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Foliicolous microfungi occurring on *Encephalartos*

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Key words

Catenulostroma
Cladophialophora
Dactylaria
ITS nrDNA
LSU nrDNA
Ochroconis
Phaeomoniella
Saccharata
systematics
Teratosphaeria

Abstract Species of *Encephalartos*, commonly known as bread trees, bread palms or cycads are native to Africa; the genus encompasses more than 60 species and represents an important component of the indigenous African flora. Recently, a leaf blight disease was noted on several *E. altensteinii* plants growing at the foot of Table Mountain in the Kirstenbosch Botanical Gardens of South Africa. Preliminary isolations from dead and dying leaves of *E. altensteinii*, *E. lebomboensis* and *E. princeps*, collected from South Africa, revealed the presence of several novel microfungi on this host. Novelties include *Phaeomoniella capensis*, *Saccharata kirstenboschensis*, *Teratosphaeria altensteinii* and *T. encephalarti*. New host records of species previously only known to occur on Proteaceae include *Cladophialophora proteae* and *Catenulostroma microsporum*, as well as a hyperparasite, *Dactylaria leptosphaeriicola*, occurring on ascomata of *T. encephalarti*.

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INTRODUCTION

Encephalartos (Zamiaceae) is a genus of cycads indigenous to Africa. Due to its edible pith, species of *Encephalartos* are commonly referred to as bread trees or bread palms (www.kew.org/plants/). Another interesting aspect that makes *Encephalartos* noteworthy is the fact that it could represent one of the oldest pot-plants in the world. A specimen of *E. altensteinii* was collected in the Eastern Cape Province of South Africa in the early 1770s, and taken to Kew Botanic Gardens in the UK by Francis Masson in 1775, where it is still to be seen in the Palm House today. Although this plant genus is endangered and known to suffer from trunk and root parasites, as well as fungal infections, very few fungi have been described from this host (Doidge 1950, Nag Raj 1993, nt.ars-grin.gov/fungaldatabases/).

Fungal biodiversity has been poorly studied from most African countries, which could explain why so few fungal taxa have thus far been reported from *Encephalartos*. In a recent attempt to estimate how many species of fungi could occur at the tip of Africa, Crous et al. (2006a) concluded that the 1.5 M estimate suggested by Hawksworth (1991) was clearly too conservative. Based on available data, South Africa alone should have at least 200 000 fungal species associated with plant species, without taking into account the number associated with insects, or other ecological habitats such as water and soil.

Because of its extremely hard, leathery leaves, microfungi are not readily observed to colonise foliage of *Encephalartos* species. In January 2008, however, a tip blight disease was observed on several *Encephalartos* palms growing in the Kirstenbosch Botanical Gardens of South Africa, as well as in the KwaZulu-Natal Province. The aim of the present study was therefore to determine if any microfungi could be isolated from these diseased leaves and also investigate symptomatic *Encephalartos* leaf samples collected from elsewhere.

MATERIALS AND METHODS

Isolates

Dead *Encephalartos* leaves, or leaves with tip blight symptoms, were chosen for study. As none of the collections had leaves that were visibly colonised, leaves were incubated in moist chambers for up to 2 wk, and inspected daily for fungi. Leaf pieces bearing ascomata were subsequently soaked in water for approximately 2 h, after which they were placed in the bottom of Petri dish lids, with the top half of the dish containing 2 % malt extract agar (MEA; Oxoid, Hampshire, England). Ascospore germination patterns were examined after 24 h, and single ascospore and conidial cultures established as described by Crous (1998). Colonies were subcultured onto 2 % potato-dextrose agar (PDA), synthetic nutrient-poor agar (SNA), MEA, and oatmeal agar (OA) (Gams et al. 2007), and incubated under continuous near-ultraviolet light at 25 °C to promote sporulation. All cultures obtained in this study are maintained in the culture collection of the CBS (Table 1). Nomenclatural novelties and descriptions were deposited in MycoBank (Crous et al. 2004b).

DNA phylogeny

Genomic DNA was isolated from fungal mycelium grown on MEA, using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) according to the manufacturer's protocols. The Primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990) were used to amplify part of the nuclear rDNA operon spanning

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Table 1 Collection details and GenBank accession numbers for fungal species isolated from *Encephalartos* spp.

Species	Strain no.[1]	Substrate	Collector(s)	GenBank Accession number	
				ITS[2]	LSU[2]
Catenulostroma abietis	CPC 14996	Dead leaf tissue of *E. altensteinii*	P.W. Crous	FJ372387	FJ372404
Cladophialophora proteae	CPC 14902	Dead leaf tissue of *E. altensteinii*	P.W. Crous	FJ372388	FJ372405
Lophiostoma sp.	CPC 15000; CBS 123543	Living leaves of *E. altensteinii*	P.W. Crous et al.	FJ372389	FJ372406
Ochroconis sp.	CPC 15461; CBS 123536	Living leaves of *E. lebomboensis*	A.R. Wood	FJ372390	FJ372407
Phaeomoniella capensis	CPC 15416; CBS 123535	Living leaves of *E. altensteinii*	A.R. Wood	FJ372391	FJ372408
Saccharata kirstenboschensis	CPC 15275; CBS 123537	Living leaves of *E. princeps*	A.R. Wood	FJ372392	FJ372409
Teratosphaeria altensteinii	CPC 15133; CBS 123539	Living leaves of *E. altensteinii*	P.W. Crous et al.	FJ372394	FJ372411
Teratosphaeria encephalarti	CPC 14886; CBS 123540	Living leaves of *E. altensteinii*	P.W. Crous et al.	FJ372395	FJ372412
	CPC 15281; CBS 123544	Living leaves of *E. altensteinii*	A.R. Wood	FJ372396	FJ372413
	CPC 15362; CBS 123541	Living leaves of *E. altensteinii*	A.R. Wood	FJ372397	FJ372414
	CPC 15413; CBS 123545	Living leaves of *E. altensteinii*	A.R. Wood	FJ372398	FJ372415
	CPC 15464; CBS 123546	Living leaves of *E. lebomboensis*	A.R. Wood	FJ372399	FJ372416
	CPC 15465	Living leaves of *E. lebomboensis*	A.R. Wood	FJ372400	FJ372417
	CPC 15466	Living leaves of *E. lebomboensis*	A.R. Wood	FJ372401	FJ372418
Teratosphaeria sp.	CPC 14997	Living leaves of *E. altensteinii*	P.W. Crous et al.	FJ372402	FJ372419

[1] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.
[2] ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; LSU: 28S nrDNA.

the 3' end of the 18S rRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region (ITS2) and the first 900 bases at the 5' end of the 28S rRNA gene (LSU). The primers ITS4 (White et al. 1990) and LR0R (Rehner & Samuels 1994) were used as internal sequence primers to ensure good quality sequences over the entire length of the amplicon. The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Crous et al. (2006b). Alignment gaps were treated as new character states. Sequence data were deposited in GenBank (Table 1) and alignments in TreeBASE (www.treebase.org). The ITS sequences were compared with the sequences available in NCBIs GenBank nucleotide database using a megablast search.

Morphology

Colony growth characteristics (surface and reverse) were assessed on MEA, PDA, OA and SNA (Gams et al. 2007), and colours determined using the colour charts of Rayner (1970). Microscopic observations were made from fungal colonies cultivated on different media, as stated with each fungus. Preparations were mounted in lactic acid and studied by means of a light microscope (× 1000 magnification). Microscopic observations were made from hyphomycetes by using the transparent tape or slide culture technique, as respectively explained by Schubert et al. (2007) and Arzanlou et al. (2007). The 95 % confidence intervals were derived from 30 observations of spores formed in culture, with extremes given in parentheses. All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands, or the working collection (CPC) of P.W. Crous (Table 1).

RESULTS

DNA phylogeny

Amplification products of approximately 1 700 bases were obtained for the isolates listed in Table 1. The LSU region of the sequences was used to obtain additional sequences from GenBank, which were added to the alignment. Due to the inclusion of the shorter *Phaeomoniella chlamydospora* (GenBank AB278179) and *Ochroconis* '*humicola*' (GenBank AB161068) sequences in the alignment, it was not possible to subject the full length of the determined LSU sequences (Table 1) to the analyses. The manually adjusted alignment contained 53 sequences (including the outgroup sequence) and, of the 563 characters used in the phylogenetic analyses, 253 were parsimony-informative, 24 were variable and parsimony-uninformative, and 286 were constant. Neighbour-joining analyses using three substitution models on the sequence data yielded trees supporting the same tree topology to one another but differed from the most parsimonious tree shown in Fig. 1 with regard to the placement of the clade containing *Ochroconis* and *Fusicladium* (in the distance analyses, this clade moves to a more basal position). Forty equally most parsimonious trees (TL = 1039 steps, CI = 0.477, RI = 0.833, RC = 0.397), one of which is shown in Fig. 1, were obtained from the parsimony analysis of the LSU alignment. The isolates from *Encephalartos* are distributed across several families and orders and taxonomic novelties are described below and specific taxa are highlighted in the Discussion. Results obtained from the BLAST searches of the ITS sequences are discussed where applicable.

Taxonomy

Several species of fungi which are believed to be new were collected, and are described in genera such as *Phaeomoniella*, *Saccharata* and *Teratosphaeria*. New records for *Encephalartos* include *Catenulostroma microsporum*, *Cladophialophora proteae*, *Dactylaria leptosphaeriicola*, and undescribed species of *Teratosphaeria*, *Lophiostoma* and *Ochroconis*.

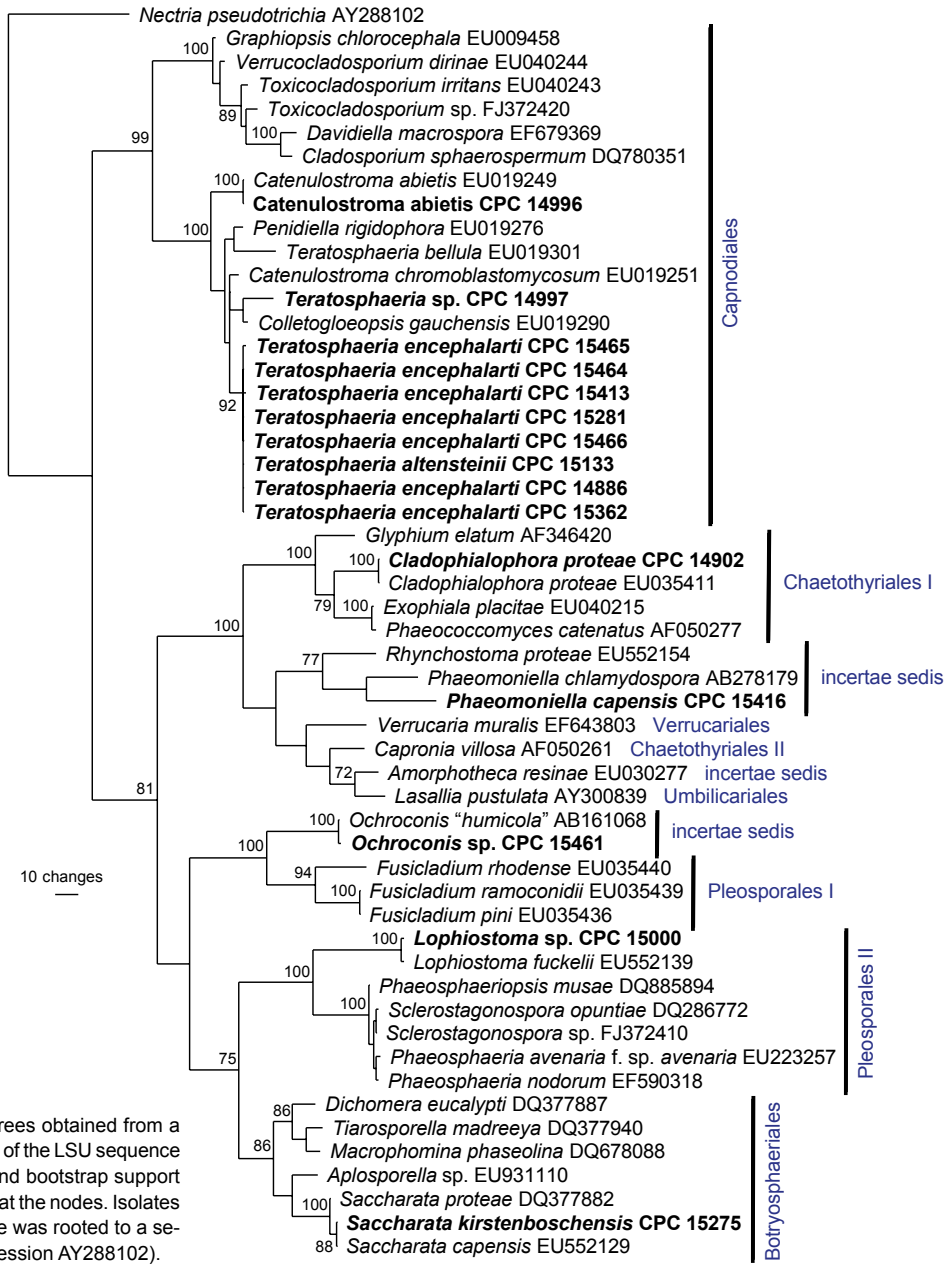

Fig. 1 One of 40 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the LSU sequence alignment. The scale bar shows 10 changes, and bootstrap support values (>70 %) from 1 000 replicates are shown at the nodes. Isolates from *Encephalartos* are shown in **bold**. The tree was rooted to a sequence of *Nectria pseudotrichia* (GenBank accession AY288102).

Phaeomoniella capensis Crous & A.R. Wood, *sp. nov.* — MycoBank MB508007; Fig. 2

Phaeomoniellae chlamydosporae similis, sed conidiis majoribus, (2–)3(–4) × 1–1.5 µm.

Etymology. Name refers to the Cape Province of South Africa, where this fungus was collected.

On SNA. *Mycelium* consisting of septate, branched, hyaline to pale brown, thick-walled hyphae, 1.5–2 µm; developing hyaline, thin-walled, swollen, globose structures. *Conidiomata* pycnidial to acervular, opening by irregular rupture, erumpent, brown, up to 250 µm diam; wall of 3–6 layers of brown *textura angularis*. *Conidiophores* hyaline, smooth, highly variable in morphology, occurring in branched structures, 2–4-septate, or solitary, ampulliform, reduced to phialides. *Conidiogenous cells* 3–10 × 2–3 µm; apical opening with minute periclinal thickening. *Conidia* hyaline, smooth, narrowly ellipsoid, straight, (2–)3(–4) × 1–1.5 µm.

Cultural characteristics — *Colonies* erumpent, spreading, lacking aerial mycelium, slimy, with folded surface and smooth, catenulate margin; on PDA salmon with patches of apricot, and apricot in reverse, reaching 10 mm diam after 1 mo; on OA salmon to flesh with brown patches due to conidiomatal formation, reaching 12 mm diam after 1 mo; on MEA salmon

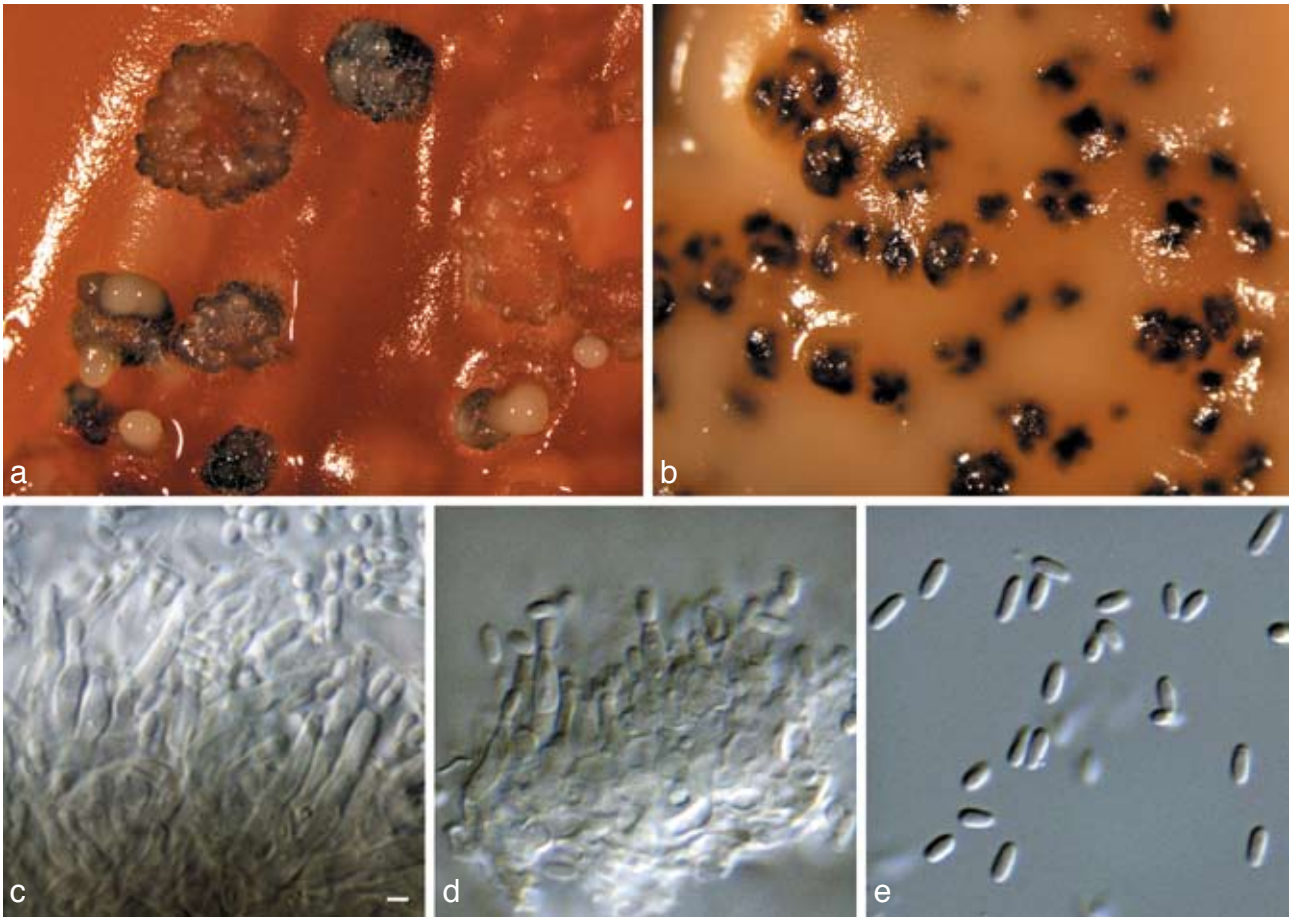

Fig. 2 *Phaeomoniella capensis* in vitro (CBS 123535). a. Colony on OA; b. colony on PDA; c, d. conidiogenous cells and conidia; e. conidia. — Scale bar = 10 µm.

with patches of apricot and flesh, apricot in reverse, reaching 15 mm diam after 1 mo.

Specimen examined. South Africa, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 22 May 2008, *A.R. Wood*, CBS H-20159, culture ex-type CPC 15416 = CBS 123535, CPC 15417–15418.

Notes — Two fungal species that have previously been described from *Encephalartos* need to be compared with *P. capensis*. *Leptothyrium evansii* forms hypophylous pycnidia with oblong, hyaline conidia, 3.5–5 × 1.5–2 µm, thus larger than observed in *P. capensis* (Sydow & Sydow 1912). The second species, *Phoma encephalarti*, is distinct in having larger, biguttulate conidia, 6.3–7.2 × 2.7–3.6 µm (Negodi 1932).

The fact that the present collection clusters in *Phaeomoniella* (hyphomycetous genus) is somewhat surprising. However, this genus also has a phoma-like synanamorph and a yeast-like growth in culture (Crous & Gams 2000), similar to *P. capensis*. Although further collections may eventually show this complex to represent more than one genus, we presently consider it best to place the *Encephalartos* fungus in *Phaeomoniella* based on current data. BLAST results of the ITS sequence revealed an identity of 89 % with *Phaeomoniella chlamydospora* (GenBank accession AY772237).

Saccharata kirstenboschensis Crous & A.R. Wood, *sp. nov.* — MycoBank MB508008; Fig. 3

Saccharatae proteae similis, sed conidiis minoribus, (16–)18–22(–24) × 3.5–4(–5) µm.

Etymology. Name refers to Kirstenbosch Botanical Gardens, South Africa, where this fungus was collected.

On WA with sterile pine needles. *Conidiomata* pycnidial, black, up to 350 µm diam, with a single, central ostiole; wall consisting of 2–3 layers of brown *textura angularis*. *Conidiophores* subcylindrical, hyaline, smooth, frequently reduced to conidiogenous cells or branched in apical part, 1–2-septate, 10–45 × 2–3.5 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, 15–20 × 2–3 µm; apex with periclinal thickening, or with 1–3 percurrent proliferations. *Paraphyses* intermingled among conidiophores, at times arising as lateral branches from conidiophores, or separate, unbranched or branched above, hyaline, smooth, 0–3-septate, 2–3 µm wide, extending above conidiophores. *Conidia* hyaline, smooth, fusiform to narrowly ellipsoid, apex subobtuse, base truncate with minute marginal frill, guttulate, thin-walled, (16–)18–22(–24) × 3.5–4(–5) µm, base 2–3 µm wide.

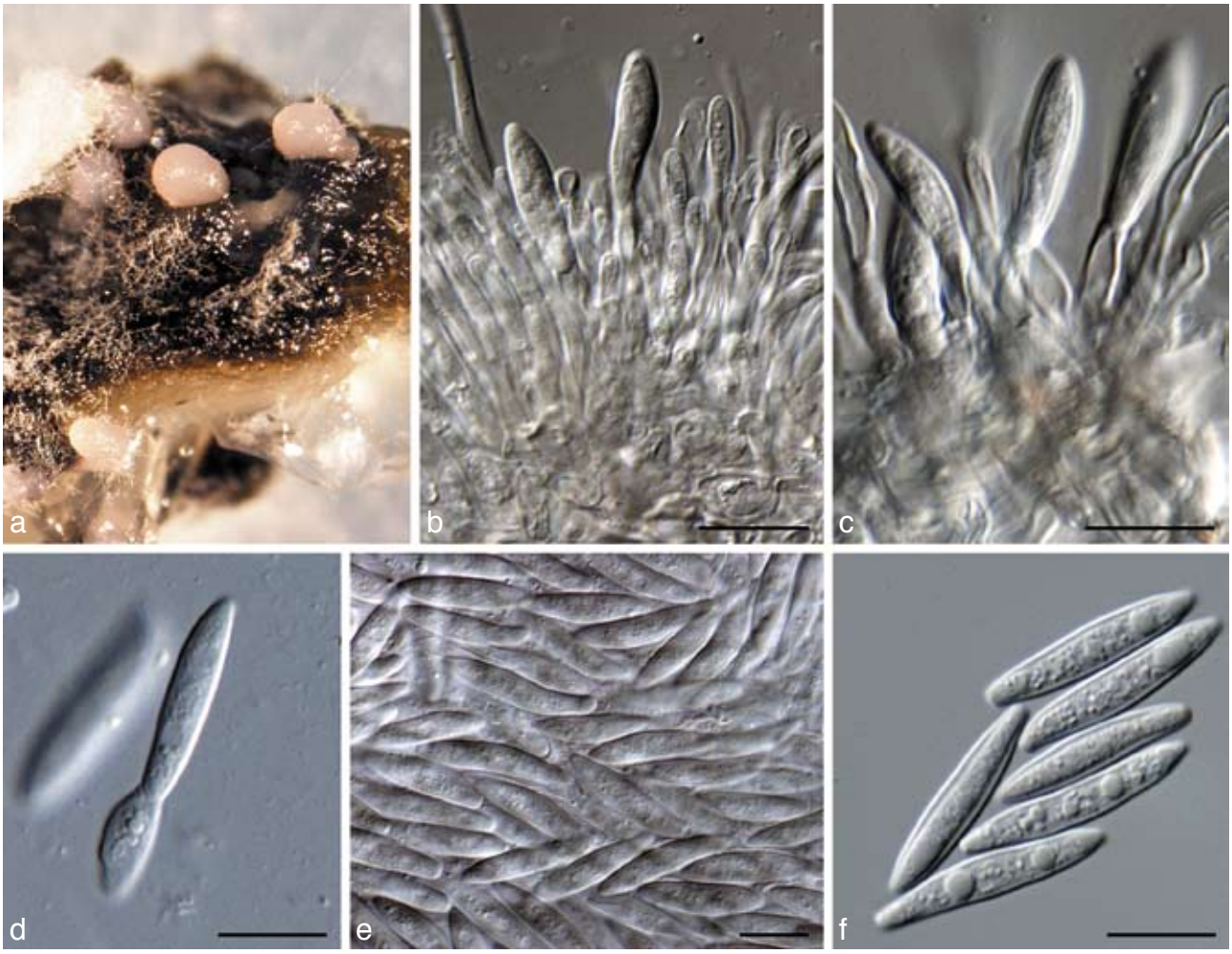

Fig. 3 *Saccharata kirstenboschensis* in vitro (CBS 123537). a. Conidiomata on WA with sterile pine needles; b, c. conidiogenous cells giving rise to conidia; d. conidium attached to conidiogenous cell; e, f. conidia. — Scale bars = 10 µm.

Cultural characteristics — *Colonies* on MEA, PDA and OA spreading, erumpent, with moderate aerial mycelium and uneven, catenulate margins; pale olivaceous-grey with patches of grey and olivaceous-grey; reverse olivaceous-grey; reaching 6 cm diam after 1 mo.

Specimen examined. South Africa, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos princeps*, 22 May 2008, *A.R. Wood*, holotype CBS H-20160, culture ex-type CPC 15275 = CBS 123537, CPC 15276–15277.

Notes — The genus *Saccharata* presently consists of two species, namely *S. proteae* (conidia 20–30 × 4.5–6 µm; Denman et al. 1999, Crous et al. 2006b) and *S. capensis* (conidia 13–18 × 3.5–5.5 µm; Marincowitz et al. 2008). *Saccharata kirstenboschensis* represents an intermediate species, having conidia 16–24 × 3.5–5 µm. Furthermore, it is the first species of *Saccharata* known to occur on a host other than Proteaceae, although all taxa described thus far appear to be endemic to South Africa. BLAST results of the ITS sequence revealed an identity of 98 % with *S. proteae* (GenBank accession EU552145; 819 of 830 bases) and *S. capensis* (GenBank accession EU552130; 803 of 816 bases).

Teratosphaeria altensteinii Crous, *sp. nov.* — MycoBank MB508010; Fig. 4

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

Etymology. Name refers to its host species, *Encephalartos altensteinii*.

Leaves with tip-blight symptoms; necrotic tissue grey-brown, separated from healthy tissue by a narrow, dark-brown border. *Ascomata* hypophyllous, black, immersed, substomatal, up to 90 µm diam; ostiole lined with periphyses; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, obovoid, straight to slightly curved, 8-spored, 35–37 × 8–9 µm. *Ascospores* bi- to triseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, prominently constricted at the septum, tapering towards both ends, but more prominently towards the lower end, 7–8(–9) × 2.5–3(–3.5) µm; germinating ascospores on MEA become brown and verruculose, germinating with multiple germ tubes irregular to the long axis of the spore, constricted at septum and distorting, up to 8 µm wide.

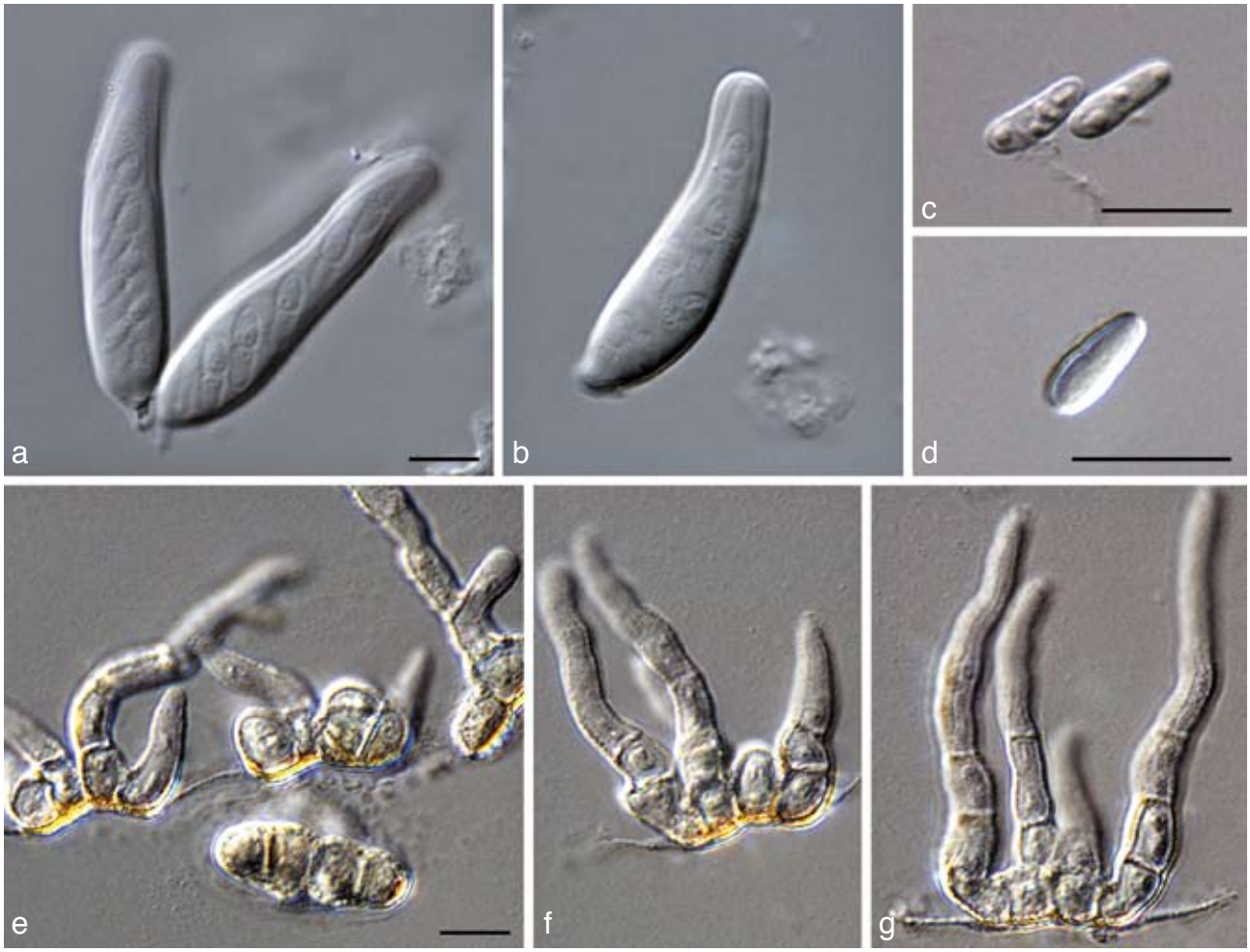

Fig. 4 *Teratosphaeria altensteinii* in vitro (CBS 123539). a, b. Asci; c, d. ascospores; e–g. germinating ascospores on MEA. — Scale bars = 10 µm.

Cultural characteristics — *Colonies* on MEA spreading, somewhat erumpent, with moderate aerial mycelium, and even, catenulate margins; surface iron-grey; reverse greenish black; reaching 20 mm diam after 1 mo; on PDA and OA similar, but olivaceous-grey on surface, and iron-grey in reverse; on MEA and PDA hyphae form terminal clusters of chlamydospore-like cells, which are catenulostroma-like in appearance, and frequently detach under squash mounts.

Specimen examined. South Africa, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 6 Jan. 2008, *P.W. Crous*, *M.K. Crous*, *M. Crous & K. Raath*, holotype CBS H-20162, culture ex-type CPC 15133 = CBS 123539, CPC 15134–15135.

Notes — *Teratosphaeria altensteinii* is phylogenetically closely related to *T. bellula* (593 of 601 bases when the ITS sequence is compared to GenBank accession EU707861), which is a pathogen of Proteaceae (Crous & Wingfield 1993, Crous et al. 2004a, 2008). Morphologically it has ascospores that are similar in shape, but are distinct in that they lack a prominent sheath and are somewhat smaller (7–9 × 2.5–3.5 µm) than those of *T. bellula* (8–11 × 2–3.5 µm; Crous & Wingfield 1993).

Teratosphaeria encephalarti Crous & A.R. Wood, *sp. nov.* — MycoBank MB508011; Fig. 5

Anamorph. *Penidiella* sp.

Teratosphaeriae bellulae similis, sed ascosporis majoribus, (9–)10–11(–14) × (3–)3.5–4 µm.

Etymology. Name refers to its host genus, *Encephalartos*.

Leaves with tip-blight symptoms; nectrotic tissue grey-brown. *Ascomata* hypophyllous, black, immersed, substomatal, up to 90 µm diam; ostiole lined with periphyses; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, obovoid to broadly ellipsoid, straight to curved, 8-spored, 30–40 × 10–13 µm. *Pseudoparaphyses* intermingled among asci, branched, septate, hyaline, 2–3 µm wide. *Ascospores* bi- to triseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest in middle of apical cell, prominently constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (9–)10–11(–14) × (3–)3.5–4 µm; turning brown and verruculose in older asci; germinating ascospores on

Fig. 5 *Teratosphaeria encephalarti* (CBS 123540). a. Diseased *Encephalartos altensteinii* palms in Kirstenbosch Botanical Gardens, South Africa; b. leaf blight symptoms; c. ascomata on leaves (arrows); d, e. asci; f. ascospores; g–k. germinating ascospores on MEA; l–o. *Penidiella* anamorph with branched conidial chains. — Scale bars = 10 µm.

MEA become brown and verruculose, germinating with several germ tubes irregular to the long axis of the spore, constricted at septum and distorting, up to 7 µm wide. On OA. *Mycelium* consisting of creeping, branched, septate, brown, smooth, 2–3.5 µm wide hyphae. *Conidiophores* solitary, erect, subcylindrical, arising from creeping hyphae, medium brown, thick-walled, smooth to finely verruculose, 1–6-septate, 15–50 × 3–4.5 µm. *Conidiogenous cells* terminal, subcylindrical, medium brown, smooth, up to 4 µm wide; scars somewhat thickened and darkened, up to 2.5 µm wide. *Ramoconidia* 0–1-septate, subcylindrical to elongate-ellipsoid, medium brown, smooth, thick-walled, with 1–3 apical loci, 10–15 × 3–4 µm. *Secondary ramoconidia* 0–1-septate, narrowly ellipsoid, 7–10 × 3–3.5 µm. *Intercalary conidia* in chains of up to 15, aseptate, fusoid-ellipsoid, medium brown, smooth, (5–)6–7(–8) × 2–3(–2.5) µm. *Terminal conidia* aseptate, ellipsoid, pale to medium brown, with truncate base, 3–4 × 2–3 µm; hila slightly thickened and darkened, 0.5–1 µm wide.

Cultural characteristics — *Colonies* on OA, MEA and PDA spreading with moderate aerial mycelium and smooth, catenulate margins; centre olivaceous-grey, outer region and reverse iron-grey; reaching 30 mm diam after 1 mo.

Specimens examined. South Africa, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 6 Jan. 2008, *P.W. Crous*, *M.K. Crous*, *M. Crous & K. Raath*, holotype CBS H-20163, culture ex-type CPC 14886 = CBS 123540, CPC 14887–14888; 22 May 2008, *A.R. Wood*, culture CPC 15413 = CBS 123545, CPC 15414–15415; CPC 15362 = CBS 123541, CPC 15363–15364; CPC 15281 = CBS 123544, CPC 15282–15283; KwaZulu-Natal, South Coast, Uvongo, Skyline Nature Reserve, arboretum, living leaves of *Encephalartos lebomboensis*, 29 May 2008, *A.R. Wood*, culture CPC 15464 = CBS 123546, CPC 15465–15466.

Notes — *Teratosphaeria encephalarti* appeared to be quite dominant on the dying leaves of *E. altensteinii* in the Western Cape Province and it is possible that this species plays a role in the recently observed leaf blight disease. Inoculation studies are required, however, to confirm its potential role in this disease. Phylogenetically *T. encephalarti* and *T. altensteinii* are distantly related (88 % based on ITS) to *T. associata*, which occurs on *Eucalyptus* and *Protea* spp. (Crous et al. 2007a, 2008). The ITS sequences of the ex-type strains of *T. altensteinii* and *T. encephalarti* have an identity of 91 % with each other (430 of 468 bases).

Undetermined species

Lophiostoma sp.

Cultural characteristics — *Colonies* on MEA, PDA and OA spreading with moderate aerial mycelium, and smooth, catenulate margins; surface olivaceous-grey; reverse iron-grey; reaching 25 mm diam after 1 mo.

Specimen examined. South Africa, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 6 Jan. 2008, *P.W. Crous*, *M.K. Crous*, *M. Crous & K. Raath*, culture CPC 15000 = CBS 123543, CPC 15001–15002.

Notes — Isolate CBS 123543 is representative of a species of *Lophiostoma* (based on ITS DNA sequence similarity to *L. macrostomum* GenBank accession EU552140). It could not be described, however, due to paucity of material. Ascospores remained hyaline upon germination on MEA, but distort prominently (up to 10 µm wide), becoming constricted, with germ tubes growing down into the agar.

Ochroconis sp. — Fig. 6

On OA. *Colonies* moderately fast-growing, flat with predominantly submerged mycelium. *Mycelium* consisting of branched, septate, hyaline to pale brown, smooth, 2–2.5 µm wide hyphae. *Conidiophores* erect, arising from creeping hyphae, unbranched, 1–6-septate, straight to flexuous, brown, thick-walled, 10–50 × 2.5–3.5 µm. *Conidiogenous cells* terminal, integrated, 10–35 µm long, polyblastic, cylindrical, straight to flexuous, pale to medium brown, with scattered pimple-shaped, subhyaline denticles, 0.5 µm wide and long. *Conidia* (5–)7–9(–10) × (2.5–)3(–3.5) µm, solitary, subhyaline, smooth to verruculose, 1-septate, thin-walled, obovoid to fusiform, apex subobtuse, base narrowly truncate with minute marginal frill, 0.5 µm wide; conidial secession rhexolytic.

Cultural characteristics — *Colonies* on MEA, PDA and OA spreading, flat, with even, smooth margins, and sparse aerial mycelium; surface olivaceous-grey, reverse iron-grey; colonies reaching 25 mm diam after 1 mo.

Specimen examined. South Africa, KwaZulu-Natal, South Coast, Uvongo, Skyline Nature Reserve, arboretum, living leaves of *Encephalartos lebomboensis*, 29 May 2008, *A.R. Wood*, culture CPC 15461 = CBS 123536, CPC 15462–15463.

Notes — Species of *Ochroconis* are known to infect cold blooded vertebrates, or to occur as saprobes on different plant substrates and in soil (de Hoog et al. 2000), suggesting that the species from *Encephalartos* is probably saprobic. Phylogenetically the present collection clusters with a strain identified as *Ochroconis humicola* (CBS 780.83), though conidia of the ex-type strain of *O. humicola* (CBS 116655) are larger and it clusters distant from these strains. Preliminary DNA sequence data suggest that many species of *Ochroconis* in fact represent species complexes, and hence it would be best to treat the *Encephalartos* collection as part of a generic revision (de Hoog et al. in prep).

Teratosphaeria sp.

Cultural characteristics — *Colonies* on MEA, PDA and OA erumpent, fluffy, with abundant aerial mycelium and even, catenulate margins; surface olivaceous-grey with patches of iron-grey and pale olivaceous-grey; reverse iron-grey; reaching 30 mm diam after 1 mo.

Specimen examined. South Africa, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 6 Jan. 2008, *P.W. Crous*, *M.K. Crous*, *M. Crous & K. Raath*, culture CPC 14997–14999.

Notes — Isolate CPC 14997 could not be described due to paucity of material. Based on the DNA similarity to an ITS sequence of *Batcheloromyces leucadendri* (accession EU552103; 739 of 801 bases identity) deposited in GenBank, however, it appears to represent a species of *Teratosphaeria*. Ascospores germinated from both polar ends with germ tubes growing parallel to the long axis of the spore. Germinating spores became

Fig. 6 *Ochroconis* sp. in vitro (CBS 123536). a, b. Conidial fascicles on MEA with light from above and below, respectively; c–j. conidiophores giving rise to conidia, with visible denticles (arrows); k. conidia. — Scale bars = 10 µm.

prominently constricted and distorted, up to 7 µm wide, pale brown, and somewhat verruculose.

DISCUSSION

Prior to the present study only four fungal species had been described from *Encephalartos*, namely *Leptothyrium evansii*, *Pestalotia encephalartos*, *Phoma encephalarti* and *Phyllosticta encephalarti* (http://nt.ars-grin.gov/fungaldatabases/). A very preliminary examination of four collections during the present study has added a further four species in genera such as *Phaeomoniella*, *Saccharata* and *Teratosphaeria*. Furthermore, due to paucity of fungal material, several other species remain to be described in future studies. At present none of these fungi are confirmed as being pathogenic, and further work is required to determine which species are pathogens of *Encephalartos* and what impact they have on the population dynamics of these plant species. Considering that many of these cycad species are endangered this could have important consequences for their conservation.

What is interesting to note, however, is that some species known from indigenous Proteaceae were also observed for the first time on *Encephalartos*. *Dactylaria leptosphaeriicola* (Fig. 7) was initially described as a hyperparasite of ascomata of *Leptosphaeria protearum* on leaves of *Protea repens*. It is interesting that this fungus was found occurring on ascomata of *Teratosphaeria encephalarti* on *Encephalartos altensteinii* in the present study. As found by Braun & Crous (1992), conidia of this species failed to germinate on MEA or PDA, stressing its close hyperparasitic relationship with its ascomycetous host. It is possible, however, that *D. leptosphaeriicola* is not a true member of *Dactylaria*, but represents yet another undescribed genus resembling *Dactylaria* in morphology. To confirm this, however, DNA will have to be isolated from fresh collections,

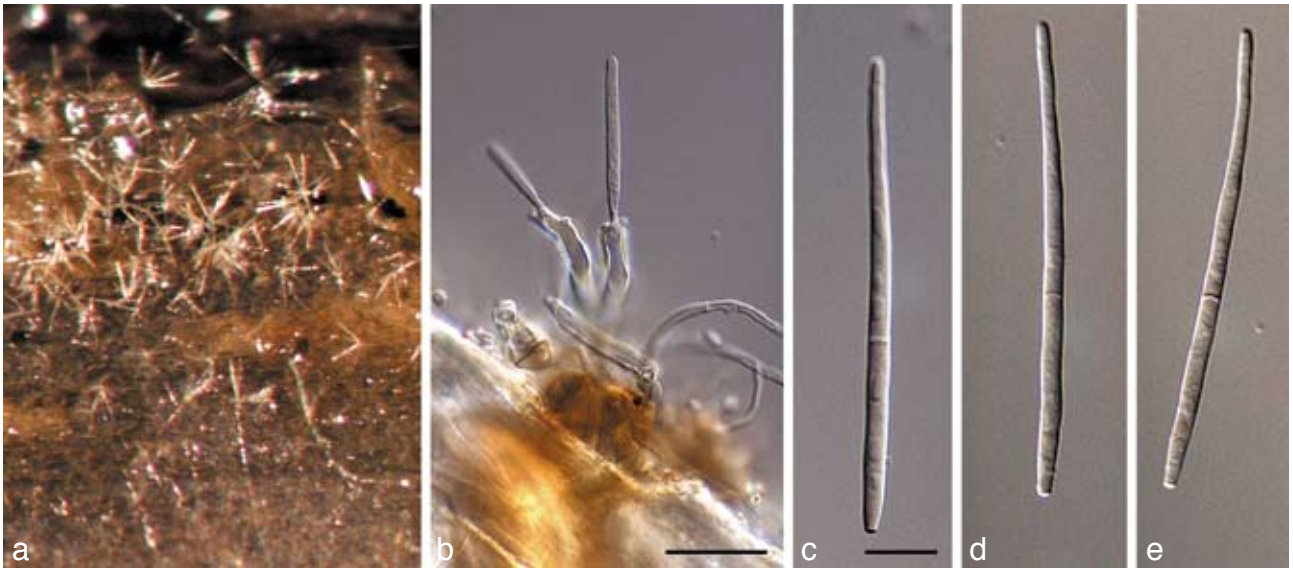

Fig. 7 *Dactylaria leptosphaeriicola* in vivo. a. Conidial fascicles on leaf; b. conidiogenous cells giving rise to conidia; c–e. conidia. — Scale bars = 10 µm.

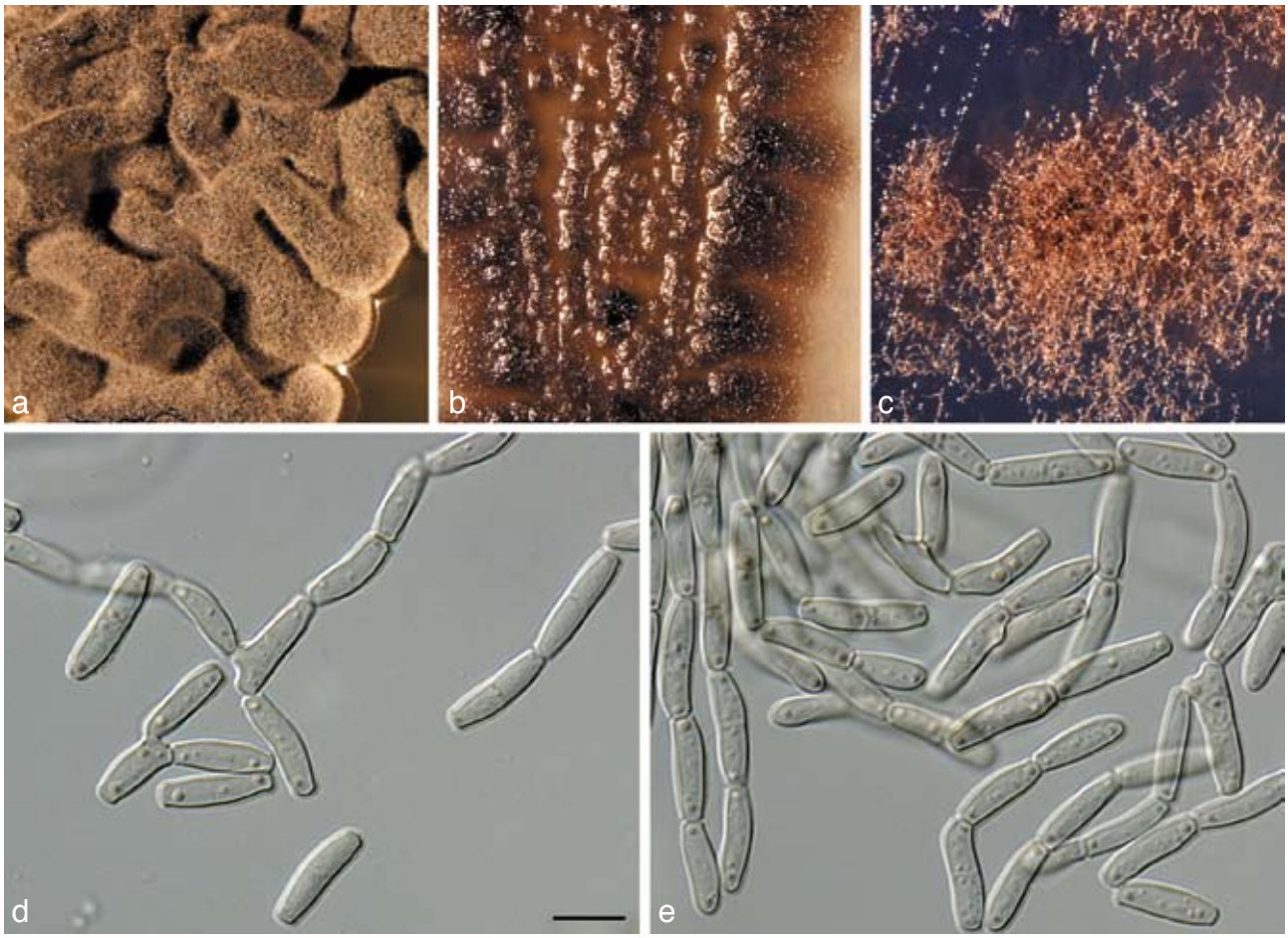

Fig. 8 *Cladophialophora proteae* in vitro (CPC 14902). a–c. Colony on MEA, OA and PDA, respectively; d, e. conidial chains. — Scale bar = 10 µm.

which would be difficult, as fascicles occur in conjunction with ascomata of other fungi, and attempts to cultivate the fungus have thus far proven to be unsuccessful.

Cladophialophora proteae was initially isolated from lesions of *Batcheloromyces proteae* on *Protea cynaroides*, to which it was assumed to be pathogenic, though no inoculation tests have ever been conducted to confirm this hypothesis (Swart et al. 1998). The status of *Cladophialophora* and *Pseudocladosporium* has been an issue of debate, and as *Cladophialophora* was used for taxa pathogenic to humans, Crous et al. (2004a) allocated the species isolated from *Protea* to *Pseudocladosporium*. However, as shown in a subsequent molecular study (Crous et al. 2007b), *Pseudocladosporium* is a synonym of *Fusicladium* (Venturiaceae) while species of *Cladophialophora* (Herpotrichiellaceae) were shown to occur on humans and plant hosts, and thus the name *Cladophialophora proteae* can be used for this fungus (Fig. 8). The fact that this species could also occur on dead leaf tissue of *Encephalartos altensteinii* (CPC 14902–14904) in the Western Cape Province is surprising, however, and again questions its possible ecological role and its potential wider host range.

The link of '*Trimmatostroma*' to '*Mycosphaerella*' was first reported on leaf spots of *Teratosphaeria maculiformis* from *Protea cynaroides* leaves collected in South Africa by Taylor & Crous (2000). After initial data suggesting that *Teratosphaeria* and *Mycosphaerella* represented a single genus (Taylor et al. 2003), a subsequent study demonstrated that these were in fact from two different families and that species of *Teratosphaeria* belonged to the Teratosphaeriaceae, in which the anamorph genus *Catenulostroma* was established for these trimmatostroma-like anamorphs (Crous et al. 2007a). Within *Catenulostroma* there is a species complex surrounding *C. abietis*, which based on DNA sequence data solely of the ITS gene region, is very difficult to distinguish. It is quite possible, therefore, that the *Encephalartos* isolates (CPC 14996), although phylogenetically similar to *Catenulostroma microsporum* (*Teratosphaeria microspora*), may very well still be shown to represent yet another cryptic species in this complex.

Africa is well known to have a high level of botanical diversity. As shown here after an initial cursory look at a few *Encephalartos* leaves, these plants were found to host numerous undescribed species of fungi. Given the high level of endemism found in African flora, it can be expected that an equally high number of these fungal species will be unique species. Unfortunately, indigenous African fungal biodiversity has never been regarded as a research priority and as such this research topic has never been well supported financially. Given the current importance placed on ecotourism and the preservation of unique African flora and fauna, it is clearly timely that more research focus and financial resources be channelled towards documenting, studying ecological roles and impacts, and conserving African mycoflora.

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Mycology

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

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ABSTRACT

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Dothistroma septosporum and *D. pini* are the two causal agents of Dothistroma needle blight of *Pinus* spp. in natural forests and plantations. Degenerate primers amplified portions of mating type genes (*MAT1-1-1* and *MAT1-2*) and chromosome walking was applied to obtain the full-length genes in both species. The mating-type-specific primers designed in this study could distinguish between the morphologically similar *D. pini* and *D. septosporum* and between the different mating types of these species. Screening of isolates from global collections of *D. septosporum* showed that only MAT2 isolates are present in Australian and New Zealand collections, where only the asexual form of the fungus has been found. In contrast, both mating types of *D. septosporum* were present in collections from Canada and Europe, where the sexual state is known. Intriguingly, collections from South Africa and the United Kingdom, where the sexual state of the fungus is unknown, included both mating types. In *D. pini*, for which no teleomorph is known, both mating types were present in collections from the United States. These results provided new insights into the biology and global distribution of two of the world's most important pine pathogens and should facilitate management of the diseases caused by these fungi.

Additional keywords: ascomycetes, heterothallic, *Mycosphaerella*, sexual reproduction.

Dothistroma needle blight, also known as red band needle blight, is one of the most important diseases of *Pinus* spp., both in natural forest ecosystems and particularly in plantations of nonnative pines (9,19,20,27). The disease owes its international notoriety to the fact that it has been one of the most important constraints to the development of plantation forestry in many countries of Africa as well as in New Zealand, Australia, Chile, and other South American countries (19,20,27). The disease is particularly severe on *Pinus radiata* D. Don. This species is highly desirable for its rapid growth and exceptional timber and, consequently, it was one of the first nonnative tree species established in intensively managed plantations in the tropics and Southern Hemisphere. Outbreaks of Dothistroma needle blight on *P. radiata* led to devastating losses and resulted in the abandonment of *P. radiata* from plantation forestry in many countries (11,31,51).

The main causal agent of Dothistroma needle blight has been a matter of considerable taxonomic confusion. Thus, in different parts of the world, the disease has been attributed to either a single pathogen, different species of a pathogen, or varieties of a species. This also has differed depending on whether the pathogen was considered introduced or native in areas where the disease has been studied. In a recent study based on DNA sequence comparisons, two distinct phylogenetic lineages for *Dothistroma* isolates were identified (2). These clearly separated *Dothistroma septosporum*, which has a worldwide distribution, and *D. pini*, until recently found only in the north-central United States. This study also showed that the disease which devastated plantations of *P. radiata* in the Southern Hemisphere is caused by *D. septosporum*. Recently, *D. pini* has been found infecting *P. palassiana* D. Don. in the Ukraine (I. Barnes, *unpublished data*) and it clearly has a distribution much wider than was believed at the time of the study of Barnes et al. (2).

Dothistroma needle blight, now known to have been caused by *D. septosporum*, resulted in huge damage to *P. radiata* plantations in the Southern Hemisphere in the 1950s and 1960s (9,19,20,27). Consequently, considerable research was conducted on the disease and great efforts were made to minimize its impact (8,19, 20,41,46). These included selection of alternative species, tree breeding, agricultural practices, and the first examples of aerial applications of chemical fungicides in forest plantations (19). Although the disease has continued to be important, it generally is considered to be under reasonable control. There has, however, been a recent resurgence of the disease in various Northern Hemisphere countries and this has raised concern that a new wave of losses might occur elsewhere in the world (5,53).

Almost nothing is known regarding the genetic diversity among isolates of *D. septosporum* and *D. pini*. *D. septosporum* first was identified in New Zealand in 1964 (21). A study by Hirst et al. (26) applied random amplified polymorphic DNA (RAPD) markers to a population of *D. septosporum* (previously described as *D. pini*) from New Zealand and the results showed no genetic variation. These results support the hypothesis that it is an introduced pathogen that has been spreading asexually ever since its introduction into that country.

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The sexual state of *D. septosporum* is a species of *Mycosphaerella* known as *Mycosphaerella pini* Rostr. (17). In most countries of the Southern Hemisphere where *D. septosporum* has long been an important forest pathogen, only the anamorph has been reported (2,5,14; M. J. Wingfield, *unpublished data*). In contrast, no sexual state has ever been reported for *D. pini*. The absence or rarity of a sexual state for either of these fungi could be the result of selection pressure and a reduced need for sexual reproduction (14). Likewise, lower frequency and limited distribution of the teleomorph compared with the anamorph suggests that the primary method of dispersal of the fungus could be an asexual cycle. Here, conidia rather than ascospores would represent the inoculum of primary epidemiological importance (10,28).

Mating type genes play an important part in the biology and evolution of fungal species. Thus, knowledge of these genes can provide insight into the potential prevalence of sexual reproduction in different species. Some heterothallic Pyrenomycetes and Discomycetes can contain up to four genes at the mating type 1 idiomorph (*MAT1-1*) of the *MAT* locus (40,43,44,55). These include the *MAT1-1-1* encoding an α domain protein, the *MAT1-1-2* encoding an amphipathic α helix protein, the *MAT1-1-3* gene encoding a high mobility group (HMG) domain protein, and the *MAT1-1-4* gene encoding a metallothionein protein. Only one gene has been characterized for the mating type 2 idiomorph (*MAT1-2*) and it encodes a regulatory protein with an HMG domain. The DNA sequences of the idiomorphs, located at the *MAT* locus of individuals of two different mating types, are unrelated and, therefore, cannot be called alleles; however, these sequences are flanked by conserved regions (32). The formal nomenclature that is proposed for mating type genes of heterothallic ascomycetes is used here for the *MAT1-1-1* and, because only a single *MAT1-2* gene has been identified for filamentous ascomycetes, this gene is referred to as *MAT1-2* (49).

DNA and amino acid sequences of the *MAT1-1-1* and *MAT1-2* genes in fungi show no obvious similarities, although the mating type locus has common flanking regions (48). Except for the HMG and α domains, the similarity of homologous mating type genes usually is very low between different species (47). The direct target genes of the mating type proteins have not yet been described, although there is evidence for the control of some genes, such as pheromone genes (4). Mating type genes have been described from various sexual and presumably asexual fungi that are close relatives of the genus *Dothistroma* (Mycosphaerellaceae). Detailed analyses have been done on the distribution of the mating types of the sexually reproducing *M. graminicola* (50,56) and the presumably asexual species *Septoria passerinii* (23), *Cercospora beticola*, *C. zeae-maydis*, and *C. zeina* (25). Equal distribution of the mating types was found in most of the populations from these five species sampled from different geographical scales, indicating that sexual stages probably exist for the latter four apparently asexual species.

D. septosporum first was described from Idaho (United States) but now is seen in many parts of the world (2). In most of the areas where this species has been introduced and causes serious disease, only the asexual state of the fungus is ever seen. This raises the interesting question as to whether this could be attributed to the introduction of only one mating type into these new environments. Thus, the aims of this study were to characterize the mating type gene or genes of the causal agents of Dothistroma needle blight and to ascertain which mating types are present in the different countries where diseases caused by these fungi occur. To achieve this objective, the full-length *MAT1-1-1* and *MAT1-2* genes of *D. septosporum* and *D. pini* were isolated and sequenced using polymerase chain reaction (PCR)-based techniques. This made it possible to develop a multiplex PCR method for the rapid screening of *MAT1-1-1* and *MAT1-2* in isolates of the pathogens. A global collection of isolates subsequently was screened to determine which mating types are present in these collections.

MATERIALS AND METHODS

Fungal isolates. In all, 230 *Dothistroma* isolates obtained from various locations in 15 countries were chosen to represent a global distribution of *Dothistroma* spp. (Table 1). Countries for which more than one isolate was screened included Austria (n = 10), Canada (n = 106), Chile (n = 10), New Zealand (n = 38), Poland (n = 11), South Africa (n = 11), Ukraine (n = 4), the United Kingdom (n = 10), and the United States (n = 17). Isolates were obtained from different culture collections and standard protocols were used to isolate the genomic DNA.

The initial screening of the mating type genes was undertaken for *D. septosporum* using two isolates. These included CBS 116489 obtained from *P. radiata* in Tzaneen, South Africa and American Type Culture Collection (ATCC) MYA-605 obtained from *P. radiata* in Rotorua, New Zealand. For *D. pini,* four isolates were used: CBS 116485, obtained from *P. nigra* in Crystal Township, MI; CBS 116487, obtained from *P. nigra* in Evergreen Township, MI; CBS 116483, obtained from River Township, MI; and CBS 117609, obtained from *P. palassiana* in Tsyurupinsk, Ukraine. The identities of the six isolates used for the screening of the mating types previously had been confirmed using comparisons of DNA sequence data for the internal transcribed spacer (ITS) regions of the ribosomal DNA (2; J. Z. Groenewald, *unpublished data*).

Isolation and characterization of *MAT1-1-1* of *Dothistroma* spp. The *MAT1-1-1*-specific degenerate primers (MgMfSpMat1-1f1 and MgMfSpMat1-1r2) (Table 2), designed by Groenewald et al. (25), were used to screen and amplify a partial region of the *MAT1-1-1* genes of the *Dothistroma* isolates.

The PCR mixtures and amplification reactions were the same as described by Groenewald et al. (25) for the amplification of the partial *MAT1-1-1* in *Cercospora* spp. The PCR products obtained were separated by electrophoresis at 80 V for 1 h on a 1% (wt/vol) agarose gel containing ethidium bromide at 0.1 μg/ml in 1× Tris-acetate-EDTA buffer (0.4 M Tris, 0.05 M sodium acetate, and 0.01 M EDTA, pH 7.85) and visualized under UV light. Amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, Netherlands) following the manufacturer's recommendations. The products were analyzed on an ABI Prism 3730 DNA Sequencer (Applied Biosystems, Foster City, CA). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNA-STAR, Madison, WI).

Internal primers were designed in the partially sequenced *MAT1-1-1* genes for each of the species (CBS 116489 for *D. septosporum* and CBS 116487 for *D. pini*). In order to obtain the full-length genes, these internal primers were used together with the appropriate primers from the DNA walking speedup kit (Seegene Inc., Rockville, MD) to determine additional sequences upstream and downstream of the partial *MAT1-1-1* sequences. The Blastx algorithm (1) was used to compare the sequences obtained from the two *Dothistroma* spp. with protein sequences of other fungi present in the National Center for Biotechnology Information (NCBI) nonredundant protein database. The geneid web server (v1.2; Research Unit on Biomedical Informatics of IMIM, Barcelona, Spain) was used to predict the gene and intron or exon boundaries using the genetic code of *Neurospora crassa*. The conversion of DNA sequences to putative amino acid sequences was done using the translation tool of the proteomics server ExPASy (18). The percentage of identities between the predicted *MAT1-1-1* gene sequences for the *Dothistroma* spp. was calculated using the alignment tool of ALIGN (37).

Isolation and characterization of *MAT1-2* of *Dothistroma* spp. The *MAT1-2*-specific degenerate primers (MgMfSpMat1-2f2 and MgMfSpMat1-2fr1) (Table 2), designed by Groenewald et al. (25), were used to screen isolates of *D. septosporum* and *D. pini*

TABLE 1. Origins of the *Dothistroma septosporum* and *D. pini* strains used during this study and the distribution of their mating types

Country, area, site	Collector	Species	Number of strains	MAT1-1-1	MAT1-2
Australia					
A.C.T. Canberra	K. Old	*D. septosporum*	10	0	10
Austria					
Thenneberg	T. Kirisits	*D. septosporum*	10	6	4
Brazil					
São Paulo	T. Namekata	*D. septosporum*	1	0	1
Canada					
Northwest British Columbia (BC)					
Brown Bear Road	K. Lewis & A. Dale	*D. septosporum*	10	5	5
Bell Irving River	K. Lewis & A. Dale	*D. septosporum*	1	0	1
Bulkley Canyon	K. Lewis & A. Dale	*D. septosporum*	9	5	4
Evelyn Pasture	K. Lewis & A. Dale	*D. septosporum*	1	0	1
Jonas Creek	K. Lewis & A. Dale	*D. septosporum*	2	0	2
Kinskutch Road	K. Lewis & A. Dale	*D. septosporum*	8	7	1
Kuldo Creek	K. Lewis & A. Dale	*D. septosporum*	7	2	5
Kisgegas Canyon	K. Lewis & A. Dale	*D. septosporum*	5	2	3
Squingula River Mine	K. Lewis & A. Dale	*D. septosporum*	8	1	7
Mosque River	K. Lewis & A. Dale	*D. septosporum*	6	1	5
Mitten Road	K. Lewis & A. Dale	*D. septosporum*	7	4	3
Nangeese Road	K. Lewis & A. Dale	*D. septosporum*	8	4	4
North Kuldo Road	K. Lewis & A. Dale	*D. septosporum*	4	1	3
Sanyam River	K. Lewis & A. Dale	*D. septosporum*	1	0	1
Nash Y	K. Lewis & A. Dale	*D. septosporum*	9	7	2
Orendo	K. Lewis & A. Dale	*D. septosporum*	7	6	1
Motaze Lake & Squingula River	K. Lewis & A. Dale	*D. septosporum*	8	6	2
Sunday Lake	K. Lewis & A. Dale	*D. septosporum*	4	1	3
Goldstream River, BC	D. Morrison	*D. septosporum*	1	0	1
Chile					
Valdivia	M. J. Wingfield	*D. septosporum*	10	0	10
France					
Meurthe-et-Moselle	M. Morelet	*D. septosporum*	1	0	1
Germany					
Bavarian Alps	L. Pehl	*D. septosporum*	1	0	1
Guatemala					
Sierra de Chuacús	Unknown	*D. septosporum*	1	0	1
New Zealand					
Bay of Plenty	M. A. Dick	*D. septosporum*	1	0	1
Golden Downs sites 1/2/3	P. Hirst	*D. septosporum*	4	0	4
Kaingora Forest	M. J. Wingfield	*D. septosporum*	10	0	10
Kaingora sites 1/2/3	P. Hirst	*D. septosporum*	11	0	11
Kinleith	P. Hirst	*D. septosporum*	5	0	5
Mt. Maunganui	K. Dobbie	*D. septosporum*	1	0	1
Rotorua	M. E. Buchanan	*D. septosporum*	2	0	2
Tongariro	J. W. Gilmour	*D. septosporum*	1	0	1
West Coast South Island	B. Doherty	*D. septosporum*	1	0	1
Poland					
Miechow Forest, Cracow	T. Kowalski	*D. septosporum*	11	3	8
Slovakia	E. Foffova	*D. septosporum*	1	1	0
South Africa					
Hogsback	J. Roux	*D. septosporum*	10	3	7
Tzaneen	I. Barnes	*D. septosporum*	1	1	0
Ukraine					
Tsyurupinsk	A. C. Usichenko	*D. pini*	4	4	0
United Kingdom					
West Midlands	A. Coggin	*D. septosporum*	1	0	1
South East England	A. V. Brown	*D. septosporum*	1	0	1
Forest of Dean	R. Beasley	*D. septosporum*	1	1	0
New Forest	A. V. Brown	*D. septosporum*	7	1	6
United States					
Bandon, Oregon	S. Cooley	*D. septosporum*	1	0	1
Michigan					
Crystal Township	G. Adams	*D. pini*	10	4	6
Evergreen Township	G. Adams	*D. pini*	1	1	0
River Township	G. Adams	*D. pini*	1	0	1
Central Minnesota	T. Nicholls	*D. pini*	1	1	0
Lincoln, Nebraska	G. Peterson	*D. pini*	3	2	1
Total	…	…	230	80	150

to obtain a partial region of the *MAT1-2* genes. The same PCR conditions described above were used to amplify the partial *MAT1-2* regions. Twelve internal primers were designed in the partially sequenced *MAT1-2* sequences for both species (ATCC MYA-605 for *D. septosporum* and CBS 116485 for *D. pini*) and the chromosome walking method also was used to obtain the full-length *MAT1-2* genes. The same procedure and programs described for the characterization and analyses of the *MAT1-1-1* sequences were used to characterize and analyze the *Dothistroma MAT1-2* sequences.

Development and screening of *D. pini* and *D. septosporum* mating-type-specific primers. *Dothistroma MAT1-1-1*-specific primers (Table 2) were designed from the aligned *MAT1-1-1* sequences of *D. pini* and *D. septosporum* (GenBank accession nos. DQ915449 and DQ915450, respectively). The forward primers were designed to be specific for *D. septosporum* (DseptoMat1f) or *D. pini* (DpiniMat1f2) and, therefore, are both species and mating type specific. The reverse primer (DotMat1r) was designed from homologous regions within the *MAT1-1-1* genes and, therefore, is only mating type specific.

Dothistroma MAT1-2-specific primers (Table 2) were designed from the aligned *MAT1-2* sequences of *D. pini* and *D. septosporum* (GenBank accession nos. DQ915451 and DQ915452, respectively). The two forward primers were designed in regions of the genes that were variable between the two species. DseptoMat2f was designed to be specific for *D. septosporum* and DpiniMat2f for *D. pini,* and both, therefore, are species and mating type specific. The reverse primer (DotMat2r) was designed from homologous regions within both the *MAT1-2* genes and, thus, is only mating type specific.

Multiplex PCR was used to screen for the *MAT1-1-1* or the *MAT1-2* of *D. pini* and *D. septosporum* in two separate reactions. The reaction mixtures had a total volume of 12.5 µl and contained 0.7 µl of diluted genomic DNA, 1× PCR buffer (Bioline, Randolph, MA), 48 µM each of the dNTPs, 4 pmol of each primer, 1 mM $MgCl_2$, and 0.7 units of *Taq* polymerase (Bioline, Randolph, MA). The amplification reactions were done on a GeneAmp PCR System 9600 (Applied Biosystems). The initial denaturation step was done at 94°C for 5 min, followed by 40 cycles of 94°C (20 s), 65°C (20 s), and 72°C (40 s). A final elongation step at 72°C (5 min) was included in the run. The resulting PCR products were visualized as described above.

Phylogenetic analyses. The nucleotide sequences of the α domain (*MAT1-1-1*) and HMG domain (*MAT1-2*) of *D. septosporum* and *D. pini* determined in this study and additional mating type sequences for other species representing different fungal orders downloaded from NCBI's GenBank database were used for phylogenetic analyses. These sequences were analyzed using the mating type gene sequences of *Magnaporthe grisea* (GenBank accession nos. AB080672 and AB080673, respectively) as the outgroup. All phylogenetic analyses were done using Phylogenetic Analysis Using Parsimony (PAUP) v4.0b 10 (Swofford, D. L. 2003. Sinauer Associates, Sunderland, MA). Maximum parsimony analyses were conducted as described by Groenewald et al. (24). All sequences generated were deposited in GenBank, and the alignments and trees were deposited in TreeBASE (TreeBASE accession no. SN3047).

RESULTS

Isolation and characterization of *MAT1-1-1* in *Dothistroma* spp. The degenerate primers MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 amplified a fragment of 914 bp for three of the six *Dothistroma* isolates tested (Fig. 1). The fragments obtained from strains CBS 116489, CBS 117609, and CBS 116487 were sequenced. The translated sequence of the fragment obtained from strain CBS 116489 (*D. septosporum*) showed 39 and 46% identity to a 229- and 63-amino-acid (aa) region of the *M. graminicola* MAT1 protein and 32% identity to a 213-aa region of the *S. passerinii* MAT1 protein using Blastx on the GenBank database. This confirmed that the 914-bp fragment is part of the *MAT1-1-1* gene of *D. septosporum*.

Sequences for the fragments obtained from the *D. pini* strains (CBS 117609 and CBS 116487) showed 100% identity to each other in this region. The translated sequences showed 39% identity to a 226-aa ($E = 2 \times 10^{-30}$) and 37% identity to a 78-aa region ($E = 2 \times 10^{-30}$) of the *M. graminicola* mating type 1-1 protein (GenBank accession no. AAL30838). It also showed 32% identity to a 218-aa region ($E = 5 \times 10^{-18}$) of the *S. passerinii* MAT-1 protein (GenBank accession no. AAO49357). This confirmed that the 914-bp fragment is part of the *MAT1-1-1* gene of *D. pini*.

Four chromosome walking steps were used to obtain the full-length *MAT1-1-1* gene sequences for *D septosporum* and *D. pini*. The geneid software predicted that the *MAT1-1-1* genes of both species contained four exons. The predicted length of the genes and the exon and intron positions are illustrated in Figure 2. Although the number of nucleotide and amino acid residues was the same for the *MAT1-1-1* of *D. septosporum* and *D. pini*, an identity of 94.1 and 94.3% was found between the 1,311-nucleotide and the 387-aa residues, respectively. All introns of the *MAT1-1-1* from both species contained a perfect lariat sequence (RCTRAC), except for the second intron of the *MAT1-1-1* of *D. septosporum*. When this intron is included in the coding region, an early stop codon is introduced in the reading frame, indicating that this is a true intron. The positions of the three predicted introns in the *Dothistroma* spp. studied correlate with those found for *Cercospora* spp. (25). The number of predicted introns (two) in the conserved α domain of the *Dothistroma* spp. correlated with the number predicted for the same region in *M. graminicola* (50) and *S. passerinii* (23).

Isolation and characterization of *MAT1-2* of *Dothistroma* spp. The degenerate primers MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1 amplified a fragment of 332 bp for the *Dothistroma* isolates that did not amplify the 914-bp fragment using the *MAT1-1-1* degenerate primers (Fig. 1). An extra 180-bp fragment also was obtained from the two *D. septosporum* strains and an extra 280-bp fragment from the four *D. pini* strains. The 332-bp fragment obtained from strain ATCC MYA-605 (*D. septo-*

TABLE 2. Primers used during this study[a]

Primer	5′–3′	Description
MgMfSpMat1-1f1	CATTNGCNCATCCCTTTG	*MAT1-1-1*-specific degenerate primer
MgMfSpMat1-1r2	GGCTTNGANACCATGGTGAG	*MAT1-1-1*-specific degenerate primer
MgMfSpMat1-2f2	CAAAGAANGCNTTCNTGATCT	*MAT1-2*-specific degenerate primer
MgMfSpMat1-2r1	TTCTTCTCNGATGGCTTGC	*MAT1-2*-specific degenerate primer
DseptoMat1f	CGCAGTAAGTGATGCCCTGAC	*Dothistroma septosporum MAT1-1-1*-specific primer
DpiniMat1f2	AGTAAGCGACGCGCTCCCATG	*D. pini MAT1-1-1MAT1*-specific primer
DotMat1r	TTGCCTGACCGGCTGCTGGTG	*Dothistroma MAT1-1-1*-specific primer
DseptoMat2f	GTGAGTGAACGCCGCACATGG	*D. septosporum MAT1-2*-specific primer
DpiniMat2f	GTAAGTGATCGTTGAACATGC	*D. pini MAT1-2*-specific primer
DotMat2r	CTGGTCGTGAAGTCCATCGTC	*Dothistroma MAT1-2*-specific primer

[a] Nucleotides specific to the given *Dothistroma* sp. are underlined.

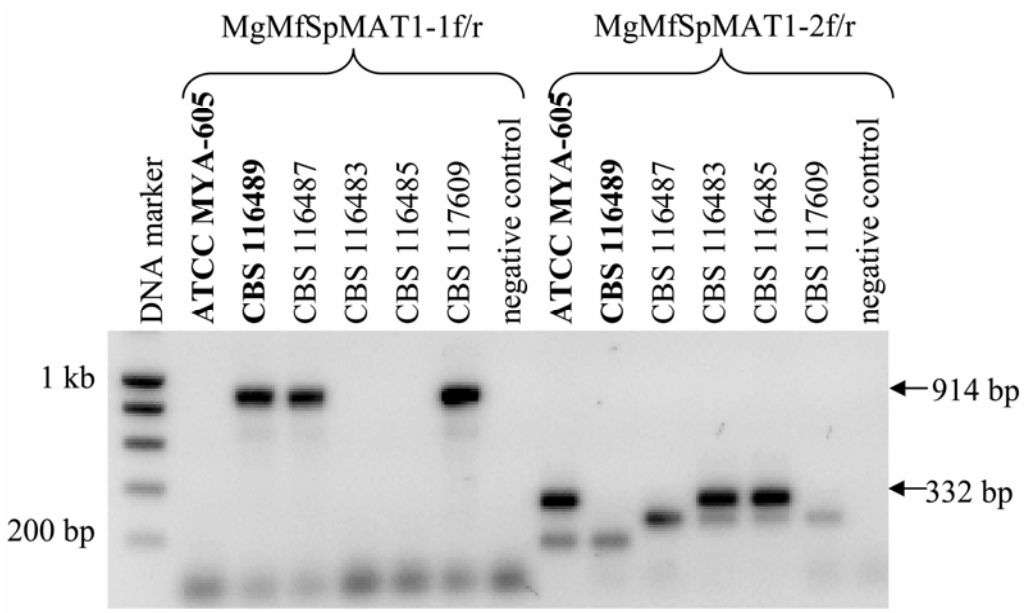

Fig. 1. Amplification products obtained from *Dothistroma septosporum* (in bold face) and *D. pini* isolates containing the partial *MAT1-1-1* (914-bp) and *MAT1-2* (332-bp) genes using the degenerate primer pairs MgMfSpMAT1-1 and MgMfSpMAT1-2, respectively.

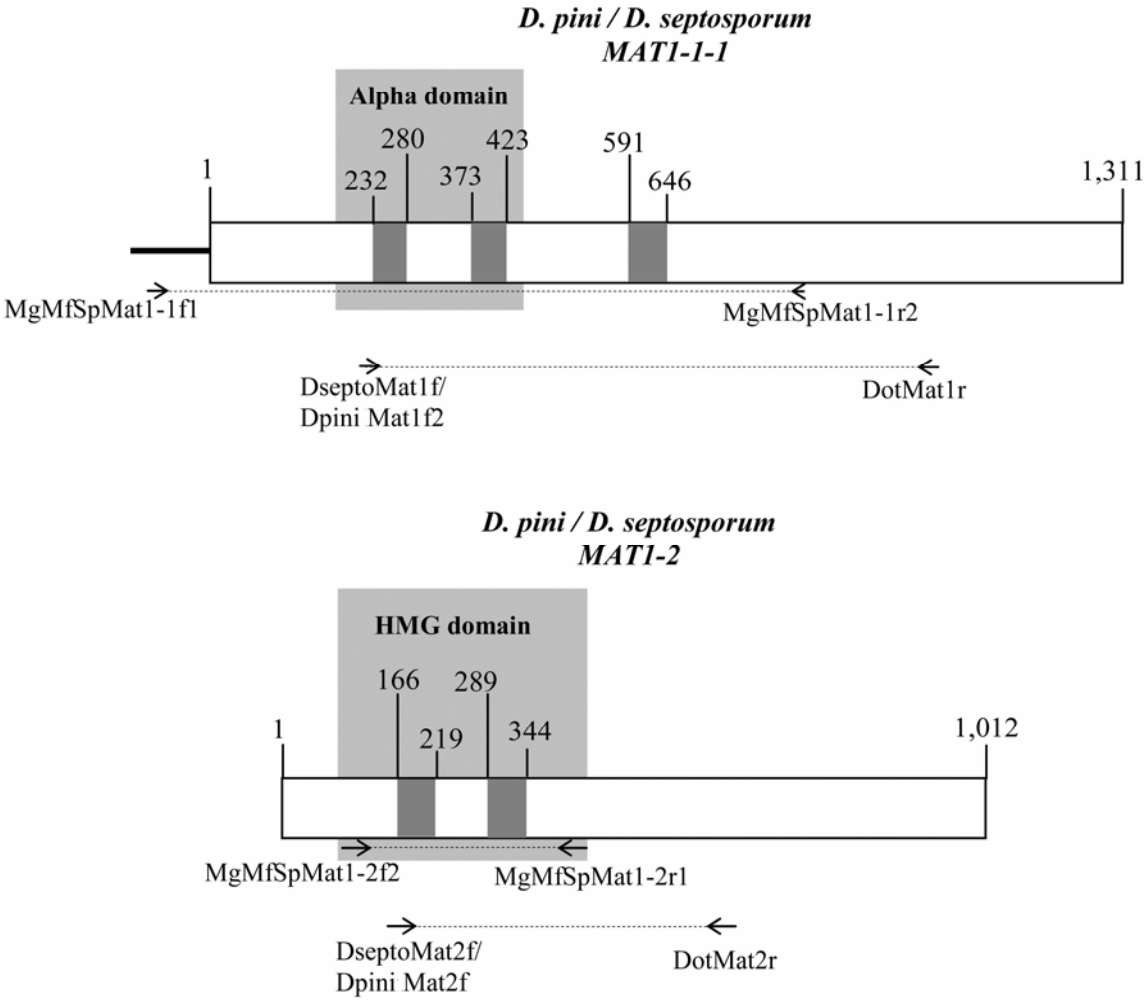

Fig. 2. Diagrammatic representation of the full-length *MAT1-1-1* and *MAT1-2* genes of *Dothistroma septosporum* and *D. pini*. The predicted sites of exons (white bars), and introns (black bars) are shown, and their locations (nucleotide position) are indicated. The areas amplified by the MgMfSpMAT1-1 and MgMfSpMAT1-2 primer sets as well as the mating-type-specific primers for each species are indicated.

sporum) was sequenced, and the translated sequence showed 55% identity to a 65-aa (E = 1×10^{-19}) and 70% identity to a 27-aa region (E = 1×10^{-19}) of the *M. graminicola* mating type 1-2 protein (GenBank accession no. AAL30836) as well as 50% identity to a 65-aa region (E = 7×10^{-17}) of the *S. passerinii* MAT-2 protein (GenBank accession no. AAO49358) using Blastx on the GenBank database. This confirmed that the 332-bp fragment is part of the *MAT1-2* gene of *D. septosporum*. The 332-bp translated sequences for the fragments obtained from the two *D. pini* strains (CBS 116483 and CBS 116485) showed 52% identity to a 65-aa (E = 1×10^{-19}) and 68% identity to a 29-aa region (E = 1×10^{-19}) of the *M. graminicola* mating type 1-2 protein (GenBank accession no. AAL30836) as well as a 47% (E = 7×10^{-17}) and 68% identity (E = 7×10^{-17}) to the same amino acid regions of the *S. passerinii* MAT-2 protein (GenBank accession no. AAO49358). This confirmed that the 332-bp fragment is part of the *MAT1-2* gene of *D. pini*. Sequences for the 180-bp (*D. septosporum*) and 280-bp (*D. pini*) fragments showed no homology to protein sequences available in GenBank.

For both of the species, four chromosome walking steps were used to obtain the full-length *MAT1-2* gene sequences. The geneid software predicted that the *MAT1-2* sequences of both species contain three exons. The predicted length of the genes, as well as exon and intron positions, is illustrated in Figure 2. Although the number of nucleotide and amino acid residues was the same for the *MAT1-2* of the two *Dothistroma* spp., an identity of 94.4 and 92.7% was found between the 1,012-nucleotide and the 302-aa residues, respectively. All the introns found for both species contained a perfect lariat sequence. The number of predicted introns (two) of the *Dothistroma* spp. studied correlates with the number predicted for *Cercospora* spp. (25), but the specific locations of these introns within the gene differed. Only one predicted intron was found in the HMG domain of species of *Cercospora* (25), *M. graminicola* (51), and *S. passerinii* (23), whereas two predicted introns were found in the same region of the *Dothistroma* spp. studied.

Screening with *D. pini* and *D. septosporum* mating-type-specific primers. In the *D. pini* MAT1 isolates, DpiniMat1f2 and DotMat1r amplified an 820-bp fragment and, in the *D. pini* MAT2 isolates, DpiniMat2f and DotMat2r amplified a 480-bp fragment (Fig. 3). Each isolate tested showed either the 820- or 480-bp fragment of the *MAT1-1-1* or *MAT1-2* genes, respectively. None of the isolates contained both fragments. The *D. pini* mating-type-specific primers did not amplify the *MAT1-1-1* and *MAT1-2* fragments in any of the *D. septosporum* isolates (Fig. 3). The majority of the *D. pini* isolates were from areas in the United States where both mating types are known to exist. Eight isolates of each mating type were found for these *D. pini* isolates, whereas only MAT1 isolates were found for the *D. pini* collection from the Ukraine (Table 1). In the *D. septosporum* MAT1 isolates, DseptoMat1f2 and DotMat1r amplified an 820-bp fragment; in the *D. septosporum* MAT2 isolates, DseptoMat2f and DotMat2r amplified a 480-bp fragment (Fig. 3). Each isolate tested showed either the 820- or 480-bp fragment of the *MAT1-1-1* or *MAT1-2* genes, respectively. None of the isolates amplified both fragments.

The *D. septosporum* mating-type-specific primers did not amplify the *MAT1-1-1* and *MAT1-2* fragments of the *D. pini* isolates (Fig. 3). In all, 20 *D. pini* and 210 *D. septosporum* isolates (Table 1) were screened with the two mating-type-specific primer sets to determine the mating type and to confirm the identity of each isolate. All *D. septosporum* isolates obtained from Chile, Australia, and New Zealand contained only the *MAT1-2*. In contrast, isolates representing both mating types were present in the Austria, Canada, Poland, South Africa, and United Kingdom collections. Only one isolate was available each from Germany, Brazil, France, Guatemala, Slovakia, and the United States. All of these isolates contained the *MAT1-2* gene, except for the isolate from Slovakia that contained *MAT1-1-1*.

Phylogenetic analyses. The alignment of partial *MAT1-1-1* nucleotide sequences (α domain) contained 21 strains, including *M. grisea* as the outgroup, and had a total length of 174 characters. Of the 174 characters, 23 were constant, 15 were variable and uninformative, and 136 were parsimony informative. The alignment of partial *MAT1-2* nucleotide sequences (HMG domain) contained 21 strains, including *M. grisea* as outgroup, and had a total length of 253 characters. Of the 249 characters, 37 were constant, 13 were variable and uninformative, and 199 were parsimony informative. Two equally parsimonious trees were obtained from each of the *MAT1-1* alignments (Fig. 4A; tree length of 638 steps; CI = 0.498, RI = 0.649, RC = 0.324) and from the *MAT1-2* alignment (Fig. 4B; tree length of 886 steps; CI = 0.512, RI = 0.659, RC = 0.338).

The topology of the phylogenetic trees using the α domain (Fig. 4A) and HMG domain (Fig. 4B) sequences were similar.

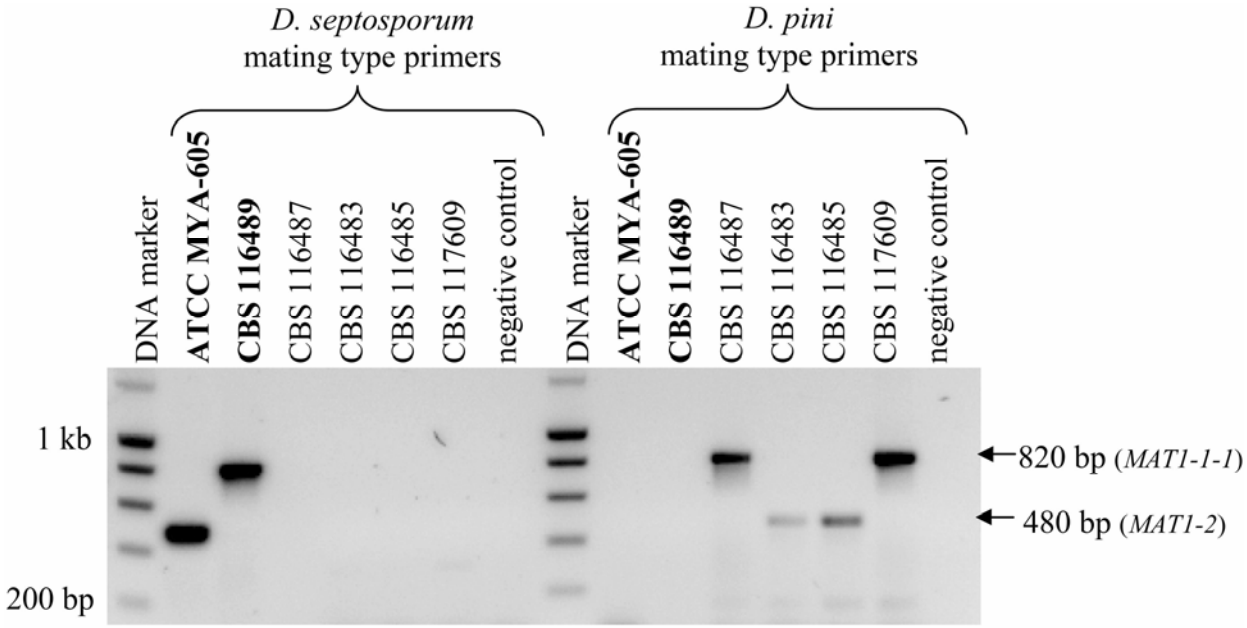

Fig. 3. *Dothistroma septosporum* (bold face) and *D. pini* isolates screened using the Dsepto/Dpini/DotMat1 primer set (820-bp fragment) and the same *Dothistroma* isolates screened with the Dsepto/Dpini/DotMat2 primer set (480-bp fragment).

The Capnodiales, Hypocreales, and Pleosporales clades showed high bootstrap support (92 to 97%) in both trees. The phylogenetic analysis using the DNA sequences in the HMG-box and α domain showed that *D. pini* and *D. septosporum*, respectively, are phylogenetically closely related to *Cercospora* spp., *M. graminicola*, and *S. passerinii* as illustrated by the 92% (*MAT1-1-1*) and 97% (*MAT1-2*) bootstrap support values.

DISCUSSION

This study represents the first attempt to ascertain which mating types are present in the different countries where diseases caused by *D. septosporum* and *D. pini* occur. In this regard, emphasis is on *D. septosporum,* because it has been introduced into numerous countries, where it has caused very damaging disease problems. Thus, the degenerate primer sets MgMfSpMAT1-1 and MgMfSpMAT1-2 (25) were used successfully to amplify portions of the mating type genes of *D. septosporum* and *D. pini*. This made it possible to characterize the full-length *MAT1-1-1* or *MAT1-2* genes of both species.

The *MAT1-1-1* and *MAT1-2* genes characterized for *D. septosporum* and *D. pini* in this study contained areas that correspond to a putative α domain and an HMG domain also described for the *MAT1-1-1* and *MAT1-2* of other ascomycetes. The two putative introns in the α domains of the *Dothistroma MAT1-1-1* also have been found in corresponding areas in *M. graminicola* (50), *S. passerinii* (23), and several *Cercospora* spp. (25). However, the third predicted intron in the downstream area flanking the α domain of the *MAT1-1-1* of both *Dothistroma* spp. is present only in the *Cercospora* sp., and not in *M. graminicola* or *S. passerinii.* The number of introns found in the HMG domain of the *MAT1-2* in both *Dothistroma* spp. differed from that of closely related species. The first predicted intron also is present in *M. graminicola* (50), *S. passerinii* (23), and *Cercospora* spp. (25). In contrast, the second predicted intron is present only in the *MAT1-2* of the *Dothistroma* spp., and not in any other members of the Mycosphaerellaceae thus far studied. These data indicate that clear differences can be found even within the conserved regions of the corresponding genes in different *Mycosphaerella* spp.

The predicted length of the encoded proteins among different *MAT1-1-1* and *MAT1-2* genes of ascomycetes varies greatly (23,25,40). In most species, the MAT1 protein is much larger than the MAT2. Results of this study have shown that this also is the case for the *Dothistroma* spp., where 387 aa were found for MAT1 and 302 aa for MAT2. Expression studies have not been done on the mating type genes of any of the above-mentioned members of the Mycosphaerellaceae. Additional studies at the mRNA and protein levels would be necessary to confirm the exact length of the coding regions and the intron and exon boundaries for the mating type genes of the *Dothistroma* spp.

Results of this study showed substantial differences between the nucleotide as well as amino acid sequences of the corresponding mating type genes and proteins of *D. septosporum* and *D. pini*. Using nucleotide sequences for phylogenetic inference in these fungi is consistent with previous studies where conserved domains within the mating type genes have been used to study the phylogenetic relationships among different fungal species and families (12,25,34,35,52). Differences in mating type sequences

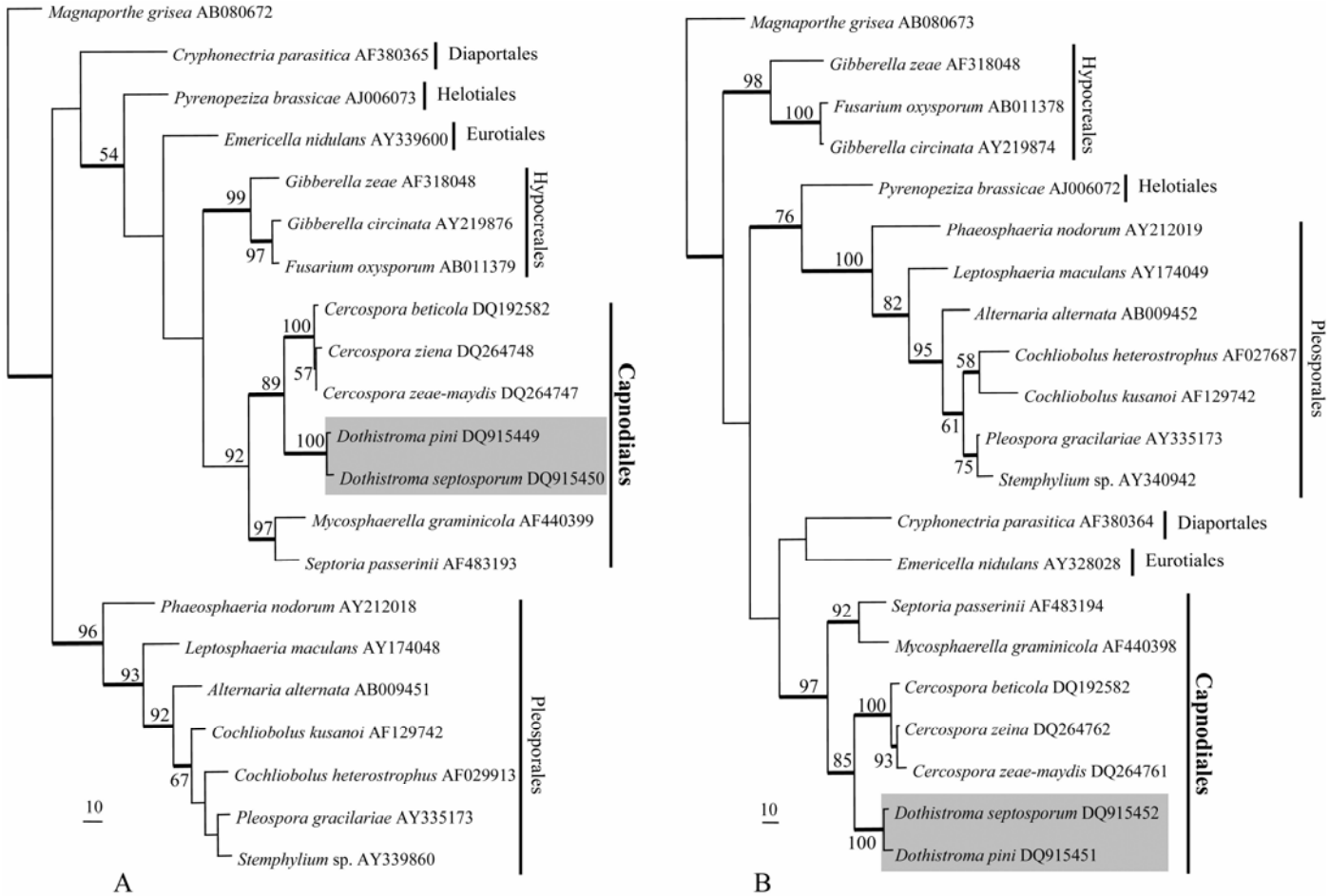

Fig. 4. One of two equally parsimonious trees obtained from each of the **A,** *MAT1-1-1* sequence alignment rooted to *Magnaporthe grisea* (AB080672) and **B,** *MAT1-2* sequence alignment rooted to *M. grisea* (AB080673). In both trees, bootstrap support values from 1,000 replicates are shown at the nodes, whereas thickened lines indicate strict consensus branches.

for *D. septosporum* and *D. pini* show that these species are distinct genetic entities and provides strong support for the results of Barnes et al. (2), who provided the first DNA-based evidence that the species are distinct.

Based on morphological characteristics, Barr (3) attempted to reclassify *Mycosphaerella pini* in a new genus outside of *Mycosphaerella*. However, molecular phylogenetic analyses have shown that *Mycosphaerella* is the most appropriate designation for this fungus classification (2,22). Phylogenetic analyses, based on the sequences of the HMG and α domains, also confirm that *Dothistroma* spp. are members of the Mycosphaerellaceae. All remaining species also grouped within their corresponding families; however, the relationship between different families is unresolved.

The mating-type-specific primer sets developed in this study, DpiniMat1 and DpiniMat2 as well as DseptoMat1 and DseptoMat2, can be used effectively in multiplex PCR assays to amplify areas within the mating type genes for *D. pini* and *D. septosporum* populations, respectively. These primers also can be used to distinguish between the two *Dothistroma* spp., making them useful tools for rapid and accurate diagnoses of two important pathogens that are morphologically similar. Prior to this study, the only diagnostic tool available to distinguish between *D. pini* and *D. septosporum,* was to amplify the ITS of the ribosomal DNA region with universal primers and then to digest the amplicon with the restriction endonuclease *Alu*I (2). Although the latter technique is useful, the ITS amplicon of *D. pini* is digested into two fragments whereas that of *D. septosporum* is not. Therefore, to prevent a false positive result for *D. septosporum*, a prior confirmation that the fungus is a *Dothistroma* sp. is required. The mating-type-specific primer sets emerging from this study are species specific and do not require a prior view on the identity of unknown isolates. They are, therefore, multifunctional and can be used for the rapid identification of the species as well as its mating type.

Although results of this study have shown that *D. pini* is probably heterothallic with a single isolate containing only one of the two mating type genes, no teleomorph has yet been linked to this species. Where both mating types were observed for the isolates from the United States, the sexual state most likely is present, but has not been observed. In contrast, the *M. pini* teleomorph of *D. septosporum* previously has been observed in some parts of the United States (9,38,39) where *D. pini* is predominantly found. Given that the anamorphs of these fungi are morphologically similar and have been confused in the past, it is possible that teleomorph structures reported for *D. septosporum* could have been linked to *D. pini* and not to *D. septosporum*.

Although a small number of isolates were screened for most countries, this study shows that *D. septosporum* probably are heterothallic and that one mating type (MAT2) seems to be more prevalent in several of the collections studied (e.g., New Zealand). Although sexual reproduction has been confirmed in *D. septosporum,* asexual reproduction happens more frequently, and the absence or rarity of the opposite mating type (MAT1) in most of the collections can explain the common occurrence of the asexual stage. Therefore, it also is possible that the teleomorph is not as rare as first believed. We found that both mating types exist within *D. septosporum* populations from Europe (Poland and Austria) and Canada, where the sexual stage (*M. pini*) has been reported in the past (7,15,17,28,29). However, the teleomorph has never been found in countries in the Southern Hemisphere such as Chile, Australia, and New Zealand, where these pathogens have long been a major problem (14,31). These are also the countries for which only one mating type (MAT2) has been observed, and this might explain the absence or rarity of the sexual stage.

Discovery in this study of only a single mating type of *D. septosporum* in New Zealand, Australian, and Chilean collections can be explained by the fact that the fungus is an introduced pathogen in those countries. For New Zealand, Hirst et al. (26) also found that no genetic variation exist among isolates of a *D. septosporum* population, which is strongly supported by the results of the present study. Dothistroma needle blight was introduced in Australia in the 1970s and it was suggested that this occurred by natural means, with conidia being blown across the Tasman Sea from New Zealand. This view was supported by the fact that the strict quarantine regulations in Australia would have made it unlikely that infected plant material entered the country (13,31,33). The presence of only one mating type shown in this study and the fact that no genetic diversity has been found yet for the pathogen in New Zealand (26) supports the view that only one genotype was introduced into or became established in Australia and New Zealand. Asexual reproduction evidently has perpetuated the spread of the fungus subsequently. We suspect that the same situation will have been true for Chile.

An intriguing result of this study has been the discovery that both mating types of *D. septosporum* exist in the South African and United Kingdom collections. This is especially interesting because the pathogen is non-native in these countries and it might have been expected that the situation would have been similar to that in other countries such as New Zealand, where the pathogen also is an alien invasive. In addition, the teleomorph of *D. septosporum* has never been observed in South Africa (M. J. Wingfield, *unpublished data*) and the United Kingdom (A. V. Brown, *unpublished data*), despite concerted efforts to detect it.

It is important to recognize that the presence of both mating types of *D. septosporum* in these two countries could indicate the presence of clandestine sex in the fungus. This would indicate the potential for the pathogen to evolve more effectively in these countries than would be true elsewhere in the world, where only a single mating type exists. Such change in the fungus could complicate efforts to develop trees resistant to Dothistroma needle blight infection in South Africa and the United Kingdom. In this regard, it has been shown previously that the introduction of the second mating type of a pathogen can cause rapid increase in virulence, gene transfer, and genetic variation, such as in *Phytophthora infestans* (16,30,42,45) and *Ophiostoma novo-ulmi* (36). This implies that the accidental introduction of the opposite mating type of *D. septosporum* into countries such as New Zealand, Australia, and Chile could seriously exacerbate red band needle disease in those countries. Thus, every effort must be made to ensure that new mating types of *D. septosporum* do not enter these countries.

There has been a dramatic increase in the impact of Dothistroma needle blight caused by *D. septosporum* in western Canada, the United States, and the United Kingdom in recent years (5,6,53). Possible reasons for this change in the disease situation in these countries are an abundance of host material or a directional climate change, as suggested by Woods et al. (54). The discovery that both mating types exist in these countries is another factor that can contribute to the change in the disease situation. The presence of both mating types increases the possibility for sexual reproduction. This, in turn, can lead to the exchange of genetic material between different strains, resulting in a possible increase in the viability of this species. Therefore, further investigation is necessary to determine whether the presence of both mating types, which could increase genetic diversity, a dramatic climate change, or possibly a combination of both these factors might account for the drastic increase in the severity of this disease.

Because only one mating type of *D. septosporum* appears to be present in most countries of the Southern Hemisphere, it is important to restrict the MAT1 isolates to their present locations. This can be achieved through refining quarantine regulations based on the knowledge that only one mating type of the pathogen is present in the country. The mating-type-specific PCR developed during this study could be implemented easily as a control method to

test for the presence of the mating types for *Dothistroma* spp. in pine plantations. One of the weaknesses of quarantine regulations internationally is that they typically rely on lists of names of pathogens rather than on knowledge of their biology and population genetics. Results of this study have provided valuable new insights into the distribution of mating types of *D. septosporum* and *D. pini* that should enhance the quality of quarantine regulations in the future.

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