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Identification of *Mycosphaerella* species associated with *Eucalyptus nitens* leaf defoliation in South Africa

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Abstract. *Eucalyptus nitens* is an important plantation tree species in South Africa, where it is grown for paper and pulp production. The growth and performance of *E. nitens* in South Africa is, however, reduced substantially by Mycosphaerella leaf blotch (MLB) disease. The aim of this study was to determine which species of *Mycosphaerella* are associated with MLB epidemics on *E. nitens* in the KwaZulu-Natal Province of South Africa. *Mycosphaerella* species were isolated from leaves from two commercial *E. nitens* plantations by inducing the active release of ascospores onto agar. All isolates were identified according to their ascospore germination patterns, anamorph associations and sequence data generated from the Internal Transcribed Spacer region of the rDNA operon. From taxonomic and sequence data, *M. nubilosa* was identified as the main cause of MLB on *E. nitens* in commercial plantations in the KwaZulu-Natal Province. *M. nubilosa* is an important *Eucalyptus* leaf pathogen, and its correct identification in South Africa is important for the future planting of *E. nitens*. This is particularly true during the initial years of tree growth where MLB results in severe defoliation.

Additional keywords: Ascomycete, *Dothideales*, *Eucalyptus* leaf blotch, *Mycosphaerella*.

Introduction

Plantations of *Eucalyptus* make up a large proportion of South Africa's commercial forestry operation, with ~600 000 ha planted to various species of this tree genus. *Eucalyptus grandis* represents ~80% of the total area planted to *Eucalyptus*. In high altitude areas where colder conditions prevail and *E. grandis* does not perform well, *E. nitens* is the preferred plantation species (Poynton 1979). One obstacle to the continued planting of *E. nitens*, however, is its susceptibility to Mycosphaerella leaf blotch (MLB) disease.

More than 30 species of *Mycosphaerella* are known to be associated with MLB of *Eucalyptus* (Carnegie and Keane 1998; Crous 1998; Dick and Dobbie 2001; Maxwell *et al*. 2003). Several *Eucalyptus* species from both the *Monocalyptus* and *Symphyomyrtus* sub-genera are susceptible to infection by *Mycosphaerella* spp. (Park and Keane 1982*b*). Infection generally results in extensive leaf spotting, decreasing photosynthetic potential and causing premature leaf abscission (Beresford 1978; Ganapathi 1979). In severe cases, trees become stunted and may eventually die.

Mycosphaerella is one of the largest ascomycete genera, including more than 2000 species. In the past, the taxonomy of this group of fungi was largely based on host affiliations (Corlett 1991). Several anamorph genera have been linked to *Mycosphaerella*, including both coelomycetes and hyphomycetes (Arx 1983). The genus includes saprophytes as well as a large number of pathogens of woody and herbaceous plants (Corlett 1991; Aptroot *et al*. 1999). The most important group of pathogens are those causing leaf spots and defoliation.

Despite the fact that MLB is one of the most important diseases of *E. nitens*, the identity of the dominant *Mycosphaerella* species causing MLB in commercial plantations in South Africa is uncertain. Lundquist and Baxter (1985) stated that *M. molleriana* (Thüm.) Lindau causes this disease. Later studies, however, referred to *M. nubilosa* (Cooke) Hansford as the fungus resulting in MLB and subsequent loss of tree growth (Lundquist and Purnell 1987). Crous *et al*. (1991) also suggested that *M. nubilosa* was the causal agent of MLB on *E. nitens* in South Africa. Although Crous *et al*. (1991) regarded

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M. nubilosa and *M. molleriana* as synonymous, fresh collections, combined with cultural and molecular studies, later showed that they are distinct (Crous and Wingfield 1997; Crous 1998; Crous *et al*. 1999). To further add to the controversy, Crous and Wingfield (1996) also described *M. juvenis* Crous & M.J. Wingf., from various provinces in South Africa, and they regarded this species as being responsible for severe outbreaks of MLB on *E. nitens* and *E. grandis* (Crous and Wingfield 1996; Crous 1998).

Although a number of other *Mycosphaerella* spp. have been reported on *Eucalyptus* spp. in South Africa, it remains unclear as to which species is the main causal organism of MLB in commercial plantations of *E. nitens*. The aim of this study was, therefore, to determine which is the dominant *Mycosphaerella* species causing MLB on *E. nitens* in commercial plantations in the country. Two plantations with active MLB outbreaks were surveyed in the KwaZulu-Natal Province of South Africa and the *Mycosphaerella* isolates associated with the disease were characterised.

Methods

Sample collection

Leaves showing MLB symptoms were collected from *E. nitens* in the Clairemont plantation near Bulwer, KwaZulu-Natal (latitude 29°45′, longitude 29°45′) and the Enon plantation near Richmond, KwaZulu-Natal (latitude 29°49′, longitude 30°13′). Seventy-one *E. nitens* trees from three compartments were sampled at the Clairemont plantation and five *E. nitens* trees were sampled from one compartment at the Enon plantation. Ten diseased leaves were collected from every tree sampled. Leaves were collected in paper bags and transported to the laboratory where *Mycosphaerella* isolations took place within 3 days.

Isolates examined

Leaves showing the most severe MLB lesions were chosen for isolations. The number of leaves used from each sample varied from two to five, depending on the number of lesions and mature fruiting bodies present on leaves. Lesions were selected and excised from diseased leaves. Excised lesions were placed in water for ~2 h after which they were placed on double sided tape and adhered to the insides of Petri dish lids, suspended over 2% malt extract agar (MEA) (2 g/L; Biolab, South Africa) with the pseudothecia facing the agar surface (Crous 1998). Petri dishes were incubated in the dark for 24 h to allow ascospore release and germination on MEA. Following overnight 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°C under continuous cool white light in a walk-in incubator. Germinating ascospores were similarly subcultured onto carnation leaf agar [CLA; sterilised carnation leaves placed onto 1% water agar (1 g/L; Biolab, South Africa)] and incubated at 25°C under continuous near-ultraviolet light (250 nm) to promote the production of asexual states.

All cultures obtained in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Symptoms and morphology

All *Mycosphaerella* isolates obtained from *E. nitens* were evaluated based on lesion characteristics, ascospore germination patterns, anamorph production and cultural characteristics. Leaf blotch symptoms were carefully examined and characterised according to their colour, shape and position of the fruiting structures.

After ascospore discharge from pseudothecia onto MEA, germinating ascospores were transferred to microscope slides and mounted in lactophenol for microscopic evaluation. Ascospores were evaluated based on characteristics known to be taxonomically relevant for these fungi (Park and Keane 1982*a*; Crous 1998). Based on these criteria, the ascospore germination patterns were grouped as outlined by Crous (1998). Pseudothecia containing ascospores were mounted in lactophenol and morphological characteristics of asci and ascospores were assessed and measurements taken at 1000× magnification using light microscopy (measurements of 20 spores and asci). Characteristics were compared with those of other *Mycosphaerella* spp. occurring on *Eucalyptus* spp. (Crous 1998).

Following the growth of cultures isolated from *E. nitens* in South Africa, comparisons were made with reference cultures of *M. molleriana* from *E. globulus* in Portugal and the USA (CMW 2734, CMW 4940, CMW 8997, CMW 8998, CMW 8999) and *M. nubilosa* from *E. globulus* in Australia (CMW 3282, CMW 6211, CMW 6210) (Table 1). Culture morphology and colour was characterised based on mycological colour charts and descriptions for *Mycosphaerella* spp. occurring on *Eucalyptus* spp. (Rayner 1970; Crous 1998). South African cultures on CLA were examined for the production of anamorph structures. Isolates of *M. molleriana* and *M. nubilosa* obtained from other countries were cultured in a similar fashion on CLA and examined for the production of anamorphs.

Following the characterisation of KwaZulu-Natal isolates based on leaf blotch symptoms, lesion characteristics, ascospore germination patterns and the production of anamorphs, representative isolates were chosen for characterisation using DNA sequencing (Table 1). Four South African isolates were chosen (two from the Clairemont plantation and two from the Enon plantation) and compared with five isolates of *M. molleriana* and three isolates of *M. nubilosa* (Table 1).

DNA isolation

Following growth of pure cultures, mycelium was scraped directly from agar plates and used for DNA isolation. Scraped mycelium was dried under vacuum and ground to a fine powder, using a pestle and mortar, in the presence of liquid nitrogen. DNA was isolated using the method described by Raeder and Broda (1985), with minor modifications. The phenol:chloroform (1:1) purification step was repeated until the interphase between the aqueous and organic phases was clean of any cellular debris. Nucleic acids were precipitated from the aqueous phase by the addition of one-tenth volume 3 M NaAc (pH 5.5) and two volumes of absolute ethanol and incubated at –20°C for 2 h. The nucleic acids were further purified by washing with 70% ethanol and dried under vacuum, after which the resulting pellet was resuspended in 50 µL water. RNaseA (10 µg/µL; Roche Diagnostics, South Africa) was added to the DNA samples and incubated at 37°C for 4 h to digest RNA. The isolated DNA was visualised on a 1% agarose gel (Boehringer Mannheim, Germany) stained with ethidium bromide and viewed under an ultraviolet light. DNA was quantified for all samples with a Beckman DU Series 60 Spectrophotometer (Beckman, Germany).

PCR amplification and purification

Isolated DNA (50–90 ng) was used as a template for the PCR. The Internal Transcribed Spacer (ITS) region of the rDNA operon was targeted for amplification using primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) (White *et al*. 1990) and LR1 (5′-GGT TGG TTT CTT TTC CT-3′) (Vilgalys and Hester 1990). The ITS1 and ITS2 regions including the 5.8S gene were amplified using these primers. DNA was amplified in a 50 µL reaction volume containing PCR buffer (10 mM

Table 1. *Mycosphaerella* isolates used for DNA sequencing studies

Isolate no.[A]	Identity	Host	Location	Isolator	Date of isolation	GenBank accession number
CMW 2734	*M. molleriana*	*E. globulus*	California, USA	M. J. Wingfield	06/1994	AF309619
CMW 4940	*M. molleriana*	*E. globulus*	Portugal	S. McCrae	07/1995	AF309620
CMW 8997	*M. molleriana*	*E. globulus*	Portugal	M. J. Wingfield	07/1995	AF449100
CMW 8998	*M. molleriana*	*E. globulus*	Portugal	M. J. Wingfield	07/1995	AF449102
CMW 8999	*M. molleriana*	*E. globulus*	Portugal	M. J. Wingfield	07/1995	AF449101
CMW 3282	*M. nubilosa*	*E. globulus*	Australia	A. J. Carnegie	09/1994	AF309618
CMW 6211	*M. nubilosa*	*E. globulus*	Australia	M. J. Wingfield	09/2000	AF449094
CMW 6210	*M. nubilosa*	*E. globulus*	Australia	M. J. Wingfield	09/2000	AF449095
CMW 9000	*M. nubilosa*	*E. nitens*	South Africa	G. C. Hunter	05/2000	AF449096
CMW 9001	*M. nubilosa*	*E. nitens*	South Africa	G. C. Hunter	05/2000	AF449097
CMW 9002	*M. nubilosa*	*E. nitens*	South Africa	G. C. Hunter	05/2000	AF449098
CMW 9003	*M. nubilosa*	*E. nitens*	South Africa	G. C. Hunter	05/2000	AF449099

[A]CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3; Roche Diagnostics, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP and dGTP; Roche Diagnostics, South Africa), 0.2 μM of primers ITS1 and LR1 (MWG Biotech, Germany) and 2.5 U *Taq* DNA polymerase (Roche Diagnostics, South Africa).

PCRs were carried out using an Eppendorf Mastercycler gradient PCR machine (Eppendorf Scientific, Germany). PCR conditions consisted of an initial denaturation step at 96°C for 2 min, followed by 40 cycles of, template denaturation for 30 s at 94°C, primer annealing for 30 s at 53°C and chain elongation for 2 min at 75°C, and a final elongation step for 7 min at 75°C. A negative control using water and no template DNA and a positive control containing DNA of a *Mycosphaerella* sp. was used for each set of reactions. PCR products were visualised in ethidium bromide-stained 2% agarose gels and viewed under ultraviolet light. Sizes of PCR products were determined against a 100 bp molecular weight marker (XIV; Roche Diagnostics, South Africa) and were purified using the High Pure PCR product purification kit (Roche Diagnostics, South Africa). After PCR purification, concentrations of purified PCR products were determined by running products on a 2% agarose gel stained with ethidium bromide with a 100 bp molecular weight marker XIV and viewed under ultraviolet 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, USA). The ABI Prism Big Dye Terminator Cycle sequencing reaction kit (Perkin Elmer Biosystems, USA) was used for the sequencing reactions. Two forwards primers, ITS1 and ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′) targeting the 3′ end of the ITS Small Subunit (SSU) and the 5.8S gene respectively, and two reverse primers, ITS2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) (White *et al*. 1990) and LR1 targeting the 5′ end of the Large Subunit (LSU) and 5.8S gene 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, Foster City, CA, USA). 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 (Phylogenetic Analysis Using Parsimony) version 4.0b1 (Swofford 1998). 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. Branch supports were investigated by performing a bootstrap search of 1000 replicates on the aligned sequences. Following the analysis, all resulting trees were rooted to an outgroup. The taxon chosen for this purpose was *Ramulispora anguioides* (Nirenberg) Crous, which resides in the genus *Tapesia* and has been shown to be an appropriate outgroup for *Mycosphaerella* (Crous *et al*. 2001).

Results

Symptoms and morphology

Leaf spots were prevalent on juvenile leaves of *E. nitens* and were pale brown in colour, often with raised brown to dark brown borders. Lesions were amphigenous extending through the leaf laminas. They were circular to irregular in shape and varied in size from a few millimetres to 1–2 cm in diameter. Black pseudothecia were amphigenous but were mainly present on the abaxial leaf surfaces. They were arranged singly over the lesion surfaces or aggregated into clusters.

Isolates examined

Ascospores were obtained from most, but not all, diseased *E. nitens* leaves sampled and resulted in a total of 180 cultures. Of these, 152 were from the Clairemont and 28 from the Enon plantations.

All cultures from KwaZulu-Natal and *M. nubilosa* in Australia shared the same culture morphology. Colonies were slow growing and had regular to irregular margins with sparse aerial mycelium. Cultures were pale olivaceous grey, but upon further incubation, became darker olivaceous grey (23”””i) (Rayner 1970). Cultures of *M. molleriana* were characterised by sectored colonies that had sparse aerial mycelium, iron grey (25”””k) (Rayner 1970) on the reverse sides of the Petri dishes and olivaceous grey (23”””i) (Rayner 1970) on the surface. These characteristics matched descriptions given for the species by Crous (1998). Although cultures of *M. nubilosa* and *M. molleriana* were similar in colour, those of *M. nubilosa* grew more slowly than those of *M. molleriana*.

No anamorph production was seen for South African *Mycosphaerella* isolates or for Australian isolates of *M. nubilosa* when grown on CLA or MEA. *M. molleriana* isolates from Portugal and the USA, however, readily produced the anamorph, *Colletogloeopsis molleriana* Crous & M. J. Wingf. in culture.

Asci of South African isolates were 8-spored, ellipsoidal to obclavate, bitunicate, frequently curved, (40) 45.5–61 (68) μm long and (11) 11–15 (19) μm wide. Discharged ascospores had truncate bases and obtuse apices and were 11–13 μm long by 2–3 μm wide. Ascospores were fusoid to ellipsoidal, 1-septate with the widest part of the spore just above the median septum (Fig. 1*A*).

Discharged ascospores from all lesions showed the same ascospore germination pattern. These were typical type C patterns as described by Crous (1998). Ascospores germinated from both poles, producing germ tubes that elongated roughly parallel to the long axis of the spore. Ascospores did not swell upon germination, although they did show a slight constriction at the median septum (Fig. 1*B*). After 24 h, however, all ascospores showed prominent distortion. Based on morphology, all isolates from *E. nitens* in South Africa were identical.

PCR amplification and sequence data analyses

Amplification of the ITS region of the rDNA operon resulted in amplification products of ~600 bp for all isolates. A total of 13 taxa were used in the analysis with 657 aligned characters. Of these characters, 388 were constant, 233 were parsimony uninformative and 36 were parsimony informative. Searches conducted in PAUP using the Heuristic search option resulted in the retention of one most parsimonious tree after 283 steps (CI = 1.000, HI = 0.000, RI = 1.000). Subsequently, 1000 bootstrap replicates

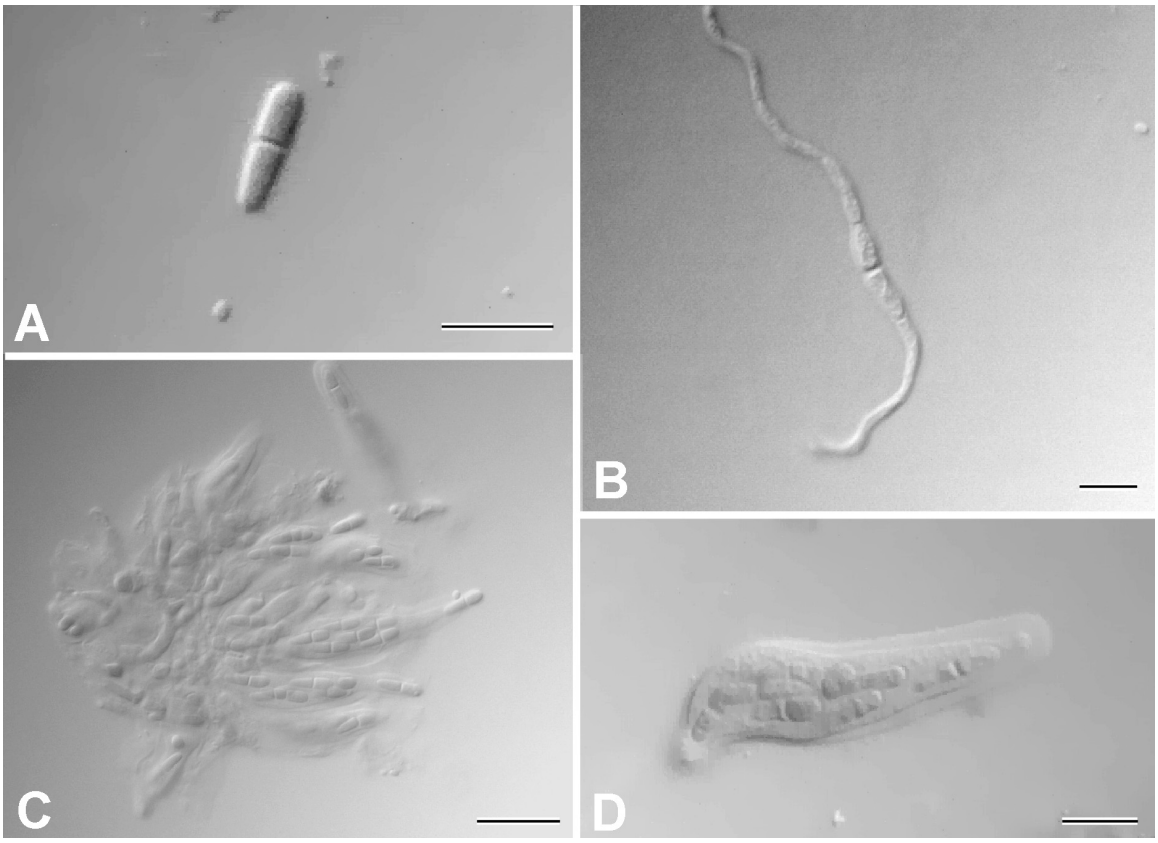

Fig. 1. Asci, ascospores and ascospore germination pattern of *Mycosphaerella nubilosa* isolated from *Eucalyptus nitens* in KwaZulu-Natal, South Africa. (*A*) One-septate fusoid to ellipsoidal ascospore prior to germination. (*B*) Typical type C ascospore germination producing two parallel germination tubes. (*C*) Fascicle of asci containing ascospores. (*D*) 8-spored bitunicate ascus. Scale bar = 10 μm.

resulted in a major consensus tree that showed the same topology as the most parsimonious tree (Fig. 2). Bootstrap analysis resolved the isolates into two well-supported clades. The first clade contained isolates representing *M. nubilosa* from Australia and those from *E. nitens* in South Africa. The DNA sequence of the ITS regions of all isolates in this clade were identical. Isolates of *M. molleriana* from Portugal and the USA made up a second clade, sister to the first.

Discussion

From results obtained in this study, it is clear that *M. nubilosa* is the dominant *Mycosphaerella* species causing MLB in commercial plantations of *E. nitens* in the KwaZulu-Natal province of South Africa. This conclusion is based on both morphological features of the fungus as well as on DNA sequence data. This result is in contrast to a previous view that *M. juvenis* is the most important cause of MLB on *E. nitens* in South Africa (Crous 1998). It is not clear why our results differ from those of Crous (1998), but it is possible that different disease situations occur in the various provinces in South Africa, and that this could also change with time. Furthermore, different species such as *M. juvenis* could also have been responsible for specific infection events in previous years. Although the present study included a considerably larger sample, based on morphology, than that examined in previous studies, it was focused only on one province. Nonetheless, this is the area where *E. nitens* is most widely grown and where MLB is most important.

M. nubilosa has been identified as being responsible for MLB on several *Eucalyptus* spp. in New Zealand and Australia (Weston 1957; Park and Keane 1982*b*; Park 1988). Confirmation of the presence of *M. nubilosa* in South Africa is of particular importance, as this species is considered to be one of the more important causal agents of MLB. There has been controversy as to whether this species occurs in South Africa (Doidge 1950; Crous 1998) and the present study provides the first conclusive evidence confirming its presence.

Sequence data based on the rDNA operon in this study, have confirmed that *M. nubilosa* is present on *E. nitens* in the KwaZulu-Natal province of South Africa. Previous studies have shown that this DNA region is useful for distinguishing *Mycosphaerella* spp. from each other (Crous *et al*. 1999,

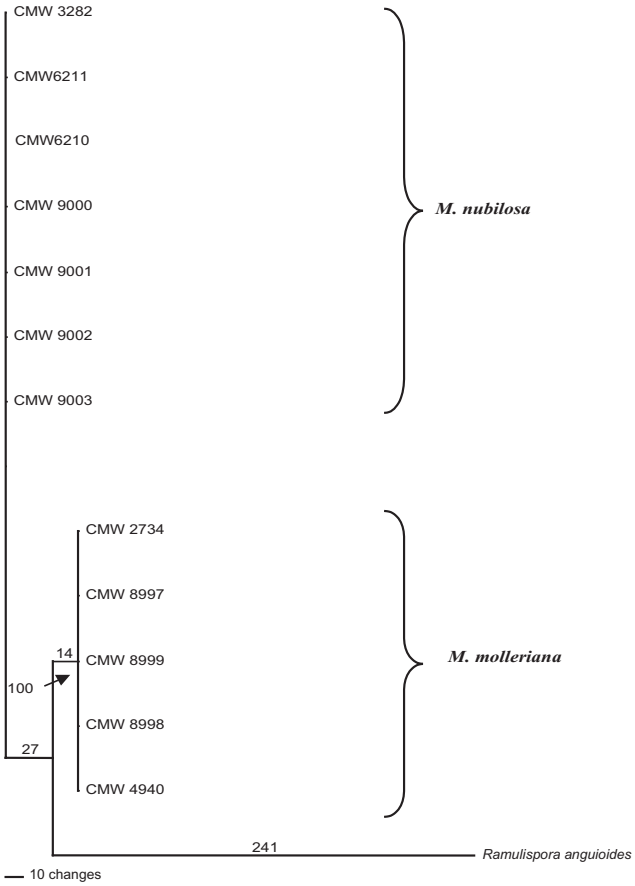

Fig. 2. Phylogram generated from aligned ITS sequence data from the rDNA operon of *Mycosphaerella nubilosa* and *M. molleriana* isolates (CI = 1.000, HI = 0.000, RI = 1.000).

2000, 2001; Goodwin *et al*. 2001). This study includes more isolates of *M. nubilosa* than previously considered. It also shows clearly that *M. nubilosa* and *M. molleriana* represent distinct species. This fact has been questioned in the past, although such studies have relied predominantly on morphological data (Crous *et al*. 1991).

Results of this study have confirmed that ascospore germination patterns represent a reliable feature to distinguish between *Mycosphaerella* spp. The ascospores of South African *M. nubilosa* isolates in this study all germinated from both poles with parallel germination tubes, and eventually led to prominent spore distortion. Thus, essentially, the germination patterns distinguishing *M. nubilosa* from *M. juvenis* do not differ dramatically as reported by Crous (1998). These two species can still be distinguished, however, based on anamorph morphology (or absence thereof) and DNA sequence data.

The fungus associated with MLB on *E. nitens* in this study did not produce an anamorph. Recent studies have shown that anamorph characteristics are important in delimiting *Mycosphaerella* spp. *M. juvenis* produces a typical *Uwebraunia* anamorph while *M. molleriana* produces a *Colletogloeopsis* anamorph (Crous and Wingfield 1996, 1997). *M. nubilosa* is not known to produce an anamorph and the lack of this stage in our cultures confirms our identification.

M. nubilosa is recognised in Australia as one of the more important *Mycosphaerella* spp., where it causes defoliation of juvenile leaves, particularly on *E. globulus* (Park and Keane 1982*a*, 1982*b*; Dick and Gadgil 1983). *M. nubilosa* has also been reported as the reason for the abandonment of *E. globulus* for afforestation in South Africa during the 1930s (Lundquist and Purnell 1987). From this study it is clear that *M. nubilosa* is a common and severe pathogen on *E. nitens* in South Africa. Our findings highlight the role and importance of quarantine, as these fungi are thought to be dispersed through seed lots. The introduction of *M. nubilosa* into areas where it presently does not occur could, therefore, result in serious losses.

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