

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

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

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

## INTRODUCTION

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

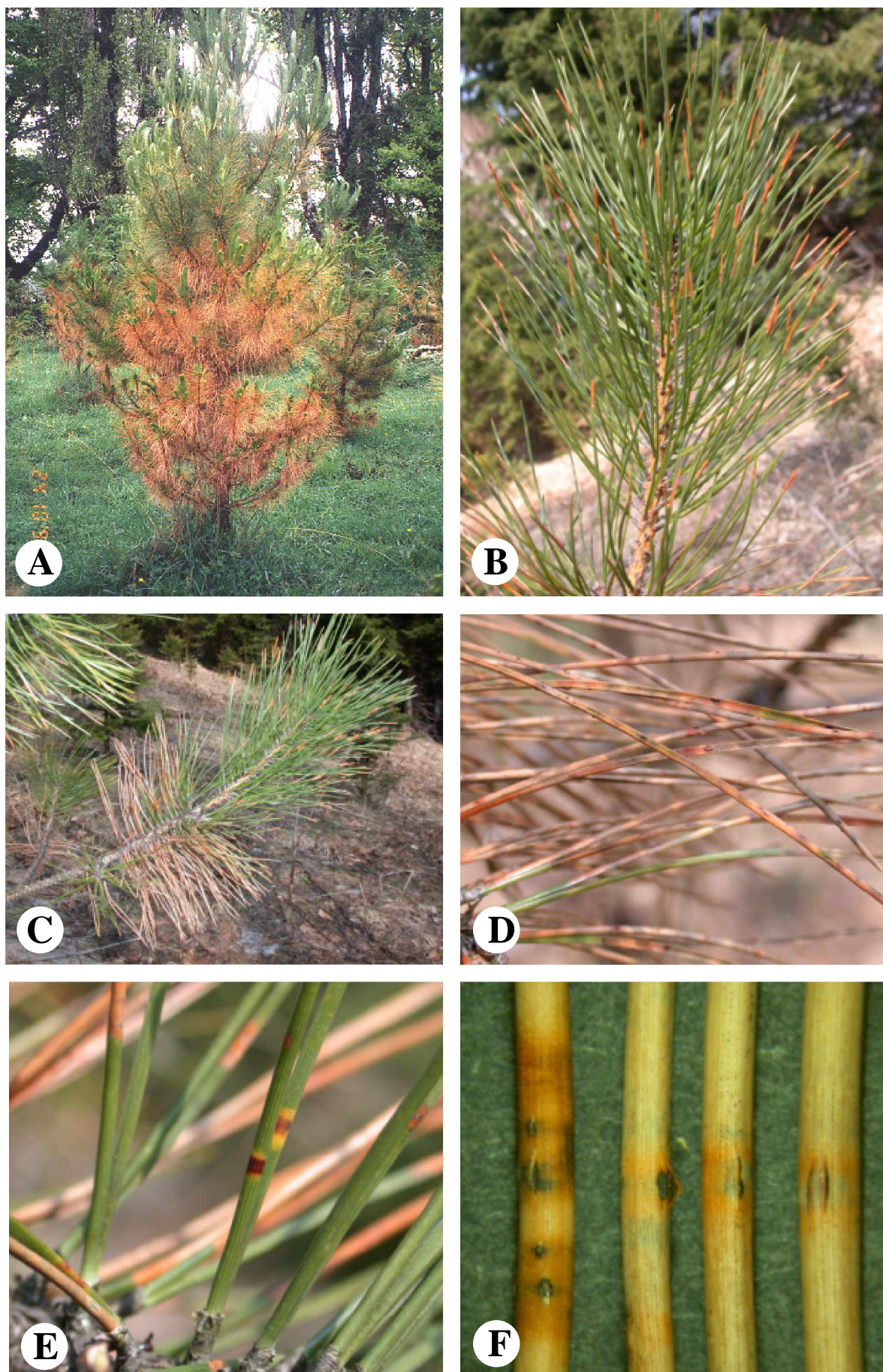
After the fungus infects via the stomata, initial symptoms appear as water-soaked lesions on the needles. Black conidiomata develop at these infection sites, which are characteristically surrounded by a red band, hence the common name of the fungus. Infected needles become necrotic and are cast (Fig.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

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

The red band needle blight pathogen has a cosmopolitan distribution, having been reported from more than 44 different countries in Eurasia, Africa, Oceania and the Americas (Data sheets on Quarantine pests: *Mycosphaerella dearnessi* and *Mycosphaerella pini*

[http://www.eppo.org/QUARANTINE/QP\\_fungi.htm](http://www.eppo.org/QUARANTINE/QP_fungi.htm), Ivory 1994). The severity of the disease appears to be related to a favourable climate in the Southern Hemisphere and to the exotic planting of susceptible host species such as *Pinus radiata* D. Don and *P. ponderosa* Laws. Thus, countries such as Chile, New Zealand and Kenya, where plantations are primarily monocultures of susceptible hosts, have experienced huge economic losses (Gibson 1974, van der Pas 1981). Control is limited to sanitary silvicultural practices, copper sprays and the planting of resistant tree species, families and clones (Carson & Carson 1989, Dick 1989, Chou 1991).





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

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

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

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

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

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

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

## MATERIALS AND METHODS

### Isolates

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

Isolates were obtained either directly from culture collections (Table 1), or from isolations made from infected needles. Infected needles collected from the field were first deposited in  $-70^\circ\text{C}$  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  $20^\circ\text{C}$  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  $20^\circ\text{C}$  until colonies had formed (approx. 4 wk, 10–15 mm diam). Colonies were scraped from the plates, excess agar removed and placed directly into Eppendorf tubes. The colonies (constituting mycelium and spores) were freeze-dried and crushed with the aid of liquid nitrogen and a glass rod.



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

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

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

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

Before DNA was extracted using the method described by Barnes *et al.* (2001), 800  $\mu$ L of extraction buffer was added to the tubes, which were then incubated in a heating block for 15 min at 85 °C followed by another 1 h at 60 °C. DNA concentrations were measured with a NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, Delaware, U.S.A.). DNA from herbarium material was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). A single conidioma was scraped from a needle and excess plant material removed. The conidioma was then crushed between two slides before DNA extraction was continued. The success of this method, using one conidioma, was first tested on the Idaho material (CMW 15077) before attempting to extract DNA from the herbarium 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  $\beta$ -tubulin gene were amplified using the primer pairs Bt2a/Bt2b and Bt1a/Bt1b (Glass & Donaldson 1995). The translation elongation factor (EF1- $\alpha$ ) gene was amplified using the forward EF1-728F and reverse primer EF1-986R (Carbone & Kohn 1999).

PCR was performed in total volumes of 25  $\mu$ L. The reaction mixtures consisted of  $\pm$  5 ng DNA template, 200 nM of the forward and reverse primers, 0.2 mM of each dNTP, 1U Taq DNA Polymerase with 10 $\times$  buffer (Roche Molecular Biochemicals, Mannheim, Germany) and 1.5 mM  $MgCl_2$ . 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  $\beta$ -tubulin gene regions and the *Puccinia graminis* sequence (GenBank X73529) for the EF1- $\alpha$  region. The random sequence (GenBank AJ544253) of *Saccharomyces cerevisiae* was used to identify the ITS1, 5.8S and ITS2 regions in our sequences.

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

### Morphology

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

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

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

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

## RESULTS

### Isolates

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

### DNA extraction, amplification and sequencing

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

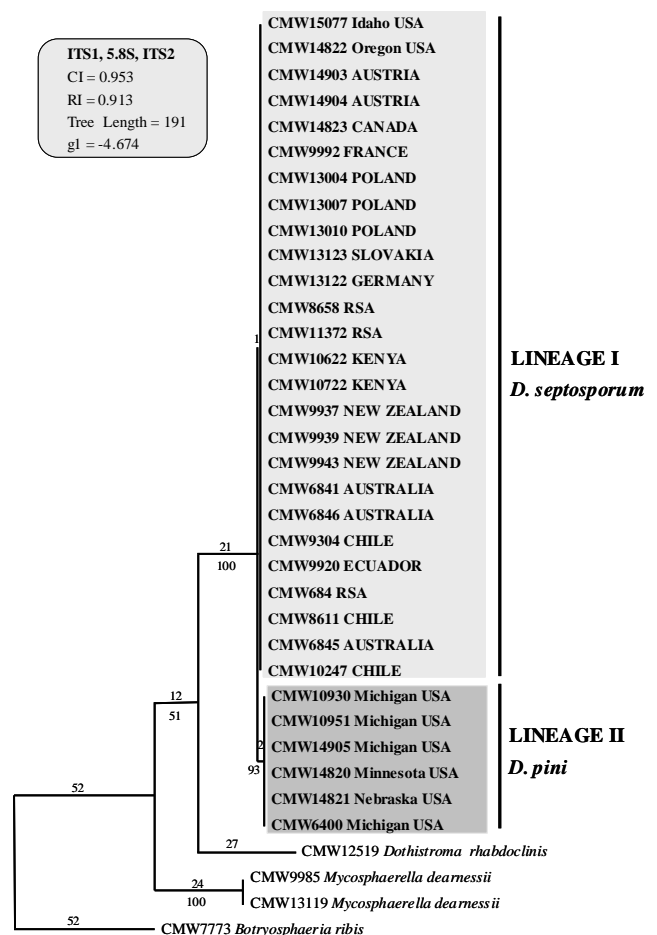
The extraction of DNA using the DNeasy Plant Mini Kit, and subsequent PCR from one conidioma from the Idaho material (less than 1-yr-old) was successful, and was thus attempted on herbarium specimens ILLS 27093 and WSP 48361. PCR of the type of *D. septosporum* var. *lineare* (*Actinothyrium marginatum*,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

### Phylogenetic analysis

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

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

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

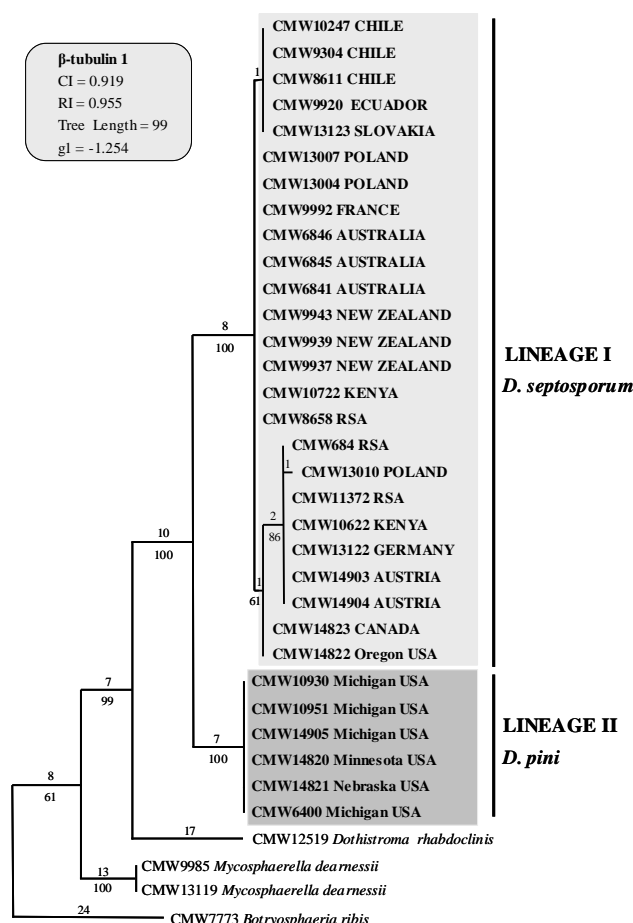


**Fig. 2.** One of 9 most parsimonious trees inferred from nuclear ribosomal internal transcribed spacer (ITS)1, 5.8S and ITS2 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 473 characters, 90 variable characters were parsimony-uninformative and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

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

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

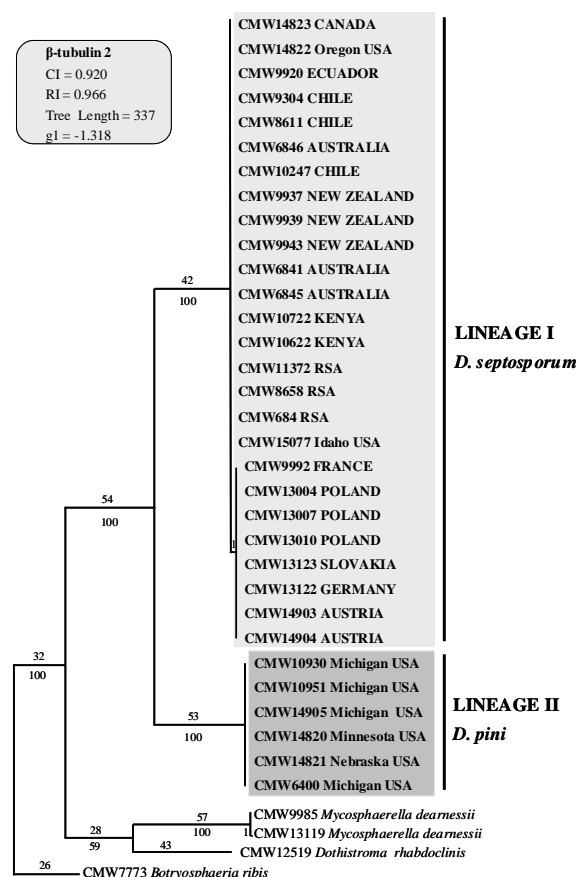
The sequence obtained from the type material of *D. septosporum* var. *lineare* (WSP 48361), was also included in this clade (Fig. 2). The ITS sequences in this lineage were identical while slight variation was observed randomly in the  $\beta$ -tubulin 1 (5 bp differences),  $\beta$ -tubulin 2 (1 bp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



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

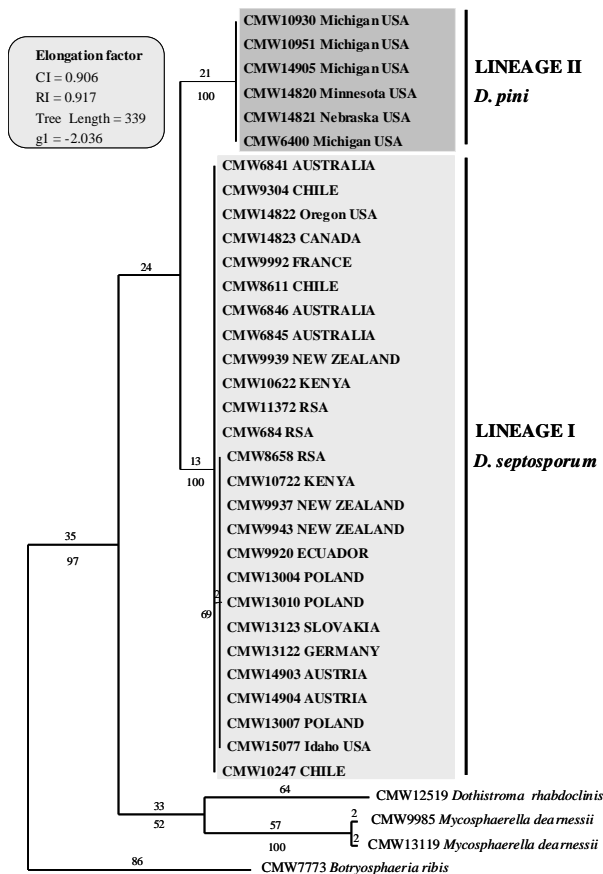
From a total of 1508 bp of aligned sequences using only *D. septosporum* isolates, there were 147 bp polymorphisms distinguishing the two lineages. Most of the variation observed between the two lineages was in the conserved exon positions. Although the ITS had only 3 bp differences between the lineages,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

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



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





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

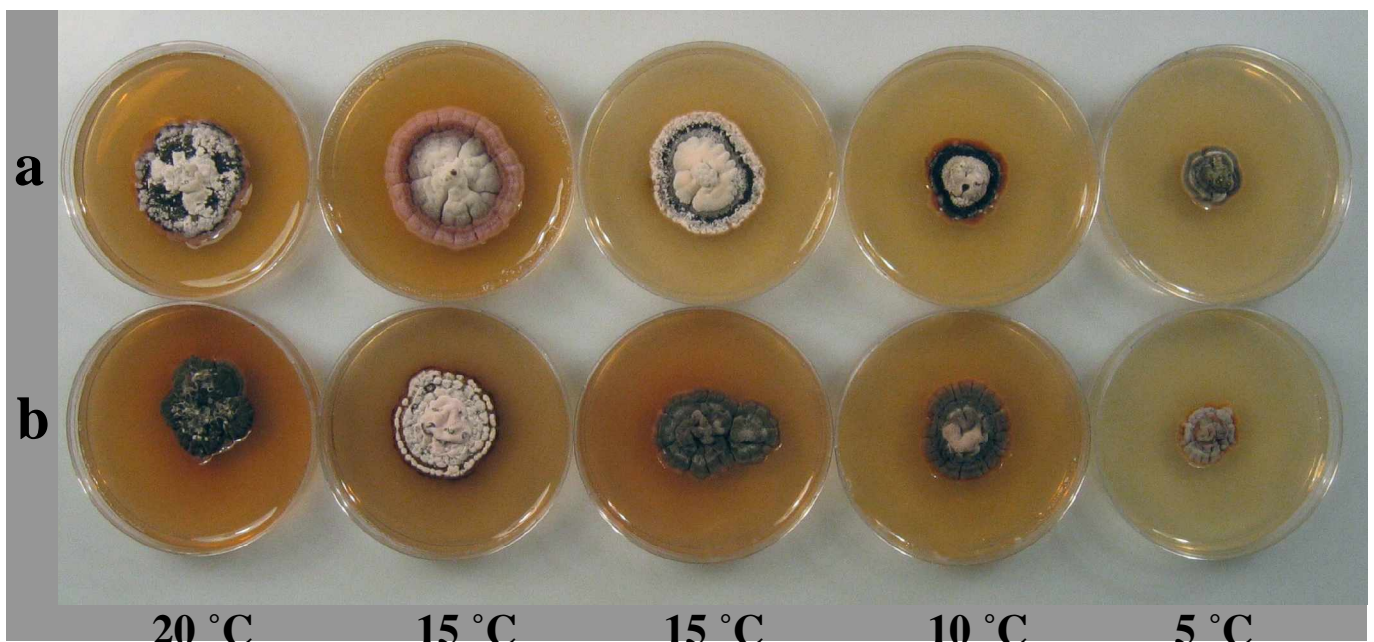
## Morphology

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

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

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

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



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

Conidial length showed extreme variation, ranging from 12–50  $\mu\text{m}$  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 (ILLIS 27093) closely matched those of other collections from the North Central U.S.A., i.e. relatively short and wide conidia.

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

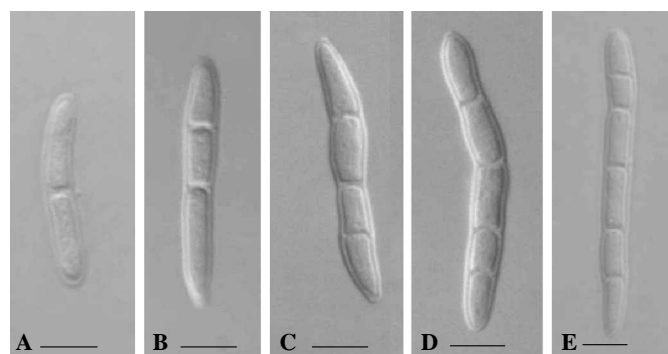
***Dothistroma pini*** Hulbary, Bull. Ill. St. nat. Hist. Surv. 21: 235. 1941. Figs 2–5, 8, 10, 12.

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

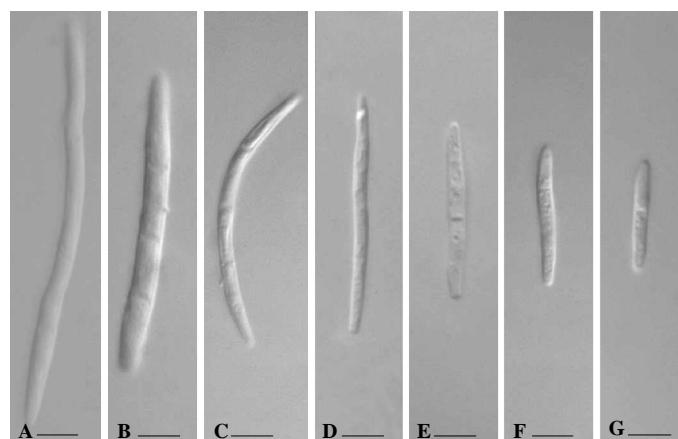
subobtuse at the apices, truncate to obconically subtruncate at the bases, (1–)3(–5) septate,  $(18\text{--})25\text{--}35$  (–45)  $\times$  3–5  $\mu\text{m}$  (av.  $30 \times 3.5 \mu\text{m}$ ) *in vivo*,  $(11\text{--})20\text{--}25$ (–27)  $\times$  (2–)2.5–3(–3.5)  $\mu\text{m}$  (av.  $22 \times 3 \mu\text{m}$ ) *in vitro*.

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

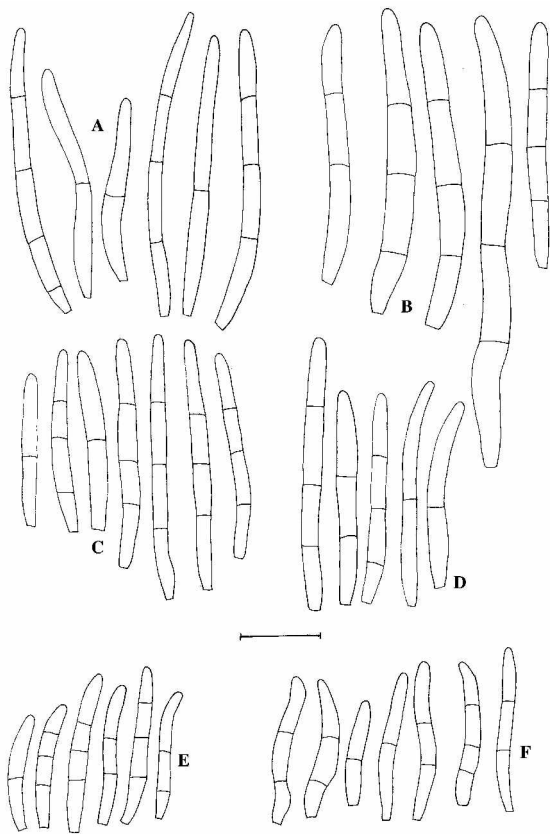
**Other specimens examined:** U.S.A., Michigan, Massaukee County, McBain, Riverside Township, isolated from *Pinus nigra*, Aug. 2001, G. Adams, herb. CBS 12203, culture CMW 14905 = CBS 116483; Michigan, Montcalm County, Stanton, Evergreen Township, from *Pinus nigra*, 2001, G. Adams, herb. CBS 12211,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



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



**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  $\mu\text{m}$ .



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

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

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

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

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

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

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

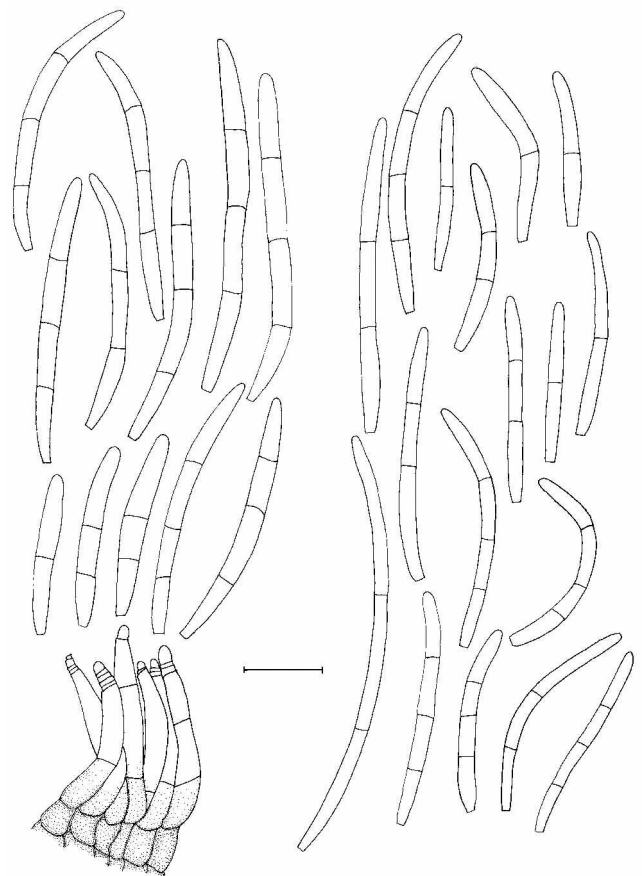
= *Dothistroma pini* var. *keniense* M.H. Ivory (as “*keniensis*”), Trans. Br. mycol. Soc. 50: 294. 1967.

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

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

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

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

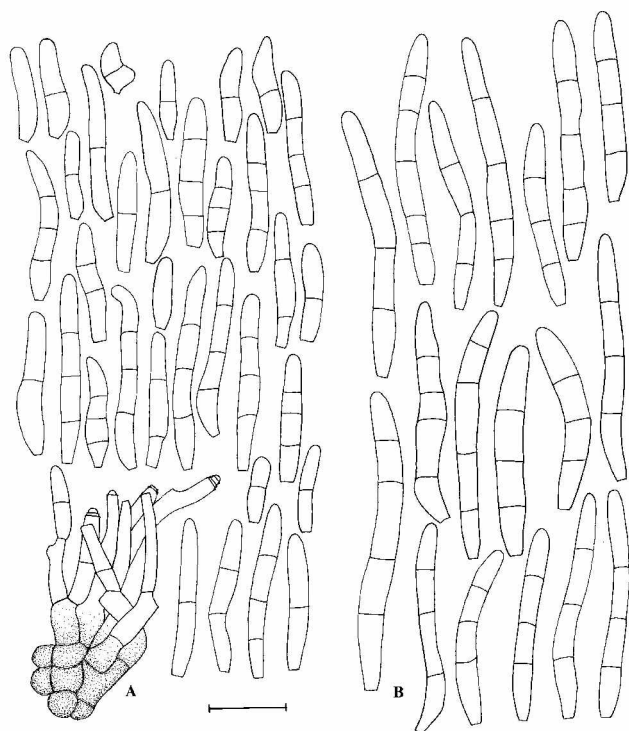


**Fig. 10.** Conidia and conidiogenous cells of *Dothistroma pini* from Michigan on *Pinus nigra* (herb. CBS 12211). On needles (left), and on oatmeal agar (right). Scale bar = 10  $\mu$ m.

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

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

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



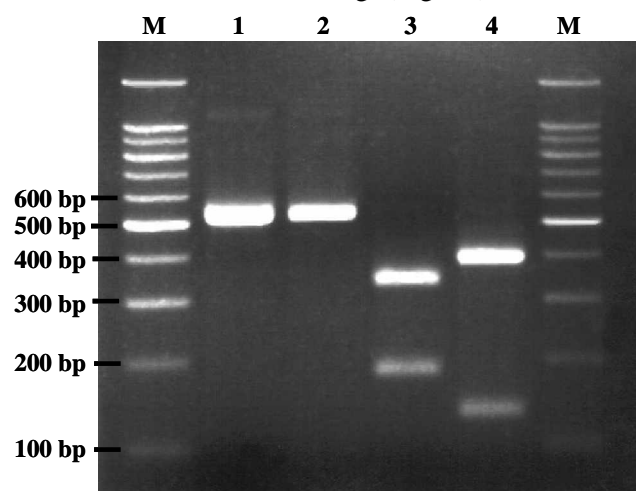
**Fig. 11.** Conidia and conidiogenous cells of *Dothistroma septosporum* from Poland on *P. nigra* (herb. CBS 12209). A. on needles. B. on oatmeal agar. Scale bar = 10 µm.

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

#### PCR-RFLP diagnostic procedure

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

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



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

## DISCUSSION

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

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



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

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

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

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

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

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

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

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

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

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

## ACKNOWLEDGMENTS

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