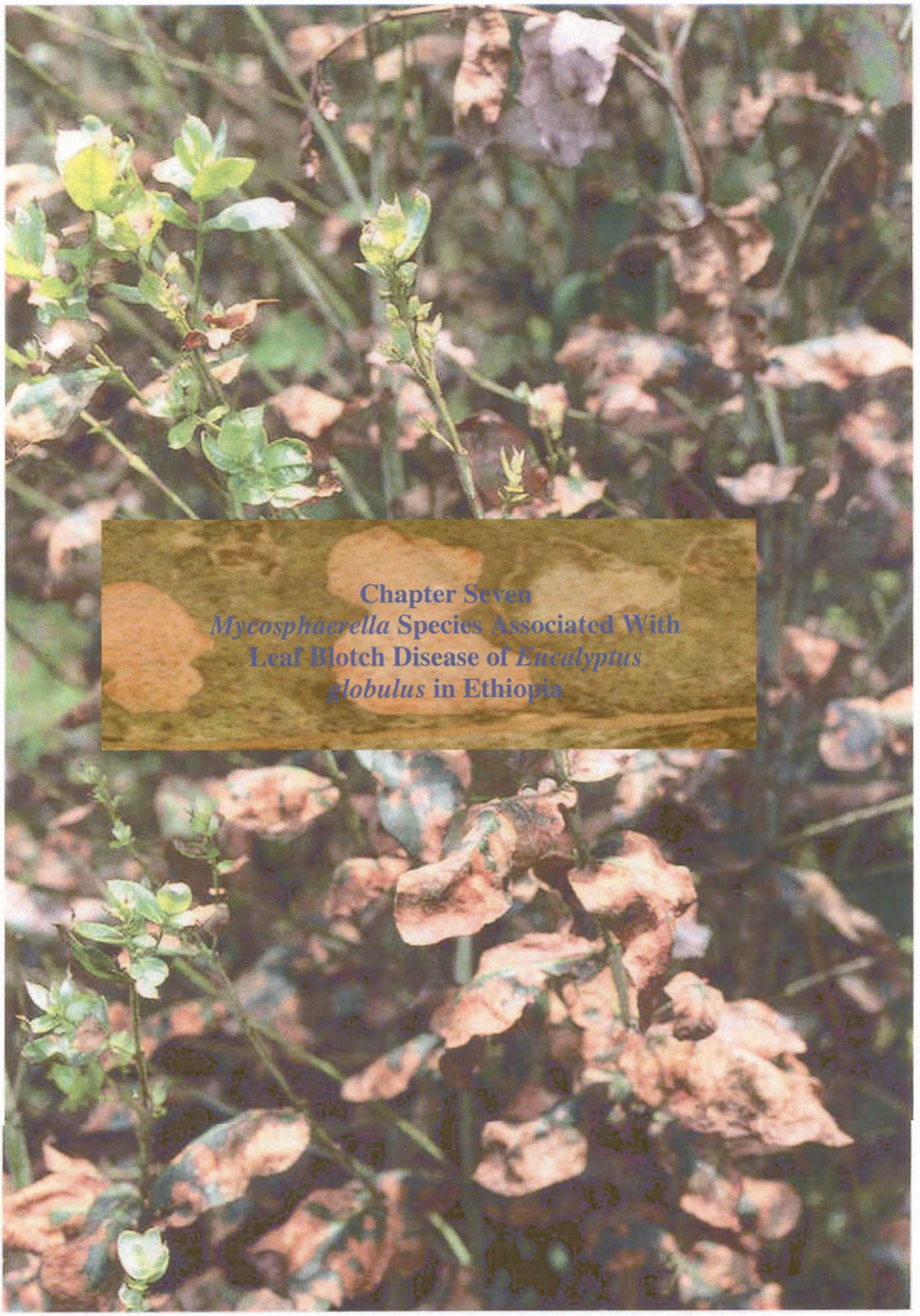

Chapter Seven
Mycosphaerella Species Associated With Leaf Blotch Disease of *Eucalyptus globulus* in Ethiopia

ABSTRACT

Several damaging leaf pathogens are known from *Eucalyptus* spp., worldwide. Of these, *Mycosphaerella* spp. are among the most important. Characteristic symptoms of Mycosphaerella leaf blotch disease (MLD) include leaf spot, premature defoliation, stunting, shoot and twig die-back as well as twig and stem cankers. Recent disease surveys conducted in Ethiopian *Eucalyptus* plantations have revealed disease symptoms similar to those caused by *Mycosphaerella* spp. These symptoms were restricted to *E. globulus* trees growing in several localities in South, South Western and Western Ethiopia. The aim of this study was to identify the fungi associated with this disease. This was achieved by examining the germination patterns of the ascospores and by sequencing the ITS region of the rRNA operon, for representative isolates. Several different ascospore germination patterns were observed, suggesting that more than one *Mycosphaerella* sp. is responsible for MLD on *E. globulus,* in Ethiopia. Analysis of sequence data showed that three *Mycosphaerella* spp., *M. marksii, M. nubilosa* and *M. grandis* were present. This is the first report of these three species from Ethiopia and it is also the first report of *M. grandis* from a country other than Australia. *M. grandis* and *M. nubilosa* were the most common species associated with leaf blotch in Ethiopia. Given the fact that these fungi are well-recognised pathogens of *Eucalyptus*, we assume that they are the most important cause of MLD on *E. globulus* in Ethiopia.

INTRODUCTION

Plantations of exotic tree species are widely utilised in the tropics and sub-tropics for the production of solid timber products and pulp. *Pinus*, *Eucalyptus*, *Cupressus* and Australian *Acacia* spp. are among the most widely planted exotic species in these situations. Plantations of *Eucalyptus* spp. alone cover approximately 10 million ha of land world-wide (Eldridge *et al.* 1997). In Ethiopia, planting of exotic species commenced with the introduction of *Eucalyptus globulus* Labill. about 110 years ago (Persson 1995). Thereafter, several *Eucalyptus* spp. including *E. camaldulensis* Dhen., *E. saligna* Sm., *E. grandis* Hill ex Maid and *E. citriodora* Hook were introduced. It has been estimated that plantations of *Eucalyptus* spp. constitute about one third of the total plantation area in the country (Anonymous 1994). The wood from plantations of *Eucalyptus* species is commonly used for construction purposes, for fuel, poles and posts and is an important resource for subsistence farmers.

Plantations of *Eucalyptus* spp., though displaying tremendous promise in areas where they have been planted as exotics, are threatened by various pathogens (Wingfield 1990, Persson 1995). Several foliage diseases have been recorded on *Eucalyptus* spp., both in their areas of origin and also in several areas where they have been introduced as plantation species. These include, for example, foliage diseases caused by *Pseudocercospora eucalyptorum* Crous, Wingfield, Marasas & Sutton (Crous *et al.* 1989), *Phaeoseptoria eucalypti* Hansf. emend. Walker (Chipompha 1987), diseases caused by *Cylindrocladium* spp. (Sharma & Mohanan 1982, Crous, Phillips & Wingfield 1991, Schoch *et al.* 1999) and leaf blotch caused by several *Mycosphaerella* spp. (Park & Keane 1982a, Crous 1998).

Mycosphaerella spp. are important leaf pathogens of *Eucalyptus* spp. and they are distributed world-wide (Corlett 1991, Crous 1998). They include both saprobes and aggressive pathogens (Von Arx 1983). Thirty-Two *Mycosphaerella* spp. have been described associated with diseases of *Eucalyptus* spp. (Crous 1998, Carnegie 2000, Milgate *et al.* 2001, Hunter *et al.* 2003). Of these, 12 have been described associated with *Eucalyptus* spp. in different African countries (Crous 1998, Hunter *et al.* 2003). For example, in South Africa nine *Mycosphaerella* species have been reported associated with different *Eucalyptus* species (Crous 1998, Hunter *et al.* 2003) and

thirteen species have been recorded from Australia (Carnegie 2000, Milgate *et al.* 2001). Similarly five *Mycosphaerella* spp. have been identified from *E. globulus* and *E. nitens* (Deane & Maid.) Maid. plantations in Chile (Ahumada 2002).

The most important symptoms of Mycosphaerella leaf disease (MLD) include leaf spot, defoliation, stunting, stem canker, twig and shoot die-back (Beresford 1978, Dick & Gadgil 1983, Lundquist & Purnell 1987, Crous 1998). MLD reduces the photosynthetic capacity of the plant, causes shoot die-back, resulting in multi-stemmed trees and reduces growth and yield of trees (Park & Keane 1982b, Dick 1982, Carnegie 2000). Lundquist & Purnell (1987) showed that MLD causes a reduction in growth of *E. nitens* trees in South Africa. Similarly, a positive correlation between severity of *M. nubilosa* infections and growth of *E. grandis* was observed in Australia (Carnegie *et al.* 1994). It has also been shown that the provenances of some *Eucalyptus* spp. such as *E. globulus*, *E. nitens* and *E. regnans* F. Muell. vary in resistance to *Mycosphaerella* infection (Dick & Gadgil 1983, Purnell & Lundquist 1986, Carnegie *et al.* 1994). In South Africa, for example, it is recommended that the New South Wales provenances of *E. nitens* are planted, as they are considerably more tolerant to infection than those from areas such as Victoria (Purnell & Lundquist 1986, Wingfield & Roux 2000).

Several different *Mycosphaerella* spp. can infect individual *Eucalyptus* trees. Similarly, more than one *Mycosphaerella* sp. can be found on a single leaf and even on the same lesion (Crous & Wingfield 1996). Milgate *et al.* (2001), for example, showed that *M. grandis* Carnegie & Keane was found associated with older lesions of *M. tasmaniensis* Crous & M.J. Wingfi., *M. nubilosa* (Cooke) Hansf. and *M. cryptica* (Cooke) Hansf. It has also been shown that lesions of *M. marksii* Carnegie & Keane coalesce with those of *M. cryptica* and *M. molleriana* (Thum.) Lindau. Park & Keane (1984) also indicated the association of *M. parva* R. F. Park & Keane, a saprophytic species, with *M. nubilosa* (Park & Keane 1982b, Crous *et al.* 1993, Carnegie & Keane 1994). In this manner, multiple infections of trees can take place, compounding the impact of MLB on susceptible trees. Such, multiple infections often result in defoliation (Park & Keane 1982b) and they also complicate identification of the causal agents.

The occurrence of *Mycosphaerella* spp. on *Eucalyptus* leaves can vary with the age of the leaves. Some *Mycosphaerella* spp. infect both juvenile as well as mature leaves and others even infect twigs and branches (Park 1988, Crous 1998). Some *Mycosphaerella* spp., including *M. nubilosa*, *M. molleriana* and *M. juvenis* Crous & M. J. Wingf. are commonly associated with severe defoliation of juvenile leaves (Crous & Wingfield 1996, Carnegie & Keane 1998), whereas *M. cryptica* and *M. suberosa* Crous, F. A. Ferreira, Alfenas & M. J. Wingf. are found mainly on mature leaves (Park & Keane 1982a, Crous *et al.* 1993, Carnegie *et al.* 1994). Succession of infections by different *Mycosphaerella* spp. thus results in susceptible trees being affected at all stages of their rotation. In cases where only juvenile leaves are attacked, for example, *M. nubilosa* on *E. nitens* in South Africa, trees can outgrow the problem as they change to their mature leaf stage, normally during their second year of growth (Lundquist & Purnell 1987).

In Ethiopia, symptoms of MLD have been reported from several plantations of *E. globulus*. It has been observed that the disease causes severe damage on juvenile *E. globulus* leaves, in most areas where this tree species is planted (Alemu, Roux & Wingfield 2003). The *Mycosphaerella* spp. involved in causing the disease have, however, not been identified. This study was, therefore, conducted to identify the fungi associated with MLD on *E. globulus* in Ethiopia. To accomplish this, a suite of identification techniques, including examination of ascospore germination patterns, cultural characteristics as well as sequencing of the Internal Transcribed Spacer (ITS) regions of the ribosomal RNA operon, were used.

MATERIALS AND METHODS

Sample collection and isolations

In a previous survey conducted in *Eucalyptus* plantations in Ethiopia, symptoms similar to those of MLD were observed in most *E. globulus* plantations investigated (Alemu *et al.* 2003). The samples used in the current study were thus collected from *E. globulus* plantations in South, South Western and Western Ethiopia (Table 1,

Figure 1). At each locality where trees showed leaf blotch symptoms, five to ten symptomatic leaves per tree were colleted from three to ten trees, depending on the size of the stand of trees.

The method described by Crous (1998), was used to isolate the *Mycosphaerella* spp. Two to four leaves were selected from each sample and four leaf discs containing lesions were excised from each leaf. These discs were then immersed in water for two hours to moisten the pseudothecia, facilitating spore release. The discs were then attached to the insides of Petri dish lids with the pseudothecia facing downwards over malt extract agar (MEA) (2% Biolab malt extract, 1.5% Biolab agar). The Petri dishes were kept in the dark at room temperature for 24 hours. After 24 hr, plates were examined for the germination of ascospores. Single germinating spores were picked up and transferred to 2% MEA plates and incubated at 25 °C in the dark. Cultures resulting from germinated ascospores were incubated at 25 °C under continuous light. Isolates obtained in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Morphological characterisation

Growth of the fungi on MEA, germination patterns and anamorph associations were used to differentiate the *Mycosphaerella* spp. associated with MLD in Ethiopia. Colony colour was determined using Rayner's (1970) colour charts. Germinating ascospores for each sample were mounted in lactophenol on microscope slides and the germination patterns noted. Ascospore germination patterns were studied using a light microscope (Zeiss Axioskope) and compared with those described for *Mycosphaerella* spp. on *Eucalyptus* (Crous 1998). To identify the anamorph states of the *Mycosphaerella* spp., isolates were grown on water agar (1.5% Biolab agar) containing sterilised carnation leaves at 25 °C under near ultra violate light (NUV) 250 nm.

DNA extraction

Isolates for DNA extraction were selected based on differences detected in culture morphology and ascospore germination patterns. Mycelium used for DNA extraction was scraped directly from the surface of cultures on agar plates. Mycelium was placed in Eppendorf tubes and freeze dried under vacuum. A modified version of the DNA extraction method described by Raeder and Broda (1985) was used to isolate DNA. A repeated Phenol:chlorophorm purification step was conducted to remove cell debris. Sodium Acetate (3M NaAc, pH 5.5) (0.1v/v) and two volumes of absolute ethanol were added to the clean aqueous phase to precipitate the nucleic acids. The precipitated DNA was washed with 70% ethanol. The DNA pellets obtained were vacuum dried to remove the remaining ethanol and the pellets were re-suspended in 50 µl sterile water. The contaminating RNA was removed by digesting the isolated DNA with RNase A (Roche, South Africa) in a 37 °C water bath overnight. The DNA in each sample was visualised under ultra-violate light after electrophoresis on a 1% agarose gel containing ethidium bromide.

PCR amplification

Specific DNA fragments from isolates included in this study were amplified using the Polymerase Chain Reaction (PCR). The Internal Transcribed Spacer (ITS) region and 5.8S genes of the ribosomal RNA operon were amplified using Primers ITS 1 (5'-TCC GTA GGT GAA CCT GCG G -3') (White *et al.* 1990) and LR 1 (5'- GGT TGG TTT CTT TTC CT-3') (Vilgalys & Hester 1990). The PCR mix consisted of 1 µL DNA, 0.25 mM dNTP's, PCR Buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) (Roche, South Africa), 0.2 mM of each primer, 2.5 U *Taq* DNA polymerase (Roche Diagnostic, South Africa) and 37 µl sterilised water. The PCR reactions consisted of an initial denaturation step at a temperature of 96 °C for 2 min, followed by 40 cycles of template denaturation at 94 °C for 30 s, primer annealing for 30 s at 55 °C and chain elongation for 2 min at 75 °C. This was followed by a final elongation at 75 °C for 7 min. PCR amplicons were electrophoresed on a 1% agarose gel, stained with ethidium bromide and viewed under UV illumination. Sizes of he PCR fragments were estimated using a 100 bp molecular weight marker (XIV)

(Roche). Prior to sequencing the PCR products were cleaned with the High Pure PCR product purification kit (Roche, South Africa).

DNA sequencing and phylogenetic analysis

The PCR products obtained were used as templates for DNA sequencing using an ABI Prism, Big Dye Terminator Cycle sequencing reaction kit (Perkin Elmer Biosystems, USA) according to the manufacturers protocol. Primers ITS 1 and LR 1 were used to sequence both strands of the amplicons. Sequencing reactions were analysed using an ABI PRISM™ 3100 automated DNA sequencer (Perkin Elmer, Norwalk, Con).

DNA sequences of the Ethiopian isolates used in this study were compared with sequences deposited in GenBank [National Centre for Biotechnology Information (NCBI), US National Institute of Health Bethesda, http:/www.ncbi.nlm.nih.gov/BLAST] for preliminary identification. Sequence Navigator (Version 1.0.1) was used to align sequences and gaps were inserted manually and treated as missing data. Sequences were aligned against those of *Mycosphaerella* spp. from and extensive in-house database emerging from previous studies (Hunter *et al.* 2003, Crous *et al.* 2001) and those obtained from GenBank (Table 1). Phylogenetic analysis of the aligned sequences was conducted using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b (Swofford 1998). The sequences were analysed using parsimony, with trees generated by heuristic searches, simple addition and Tree Bisection Reconstruction (TBR) branch swapping. Bootstrap values for the branching points were calculated using 1000 replicates (Felsenstein 1993). In the phylogenetic analysis, *Ramulispora anguoides* (Nirenberg) Crous was used as the outgroup taxon.

RESULTS

Sample collection and isolation

Symptoms of MLB were found on *E. globulus* at several localities, including Wondo Genet, Hossana, Endibir, Bedele, Menagesha, Holeta and Addis Alem (Table 1).

Disease symptoms, including shoot die-back and leaf blotch (Figure 2a-2d) were common. In some cases, nearly 100% of the leaves on a tree and nearly 100% of the leaf surfaces of these trees were affected (Figure 2c). Lesions varied in size from small to large spots spreading over the whole leaf surface. Some lesions coalesced to form larger lesions (Figure 2a, 2b). The lesions were light brown in colour and had raised brown margins. On some leaves, lesions were confined to the margins of the leaves. Other samples had leaf spots that extended through the leaf laminas, with lesions visible on both leaf surfaces with a light brown colour and a faint red margin (Figure 2).

Ascospores germinated within 24 hours. *Mycosphaerella* spp. were successfully isolated from samples collected from 16 trees. Ascospores from a number of samples failed to germinate, while some isolates died shortly after germination. Representative isolates were, however, obtained from all areas sampled.

Morphological characterisation

When the growth of the fungi on MEA was considered, three culture morphologies were found (Figure 3a, b, c). Four *Mycosphaerella* isolates (CMW10186, CMW10189, CMW10376, CMW10187) obtained from *E. globulus* leaves collected from Addis Alem, Endibir and Hossana (Table 1) had similar colony morphology and constituted one group designated as Group I (Figure 3a). The colony colour of this group is olivaceous black 27''''m. Group II isolates (CMW11148, CMW11149, CMW10377, CMW11150), obtained from Hossana, Endibir, Holeta, and Bedele (Table 1) showed a dark olivaceous grey colour, 23''''i (Figure 3b). The third group included only one isolate (CMW10190) obtained near Hossana. This isolate had a pale olivaceous grey colour (23''''f) (Figure 3c).

Examination of the ascospore germination of *Mycosphaerella* isolates obtained from Ethiopia showed three different germination patterns. These germination patterns could be directly correlated to the morphological groups defined based on culture morphology (Figure 4). Isolates belonging to morphotype I (CMW10186, CMW10189, CMW10376 and CMW10187) had an ascospore germination pattern

closely resembling a Type F pattern (Figure 4a). This pattern is characteristics of *M. juvenis*. The four isolates in morphotype Group II (CMW11148, CMW11149, CMW10377, CMW11150) had Type C (Crous 1998) germination patterns (Figure 4b). This type of germination is characteristic of *M. heimii, M. gregaria* Carnegie & Keane, *M. molleriana* and *M. nubilosa* (Crous 1998). The isolate obtained from Hossana (CMW10190) had a Type B germination pattern (Figure 4c) which is associated with *M. gracilis* Crous & Alfenas and *M. marksii* (Crous 1998). No anamorph structures were found for any of the Ethiopian *Mycosphaerella* isolates.

DNA sequencing and phylogenetic analysis

Amplification of the ITS region of the rRNA operon produced a similar sized fragment of approximately 600 bp for all *Mycosphaerella* isolates obtained from Ethiopia. A BLAST search using sequences of Ethiopian *Mycosphaerella* isolates showed that these isolates were closely related to three different *Mycosphaerella* species. When the sequence data were incorporated into a larger data base of sequences from previous studies including those in GenBank and analysed, 12 trees were generated. These trees had the same topology. The number of characters in the analysed data set was 705 bp's, of which 267 characters were constant, 131 variable characters were parsimony uninformative and 307 characters were parsimony informative. The phylogenetic tree generated using a heuristic search had CI and RI values of 0.698 and 0.861 respectively. In all parsimonious phylogenetic trees (Figure 5) one of the Ethiopian *Mycosphaerella* isolates (CMW10190), grouped with *M. marksii* with 100% bootstrap support. Four of the isolates (CMW10186, CMW10189, CMW10376 and CMW10187) grouped with *M. grandis* (100% bootstrap support) and the remaining four isolates, (CMW11148, CMW11149, CMW10377 and CMW11150) resided in the *M. nubilosa* clade (100% bootstrap support).

DISCUSSION

Mycosphaerella leaf blotch was the most common foliage disease observed on *E. globulus* in Ethiopia, during surveys in 2000 and 2001 (Alemu *et al.* 2003). Results

of the present study provide the first identification of this group of fungi on *Eucalyptus* in Ethiopia. Three *Mycosphaerella* spp., namely *M. grandis, M. nubilosa* and *M. marksii* were thus identified and this study represents the first report of these *Mycosphaerella* spp. on *Eucalyptus* spp. from Ethiopia. This study also represents the first report of *M. grandis* from a country other than Australia.

Ascospore germination patterns present a useful method to differentiate between *Mycosphaerella* spp. (Park & Keane 1982a). Crous (1998) described 14 types of ascospore germination patterns for *Mycosphaerella* spp. Examination of the ascospore germination patterns revealed that three different species of *Mycosphaerella* were linked to MLD in Ethiopia. The occurrence of 3 different species was supported by DNA sequence data confirming the value that germination patterns have when identifying *Mycosphaerella* spp.

Mycosphaerella marksii was found only from a single leaf sample collected from *E. globulus* near Hossana. Previous studies have shown that *M. marksii* occurs on several *Eucalyptus* spp., including *E. globulus, E. grandis, E. nitens* and *E. saligna* (Carnegie *et al.* 1994). This fungus was first described in Australia and it is now known to occur in South Africa, Indonesia, Portugal and Uruguay (Carnegie *et al.* 1994, Crous & Wingfield 1996, Carnegie & Keane 1997, Crous 1998). This fungus is common in Australia and South Africa, but has not been reported to cause significant damage (Carnegie 2000, Hunter *et al.* 2003). Because the fungus was collected only from a single leaf, it is probably not an important component of the MLD problem in Ethiopia.

Mycosphaerella grandis was found on samples collected from Addis Alem, Endibir and Hossana. This fungus was first described from Australia on *E. grandis*, *E. globulus* and *E. nitens* (Carnegie & Keane 1994, Carnegie 2000). According to Carnegie & Keane (1994), this pathogen is a common cause of necrotic lesions at the margins of leaves. This type of symptom was common in Ethiopia, suggesting that *M. grandis* is one of the more important components of MLD in the country. This is the first report of this species outside of Australia, and given its occurrence in Ethiopia, it might be expected to be found in neighbouring countries in the future.

Mycosphaerella nubilosa was found in several areas including Endibir, Holeta, Hossana, and Bedele. This species mostly affects juvenile leaves of *E. globulus* (Park & Keane 1982a, Purnell & Lundquist 1986, Carnegie *et al.* 1994). This is also one of the most common and destructive foliage pathogens of *Eucalyptus* in Australia, New Zealand and South Africa (Park & Keane 1982a, Dick & Gadgil 1983, Purnell and Lundquist 1986, Hunter *et al.* 2003). *M. nubilosa* and *M. molleriana* were once regarded as the same fungus (Crous, Wingfield & Park 1990), but it has been shown that they represent distinct species (Crous & Wingfield 1997, Crous 1998, Crous *et al.* 1999). The presence of *M. nubilosa* in Ethiopia explains the serious defoliation of *E. globulus* in this country. This fungus should be placed on the list of more important constraints to *E. globulus* propagation in the future.

This study has shown that MLD is common, wherever *E. globulus* is grown in Ethiopia. Previous studies have shown that infection by *Mycosphaerella* spp. not only causes premature defoliation and retarded growth, but can also lead to the abandonment of planting certain *Eucalyptus* spp. (Lundquist & Purnell 1987). In South Africa, for example, the planting of *E. globulus* was terminated in the 1940's as a result of MLB (Lundquist & Purnell 1987). As *E. globulus* is a widely planted species in Ethiopia, the discovery of *M. nubilosa* is of concern, especially given the fact that plantations of this species are likely to be expanded in the future. Selection of species and provenances with tolerance to MLB might help to minimise loss of yield caused by *Mycosphaerella* species in the future.

REFERENCES

Ahumada, R (2002) *Diseases in commercial Eucalyptus plantations in Chile, with special reference to Mycosphaerella and Botryosphaeria species.* M. Sc. Thesis. The Faculty of Natural and Agricultural Science. University of Pretoria, South Africa.

Alemu Gezahgne, Roux, J. & Wingfield, M. J. (2003) Diseases of exotic plantation *Eucalyptus* and *Pinus* species in Ethiopia. *South African Journal of Science* **99**:29-33.

Anonymous (1994) *Ethiopian Forestry Action Program*. Final Report, Vol. I, pp14 and vol. II, pp34. Addis Ababa Ethiopia.

Beresford, R. M. (1978) *Mycosphaerella nubilosa (Cooke) Hansf. on Eucalyptus delegatensis R. T. Baker: Further studies of epidemiology in the northern island of New Zealand.*, Ph.D Thesis. University of Auckland, New Zealand.

Carnegie, A. J. & Keane, P. J. (1994) Further *Mycosphaerella* species associated with leaf diseases of *Eucalyptus*. *Mycological Research* **98**: 413-418.

Carnegie, A. J., Keane, P. J., Ades, P. K. & Smith, I. W. (1994) Variation in susceptibility of *Eucalyptus globulus* provenances to Mycosphaerella leaf disease. *Canadian Journal of Forestry Research* **24**: 1751-1757.

Carnegie, A. J. & Keane, P. J. (1997) A revised *Mycosphaerella gregaria* nom. nov. for *M. aggregata* on *Eucalyptus*. *Mycological Research* **101**:843-844.

Carnegie, A. J. & Keane, P. J. (1998) *Mycosphaerella vespa* sp. nov. from diseased *Eucalyptus* leaves in Australia. *Mycological Research* **102**:1274-1276.

Carnegie, A. J. (2000) *A study of the species of Mycosphaerella on Eucalyptus in Australia and the impact of the Mycosphaerella leaf diseases on Eucalyptus globulus Labill.* Ph. D Thesis. The University of Melbourne, Australia.

Chipompha, N. W. S. (1987) *Phaeoseptoria eucalypti*: A new pathogen of *Eucalyptus* in Malawi. *South African Journal of Science* **142**: 10-12.

Corlett, M. (1991) An annotated list of the published names of *Mycosphaerella* and *Sphaerella*. *Mycologia Memoir* **18**: 1-328.

Crous, P. W., Wingfield, M. J., Marasas, W. F. O. & Sutton, B. C. (1989) *Pseudocercospora eucalyptorum* sp. nov. on *Eucalyptus* leaves. *Mycological Research* **93**: 394-398.

Crous, P. W., Wingfield, M. J. & Park, R. F. (1990) *Mycosphaerella nubilosa*, a synonym of *M. molleriana*. *Mycological Research* **95**: 628-632.

Crous, P.W., Phillips, A. J. L. & Wingfield, M. J. (1991) The genera *Cylindrocladium* and *Cylindrocladiella* in South Africa, with special reference to forestry nurseries. *South African Forestry Journal* **157**: 68-85.

Crous, P. W., Ferreria, F. A., Alfenas, A. C. & Wingfield, M. J. (1993) *Mycosphaerella suberosa* associated with corky leaf spots on *Eucalyptus* in Brazil. *Mycologia* **85**: 705-710.

Crous, P. W. & Wingfield, M. J. (1996) Species of *Mycosphaerella* and their

anamorphs associated with leaf blotch disease of *Eucalyptus* in South Africa. *Mycologia* **88**: 441-458.

Crous, P. W. & Wingfield, M. J. (1997) *Colletogloeopsis*, a new Coelomycetes genus to accommodate anamorphs of two species of *Mycosphaerella* on *Eucalyptus*. *Canadian Journal of Botany* **75**: 781-790.

Crous, P. W. (1998) *Mycosphaerella* spp. and their anamorphs associated with leaf spot diseases of *Eucalyptus*. *Mycologia Memoir* **21**: 1-170.

Crous, P. W., Hong, L., Wingfield, M. J., Wingfield, B. D. & Kang, J. C. (1999) *Uwebraunia* and *Dissoconium*, two morphologically similar anamorph genera with different teleomorph affinity. *Sydowia* **51**: 155-166.

Crous, P. W., Hong, L., Wingfield, B. D. & Wingfield, M. J. (2001) ITS rDNA phylogeny of selected *Mycosphaerella* species and their anamorphs occurring on *Myrtaceae*. *Mycological Research* **105**: 425-431.

Dick, M. (1982) Leaf inhabiting fungi of *Eucalyptus* in New Zealand. *Journal of Forest Science* **12**: 525-537.

Dick, M. & Gadgil, P. D. (1983) *Eucalyptus* leaf spots. *Forest Pathology in New Zealand* **1**: 1-7.

Eldridge, K., Davidson, J., Harwood, C. & Van Wyk, G. (1997) *Eucalyptus* domestication and breeding. Oxford Science Publications.

Felsentein, J. (1993) DNABOOT-Bootstrap Confidence Interval on DNA parsimony 3.1. Seattle, University of Washington DC.

Hunter, G. C., Crous, P. W., Roux, J., Coutinho, T. A., Wingfield, B. D. & Wingfield M. J. (2003) Identification of *Mycosphaerella* spp. associated with leaf disease of *Eucalyptus nitens* in South Africa. *Australian Journal of Pathology* (Submitted).

Lundquist, J. E. & Purnell, R. C. (1987) Effects of Mycosphaerella leaf spot on growth of *Eucalyptus nitens*. *Plant Disease* **71**: 1025-1029.

Milgate, A. W., Yuan, Z. Q., Vailancourt, R. E. & Mohammed C. (2001) *Mycosphaerella* species occurring on *Eucalyptus globulus* and *Eucalyptus nitens* plantations of Tasmania, Australia. *Forest Pathology* **31**: 53-63.

Park, R. F. & Keane, P. J. (1982a) Three *Mycosphaerella* species from leaf diseases of *Eucalyptus*. *Transactions of the British Mycological Society* **79**: 95-100.

Park, R. F. & Keane, P. J. (1982b) Leaf disease of *Eucalyptus* associated with

Mycosphaerella species. *Transactions of the British Mycological Society* **79**: 101-115.

Park, R. F. & Keane, P. J. (1984) Further *Mycosphaerella* species causing leaf diseases of *Eucalyptus*. *Transactions of the British Mycological Society* **83**: 93-105.

Park, R. F. (1988) Effects of certain hosts, inoculum and environmental factors on infections of *Eucalyptus* species by two *Mycosphaerella* species. *Transactions of the British Mycological Society* **91**: 261-266.

Persson, A. (1995) Exotics-prospects and risks from European and African view point. *Agricultural Science* **9**: 47-62.

Purnell, R. C. & Lundquist, J. E. (1986) Provenance variation of *Eucalyptus nitens* on the Eastern Transvaal Highveld in South Africa. *South African Forestry Journal* **138**: 23-31.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Applied Microbiology* **1**: 17-20.

Rayner, R. W. (1970) *A mycological colour chart*. Commonwealth Mycological Institute, Kew.

Schoch, C. L., Crous, P. W., Wingfield, B. D. & Wingfield, M. J. (1999) The *Cylindrocladium candelabrum* species complex includes distinct mating populations. *Mycologia* **91**: 286-298.

Sharma, J. K. & Mohanan, C. (1982) *Cylindrocladium* spp. associated with various diseases of *Eucalyptus* in Kerala. *European Journal of Forest Pathology* **12**: 129-136

Swofford, D. L. (1998) PAUP*. *Phylogenetic Analysis Using Parsimony* (*and other methods). Version 4.0. Beta version. Sinauer Associates. Sunderland, Massachusetts.

Vilgalys, R. & Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238-4246.

Von Arx, J. A. (1983) *Mycosphaerella* and its anamorphs. *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen, Series* C **86**: 15-54.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR*

protocols: a guide to methods and applications. San Diego, Academic Press.

Wingfield, M. J. (1990) Pathogens in exotic plantation forestry. *International Forestry Review* **1**: 163-168.

Wingfield, M. J. & Roux, J. (2000) Plant disease of South African forest plantations. In *South African Forestry Handbook* (D. L. Owen ed):pp 241-252. The South African Forestry Institute. Pretoria, South Africa.

Table 1. *Mycosphaerella* isolates used in this study.

Isolates	Species	Host	Origin	Collector	Accession No.
CMW10190[a]	*M. marksii*	*Eucalyptus globulus*	Ethiopia	Alemu Gezahgne & J. Roux	AY244404
CMW10186[a]	*M. grandis*	,,	Ethiopia	Alemu Gezahgne & J. Roux	AY244405
CMW10187[a]	*M. grandis*	,,	Ethiopia	Alemu Gezahgne & J. Roux	AY244406
CMW10377[a]	*M. nubilosa*	,,	Ethiopia	Alemu Gezahgne & J. Roux	AY244408
CMW10189[a]	*M.grandis*	,,	Ethiopia	Alemu Gezahgne & J. Roux	AY244412
CMW10376[a]	*M. grandis*	,,	Ethiopia	Alemu Gezahgne & J. Roux	AY244407
CMW11148[a]	*M. nubilosa*	,,	Ethiopia	Alemu Gezahgne & J. Roux	AY244409
CMW11149[a]	*M. nubilosa*	,,	Ethiopia	Alemu Gezahgne & J. Roux	AY244411
CMW11150[a]	*M. nubilosa*	,,	Ethiopia	Alemu Gezahgne & J. Roux	AY244410
CMW9090	*M. marksii*	*E. grandis*	South Africa	M. J. Wingfield	AF468870
CMW9091	*M. marksii*	,,	,,	M. J. Wingfield	AF468871
CMW9092	*M. marksii*	,,	,,	M. J. Wingfield	AF468872
CMW3358	*M. parkii*	*E. grandis*	Australia	A. J. Carnegie	AF309590
CMW4945	*M. africana*	*E. viminalis*	South Africa	P. W. Crous	AF309602
CMW4942	*M. heimii*	*Eucalyptus* spp.	Madagascar	P. W. Crous	AF309606
CMW5705	*M. heimii*	*Eucalyptus* spp.	Brazil	P. W. Crous	AF452508
CMW5224	*M. flexuosa*	*E. globulus*	Colombia	M. J. Wingfield	AF309603
CMW4937	*M. juvenis*	*E. grandis*	South Africa	M. J. Wingfield	AF309604
CMW4036	*M. juvenis*	,,	,,	M. J. Wingfield	AF309605
CMW3282	*M . nubilosa*	*E. globulus*	Australia	A. J. Carnegie	AF309618
CMW4940	*M. molleriana*	*E. globulus*	Portugal	S. McCare	AF309620
CMW2734	*M. molleriana*	*E. globulus*	California (USA)	M. J. Wingfield	AF309619

[a]Isolates collected from *E. globulus* in Ethiopia and sequenced in this study. All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. All other sequences are obtained from Crous *et al.*, (2001) and Hunter *et al.* (2003).

Figure 1. Map of Ethiopia showing the plantation areas where samples were collected.

Figure 2. Symptoms of Mycosphaerella leaf blotch. (A) Extensive leaf spotting on leaf surfaces, (B) Necrotic leaf lesions especially on the edges of young leaves (C) senescing leaves as a result of severe infection, (D) small round leaf spots showing pseudothecia.

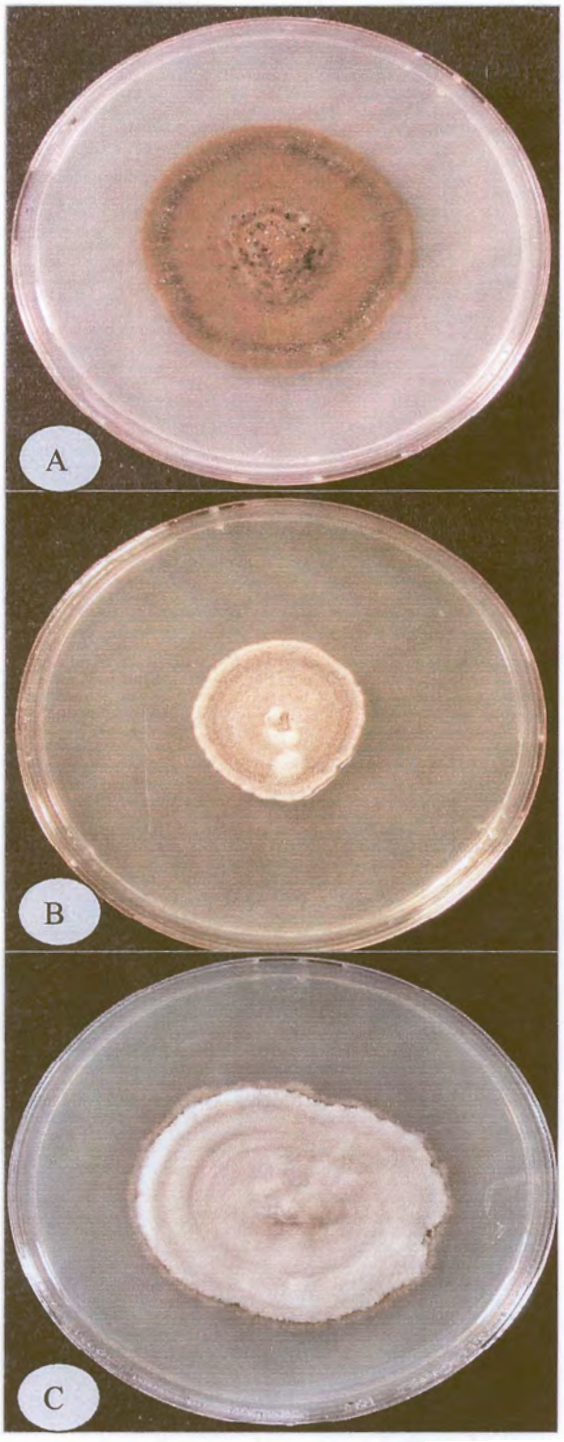

Figure 3. Cultural characteristics of *Mycosphaerella* spp. (A) Culture of *Mycosphaerella sp.* of group I on MEA, (B) Culture of *Mycosphaerella* spp. of group II on MEA and (C) Culture of *Mycosphaerella sp.* of group III. on MEA.

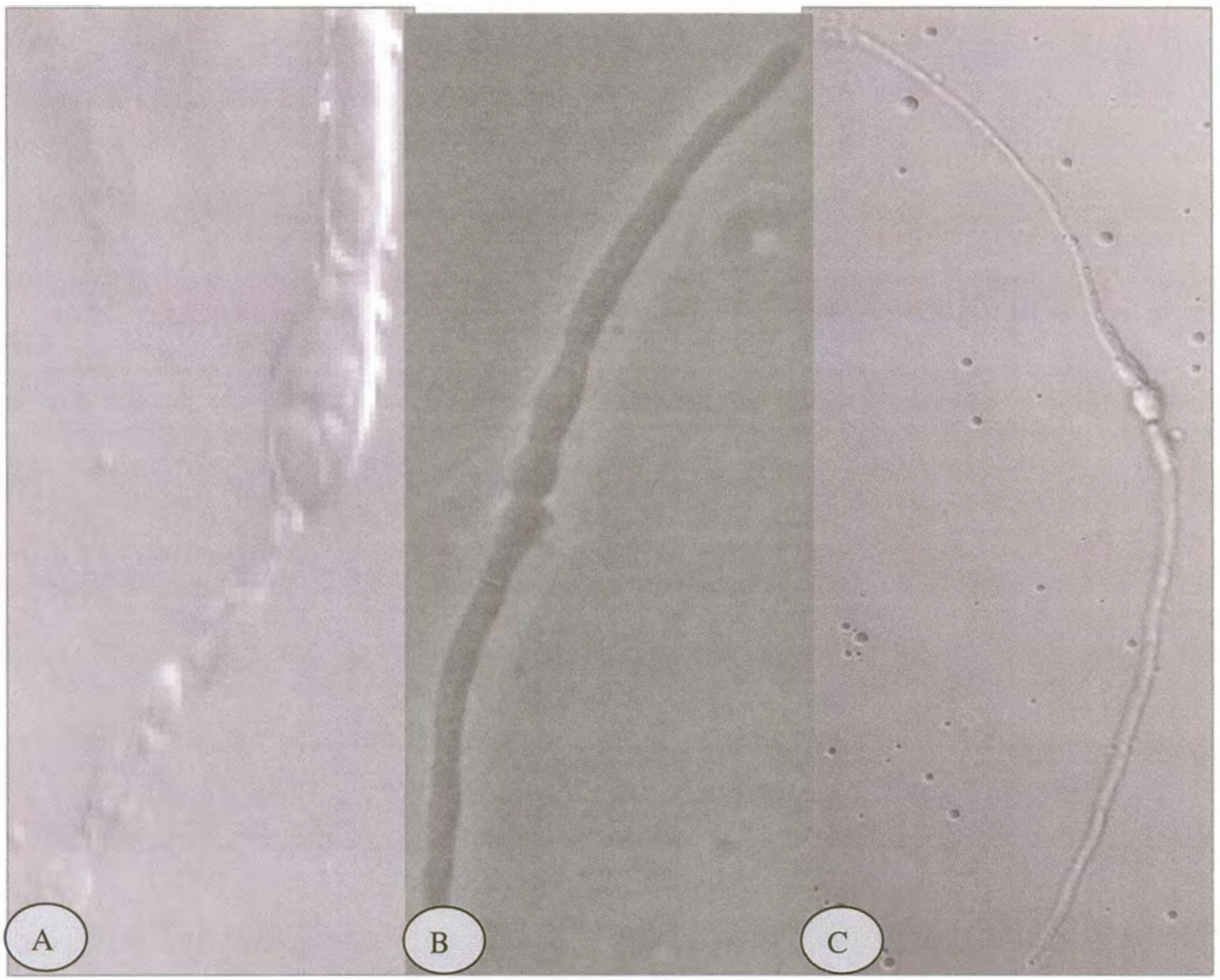

Figure 4. Ascospore germination patterns of *Mycosphaerella* spp. (A) Type F ascospore germination pattern of Group I, (B) Type C ascospore germination pattern ofGroup II, (C) Type B ascospore germination pattern of Group III.

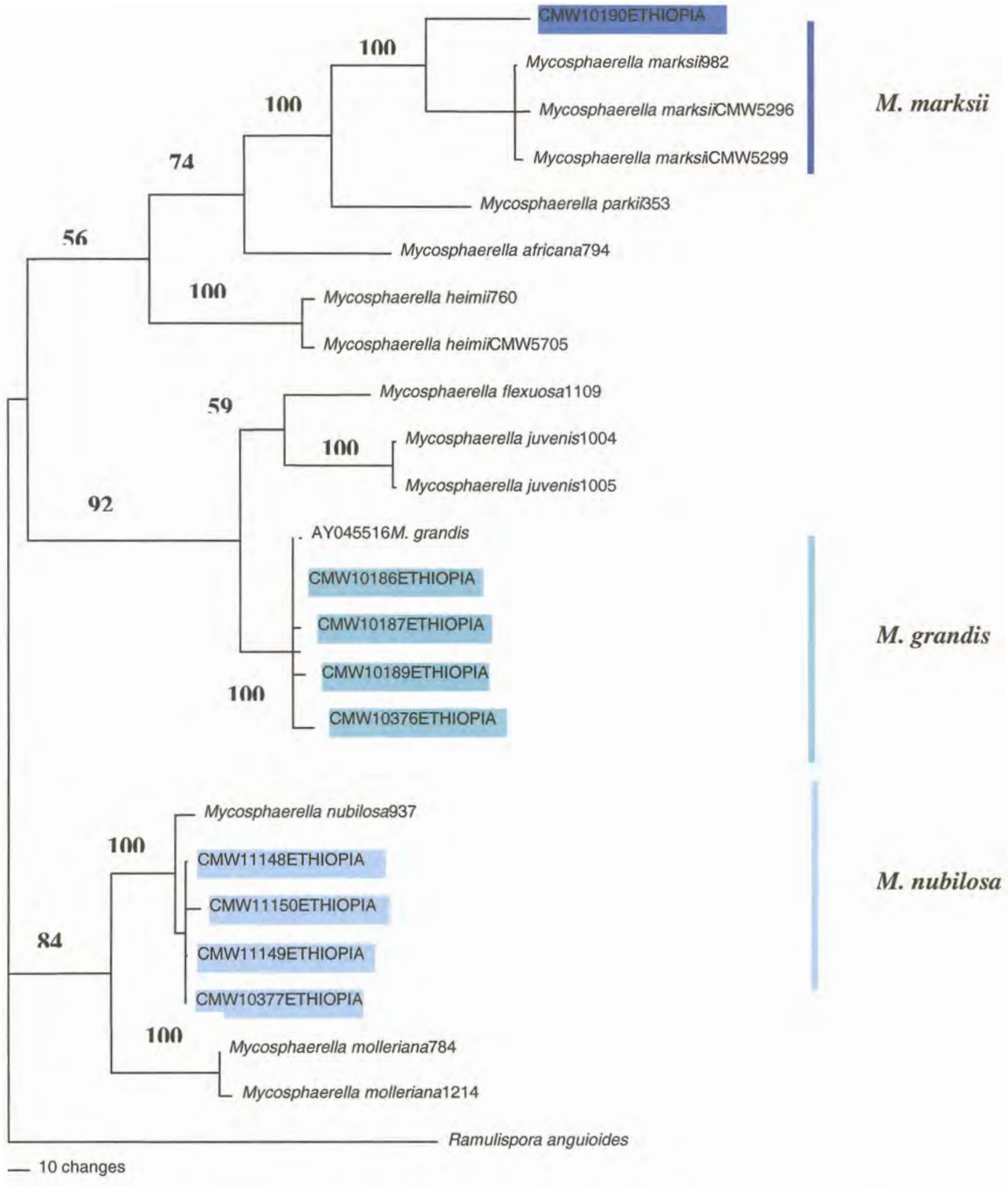

Figure 4. Phylogenetic tree of the ITS sequence data of *Mycosphaerella* spp. Number of characters = 705, CI = 0.691 and RI = 0.861. Bootstrap values are shown at each branch.

Figure 6. Aligned ITS sequence of *Mycosphaerella* species. (-)=Gaps, (.)= Homologous nucleotides (N)= unknown bases

```
                                  10        20        30        40        50        60        70
CMW10190Ethiopia                  TCCGTAGGTG AACC-TGCGG AGGGATCATT ACC-GAG--C GGAGGGCCC- -CGG-CCCG- -----ACCTC
Mycosphaerella_marksiiCMW9090     .......... .......G.. .......... .........- T.....TTT. .......... ..........
Mycosphaerella_marksiiCMW9091     ..G....... .........A .......... .........- T.....TTT. CG...-.... ..........
Mycosphaerella_marksiiCMW9092     .......... .......... .......... .........- T.....TTT. .......... ..........
Mycosphaerella_parkii353          .......... .......... .......... ..T......- T.....TTTC A.C..-.... ..........
Mycosphaerella_africana794        .......... .......... .......... ..T......- T......-TC A..-...... ..........
Mycosphaerella_flexuosa1109       ..G....... .......... .......... ..T......- T.....-.TC CG.-...... ..........
AY045516M.grandis                 .......... .......... .......... .........- T.......TC CG....T... ..........
CMW10186Ethiopia                  .......... .......... .......... .........- T.......TC CG....T... ..........
CMW10187Ethiopia                  .......... .......... .......... -........- T.......TC CG....T... ..........
CMW10189Ethiopia                  N......... G.AAC..... .......... .........- T.......TC CG....T... .........T
Mycosphaerella_juvenis1004        .......... .......... .......... .........- T......-TC CG.-...... ..........
Mycosphaerella_juvenis1005        .......... .......... .......... .........- T......-TC CG.-...... ..........
Mycosphaerella_heimii760          .......... .......... .......... ..T......- T......TA. .G.-.T.... ..........
Mycosphaerella_heimiiCMW5705      .......... .......... .......... ..T......- T......TA. CG.-.T.... ..........
Mycosphaerella_nubilosa937        .......... .......... .......... .........- T......--G G.A....... ..........
CMW11148Ethiopia                  .......... G.A....... .......... ..T......- T.C....--G C.A....... ..........
CMW11149Ethiopia                  NNNNNNNNNN NNNNC..... .......... ..T......- T.C....--G C.A....... .........CT
CMW11150Ethiopia                  NNNNNNNNNN N..-...... .......... ..T......- T.C....--G C.A....... ..........
CMW10377Ethiopia                  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNG C.A....... ..........
CMW10376Ethiopia                  .......... .......... .......... .........- T.......T. C...G.T... ..........
Mycosphaerella_molleriana784      .......... .......... .......... ..T......- T......--G CAA....... ..........
Mycosphaerella_molleriana1214     .......... .......... .......... ..T......- T......--G CAA....... ..........
Ramulispora_anguioides            .......... .......... .A........ .ATA...CAA T...C.T.AG .-C.C....G GAGCA.-TC.
```

```
                                  80         90         100        110        120        130        140
CMW10190Ethiopia                  ------CAA- CCCT------ -----TT--- --GT------ -------GAA ---------- -----TCA--
Mycosphaerella_marksiiCMW9090     .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9091     .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9092     .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_parkii353          .......... .......... .......... .......... .......... .......... .....C...C
Mycosphaerella_africana794        .......... .......... .......... .......... .......... .......... .....C....
Mycosphaerella_flexuosa1109       .......... .......... .......... .......... .......... ......TTCG ACCT.---..
AY045516M.grandis                 .......... ...C...... ....A..... .........A TTCC.....- .......... .....---..
CMW10186Ethiopia                  .......... ...C...... ....A..... .........A TTCC.....- .......... .....---..
CMW10187Ethiopia                  .......... ...C...... ...CA..... .........A TTCC.....- .......... .....---..
CMW10189Ethiopia                  .....C.... ...C...... ....A..... .........A TTCC.....- .......... .....---..
Mycosphaerella_juvenis1004        .......... ...C...... ....A.-... .......... .......... .......... ....TCT-.C
Mycosphaerella_juvenis1005        .......... ...C...... ....A.-... .......... .......... .......... ....TCT-.C
Mycosphaerella_heimii760          .......... .......... .......... .......... .......... .......... .....C....
Mycosphaerella_heimiiCMW5705      .......... .......... .......... .......... .......... .......... .....C....
Mycosphaerella_nubilosa937        CT....--.. ...C...... ....A.-... .......... TTTC...--- .......... .....C..CC
CMW11148Ethiopia                  C.....-..A ...C...... ....A.-... .......... TTTC...--- .......... .....C..CC
CMW11149Ethiopia                  CC....-..A ...C...... ....A.-... .........T TTTC...--- .......... .....-..CC
CMW11150BEthiopia                 C.....-..A ...C...... ....A.-... .......... TTTC...--- .......... .....C..CC
CMW10377Ethiopia                  C.....-..A ...C...... ....A.-... .......... TTTC...--- .......... .....C..CC
CMW10376Ethiopia                  C.....--.A ...C...... ....A..... .........A TT.....--- .......... .....C.G..
Mycosphaerella_molleriana784      ......-C.A ...C...... ....A.-... .......... .TTC...--- .......... ....CAA.CC
Mycosphaerella_molleriana1214     ......-C.A ...C...... ....A.-... .......... .TTC...--- .......... ....CAA.CC
Ramulispora_anguioides            TGGGGG.C.C ..TCCTCGGA GGGTT.AGAG AC..CGAGCC TCTCGGA... GCTCGGTTCA GACCT---CC
```

```
                                  150        160        170        180        190        200        210
CMW10190Ethiopia                  -AACCT---- ---------- -GTTGCTTCG G--GGGCGAC CCT-GCC--- --G-TTC-GC GGCGA-----
Mycosphaerella_marksiiCMW9090     A.C.T-.... .......... .......... .......... .......... .......... ....-C....
Mycosphaerella_marksiiCMW5296     A.C..-.... .......... .......... .......... .......... .......... ....-C....
Mycosphaerella_marksiiCMW5299     A.C.T-.... ........GT T......... .......... .......... .......... ....-C....
Mycosphaerella_parkii353          A.-.T-.... .......... T......... .......... .......... .......... ...ATC....
Mycosphaerella_africana794        ..-.T-.... ........C. T......... .......... ..C....... ...T...G.. .A.--.....
Mycosphaerella_flexuosa1109       .--.T-.... .......... ......C... .......... ..G....CTC T...---G.T .-.--C....
AY045516M.grandis                 .-C.T-.... ........CT T.....C... .......... ..G....TTC .G.GC--G-- ---TC.....
CMW10186Ethiopia                  .-C.T-.... ........CT T.....C... .......... ..G....TTC .G.GC--G-- ---TC.....
CMW10187Ethiopia                  .-C.T-.... ........CT T.....-... .......... ..G....TTC .G.GC--G-- ---TC.....
CMW10189Ethiopia                  .-C.T-.... ........CT T.....C... .......... ..G....TTC .G.GC--G-- ---TC.....
Mycosphaerella_juvenis1004        ..C.T-.... ........C. T.....C... ......T... ..G....CTC TG.G.--G-- ---CC.....
Mycosphaerella_juvenis1005        ..C.T-.... ........C. T.....C... ......T... ..G....CTC TG.G.--G-- ---CC.....
Mycosphaerella_heimii760          A.-.T-.... .......... T......... .......... .......... .G-.C.TG.G C.GT-....G
Mycosphaerella_heimiiCMW5705      A.-.T-.... .......... T......... .......... .......... .G-.C.TC.G C.GT-....G
Mycosphaerella_nubilosa937        A-C---.... .......... ......C... .......... ..G....CCC .G-.---C.- ---CC.....
CMW11148Ethiopia                  A-C---.... .......... ......C... .......... ..G....CCC .G-.---C.- ---CC.....
CMW11149Ethiopia                  A-C---.... .......... ......C... .......... ..G....CCC .G-.---C.- ---CC.....
CMW11150Ethiopia                  A-C---.... .......... ......C... .......... ..G....CCC .G-.---C.- ---CC.....
CMW10377Ethiopia                  A-C---.... .......... ......C... .......... ..G....CCC .G-.---C.- ---CC.....
CMW10376Ethiopia                  ..-....... ........CT T.....C... .......... ..G......T TC..---..G C.-TC.....
Mycosphaerella_molleriana784      A-C---.... .......... ......C... .......... ..G....... .G-.---C-- C...CC....
Mycosphaerella_molleriana1214     A-C---.... .......... ......C... .......... ..G....... .G-.---C-- C...CC....
Ramulispora_anguioides            ACC.T.GAAT AAAAAACCTT T.......T. .CA..A..C. T.GC...AGC GG-.C.TC.G C-T.TTGAG.
```

```
                                  220        230        240        250        260        270        280
CMW10190ETHIOPIA                  -GGC-GCCCC CGGGGGAAA- ----TCAAAC A-CTGCGTCA ATTTG-TGTC GGAGTA---- -CTT------
Mycosphaerella_marksiiCMW9090     ......... ......... ......... ......... ......... ......... .........
Mycosphaerella_marksiiCMW9091     ......... ......... ......... ......... ......... ......... .........
Mycosphaerella_marksiiCMW9092     ......... ......... ......... ......... ......... ......... .........
Mycosphaerella_parkii353          .-....... ...A...--T AC.T.--... C.....A... --....C... ......A... TT..A.....
Mycosphaerella_africana794        ....G..... ...A..--TC A......... ......A..- -.....C... .....-.... ....AAAGTA
Mycosphaerella_flexuosa1109       ...-G..... ...C...--C ACC...-... -T....A..- -.....C... T.....TGAT AT.....GAA
AY045516M.grandis                 ...-G..... ...T...CC. A......... -T....A..- -....AC... T.....AA.T AT.-...GAA
CMW10186Ethiopia                  ...-G..... ...T...CC. A......... -T....A..- -....AC... T.....AA.T AT.-...GAA
CMW10187Ethiopia                  ...-G..... ...T...CC. A......... -T....A..- -....AC... T.....AA.T AT.-...GAA
CMW10189Ethiopia                  ...-G..... ...T...CC. A......... -T....A..- -....AC... T.....AA.T AT.-...GAA
Mycosphaerella_juvenis1004        ...-G..... ...C...CC. A..C..-... -T....A..- -.G...C... T.....AA.T AT.....GAA
Mycosphaerella_juvenis1005        ...-G..... ...C...CC. A..C..-... -T....A..- -.G...-... T.....AA.T AT.....GAA
Mycosphaerella_heimii760          C......... ...A..-CC. ATT.--.... ......A... -.-...C... ......AA.. .---..AG..
Mycosphaerella_heimiiCMW5705      C......... ...A..-CC. ATT.--.... ......A... -.-...C... ......AA.. .---..AG..
Mycosphaerella_nubilosa937        ...-G....T ..CA.A.CCC CTC.---... GG...GA..- -.G...C..- ......A.TA C---.AA...
CMW11148Ethiopia                  ...-G....T ..CA...CCC CTC.---... G.....A..- -.G...C... ......A.TA C---.AA...
CMW11149Ethiopia                  ...-G....T ..CA...CCC CTC.---... G.....A..- -.G...C... ......A.TA C---.AA...
CMW11150Ethiopia                  ...-G....T ..CA...CCC CTC.---... G.....A..- -.G...C... ......A.TA C---.AA...
CMW10377Ethiopia                  ...-G....T ..CA...CCC CTC.---... G.....A..- -.G...C... ......A.TA C---.AA...
CMW10376ETHIOPIA                  ...-G..... ...T...CC. ATC.---..A C.TCTGCATC --..TGACGT CTGAGTAAAT AT.-....GA
Mycosphaerella_molleriana784      ...-G..... ...T...CCC .TC.---... T.....A..- -.C...C... T....-..CA C---.AA...
Mycosphaerella_molleriana1214     ...-G..... ...T...CCC .TC.---... T.....A..- -.C...C... T....-..CA C---.AA...
Ramulispora_anguioides            .--TGC.TG. .A.A...CCA C...---... TCT..TT.TT .-G..A.... T......CTA TA.AATAG..
```

```
                                290        300        310        320        330        340        350

CMW10190Ethiopia                -----GTTAA -TAAA-CAAA AC-------- TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA
Mycosphaerella_marksiiCMW9090   .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9091   .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9092   .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_parkii353        .....-.... ...C.T-... .......... .......... .......... .......... ..........
Mycosphaerella_africana794      ..AA.-.... A----..... .......... .......... .......... .......... ..........
Mycosphaerella_flexuosa1109     TCAA.----- .----T.... .......... .......... .......... .......... ..........
AY045516M.grandis               TCAA.----- .----T.... .......... ...T...... .......... .......... ..........
CMW10186Ethiopia                TCAA.----- .----T.... .......... ...T...... .......... .......... ..........
CMW10187Ethiopia                TCAA.----- .----T.... .......... ...T...... .......... .......... ..........
CMW1089Ethiopia                 TCAA.----- .----T.... .......... ...T...... .......... .......... ..........
Mycosphaerella_juvenis1004      TCAAA----- .----T.... .......... .......... .......... .......... ..........
Mycosphaerella_juvenis1005      TCAAA----- .----T.... .......... .......... .......... .......... ..........
Mycosphaerella_heimii760        T.AAA-.... A----..... .......... .......... .......... ....CA.... ..........
Mycosphaerella_heimiiCMW5705    T.AAA-.... A----..... .......... .......... .......... ....CA.... ..........
Mycosphaerella_nubilosa937      CCAA.----- .----T...- --.TTAAAAC .......... .......... .......... ..........
CMW11148Ethiopia                CCAA.----- .----T...- --.TTAAAAC .......... .......... .......... ..........
CMW11149Ethiopia                CCAA.----- .----T...- --.TTAAAAC .......... .......... .......... ..........
CMW11150Ethiopia                CCAA.----- .----T...- --.TTAAAAC .......... .......... .......... ..........
CMW10377Ethiopia                CCAA.----- .----T...- --.TTAAAAC .......... .......... .......... ..........
CMW10376Ethiopa                 ATCAA----- .----T.... .......... ...T...... .......... .......... ..........
Mycosphaerella_molleriana784    ..AA.----- .----T...T CAATCAAAAC .......... .......... .......... ..........
Mycosphaerella_molleriana1214   ..AA.----- .----T...T CAATCAAAAC .......... .......... .......... ..........
Ramulispora_anguioides          .....----- .----.---- --........ ..AA..TTT CA.CAA.GGA TC...T.GT. .TGGC.TCG.
```

```
                                  360        370        380        390        400        410        420
CMW10190Ethiopia                  CGCAGCGAAA TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA ACGCACATTG
Mycosphaerella_marksiiCMW9090     .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9091     .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9092     .......... .......... .......... ...C...... .......... .......... ..........
Mycosphaerella_parkii353          .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_africana794        .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_flexuosa1109       .......... .......... .......... .......... .......... .......... ..........
AY045516M.grandis                 .......... .......... ....-..... .......... .......... .......... ..........
CMW10186Ethiopia                  .......... .......... .......... .......... .......... .......... ..........
CMW10187Ethiopia                  .......... .......... .......... .......... .......... .......... ..........
CMW10189Ethiopia                  .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_juvenis1004        .......... .......... .........C .......... .......... .......... ..........
Mycosphaerella_juvenis1005        .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_heimii760          .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_heimiiCMW5705      .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_nubilosa937        .......... .......... .......... .......... .......... .......... ..........
CMW11148Ethiopia                  .......... .......... .......... .......... .......... .......... ..........
CMW11149Ethiopia                  .......... .......... .......... .......... .......... .......... ..........
CMW11150Ethiopia                  .......... .......... .......... .......... .......... .......... ..........
CMW10377Ethiopia                  .......... .......... .......... .......... .......... .......... ..........
CMW10376Ethiopia                  .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_molleriana784      .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_molleriana1214     .......... .......... .......... .......... .......... .......... ..........
Ramulispora_anguioides            T.A..AACGC A....A.T.C G..AA.T.A. .TGA.T.G.. .AATTCAG.G A.TCA.CGA. T.TTTG.AC.
```

```
                                430       440       450       460       470       480       490

CMW10190Ethiopia                CGCCCCGTGG TATTCCGCGG GGCATGCCTG TTCGAGCGTC ATTTCA-CCA CTCGAG--TC TGACTCGGTA
Mycosphaerella_marksiiCMW9090   .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9091   .......... .......... ..T....... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9092   .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_parkii353        .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_africana794      .......... .......... .......... .......... .......... ...A....C. .AG..T....
Mycosphaerella_flexuosa1109     ......T... .......G.. .......... .......... .......... ...A....C. ..G..T....
AY045516M.grandis               ......TC.. .......A.. .......... .......... .......... ...A....C. ..G..T....
CMW10186Ethiopia                ......TC.. .......A.. .......... .......... .......... ...A....C. ..G..T....
CMW10187Ethiopia                ......T... .......A.. .......... .......... .......... ...A....C. ..G..T....
CMW10189Ethiopia                ......TC.. .......A.. .......... .......... .......... ...A....C. ..G..T....
Mycosphaerella_juvenis1004      ......T... .......G.. .......... .......... .......... ...A....C. ..G..T....
Mycosphaerella_juvenis1005      ......T... .......G.. .......... .......... .......... ...A....C. ..G..T....
Mycosphaerella_heimii760        .....TC... .......G.. .......... .......... .......... ...A....C. ..G..T....
Mycosphaerella_heimiiCMW5705    .....TC... .......G.. .......... .......... .......... ...A....C. ..G..T....
Mycosphaerella_nubilosa937      .....TC... .......GA. .......... .......... .......... ...C....C. CCG..T....
CMW11148Ethiopia                .....TC... .......GA. .......... .......... .......... ...C....C. CCG..G...C
CMW11149Ethiopia                .....TC... .......GA. .......... .......... .......... ...C....C. CCG..G...C
CMW11150Ethiopia                .....TC... .......GA. .......... .......... .......... ...C....C. CCG..G...C
CMW10377Ethiopia                .....TC... .......GA. .......... .......... .......... ...C....C. CCG..G...C
CMW10376Ethiopia                ......TC.. .......A.. .......... .......... .......... ...A....C. ..G..T....
Mycosphaerella_molleriana784    .....TC... .......GA. .......... .......... ...A...... ...CG.C.CT C-G..G....
Mycosphaerella_molleriana1214   .....TC... .......GA. .......... .......... ...A...... ...CG.C.CT C-G..G....
Ramulispora_anguioides          .A.ATT.C.C CC.CTG.TAT TC.GG.GGGC A.GCCTGT.. GAGCGTCATT A.AACCAC.. AAG...TC-G
```

```
                                  500        510        520        530        540        550        560

CMW10190Ethiopia                  TTGGGCGCCG CGTT-T--CG -ACGCGCGCC -------TTA AAGTTT-CCG GCTG-GACCG TCCGTCTCCG
Mycosphaerella_marksiiCMW99090    .......T.. .......... ..T....... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9091     .......T.. .......... ..T....... .......... .......... .......... ..........
Mycosphaerella_marksiiCMW9092     .......T.. .......... ..T....... .......... .......... .......... ..........
Mycosphaerella_parkii353          .......T.. ..GCT..C.. .C........ ........C. ....C..... ......G.A. C.........
Mycosphaerella_africana794        .......T.. ..G-T..C.. .-...--... .......... ....C..... .....AG.A. .T......TA
Mycosphaerella_flexuosa1109       .......AG. ..GCT..C.. GC...C...- ........C. ....C..... -......... A.......TA
AY045516M.grandis                 .......... ..G.T.GC.. .-...--... ........C. ....C..... .....AG..A A.T.....TA
CMW10186Ethiopia                  .......... ..G.T.GC.. .-...--... ........C. ....C..... .....AG..A A.T.....TA
CMW10187Ethiopia                  .......... ..G.T.GC.. .-...--... ........C. ....C..... .....AG..A A.T.....TA
CMW10189Ethiopia                  .......... ..G.T.GC.. .-...--... ........C. ....C..... .....AG..A A.T.....TA
Mycosphaerella_juvenis1004        .......AG. ..GCT...-- .-....-... GCCCGCC.C. ....C..... ........G. AT......TA
Mycosphaerella_juvenis1005        .......AG. ..GCT...-- .-....-... GCCCGCC.C. ....C..... ........G. AT......TA
Mycosphaerella_heimii760          .......T.. ..GCT-.C.. .-...--... .......... ....C.T... .....AG.T. ........TA
Mycosphaerella_heimiiCMW5705      .......T.. ..GCT-.C.. .-......C-- .......... ....C.T... .....AG.T. ........TA
Mycosphaerella_nubilosa937        .......... ..GCC..C.. .-...-GC.- ........C. .T..C..... ..C..AG... A.......TC
CMW11148Ethiopia                  .......... ..GCC..C.. .-...-GC.- ........C. .T..C..... ..C..AG... A.......TC
CMW11149Ethiopia                  .......... ..GCC..C.. .-...-GC.- ........C. .T..C..... ..C..AG... A.......TC
CMW11150Ethiopia                  .......... ..GCC..C.. .-...-GC.- ........C. .T..C..... ..C..AG... A.......TC
CMW10377Ethiopia                  .......... ..GCC..C.. .-...-GC.- ........C. .T..C..... ..C..AG... A.......TC
CMW10376Ethiopia                  .......... ..G.T.GC.. .-......C-- ........C. ....C..... .....AG..A A.T.....TA
Mycosphaerella_molleriana784      .......... ..GC.-...- TC....---- ...CGCC.CG ....C..... ..C..AG... A........A
Mycosphaerella_molleriana1214     .......... T.GC.-...- TC....---- ...CGCC.CG ....C..... ..C..AG... A...G....A
Ramulispora_anguioides            C.T..TATT. G.G.TCGCG. TTT-..---- ..CGGCC--- ---.C.AAAC T.A.T.G.G. .G.C.G..G.
```

```
                                570        580        590        600        610        620        630
CMW10190Ethiopia                AGCGTTGTGG CATCTGTC-- ---------- TC-GCT---A GG--GAGT-C GCGGAGGGCG -TT---GGCC
Mycosphaerella_marksiiCMW9090   .......... .......... .......... .......... .......C.. .......... ..........
Mycosphaerella_marksiiCMW9091   .......... .......... .......... .......... .......C.. .......... ..........
Mycosphaerella_marksiiCMW9092   .......... .......... .......... .......... .......C.. .......... ..........
Mycosphaerella_parkii353        .......... ....----.. ACA.GTTC.. .......... .......... .....C.... ..C.......
Mycosphaerella_africana794      .......... ..-------.T ATA..TT... .......G.. --AA....T. .-...C...- ...TT.....
Mycosphaerella_flexuosa1109     .........A .--------.T AAA..TTGGA -.C...TGT- --......AT .-...C.T.- .C...C....
AY045516M.grandis               .......... -------TTT AATCAT.... -.C...TGT- --.....AT. .---.A.... A--..C....
CMW10186Ethiopia                .......... -------TTT AATCAT.... -.C...TGT- --.....AT. .---.A.... A--..C....
CMW10187Ethiopia                .......... -------TTT AATCAT.... -.C...TGT- --.....AT. .---.A.... A--..C....
CMW10189Ethiopia                .......... -------TTT AATCAT.... -.C...TGT- --.....AT. .---.A.... A--..C....
Mycosphaerella_juvenis1004      .........A .T..G---.. .....TTGGA -.C...TGC- --......A. .-...---.. ..CCTC....
Mycosphaerella_juvenis1005      .........A .T..G---.. .....TTGGA -.C...TGC- --......A. .-...---.. ..CCTC....
Mycosphaerella_heimii760        .......... .--------.. AACTAT.... -TC...T.C- ..AG.--... .-..T..C.. .--..C....
Mycosphaerella_heimiiCMW5705    .......... .--------.. AACTAT.... -TC...T.C- ..AG.--... .-..T..C.. .--..C....
Mycosphaerella_nubilosa937      .......... ..------.. ..CTACTGTT ..GCT-.G.. C..G.GAC.. .-.TCT.... .--.CGC...
CMW11148Ethiopia                .......... ..------.. ..CTACTGTT ..GCT-.G.. C..G.GAC.. .-.TCT.... .--GCGC...
CMW11149Ethiopia                .......... ..------.. ..CTACTGTT ..GCT-.G.. C..G.GAC.. .-.TCT.... .--GCGC...
CMW11150Ethiopia                .......... ..------.. ..CTACTGTT ..GCT-.G.. C..G.GAC.. .-.TCT.... .--GCGC...
CMW10377Ethiopia                .......... ..------.. ..CTACTGTT ..GCT-.G.. C..G.GAC.. .-.TCT.... .--GCGC...
CMW10376ETHIOPA                 .......... -------TTT AATCAT.... -.C...TGT- -.AG-.-... .---.A.... A--..C....
Mycosphaerella_molleriana784    .......... ..CAAC.GTT T.CG...CTT ...--GGG.. C-..----.. .-.TCT.... .--GCGC...
Mycosphaerella_molleriana1214   .......... ..CAAC.GTT T.CG...CTT ...--GGG.. C-..----.. .-.TCT.... .--GCGC...
Ramulispora_anguioides          CT.TAC.C.T AG.AATA-CT CC.....TCG -.GAT....- --..----.G A-.TCC..TA .--GGTTTA.
```

```
                                    640        650        660        670        680        690      697

CMW101901Ethiopia                    --------GT TAAAC----- ---------A CCCCATCA-A AGGTTGACCT CGGATCAGGT AGGGATA
Mycosphaerella_marksiiCMW9090       .......... .......... .......... .......... .......... .......... .......
Mycosphaerella_marksiiCMW9091       .......... .......... .......... .......... .......... .......... .......
Mycosphaerella_marksiiCMW9092       .......... .......... .......... .......... .......... .......... .......
Mycosphaerella_parkii353            .......... ....T..... .......... .......... .......... .......... .......
Mycosphaerella_africana794          .......... ....TC.... TTTC...TT. --------.. .......... .......... .......
Mycosphaerella_flexuosa1109         .......... .......... ..TTATTAC. .-------.. .......... .......... .......
AY045516M.grandis                   .......... .......... ..TTATT.C. --------.. .......... .......... .......
CMW10186Ethiopia                    .......... .......... ..TTATT.C. --------.. .......... .......... .......
CMW10187Ethiopia                    .......... .......... ..TTATT.C. --------.. .......... .......... .......
CMW10189Ethiopia                    .......... .......... ..TTATT.C. --------.. .......... .......... .......
Mycosphaerella_juvenis1004          .......... .....CC... TTTTAT..C. --------.. .......... .......... .......
Mycosphaerella_juvenis1005          .......... .....CC... TTTTAT..C. --------.. .......... .......... .......
Mycosphaerella_heimii760            .......... ....TC.... TTT.....C. .-------.. .......... .......... .......
Mycosphaerella_heimiiCMW5705        .......... ....TC.... TTT.....C. .-------.. .......... .......... .......
Mycosphaerella_nubilosa937          .......... .....CC... TTT.....C. ..------A. .......... .......... .......
CMW11148Ethiopia                    .......... .....CC... TTT.....C. ..------A. .......... .......... .......
CMW11149Ethiopia                    .......... .....CC... TTT.....C. ..------A. .......... .........- .......
CMW11150Ethiopia                    .......... .....CC... TTT.....C. ..------A. .......... .......... .......
CMW10377Ethiopia                    .......... .....CC... TTT.....C. ..------A. .......... .......... .......
CMW10376Ethiopa                     .......... ....-C.... ..TTATT.C. --------.. .......... .......... .......
Mycosphaerella_molleriana784        .......... .....CC... TT...T..C. .-------A. .......... .......... .......
Mycosphaerella_molleriana1214       .......... .....CC... TT...T..C. .-------A. .......... .......... .......
Ramulispora_anguioides              TTGCCAAC-- -..C.CCCAA TTTTTT.AC- --------.- .......... .......... .......
```

SUMMARY

In Ethiopia, the planting of exotic species commenced with the introduction of *Eucalyptus globulus* approximately 110 years ago. Today several different *Eucalyptus*, *Pinus*, *Cupressus* and Australian *Acacia* species are planted to provide wood for fuel/energy and raw material for furniture and construction. In many areas, people are dependent solely on wood to provide for their basic fuel and construction needs. Despite this, little attention has been given to improve the silvicultural and management practices of plantations in Ethiopia. In particular, disease surveillance and management has never received due attention. The aim of the studies that make up this thesis have been to address the issue of diseases of plantation trees in Ethiopia. Studies have thus focused on the prevalence, identity and importance of major diseases of especially *Eucalyptus* and *Pinus* spp.

As a background to this thesis, available information on diseases of exotic tree species in Africa has been reviewed and this is presented in the first chapter. In the review, diseases of the major exotic plantation species including *Eucalyptus*, *Pinus*, *Cupressus* and *Acacia* species have been considered. A section was also devoted to highlight tree diseases reported from Ethiopia. The review shows clearly that there is a great lack of information on diseases of exotic plantation species in most African countries, with the exception of South Africa. This suggests the need for more pathology studies in African plantations. The review also highlights the importance of diseases in plantation forests.

In Ethiopia, little information is available on tree diseases in plantation forests. To partially address this problem, disease surveys were conducted in 2000 and 2001 in *Eucalyptus* and *Pinus* plantations in South and South Western Ethiopia. The results of this survey showed that a number of pathogens, known from other countries, including Armillaria root rot, stem canker and foliage diseases are found in plantations of Ethiopia. The major diseases discovered during the survey are discussed in Chapter two of this thesis and an indication is given of their impact and distribution.

During the disease survey, Armillaria root rot was found to be associated with both exotic and native tree species. Morphological and molecular identification techniques revealed that the *Armillaria* sp. collected in this study is *A. fuscipes*. This is discussed in chapter three, where I also provide preliminary data regarding the host range and distribution of Armillaria root rot in Ethiopia. Prior to this study it was suggested that *A. mellea* is responsible for Armillaria root rot of

hard woods in Ethiopia. The current study, however, showed that at least two *Armillaria* spp., *A. mellea* and *A. fusicpes* are causing Armillaria root rot in the country. Of significance is the fact that *A. fuscipes* was isolated from two indigenous tree species, *A. abyssinica* and *J. excelsa.*

Chapter four of this thesis deals with the identity of the fungus causing stem canker on *Eucalyptus camaldulensis.* Disease symptoms identical to those caused by *Coniothyrium zuluense* were commonly found on *E. camaldulensis* in restricted areas in Western Ethiopia. The causative agent was determined based on DNA sequence analysis of the ITS 1, ITS 2 and 5.8S gene region and β-tubulin genes. According to the phylogenetic tree generated for these sequence data, the Ethiopian *Coniothyrium* isolates seem to be closely related to *C. zuluense,* however, the Ethiopian isolates formed a separate group. This may suggest that *C. zuluense* represents a species complex, but this needs further investigation. Coniothyrium canker is considered to be one of the most serious diseases of *Eucalyptus* spp. especially to the sawn timber and construction industry as it weakens and flaws the timber. It occurrence in Ethiopia is, therefore, of great importance.

Disease symptoms similar to those of Botryosphaeria canker on *Eucalyptus* were commonly observed in all the areas where surveys were conducted. *Botryosphaeria* spp. are known as opportunistic stress related and endophytic pathogens on a wide range of woody plants, worldwide. In Ethiopia, symptoms similar to those associated with *Botryosphaeria* infection elsewhere, were found in almost all plantations surveyed. The disease was found on several *Eucalyptus* spp. including *E. globulus*, *E. saligna*, *E. grandis* and *E. citriodora.* Both morphological and molecular identification techniques were used to determine the identity of the fungus and the results are presented in chapter five. It was shown that *B. parva* is responsible for Botryosphaeria stem canker of *Eucalyptus* spp. in Ethiopia and the pathogenicity of Ethiopian isolates was also tested. This pathogen can have a serious effect on *Eucalyptus* in Ethiopia, as growing conditions in the country are often harsh and many people rely on coppicing to reproduce their stands. All these factors are conducive to stress and thus to *Botryosphaeria* infection.

Diplodia pinea is a fungus that commonly resides in the cones of *Pinus* spp. and it tends to move from these sites to infect stems, when trees are under stress. Therefore, isolations were made from *Pinus patula* cones to determine whether *D. pinea* was present in these structures in Ethiopia. Chapter 6 of the thesis provides results of this study. It was expected that *D. pinea* would be the most common inhabitant of the cones. Contrary to this, a *Fusicoccum* sp. was found more

frequently than *D. pinea*. The results presented in this chapter show clearly that the A morphotype of *D. pinea* is found in cones of *P. patula* in Ethiopia. The *Fusicoccum* sp. found associated with *P. patula* cones is most closely related to *B. parva*. Results of greenhouse inoculation studies showed that both these fungi are pathogenic to *Pinus tadea*, with *D. pinea* being the more pathogenic.

Serious leaf spot and shoot die-back symptoms were observed on leaves of *E. globulus* at several localities. The leaf blotch symptoms closely resemble those caused by *Mycosphaerella* spp. Even though 30 different *Mycosphaerella* spp. are known to be associated with *Eucalyptus* species world-wide, the cause of Mycosphaerella leaf blotch on *E. globulus* in Ethiopia is not known. Morphological and DNA based comparisons were used to determine the identity of the species found in Ethiopia and the results are provided in chapter seven. I was thus able to show that three *Mycosphaerella* spp. namely, *M. marksii, M. grandis* and *M. nubilosa* are involved in causing Mycosphaerella leaf disease of *E. globulus* in Ethiopia. This is the first report of these species from Ethiopia and the first report of *M. grandis* from a country other than Australia.

The results presented in the various chapters making up this thesis provide the first detailed studies on diseases of plantation trees in Ethiopia. Most tree diseases discussed in the thesis are first reports for the country. The thesis provides information on the identity of the pathogens and their significance in plantation development in Ethiopia. It also highlights the need for adequate management and silvicultural practices, as well as the need for selecting disease tolerant provenances and/or individuals. The information presented in the thesis also expands the host range and geographic distribution of all the pathogens included in the study, giving the study international significance.