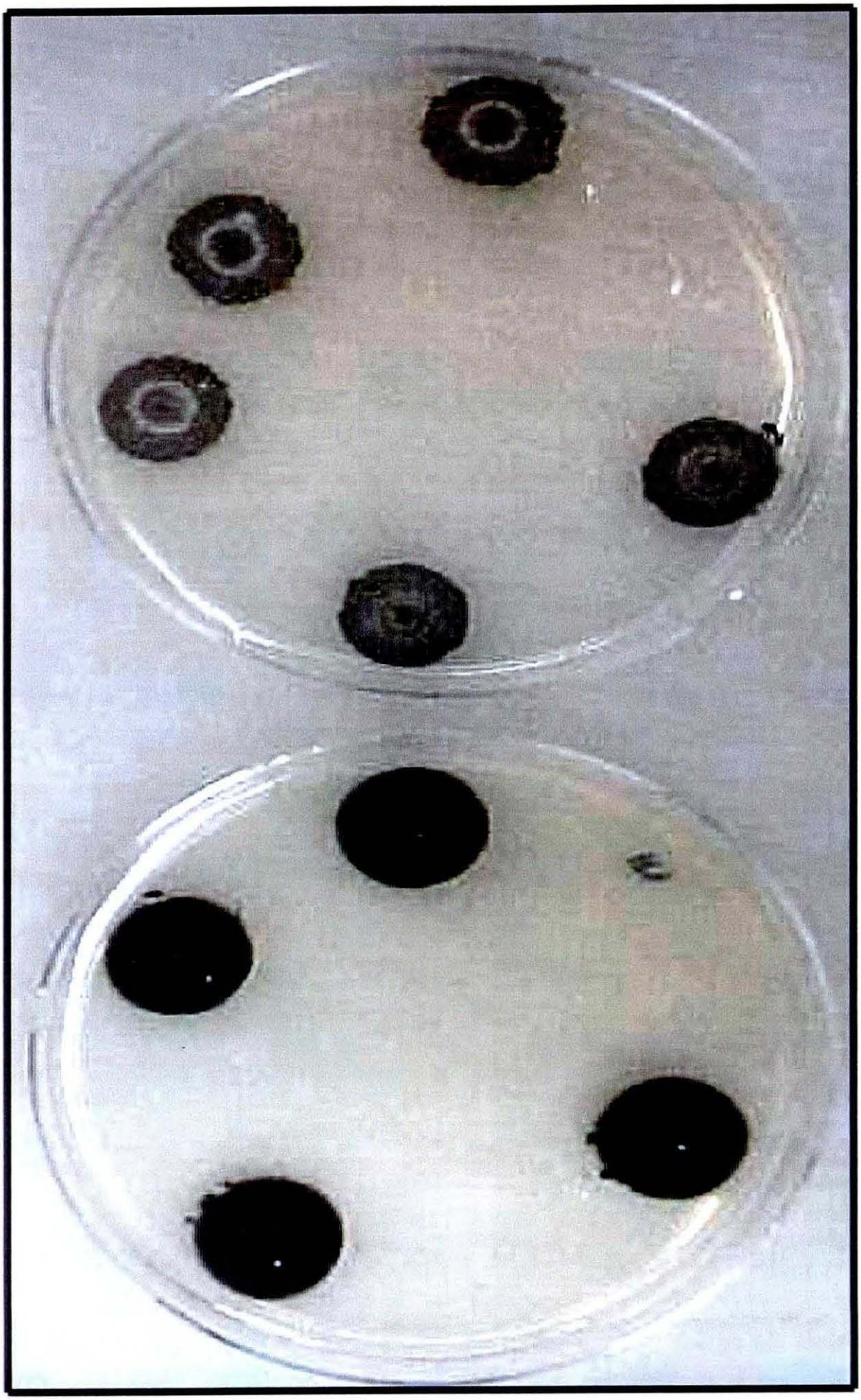
Chapter 5

First report of *Mycosphaerella heimii* from Brazil and Hawaii



ABSTRACT

Mycosphaerella leaf disease causes serious damage to commercially grown *Eucalyptus* species. Several species of *Mycosphaerella* are recognized as contributing to, or causing this disease. Recent surveys of *Eucalyptus* spp. in Brazil and Hawaii have resulted in a collection of several *Mycosphaerella* isolates from diseased leaves. *Mycosphaerella* spp. on *Eucalyptus* are difficult to identify due to many species present and the paucity of morphological characteristics on which to base identifications. The aim of this study was to identify these *Mycosphaerella* species. This was achieved by sequencing the Internal Transcribed Spacer regions of the rDNA operon. Results from this study have enabled us to identify the *Mycosphaerella* species from Brazil and Hawaii as *M. heimii*. This represents the first report of *M. heimii* from these countries. *Mycosphaerella heimii* is common in Indonesia and Madagascar where it causes severe leaf spots on a number of *Eucalyptus* spp. and its presence in Brazil and Hawaii is of concern. The fungus could also threaten *Eucalyptus* plantations in other South American countries in the future.

INTRODUCTION

Eucalyptus spp. are commercially propagated as exotics in plantations in many parts of the world. In Brazil, the estimated area planted to plantations is 6.2 million ha, of which *Eucalyptus* spp. make up 52 % (WFI 1999). Due to this extensive establishment of plantations, the paper and pulp industry in Brazil has become one of the largest in the world (WFI 1999). In Hawaii, over 100 *Eucalyptus* spp. are grown as exotics where they are used for furniture, reforestation of damaged pasture lands, floor boards and boat framing (HFOI 2000).

The continuous expansion of *Eucalyptus* planting has resulted in a growing movement of seed and to a lesser extent, plant material between countries and continents. Pathogens such as *Mycosphaerella* spp. are thought to be moved on seed, thus quarantine measures to prevent such spread are important. *Mycosphaerella* represents a large genus of Ascomycetes, including approximately 2000 species (Corlett 1991, Corlett 1995). A large number of *Mycosphaerella* spp. are frequently isolated from *Eucalyptus* leaves, on which they cause a disease known as Mycosphaerella leaf disease (MLD) (Crous 1998). Many *Eucalyptus* spp. that belong to the sub-genera *Monocalyptus* and *Symphyomyrtus* have been identified as hosts of *Mycosphaerella* spp. The distribution of MLD is extensive and virtually every country where *Eucalyptus* spp. are grown has reported its presence where infection can cause defoliation and growth retardation.

Mycosphaerella spp. are difficult to identify, and in the past names have largely been linked to hosts (Corlett 1991). The many species found on *Eucalyptus* have been defined based on teleomorph and anamorph characters (Crous 1998), and more recently DNA sequence data (Crous *et al.* 2000, Crous *et al.* 2001). Ascospore germination patterns, especially, have proven to be valuable for the grouping and identification of *Mycosphaerella* spp. Today fourteen types of ascospore germination patterns have been distinguished for species occurring on *Eucalyptus* (Park & Keane 1982, Crous 1998). Currently 23 anamorph genera from the coelomycetes and hyphomycetes have been linked to *Mycosphaerella,* and seven of these anamorph genera are known to be associated with those species occurring on *Eucalyptus* (Arx 1983, Crous 1998). Sequence data obtained especially from the ribosomal DNA operon, have been helpful in delimiting species and recognising lineages within *Mycosphaerella* (Crous *et al.* 2000, Crous *et al.* 2001).

Integrating with morphology, these characteristics have allowed for the classification and identification of *Mycosphaerella* spp. associated with *Eucalyptus* and other hosts.

During a survey of *Eucalyptus* spp. in Brazil and Hawaii, collections of *Mycosphaerella* species were obtained from diseased leaves. The aim of this study was to determine the identity of these isolates. This was achieved by sequencing the Internal Transcribed Spacer region (ITS) of the rDNA operon and comparing sequence data from these isolates with those of known *Mycosphaerella* spp. occurring on *Eucalyptus* spp. (Crous *et al.* 2001).

MATERIALS AND METHODS

Isolates and culture growth

A collection of *Mycosphaerella* isolates was obtained from diseased *Eucalyptus* leaves from four locations in Brazil (Veracruz, Texeira de Freitas, Aracruz and Jari) and the island of Hawaii (Table 1). All cultures are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Cultures were grown on 2% MEA (Malt Extract Agar) (wt/v) (Biolab, South Africa) and incubated at 25°C under continuous cool white light in an incubator. The cultural morphology of axenic colonies was evaluated based on descriptions for known *Mycosphaerella* spp. occurring on *Eucalyptus* spp. (Crous 1998). Characterisation of culture colour was based on the colour charts of Rayner (1970). Fungal isolates were also grown on Carnation Leaf Agar (CLA) [1% water agar (wt/v) (Biolab, South Africa) with sterilized carnation leaves placed onto medium] and incubated at 25°C under continuous near-ultra-violet light (nuv, 250 nm) to promote the formation of anamorphs. Following sufficient colony growth, ten isolates were selected based on culture colour and morphology for DNA extraction and molecular identification using DNA sequencing.

Molecular characterization

DNA Isolation

Following growth of pure cultures, mycelium was scraped directly from agar plates and dried under vacuum. The dried mycelium was lypholized with liquid nitrogen and DNA isolated according to the method of Raeder & Broda (1985) with minor variations. The 1:1 phenol:chloroform purification step was repeated until the interphase between the two aqueous phases was clean of any cellular debris. Nucleic acids were precipitated by the addition of 10% 3M NaAc and 2 volumes of absolute ethanol and incubated at -20°C for 2 hours. DNA was further purified by washing with 70% ethanol and dried under vacuum and the resulting DNA pellet resuspended in 50 µl SABAX water. RnaseA (10 µg/µl) was added to the DNA samples, and incubated at 37°C for three to four hours to digest any residual protein or RNA. DNA was visualized on a 1% agarose gel (wt/v) (Boehringer Mannheim, Germany) stained with ethidium bromide and viewed under an ultra-violet 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 Polymerase Chain Reaction (PCR). The Internal Transcribed Spacer (ITS) region of the ribosomal DNA operon, was targeted for amplification using primers ITS 1 (5'- TCC GTA GGT GAA CCT GCG G – 3') and LR1 (5'- GGT TGG TTT CTT TTC CT –3') (White *et al.* 1990). The ITS 1 and ITS 2 regions including the 5.8S gene were amplified. DNA was amplified in a 50 µl reaction volume containing PCR buffer (10 mM Tris-HCL, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) (Roche Diagnostics, 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 2.5U Taq DNA polymerase (Roche Diagnostics, South Africa). SABAX water was added to achieve a total volume of 50 µl.

PCR reactions were carried out using an Eppendorf Mastercycler gradient PCR machine (Eppendorf Scientific, Germany). PCR reaction conditions consisted of an initial

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

DNA sequencing and data analysis

Purified PCR products were used as template DNA for sequencing reactions on an ABI PRISM™ 377 Automated DNA sequencer (Perkin Elmer, Norwalk, CON). The ABI Prism Big Dye Terminator Cycle sequencing reaction kit (Perkin Elmer Biosystems, USA) was used for the sequencing reactions. Two forward 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 analyzed using Sequence Navigator version 1.0.1 (Perkin-Elmer, Applied Biosystems, Foster City, CA). 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. Published sequences of *Mycosphaerella* spp. from *Eucalyptus* spp. were obtained from Genebank and compared with the ten sequences obtained for the *Mycosphaerella* spp. from Brazil and Hawaii (Table 1, Also see Chapter 3, Table 1). Following the analysis, all resulting trees were rooted to an outgroup taxon, *Ramulispora anguoides* (Nirenberg) Crous, which has been shown to be an appropriate outgroup for *Mycosphaerella* (Crous *et al*. 2001).

RESULTS

Isolates and culture growth

All ten isolates examined in this study showed the same cultural morphology. Fungal colonies were slightly raised above the agar surface and did not exhibit any sectoring or folding. Colonies were even edged with regular margins. Aerial mycelium varied from medium to profuse. Colonies were olivaceous grey 23’’’’’i (surface) and greenish black 33’’’’’k (reverse). Red crystal production was observed in water agar medium supplemented with carnation leaves for all the isolates. Growth of isolates on carnation leaf agar resulted in the formation of an anamorph that resembled *Pseudocercospora heimii* Crous (Crous 1998) (Figure 4).

Molecular characterization

PCR Amplification and DNA Sequence analysis

Amplification of the ITS region of the ten representative isolates from Brazil and Hawaii resulted in amplification products of approximately 600 base pairs for all isolates. Analysis of the data set containing *Mycosphaerella* isolates from Brazil and Hawaii and species of *Mycosphaerella* occurring on *Eucalyptus* and other *Myrtaceae*, resulted in the generation of two most parsimonious trees with a length of 1555 steps (CI = 0.504, RI = 0.693, HI = 0.496). A total of 657 characters were analyzed, of which 261 characters were constant, 104 characters parsimony-uninformative and 292 characters parsimony informative. A bootstrap analysis of 1000 replicates resulted in the generation of a tree

similar to the most parsimonious tree generated from the heuristic search. All isolates from Brazil and Hawaii grouped with *M. heimii* with a well-supported clade (Figure 1). *M. heimii*, *M. heimioides* and *M. irregulariramosa* all grouped within the larger monophyletic *Mycosphaerella* clade (Figure 2). All of these species, and those from Brazil and Hawaii formed a group, within *Mycosphaerella sensu stricto*, characterized by having *Pseudocercospora* anamorphs (Crous *et al.* 2001).

Following identification of the isolates used in this study against all other *Mycosphaerella* spp. on *Myrtaceae*, the isolates from Brazil and Hawaii were reconsidered in a smaller data set, specifically with species in the *M. heimii* complex. The objective here was to increase the resolution between species. This data set produced one most parsimonious tree with a length of 261 steps (CI = 0.9923, RI = 0.8750, HI = 0.0077). There were a total of 657 characters of which, 408 were constant, 237 variable characters were parsimony-uninformative and 12 parsimony informative characters. The major consensus tree showed the same topology as the most parsimonious tree. This ITS data set again resolved the *Mycosphaerella* isolates from Brazil and Hawaii into *M. heimii*. All these isolates grouped together with *M. heimii* (CMW 4942) in a clade that was strongly supported (Figure 3). A second clade, sister to the *M. heimii* clade included *M. irregulariramosa* isolates (CMW 4943, CMW 5149). *Mycosphaerella heimioides* (CMW 3046) grouped distantly to isolates of *M. heimii* and *M. irregulariramosa*. This species was, however, closely related to *M. heimii* and *M. irregulariramosa* with strong bootstrap support (94%).

DISCUSSION

This study represents the first report of *M. heimii* from Brazil and Hawaii. The fungus appears to be common on various *Eucalyptus* spp. (Table 1) in these countries and is considered to be a potentially important pathogen. *Mycosphaerella heimii* is known to occur in Madagascar and Indonesia where it was isolated from various *Eucalyptus* spp. Infection results in medium brown, elongated lesions that are surrounded by raised brown margins (Crous & Swart 1995, Crous & Wingfield 1997, Crous 1998). The anamorph of *M. heimii*, *P. heimii* was readily produced on CLA, confirming that anamorph associations of *Mycosphaerella* spp. are helpful in the initial identification of *Mycosphaerella* spp. However, without DNA sequence comparisons, it would not have been possible to conclusively identify the Brazil and Hawaii collections as *M. heimii*.

Mycosphaerella heimii was originally described by Bouriquet (1946) from Madagascar, where it causes leaf spots on *E. obliqua* L' Heritier. Bouriquet did not, however, provide a Latin description for this fungus and it was never validly published. During subsequent surveys of *Eucalyptus* species in Madagascar, Crous & Swart (1995) isolated *M. heimii* from Moramanga and Tamatave. A Latin description was provided together with a description of the anamorph, *Pseudocercospora heimii*. In this study, we have expanded the known distribution of *M. heimii* considerably and suspect that the fungus has been moved between *Eucalyptus* growing countries with seed.

Mycosphaerella heimii, together with *M. heimioides* Crous & M. J. Wingf. and *M. irregulariramosa* are all members of what is referred to as the *M. heimii* complex (Crous 1998, Crous *et al.* 2001). These species have similar ascospore morphology and all produce a *Pseudocercospora* Speg. anamorph (Crous & Wingfield 1997, Crous *et al.* 2001). They do, however, have different geographical distributions. *M. heimii* has previously been known to occur in Indonesia and Madagascar, *M. heimioides* is known only from Indonesia and *M. irregulariramosa* only from South Africa (Crous 1995, Crous & Swart 1995, Crous & Wingfield 1997).

Isolates of *M. heimii* used in this study produced distinct cultural characteristics. They produced slightly raised colonies with even edges, which did not sector or fold considerably. Colonies were olivaceous grey on the top and greenish brown on the bottom. These characteristics fit those described by Crous (1998), distinguishing *M. heimii* from other species in the *M. heimii* complex. *Mycosphaerella heimioides* produces cultures that grow in concentric rings and at different elevations, producing red crystals in culture (Crous & Wingfield 1997), which was also observed for isolates from Brazil and Hawaii. *Mycosphaerella irregulariramosa* has a similar cultural morphology to that of *M. heimii*, but can be distinguished by its more grayish colonies. Cultural morphology and crystal formation is variable, and cannot be used as sole characteristics to distinguish *M. heimii* from other morphologically similar species within the *M. heimii* complex.

Pseudocercospora spp. within the *M. heimii* complex are characterized as having pale brown, smooth to finely verruculose, obclavate to subcylindrical conidia, and small fascicles with conidiophores that are usually reduced to conidiogenous cells (Crous *et al.*

2001). The anamorph of *M. heimii*, *P. heimii* was readily produced in culture in this study and can be distinguished from the other anamorphs in the *M. heimii* complex (Figure 4). *Pseudocercospora heimii* produces long irregularly curved conidia that are 55–300 × 2.5–3 µm, distinguishing it from the shorter conidia produced by *P. heimioides* [(25-)40 –90 (-150) × (2-) 2.5 – 3(-3.5) µm] (Crous & Swart 1995, Crous & Wingfield 1997, Crous 1995). The conidia of *P. irregulariramosa* are produced from conidiophores that form from a well developed stroma, whereas, the conidiophores of *P. heimioides* are reduced to conidiogenous cells. Those of *M. heimii* are fasciculate or produced as lateral projections on secondary mycelium (Crous & Swart 1995, Crous & Wingfield 1997).

From sequence data obtained in this study, two clades with *Pseudocercospora* Speg. anamorphs could be identified within the larger monophyletic *Mycosphaerella* clade. The first clade contained *Mycosphaerella* species from *Syzygium* and *Eucalyptus*. The second clade included *M. heimii*, *M. irregulariramosa*, *M. crystallina* Crous & M. J. Wingf, *M. heimioides* and *M. colombiensis* Crous & M. J. Wingf., as well as isolates of *M. heimii* from Brazil and Hawaii. All of the species in the second clade are only known to occur on *Eucalyptus* spp. This finding is consistent with results of Crous *et al.* (2001), who found that *Pseudocercospora* anamorphs have evolved more than once in *Mycosphaerella*. Sequence data in this study show clearly that *M. heimii* is phylogenetically close to other species within the *M. heimii* complex. These data support the view of Crous *et al.* (2001), that species within the *M. heimii* complex may represent different varieties of *M. heimii*, rather than distinct species.

Mycosphaerella heimii poses a threat to the future afforestation of *Eucalyptus* spp. It has the ability to cause severe leaf spotting and defoliation (Crous & Swart 1995, Crous & Wingfield 1997). Currently, the origin of this fungus is not known. It is possible that it originated on a native plant in any one of the countries where it has been found. In this case, it could pose a threat to areas where *Eucalyptus* are native. Alternatively, the fungus originated on *Eucalyptus* in its native range, and is slowly moving internationally. The area where *M. heimii* is found closest to the native range of *Eucalyptus* is Indonesia. Our collections originate from Northern Sumatra and it is possible that the fungus originated on native trees in the Southern part of the Indonesian archipelago. All areas where *M. heimii* has been found have tropical climates and it appears that this species is suited to a hot

humid environment. In these areas, *E. urophylla* or hybrids of this species are commonly planted. It is thus interesting to consider that *E. urophylla* has an Indonesian origin, and that Indonesia is one of the areas where *M. heimii* is commonly found.

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Table 1: *Mycosphaerella* isolates used for DNA sequence analysis

Isolate No.	Teleomorph	Host	Location	Collector	GenBank Accession No.
CMW 4942	*M. heimii*	*Eucalyptus* spp.	Madagascar	P.W. Crous	AF309606
CMW 4943	*M. irregulariramosa*	*E. saligna*	South Africa	M.J. Wingfield	AF309607
CMW 5149	*M. irregulariramosa*	*E. saligna*	South Africa	M.J. Wingfield	AF309608
CMW 3046	*M. heimioides*	*Eucalyptus* spp.	Indonesia	M.J. Wingfield	AF309609
CMW 5705*	*Mycosphaerella* spp.	*Eucalyptus* spp.	Veracruz, Brazil	P.W. Crous	AF452508
CMW 5707*	*Mycosphaerella* spp.	*Eucalyptus* spp.	Texta de Freitas Brazil	P.W. Crous	AF452509
CMW 5714*	*Mycosphaerella* spp.	*Eucalyptus* spp.	Jari, Brazil	P.W. Crous	AF452510
CMW 5711*	*Mycosphaerella* spp.	*E. grandis× urophylla*	Veracruz, Brazil	P.W. Crous	AF452511
CMW 5713*	*Mycosphaerella* spp.	*Eucalyptus* spp.	Aracruz, Brazil	P.W. Crous	AF452512
CMW 5706*	*Mycosphaerella* spp.	*Eucalyptus* spp.	Veracruz, Brazil	P.W. Crous	AF452513
CMW 5710*	*Mycosphaerella* spp.	*E. grandis× urophylla*	Veracruz, Brazil	P.W. Crous	AF452514
CMW 5720*	*Mycosphaerella* spp.	*E. urophylla*	Hawaii, U.S.A	P.W. Crous	AF452515

CMW : Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria

CMW*: Cultures sequenced in this study, other sequences as from a study by Crous *et al.* (2001)

Figure 1: Cladogram showing the ITS phylogeny of *Mycosphaerella* species from *Eucalyptus* and *Syzygium* including isolates from Brazil and Hawaii. One of the most parsimonious trees (length 1555, CI = 0.504, RI = 0.693, HI = 0.496) inferred from heuristic searches using PAUP. Bootstrap support of 1000 replicates is shown above the branches.

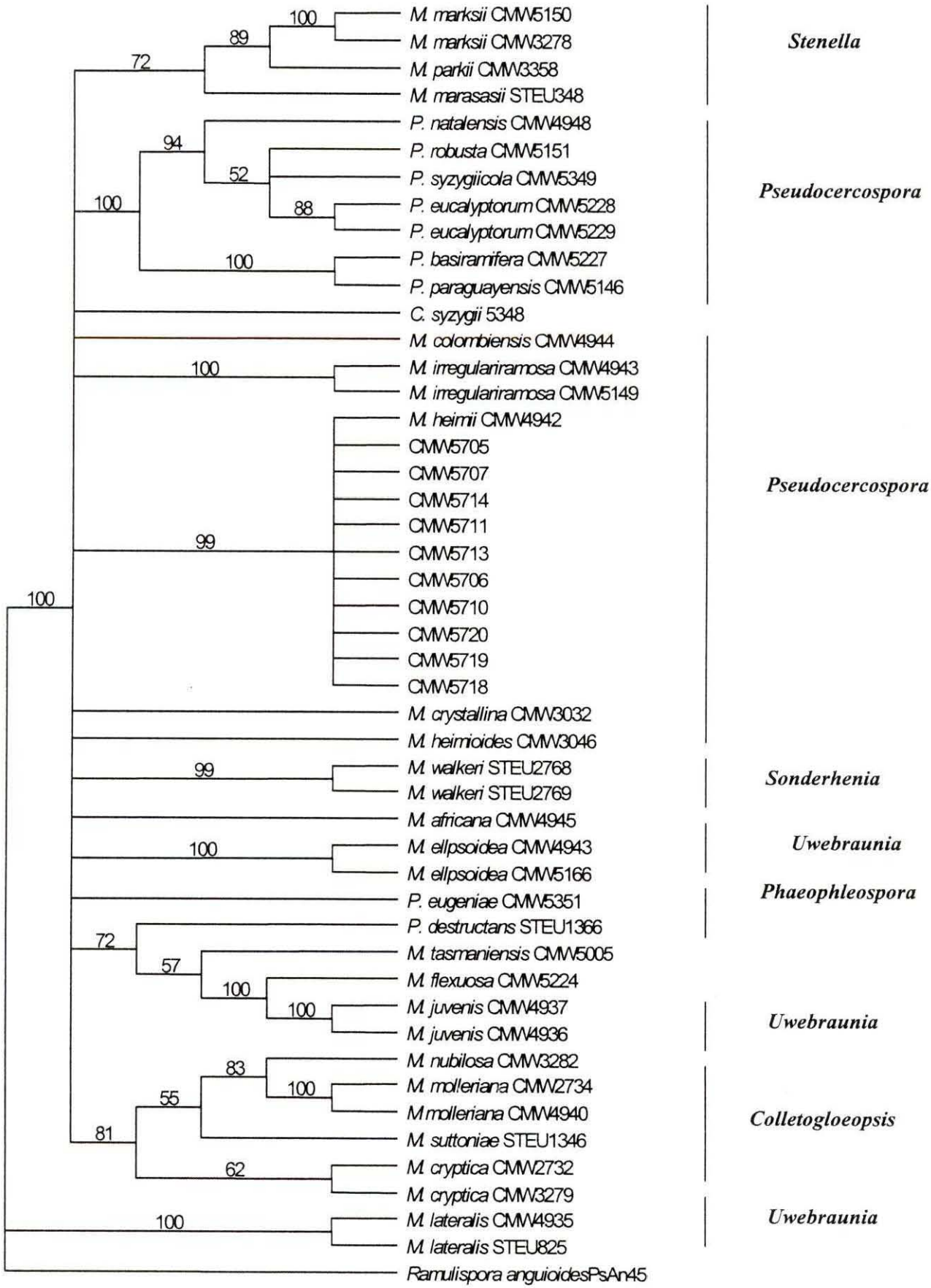

Figure 2: Cladogram showing the position of *Mycosphaerella* isolates from Brazil and Hawaii within the *M. heimii* complex. Outgroup taxon, *Ramulispora anguoides*. Most parsimonious tree (length = 261, CI = 0.8889, RI = 0.8750, HI = 0.0077) inferred using heuristic and branch swapping options of PAUP Version 4.0. Bootstrap support of 1000 replications shown above branches.

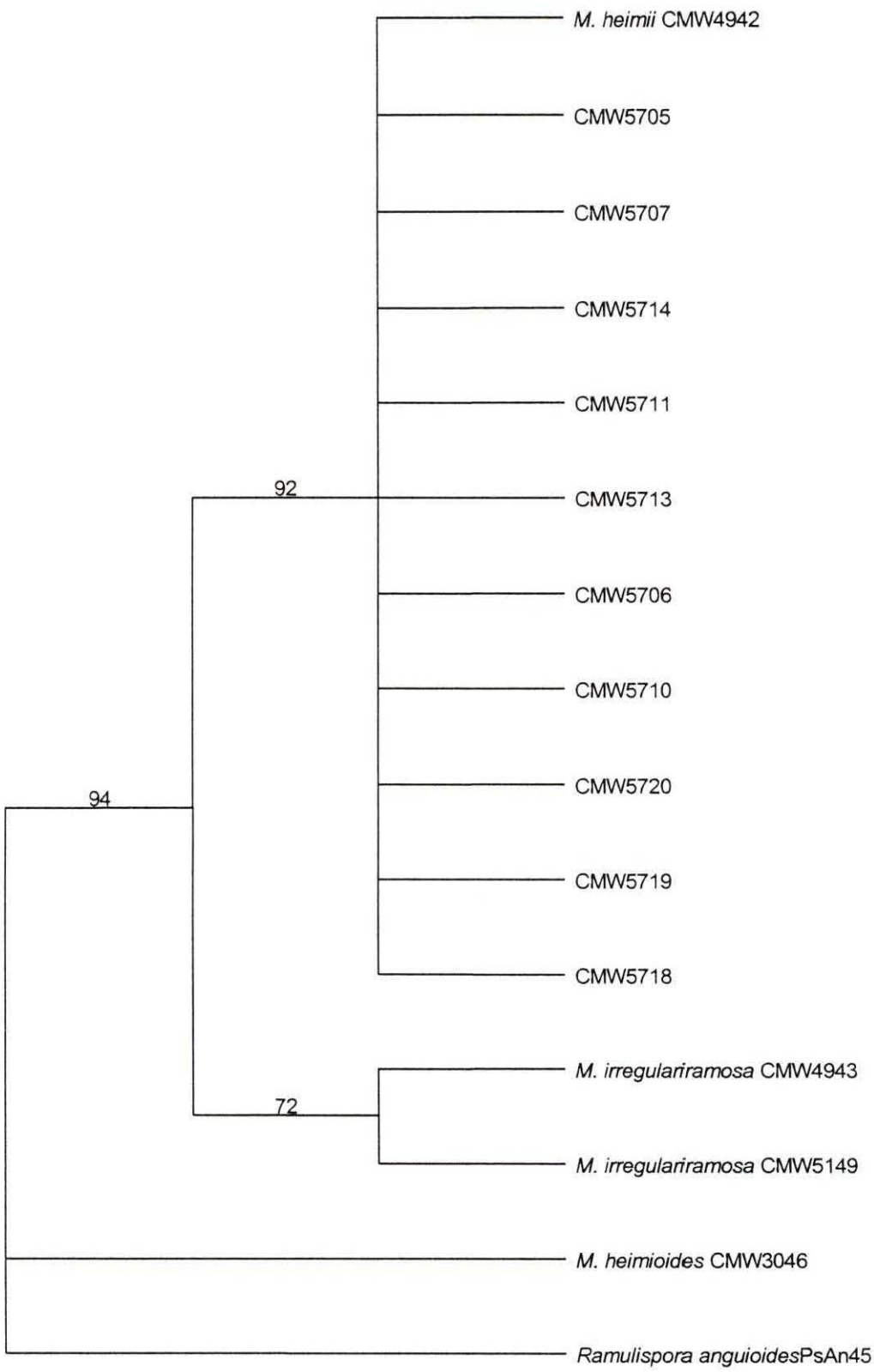
M. heimii CMW4942
CMW5705
CMW5707
CMW5714
CMW5711
92
CMW5713
CMW5706
CMW5710
94
CMW5720
CMW5719
CMW5718
M. irregulariramosa CMW4943
72
M. irregulariramosa CMW5149
M. heimioides CMW3046
Ramulispora anguioidesPsAn45

Figure 3: Morphological features of Brazilian and Hawaiian *Mycosphaerella heimii*. **(A)** 8-spored bitunicate fasciculate asci, **(B)** guttulate straight ellipsoidal ascospores, **(C)** Type C ascospore germination with two or more germination tubes parallel to the long axis of the spore, (D) Solitary, guttulate irregularly curved conidia, **(E)** Smooth, branched septate hyphae.

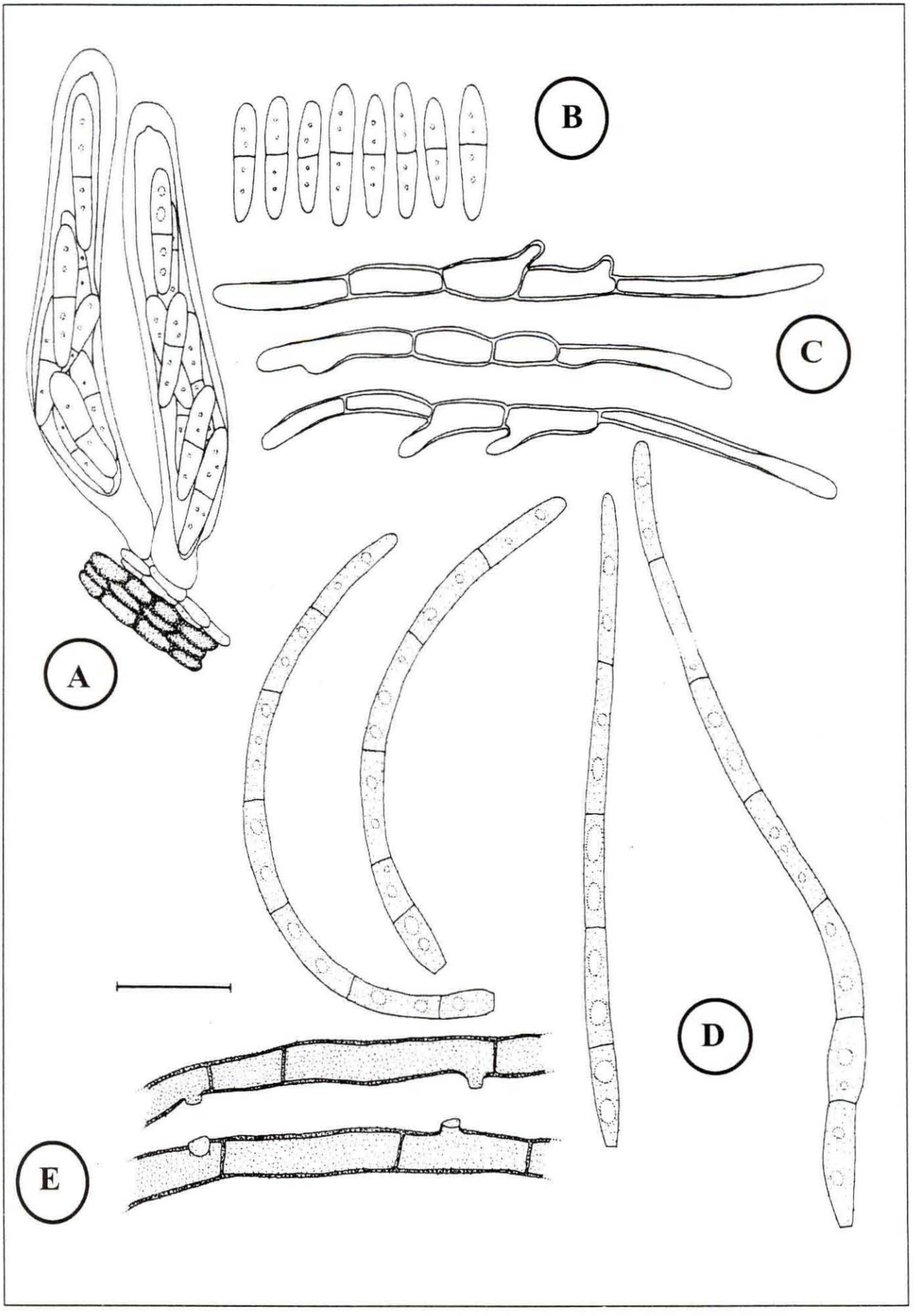

Figure 4: Aligned sequence data from the Internal Transcribed Spacer (ITS) region of the rDNA operon for isolates of *Mycosphaerella* obtained from Brazil and Hawaii together with isolates of members of the *M. heimii* complex (Table 1). Sequence data was generated using primers ITS1 and LR1 together with two internal primers namely, ITS2 and ITS3. Gaps inserted during alignments are indicated with a dash **(-)**.

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                                10         20         30         40
M._heimiiCMW4942                TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
CMW5705                         TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
CMW5707                         TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
CMW5714                         TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
CMW5711                         TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
CMW5713                         TCCGTAGGTG AACC----TG CGGAGGGATC ATTAAT-GAG
CMW5706                         TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
CMW5710                         TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
CMW5720                         TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
M._irregulariramosaCMW4943      TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
M._irregulariramosaCMW5149      TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
M._heimioidesCMW3046            TCCGTAGGTG AACC----TG CGGAGGGATC ATTACT-GAG
Ramulispora_anguioides          TCCGTAGGTG AACC----TG CGGAAGGATC ATTAATAGAG

                                   50         60         70         80
M._heimiiCMW4942                ---TGAGGGC TA--CGGTCC G--------A CCTC----CA
CMW5705                         ---TGAGGGC TA--CGGTCC G--------A CCTC----CA
CMW5707                         ---TGAGGGC TA--CGGTCC G--------A CCTC----CA
CMW5714                         ---TGAGGGC TA--CGGTCC G--------A CCTC----CA
CMW5711                         ---TGAGGGC TA--CGGTCC G--------A CCTC----CA
CMW5713                         ---TGAGGGC TA--CGGTCC G--------A CCTC----CA
CMW5706                         ---TGAGGGC TA--CGGTCC G--------A CCTC----CA
CMW5710                         ---TGAGGGC TA--CGGTCC G--------A CCTC----CA
CMW5720                         ---TGAGGGC TA--CGGTCC G--------A CCTC----CA
M._irregulariramosaCMW4943      ---TGAGGGC TTC--GGTCC G--------A CCTC----CA
M._irregulariramosaCMW5149      ---TGAGGGC TTC--GGTCC G--------A CCTC----CA
M._heimioidesCMW3046            ---TGAGGGC TTC--GGTCC G--------A CCTC----CA
Ramulispora_anguioides          CAATGAGCGT CAGCGCCCCG GGA--GCAAT CCTGGGGGCC

                                   90        100        110        120
M._heimiiCMW4942                ACCCT----- ------TT-- ----GT---- ---------G
CMW5705                         ACCCT----- ------TT-- ----GT---- ---------G
CMW5707                         ACCCT----- ------TT-- ----GT---- ---------G
CMW5714                         ACCCT----- ------TT-- ----GT---- ---------G
CMW5711                         ACCCT----- ------TT-- ----GT---- ---------G
CMW5713                         ACCCT----- ------TT-- ----GT---- ---------G
CMW5706                         ACCCT----- ------TT-- ----GT---- ---------G
CMW5710                         ACCCT----- ------TT-- ----GT---- ---------G
CMW5720                         ACCCT----- ------TT-- ----GT---- ---------G
M._irregulariramosaCMW4943      ACCCT----- ------TT-- ----GT---- ---------G
M._irregulariramosaCMW5149      ACCCT----- ------TT-- ----GT---- ---------G
M._heimioidesCMW3046            ACCCT----- ------TT-- ----GT---- ---------G
Ramulispora_anguioides          ACCCTCCTCG GAGGGTTTAG AGACGTCGAG CCTCTCGGAG

                                  130        140        150        160
M._heimiiCMW4942                AA-------- -------CCA AA-CT----- ----------
CMW5705                         AA-------- -------CCA AA-CT----- ----------
CMW5707                         AA-------- -------CCA AA-CT----- ----------
CMW5714                         AA-------- -------CCA AA-CT----- ----------
CMW5711                         AA-------- -------CCA AA-CT----- ----------
CMW5713                         AA-------- -------CCA AA-CT----- ----------
CMW5706                         AA-------- -------CCA AA-CT----- ----------
CMW5710                         AA-------- -------CCA AA-CT----- ----------
CMW5720                         AA-------- -------CCA AA-CT----- ----------
M._irregulariramosaCMW4943      AA-------- -------CCA AA-CT----- ----------
M._irregulariramosaCMW5149      AA-------- -------CCA AA-CT----- ----------
M._heimioidesCMW3046            AA-------- -------CCA AA-CT----- ----------
Ramulispora_anguioides          AAGCTCGGTT CAGACCTCCA CCCTTGAA-T AAAAAACCTT
```

```
                                170         180         190         200
M._heimiiCMW4942                TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTGGGCG
CMW5705                         TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
CMW5707                         TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
CMW5714                         TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
CMW5711                         TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
CMW5713                         TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
CMW5706                         TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
CMW5710                         TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
CMW5720                         TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
M._irregulariramosaCMW4943      TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
M._irregulariramosaCMW5149      TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
M._heimioidesCMW3046            TGTTGCTTCG G--GGGCGAC CCT-GCCG-- --CTTCGGCG
Ramulispora_anguioides          TGTTGCTTTG GCAGGACGCC TCGCGCCAGC GGCTTCGGCT

                                210         220         230         240
M._heimiiCMW4942                GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
CMW5705                         GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
CMW5707                         GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
CMW5714                         GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
CMW5711                         GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
CMW5713                         GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
CMW5706                         GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
CMW5710                         GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
CMW5720                         GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
M._irregulariramosaCMW4943      GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
M._irregulariramosaCMW5149      GT-GCGGC-G CCCCCGGAGG CCATTA---- AACACTGCAT
M._heimioidesCMW3046            GT-GCGGC-G CCCCCGGAGG CCAT-----A AACACTGCAT
Ramulispora_anguioides          GTTGAGTG-C CTGCCAGAGG ACCA----CA ACTCTTGTTT

                                250         260         270         280
M._heimiiCMW4942                CA--TTG-CG TCGGAGTAA- -------A-- AGTAAAT-TA
CMW5705                         CA--TTG-CG TCGGAGTAA- -------A-- AGTAAAT-TA
CMW5707                         CA--TTG-CG TCGGAGTAA- -------A-- AGTAAAT-TA
CMW5714                         CA--TTG-CG TCGGAGTAA- -------A-- AGTAAAT-TA
CMW5711                         CA--TTG-CG TCGGAGTAA- -------A-- AGTAAAT-TA
CMW5713                         CA--TTG-CG TCGGAGTAA- -------A-- AGTAAAT-TA
CMW5706                         CA--TTG-CG TCGGAGTAA- -------A-- AGTAAAT-TA
CMW5710                         CA--TTG-CG TCGGAGTAA- -------A-- AGTAAAT-TA
CMW5720                         CA--TTG-CG TCGGAGTAA- -------A-- AGTAAAT-TA
M._irregulariramosaCMW4943      CA--TTG-CG TCGGAGTTA- -------A-- AGTAAAT-TA
M._irregulariramosaCMW5149      CA--TTG-CG TCGGAGTTA- -------A-- AGTAAAT-TA
M._heimioidesCMW3046            CA--TTG-CG TCGGAGT--- -------AAA AGTAAAT-TA
Ramulispora_anguioides          TTAGTG-ATG TCTGAGTACT AT-------- --ATAAT--A

                                290         300         310         320
M._heimiiCMW4942                AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
CMW5705                         AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
CMW5707                         AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
CMW5714                         AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
CMW5711                         AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
CMW5713                         AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
CMW5706                         AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
CMW5710                         AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
CMW5720                         AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
M._irregulariramosaCMW4943      AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
M._irregulariramosaCMW5149      AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
M._heimioidesCMW3046            AACAAAACTT TCAACAACGG ATCTCTTGGT TCCAGCATCG
Ramulispora_anguioides          GTTAAAACTT TCAACAACGG ATCTCTTGGT TCTGGCATCG
```

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                                330        340        350        360
M._heimiiCMW4942                ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
CMW5705                         ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
CMW5707                         ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
CMW5714                         ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
CMW5711                         ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
CMW5713                         ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
CMW5706                         ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
CMW5710                         ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
CMW5720                         ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
M._irregulariramosaCMW4943      ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
M._irregulariramosaCMW5149      ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
M._heimioidesCMW3046            ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC
Ramulispora_anguioides          ATGAAGAACG CAGCGAAATG CGATAAGTAA TGTGAATTGC

                                370        380        390        400
M._heimiiCMW4942                AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
CMW5705                         AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
CMW5707                         AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
CMW5714                         AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
CMW5711                         AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
CMW5713                         AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
CMW5706                         AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
CMW5710                         AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
CMW5720                         AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
M._irregulariramosaCMW4943      AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
M._irregulariramosaCMW5149      AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
M._heimioidesCMW3046            AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG
Ramulispora_anguioides          AGAATTCAGT GAATCATCGA ATCTTTGAAC GCACATTGCG

                                410        420        430        440
M._heimiiCMW4942                CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
CMW5705                         CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
CMW5707                         CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
CMW5714                         CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
CMW5711                         CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
CMW5713                         CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
CMW5706                         CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
CMW5710                         CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
CMW5720                         CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
M._irregulariramosaCMW4943      CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
M._irregulariramosaCMW5149      CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
M._heimioidesCMW3046            CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT
Ramulispora_anguioides          CCCTCTGGTA TTCCGGGGGG CATGCCTGTT CGAGCGTCAT

                                450        460        470        480
M._heimiiCMW4942                TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
CMW5705                         TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
CMW5707                         TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
CMW5714                         TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
CMW5711                         TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
CMW5713                         TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
CMW5706                         TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
CMW5710                         TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
CMW5720                         TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
M._irregulariramosaCMW4943      TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
M._irregulariramosaCMW5149      TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
M._heimioidesCMW3046            TTCACCAC-T CAAGC-CTG- -GCTTGGTAT TGGGCGTCG-
Ramulispora_anguioides          TATAACCACT CAAGCTCTC- -GCTTGGTAT TGGGGTTCG-
```

```
                                490         500         510         520
M._heimiiCMW4942                -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
CMW5705                         -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
CMW5707                         -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
CMW5714                         -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
CMW5711                         -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
CMW5713                         -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
CMW5706                         -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
CMW5710                         -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
CMW5720                         -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
M._irregulariramosaCMW4943      -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
M._irregulariramosaCMW5149      -CGGCT----  --CCGCG---  ---CGCCTTA  AAGTCTT--C
M._heimioidesCMW3046            -CGGCTT---  ---CGCG---  ---CGCCTTA  AAGTCTT--C
Ramulispora_anguioides          -CG-GTTTC-  GCG-GC----  ----CTCT-A  AACTCA---G

                                530         540         550         560
M._heimiiCMW4942                CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
CMW5705                         CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
CMW5707                         CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
CMW5714                         CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
CMW5711                         CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
CMW5713                         CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
CMW5706                         CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
CMW5710                         CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
CMW5720                         CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
M._irregulariramosaCMW4943      CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
M._irregulariramosaCMW5149      CGGCTG-AGC  TGTC-CGTCT  CTAAGCGTTG  TGGCAACTAT
M._heimioidesCMW3046            CGGCTG-AGC  TGTC-CGTCT  CTAAGCGATG  TGGCAACTAT
Ramulispora_anguioides          TGGCGG--TG  CCTGTCGGCT  CTACGCGTAG  TAATA-CTCC

                                570         580         590         600
M._heimiiCMW4942                ------TCGC  TTCG---GAG  G-T-CGGG-T  GGC--CGCGG
CMW5705                         ------TCGC  TTCG---GAG  G-T-CGGG-T  GGC--CGCGG
CMW5707                         ------TCGC  TTCG---GAG  G-T-CGGG-T  GGC--CGCGG
CMW5714                         ------TCGC  TTCG---GAG  G-T-CGGG-T  GGC--CGCGG
CMW5711                         ------TCGC  TTCG---GAG  G-T-CGGG-T  GGC--CGCGG
CMW5713                         ------TCGC  TTCG---GAG  G-T-CGGG-T  GGC--CGCGG
CMW5706                         ------TCGC  TTCG---GAG  G-T-CGGG-T  GGC--CGCGG
CMW5710                         ------TCGC  TTCG---GAG  G-T-CGGG-T  GGC--CGCGG
CMW5720                         ------TCGC  TTCG---GAG  G-T-CGGG-T  GGC--CGCGG
M._irregulariramosaCMW4943      ------TCGC  TTCG---GAG  G-C-CGGG-T  GGC--CGCGG
M._irregulariramosaCMW5149      ------TCGC  TTCG---GAG  G-C-CGGG-T  GGC--CGCGG
M._heimioidesCMW3046            C------CGC  TTTG---GAG  ---GCGGG-T  GGC----CGG
Ramulispora_anguioides          ------TCGC  GAT---TGAG  TCCGGTA---  GGTTTACTTG

                                610         620         630         640
M._heimiiCMW4942                CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
CMW5705                         CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
CMW5707                         CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
CMW5714                         CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
CMW5711                         CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
CMW5713                         CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
CMW5706                         CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
CMW5710                         CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
CMW5720                         CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
M._irregulariramosaCMW4943      CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
M._irregulariramosaCMW5149      CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
M._heimioidesCMW3046            CC--------  GTTAAATCTT  T---CACAA-  GGTTGAC-CT
Ramulispora_anguioides          CCAACAACC-  -----CCCAA  TTTTTTACA-  GGTTGAC-CT
```

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                                650
M._heimiiCMW4942                CGGATCAGGT AGGGATA
CMW5705                         CGGATCAGGT AGGGATA
CMW5707                         CGGATCAGGT AGGGATA
CMW5714                         CGGATCAGGT AGGGATA
CMW5711                         CGGATCAGGT AGGGATA
CMW5713                         CGGATCAGGT AGGGATA
CMW5706                         CGGATCAGGT AGGGATA
CMW5710                         CGGATCAGGT AGGGATA
CMW5720                         CGGATCAGGT AGGGATA
M._irregulariramosaCMW4943      CGGATCAGGT AGGGATA
M._irregulariramosaCMW5149      CGGATCAGGT AGGGATA
M._heimioidesCMW3046            CGGATCAGGT AGGGATA
Ramulispora_anguioides          CGGATCAGGT AGGGATA
```

SUMMARY

Studies presented in this thesis, highlight the complexity and importance of Mycosphaerella leaf disease (MLD) on *Eucalyptus* spp., especially in South Africa. In Chapter 1, a review of the literature dealing with *Mycosphaerella* and MLD of *Eucalyptus* spp. is presented. It is clear from this review that the disease is prevalent in most countries where *Eucalyptus* spp. are commercially grown, including Australia where they are native. The number of *Mycosphaerella* species known from *Eucalyptus* spp. is increasing and this suggests that their economic effect on commercial *Eucalyptus* forestry, will probably increase. It will thus become important to effectively identify species responsible for MLD. To do this, the existing complex taxonomy of this group of fungi, will undoubtedly prove to be an obstacle. However, DNA based identification methods are proving useful in identifying species and delimiting lineages within *Mycosphaerella*. Future commercial propagation of *Eucalyptus* spp. will need to seriously consider the use of hybrids resistant to infection by *Mycosphaerella* spp. Furthermore, there will be a serious need for effective quarantine measures to prevent the introduction of new, perhaps more pathogenic, *Mycosphaerella* spp. into areas where they do not already occur.

Three species of *Mycosphaerella*, *M. molleriana*, *M. nubilosa* and *M. juvenis* have traditionally been regarded as the most important *Mycosphaerella* spp. in South Africa. At various times, each species has been considered to be the only pathogen causing MLD in the country. Results from Chapter 2 and Chapter 3 have shown that *M. nubilosa* is the main pathogen responsible for MLD, especially, on *E. nitens* in the KwaZulu-Natal province of South Africa. This is interesting as *M. molleriana*, which was originally thought to be the only species in South Africa, was not isolated. Moreover, the susceptibility of *E. nitens* to *M. nubilosa* appears to be high, resulting in severe defoliation. Considering that *E. nitens* is a popular species grown at higher altitudes of South Africa, the recognition of *M. nubilosa* is important. This fungus is well recognized in Australia as an important pathogen and comparisons of data from that country will be useful in the future.

Several *Mycosphaerella* spp. have, in the past been found to occur within single stands of commercial *Eucalyptus* spp. As part of the research presented in Chapter 3, surveys conducted in South Africa showed that there are seven species of *Mycosphaerella*

occurring in plantations. These include: *M. ellipsoidea*, *M. irregulariramosa*, *M. juvenis*, *M. lateralis*, *M. marksii*, *M. nubilosa* and one newly described species *M. fori*. All of these species, apart from *M. fori*, were previously known to occur in South Africa. It is interesting that *M. juvenis*, previously thought to be one of the main species causing MLD, was found only to occur in a low numbers. This suggests that species causing epidemics may change over time. The identification of *M. fori* from a previously well surveyed area was unexpected. This new species was dominant in Tzaneen and future surveys will be conducted to determine its distribution and host range within South Africa. The identification of a new species also highlights the need for additional surveys in South Africa to identify new species and to recognize possible new introductions of exotic *Mycosphaerella* spp. The presence of *M. ellipsoidea*, *M. irregulariramosa*, *M. lateralis* and *M. marksii* in this survey was not unusual, as they were previously known in South Africa. However, they were found only to occur at low levels and, as such, do not seem to contribute significantly to outbreaks of MLD.

Various taxonomic and DNA-based methods have been used for the identification of *Mycosphaerella* spp. However, some taxonomic characters are of little value at the species level. In Chapter 4, RFLP's were considered as an option to differentiate between species of *Mycosphaerella* on *Eucalyptus*. Results of this study showed that the restriction enzyme *Hae*III may be used for RFLP identification of *Mycosphaerella* spp. From a total of twenty-one *Mycosphaerella* spp. tested, RFLP digestion with *Hae*III could resolve six of these species to species level. However, other species formed groups that had similar restriction profiles. They could be further separated based on ascospore germination patterns. This study forms a foundation for future studies in which other enzymes may be used together with *Hae*III to elucidate groups of species. It is suggested though, that this technique be combined with existing methods such as ascospore germination patterns and anamorph associations to identify species of *Mycosphaerella* occurring on *Eucalyptus* spp. with confidence. This should negate the use of expensive sequencing techniques, which are currently necessary.

In virtually every country where *Eucalyptus* is grown commercially, MLD is prevalent. However, the specific *Mycosphaerella* spp. in countries are generally not the same. In Chapter 5, I used DNA sequence data from the ITS region of the rDNA operon as well as morphological data to identify *M. heimii* from Brazil and Hawaii, U.S.A. This represents

the first report of the species from these countries. *M. heimii* was previously thought to occur only in Madagascar and Indonesia, where it is recognized as a primary pathogen of several *Eucalyptus* spp, including *E. urophylla*. This is one of the main *Eucalyptus* spp. in Brazil. It has thus been suggested that this species may have been introduced into these countries via infected seed lots. This highlights the need for effective quarantine measures within these and other South American countries to inhibit the further spread of this pathogen through South America.

OPSOMMING

Studies wat in hierdie tesis aangebied is, beklemtoon die kompleksiteit en belangrikheid van Mycosphaerella blaarsiekte MLD op *Eucalyptus* spp., veral in Suid-Afrika. Hoofstuk 1 verteenwoordig 'n oorsig van die literatuur op *Mycosphaerella* en MLD van *Eucalyptus* spp. Dit is duidelik vanuit die literatuur dat die siekte algemeen voorkom in lande waar *Eucalyptus* spp. kommersieël verbou word insluitende die inheemse bome van Australia. Die aantal bekende *Mycosphaerella* spesies op *Eucalyptus* spp. is besig om toe te neem, en dit impliseer 'n toename in die ekonomiese impak op die kommersiële *Eucalyptus* bosbou. Die korrekte identifikasie van spesies verantwoordelik vir MLD is dus baie belangrik. Die bestaande komplekse taksonomie van hierdie groep swamme bemoelik die taak van identifikasie, alhoewel DNS gebaseerde metodes vir die identifisering van spesies en bepaling van verwantskappe binne *Mycosphaerella* al as bruikbaar bewys is. Toekomstige kommersiële verbouing van *Eucalyptus* spp. sal die gebruik van hibriede wat weerstandbiedend teen *Mycosphaerella* spp. is, êrenstig moet oorweeg. Verder is daar 'n dringende behoefte vir effektiewe kwarantyn maatreëls om die binnedringing van nuwe, dalk meer patogeniese, *Mycosphaerella* spp. in ongeïnfekteerde areas te voorkom.

Drie spesies van *Mycosphaerella*, naamlik *M. molleriana*, *M. nubilosa* and *M. juvenis*, was oorspronklik gereken as die belangrikste *Mycosphaerella* spp. in Suid-Afrika. Op verskillende tye is al aangeneem dat een van die spesies die enigste patogeen is wat MLD in Suid-Afrika veroorsaak. Resultate van Hoofstuk 2 en Hoofstuk 3 bewys dat *M. nubilosa* die hoof patogeen is wat MLD veroorsaak, veral op *E. nitens* in die provinsie KwaZulu-Natal in Suid-Afrika. Dit is interessant dat *M. molleriana*, wat oorspronklik gereken is as die enigste spesie in Suid-Afrika, nie geisoleer kon word nie. Daarby, was *E. nitens* baie vatbaar vir *M. nubilosa*, wat aanleiding gegee het tot êrnstige terugsterwing. Die identifikasie van *M. nubilosa* is veral baie belangrik in die lig daarvan dat *E. nitens* 'n populêre spesie in areas in Suid-Afrika wat hoog bo seespieël is. Hierdie swam is bekend in Australië as 'n belangrike patogeen, en vergelyking van data tussen Suid-Afrika en Australië sal baie bruikbaar wees in die toekoms.

Verskeie *Mycosphaerella* spp. is al in die verlede saam gevind in 'n enkel kommersiële *Eucalyptus* plantasie. Die navorsing in Hoofstuk 3, wys dat daar sewe spesies van *Mycosphaerella* voorkom in Suid-Afrikaanse plantasies. Hulle sluit in: *M. ellipsoidea*, *M.*

irregulariramosa, *M. juvenis*, *M. lateralis*, *M. marksii*, *M. nubilosa* en een nuut beskryfde spesie *M. fori*. Al die spesies, behalwe *M. fori*, is al voorheen in Suid-Afrikaanse plantasies gevind. Dit is interesant dat *M. juvenis*, wat voorheen bestempel is as een van die belangrikste spesies wat MLD veroorsaak in Suid-Afrika, in lae getalle voorgekom het. Dit impliseer dat spesies wat voorheen aanleiding gegee het tot epidemies, oor tyd kan verander. Die identifikasie van *M. fori* van 'n area wat vroeër deeglik ondersoek is, was onverwags. Die nuwe spesie was dominant in Tzaneen en toekomstige opnames sal uitgevoer word om die verspreiding en die verskillende gashere binne Suid-Afrika te bepaal. Die identifikasie van 'n nuwe spesie beklemtoon ook die noodsaaklikheid vir addisionele soektogte in Suid-Afrika om spesies te identifiseer en infeksies deur eksotiese *Mycosphaerella* spp. te kan uitken. Die teenwoordigheid van *M. ellipsoidea*, *M. irregulariramosa*, *M. lateralis* and *M. marksii* in hierdie opname was nie ongewoon nie, omdat hulle al bekend was in Suid-Afrika. Daar is wel gevind dat hulle slegs in lae getalle voorkom, en hulle dus nie noemenswaardig bydra tot die uitbraak van MLD in Suid-Afrika nie.

Verskeie taksonomiese en DNS gebaseerde metodes is gebruik vir die identifikasie van *Mycosphaerella* spp. Sommige taksonomiese karakters is egter nie baie betekenisvol vir identifikasie van spesies nie. In Hoofstuk 4, is Restriksie Fragment Lengte Polimorfismes (RFLP's) ondersoek as 'n opsie om te onderskei tussen spesies van *Mycosphaerella* op *Eucalyptus*. Resultate van die studie het bewys dat die restriksie ensiem *Hae*III gebruik kan word vir RFLP identifikasie van *Mycosphaerella* spp. Uit 'n totaal van een-en-twintig *Mycosphaerella* spp. wat getoets is, kon RFLP na snying met *Hae*III ses van die spesies onderskei. Daar was egter ook gevind dat sommige RFLP profiele spesie groepe verteenwoordig. Spesies binne die groepe kon verder onderskei word deur askospoor ontkiemingspatrone. Hierdie studie vorm 'n basis vir toekomstige studies, waarin ander ensieme saam met *Hae*III gebruik kan word om spesies binne spesie-groepe te onderskei. Hierdie tegniek moet gekombineer word met bestaande identifikasie metodes soos, askospoor ontkiemings patrone, om spesies van *Mycosphaerella* op *Eucalyptus* spp. met sekerheid te identifiseer.

In byna elke land waar *Eucalyptus* kommersieël geproduseer word, kom MLD volop voor. Die spesifieke *Mycosphaerella* spp. in hierdie lande is oor die algemeen nie dieselfde nie. In Hoofstuk 5, is DNS basispaar volgorde data van die ITS gebied van die ribosomale DNS

operon, sowel as morfologiese data, gebruik om *M. heimii* van Brazilië en Hawaii, V.S.A te identifiseer. Dit was voorheen aanvaar dat *M. heimii* slegs in Madagaskar en Indonesië voorkom, waar dit as 'n primêre patogeen van verskeie *Eucalyptus* spp, soos *E. urophylla*, erken is. *Eucalyptus urophylla* is ook een van Brazilië se belangrikste *Eucalyptus* spp. Dit is moontlik dat *M. heimii* die lande binne gekom het deur middel van geinfekteerde saad. Dit beklemtoon die belang van kwarantyn maatreëls om verdere verspreiding van hierdie patogeen binne hierdie en ander Suid-Amerikaanse lande te voorkom.