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Mycosphaerella species causing leaf disease in South African *Eucalyptus* plantations

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Commercial *Eucalyptus* plantations provide an important source of hardwood for forestry industries, worldwide. Several species of *Mycosphaerella* are associated with a destructive *Eucalyptus* leaf disease known as Mycosphaerella Leaf Blotch (MLB). During 2000, a survey was undertaken in several commercial *Eucalyptus* growing areas of South Africa to determine the identity of the *Mycosphaerella* spp. contributing to outbreaks of MLB. Symptomatic leaf samples were collected from three major *Eucalyptus* growing areas and the *Mycosphaerella* spp. were isolated. Isolates were identified using morphology, ascospore germination patterns and sequence data from the ribosomal DNA operon. Six species, namely *M. ellipsoidea*, *M. irregulariramosa*, *M. juvenis*, *M. lateralis*, *M. marksii*, *M. nubilosa*, as well as a new species, described here as *M. fori* sp. nov., were recognized. *Mycosphaerella nubilosa* was the most common species isolated from commercial plantations, particularly on *E. nitens*, and appears to be the dominant species contributing to MLB. Data obtained in this study show that MLB is caused by a complex of species contributing to disease outbreaks in South Africa.

INTRODUCTION

Eucalyptus is a large genus in the *Myrtaceae*, including approximately 700 species (Potts & Pederick 2000). Most of these species are native to Australia, Papua New Guinea, Indonesia and the Philippines where they occur in natural forests (Turnbull 2000). *Eucalyptus* spp. are grown commercially in intensively managed plantations in both the Northern and Southern Hemispheres. In South Africa, *Eucalyptus* spp. make up 47% of the ~1.5 M ha of commercial plantations (Edwards 2000). These trees are now amongst a small number of favoured forestry species throughout the world. As such, they rival *Pinus* spp. in their use as a commercial forestry resource. *Eucalyptus* spp. are, however, susceptible to infection by many pathogens that threaten plantations (Wingfield *et al.* 1995, Wingfield 1999, Keane *et al.* 2000). Of these, species of *Mycosphaerella* represent some of the most important *Eucalyptus* leaf pathogens (Park *et al.* 2000).

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The genus *Mycosphaerella* is large and includes approximately 2000 named species (Corlett 1991, 1995) although the status of many of these have not been confirmed by modern phylogenetic approaches. Species of *Mycosphaerella* include both saprophytes and parasites that infect woody and herbaceous hosts and generally cause leaf diseases (von Arx 1983). Several species of *Mycosphaerella* are associated with Mycosphaerella Leaf Blotch (MLB) of *Eucalyptus* spp. in many countries where these trees are grown as a commercial hardwood resource (Crous 1998, Carnegie 2000).

Symptoms associated with MLB are variable and can differ depending on the host and *Mycosphaerella* sp. involved. The primary symptoms are leaf spots that reduce the photosynthetic capacity of leaves and result in defoliation (Beresford 1978, Ganapathi 1979). In cases of severe infection, the disease can spread to young shoots and branches, where cankers are formed, resulting in gum exudation and eventual twig die-back (Dick 1982, Dick & Gadgil 1983). This symptom leads to the formation of multi-stemmed trees, which are

unsuitable for milling (Beresford 1978). Trees often become physiologically stressed and stunted, resulting in reduced growth, infection by secondary pathogens, and increased silvicultural costs.

The taxonomy and identification of *Mycosphaerella* species is complicated and beset with problems. Traditionally, host affiliations have been the primary factor considered for identification. This approach has, however, been recognized as unreliable (Corlett 1991, Aptroot & Lucking 2001). Recently, cultural characteristics and anamorph–teleomorph connections have received attention in the classification of *Mycosphaerella* spp. Presently 23 anamorph genera, including coelomycetes and hyphomycetes, are accepted as *Mycosphaerella* anamorphs (von Arx 1983, Crous 1998, Crous *et al*. 2000, Crous & Braun 2003). Ascospore germination patterns have also provided useful taxonomic characters (Park & Keane 1982a, Crous 1998). More recently, DNA sequence data have contributed substantially to the identification of *Mycosphaerella* spp. occurring on *Eucalyptus* spp. and other hosts (Crous *et al*. 1999, 2001). From these data, evidence is also starting to emerge to support the recognition of certain *Mycosphaerella* clades as distinct holomorphs, such as *Davidiella*, which has *Cladosporium s. str*. anamorphs (Braun *et al*. 2003).

Several *Mycosphaerella* spp. are readily isolated from diseased *Eucalyptus* leaves in South Africa (Crous 1998). During initial studies of MLB in this country, the disease was attributed to *M. molleriana* (Doidge 1950). Subsequent studies, particularly on *E. nitens*, suggested that *M. nubilosa* was the only *Mycosphaerella* spp. causing MLB in South Africa (Purnell & Lundquist 1986, Crous *et al*. 1989). More recently, several new species of *Mycosphaerella* were identified on *Eucalyptus* spp., including *M. africana*, *M. crystallina*, *M. ellipsoidea* and *M. juvenis* (Crous & Wingfield 1996, Crous 1998). The impact, relative occurrence and importance of these *Mycosphaerella* spp. on commercial forestry in South Africa, however, remains unclear.

The aim of this study was to consider the occurrence of *Mycosphaerella* spp. on *Eucalyptus*, specifically in commercial plantations experiencing outbreaks of MLB. To achieve this goal, surveys were conducted and samples collected from plantations in three major *Eucalyptus* growing areas of South Africa. *Mycosphaerella* spp. were recovered from diseased leaves and identified based on morphology, ascospore germination patterns, symptomatology, cultural characteristics and sequence data from the ITS region of the rDNA operon.

MATERIALS AND METHODS

Sample collection

Samples of diseased *Eucalyptus* leaves were collected from various plantations in three major *Eucalyptus* growing areas of South Africa, namely the KwaZulu-Natal Midlands, Tzaneen in the Northern Province, and Umtata in the Eastern Cape Province. From KwaZulu-Natal, samples were collected from several different *Eucalyptus* spp. including *E. nitens*, *E. bicostata*, *E. macarthurii*, *E. smithii*, *E. dunnii*, *E. grandis* and one clone of *E. grandis* × *E. nitens*. Samples from the Northern Province were collected from *E. grandis* and *E. grandis* × *E. camaldulensis*. All samples from the Eastern Cape Province were collected from naturally regenerated *E. grandis* trees.

Isolation procedures and isolates examined

Leaves displaying a wide array of different lesion forms were chosen for isolations. Excised lesions were soaked in water for approximately two hours. Thereafter, lesions were placed on double-sided adhesive tape, and adhered to the insides of Petri dish lids over 2% malt extract agar (MEA) (w/v) (Biolab, Johannesburg), with the pseudothecia facing the agar surface (Crous 1998). Petri dishes were incubated in darkness for 24 h to allow for ascospore release and germination on MEA. Following incubation, individual germinating ascospores were subcultured onto 2% MEA and incubated at 25 °C in the dark.

Once colonies had formed, they were incubated at 25 ° under continuous cool white light in an incubator. Germinating ascospores were also sub cultured onto Carnation Leaf Agar (CLA, 1% water agar (w/v) (Biolab) with sterilized carnation leaves placed onto medium, and incubated at 25 ° under continuous near-UV-light (*nuv*, 250 nm) to promote sporulation. All cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI, Pretoria), and duplicate cultures have been deposited with the Centraalbureau voor Schimmelcultures (CBS, Utrecht) (Table 1).

Germinating ascospores were transferred to microscope slides and mounted in lactophenol, without cotton blue, for examination. Ascospores were evaluated based on characteristics known to be taxonomically relevant for *Mycosphaerella* spp. (Park & Keane 1982a, Crous 1998). From these criteria, the ascospore germination patterns were grouped as outlined by Crous (1998). Characteristics were compared with those of other *Mycosphaerella* spp. described from *Eucalyptus* spp. (Crous 1998). Following isolation, cultures of *Mycosphaerella* spp. were grouped according to culture colour (Rayner 1970) and morphology. Two to four representative isolates, grouped according to morphology, cultural characteristics and ascospore germination patterns, were chosen for DNA sequencing (Table 1). The sequences of these isolates were compared with those of other *Mycosphaerella* spp. known to cause MLB on *Eucalyptus* spp. (Crous *et al*. 2001) (Table 1).

DNA isolation

Following growth of axenic cultures, mycelium was scraped directly from agar plates and used for DNA

Table 1. Isolates included in the sequence analysis of *Mycosphaerella* species.

Isolate[a]	Teleomorph (*Mycosphaerella*)	Anamorph	GenBank accession no.	Origin
CMW 4945^T	*M. africana*	Unknown	AF309602	*Eucalyptus*, RSA
CMW 4944^T	*M. colombiensis*	*Pseudocercospora colombiensis*	AF309612	*Eucalyptus*, Colombia
CMW 2732	*M. cryptica*	*Colletogloeopsis nubilosum*	AF309622	*Eucalyptus*, Chile
CMW 3279^T	*M. cryptica*	*C. nubilosum*	AF309623	*Eucalyptus*, Australia
CMW 3042^T	*M. crystallina*	*Pseudocercospora crystallina*	AF309611	*Eucalyptus*, RSA
CMW 4934, 5166	*M. ellipsoidea*	*Uwebraunia ellipsoidea*	AF309592, AF309593	*Eucalyptus*, RSA
CMW 9098*	*M. ellipsoidea*	*U. ellipsoidea*	AF468874	*Eucalyptus*, KwaZulu-Natal, RSA
CMW 9099*	*M. ellipsoidea*	*U. ellipsoidea*	AF468875	*Eucalyptus*, KwaZulu-Natal, RSA
CMW 9100*	*M. ellipsoidea*	*U. ellipsoidea*	AF468876	*Eucalyptus*, KwaZulu-Natal, RSA
CMW 5224^T	*M. flexuosa*	Unknown	AF309603	*Eucalyptus*, Colombia
CMW 9094*	*M. fori*	*Pseudocercospora* sp.	AF468868	*Eucalyptus*, Tzaneen, RSA
CMW 9095*	*M. fori*	*Pseudocercospora* sp.	AF468869	*Eucalyptus*, Tzaneen, RSA
CMW 4942^T	*M. heimii*	*Pseudocercospora heimii*	AF309606	*Eucalyptus*, Madagascar
CMW 3046^T	*M. heimioides*	*P. heimioides*	AF309609	*Eucalyptus*, Indonesia
CMW 4943^T, 5149^T	*M. irregulariramosa*	*P. irregulariramosa*	AF309607, AF309608	*Eucalyptus*, RSA
CMW 9097*	*M. irregulariramosa*	*P. irregulariramosa*	AF468877	*Eucalyptus*, Tzaneen, RSA
CMW 5825*	*M. irregulariramosa*	*P. irregulariramosa*	AF468878	*Eucalyptus*, Tzaneen, RSA
CMW 4937, 4936	*M. juvenis*	*U. juvenis*	AF309604, AF309605	*Eucalyptus*, RSA
CMW 9101*	*M. juvenis*	*U. juvenis*	AF468879	*Eucalyptus*, Umtata, RSA
CMW 9102*	*M. juvenis*	*U. juvenis*	AF468880	*Eucalyptus*, KwaZulu-Natal, RSA
CMW 9103*	*M. juvenis*	*U. juvenis*	AF468881	*Eucalyptus*, KwaZulu-Natal, RSA
STE-U 825^T	*M. lateralis*	*Dissoconium dekkeri*	AF309624	*Eucalyptus*, RSA
CMW 4935	*M. lateralis*	*D. dekkeri*	AF309625	*Eucalyptus*, Zambia
CMW 9106*	*M. lateralis*	*D. dekkeri*	AF468882	*Eucalyptus*, Tzaneen, RSA
CMW 9107*	*M. lateralis*	*D. dekkeri*	AF468883	*Eucalyptus*, KwaZulu-Natal, RSA
STE-U 348	*M. marasasii*	*Stenella marasasii*	AF309591	*Syzygium*, South Africa
CMW 5150, 3278	*M. marksii*	Unknown	AF309588, AF309598	*Eucalyptus*, Australia
CMW 9090*	*M. marksii*	Unknown	AF468870	*Eucalyptus*, KwaZulu-Natal, RSA
CMW 9091*	*M. marksii*	Unknown	AF 468871	*Eucalyptus*, KwaZulu-Natal, RSA
CMW 9092*	*M. marksii*	Unknown	AF468872	*Eucalyptus*, Tzaneen, RSA
CMW 9093*	*M. marksii*	Unknown	AF468873	*Eucalyptus*, Tzaneen, RSA
CMW 2734	*M. molleriana*	*Colletogloeopsis molleriana*	AF309619	*Eucalyptus*, USA
CMW 4940	*M. molleriana*	*C. molleriana*	AF309620	*Eucalyptus*, Portugal
CMW 3282	*M. nubilosa*	Unknown	AF309618	*Eucalyptus*, Australia
CMW 9104*	*M. nubilosa*	Unknown	AF449096	*Eucalyptus*, KwaZulu-Natal, RSA
CMW 9105*	*M. nubilosa*	Unknown	AF449097	*Eucalyptus*, Umtata, RSA
CMW 3358^T	*M. parkii*	*Stenella parkii*	AF309590	*Eucalyptus*, Brazil
CMW5348^T	*M. suttoniae*	*Phaeophleospora eppicocoides*	AF309621	*Eucalyptus*, Indonesia
CMW 5348	*M. syzygii*	*Cercostigmina punctata*	AF309610	*Syzygium*, RSA
CMW 5005^T	*M. tasmaniensis*	*Mycovellosiella tasmaniensis*	AF309617	*Eucalyptus*, Australia
STE-U 2768, 2769	*M. walkeri*	*Sonderhenia eucalypticola*	AF309615, AF309616	*Eucalyptus*, Uruguay
CMW 5129	*Mycosphaerella* state unknown	*Phaeophleospora destructans*	AF309614	*Eucalyptus*, Indonesia
CMW 5351	*Mycosphaerella* state unknown	*P. eugeniae*	AF309613	*Eugenia*, Brazil
CMW 5227^T	*Mycosphaerella* state unknown	*Pseudocercospora basiramifera*	AF309595	*Eucalyptus*, Thailand
CMW 5228^T, 5229^T	*Mycosphaerella* state unknown	*P. eucalyptorum*	AF309598, AF309599	*Eucalyptus*, RSA
CMW 4948	*Mycosphaerella* state unknown	*P. natalensis*	AF309594	*Eucalyptus*, RSA
CMW 5146	*Mycosphaerella* state unknown	*P. paraguayensis*	AF309596	*Eucalyptus*, Brazil
CMW 5151^T	*Mycosphaerella* state unknown	*P. robusta*	AF309597	*Eucalyptus*, Malaysia
CMW 5349	*Mycosphaerella* state unknown	*P. syzgiicola*	AF309600	*Syzygium*, RSA

[a] STE-U, Culture collection of the Department of Plant Pathology, University of Stellenbosch; CMW, Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

* Isolates sequenced during this study.

^{T} Ex-type cultures.

isolation. Harvested mycelium was dried under vacuum and ground to a fine powder using liquid nitrogen. DNA was isolated using the method of Raeder and Broda (1985) with minor variation. The 1:1 phenol: chloroform purification step was repeated until the interphase between the two aqueous phases was clean of any cellular debris. Nucleic acids were precipitated by the addition of 10% 3 M NaAc and 2 volumes of absolute ethanol and incubated at −20 ° for 2 h. DNA was further purified by washing with 70% ethanol and dried under vacuum, after which the resulting DNA pellet was resuspended in 50 µl SABAX water. RNaseA (10.0 µg µl^{-1}) was added to the DNA samples, and incubated at 37 ° for 3–4 h to digest any residual protein or RNA. DNA was visualized on a 1% agarose gel (w/v) (Roche Diagnostics, Mannheim) stained with ethidium bromide and viewed under an UV-light. DNA was quantified for all samples with an Eppendorf BioPhotometer (Eppendorf Scientific, Hamburg).

PCR amplification and purification

Isolated DNA (50–90 ng) was used as a template for PCR. The ITS region of the rDNA operon was targeted for amplification using primers ITS 1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and LR1 (5′-GGT TGG TTT CTT TTC CT-3′) (Vilgalys & Hester 1990, White *et al*. 1990). The ITS 1 and ITS 2 regions including the 5.8S gene were amplified. DNA was amplified in a 50 µl reaction volume containing PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) (Roche Diagnostics, Johannesburg), 2.5 mM of each dNTP (dATP, dTTP, dCTP and dGTP) (Roche Diagnostics), 0.2 µM of primers ITS1 and LR1 (MWG Biotech, Ebersberg) and 2.5 U Taq DNA polymerase (Roche Diagnostics). SABAX water was used to achieve the total volume of 50 µl.

PCR reactions were carried out using an Eppendorf Mastercycler gradient PCR machine (Eppendorf Scientific). PCR reaction conditions included an initial denaturation temperature of 96 ° for 2 min. Following initial denaturation, 40 cycles of template denaturation for 30 s at 94 °, primer annealing for 30 s at 53 ° and chain elongation for 2 min at 75 ° were carried out with a final elongation at 75 ° for 7 min. A negative control using water and no template DNA and a positive control containing DNA of a *Mycosphaerella* sp., was used for each reaction. PCR products were visualized in 2% agarose gels stained with ethidium bromide and viewed under UV-light. Sizes of PCR products were determined against a 100 bp molecular weight marker XIV (Roche Diagnostics). PCR products were purified using the High Pure PCR product purification kit (Roche Diagnostics). After PCR purification, concentrations of purified PCR products were determined by running products on a 2% agarose gel stained with ethidium bromide, together with a 100 bp molecular weight marker XIV and viewed under UV-light.

DNA sequencing and data analysis

Purified PCR products were used as template DNA for sequencing reactions on an ABI PRISM™ 377 Automated DNA sequencer (Perkin Elmer, Norwalk, CON). The ABI Prism Big Dye Terminator Cycle sequencing reaction kit (Perkin Elmer Applied Biosystems, Foster City, CA) was used for the sequencing reactions. Sequencing reactions were conducted with the same primers used for the PCR reaction. However, two internal primers ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′) and ITS2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) (White *et al*. 1990) were used to completely sequence both DNA strands of the ITS region.

Sequences were analysed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied Biosystems). Sequence alignments were done using the Clustal function of Sequence Navigator and gaps were inserted manually where necessary. Phylogenetic analysis of aligned sequences was conducted using PAUP version 4.0b10 (Swofford 2002). The Heuristic search function was used to generate the most parsimonious trees. Starting trees for the analyses were obtained by stepwise addition, with the MULPAR function effective. Tree Bisection Reconnection (TBR) was used as the swapping algorithm with maximum parsimony as an optimal criterion. All characters in the analysis were of equal weight. Gaps were coded as fifth character. Branch support was evaluated by performing a Bootstrap search of 1000 replicates on the aligned sequences. Published sequences for *Mycosphaerella* spp. from *Eucalyptus* spp. were obtained from GenBank and compared with those obtained for the *Mycosphaerella* spp. isolated in this study. Following the analysis all resulting trees were rooted to an outgroup. The taxon chosen for this purpose was *Ramulispora anguoides* (Nirenberg) Crous, which resides in the genus *Tapesia* and has been shown to be an appropriate outgroup for *Mycosphaerella* spp. (Crous *et al*. 2001).

RESULTS

Isolation procedures and isolates examined

Mycosphaerella ascospores were obtained from most, but not all lesions examined. Isolates of *Mycosphaerella* were, however, recovered from a wide variety of *Eucalyptus* spp. and all of the three areas sampled. These isolates could be separated into seven groups based on lesion type, cultural morphology and ascospore germination patterns. Based on symptoms, cultural characteristics and germination patterns, five species of *Mycosphaerella* were identified from the KwaZulu-Natal Midlands (Kwa-Zulu Natal Province). These included *M. ellipsoidea*, *M. juvenis*, *M. lateralis*, *M. marksii* and *M. nubilosa*. Four species were identified from Tzaneen (Northern Province), namely *M. irregulariramosa*, *M. lateralis* and *M. marksii* and an apparently undescribed *Mycosphaerella* species.

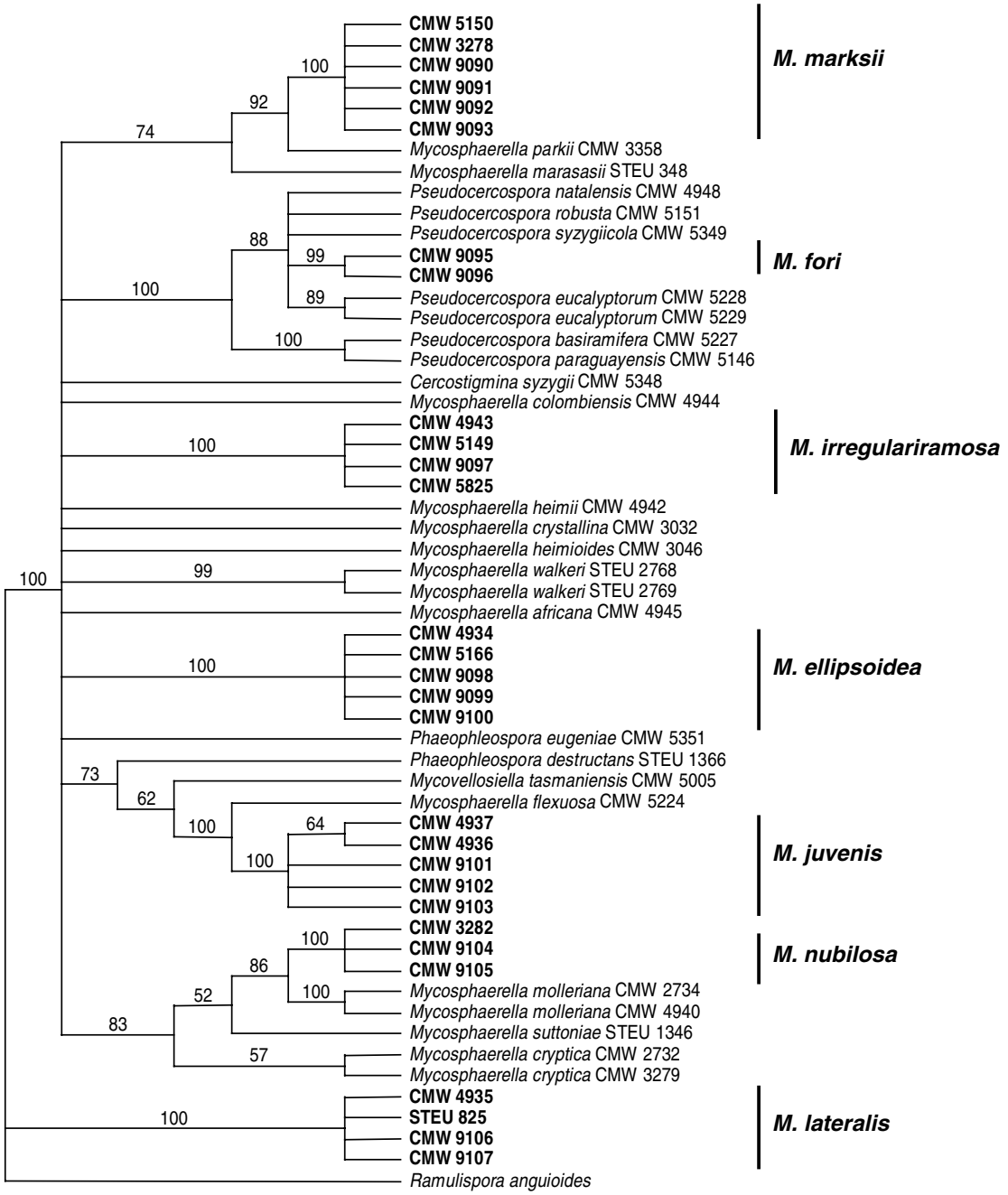

Fig. 1. Cladogram of *Mycosphaerella* species on *Myrtaceae* and *Mycosphaerella* species isolated from various *Eucalyptus* spp. One of four most parsimonious trees (length = 1562, CI = 0.5038, RI = 0.7879, HI = 0.4962) inferred from heuristic searches using PAUP version 4.0b1. Bootstrap support values of 1000 replicates is listed above branches.

Two species, *M. nubilosa* and *M. juvenis*, emerged from samples originating in Umtata (Eastern Cape Province).

PCR amplification and sequence data analysis

Amplification of the ITS region of the rDNA operon resulted in amplification products of approximately 600 bp for all isolates. Sequence and parsimony analysis of representative isolates combined with published sequences of *Mycosphaerella* spp. on *Eucalyptus* spp. produced four most parsimonious trees with a length of 1562 steps (CI = 0.5038, RI = 0.7879, HI = 0.4962) using the heuristic search option. Of the 657 characters that were analyzed; 257 of these were constant, 106 were parsimony-uninformative and 295 were parsimony-informative. A bootstrap search of 1000 replicates produced a tree of the same topology as the most parsimonious tree (Fig. 1). The total data set could be resolved into two major clades. The first clade comprised a larger monophyletic *Mycosphaerella* clade and the second clade was represented by species producing a *Dissoconium* anamorph. Within the larger *Mycosphaerella* clade, several anamorph genera

associated with those *Mycosphaerella* spp. occurring on *Eucalyptus* were represented. These included *Stenella*, *Pseudocercospora*, *Cercostigmina*, *Sonderhenia*, *Uwebraunia*, *Phaeophleospora*, *Mycovellosiella* and *Colletogloeopsis*.

Based on isolations made from South African *Eucalyptus* leaves, six previously recognized species and one new species of *Mycosphaerella* could be identified from the sequence data. These taxa correlated with those identified based on morphology. Thus, sequence data clearly separated *M. marksii*, *M. irregulariramosa*, *M. ellipsoidea*, *M. juvenis*, *M. nubilosa*, *M. lateralis* and *M. fori*. Four of the species isolated during this survey, *M. marksii*, *M. irregulariramosa*, *M. ellipsoidea* and *M. lateralis* were supported with a 100% bootstrap support, and showed no base differences when compared with their respective reference isolates. *Mycosphaerella nubilosa* isolates also had strong bootstrap support and had four base differences when compared with the reference isolate of *M. nubilosa* (CMW 3282) originally collected from Australia. Isolates of *M. juvenis* grouped within the *Uwebraunia* sub-clade, together with two reference isolates of *M. juvenis* (CMW 4936 and CMW 4937) from South Africa, and showed eight base pair differences when compared with the reference strains. Isolates of *M. lateralis* grouped outside of the larger *Mycosphaerella* clade within a smaller, well-supported second clade, together with reference isolates from South Africa (CMW 5164) and Zambia (CMW 4935). This smaller clade is represented by species with a *Dissoconium* anamorph, of which *M. lateralis* is the only *Mycosphaerella* species associated with this genus (Crous *et al*. 1999).

Isolates CMW 9094 and CMW 9095, representing *M. fori* from Tzaneen, grouped in a clade comprising *Pseudocercospora* species from *Eucalyptus* and *Syzygium*. These isolates grouped close to *Pseudocercospora eucalyptorum* isolates, which is known to occur on *Eucalyptus* leaves in South Africa. They were, however, distinct from this species, grouping within their own clade with high bootstrap support (99%).

TAXONOMY

An apparently undescribed species of *Mycosphaerella* was collected from 43 *Eucalyptus grandis* trees in Tzaneen (Northern Timbers Plantation). This species was dominant in Tzaneen and was represented by a total of 45 isolates. This fungus was not found in other forestry areas surveyed. Based on all key morphological characteristics used to identify *Mycosphaerella* spp. from *Eucalyptus*, it was concluded that this fungus represented a new taxon. It is thus described below:

Mycosphaerella fori G. C. Hunter, Crous & M. J. Wingf., **sp. nov.** (Figs 2–3)
Anamorph: *Pseudocercospora* sp.

Etym.: Raper (1987) states that Tzaneen is a North Sotho term indicating 'where the people used to meet' or 'in a basket'. The epithet, therefore refers to 'of the forum', or by extension 'of Tzaneen'.

Asci fasciculati, bitunicati, subsessiles, subcylindrici vel anguste obclavati, recti vel subincurvi, 8-sporati, 35–55 × 6–10 μm. Ascosporae 3-vel multiseriatae, superpositae, hyalinae, guttulatae, crassiparietales, rectae vel subfalcatae, anguste ellipsoideae apicibus subobtusis, medio uniseptatae, in septo inconstricto latissimae, pariter angustatae ad fines ambas, in vivo (12–)14–16(–20) × 3(–3.5) μm, *in vitro* 11–14 × 2.5–3.5 μm.

Typus: **South Africa**: *Northern Province*: Tzaneen, on leaves of *Eucalyptus grandis* 2000, *G. C. Hunter* (PREM 57305 – holotypus; cultura viva CBS 113285).

Leaf spots amphigenous, subcircular to irregular, 2–30 mm diam, becoming confluent along leaf margins, grey, surrounded by a dark brown outer zone, and a thin red-purple border, confluent with the leaf surface; abaxial surface medium brown with a dark brown outer zone. *Ascomata* predominantly epiphyllous, single, black, immersed becoming erumpent, globose, 50–100 μm diam; apical ostiole 5–10 μm diam; wall of 2–3 layers of medium brown textura angularis. *Asci* fasciculate, bitunicate, subsessile, subcylindrical to narrowly obclavate, straight or slightly incurved, 35–55 × 6–10 μm, 8-spored. *Ascospores* 3- to multiseriate, overlapping, hyaline, guttulate, thick-walled, straight to slightly curved, narrowly ellipsoid with subobtuse apices, medianly 1-septate, widest at unconstricted septum, tapering equally toward both ends, (12–)14–16(–20) × 3(–3.5) μm *in vivo*, 11–14 × 2.5–3.5 μm *in vitro*.

Mycelium immersed and superficial, of smooth, branched, septate, pale brown hyphae, 3–4 μm diam. *Caespituli* fasciculate, predominantly epiphyllous, medium brown on leaves, 150 μm wide and 90 μm high. *Conidiophores* occurring singly on secondary mycelium as lateral projections, or arranged in fascicles; fascicles arising from the upper cells of a medium brown stroma 60 μm wide and 50 μm high; conidiophores smooth, unbranched or rarely branched below, 1–3-septate, subcylindrical, straight to geniculate-sinuous, pale brown, 20–60 × 2.5–4 μm. *Conidiogenous cells* terminal, subcylindrical, straight or with several geniculations, pale brown, monoblastic or polyblastic, sympodial, or proliferating 1–3 times percurrently near the apex, 15–30 × 2.5–3.5 μm, with truncate apices; conidial scars unthickened. *Conidia* solitary, subulate-subcylindrical, pale brown, smooth, variously curved, apex subobtuse, base truncate, (50–)70–90(–100) × 2–3(–3.5) μm, indistinctly 1–3-septate; hilum unthickened, 1.5–2 μm wide.

Ascospore germination on MEA: Type C (Crous 1998). Ascospores do not darken on MEA, and germinate from both ends, with germ tubes parallel to the long axis of spore, and with no visible distortion. Some constriction occurs at the original ascospore septum, with ascospores becoming 3–4 μm diam.

Cultures: Colonies 35–39 mm diam on MEA after 1 month at 25 °C. Colonies olivaceous, 21″k (reverse) and smoke grey, 21‴f to grey olivaceous, 21‴i (surface)

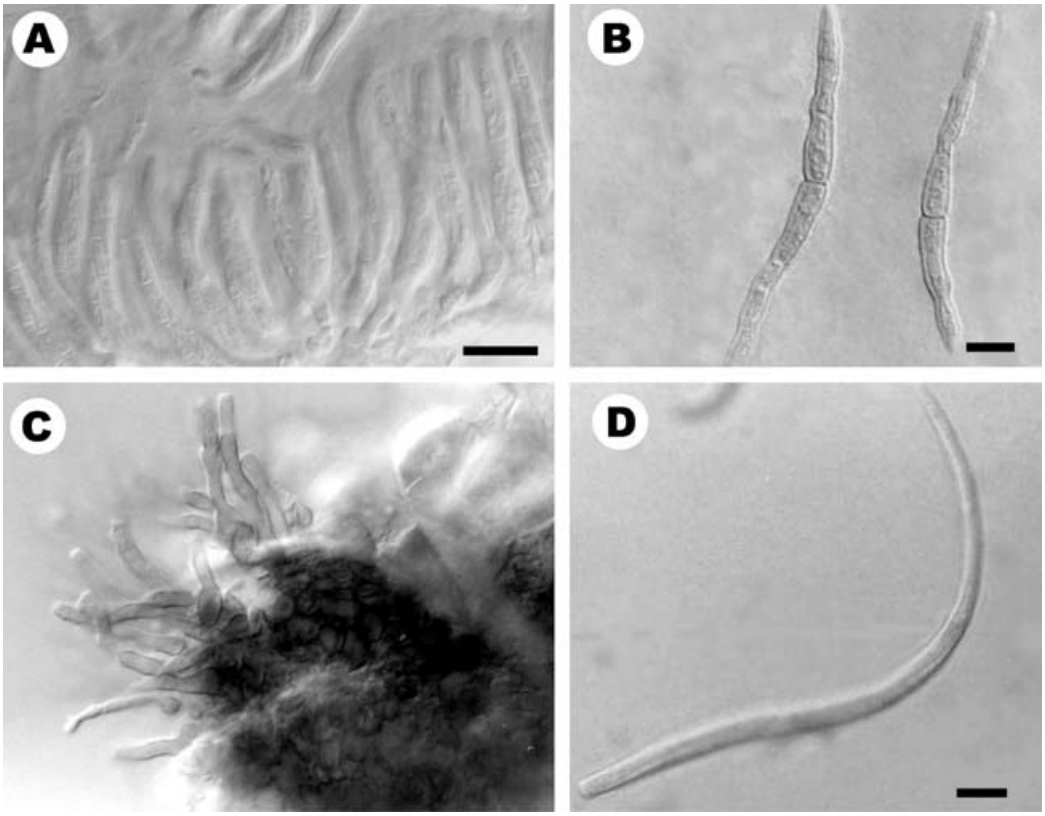

Fig. 2. Teleomorph and anamorph structures of *Mycosphaerella fori* (PREM 57305) from leaves of *Eucalyptus grandis* in Tzaneen, South Africa. (A) Bitunicate 8-spored asci. (B) Type C ascospore germination pattern, with spores germinating from both spore poles and producing parallel germ tubes with no visible constriction. (C) Fascicle of conidiophores arising from a brown stroma. (D) Solitary, subcylindrical conidia showing a subobtuse apex and a truncate base. Bar = 10 μm.

(Rayner 1970). Colony centre smoke grey, while colony border is grey olivaceous. Aerial mycelium profuse. Margins regular and smooth. Colony surface not sectored and no folding occurs. Only the *Pseudocercospora* anamorph is observed in culture.

Cardinal Temperatures: Min. above 5 °, opt. 20–25 °, max. below 35 °.

Host: *Eucalyptus grandis*.

Distribution: Tzaneen (Northern Province, South Africa).

Notes: *Mycosphaerella fori* is most similar to *M. gracilis*, but can be distinguished by its ascospore germination pattern, where ascospores remain unconstricted in *M. gracilis* (type B). Furthermore, conidia of the *Pseudocercospora* anamorph of *M. fori* taper towards their apices and are prominently curved, whereas conidia of *P. gracilis* are cylindrical and straight. Conidial shape and dimensions of *M. fori* agree with those of *P. natalensis*, but can be distinguished by the latter having pluriseptate conidia (Crous 1998).

DISCUSSION

Results of this study have provided substantial clarification regarding our understanding of the occurrence of *Mycosphaerella* spp. in commercial *Eucalyptus* plantations in South Africa. While a relatively large number of species of *Mycosphaerella* have previously been reported from South Africa (Crous & Wingfield 1996, Crous 1998), there has been very little information available regarding their relative importance and distribution. The focus of the present study was to include the most important *Eucalyptus* spp. in South Africa and to concentrate collections in plantations where leaf spot problems were being experienced. As a result, we were able to detect six of the ten species previously reported on *Eucalyptus* in South Africa, and a previously undescribed new species was also collected.

It is clear from this study that one species, *M. nubilosa*, is dominant in disease outbreaks throughout the sampled areas, particularly KwaZulu-Natal and Umtata. *M. nubilosa* is one of the main pathogens responsible for MLB in Australia and New Zealand (Park & Keane 1982a, b, Dick & Gadgil 1983). It is now clear that it is also an important pathogen in South Africa, where it causes severe leaf spotting and defoliation of young *Eucalyptus* trees, especially *E. nitens*. This species was, however, also isolated from other *Eucalyptus* spp., including *E. bicostata*, *E. dunnii* and *E. grandis*. Among these species, *E. grandis* and *E. dunnii* are particularly important in South Africa, but *M. nubilosa* does not appear to cause severe damage on them.

M. marksii was first identified from the Victoria Province in Australia where it was infecting both adult

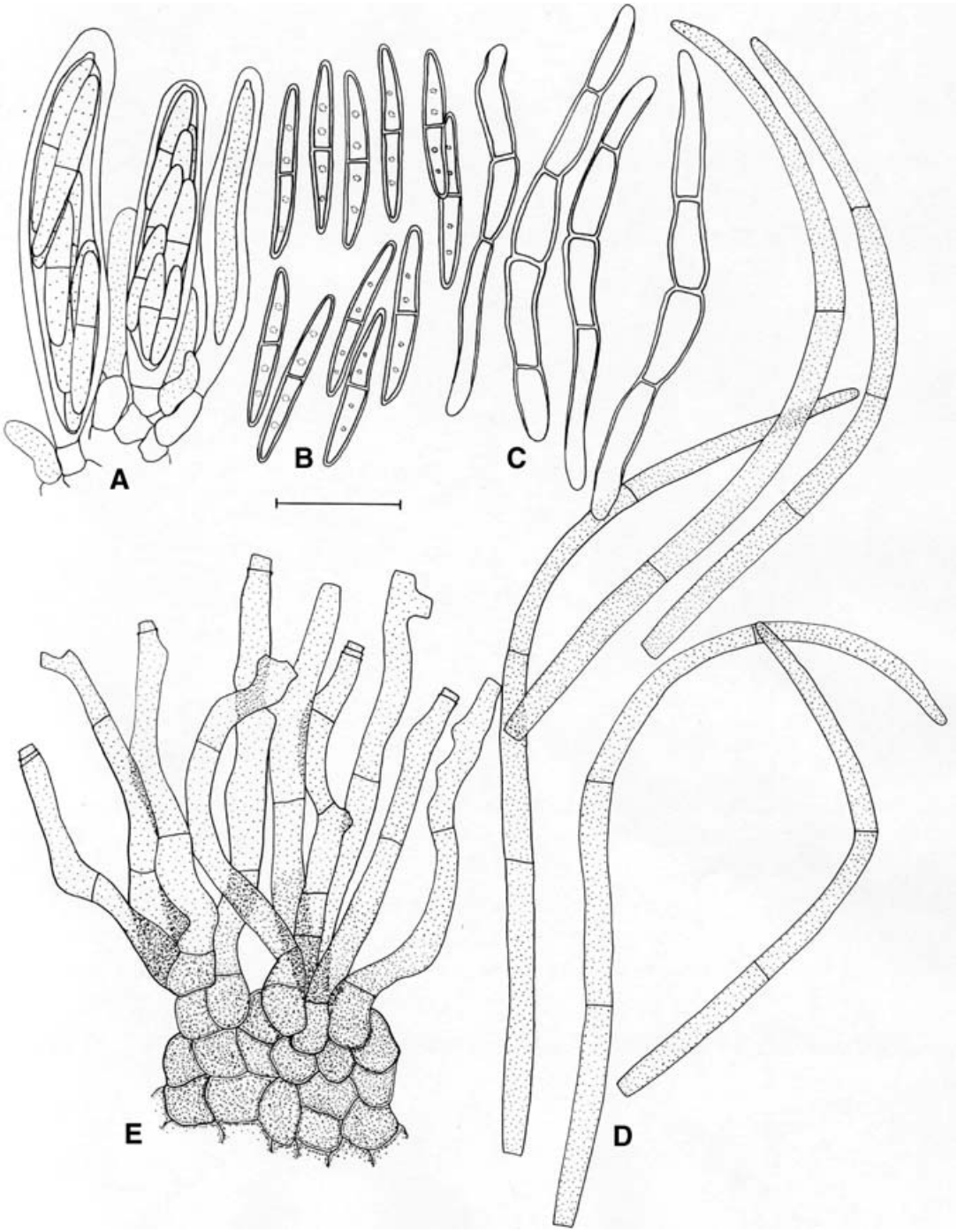

Fig. 3. *Mycosphaerella fori* (PREM 57305) and its *Pseudocercospora* anamorph. (A) Subcylindrical to narrowly obclavate, straight or incurved asci. (B) Ascospores. (C) Type C ascospore germination patterns. (D) Pale brown variously curved solitary conidia. (E) Conidiophores and terminal pale brown conidiogenous cells showing several geniculations. Bar = 10 μm.

and juvenile leaves of several *Eucalyptus* spp. (Carnegie & Keane 1994). Previous surveys of *Eucalyptus* spp. in South Africa showed that *M. marksii* was present in the Western Cape, KwaZulu-Natal and Gauteng Provinces (Crous & Wingfield 1996). Results of the present study have expanded our knowledge of the geographic distribution of this fungus to now include the Northern Province. The known host range of *M. marksii* includes *E. botryoides*, *E. fraxinoides*, *E. globulus*, *E. grandis*, *E. nitens*, *E. quadrangulata* and *E. saligna* (Crous 1998). In this study, *M. marksii* was also found on *E. bicostata* and *E. smithii*, the last of which is an important plantation species. However, the low number of isolates of *M. marksii* collected suggests that this fungus is not a major contributor to MLB outbreaks.

In this study, *M. lateralis* was found on diseased leaves of *E. nitens* and *E. grandis* leaves from KwaZulu-Natal and Tzaneen. This identification is not surprising, as the fungus was previously known to occur in these

areas (Crous & Wingfield 1996). However, our results have expanded the known host range of this fungus to include *E. grandis*. Other than in South Africa, *M. lateralis* is known to occur in Zambia, and has recently been found on eucalypts in Queensland, Australia (Crous 1998, Maxwell *et al*. 2000).

M. juvenis was previously considered as the most important species contributing to outbreaks of MLB in South Africa (Crous & Wingfield 1996, Crous 1998). It was thus surprising that this fungus was encountered relatively infrequently in the present study. The reason for the apparent change in status of this fungus is not clear. It is possible that species of *Mycosphaerella* causing leaf disease differ in their distributions and occurrence in different years and this matter deserves further study. From the samples considered in the present study it is clear that *M. nubilosa* is the dominant species in commercial plantations. This finding has emerged from a study that included considerably more material from a wider geographical area than has ever previously been sampled. Unlike *M. nubilosa*, *M. juvenis* has never been found in Australia, and it is thus difficult to assess its relative importance. However, due to its perceived importance in South Africa, *M. juvenis* is considered an important quarantine pathogen in Australia.

M. ellipsoidea is a relatively newly described *Mycosphaerella* species that has previously been known only from the Western Cape Province of South Africa (Crous & Wingfield 1996). In this study, *M. ellipsoidea* was collected from KwaZulu-Natal from leaves of *E. nitens*, although its incidence was low. Its appearance, for the first time in a commercially important forestry area, deserves consideration.

In this study, *M. irregulariramosa* was isolated from leaves of *E. grandis* from Tzaneen. Although this fungus has previously been reported from Tzaneen, this is the first report of its occurrence on *E. grandis*. Only eight isolates of this species were recovered, and relative to other species, it was the least common in the area sampled.

The discovery of a new species of *Mycosphaerella* was surprising. This is because extensive collections have been made in the past, resulting in the unusual situation that the largest number of *Mycosphaerella* spp. from *Eucalyptus* are presently known from South Africa. *M. fori* appears to be the most important species responsible for leaf spot in the Tzaneen area. It was found on the most important *Eucalyptus* sp. grown in South Africa, and there is concern that it might spread to other areas in the future.

A large number of *Mycosphaerella* spp. have been reported on *Eucalyptus* spp. in South Africa. These include, *M. africana*, *M. ellipsoidea*, *M. endophytica*, *M. irregulariramosa*, *M. juvenis*, *M. lateralis*, *M. marksii*, *M. crystallina* and *M. nubilosa* (Crous 1998). Six of these species were collected during the present study; the three species not collected were *M. africana*, *M. endophytica*, and *M. crystallina*. *M. africana* and *M. endophytica* were originally identified from Stellenbosch in the Western Cape Province (Crous 1998), which is outside the commercial *Eucalyptus* growing area. These fungi have either been isolated from the main *Eucalyptus* areas, or they are marginally important. The other species not collected was *M. crystallina*, which is known to occur in the KwaZulu-Natal province on leaves of *E. bicostata* and E. *grandis* × *E. camaldulensis* (Crous & Wingfield 1996). The absence of this species in this study suggests that it is probably not an important pathogen in commercial plantations.

Determining the relative importance of *Mycosphaerella* spp. causing MLD on *Eucalyptus* is difficult. This is due to the fact that many species are associated with the disease and it is extremely difficult to identify them. Although results of this study have shown that it is possible to define species based on morphology, these characters are variable. Generally identifications made in the absence of DNA sequence data are viewed with some circumscription. Clearly, it is impossible to obtain DNA sequences for the large number of isolates that emerge from extensive surveys. Rapid DNA-based procedures for the identification of *Mycosphaerella* spp. on *Eucalyptus* are thus needed. These would not only facilitate efforts to improve disease management but they would be extremely valuable in the application of more stringent and meaningful quarantine.

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