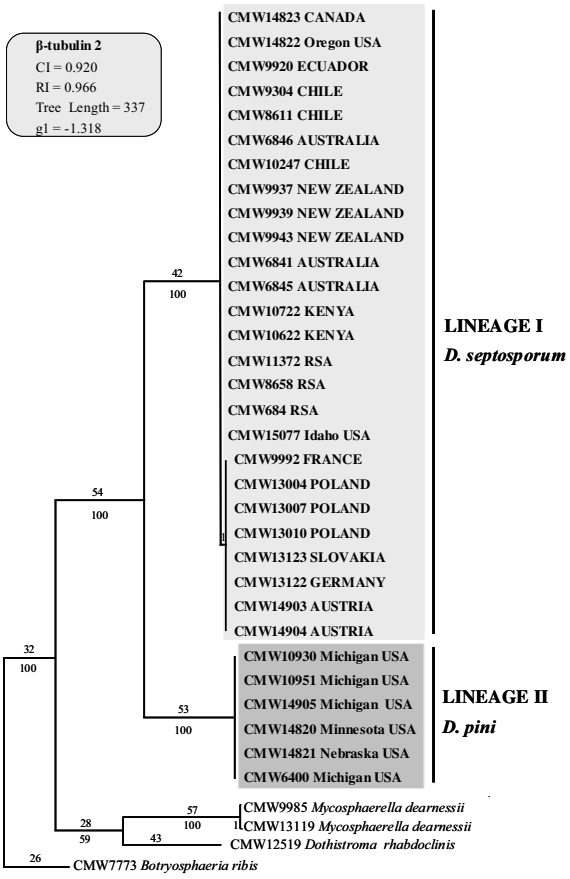

Fig. 4. Phylogeny of the red band needle blight fungi based on the β-tubulin-2 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Slight variation is observed within Lineage I while no variation is observed within Lineage II. Of 418 characters, 30 variable 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.

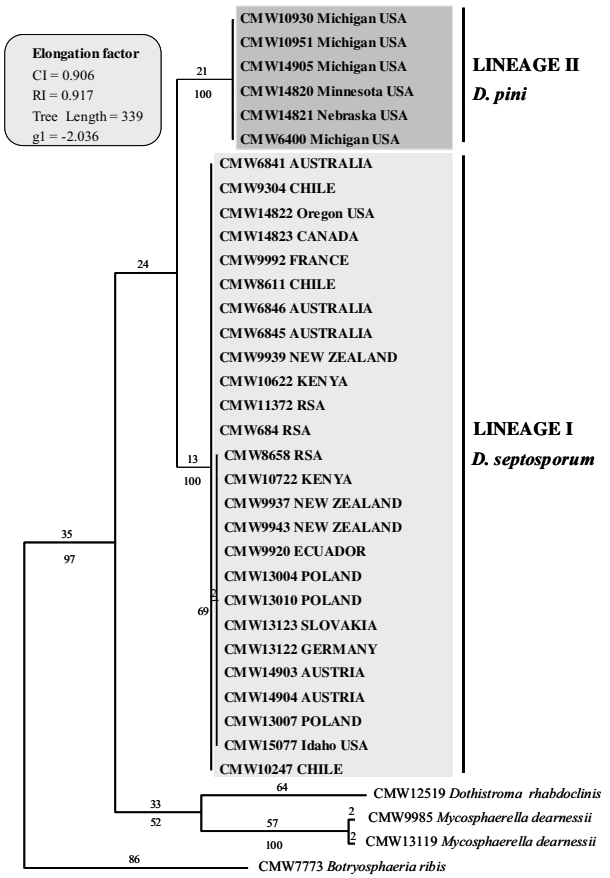

Fig. 5. One of 12 most parsimonious trees inferred from the EF1-α sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 346 characters, 87 variable characters were parsimony-uninformative and 130 were parsimony-informative. Bootstrap values are indicated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

Morphology

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

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

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

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

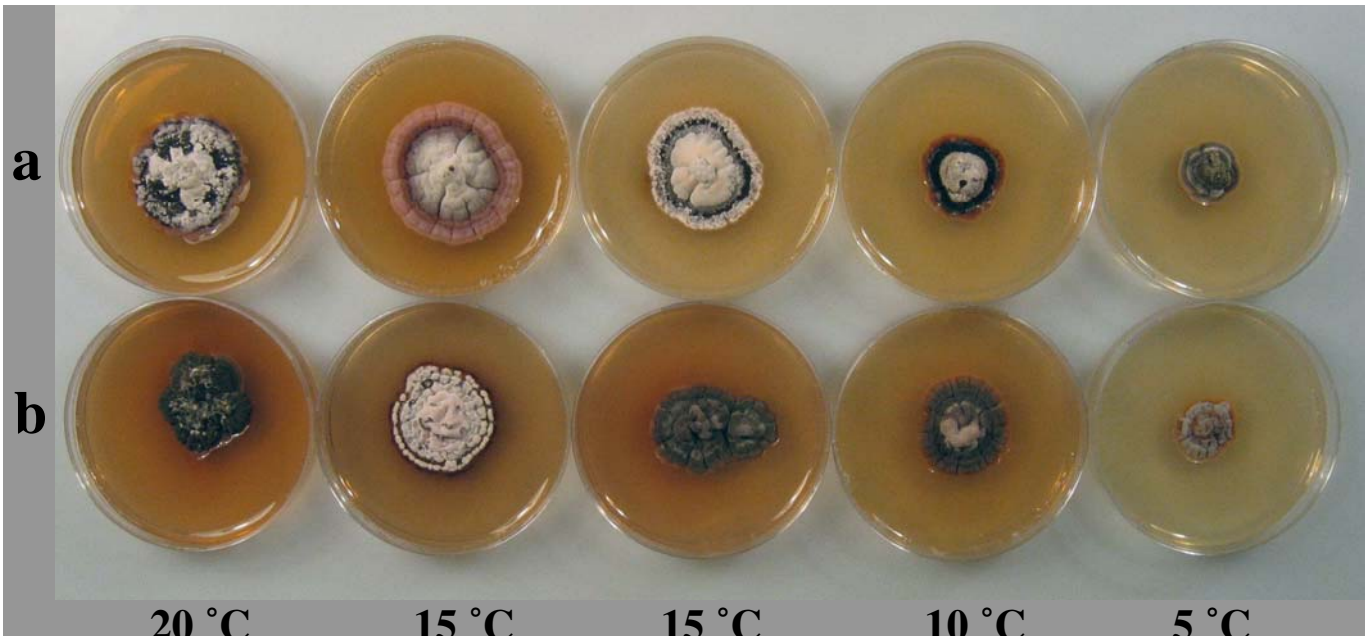

Fig. 6. Culture morphology of *Dothistroma* isolates from Lineages I (*D. septosporum s. str.*) and II (*D. pini*). Cultures, grown on 2 % MEA, have approximately the same amount of growth at their respective temperatures after a six week period. Cultures vary considerably in morphology and colour within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

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

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

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

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

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

Conidiomata predominantly occurring in red bands on the upper and lower needle surfaces, separate to aggregated, sub-epidermal, becoming erumpent, and splitting the needle surface with one or two longitudinal slits; at maturity acervular, black, up to 1 mm in length, lined internally with pseudoparenchymatous cells giving rise to conidiophores; these cells brown, becoming paler at the point of conidiophore attachment. *Conidiophores* pale brown to hyaline, smooth, densely aggregated, subcylindrical to irregular, 1–4-septate, branched or simple, 15–27 × 2–3 µm. *Conidiogenous cells* integrated, hyaline, smooth, subcylindrical, tapering towards the bluntly rounded apices, proliferating sympodially or percurrently near the apex, 7–12 × 2–3 µm. *Conidia* aggregated in cream to pale brown, slimy masses; smooth, thin-walled, hyaline, subcylindrical to narrowly obclavate or irregular, subobtuse at the apices, truncate to obconically subtruncate at the bases, (1–)3(–5) septate, (18–)25–35 (–45) × 3–5 µm (av. 30 × 3.5 µm) *in vivo,* (11–)20–25(–27) × (2–)2.5–3(–3.5) µm (av. 22 × 3 µm) *in vitro*.

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

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

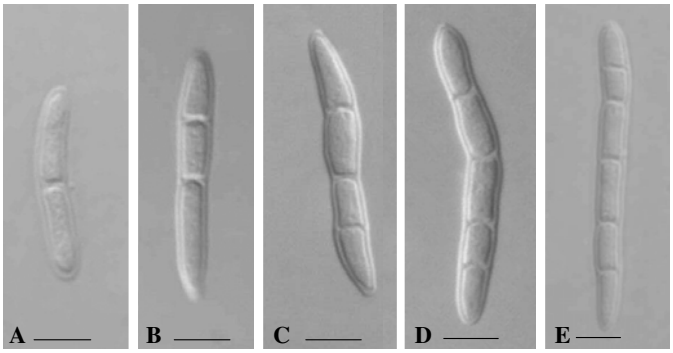

Fig. 8. Variation observed in conidial dimensions and number of septa within isolate CBS 116487 (Michigan, U.S.A.), from Lineage II (*D. pini*). Scale bars = 5 µm.

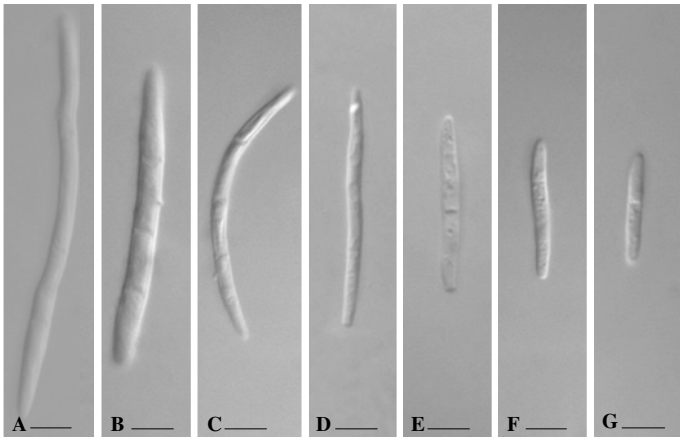

Fig. 7. Variation in conidial dimensions found within isolates from Lineage I (*D. septosporum s. str.*). Conidia obtained directly from infected hosts. A–C. Austria. D, E. New Zealand. F, G. Ecuador. Scale bars = 5 µm.

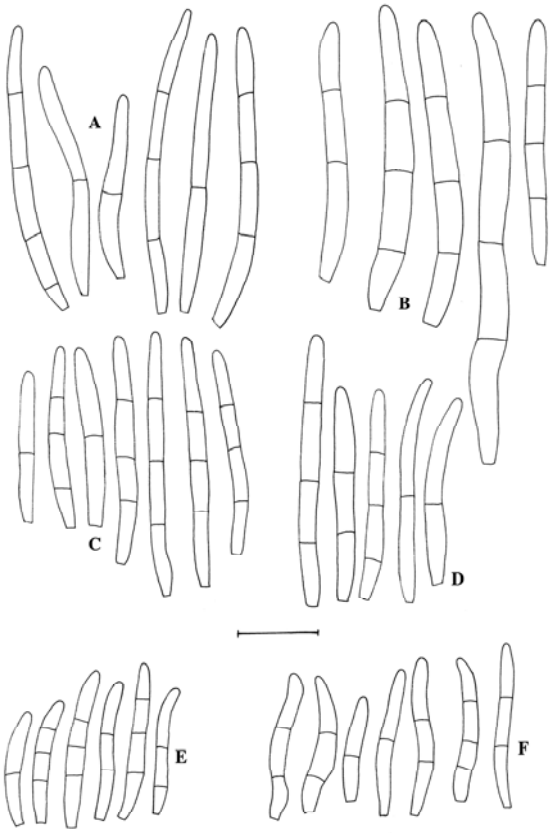

Fig. 9. Variation in conidial morphology of *Dothistroma septosporum s. str.* on needles. A. Idaho, WSP 48361, type of *Actinothyrium marginatum*. B. Idaho, herb. CBS 12204. C. Chile, herb. CBS 12206. D. Austria, herb. CBS 12205. E. Ecuador, herb. CBS 12207. F. New Zealand, herb. CBS 12208. Scale bar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

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

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

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

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

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

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

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

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

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

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

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

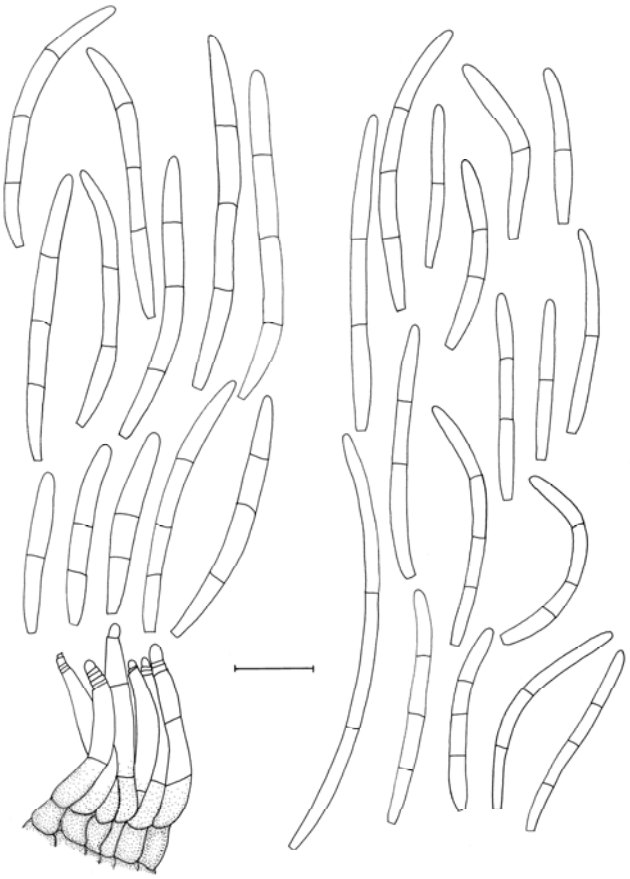

Fig. 10. Conidia and conidiogenous cells of *Dothistroma pini* from Michigan on *Pinus nigra* (herb. CBS 12211). On needles (left), and on oatmeal agar (right). Scale bar = 10 µm.

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

Conidiomata predominantly occurring in red bands on the upper and lower needle surface, separate to aggregated, sub-epidermal, becoming erumpent, and splitting the needle surface with one or two longitudinal slits; at maturity acervular, black, up to 1 mm in length, lined internally with pseudoparenchymatous cells giving rise to conidiophores; these cells brown, becoming paler at the point of conidiophore attachment. *Conidiophores* pale brown to hyaline, smooth, densely aggregated, subcylindrical to irregular, 0–4-septate, branched or simple, 7–25 × 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*I restriction site, and thus, upon exposure of the PCR product to *Alu*I, the fragment retains its original length of 520 base pairs.

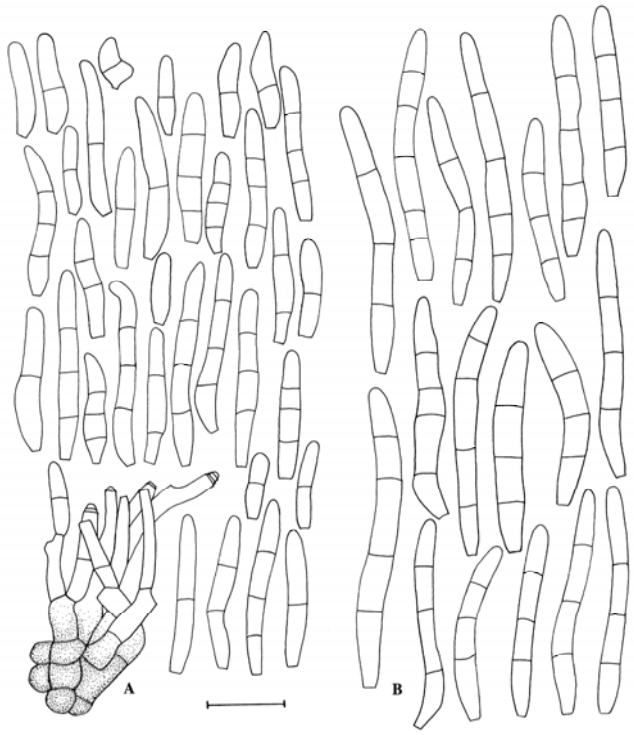

Fig. 11. Conidia and conidiogenous cells of *Dothistroma septosporum* from Poland on *P. nigra* (herb. CBS 12209). A. on needles. B. on oatmeal agar. Scale bar = 10 µm.

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

PCR-RFLP diagnostic procedure

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

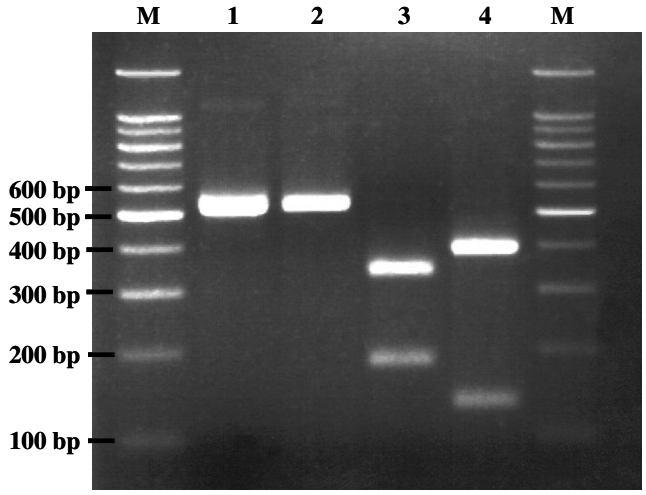

Fig. 12. PCR-restriction fragment length polymorphism (RFLP) pattern of the three *Dothistroma* species digested with the restriction enzyme *Alu*I. A 100 bp marker (M) is on either side of the gel. Lane 1: uncut PCR amplicon (CMW 14822) used as a control; Lane 2: *D. septosporum* (CMW 6841) from Lineage I is not digested by *Alu*I, 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. septospora* var. *lineare* (Ivory 1967). This dictates that *M. pini* is connected to the fungus reflected by phylogenetic Lineage I with the anamorph *D. septosporum*. The separation of *M. pini* into a separate genus, *Eruptio* M.E. Barr (Barr 1996), was refuted by Crous *et al*. (2001), who showed that *Eruptio* is a synonym of *Mycosphaerella*.

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

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

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

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REFERENCES

Barnes I, Roux J, Coetzee MPA, Wingfield MJ (2001). Characterization of *Seiridium* spp. associated with cypress canker based on β-tubulin and histone sequences. *Plant Disease* **85**: 317–321.

Barr ME (1996). *Planistromellaceae*, a new family in the *Dothideales*. *Mycotaxon* **60**: 433–442.

Bassett C (1969). *Larix decidua* a new host for *Dothistroma pini*. *Plant Disease Reporter* **53**: 706.

Bradshaw R E, Ganley RJ, Jones WT, Dyer P S (2000). High levels of dothistromin toxin produced by the forest pathogen *Dothistroma pini*. *Mycological Research* **104**: 325–332.

Butin H, Kehr R, Pehl L (2000). *Dothistroma rhabdoclinis* sp. nov. associated with *Rhabdocline pseudotsugae* on Douglas fir. *Forest Pathology* **30**: 195–203.

Carbone I, Kohn LM (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553–556.

Carson SD, Carson MJ (1989). Breeding for resistance in forest trees – a quantitative genetic approach. *Annual Review of Phytopathology* **27**: 373–395.

Chou CKS (1991). Perspectives of disease threat in large-scale *Pinus radiata* monoculture – The New Zealand experience. *European Journal of Forest Pathology* **21**: 71–81.

Couch BC, Kohn LM (2002). A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* **94**: 683–693.

Crous PW, Kang J-C, Braun U (2001). A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequence and morphology. *Mycologia* **93**: 1081–1101.

Dearness J (1928). New and noteworthy fungi IV. *Mycologia* **18**: 236–255.

Dick AMP (1989). Control of Dothistroma needle blight in the *Pinus radiata* stands of Kinleith forest. *New Zealand Journal of Forestry Science* **19**: 171–179.

Doroguine G (1911). Une maladie cryptogamique du pin. *Bulletin Trimestriel de la Société Mycologique de France* **27**: 105–106.

Dubin H J, Walper S (1967). *Dothistroma pini* on *Pseudotsuga menziesii*. *Plant Disease Reporter* **51**: 454.

Evans HC (1984). The genus *Mycosphaererella* and its anamorphs *Cercoseptoria*, *Dothistroma* and *Lecanosticta* on pines. *Mycological Papers* **153**: 1–102.

Funk A, Parker AK (1966). *Scirrhia pini* n. sp., the perfect state of *Dothistroma pini* Hulbary. *Canadian Journal of Botany* **44**: 1171–1176.

Gadgil PD (1967). Infection of *Pinus radiata* needles by *Dothistroma pini*. *New Zealand Journal of Botany* **5**: 498–503.

Gadgil PD (1984). Dothistroma needle blight. *Forest Pathology in New Zealand* No **5**.

Gibson IAS (1974). Impact and control of Dothistroma blight of pines. *European Journal of Forest Pathology* **4**: 89–100.

Gibson IAS, Christensen PS, Munga FM (1964). First observations in Kenya of a foliage disease of pines caused by *Dothistroma pini* Hulbary. *Commonwealth Forestry Review* 31–42.

Glass NL, Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Goodwin SB, Dunkle LD, Zismann VL (2001). Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. *Phytopathology* **91**: 648-658.

Gremmen J (1968). The presence of *Scirrhia pini* Funk et Parker in Romania (Conidial stage: *Dothistroma pini*

Hulb.) *Bulletin Trimestiel de la Société Mycologique de France* **84**: 489–492.

Hedgcock GG (1929). *Septoria acicola* and the brown-spot disease of pine needles. *Phytopathology* **19**: 993–999.

Hulbary RL (1941). A needle blight of Austrian pine. *Illinois Natural History Survey Bulletin* **21**: 231–236.

Ivory MH (1967). A new variety of *Dothistroma pini* in Kenya. *Transactions of the British Mycological Society* **50**: 289–297.

Ivory MH (1994). Records of foliage pathogens of *Pinus* species in tropical countries. *Plant Pathology* **43**: 511–518.

Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifert KA, Bright DE, Wingfield BD (2004). *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411–418.

Karadži DM (1994). *Picea omorika* – a new host of *Dothistroma septospora*. *European Journal of Forest Pathology* **24**: 300–303.

Kowalski T, Jankowiak R (1998). First record of *Dothistroma septospora* (Dorog.) Morelet in Poland: a contribution to the symptomology and epidemiology. *Phytopathologia Polonica* **16**: 15–29.

Lang KJ (1987). *Dothistroma pini* on young Norway spruce (*Picea abies*). *European Journal of Forest Pathology* **17**: 316–317.

Morelet M (1968). De aliquibus in mycologia novitatibus (3e note). *Bulletin de la Société des Sciences Naturelles et d'Archéologie de Toulon et du Var* **177**: 9.

O'Donnell K, Ward TJ, Geiser DM, Kistler HC, Aoki T (2004). Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology* **41**: 600–623.

Pas JB van der (1981). Reduced early growth rates of *Pinus radiata* caused by *Dothistroma pini*. *New Zealand Journal of Forestry Science* **11**: 210–220.

Peterson GW (1974). *Dothistroma* needle blight of pines. *Forest Insect and Disease Leaflet 143 U.S. Department of Agriculture, Forest Service.*

Saccardo PA (1920). Mycetes Boreali-Americani. *Nuovo Giornale Botanico Italiano* **27**: 75–88.

Siggers PV (1944). The brown spot needle blight of pine seedlings. *U.S.D.A. Technical Bulletin* **870**: 1–36.

Sutton BC (1980). The *Coelomycetes*: *Fungi imperfecti with pycnidia, acervuli and stromata*. Kew, Surrey, UK: Commonwealth Mycological Institute.

Swofford DL (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods).* Version 4. Sunderland, Massachusetts: Sinauer Associates.

Sydow H, Petrak F (1924). Zweiter Beitrag zur Kenntnis der Pilzflora Nordamerikas, insbesondere der nordwestlichen Staaten. *Annales Mycologici* **22**: 387–409.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876–4882.

Thyr BD, Shaw CG III (1964). Identity of the fungus causing redband disease on pines. *Mycologia* **56:** 103–109.

Trotter A. (1931). P.A. Saccardo's Supplementum Universale. *Sylloge Fungorum* **25**: 480.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A sequencing guide to methods and applications*. (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds.). San Diego, Academic Press: 315–322.

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

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

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

For isolates obtained in 2000, template DNA for polymerase chain reaction (PCR) was obtained by scraping mycelia with a pipette tip from 4- to 6 wk old cultures grown on PDA (Harrington and Wingfield 1995). For the isolates obtained in 2005, DNA was extracted from mycelia with Prepman Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California). Primer pairs used for amplification and sequencing of the ITS region were ITS-1F/ITS4 (White et al 1990), and primer pairs used for amplification and sequencing of LSU were respectively LR0R/LR5 and LR0R/LR3 (Vilgalys and Hester 1990). Amplification reactions consisted of 4 mM $MgCl_2$, 5% DMSO, 1× Sigma buffer, 200 μM dNTPSs, 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.

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

Species	Strain CBS Accession No.	Herbarium Accession No.	GenBank Accession	
			LSU	ITS
Schizothyrium pomi	CUA1a, CBS 118957	438789, CBS-H19787	AY598895	EF164898
	ZJ001		AY598894	AY598848
	ZJ002			AY598849
	ZJ003			AY598850
	AHA2a			AY598851
	GTA1a			AY598852
	CBS 228.57		EF134947	EF134947
	CBS 406.61		EF134949	EF134949
	CBS 486.50		EF134948	EF134948
Zygophiala cryptogama	FVA2a, CBS 118949	438791, CBS-H19785	AY598896	AY598854
	MWA8a			EF164899
	KY1 1.2A1c		EF164902	EF164900
Zygophiala tardicrescens	MWA1a, CBS 118946	438792, CBS-H19788	EF164901	AY598856
Zygophiala wisconsinensis	MSTA8a, CBS 118950	438790, CBS-H19786	AY598897	AY598853
	GTA4b			AY598855

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

Morphology of SBFS isolates on apple and in vitro.—Signs of SBFS on preserved apple peels were described, including mycelial growth patterns and fruiting body size and density. Colony descriptions were made after 1 mo growth on oatmeal agar (OA) at 21–24 C under intermittent ambient light. Fungal structures were mounted in clear lactic acid and examined at 1000× magnification. Thirty measurements were determined for each structure. For conidial measurements, the 95% percentiles are presented and extremes given in brackets.

RESULTS

Phylogenetic analysis.—The ITS alignment contained 83 taxa (including outgroup), and 481 characters were used for the analyses. Of these characters, 33 were parsimony informative, 101 were variable and parsimony uninformative and 347 were constant. The 24 equally parsimonious trees obtained from ITS analysis delimited four putative species of *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, Virginia and Missouri and is described as *Zygophiala wisconsinensis* sp. nov. Isolates from the last clade (100% bootstrap support and sister of *Z. wisconsinensis*) were obtained from a

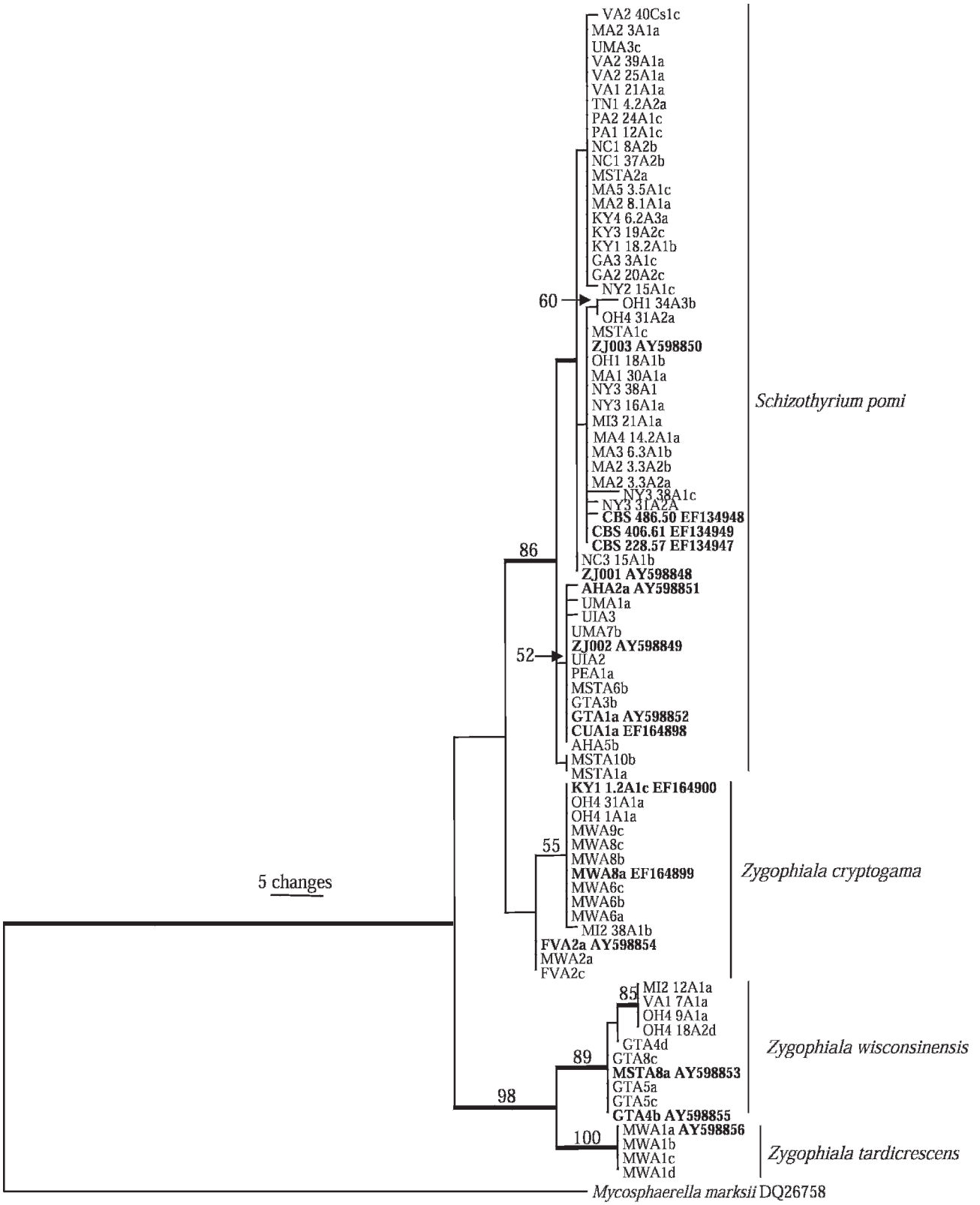

FIG. 1. One of 24 equally most parsimonious trees determined from ITS sequences obtained from isolates taken from flyspeck signs on apple fruit from eastern and midwestern orchards. Bootstrap support values (>50%) based on 1000 replicates are shown at the nodes, and strict consensus branches are thickened.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

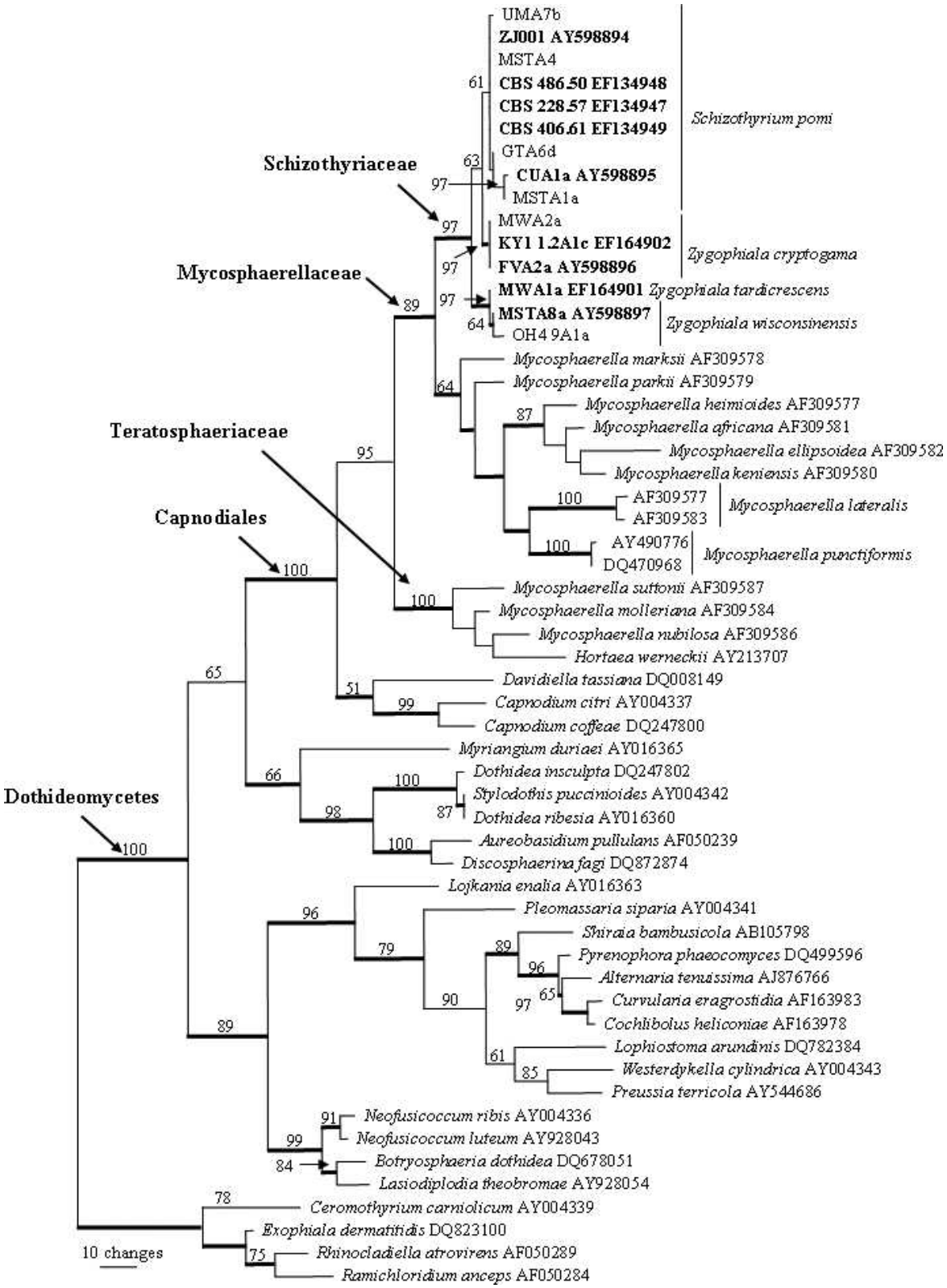

FIG. 2. One of 10 equally most parsimonious trees of partial sequences of the 28S large subunit (LSU) region of rDNA from flyspeck isolates on apple fruit from eastern and midwestern orchards and other ascomycetes. Bootstrap support values (>50%) based on 1000 replicates are shown at the nodes, and strict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

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

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

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

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

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

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

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

Ascomata black, shiny, dimidiate, in random clusters, but frequently in circles, superficial on leaves, stems or fruit, appressed to the cuticle, 150–375 µm diam, 30–50 µm high, with irregular margins; upper layer consisting of interwoven mycelium, forming 2–4 layers of thick-walled, brown, pseudoparenchymatal cells, 4–8 µm thick; ostiole central, but upper layer splitting at maturity via irregular ruptures from the elevated center; ascomata situated on a thin, hyaline, basal stroma. *Hamathecium* hyaline, consisting of branched, septate, pseudoparaphysoid-like filaments, 3–5 µm wide. *Asci* bitunicate, 8-spored, ovoid to subglobose or ellipsoid to clavate, apical chamber present but inconspicuous at maturity, 20–45 × 8–16 µm; formed in a single layer in the hamathecial tissue. *Ascospores* hyaline, guttulate, thick-walled, medianly 1-septate, constricted at septum, fusoid-ellipsoidal, widest in the middle of the apical cell, which is acutely rounded, while the lower cell is subobtusely rounded, (10–)12–13(–14) × (3–)3.5–4(–5) µm. Ascospores germinating after 24 h on MEA, becoming brown and verruculose, with a visible mucoid sheath surrounding the spore on the agar surface, slightly or not constricted at the septum, 4–5 µm wide, not distorting, germinating from both ends, with 2–3 germ tubes; cultures are homothallic.

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

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

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

←

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

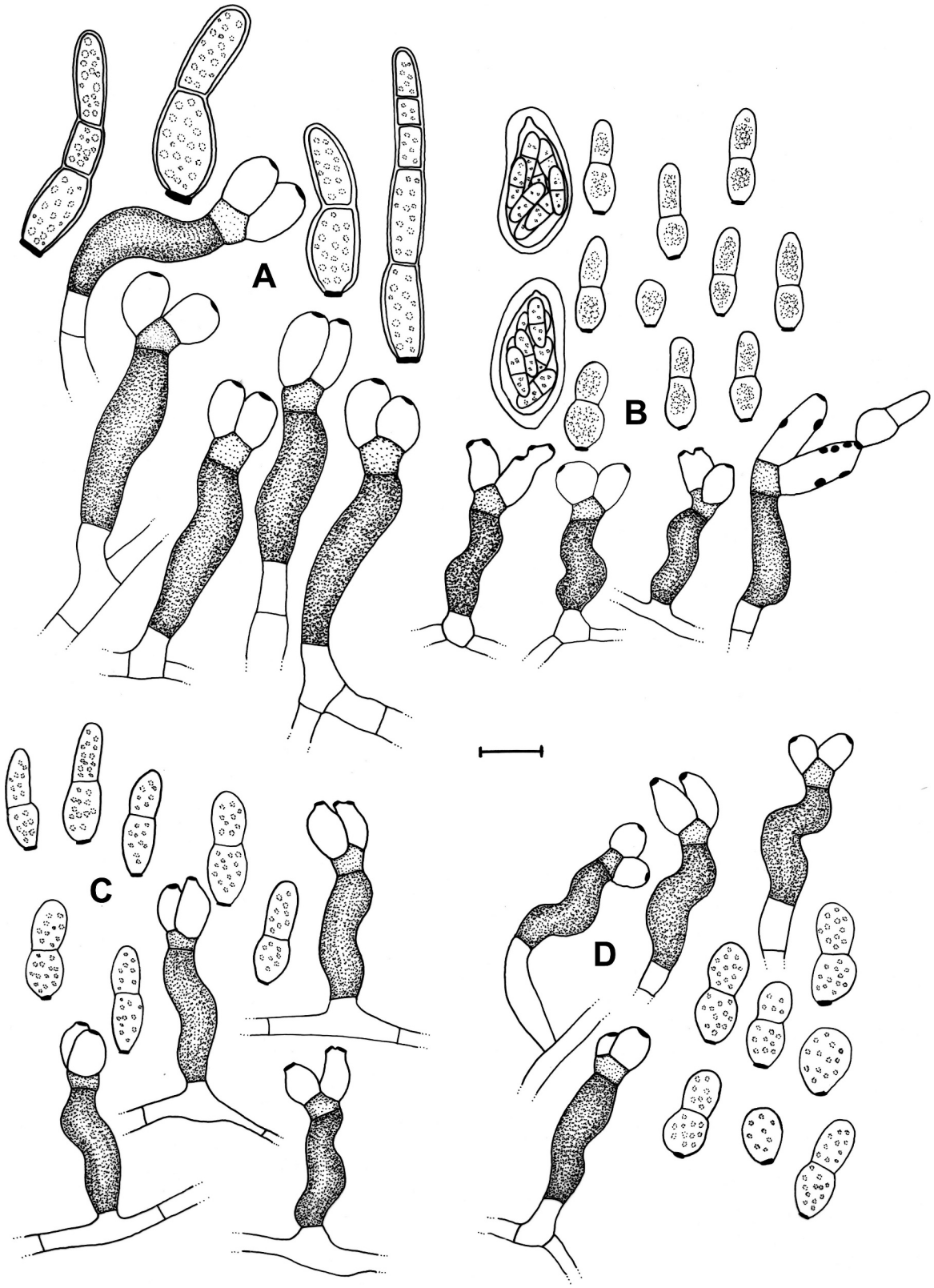

FIG. 3. *Zygophiala* spp. sporulation on oatmeal agar. A. Conidiophores and conidia of the *Zygophiala* anamorph of *Schizothyrium pomi* (CBS 118957). B. Asci, conidiophores and conidia of *Z. cryptogama* (CBS 118949). C. Conidiophores and conidia of *Z. tardicrescens* (CBS 118946). D. Conidiophores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

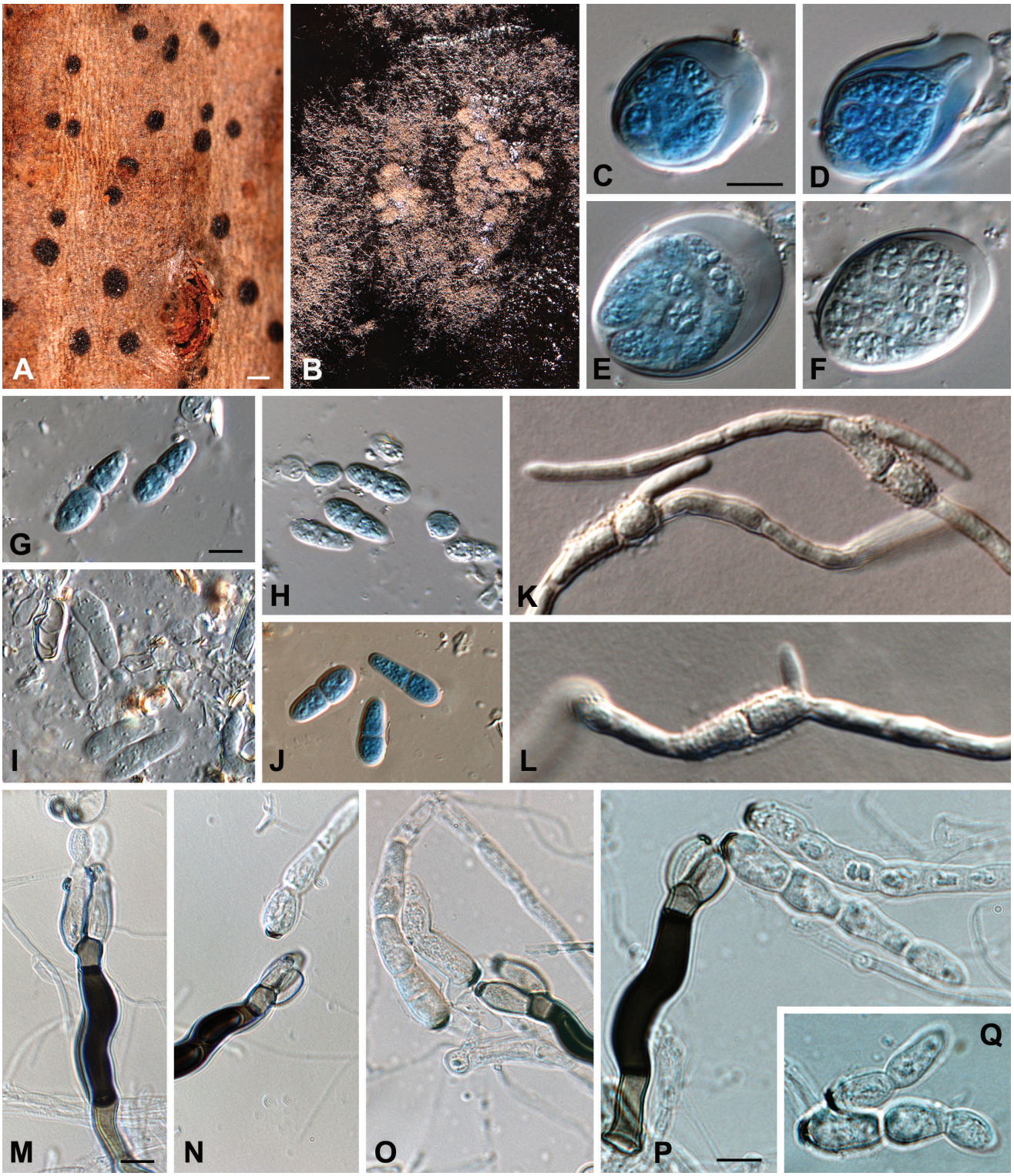

FIG. 4. *Schizothyrium pomi* and its *Zygophiala* anamorph. A. Thyrothecia occurring on a *Rhus* stem. B. Ascomatal initials forming on oatmeal agar. C–F. Asci. G–J. Ascospores. K–L. Germinating ascospores. M–Q. Conidiophores and conidia in vitro. Bars: C = 6, G = 5, M, *P* = 8 µm.

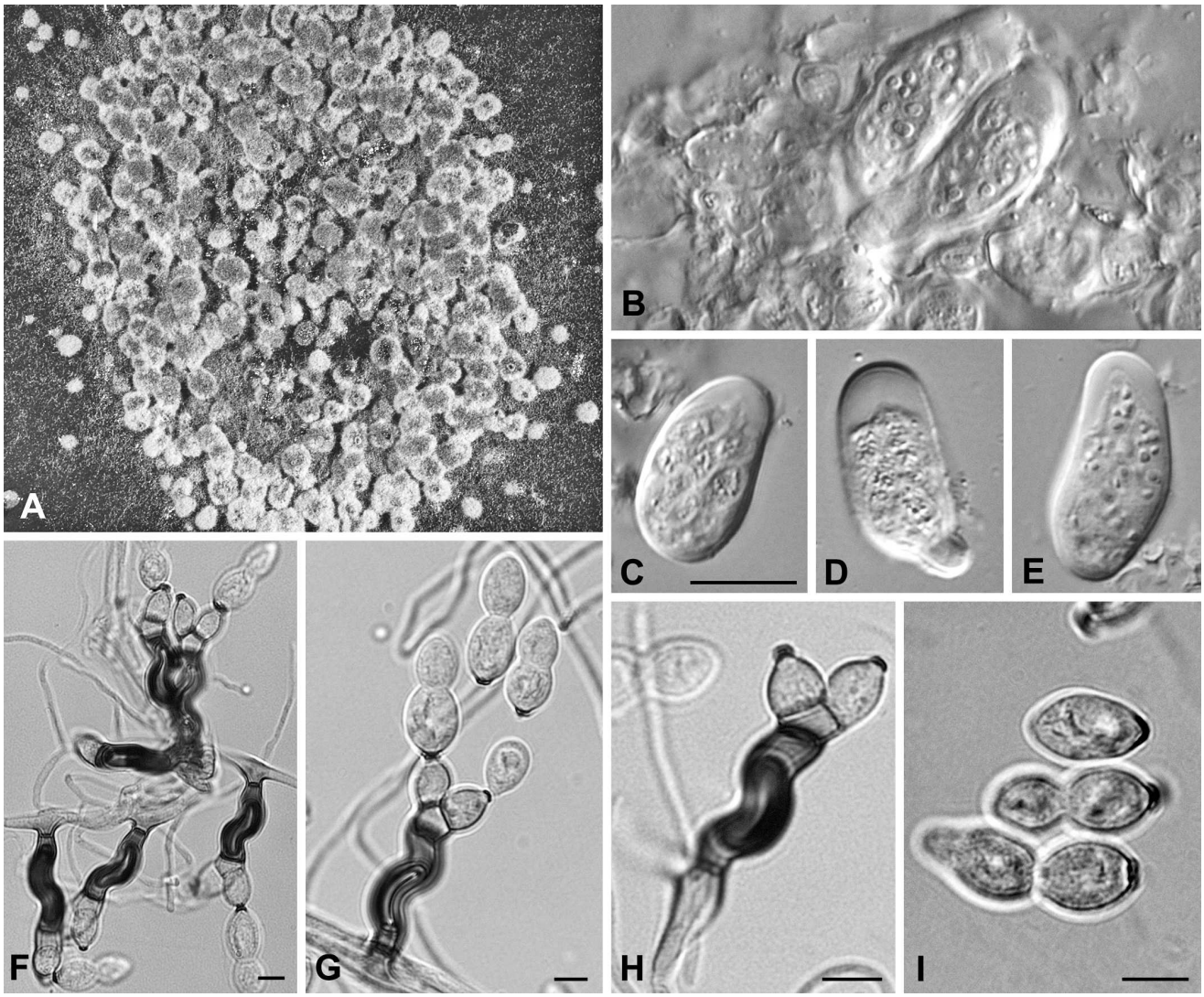

FIG. 5. *Zygophiala cryptogama* on oatmeal agar (CBS 118949). A. Ascomatal initials. B–E. Asci. F–H. Conidiophores. I. Conidia. Bars: C = 13, F = 4, G, H = 5, I = 6 µm.

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 × 4–5 µm and conidia 15–18 × 4–5 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. FIGS. 3, 5.

MycoBank MB501243.

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

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

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

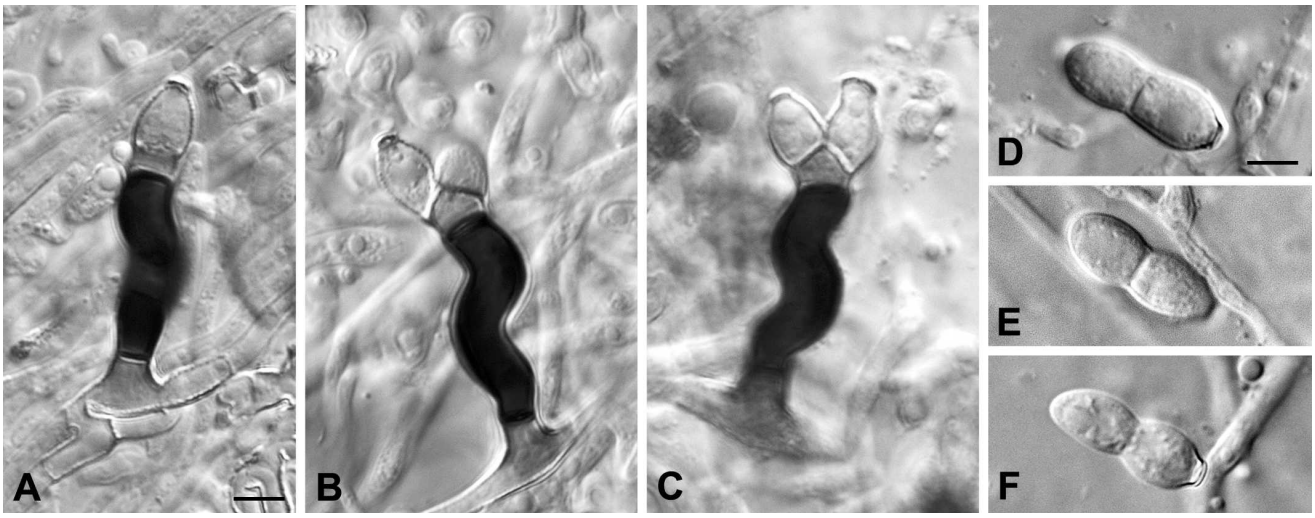

FIG. 6. *Zygophiala tardicrescens* on oatmeal agar (CBS 118946). A–C. Conidiophores. D–F. Conidia. Bars: A = 6, D = 7 µm.

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

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

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

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

Zygophiala tardicrescens Batzer & Crous, sp. nov.

FIGS. 3, 6. MycoBank MB501244.

Etymology. Named after its slow growth.

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

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

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

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

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

Zygophiala wisconsinensis Batzer & Crous, sp. nov.

FIGS. 3, 7. MycoBank MB501245.

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

Zygophialae jamaicensi similis, sed coloniis celerius

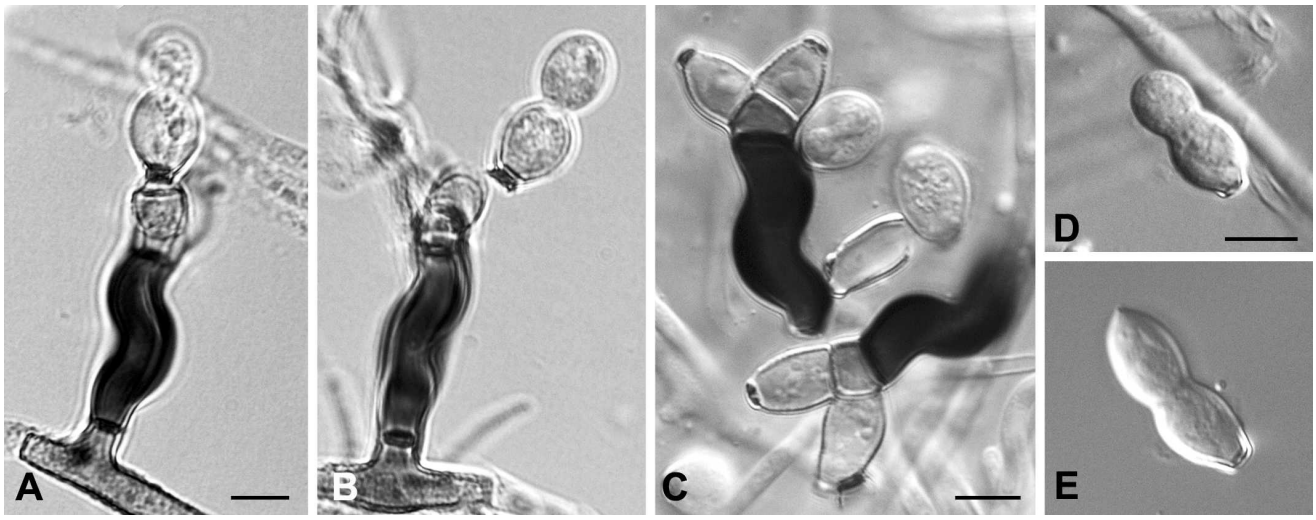

FIG. 7. *Zygophiala wisconsinensis* on oatmeal agar (CBS 118950). A–C. Conidiophores. D–E. Conidia. Bars: A, C = 6, D = 7 µm.

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, doliiform to ellipsoidal, polyblastic conidiogenous cells, 7–11 × 5–6 µm, with 1–2 prominent scars, apical and lateral, darkened, thickened, somewhat refractive, 2 µm wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, granular, aseptate, 6–8 × 6–8 µm, or transversely 1-septate (rarely median), (13–)15–18(–23) × (6–)7–8 µm, prominently constricted at the septum; apex obtuse, base subtruncate, with a darkened, thickened hilum, 2–3 µm wide.

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

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

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

DISCUSSION

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

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

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

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

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

KEY TO SPECIES OF *ZYGOPHIALA*

1. Conidia (0–)1 to multiseptate on OA 2
1. Conidia (0–)1-septate on OA 3
2. One-septate conidia (20–)22–25(–30) × 5–7(–8) µm. . . . . *Schizothyrium pomi*
2. One-septate conidia (12–)14–18(–20) × (4–)5–6(–8) µm . . . . . *Zygophiala cryptogama*
3. One-septate conidia shorter than 20 µm, and narrower than 6 µm; conidia 15–18 × 4–5 µm . . . . . *Zygophiala jamaicensis*
3. One-septate conidia 20 µm or longer, and 6 µm or wider; conidia 13–23 × 6–8 µm 4
4. Colonies fast-growing, reaching 13.5–22.5 mm diam on MEA after 2 wk at 25 C. . . . . *Zygophiala wisconsinensis*
4. Colonies slow-growing, reaching 2.5–4.5 mm diam on MEA after 2 wk at 25 C. . . . . *Zygophiala tardicrescens*

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LITERATURE CITED

Baines RC. 1940. Pathogenicity and hosts of the flyspeck fungus of apple. Phytopathology 30:2.

Baker KF, Davis LH, Durbin RD, Snyder WC. 1977. Greasy blotch of carnation and flyspeck disease of apple: diseases caused by *Zygophiala jamaicensis*. Phytopathology 67:580–588.

Batzer JC, Gleason ML, Harrington TC, Tiffany LH. 2005. Expansion of the sooty blotch and flyspeck complex on apples based on analysis of ribosomal DNA gene sequences and morphology. Mycologia 97:1268–1286.

Belding RD, Sutton TB, Blankenship SM, Young E. 2000. Relationship between apple fruit epicuticular wax and growth of *Peltaster fructicola* and *Leptodontidium elatius*, two fungi that cause sooty blotch disease. Plant Dis 84: 767–772.

Colby AS. 1920. Sooty blotch of pomaceous fruits. Trans III Acad Sci 13:139–179.

Crous PW. 1998. *Mycosphaerella* spp. and their anamorphs associated with leaf spot diseases of *Eucalyptus*. Mycol Mem 21:1–170.

———, Denman S, Taylor JE, Swart L, Palm ME. 2004a. Cultivation and diseases of Proteaceae: *Leucadendron*, *Leucospermum* and *Protea*. CBS Biodiv Ser 2:1–228.

———, Groenewald JZ, Mansilla JP, Hunter GC, Wingfield MJ. 2004b. Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. Stud Mycol 50:195–214.

———, ———, Wingfield MJ, Aptroot A. 2003. The value of ascospore septation in separating *Mycosphaerella* from *Sphaerulina* in the Dothideales: a Saccardoan myth? Sydowia 55:136–152.

———, Wingfield MJ, Mansilla JP, Alfenas AC, Groenewald JZ. 2006. Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus* II. Stud Mycol 55:99–131.

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

imperfect stage of *Mycothyriella rubi*, cause of flyspeck of apple. Phytopathology 43:470–471.

Duggar BM, ed. 1909. Sooty blotch and flyspeck of apple and other plants. *Leptothyrium pomi* (Mont. & Fr.) Sacc. In: Fungous diseases of plants with chapters on physiology, culture methods and technique. Boston: Ginn & Co. p 367–369.

Ellis MB. 1971. Dematiaceaous Hyphomycetes. Surrey, England: Commmonwealth Mycological Institute. 608 p.

Eriksson OE, ed. 2006. Classification of the Ascomycetes. Myconet 12:1–82. Available from www.fieldmuseum.org/myconet/

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acid Symp Ser 41:95–98.

Harrington TC, Wingfield BD. 1995. A PCR-based identification method for species of *Armillaria*. Mycologia 87: 280–288.

Johnson EM, Sutton TB. 1994. First report of *Geastrumia polystigmatis* on apple and common blackberry in North America. Plant Dis 78:1219.

———, ———, Hodges CS. 1996. *Peltaster fructicola*: a new species in the complex of fungi causing apple sooty blotch disease. Mycologia 88:114–120.

———, ———, ———. 1997. Etiology of apple sooty blotch disease in North Carolina. Phytopathology 87:88–95.

Kirk PM, Cannon PF, David JC, Stalpers JA, eds. 2001. Ainsworth and Bisby's Dictionary of the Fungi. 9th ed. Cambridge, UK: CAB International. 655 p.

Le Corronc F, Batzer JC, Gleason ML. 2006. Effect of apple juice on in vitro morphology of four newly discovered fungi in the sooty blotch and flyspeck complex. Phytopathology 96:S65.

Lerner SM. 1999. Studies on biology and epidemiology of *Schizothyrium pomi*, causal agent of flyspeck disease of apple (Master's thesis). Amherst: University of Massachusetts.

Martyn EB. 1945. Note on banana leaf speckle in Jamaica and some associated fungi. Mycol Pap 13:1–5.

Montagne C. 1834. Notice sur les plantes crytogames récemment découverts en France. Ann Sci Nat, sér 2 Bot 1:295–349.

Nasu H, Fujii S, Yokoyama T. 1985. *Zygophiala jamaicensis* Mason, a casual fungus of flyspeck of grape, Japanese persimmon and apple. Ann Phytopathol Soc Jap 51: 536–545.

———, Konoh H. 1987a. Distribution of *Zygophiala jamaicensis* in Okayama Prefecture, Japan. Trans Mycol Soc Jap 28:209–213.

———, ———. 1987b. Scanning electron microscopy of flyspeck of apple, pear, Japanese persimmon, plum, Chinese quince, and pawpaw. Plant Dis 71:361–364.

———, ———. 1993. The pathological anatomy of *Zygophiala jamaicensis* on fruit surfaces. In: Biggs AR, ed. Handbook of cytology, histology, and histochemistry of fruit tree diseases. Boca Raton: CRC Press. p 137–154.

Schoch C, Shoemaker RA, Seifert K, Hambleton S, Spatafora JW, Crous PW. 2006. A multigene phylogeny of the Dothideomycetes using four nuclear loci. Mycologia (In press).

Schweinitz LD. 1834. *Dothidea pomigena*. Trans Am Philos Soc n.s. 4:232.

Sprague CJ. 1856. Contributions to New England mycology. Proc Boston Soc Nat History 5:325–329.

Sutton TB, Bond JJ, Ocamb-Basu CM. 1988. Reservoir hosts of *Schizothyrium pomi*, cause of flyspeck of apple, in North Carolina. Plant Dis 72:801.

Swofford DL. 2002. PAUP* Phylogenetic analysis using parsimony (*and other methods). Version 4.0. Sunderland, Massachusetts: Sinauer Associates.

Tarnowski TB, Batzer JC, Gleason ML, Helland S, Dixon P. 2003. Sensitivity of newly identified clades in the sooty blotch and flyspeck complex on apple to thiophanate-methyl and ziram. Online. Plant Health Progress doi:10.1094/PHP-2003-12XX-01-RS.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The Clustal X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Res 25: 4876–4882.

Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172:4239–4246.

von Arx JA. 1959. Ein beitrag zur kenntnis der fliegenfleckenpilze. Proc Koninkl Nederl Akad, ser C 62:333–340.

———, Müller E. 1975. A re-evaluation of the bitunicate ascomycetes with keys to families and genera. Stud Mycol 9:1–159.

Williamson SM, Sutton TB. 2000. Sooty blotch and flyspeck of apple: etiology, biology and control. Plant Dis 84: 714–724.

White TJ, Bruns T, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White JW, eds. PCR protocols: a guide to molecular methods and amplifications. New York: Academic Press. p 315–322.

Wrona BR. 2004. Influence of fruit surface sugars on the growth of fungi that cause sooty blotch of apple. J Plant Protect Res 44:283–288.

———, Gleason ML. 2005. Effect of surface amino acids on the growth of *Peltaster fructicola*—fungus associated with sooty blotch complex. J Plant Protect Res 45:273–278.

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

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

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

The genus *Cladosporium* was described by Link (1816) with *Cladosporium herbarum* as type species. Surveys of the generic history of *Cladosporium* were given by de Vries (1952) and David (1997). Early descriptions of *Cladosporium* were rather vague and the delimitations from similar genera obscure (Nees 1817, Corda 1837, 1842, Fries 1832, 1849, Saccardo 1886, Lindau 1907, etc.). Since its introduction, more than five hundred taxa have been attributed to *Cladosporium*. Due to the imprecise circumscription of *Cladosporium*, it is not surprising that numerous superficially similar but unrelated hyphomycetes have been assigned to this genus, making it very heterogeneous. De Vries (1952) and Ellis (1971, 1976) maintained broad concepts of *Cladosporium* and did not contribute towards a reduction of its heterogeneity, which was later discussed in detail by von Arx (1983), Morgan-Jones & Jacobsen (1988), McKemy & Morgan-Jones (1990), Morgan-Jones & McKemy (1990), and David (1997).

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

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

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sphaerella (Crous et al. 2000, Crous, Kang & Braun 2001), *Venturia* (Ritschel 2001, Schubert 2001), *Botryosphaeria* (Denman et al. 2000), and *Calonectria* (Crous 2002).

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

Material and methods

DNA isolation, amplification and phylogeny

The isolation protocol of Crous et al. (2000) was used to isolate genomic DNA from fungal mycelia grown on 2 % malt extract agar (MEA; Biolab, Midrand, Johannesburg) plates. The primers ITS1 (5'-TTT CCG TAG GTG AAC CTG C-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al. 1990) were used to amplify part of the nuclear rRNA operon spanning the 3' end of the 18S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS (ITS2) region and the 5'end of the 28S (large subunit) of the rRNA gene. The reaction mixture contained 5 μL of diluted sample, 1 x buffer, 8 mM $MgCl_2$, 500 μM of each of the dNTPs, 2.5 U (Bioline) Taq polymerase and 10 pM of each primer and made up to a total volume of 25 μl with sterile water. The cycling conditions comprised denaturing at 96 °C for 5 min, followed by 30 cycles of denaturation at 96 °C (30 s), annealing 50 °C (30 s) and elongation at 72 °C (90 s). A final elongation step at 72 °C for 7 min was included. The 5' end of the 18S rRNA gene was amplified with primers NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') and NS4 (5'-CTT CCG TCA ATT CCT TTA AG-3') (White et al. 1990). PCR conditions were the same for this region, except for the $MgCl_2$ concentration, which was lowered to 1.5 mM. PCR products were separated by electrophoresis at 75 V for 1 h in a 0.8 % (w/v) agarose gel in 0.5 x TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK) following ethidium bromide staining.

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

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

Morphology

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

Results

Phylogenetic analysis

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

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

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

The NJ tree for the SSU sequencing data (Fig. 2) contained isolates from the Mycosphaerellaceae, Pleosporaceae, Venturiaceae, as well as Dothioraceae, Dothideaceae, Botryosphaeriaceae, Leptosphaeriaceae and Pleosporales *inc. sed.* The Mycosphaerellaceae isolates consisted of isolates of *Mycosphaerella* and a strongly supported clade (90 %) of *Davidiella* containing *Cladosporium* spp. and a single isolate of *Sphaerulina polyspora* (STE-U 4301). *Mycosphaerella* isolates were present in a poorly supported (55 %) group, and contained, amongst others, *'Cladophialophora hachijoensis'* (STE-U 5121), *Passalora fulva* (STE-U 3688), *'Cladosporium' staurophorum* (STE-U 3687) and *Mycosphaerella* spp. The Dothideaceae clade was well-supported (100 %) and was grouped inside a clade with a 98 % bootstrap support value that contained a single isolate of the 'Dothioraceae'. The Venturiaceae clade (100 % bootstrap support) consisted of *Pseudocladosporium hachijoense* (STE-U 5391), *Fusicladium convolvulorum* (STE-U 3884), as well as isolates of *Fusicladium effusum*. *Anungitopsis amoena* (CBS 254.95) grouped with 99 % bootstrap support outside the Venturiaceae clade. The Pleosporaceae clade consisted of *Pleospora betae* U43465 (100 % bootstrap support) and a well-supported (100 %) clade containing *Pleospora herbarum* (U43458), isolates of *Alternaria malorum* and species of *Alternaria* and *Lewia*. The Paraphaeosphaeriaceae clade was well supported (100 %), and was grouped inside a clade that also contained a single isolate of the Leptosphaeriaceae (100 % bootstrap support).

Morphology

Cladosporium malorum (Pleosporaceae) clade

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

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

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

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

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

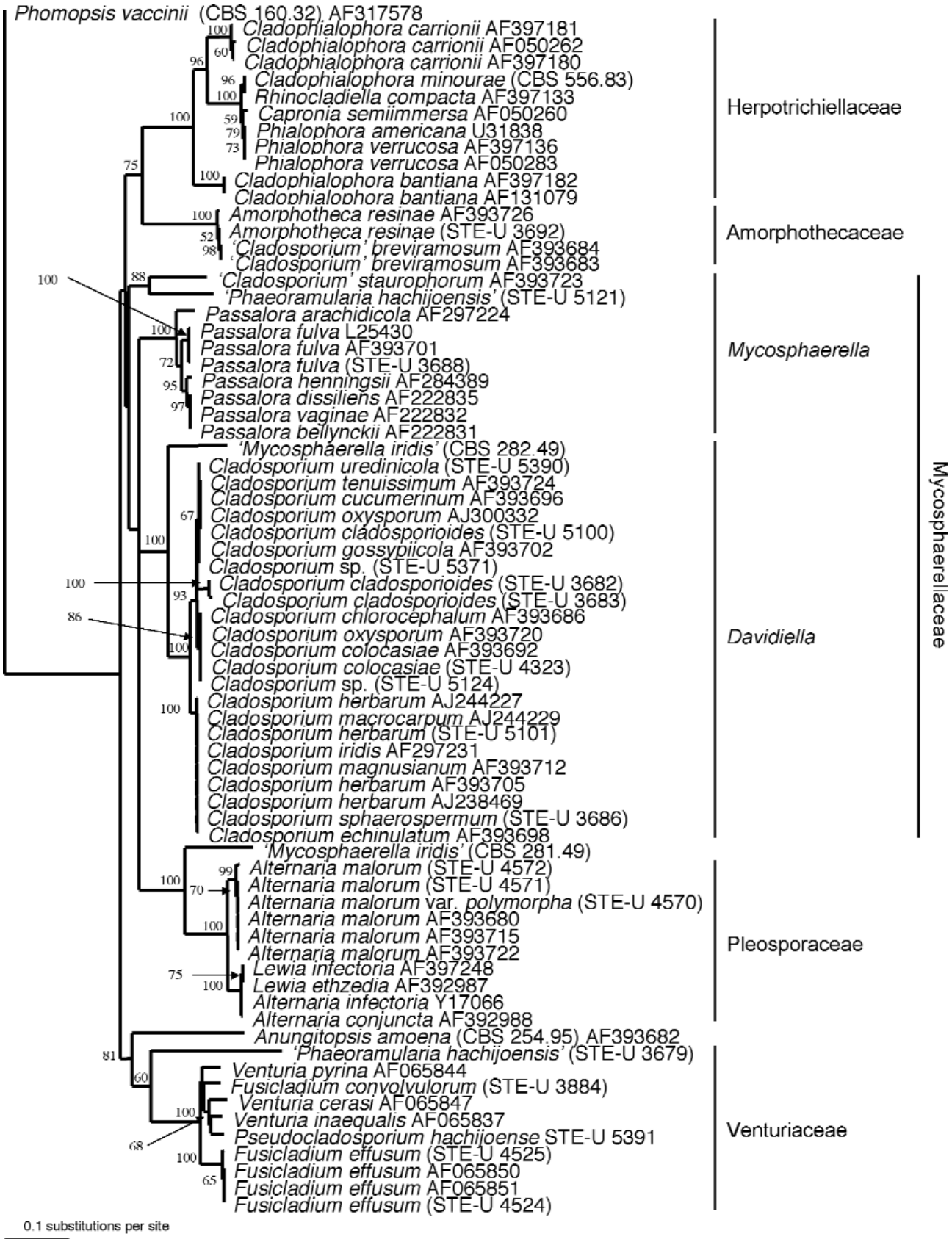


Fig. 1. Phylogram of neighbour joining tree obtained from ITS sequencing data using the uncorrected 'p' model of substitution. Bootstrap support values from 1000 replicates are shown at nodes. Due to the fact that not all nodes are clearly visible, some bootstrap values are not shown. The GenBank sequence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

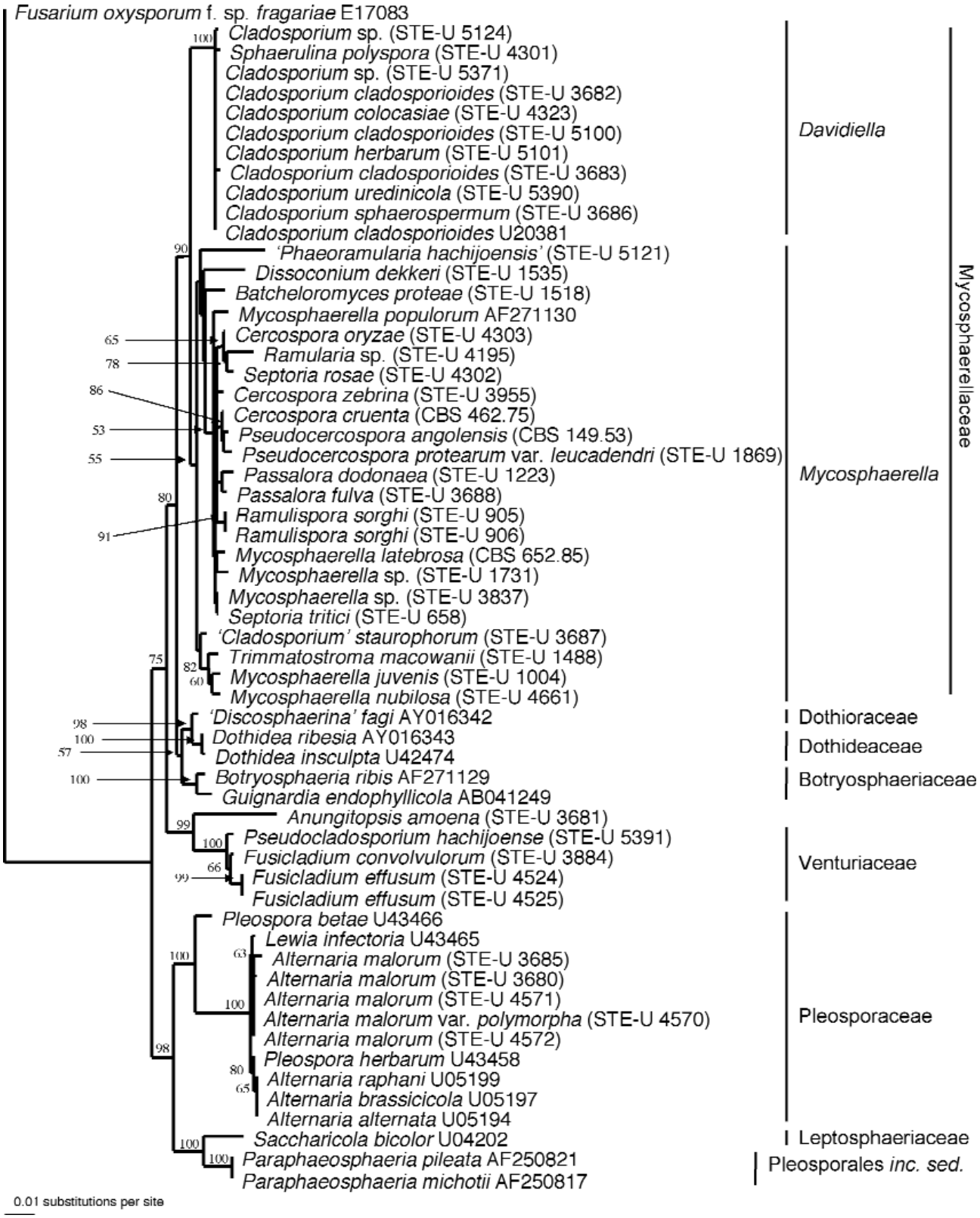


Fig. 2. Phylogram of neighbor joining tree obtained from small subunit rRNA gene sequencing data using the uncorrected 'p' model of substitution. Bootstrap support values from 1000 replicates are shown at nodes. Due to the fact that not all nodes are clearly visible, some bootstrap values are not shown.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

intercalary or terminal, chlamydospore-like (Fig. 4). Conidiophores pleurogenous and terminal, erect, straight, subcylindrical or somewhat attenuated towards the apex, slightly geniculate-sinuous, unbranched or rarely branched, 5–50 x 2–5(7) µm, 0–2(3)-septate, pale olivaceous to olivaceous-brown, thin-walled, smooth or almost so; conidiogenous cells integrated, terminal or conidiophores reduced to conidiogenous cells, 5–15 µm long, monotretic, determinate or polytretic, sympodial, usually with 1–2 conspicuous loci, 0.5–1.5 µm wide, unthickened, with minute central pori, 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* spp., *Triticum aestivum*), stored grains, Bing cherry fruit, fruits of *Malus domestica*, *Prunus persica,* and an old polypore on *Picea* sp., Canada, Lebanon, Libya, Pakistan, South Africa, Syria, Turkey, and the USA. Pathogenic in ripe apples (Ruehle 1931) and ripe cherries (Dugan & Roberts 1994). Once recorded as the principal fungal contaminant in market wheat in Washington state, USA (Schnellhardt & Heald 1936).

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

Alternaria malorum var. ***polymorpha*** Dugan, **var. nov.**
Figs. 3–12

Differt a var. *malorum* conidiis latioribus, *ca* 3.5–6 µm latis, atrioribus, leviter crassitunicatis, interdum longitudine septatis, raro alternarioidibus intermixtis.

Etymology: Referring to its variable conidial shape.

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

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

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

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

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

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

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

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

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

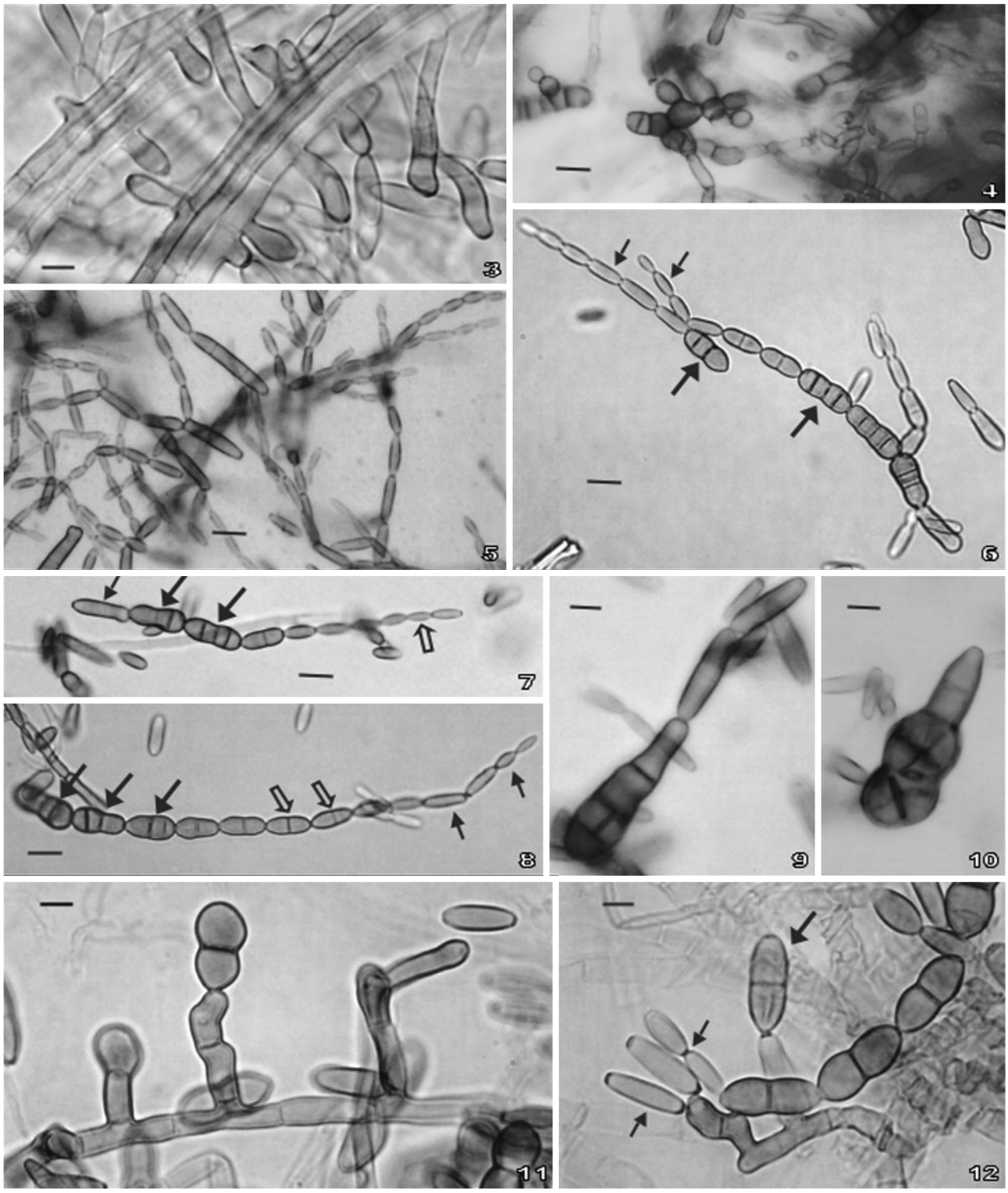
Davidiella tassiana (De Not.) Crous & U. Braun, **comb. nov.**

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

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

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

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



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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Basionym: *Heterosporium ornithogali* Klotzsch ex Cooke, Grevillea 5: 123, 1877.

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

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

Discussion

This study has provided further evidence for the separation of *Cladosporium s. str.* anamorphs from the main *Mycosphaerella* clade, and has provided the basis for the introduction of a new teleomorph genus, *Davidiella*, for this group of fungi. Furthermore, it has also shown that several *Cladosporium*-like fungi are clearly not congeneric with *Cladosporium s. str.*, and that the relatively minor differences in the scars and conidial loci, are supportive of their different phylogenetic affinities. Similarily, *C. malorum* appears to be best assigned to *Alternaria* based on its ITS and SSU phylogenetic placement, and such placement is also supported by its unique mode of conidiogenesis. As in other hyphomycetes in this complex (CROUS, KANG & BRAUN 2001), conidial septation, and the presence of oblique septa, are of less importance at the generic level. HÖLLER, GLOER & WICKLOW (2002) identified various metabolites produced by an undetermined *Cladosporium*-like hyphomycete, which was isolated from a resupinate polypore in the USA. These metabolites, which included altersolanol and macrosporin, are commonly produced by *Alternaria* spp. A culture derived from this isolate, and which was deposited at IMI, was examined by U. Braun, and identified as *C. malorum*. The taxonomic decision to place this species in *Alternaria* is thus further supported by these metabolite data from HÖLLER, GLOER & WICKLOW (2002).

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

Herpotrichiellaceae and *Venturiaceae*

Of particular interest in the *Herpotrichiellaceae* are those species pathogenic to humans, which are presently placed in *Cladophialophora* (Fig. 1). Human-pathogenic cladosporioid

hyphomycetes have previously been placed in *Cladosporium s. lat.* and confused with true *Cladosporium* species. There is a large number of publications dealing with all aspects of these fungi, including morphology, biology/ecology, physiology and molecular data (Masclaux et al. 1995, Untereiner 1997, Gerrits van den Ende & de Hoog 1999, Untereiner & Naveau 1999, Untereiner, Gerrits van den Ende & de Hoog 1999, de Hoog et al. 2000). It has been clearly demonstrated in all phylogenetic analyses that the truly human-pathogenic *Cladosporium* species are *Capronia* anamorphs belonging to the *Herpotrichiellaceae*, and all species concerned have been placed in *Cladophialophora*. The morphological distinction between *Cladophialophora* and *Cladosporium s. str.* has also been demonstrated by several authors (Braun & Feiler 1995, Braun 1998, de Hoog et al. 2000). *Cladophialophora* species are characterised by truncate, unthickened, barely darkened, often somewhat denticle-like conidiogenous loci, whereas *Cladosporium* loci are 'coronate' (David 1997), e.g. protuberant and with raised periclinal rims that surround a central convex dome. True *Cladosporium* species also differ from *Cladophialophora* physiologically in their ability to liquefy gelatine (de Hoog et al. 1995).

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

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

Several authors have dealt with *Phaeoramularia hachijoensis* (Matsushima 1975), but all reassessments of this species were based on non-type material, since type material and strains were not available and are possibly not extant any longer. Cultures assigned to this species are undoubtedly heterogeneous. Braun & Feiler (1995) considered CBS 462.82 and ATCC 96019 to be representative of *P. hachijoesis* and placed the species in *Cladophialophora*. Dugan, Roberts & Hanlin (1995) found the teleomorph of ATCC 96019, and described it as *Capronia hystricoides*. A German strain was similar, but differed by having paler structures, finer conidia and a distinct habit of the colonies (Braun & Feiler 1995). Untereiner & Naveau (1999) provided 28S rDNA sequence data to support the fact that the BBA strain was not conspecific with *P. hachijoensis* sensu Braun & Feiler (1995) and Dugan et al. (1995), but even quite unrelated. Of the three isolates of *P. hachijoensis* studied, it appears that each isolate represents a different species in distinct genera. Hence, the application of the name *P. hachijoensis* must be based on an interpretation. We propose to follow the treatment and application of this name by Dugan, Roberts & Hanlin (1995) as anamorph of *Capronia hystricoides* (Syn. *Caproventuria hystricoides*). Untereiner (1997) reduced the latter species to synonymy with *Capronia hanliniana* (anamorph *Cladophialophora brevicatenata*), assigned it to the *Venturiaceae* and proposed the combination *Venturia hanliniana*. In the present study, the isolate of *P. hachijoensis* used by Dugan, Roberts & Hanlin (1995) (ATCC 96019 = STE-U 5391) also clustered in *Venturia*, thus supporting the conclusion by Untereiner (1997). Braun (1998) recognised Untereiner´s (1997) exclusion of this species from *Capronia*. He discussed some distinctive features supporting *C. hanliniana* and *C. hystricoides*, which are well-distinguished by their anamorphs, and also from true *Venturia* species. Braun (1998) therefore introduced the new genus *Caproventuria* for the teleomorphs, and *Pseudocladosporium* for the anamorphs. In the present phylogram, it can be seen that *Caproventuria*/*Pseudocladosporium* is unrelated to the *Herpotrichiellaceae* (*Capronia*/*Cladophialophora*), but rather clusters within *Venturiaceae* (Figs. 1–2). The genus *Pseudocladosporium* is tentatively maintained and confined to anamorphs of *Caproventuria*, awaiting the treatment of more taxa.

Amorphothecaceae

Sorocybe resinae (syn.: *Hormoconis resinae*; teleomorph *Amorphotheca resinae*) belongs to a group of hyphomycetes characterised by having more or less distinctly denticulate, pigmented conidiogenous cells and 0–2-septate, pigmented conidia formed in long, often branched chains. This assemblage of anamorphs can be considered as a counterpart to the *Dactylaria* (de Hoog 1985) complex distinguished by catenate conidia. The delimitations of these genera and some allied ones, e.g., *Anungitea*, *Pleurotheciopsis* and *Polyscytalum*, is difficult and partly vague, since morphology and conidiogenesis are very similar to each other. It is still unclear in this complex which characteristics are appropriate for a generic delimitation. Partridge & Morgan-Jones (2002) reduced *Hormoconis* (von Arx 1973) to synonymy with *Sorocybe*.

They considered *H. resinae* to be the mononematous form of *S. resinae*, and noted that the connection between *Amorphotheca* (PARBERY 1969) as teleomorph and *S. resinae* as anamorph, remains to be resolved. *Sorocybe resinae*, the type species of this genus, differs from species of allied genera in having rather inconspicuous, not distinctly denticle-like conidiogenous loci (DE VRIES 1952; PARTRIDGE & MORGAN-JONES 2002). The clustering of two isolates of '*Cladosporium*' *breviramosum* (AF393683, 393684) in the *Amorphothecaceae* is unusual, and the original strains will have to be re-examined to resolve their identity and position.

Incertae sedis

The status of *Anungitopsis amoena* (syn. *Cladosporium amoenum*) (HO et al. 1999) is unclear, and the correct placement of this species in *Anungitopsis* is not certain. The type species of the latter genus and the other species assigned to it have long rachis-like conidiogenous cells with numerous, dense, rather inconspicuous conidiogenous loci. The loci in *A. amoena* are less numerous, scattered and more distinct, partly almost denticle-like.

Pleosporaceae

This study has shown that *Cladosporium malorum* belongs to *Alternaria* (Figs. 1–2). Conidiogenesis and the structure of the conidiogenous loci of this fungus were undoubtedly misinterpreted by all previous mycologists, who placed this fungus in *Cladosporium, Cladophialophora, Phaeoramularia* or *Pseudocladosporium*, suggesting that the conidiogenesis was holoblastic. These treatments were undoubtedly influenced by the cladosporioid habit of this fungus, e.g., pigmented, 0–2-septate conidia formed in long acropetal chains (Fig. 5). However, the conidiogenous cells possess minute, but conspicuous pores, and should rather be regarded as poroconidia. Within the genus *Alternaria*, however, *A. malorum* is not totally unique in having largely aseptate, cylindrical conidia, as this is also found in other species of *Alternaria*, e.g. *A. cetera* (SIMMONS 1996).

Alternaria malorum var. *polymorpha* is distinguished from var. *malorum* by the production of an additional class of 1(–3)-septate conidia which differ from normal *A. malorum* conidia largely by the degree of septation, greater width, deeper colour and somewhat thicker walls (Figs. 6–8). In addition, these alternative conidia could become longitudinally septate and, in rare instances, distinctly alternarioid (Figs. 9–10). The alternative conidia are borne on the same kinds of conidiophores as those bearing regular conidia (Fig. 11), and sometimes from a single, branched conidiophore (Fig. 12). The alternative forms of conidia could occur together with the regular conidia in the same chain (Figs. 6–8) and could be subtended by normal ramo-conidia within the chain (Fig. 7). That the division between the regular conidia and those with alternative morphologies is not absolute can be seen by occasional production of intermediate types (Fig. 8). A small minority of the dictyoconidia were regularly (Fig. 9) or irregularly (Fig. 10) alternarioid in shape. Conidiogenesis is the same for normal conidia and those characterising var. *polymorpha,* and the alternative conidia occur mixed together with normal *P. malorum* conidia, so that classification as a variety seems to be appropriate. The two varieties appeared similar, however, based on the molecular data presented here.

Mycosphaerella (*Mycosphaerellaceae*)

This clade contains *Mycosphaerella* species and cercosporoid anamorphs that are now placed in *Passalora s.lat.* (incl. *Fulvia*, *Mycovellosiella* and *Phaeoramularia*). Comprehensive morphological and molecular analyses of this fungal group were recently conducted (CROUS et al. 2000, 2001), in which it was shown that *Mycosphaerella* isolates form a single large monophyletic clade, with species of *Mycosphaerella* with *Cladosporium s. str.* anamorphs in a distinct subclade. These molecular data further showed that *Passalora fulva* [= *Fulvia fulva*, *Cladosporium fulvum, Mycovellosiella fulva*] is also a part of the *Mycosphaerella* clade, clustering together with other taxa with *Passalora s.lat.* anamorphs. Furthermore, the conidiogenous loci of *P. fulva* are quite distinct from *Cladosporium s. str.* scars, and agree better with cercosporoid scar types (BRAUN 1995).

Various authors confused *Cladosporium* with *Biharia*, *Fulvia*, *Mycovellosiella* and *Stenella*. For instance, VON ARX (1981) reduced these names to synonymy with *Cladosporium*. ELLIS (1971) listed *Biharia* as a synonym of *Mycovellosiella*, but since the superficial hyphae of the type species, *B. vangueriae*, are verruculose, DEIGHTON (1979) reduced *Biharia* to synonymy with *Stenella*. VON ARX (1983) recognised *Mycovellosiella*, including *Fulvia*, but maintained *Biharia* and *Stenella* as synonyms of *Cladosporium*. However, *Passalora s. lat.* and *Stenella* are easily distinguishable from *Cladosporium s. str.* by their distinct conidiogenous loci (scars) and conidial hila, which are truncate to pileate, barely protuberant, somewhat thickened and darkened, but always without a raised periclinal rim. Furthermore, the separation of *Cladosporium, Passalora s.lat.* and *Stenella* is also supported by molecular data (CROUS et al. 2000, 2001, Crous unpubl.).

Davidiella (*Mycosphaerellaceae*)

Cladosporium herbarum, the lectotype species of *Cladosporium* (CLEMENTS & SHEAR 1931), is the anamorph of *Davidiella tassiana* (VON ARX 1950, BARR 1958), which has also been confirmed by molecular examinations (MASCLAUX et al. 1995, DE HOOG et al. 1999). All species of *Cladosporium s. str.* examined represent a monophyletic clade (DE HOOG et al. 1999, UNTEREINER & NAVEAU 1999, CROUS et al. 2000, 2001) (Figs. 1–2).

True *Cladosporium* species are easily separable from all other *Cladosporium*-like hyphomycetes by their distinctive conidiogenous loci, which were described in detail by DAVID (1997), who pointed out that this scar type is a significant ge-

neric character. The first detailed examinations of *Cladosporium* scars were published by ROQUEBERT (1981). The conidiogenous loci (scars) and conidial hila are usually distinctly protuberant, thickened, darkened and composed of a raised periclinal rim that surrounds a central convex part (dome or mound, DAVID 1997). This type of scar has been called 'coronate' (DAVID 1997) or it may simply be described as '*Cladosporium*-type', since it is so characteristic and unique. *Cladosporium s. str.* should be confined to *Davidiella* anamorphs with coronate conidiogenous loci. The first clear circumscription in this sense, including a clear description of the peculiar scars has been published by DAVID (1997).

The genus *Heterosporium* was reduced to synonymy with *Cladosporium* by DE VRIES (1952), a view endorsed by HUGHES (1958) and ELLIS (1971, 1976). VON ARX (1981, 1983) reinstated *Heterosporium* and various authors followed his decision. DAVID (1997) examined the conidiogenous loci (scars) and conidial hila of *Cladosporium* and *Heterosporium* species, showed that these structures are generally uniform in all species of the two 'genera', and so reduced *Heterosporium* to synonymy with *Cladosporium*. DAVID´S (1997) taxonomic decisions are fully supported by our study, in which several *Heterosporium* species that have *Davidiella* teleomorphs, cluster within the *Cladosporium* clade.

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References

ARX JA VON (1949) Beiträge zur Kenntnis der Gattung *Mycosphaerella*. – Sydowia **3**: 28-100.

ARX JA VON (1950) Über die Ascusform von *Cladosporium herbarum* (Pers.) Link. – Sydowia **4**: 320-324.

ARX JA VON (1973) Centraalbureau voor Schimmelcultures Baarn and Delft. Progress Reports 1972. – Verhandelingen der Koninklijke Nederlandsche Akademie van Wetenschappen, Afdeeling Natuurkunde **61**: 59-81.

ARX JA VON (1981) The Genera of Fungi Sporulating in Pure Culture. 3rd edn. J Cramer, Vaduz.

ARX JA VON (1983) *Mycosphaerella* and ist anamorphs. – Proceedings Koninklijke Nederlandse Akademie van Wetenschappen, Series C, **86**(1): 15–54.

BARR ME (1958) Life history studies of *Mycosphaerella tassiana* and *M. typhae*. – Mycologia **50**: 501–513.

BRAUN U (1995) A monograph of *Cercosporella, Ramularia* and allied genera (Phytopathogenic Hyphomycetes). Vol. 1. IHW-Verlag, Eching

BRAUN U (1998) A monograph of *Cercosporella, Ramularia* and allied genera (Phytopathogenic Hyphomycetes). Vol. 2. IHW-Verlag, Eching.

BRAUN U, FEILER U (1995) *Cladophialopora* and its teleomorphs. – Microbiological Research **150**: 81–91.

BRAUN U, TAKAMATSU S (2000) Phylogeny of *Erysiphe*, *Microsphaera*, *Uncinula* (*Erysipheae*) and *Cystotheca*, *Podosphaera*, *Sphaerotheca* (*Cystotheceae*) inferred from rDNA ITS sequences – some taxonomic consequences. – Schlechtendalia **4**: 1-33.

CLEMENTS FE, SHEAR C L (1931) The Genera of Fungi. H. W. Wilson, New York.

CORDA ACJ (1837) Icones fungorum hucusque cognitorum. Vol. 1. Prague.

CORDA ACJ (1842) Anleitung zum Studium der Mykologie. Prague.

CROUS PW (2002) Taxonomy and pathology of *Cylindrocladium* (*Calonectria*) and allied genera. American Phytopathological Society Press, St. Paul, MN.

CROUS PW, APTROOT A, KANG JC, BRAUN U, WINGFIELD MJ (2000) The genus *Mycosphaerella* and its anamorphs. – Studies in Mycology **45**: 107–121.

CROUS PW, KANG JC, BRAUN U (2001) A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequences. – Mycologia **93**: 1081-1101.

DAVID JC (1997) A contribution to the systematics of *Cladosporium*. Revision of the fungi previously referred to *Heterosporium*. – Mycological Papers **172**: 1-157.

DE HOOG GS (1985) Taxonomy of the *Dactylaria* complex, IV–VI. – Studies in Mycology **26**: 1-124.

DE HOOG GS, GUARRO J, GENÉ J, FIGUERAS M J (2000) Atlas of Clinical Fungi. 2nd edn. Centraalbureau voor Schimmelcultures, Utrecht.

DE HOOG GS, GUÉHO E, MASCLAUX F, GERRITS VAN DEN ENDE AHG, KWON-CHUNG KJ, MCGINNIS MR (1995) Nutritional physiology and taxonomy of human-pathogenic *Cladosporium-Xylohypha* species. – Journal of Medical and Veterinary Mycology **33**: 339-347.

DE HOOG GS, ZALAR P, URZÌ C, DE LEO F, YURLOVA NA, STERFLINGER K (1999) Relationships of dothideaceous black yeasts and meristematic fungi based on 5.8S and ITS2 rDNA sequence comparison. – Studies in Mycology **43**: 31-37.

DE VRIES GA (1952) Contribution to the Knowledge of the Genus *Cladosporium* Link ex Fr. Centraalbureau voor Schimmelcultures, Baarn.

DEIGHTON FC (1979) Studies on *Cercospora* and allied genera. VII. New species and redispositions. – Mycological Papers **144**: 1-56.

DENMAN S, CROUS PW, TAYLOR JE, KANG JC, PASCOE I, WINGFIELD MJ (2000) An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. – Studies in Mycology **45**: 129-140.

DUGAN FM, ROBERTS RG (1994) Etiology of preharvest colonization of Bing cherry fruit by fungi. – Phytopathology **84**: 1031-1036.

DUGAN FM, ROBERTS RG, HANLIN RT (1995) New and rare fungi from cherry fruit. – Mycologia **87**: 713-718.

ELLIS MB (1971) Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew.

ELLIS MB (1976) More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew.

FRIES EM (1832) Systema mycologicum. Vol. 3. E. Mauritius, Greifswald.

FRIES EM (1849) Summa vegetabilium Scandinaviae. Sect. 1. A. Bonnier, Uppsala.

GERRITS VAN DEN ENDE AHG, DE HOOG GS (1999) Variability and molecular diagnostics of the neurotrophic species *Cladophialophora bantiana*. – Studies in Mycology **43**: 151-162.

HILLIS DM, BULL JJ (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. – Systematic Biology **42**: 182-192.

HO MHM, CASTAÑEDA RF, DUGAN FM, JONG SC (1999) *Cladosporium* and *Cladophialophora* in culture: descriptions and an expanded key. – Mycotaxon **72**: 115-157.

HÖHNEL FXR VON (1918) Mykologische Fragmente. Annales Mycologici **16**: 35-174.

HÖLLER U, GLOER JB, WICKLOW DT (2002) Biologically active polyketide metabolites from an undetermined fungicolous hyphomycete resembling *Cladosporium*. – Journal of Natural Products **65**: 876-882.

HUGHES SJ (1958) Revisiones hyphomycetum aliquot cum appendice de nominibus rejiciendis. – Canadian Journal of Botany **36**: 727-836.

KENDRICK WB (1980) The generic concept in hyphomycetes. – Mycotaxon **11**: 339-364.

LINDAU G (1907) *Mycosphaerella tulasnei* und *Sphaerulina intermixta*, bezw. *Cladosporium herbarum* und *Dematium pullans*. In Lafar H (ed) Handbuch der technischen Mykologie. 2nd edn. pp. 270-279. Gustav Fischer, Jena.

LINK HF (1816) Observationes in ordines plantarum naturales. Dissertatio II, complectens Anandrarum ordines Epiphytas, Mucedines, Gastromycos et Fungos. – Magazin der Gesellschaft naturforschender Freunde zu Berlin **7**: 25-45.

MARASAS WFO, BREDELL IH (1974) A new species of *Phaeoramularia* (*Fungi imperfecti: Dematiaceae*) from South Africa. – Bothalia **11**: 217-219.

MASCLAUX F, GUÉHO E, DE HOOG GS, CHRISTEN R (1995) Phylogenetic relationship of human-pathogenic *Cladosporium* (*Xylohypha*) species. – Journal of Medical and Veterinary Mycology **33**: 327-338.

MATSUSHIMA T (1975) Icones Microfungorum a Matsushima Lectorum. T. Matsushima, Kobe.

MCKEMY JM, MORGAN-JONES G (1990) Studies in the genus *Cladosporium* sensu lato. II. Concerning *Heterosporium gracile*, the causal organism of leaf spot disease of *Iris* species. – Mycotaxon **39**: 425-440.

MORGAN-JONES G, JACOBSEN BJ (1988) Notes on hyphomycetes. LVIII. Some dematiaceous taxa, including two undescribed species of *Cladosporium*, associated with biodeterioration of carpet, plaster and wallpaper. – Mycotaxon **32**: 223-236.

MORGAN-JONES G, MCKEMY JM (1990) Studies in the genus *Cladosporium* sensu lato. I. Concerning *Cladosporium uredinicola*, occurring on telial columns of *Cronartium quercuum* and other rusts. – Mycotaxon **39**: 185-202.

NEES CGD (1817) Das System der Pilze und Schwämme. Stahelsche Buchhandlung, Würzburg.

PAGE RDM (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. – Computer Applications in the Biosciences **12**: 357-358.

PARBERY DG (1969) *Amorphotheca resinae* gen. nov., sp. nov., the perfect state of *Cladosporium resinae*. – Australian Journal of Botany **17**: 331-357.

PARTRIDGE EC, MORGAN-JONES G (2002) Notes on hyphomycetes, LXXXVIII. New genera in which to classify *Alysidium resinae* and *Pycnostysanus azaleae*, with a consideration of *Sorocybe*. – Mycotaxon **83**: 335-352.

RAMBAUT A (2002) Sequence Alignment Editor. Version 2.0. Department of Zoology, University of Oxford, Oxford.

REYNOLDS DR (1993) The fungal holomorph: an overview. In Reynolds DR, Taylor JW (eds) The Fungal Holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics, pp. 15-25. CAB International, Wallingford.

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

RITSCHEL A (2001) Taxonomische Revision der Gattungen *Pollaccia* and *Spilocaea* (Hyphomycetes, *Venturia*-Anamorphen). Dipl.-Arbeit thesis, Martin-Luther-Universität, Halle.

ROQUEBERT MF (1981) Conidiogenèse et conidiophores chez *Cladosporium herbarum* (Pers.) Link et *Cladosporium cladosporioides* (Fresen.) de Vries. – Cryptogamie Mycologie **2**: 269-278.

RUEHLE GD (1931) New apple-rot fungi from Washington. – Phytopathology **21**: 1141-1152.

SACCARDO PA (1886) Sylloge Fungorum. Vol. 4. Seminarium, Padua.

SCHNELLHARDT O, HEALD FD (1936) The spore load of market wheat. – Transactions of the American Microscopical Society **55**: 281-285.

SCHUBERT K (2001) Taxonomische Revision der Gattung *Fusicladium* (Hyphomycetes, *Venturia*-Anamorphen). Dipl.-Arbeit thesis, Martin-Luther-Universität, Halle.

SIMMONS EG (1996) *Alternaria* themes and variations (145–149). – Mycotaxon **57**: 391-409.

STEVENS RB (1981) Mycology Guidebook. University of Washington Press, Seattle.

SUTTON BC, HENNEBERT GL (1994) Interconnections amongst anamorphs and their possible connections to ascomycete systematics. In Hawksworth DL (ed) Ascomycete Systematics: problems and prospects in the nineties, pp. 77-98. Plenum Press, New York.

SWOFFORD DL (2000) PAUP* 4.0: phylogenetic analysis using parsimony. Sinauer Associates, Sunderland, MA.

UNTEREINER WA (1997) Taxonomy of selected members of the ascomycete genus *Capronia* with notes on anamorph-teleomorph connection. – Mycologia **89**: 120-131.

UNTEREINER WA, GERRITS VAN DEN ENDE AHG, DE HOOG GA (1999) Nutritional physiology of species of *Capronia*. – Studies in Mycology **43**: 98-106.

UNTEREINER WA, NAVEAU FA (1999) Molecular systematic of the *Herpotrichiellaceae* with an assessment of the phylogenetic position of *Exophiala dermatitidis* and *Phialophora americana*. – Mycologia **91**: 67-83.

WHITE TJ, BRUNS T, LEE S, TAYLOR J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: a guide to methods and applications, pp. 315–322. Academic Press, San Diego.

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Tab. 1: Isolates of *Cladosporium* and allied genera studied

Sequence data	Species	Accession no.[a]	GenBank accession no.	Substrate	Origin
SSU	*Alternaria alternata*	–	U05194	*Brassica rapa* ssp. *oleifera*	Alberta, Canada
SSU	*Alternaria brassicicola* (syn. *Helminthosporium brassicicola*)	–	U05197	*Brassica oleracea* ssp. *capitata*	British Columbia, Canada
ITS	*Alternaria conjuncta*	'EGS' 37-139	AF392988	*Pastinaca sativa*	Switzerland
ITS	*Alternaria ethzedia / Lewia ethzedia*	'EGS' 37-143	AF392987	*Brassica* sp.	Switzerland
ITS	*Alternaria infectoria / Lewia infectoria*		Y17066	Linseed	–
SSU	*Alternaria infectoria / Lewia infectoria*	IMI 303186	U43465	*Triticum* sp.	UK
ITS	*Alternaria infectoria / Lewia infectoria*	STE-U 4271	AF397248	*Triticum* sp.	UK
ITS	*Alternaria malorum* (syn. *Cladosporium porophorum*)	ATCC 36953 CBS 135.31	AF393715	Fruit of *Malus sylvestris*	USA
ITS; SSU	*Alternaria malorum* (syn. *Cladosporium porophorum*)	ATCC 200939 CBS 173.80 STE-U 3685	AF393722; AY251128	Agricultural soil	Syria
ITS; SSU	*Alternaria malorum* (syn. *Phaeoramularia kellermaniana*)	ATCC 28332 CBS 266.75 STE-U 3680	AF393680; AY251127	Straw of *Triticum aestivum*	Western Cape, South Africa
ITS; SSU	*Alternaria malorum*	STE-U 4572	AY251079; AY251131	*Festuca idahoensis*	Washington, USA
ITS; SSU	*Alternaria malorum*	STE-U 4571	AY251081; AY251130	*Bromus tectorum*	Washington, USA
ITS; SSU	*Alternaria malorum* var. *polymorpha*	STE-U 4570 CBS 112048[b]	AY251080; AY251129	*Vitis vinifera*	Washington, USA
SSU	*Alternaria raphani*	–	U05199	*Brassica rapa* ssp. *oleifera*	Saskatchewan, Canada
ITS; SSU	*Anungitopsis amoena* (syn. *Cladosporium amoenum*)	ATCC 200947 CBS 254.95 STE-U 3681	AF393682; AY251122	*Eucalyptus grandis*	Cuba
SSU	*Batcheloromyces proteae*	STE-U 1518	AY251102	*Protea cynaroides*	Western Cape, Stellenbosch, South Africa
ITS	*Capronia semiimmersa*	MUCL 39979	AF050260	Rotten wood, *Acer* sp.	USA
SSU	*Cercospora zebrina*	STE-U 3955	AY251104	*Trifolium pratense*	Canada
ITS	*Cladophialophora bantiana* (syn. *Xylohypha bantiana*)	WC 2907	AF397182	–	USA
ITS	*Cladophialophora bantiana* (syn. *Xylohypha bantiana*)	UTHSC 94-986	AF131079	–	–
ITS	*Cladophialophora carrionii* (syn. *Cladosporium carrionii*)	ATCC 16264 CBS 160.54	AF050262	Man, chromoblasto-mycosis	Australia
ITS	*Cladophialophora carrionii* (syn. *Cladosporium carrionii*)	FMC 248	AF397181	–	Venezuela
ITS	*Cladophialophora carrionii* (syn. *Cladosporium carrionii*)	IMTSP 690	AF397180	–	USA
ITS	*Cladophialophora minourae* (syn. *Cladosporium minourae*)	ATCC 52853 CBS 556.83[b]	AY251087	Decaying wood	Japan
ITS	*'Cladosporium' breviramosum*	ATCC 64696	AF393684	Vinyl wallpaper	Georgia, USA
ITS	*'Cladosporium' breviramosum*	ATCC 76215	AF393683	Discolored wallpaper	Georgia, USA
ITS	*Cladosporium chlorocephalum* (syn. *Periconia chlorocephala*)	ATCC 38011	AF393686	*Paeonia suffruticosa* leaf	Japan
SSU	*Cladosporium cladosporioides*	–	U20381	–	–
ITS; SSU	*Cladosporium cladosporioides*	CBS 109.21 ATCC 11277 STE-U 3682	AY251073; AY251093	*Hedera helix*	UK
ITS; SSU	*Cladosporium cladosporioides*	CBS 401.80 ATCC 200941 STE-U 3683	AY251074; AY251091	*Triticum aestivum*	Netherlands
ITS; SSU	*Cladosporium cladosporioides*	ATCC 66669 STE-U 5100	AY251070; AY251094	Creosote-treated southern pine pole	Binghamton, New York, USA
ITS	*Cladosporium colocasiae*	ATCC 38014	AF393692	*Colocasia esculenta* leaf	Japan

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

Sequence data	Species	Accession no.[a]	GenBank accession no.	Substrate	Origin
ITS; SSU	*Cladosporium colocasiae*	STE-U 4323	AY251075; AY251092	*Colocasia esculenta*	Fiji islands
ITS	*Cladosporium cucumerinum*	ATCC 26211	AF393696	*Cucumis sativa*	–
ITS	*Cladosporium echinulatum* / *Davidiella dianthi* (syn. *Mycosphaerella dianthi*)	ATCC 56129	AF393698	*Dianthus caryophyllus* leaves	Portugal
ITS	*Cladosporium gossypiicola*	ATCC 38026 CBS 674.82	AF393702	Seed of *Gossypium* sp.	Jaffa, Israel
ITS	*Cladosporium herbarum* / *Davidiella tassiana* (syn. *Mycosphaerella tassiana*)	ATCC 201090	AF393705	Asymptomatic cherry fruits, *Prunus avium* cv. 'Bing'	Wenatchee, Washington, USA
ITS	*Cladosporium herbarum* / *Davidiella tassiana* (syn. *Mycosphaerella tassiana*)	CBS 399.80	AJ244227	Skin of man, foot	Geleen, Netherlands
ITS	*Cladosporium herbarum* / *Davidiella tassiana* (syn. *Mycosphaerella tassiana*)	CBS 111.82	AJ238469	*Arctostaphylos uva-ursi*	Alvaneu, Graubünden, Switzerland
ITS; SSU	*Cladosporium herbarum* / *Davidiella tassiana* (syn. *Mycosphaerella tassiana*)	ATCC 66670 STE-U 5101	AY251078; AY251096	CCA-treated Douglas-fir pole	Geneva, New York, USA
ITS	*Cladosporium iridis* / *Davidiella macrospora* (syn. *Mycosphaerella macrospora*)	–	AF297231	*Iris germanica*	Indiana, USA
ITS	*Cladosporium macrocarpum*	CBS 175.62	AJ244229	Grain of *Hordeum vulgare*	Netherlands
ITS	*Cladosporium magnusianum* (syn. *Heterosporium magnusianum*)	ATCC 200946 CBS 842.91	AF393712	Green leaf of *Narthecium ossifragum*	Bjerkreim County, Norway
ITS	*Cladosporium oxysporum*	CBS 125.80	AJ300332	Seedcoat of *Cirsium vulgare*	Netherlands
ITS	*Cladosporium oxysporum*	ATCC 76499	AF393720	Decayed leaf, *Lespedeza bicolor*	Lee Co., Alabama, USA
ITS; SSU	*Cladosporium* sp.	STE-U 5371	AY251072; AY251099	*Spinacia* sp.	Gaborone, Botswana
ITS; SSU	*Cladosporium* sp.	STE-U 5124	AY251076; AY251090	*Apium graveolens*	New Zealand
ITS; SSU	*Cladosporium sphaerospermum*	ATCC 11290 CBS 188.54 STE-U 3686	AY251077; AY251098	–	–
ITS; SSU	*Cladosporium staurophorum* (syn. *Hormodendrum staurophorum*)	ATCC 200934 CBS 375.81 STE-U 3687	AF393723; AY251121	Soil	Cruz Verde, Cundinamarca, Colombia
ITS	*Cladosporium tenuissimum*	ATCC 38027	AF393724	Soil	New Caledonia
ITS; SSU	*Cladosporium uredinicola*	ATCC 46649 STE-U 5390	AY251071; AY251097	Hyperparasite on *Cronartium fusiforme* f. sp. *quercum* on *Quercus nigra* leaves	Alabama, USA
SSU	'*Discosphaerina' fagi* (syn. *Guignardia fagi*)	CBS 171.93 IMI 189460A	AY016342	Leaf of *Populus* sp.	UK
SSU	*Dissoconium dekkeri* / *Mycosphaerella lateralis*	CBS 567.89 STE-U 1535	AY251101	*Juniperus chinensis*, 'Old Gold'	Maarssen, Netherlands
SSU	*Dothidea insculpta*	–	U42474	–	–
SSU	*Dothidea ribesia*	CBS 195.58	AY016343	*Ribes* sp.	Gunzgen, Kt. Solothurn, Switzerland
ITS	*Fusicladium effusum* (syn. *Cladosporium caryigenum*)		AF065850	Pecan nuts	Georgia, USA
ITS; SSU	*Fusicladium effusum* (syn. *Cladosporium caryigenum*)	STE-U 4524	AY251084; AY251125	Pecan nuts	Georgia, USA
ITS; SSU	*Fusicladium effusum* (syn. *Cladosporium caryigenum*)	STE-U 4525	AY251085; AY251126	Pecan nuts	Georgia, USA

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

Sequence data	Species	Accession no.[a]	GenBank accession no.	Substrate	Origin
ITS	*Fusicladium effusum* (syn. *Cladosporium caryigenum*)		AF065851	Pecan nuts	Louisiana, USA
ITS; SSU	*Fusicladium convolvulorum*	IMI 383037 STE-U 3884	AY251082; AY251124	–	New Zealand
ITS	*Fusicladium pomi* (syn. *Spilocaea pomi*) / *Venturia inaequalis*	–	AF065837	*Malus* sp.	–
ITS	*Fusicladium pyrorum* / *Venturia pyrina*	–	AF065844	Pear	Israel
SSU	*Fusicoccum* sp. / *Botryosphaeria ribis*	–	AF271129	–	–
SSU	*Guignardia endophyllicola*	CBS 398.80 IFO 33062	AB041249	–	New Zealand
SSU	*Saccharicola bicolor* (syn. *Leptosphaeria bicolor*)	ATCC 42652	U04202	*Saccharum officinarum*	Kenya
ITS	*Mycosphaerella iridis* (syn. *Sphaerella iridis*)	CBS 282.49	AY251088	Leaf spot in *Iris pseudacorus*	Baarn, Netherlands
ITS	*Mycosphaerella iridis* (syn. *Sphaerella iridis*)	CBS 281.49	AY251089	Leaf spot in *Iris pseudacorus*	Glattfelden, Zürich, Switzerland
SSU	*Mycosphaerella latebrosa* (syn. *Sphaerella latebrosa*)	CBS 652.85	AY251114	Leaf spot in *Acer pseudoplatanus*	Baarn, Netherlands
SSU	*Mycosphaerella nubilosa* (syn. *Sphaerella nubilosa*)	STE-U 4661	AY251120	*Eucalyptus globulus*	Ponte Areas, Spain
SSU	*Mycosphaerella populorum*	–	AF271130	–	–
SSU	*Mycosphaerella* sp.	STE-U 1731	AY251115	*Protea* sp.	Drakensberg, Kwazulu-Natal, South Africa
SSU	*Mycosphaerella* sp.	STE-U 3837	AY251116	*Acacia* sp.	Venezuela
SSU	*Paraphaeosphaeria michotii* (syn. *Sphaeria michotii*)	CBS 591.73	AF250817	*Juncus squarrosus*	France
SSU	*Paraphaeosphaeria pilleata*	CBS 102207	AF250821	*Juncus roemerianus*	North Carolina, USA
ITS	*Passalora arachidicola* (syn. *Cercospora arachidis*) / *Mycosphaerella arachidis*	–	AF 297224	*Arachis hypogaea*	USA
ITS	*Passalora bellynckii* (syn. *Mycovellosiella bellynckii*)	CBS 150.49 STE-U 3635	AF222831	*Cynanchum vincetoxicum*	Switzerland
ITS	*Passalora dissiliens* (syn. *Phaeoramularia dissiliens*)	CBS 219.77	AF222835	Living leaf of *Vitis vinifera*	Basrah Province, Iraq
SSU	*Passalora dodonaeae*	STE-U 1223[b]	AY251108	*Dodonaea* sp.	Western Cape, South Africa
ITS	*Passalora fulva* (syn. *Cladosporium fulvum*)	ATCC 44960	AF393701	Tomato	Netherlands
ITS	*Passalora fulva* (syn. *Cladosporium fulvum*)	IMI 050487	L25430	*Lycopersicon esculentum*	Zimbabwe
ITS; SSU	*Passalora fulva* (syn. *Cladosporium fulvum*)	CBS 119.46 STE-U 3688	AY251069; AY251109	*Lycopersicon esculentum*	Netherlands
ITS	*Passalora henningsii* (syn. *Cercospora henningsii*)	–	AF284389	*Manihot esculenta*	Pernambuco, Brazil
SSU	*Passalora janseana* (syn. *Napicladium janseanum*)	CBS 145.37 IMI 303642 STE-U 4303	AY251103	*Oryza sativa*	Arkansas, USA
ITS	*Passalora vaginae* (syn. *Mycovellosiella vaginae*)	CBS 140.34	AF222832	*Saccharum officinarum*	Taiwan
ITS; SSU	'*Phaeoramularia hachijoensis*'	ATCC 96545 STE-U 5121	AY251068; AY251100	Air	Long Island, New York, USA
ITS	'*Phaeoramularia hachijoensis*'	CBS 462.82 STE-U 3679	AY251086	*Pinus* sp	De Vuursche, Baarn, Netherlands
ITS	*Phialophora americana*	CDC 10	U31838	Paper pulp	Wisconsin, USA

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

Sequence data	Species	Accession no.[a]	GenBank accession no.	Substrate	Origin
ITS	*Phialophora americana*	FMC 2214	AF397136	–	Colombia
ITS	*Phialophora americana*	CBS 840.69 MUCL 15537	AF050283	Decaying timber	Helsinki, Finland
SSU	*Pleospora betae*	IMI 156653	U43466	Seed of *Beta* sp.	UK
SSU	*Pseudocercospora cruenta* (syn. *Cercospora cruenta*)	CBS 462.75	AY251105	*Phaseolus* sp.	Labasa, Fiji
SSU	*Pseudocercospora protearum* var. *leucadendri* (syn. *Cercospora protearum* var. *leucadendri*)	STE-U 1869	AY251107	*Leucadendron* sp.	Western Cape, Stellenbosch, South Africa
ITS; SSU	*Pseudocladosporium hachijoense*	ATCC 96019 STE-U 5391	AY251083; AY251123	*Prunus avium*	Wenatchee, Washington, USA
SSU	*Pseudocercospora angolensis* (syn. *Cercospora angolensis*)	ATCC 11669 CBS 149.53	AY251106	Leaf of *Citrus sinensis*	Bié, Angola
SSU	*Ramularia* sp.	STE-U 4195	AY251112	–	–
SSU	*Ramulispora sorghi*	STE-U 905	AY251110	*Sorghum* sp.	KwaZulu-Natal, South Africa
SSU	*Ramulispora sorghi*	STE-U 906	AY251111	*Sorghum* sp.	KwaZulu-Natal, South Africa
ITS	*Rhinocladiella compacta* (syn. *Hormodendrum compactum*)	IMTSP 373	AF397133	–	–
SSU	*Septoria rosae*	ATCC 24311 CBS 355.58 STE-U 4302	AY251113	Leaf of *Rosa* sp.	–
SSU	*Septoria tritici*	STE-U 658	AY251117	*Triticum* sp.	Western Cape, South Africa
SSU	*Sphaerulina polyspora*	CBS 354.29 STE-U 4301	AY251095	–	–
SSU	*Stemphyllium herbarum* / *Pleospora herbarum*	ATCC 11681	U43458	Onion leaf	Colorado
ITS	*Sorocybe resinae* (syn. *Hormodendrum resinae*) / *Amorphotheca resinae*	ATCC 200942 CBS 406.68	AF393726	Soil	UK
ITS	*Sorocybe resinae* (syn. *Hormodendrum resinae*) / *Amorphotheca resinae*	ATCC 11841 CBS 184.54 STE-U 3692	AY251067	Creosote-treated wooden pole	St Louis, Missouri, USA
SSU	*Trimmatostroma macowanii*	STE-U 1488	AY251118	*Protea* sp.	Hermanus, Western Cape, South Africa
SSU	*Uwebraunia juvenis* / *Mycosphaerella juvenis*	STE-U 1004[b]	AY251119	Leaves of *Eucalyptus grandis*	Hazyview, Gauteng, South Africa
ITS	*Venturia cerasi*	ATCC 12119 CBS 444.54	AF065847	*Prunus cerasus*	East Germany

[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 of Tropical Medicine of São Paulo;
CDC: Centre for Disease Control and Prevention, U.S. Department of Health and Human Services.
IFO: Institute for Fermentation, Osaka, Japan.
[b] Ex-type isolates.