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PREFACE

Species of *Eucalyptus* are native to Australia, Indonesia, Papua New Guinea and the Philippines where they occur in natural forests. Numerous of these *Eucalyptus* spp. are propagated commercially in tropical and sub-tropical countries worldwide. In South Africa, *Eucalyptus* plantations form a large part of the commercial forestry sector and approximately 700 000 hectares are now planted to various *Eucalyptus* spp. and their hybrids. Timber produced from *Eucalyptus* spp. grown in commercial plantations has many uses, primarily in the paper and pulp industries. Revenue generated from such commercial forestry operations contributes to the overall GDP of the country and provides employment for several thousand individuals.

Eucalyptus spp. are susceptible to many pests and pathogens. Some of the most destructive *Eucalyptus* leaf pathogens are ascomycete fungi belonging to the genus *Mycosphaerella*. Approximately 100 species belonging to this genus are recognised as causing leaf diseases of *Eucalyptus* spp., which are collectively known as Mycosphaerella Leaf Disease (MLD). Infection by species of *Mycosphaerella* results in several symptoms including leaf spots, twig cankers, twig dieback and growth stunting. Due to these infections *Eucalyptus* trees can be prematurely defoliated and in such cases, they cannot achieve their full growth potential.

Much research has been conducted on *Mycosphaerella* spp. causing MLD on *Eucalyptus* spp. This research has focussed on the taxonomy, epidemiology and phylogeny of these fungi, but also tree improvement. Classical morphological and DNA-based phylogenetic studies of *Mycosphaerella* spp. occurring on *Eucalyptus* have given rise to a great increase in the number of *Mycosphaerella* spp. being described from this host. This work has resulted in a broader understanding of *Mycosphaerella* species concepts. The first chapter of this thesis, therefore, serves to critically review and analyse the existing body of research on *Mycosphaerella* spp. known to occur on *Eucalyptus* and to place this research within the context of this thesis.

Identification of *Mycosphaerella* spp. is notoriously difficult. Early taxonomic studies on these fungi used teleomorph morphology to identify species. Later studies focussed on morphological characteristics of the anamorphs as these offered additional characters to identify and discriminate between *Mycosphaerella* spp. These were, however, also not completely satisfactory and most recently, DNA sequence comparisons have become the preferred technique used to identify and consider species relationships within

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Mycosphaerella. Here, the Internal Transcribed Spacer (ITS) region of the ribosomal DNA operon has been the predominant gene region used when identifying species of *Mycosphaerella*. The ITS gene region does not, however, offer sufficient genetic resolution to study deeper nodes within *Mycosphaerella*, nor does it elucidate species complexes. To address this problem, the second chapter of this thesis employs DNA sequence data from four nuclear gene regions in order to offer more genetic resolution for *Mycosphaerella* spp. The aim in this chapter is to produce a framework wherein both deeper nodes and, therefore, anamorph associations, of *Mycosphaerella* may be effectively characterised and studied. Furthermore, terminal nodes, species boundaries and species complexes can be elucidated with greater confidence when using DNA sequence results from several gene regions.

Eucalyptus spp. are grown on a large scale in south-east Asia. In Thailand several thousand hectares are planted to *Eucalyptus camaldulensis* and hybrids of this species. In recent years, a serious leaf disease of *E. camaldulensis* and its hybrids has been observed on these trees. This disease is well-known and causes distinctive chlorotic leaf lesions with abundant caespituli bearing conidiophores and conidia of a *Pseudocercospora* species. However, this disease has incorrectly been ascribed to a well-known cosmopolitan *Pseudocercospora* species, namely *Pseudocercospora eucalyptorum*. Recent surveys in Thailand have resulted in several collections of the causal agent of the disease. The aim of the research presented in chapter three was, therefore, to characterise and name this species. This was achieved by classical taxonomic evaluation and comparison with other known *Pseudocercospora* spp. known to occur on *Eucalyptus* spp. DNA sequence data from four nuclear gene regions of the unidentified *Pseudocercospora* sp. were also compared with those of *Mycosphaerella* and *Pseudocercospora* known to occur on *Eucalyptus* to study the phylogenetic relationship between this and other species known from this host.

Mycosphaerella nubilosa causes severe defoliation and growth retardation of several *Eucalyptus* spp. Many techniques have been used to study this pathogen including classical morphology, DNA sequence data, Randomly Amplified Polymorphic DNA (RAPD's) and Restriction Fragment Length Polymorphisms (RFLP's). However, microsatellites or simple sequence repeats (SSR's) have never been evaluated for their use in the study of this important *Eucalyptus* pathogen. The aim of the research presented in chapter four was to produce highly polymorphic DNA-based microsatellite markers for *M. nubilosa*, which could then be applied in population genetic studies.

Population biology studies have found wide application in the study of several important forestry fungal pathogens. The majority of research that has been conducted on

Mycosphaerella nubilosa has focussed on its epidemiology, host susceptibility, taxonomy and phylogenetic placement within *Mycosphaerella*. Little is known regarding the population dynamics of this important leaf pathogen with respect to its genetic structure, population differentiation or the mode of reproduction that is used by this species. My aim in chapter five was to investigate the genetic structure of *M. nubilosa* populations from several different countries and to determine if and how these populations were connected. Furthermore, the reproduction strategy of *M. nubilosa* is unknown and I, therefore, investigated the potential reproductive strategy employed by this pathogen. These aims were achieved by using alleles size data from 10 polymorphic microsatellite markers. By using statistical algorithms, I investigated the gene and genotypic diversity, population differentiation, gene flow and mode of reproduction of *M. nubilosa*.

Mycosphaerella nubilosa is one of the most pathogenic and well-known *Mycosphaerella* species causing MLD on *Eucalyptus*. The taxonomy of this species has undergone many revisions in the past. However, the taxonomy and phylogenetic placement of this species is still uncertain. Recent surveys conducted on *Eucalyptus* spp. from several countries have resulted in an extensive collection of *M. nubilosa* isolates. Furthermore, slight differences in morphology and epidemiology of this species have been detected. I believe that this species may actually represent a species complex consisting of two species that are morphologically very similar. The aim of the study presented in chapter six was to determine whether *M. nubilosa* represents a species complex, and if so to consider how many phylogenetic lineages are supported within this complex. This was determined by employing DNA sequence data from three nuclear gene regions. The culture morphology of this species was also intensively studied to determine if and what morphological differences exist between members of the *M. nubilosa* species complex.

The work presented in this thesis is focussed on the phylogeny, taxonomy and population biology of *Mycosphaerella* spp. occurring on *Eucalyptus*. Results generated from this thesis are aimed at increasing our understanding of *Mycosphaerella* spp. occurring on *Eucalyptus*, particularly from a phylogenetic and population structure perspective. It is my hope that results arising from this thesis will increase the understanding of species concepts and boundaries within *Mycosphaerella* and broaden general knowledge with respect to populations of *Mycosphaerella* spp. Such results should be incorporated into quarantine action lists and could, therefore, facilitate the effective trade of wood products between South Africa and other countries.

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SUMMARY

Much research has been published on *Mycosphaerella* spp. causing Mycosphaerella Leaf Disease (MLD) on *Eucalyptus* spp. The first chapter of this thesis presents a review of the literature on this topic and focuses on the taxonomy, phylogeny and population biology of *Mycosphaerella* spp. occurring on *Eucalyptus*. From the published literature, it is clear that the majority of research conducted on MLD has focussed on the epidemiology and taxonomy of *Mycosphaerella* spp and the susceptibility of *Eucalyptus* hosts to species of *Mycosphaerella*. Advances in DNA-based technologies have, however, lead to extensive DNA sequence datasets of *Mycosphaerella* spp occurring on *Eucalyptus*. These datasets have provided substantial insight into species concepts for *Mycosphaerella* and have led to the realisation that many morphological species are complexes of several cryptic phylogenetic taxa. Furthermore, a recent application to the study of *Mycosphaerella* spp. occurring on *Eucalyptus* is that concerning their population dynamics. Such studies will aid in our understanding of the genetic structure of *Mycosphaerella* populations and their movement between countries. These population-based studies will aid forestry companies in establishing *Eucalyptus* breeding programmes to produce tolerant *Eucalyptus* genotypes that may be deployed in commercial forestry operations.

Mycosphaerella spp. are difficult to identify, due to their conserved teleomorph morphology and the lack of natural occurrences of anamorph structures. DNA sequence data have, therefore, become the definitive technique used to identify *Mycosphaerella* spp. The Internal Transcribed Spacer (ITS) region of the ribosomal RNA operon has traditionally been targeted for DNA sequence comparisons. However, this gene region does not offer sufficient resolution to discriminate cryptic taxa or resolve deeper nodes within *Mycosphaerella*. Results presented in chapter two of this thesis present a multi-gene phylogeny for the identification of *Mycosphaerella* spp. occurring on *Eucalyptus*. This is based on DNA sequence data from four nuclear gene regions. The generation of these sequence datasets has allowed for competent elucidation of cryptic taxa, species complexes and the greater resolution of deeper nodes within *Mycosphaerella*. Furthermore, these results have also led to recognising that *Mycosphaerella ambipyhlla* and *M. vespa* is a synonym of *Mycosphaerella molleriana* and *Pseudocercospora epispermogonia* is recognised as the asexual state of *Mycosphaerella marksii*.

A serious foliar disease of *Eucalyptus camaldulensis* and hybrids of this species has

been known from Thailand and Vietnam for many years. This disease has been known to be caused by a species of *Pseudocercospora* and was attributed to the cosmopolitan *Pseudocercospora eucalyptorum*. Results of a study presented in chapter three of this thesis have, however, clearly shown that *P. eucalyptorum* is not the causal agent of the disease observed on *E. camaldulensis* in Thailand. By employing classical morphological techniques and DNA sequence data from four nuclear gene regions, I have shown that an undescribed species of *Pseudocercospora* is responsible for epidemics of this leaf disease. This species is formally described as *Pseudocercospora flavomarginata*. *P. flavomarginata* is only known from Thailand and Vietnam. However, considering that *E. camaldulensis* is planted in other south-east Asian countries and that *E. camaldulensis* is the most commonly found *Eucalyptus* sp. in Australia, further surveys in these areas will most likely lead to the discovery of the pathogen from these countries.

Techniques that have been used to identify *Mycosphaerella* spp. include classical morphological comparisons and analyses of DNA sequence data. These techniques have, however, allowed only for the study of the evolutionary history within *Mycosphaerella* and for species identification. Recent advances in the field of population biology have led to the study of many fungal pathogens at a population level. One of the main tools used to study population biology involves applying DNA-based microsatellite markers. Chapter four of this thesis focuses on the development of DNA-based microsatellite markers for the *Eucalyptus* leaf pathogen *Mycosphaerella nubilosa*. By employing specific enrichment protocols, I was able to develop ten polymorphic microsatellite markers for *M. nubilosa*. These microsatellite markers exhibit high specificity for *M. nubilosa* and did not cross amplify with other *Mycosphaerella* spp. that are closely related to *M. nubilosa*.

Mycosphaerella nubilosa has been extensively studied with respect to its taxonomy and epidemiology. However, nothing is known regarding the population biology of this important *Eucalyptus* leaf pathogen. Therefore, DNA-based microsatellite markers developed in chapter four of this thesis were used to study the population biology of *M. nubilosa* from several different geographic locations. Results presented in chapter five of this thesis show that populations of *M. nubilosa* from eastern Australia are genetically more diverse than those populations from western Australia, Africa and Europe. This indicates that eastern Australia is the likely centre of origin for *M. nubilosa*. Furthermore, based on shared haplotypes between *M. nubilosa* populations used in this study, I have proposed a pathway of gene flow of *M. nubilosa*. This suggests that the pathogen moved from eastern Australia to both western

Australia and South Africa and then from South Africa into other countries in Africa and finally into Europe.

An interesting result emerging from the population biology study presented in chapter five, is the finding that *M. nubilosa* appears to employ a homothallic mating strategy. Thus, opportunities exist, in countries with limited genetic diversity of *M. nubilosa*, to breed for *Eucalyptus* resistance. From the high number of *M. nubilosa* haplotypes observed in Australia and South Africa, it is also important that this pathogen be added to quarantine action lists to prevent the movement of contaminated *Eucalyptus* germplasm. This is necessary to prevent novel *M. nubilosa* haplotypes from moving into new environments where susceptible *Eucalyptus* spp. are propagated.

Mycosphaerella nubilosa is one of the most pathogenic *Mycosphaerella* spp. causing MLD on *Eucalyptus*. Surveys of diseased *Eucalyptus* plantations from several countries where this pathogen occurs, have resulted in an extensive collection of *M. nubilosa* isolates. Recently, DNA-based studies have led to the hypothesis that *M. nubilosa* may represent two distinct taxa. Results of studies presented in chapter six of this thesis indicate that two distinct ITS phylogenetic lineages are represented by *M. nubilosa sensu lato*. These are characterised by defined geographic distributions and *Eucalyptus* host associations. *M. nubilosa* ITS lineage 1 is found exclusively in New Zealand, Tasmania and Victoria, eastern Australia occurring on *E. globulus*. *M. nubilosa* ITS lineage 2 has a broader geographic distribution and can be found in Spain, Portugal, Tanzania, Kenya, Ethiopia, South Africa, western Australia, Victoria and New South Wales, eastern Australia, where it occurs on *E. globulus* and several other *Eucalyptus* spp. that are used in commercial forestry including *E. nitens*. It is envisaged that results presented in chapter six will lead to more extensive studies into *M. nubilosa sensu lato* that may result in the description of a new *Mycosphaerella* sp. represented by *M. nubilosa* ITS lineage 1.

1

Taxonomy, phylogeny and population biology of *Mycosphaerella* species occurring on *Eucalyptus*. A literature review

1.0 INTRODUCTION

Species of *Eucalyptus sensu stricto* (excluding *Corymbia* and *Angophora*) are native to Australia, Indonesia, Papua New Guinea and the Philippines where they grow in natural forests (Ladiges 1997, Potts & Pederick 2000, Turnbull 2000). From these natural environments, various *Eucalyptus* spp. have been selected and planted as non-natives in many tropical and sub-tropical countries where they are among the favoured tree species for commercial forestry (Poynton 1979, Turnbull 2000). Commercial plantations of *Eucalyptus* spp. are second only to *Pinus* spp. in their usage and productivity worldwide and several million hectares of *Eucalyptus* spp. and their hybrids are grown in intensively managed plantations (Old *et al*. 2003). *Eucalyptus* spp. offer the advantage of desirable wood qualities and relatively short rotation periods in commercial forestry programmes where rotations range from 5−15 years with appropriate silvicultural and site practices (Zobel 1993, Turnbull 2000).

Although *Eucalyptus* spp. are favoured commercial forestry species, they are threatened by many pests and diseases (Elliott *et al*. 1998, Keane *et al*. 2000). There are many native and non-native fungal pathogens that can infect the roots, stems and leaves of *Eucalyptus* trees (Park *et al*. 2000, Old & Davison 2000, Old *et al*. 2003). Consequently there are many pathogens that can infect and cause disease on *Eucalyptus* trees simultaneously. It is important, therefore, to identify and understand the biology of such pathogens in order to develop effective management strategies for commercial *Eucalyptus* forestry.

Some of the most important *Eucalyptus* leaf diseases are caused by species of *Mycosphaerella* Johanson. More than 100 species of this genus are recognised for causing leaf diseases on *Eucalyptus* spp. (Crous 1998, Crous *et al*. 2004a, Crous *et al*. 2006). These leaf diseases are collectively known as Mycosphaerella Leaf Disease (MLD). *Mycosphaerella* spp. have been identified from both natural *Eucalyptus* stands and commercial *Eucalyptus* plantations where they cause a range of symptoms including leaf spots, leaf withering, twig cankers, premature defoliation, multi-leadered stems, seedling blight and in severe cases death of young trees (Park & Keane 1982b, Crous 1998). The majority of *Eucalyptus* spp. become

infected by *Mycosphaerella* spp. during their juvenile leaf phase and the leaf spots caused by *Mycosphaerella* spp. reduce the photosynthetic capacity of *Eucalyptus* leaves, leading to premature leaf defoliation and overall growth stunting (Park & Keane 1982b, Lundquist & Purnell 1987, Carnegie & Ades 2003, Pinkard & Mohammed 2006).

Mycosphaerella is one of the largest ascomycete genera. Over 3000 taxa are currently accommodated in this genus, with the majority of species recognised as saprobes or pathogens of woody and herbaceous hosts (von Arx 1983, Corlett 1991, Aptroot 2006). The sexual stage of *Mycosphaerella* is morphologically conserved and species of the genus are difficult to culture, thus making species identification particularly complex (Crous 1998, Crous *et al*. 2004a). There are, however, approximately 30 anamorph genera that are associated with *Mycosphaerella* (Crous *et al*. 2000, 2004a, 2006). These anamorph states are morphologically variable and provide greater information for species delineation (Crous & Wingfield 1996, Crous *et al* 2000, 2006, Verkley & Priest 2000). Ultimately, results from DNA sequence analyses is the most effective method used in *Mycosphaerella* species identification. Therefore, morphological characteristics, combined with recent advances in DNA-based technologies have served to elucidate species concepts within *Mycosphaerella* and aid in competent species identification.

An extensive body of research on *Mycosphaerella* has been published in recent years. This literature review, therefore, serves to critically analyse the existing research on *Mycosphaerella* and place it into context with the particular aims of this thesis, which are to study those species of *Mycosphaerella* involved in MLD of *Eucalyptus*.

2.0 MYCOSPHAERELLA LEAF DISEASE (MLD)

2.1 DISEASE EPIDEMIOLOGY

An understanding of the epidemiology of MLD provides valuable information for the management of this disease. Epidemiological knowledge, such as the infection process and disease development of *Mycosphaerella* spp. occurring on *Eucalyptus* spp., is based largely on studies of *Mycosphaerella cryptica* (Cooke) Hansf. and *Mycosphaerella nubilosa* (Cooke) Hansf., two of the most important *Eucalyptus* leaf pathogens (Park & Keane 1982a, b, Park 1988a, b). The infection process and disease development of these *Mycosphaerella* spp. on *Eucalyptus* can be considered in terms of three phases namely, spore deposition and infection, fungal growth and formation of reproductive structures and finally spore liberation.

2.1.1 *Spore deposition and infection*

Both the ascospores and conidia of *Mycosphaerella* spp. can be involved in disease development on *Eucalyptus* leaves. However, ascospores act as the primary source of inoculum in many species (Beresford 1978, Park & Keane 1987). The primary inoculum source is from attached infected leaves or from fallen overwintered leaf litter, which is the general trend for many ascomycete leaf-inhabiting fungi (Luley & McNabb 1989, Patton & Spear 1983, Park & Keane 1987). Ascogonia or conidiomata of *Mycosphaerella* spp. have been shown to remain viable for a period of several months, providing sufficient inoculum for successive infection cycles (Cheah & Hartill 1987, Park & Keane 1987, Park 1988b). In certain *Mycosphaerella* spp. such as *Mycosphaerella citri* Whiteside, the cause of citrus greasy spot, it is known that ascospore production can occur throughout the entire year ensuring a continual inoculum source (Mondal & Timmer 2002).

Infection of *Eucalyptus* leaves by *Mycosphaerella* spp. predominantly occurs during the vegetative period of the host during the summer and autumn months (Ganapathi 1979, Cheah & Hartill 1987). Park (1988a), showed that the young expanding leaves, less than 46 days old, of *E. globulus*, were particularly susceptible to *M. nubilosa*, while Ganapathi (1979), showed that leaves of *Eucalyptus delegatensis* were most susceptible to infection during the first 21 days after they unfold. However, as the leaves of *Eucalyptus* spp. age they become more resistant to infection as a result of the deposition of resistant compounds such as lignin (Park 1988a).

Infection of the leaf surface by the spores may either be direct or indirect. Germ tubes of *M. cryptica* ascospores are able to penetrate both directly, through the cuticle, or indirectly through stomata (Park & Keane 1982b, Park 1988a). During direct penetration, a protoappresorium is formed alongside the ascospore or at the end of the germ tube (Ganapathi 1979, Park & Keane 1982b). The protoappresorium forms an infection peg that penetrates the cuticle allowing the spore to form invasive hyphae that grow between the cuticle and epidermal cells. Branching hyphae are subsequently formed that grow intercellularly throughout the epidermal layer (Park 1988a). In the case of indirect penetration, infection occurs through the stomata where the germ tubes produce hyphal swellings within the stomatal pores and substomatal cavities (Park & Keane 1982b, Niyo *et al*. 1986). Conidia of *Mycosphaerella lateralis* Crous & M.J. Wingf. have been shown to germinate on both adaxial

and abaxial leaf surfaces, but only penetrate the leaf through stomata on the abaxial leaf surface and do not produce hyphal swellings or appresoria (Jackson *et al*. 2004). Crous *et al*. (1989c), showed from growth room inoculations how conidia of *Phaeophleospora epicoccoides* (Cooke & Massee) Crous, F.A. Ferreira & B. Sutton (as *Phaeoseptoria eucalypti* Hansf.) were able to penetrate leaves of *Eucalyptus* spp., from the subgenus *Symphyomyrtus*, through leaf stomata. Due to the larger number of stomata on abaxial leaf surfaces there is an increase in infection and subsequent pseudothecial development on abaxial leaf surfaces (Niyo *et al*. 1986).

Levels of moisture in the environment affect the ability of the fungal spores to infect host material. Higher levels of infection, and consequently disease development, have been noted for spores of *Mycosphaerella populorum* G.E. Thomps., on *Populus*, after periods of rainfall and leaf wetting (Luley & McNabb 1989). Conidial germination of *Mycosphaerella fijiensis* var. *difformis* J.L. Mulder & R.H. Stover decreased as levels of relative humidity decreased and maximum germ tube development was observed in the presence of free water (Jacome *et al*. 1991). Temperature also affects the ability of spores to infect the leaf surface. It has been shown that ascospores and conidia of *M. fijiensis* var. *difformis* germinate at temperatures ranging from 20–35 °C with maximum germination occurring at 25 °C (Jacome *et al*. 1991).

Once ascospores or conidia are deposited onto the leaf surface they germinate and form germ tubes. Germination of ascospores and conidia generally occurs in surface moisture, although it has been shown that spores of some *Mycosphaerella* spp. occurring on *Eucalyptus*, such as *M. nubilosa* and *M. cryptica*, can survive periods of desiccation on the leaf surface and still remain viable and infective (Beresford 1978, Park 1988a).

2.1.2 *Fungal growth and formation of reproductive structures*

Upon entry into the leaf, fungal hyphae grow along the vascular bundles and colonize the leaf tissue, becoming established throughout the leaf. Following chlorosis, hyphae grow intercellularly throughout the spongy and palisade mesophyll and eventually aggregate in the substomatal cavities as has been shown in *M. cryptica* and *M. nubilosa* occurring on *Eucalyptus* (Park & Keane 1982b). These hyphal aggregates then develop into immature ascomata with trichogynes (Park & Keane 1982b).

Ganapathi (1979) described the development of pseudothecial ascomata of *M. cryptica* (as *M. nubilosa*) in detail. His studies showed that the ascocarp initials comprise a group of

cells. This developing ascoma has the appearance of a stroma with the presence of developing trichogynes, which grow toward the stromatal apex. During ascogonial development, the stroma matures and breaks through the host surface. The developing trichogynes grow through the top of the stroma and are fertilised by spermatia of the genus *Asteromella* Pass. & Thüm. Spermatia are formed in a gelatinous matrix that seaps from the ostiole onto the leaf surface (Ganapathi & Corbin 1979). After fertilisation, ascogonia mature and through successive developmental steps form asci and ascospores. Mature ascogonia of *Mycosphaerella* spp. generally have large thick, elongated cells impregnated with melanin that form the outer layers of the ascocarp wall (Niyo *et al.* 1986). Although cells making up the inner ascocarp walls generally contain lower melanin levels than those of the outer ascogonial walls, similar cellular organelles are observed in both cell types (Niyo *et a*l. 1986).

2.1.3 *Spore liberation*

Liberation of ascospores is dependant on moisture. For example, in *M. cryptica* and *M. nubilosa*, ascospores are discharged when the relative humidity is greater than 95 % and are not discharged when the relative humidity is below 90 % (Beresford 1978, Park & Keane 1982b, Cheah & Hartill 1987). Cheah & Hartill (1987) found that ascospores of *M. cryptica* are discharged after rainfall and that discharge continues for up to two hours after rainfall has ceased. They also suggest that longer periods of light rainfall will lead to more ascospores being discharged because this allows for ascospore maturation in the asci. Discharge of ascospores in this case will continue in the presence of sufficient moisture and relative humidity until the asci within the pseudothecia are exhausted of ascospores (Cheah & Hartill 1987). Mondal *et al.* (2003), observed that ascospore release of *M. citri* under field conditions occurred 1 to 2 hours after rainfall had begun, with peak ascospore release occurring 6 to 8 hours after the onset of rainfall. Furthermore, ascospore release continued for up to 16 hours after rainfall. Dew may also serve as a stimulus for ascospore release, but fewer ascospores are found to be released under these conditions as has been shown in *M. populorum* on mixed hybrids of *Populus* and suggested for *M. citri* on *Citrus* (Luley & McNabb 1989, Mondal *et al*. 2003).

Park & Keane (1982b), found that the optimum temperature for ascospore discharge in *M. nubilosa* and *M. cryptica* was 25 °C and 20 °C, respectively, and that they may be ejected up to a distance of 12–15 mm above the pseudothecia. This would allow the spores to be wind dispersed. Ascospores are likely to be dispersed by wind for considerable distances as has

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been suggested for ascospores of *M. citri* that may be wind dispersed for a distance of up to 80 meters (Mondal *et al*. 2003). However, conidia of *Mycosphaerella* spp. would not be dispersed for long distances, for example, conidia of *M. cryptica* are usually produced in a gelatinous matrix on the leaf surface and as such would are splash-dispersed over short distances within the same tree (Beresford 1978, Cheah & Hartill 1987).

3.0 SYMPTOMATOLOGY

Infection of *Eucalyptus* leaves by *Mycosphaerella* spp. results in the formation of various symptoms. These symptoms include leaf spots, leaf blotches, leaf blight, leaf withering, lamina distortion, twig and stem cankers, tip die-back, growth stunting, multi-leadered trees and in severe cases death of the sapling or tree (Dick 1982, Crous *et al*. 1989b, Wingfield *et al*. 1996, Crous 1998, Park *et al*. 2000).

Infection by several *Mycosphaerella* spp. occurs during the juvenile phase of tree growth, resulting in severely infected and spotted leaves. Due to the leaf spotting, there is a loss in the photosynthetic capability of the leaves and they are prematurely shed as has been shown for *M. nubilosa* on *E. globulus* (Pinkard & Mohammed 2006). Furthermore, almost complete defoliation of juvenile and intermediate leaves of *E. globulus* subsp. *globulus* by *M. nubilosa* has been reported from Australia (Park & Keane 1982b). Through inoculation of potted *Eucalyptus camaldulensis* with spore suspensions of *P. epicoccoides* (teleomorph: *Mycosphaerella suttonii* Crous & M.J. Wingf.), Crous *et al*. (1989c) were able to show how this fungus causes premature defoliation after 12 weeks.

The most common symptom of *Mycosphaerella* infection is the development of leaf spots on *Eucalyptus* leaves. Leaf spots may be of varying shapes, for example circular to irregular (*M. ambiphylla* A. Maxwell, *M. cryptica*, *M. vespa* Carnegie & Keane, *Coniothyrium ovatum* H.J. Swart) (Dick 1982, Crous *et al*. 1988, Carnegie & Keane 1998, Maxwell *et al*. 2003), irregular (*M. nubilosa*) (Dick 1982), round or slightly irregular (*M. parkii* Crous, M.J. Wingf., F.A. Ferreira & Alfenas) (Crous & Alfenas 1995, Crous *et al*. 1993b), small and discrete (*M. heimii* Crous) (Crous & Swart 1995), sub-circular to irregular (*M. suttonii*) (Crous & Wingfield 1997), sub-circular (*M. aurantia* A. Maxwell, *M. irregulariramosa* Crous & M.J. Wingf.) (Crous & Wingfield 1997, Maxwell *et al*. 2003), sub-circular to confluent (*Pseudocercospora eucalyptorum* Crous, M.J. Wingf., Marasas & B. Sutton) (Crous *et al*. 1989d) and leaf spots may be absent (*M. heimioides* Crous & M.J. Wingf.) (Crous & Wingfield 1997, Crous 1998). Several species of *Mycosphaerella* have also

been shown to have an endophytic growth phase, for instance *M. endophytica* Crous & H. Smith on *Eucalyptus* (Crous 1998), and *M. punctiformis* (Pers.) Starbäck on *Quercus* (Verkley *et al*. 2004a). It has, for instance, been shown that *M. punctiformis* is able to colonise and grow endophytically in living and dead leaves of *Quercus* and is only detected by the presence of spermagonia in senescent *Quercus* leaves (Verkley *et al*. 2004a).

Leaf spots may vary in colour on leaf surfaces. They may be brown (*M. ambiphylla*) (Maxwell *et al*. 2003), dark brown with a yellow-red margin (*M. suberosa* Crous, F.A. Ferreira, Alfenas & M.J. Wingf.) (Dick & Dobbie 2001), yellow to brown (*M. nubilosa*) (Crous *et al*. 1989a, Crous 1998), grey to pale brown (*M. tasmaniensis* Crous & M.J. Wingf., *P. eucalyptorum*) (Crous *et al*. 1989a, Crous *et al*. 1998), pale brown to red-brown (*M. vespa*) (Carnegie & Keane 1998), dark purple to black (*C. ovatum*) (Crous *et al*. 1988, Crous *et al*. 1989a), purple to brownish (*P. epicoccoides*) (Crous *et al*. 1998) pale brown surrounded by a red-purple margin (*M. suttonii*) (Crous & Wingfield 1997) or rust-brown (*M. intermedia* M.A. Dick & Dobbie) (Dick & Dobbie 2001).

Leaf lesions on *Eucalyptus* caused by *Mycosphaerella* spp. may have distinct lesion margins and lesion zones of various colours that are usually darker than the centre of the lesions. Lesions of *M. intermedia* form lesions with raised, dark brown margins that are surrounded by a red-purple zone (Dick & Dobbie 2001). Lesions of *P. epicoccoides* form lesions that are surrounded by a distinct purple discolouration (Crous *et al*. 1989a). Lesions of *M. ambiphylla* on the other hand are known to be suberized with red margins (Maxwell *et al*. 2003). Lesions may also be surrounded by red-purple borders (*M. suberosa*) (Crous *et al*. 1993a). *Eucalyptus* leaves may in certain instances become prominently buckled with lesion development as has been shown for *M. cryptica* (Park & Keane 1982b, Crous 1998).

Aggressive *Mycosphaerella* spp. may move from the leaf onto young *Eucalyptus* stems and cause cankers. *Mycosphaerella cryptica* has been observed to cause stem cankers on young branches and shoots of *E. obliqua* and *E. globulus* subsp. *globulus* (Park & Keane 1982b). However, these cankers were only caused by the acervuli of *Colletogloeopsis nubilosum* (Ganap. & Corbin) Crous & M.J. Wingf., the anamorph of *M. cryptica* (Park & Keane 1982b). Dick (1982) reported the occurrence of cankers on young petioles, shoots and twigs caused by *M. cryptica*. Such cankers eventually lead to twig girdling and die-back of the young stem and also the thinning of crowns and the death of young tree tops (Dick 1982). Cankers also result in growth stunting, multi-leadered trees and a bushy appearance of the young *Eucalyptus* tree (Dick 1982).

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4.0 TELEOMORPH CONCEPTS

Mycosphaerella is one of the largest ascomycete genera. More than 3000 taxa, that are characterised as parasites or saprobes of various vascular and woody hosts, are currently accommodated within *Mycosphaerella* (von Arx 1983, Corlett 1991, Corlett 1995, Aptroot 2006). Morphologically, *Mycosphaerella* is characterised by the formation of small, spherical, ostiolate ascomata, 8-spored, bitunicate asci without filamentous paraphyses and 2-celled, hyaline ascospores without appendages, but that can have a mucous sheath (von Arx 1983, Crous *et al*. 2000). The spermatial state of *Mycosphaerella* spp. is widely accepted to be accommodated within *Asteromella*. This genus is characterised by the formation of hyaline rod-shaped, ellipsoidal, cylindrical or allantoid spermatia formed on phialides that line the inner walls of *Mycosphaerella* spermagonia (von Arx 1983, Crous & Wingfield 1996, Verkley *et al*. 2004a).

Barr (1972) separated *Mycosphaerella* into two sub-genera based on ascus shape and anamorph associations. Each sub-genus was further separated into sections based on ascospore type and habitat. Sub-genus *Mycosphaerella* was characterised by oblong, elongate or clavate asci occurring in broad fascicles, arising from the basal layer of small cells. This sub-genus was further divided into the sections *Mycosphaerella* (saprobic ascomata, obovate, oblong to ellipsoidal ascospores with rounded apices), *Macula* M.E. Barr (parasitic ascomata, leaf spots exhibiting marginal zones, obovate, oblong to ellipsoidal ascospores with rounded apices), *Caterva* M.E. Barr (saprobic species with fusoid ascospores that have pointed ends), *Longispora* M.E. Barr (saprobic species, fusoid to elongate ascospores with a 6 : 1 or more length to width ratio) and *Plaga* M.E. Barr (parasitic species, lesions exhibiting marginal zone, fusoid ascospores with pointed ends) (Barr 1972). The sub-genus *Didymellina* (Höhn.) M.E. Barr was characterised by asci that are saccate, ovoid, oblong, few in fascicles, arising from an arched basal cushion of hyaline cells, ascospores mostly crowded in the ascus. Sub-genus *Didymellina* was further sub-divided in sections *Didymellina* (parasitic species forming leaf spots), *Cymadothea* (F.A. Wolf) Arx (multiloculate ascomata, obovate ascospores, conidial state *Polythrincium* Kunze, parasitic species causing leaf blotches), *Stigmina* M.E. Barr (uniloculate ascomata, obovate ascospores, conidial state *Stigmina*, parasitic species causing leaf blotches), *Tassiana* M.E. Barr (saprobic ascomata, obovate ascomata) and *Fusispora* M.E. Barr (saprobic ascomata, fusoid ascospores with pointed ends) (Barr 1972). Von Arx (1983) disagreed with Barr's (1972) circumscription of *Mycosphaerella* because the

delimiting characteristics were too divergent. This was further substantiated by Crous (1998), who found that the delimitation of Barr (1972) did not agree with the observed anamorph/teleomorph connections within *Mycosphaerella*.

Crous *et al*. (2000) separated *Mycosphaerella* into six sections based on morphological characteristics of the asci, ascospores and anamorph associations. Sections accepted by Crous *et al*. (2000) include the sections *Mycosphaerella* (cylindrical uniseriate asci, inequilateral, uniseriate, thin-walled, small ascospores with rounded apices, *Ramularia* Unger anamorphs), *Tassiana* (pyriform asci, thick-walled, equilateral, large ascospores that are constricted at the septum and have rounded apices, *Cladosporium* Link anamorphs), *Caterva* (cylindrical asci, thin-walled, inequilateral, medium sized ascospores with more or less pointed ends, *Asteromella* spermatial states), *Longispora* (cylindrical asci, aggregated, thin-walled, long, equilateral ascospores rarely constricted at the median septum, with rounded apices and pointed bases, *Phleospora* Wallr. or *Septoria* Sacc. anamorphs), *Fusispora* (pyriform asci, thin-walled, equilateral, fusiform ascospores that are rarely constricted at the septum and that are pointed at both ends, unknown anamorphs) and section *Plaga* (endophytic species, obovoid to ellipsoidal or cylindrical asci, small to medium fusiform to obovoid ascospores with rounded apices, anamorphs: *Colletogloeopsis* Crous & M.J. Wingf., *Mycovellosiella* Rangel, *Phaeophleospora* Rangel, *Pseudocercospora* Speg., *Pseudocercosporella* Deighton, *Sonderhenia* H.J. Swart & J. Walker, *Stenella* Syd. and *Uwebraunia* Crous & M.J. Wingf.) (Crous *et al*. 2000). According to this circumscription, those species of *Mycosphaerella* occurring on *Eucalyptus* can, therefore, be accommodated in section *Plaga*.

Braun *et al*. (2003) separated section *Tassiana* of *Mycosphaerella* into a new and separate teleomorph genus *Davidiella* Crous & U. Braun. Through a phylogenetic study of the Internal Transcribed Spacer (ITS) and Small Subunit (SSU) gene regions it was evident that *Mycosphaerella* spp. with *Cladosporium* anamorphs grouped sister to the larger *Mycosphaerella sensu stricto* clade. Due to this, *Davidiella* (*Davidiellaceae*, *sensu* Schoch *et al*. 2006), with the type species *Davidiella allicina* (Fr.) Aptroot (Aptroot 2006), was erected for those species of *Mycosphaerella* with *Cladosporium* anamorphs. Morphologically, *Davidiella* produces ascomata that are identical to *Mycosphaerella* section *Tassiana sensu* Barr (1972), but has distinct *Cladosporium* anamorphs (Braun *et al*. 2003).

Mycosphaerella is morphologically very similar to another ascomycete genus, *Didymella* Sacc. *Didymella* can, however, be distinguished from *Mycosphaerella* based on characters of the ascospores, presence of filamentous paraphyses and anamorph associations

(von Arx 1983). *Sphaerulina* Sacc. and *Microcyclus* Sacc., Syd. & P. Syd. were also once considered closely related to *Mycosphaerella*, but they have been classified into a different family (von Arx 1983). *Sphaerulina* is polyphyletic, however, and some taxa with 3-septate ascospores are presently accommodated in *Mycosphaerella* (Crous *et al*. 2003). Sivanesan (1984) placed *Mycosphaerella* within the order *Dothideales* and in the family *Dothideaceae*. However, Sutton & Hennebert (1994) placed *Mycosphaerella* within the order *Dothideales* and the family *Mycosphaerellaceae*. Despite many revisions and divisions within *Mycosphaerella*, the teleomorph remains morphologically conserved (Crous *et al*. 2000). Due to the conserved nature of the teleomorph, few morphological features are phylogenetically informative for species delimitation within *Mycosphaerella*. Through extensive phylogenetic analyses using DNA sequence data from four nuclear gene regions combined with parsimony and bayesian phylogenetic inferences, Schoch *et al*. (2006) have shown that *Mycosphaerella* does not group within the *Dothideales* but is rather accommodated within the *Capnodiales* (*Dothideomycetes*). Schoch *et al*. (2006) further proposed the Ascomycete sub-class *Dothideomycetidae*, containing the orders *Dothideales*, *Capnodiales* and *Myriangiales*. Following this classification, the taxonomic position of *Mycosphaerella* is as follows:

Phylum: *Ascomycota*
Class: *Dothideomycetes*
Sub-class: *Dothideomycetidae*
Order: *Capnodiales*
Family: *Mycosphaerellaceae*
Genus: *Mycosphaerella*

5.0 ANAMORPH ASSOCIATIONS OF *MYCOSPHAERELLA* SPP. OCCURRING ON *EUCALYPTUS* LEAVES

The use of anamorph morphology in mycological classification is, in many cases, more useful than that of the teleomorph. This is due to the diverse and heterogeneous nature of the anamorphs in comparison to the relatively conserved morphology of the teleomorph states. Crous & Mourichon (2002) stated that the anamorph states of *Mycosphaerella* spp. were not phylogenetically informative, but did acknowledge that anamorph morphology was still the most informative feature to be used to distinguish among species.

Anamorph genera of *Mycosphaerella* are morphologically diverse and are classified in both the coelomycetes and hyphomycetes (von Arx 1983, Crous & Wingfield 1996, Crous 1998). Barr (1972) noted that the anamorph genera of *Mycosphaerella* were morphologically variable and accepted eleven anamorph genera based on conidial septation, pigmentation and the formation of conidial chains. Von Arx (1983), studying the conidiomatal structure, type of scars on the conidiogenous cells and position on the host of various species representing anamorphs of *Mycosphaerella* eventually accepted 23 anamorph form genera. Sutton & Hennebert (1994) studied the mode of conidiogenesis of *Mycosphaerella* anamorphs and eventually also accepted 23 anamorph genera for *Mycosphaerella*. Crous *et al*. (2000) also recognised 23 anamorph genera of *Mycosphaerella* and separated these genera based on characters of the mycelium (presence or absence of superficial mycelium), conidiophores (arrangement, branching, pigmentation), conidiogenous cells (placement, proliferation and scar type) and conidia (formation, shape, septation, wall and pigmentation).

Von Arx (1983) noted that some species of *Mycosphaerella* that have known anamorphs are indistinguishable from species without anamorphs. He, therefore, believed that anamorphs should not be used to distinguish between genera, sub-genera or sections of *Mycosphaerella*. Braun (1990), disagreed with the view of von Arx (1983) and stated that anamorph characters that should be used for generic separation include characteristics of the caespituli (fasciculate/synnematous), nature of the conidial scars (thickened, conspicuous/unthickened, obscure) and conidial formation. Braun (1990) did, however, propose that anamorph genera that have colourless or coloured conidiophores or conidia should not be merged. Crous (1998) evaluated morphological features of *Mycosphaerella* spp. occurring on *Eucalyptus* trees using multiple correspondence analysis (MCA), and found that the species of *Mycosphaerella* grouped according to their anamorph associations. He, therefore, suggested that species of *Mycosphaerella* should be separated into groups according to their anamorph affiliations. Stewart *et al*. (1999) were able to effectively separate the cercosporoid genera *Cercospora* Fres., *Pseudocercospora* and *Passalora* Fr. through sequence data from the ITS region and therefore agreed with the view of Crous (1998).

Mycosphaerella has been linked to approximately 30 different anamorph genera including coelomycetes and hyphomycetes. However, when considering those *Mycosphaerella* spp. occurring on *Eucalyptus* leaves, only 14 anamorph genera are recognised (Crous 1998, Crous *et al*. 2006). For the purpose of this review only those *Mycosphaerella* anamorph genera occurring on *Eucalyptus* will be discussed further.

5.1 *Colletogloeopsis*

Ganapathi & Corbin (1979) described *Colletogloeum nubilosum* as the anamorph of *M. cryptica* (which they referred to as *M. nubilosa*) from *E. delegatensis* in New Zealand. They found it difficult to place *C. nubilosum* because the conidiogenous cells were annellidic and the conidia were aseptate and produced sympodially. They noted that the anamorph genera *Pollaccia* E. Bald. & Cif. and *Colletogloeum* Petr. were the genera most appropriate in which to place *C. nubilosum*. They thus placed *C. nubilosum* in *Colletogloeum* because the sexual state of *Pollaccia* was *Venturia* Sacc. and no sexual states had been found for other *Colletogloeum* species. They noted that *C. nubilosum* was distinct from other species of *Colletogloeum* in that it has aseptate conidia.

Crous & Wingfield (1997) re-evaluated the species of *Colletogloeum* on *Eucalyptus* and found that these species did not agree with the morphological description of the type of *Colletogloeum* and that these species formed a separate group that were characterised by brown, verruculose, thick-walled, aseptate conidia, from sympodially or percurrently proliferating brown, verruculose conidiogenous cells (Crous & Wingfield 1997). Crous & Wingfield (1997) therefore, established a new genus, *Colletogloeopsis* for anamorphs of *M. cryptica* (*Colletogloeopsis nubilosum*) and *Mycosphaerella molleriana* (Thüm) Lindau (*Colletogloeopsis molleriana* Crous & M.J. Wingf.). Recently, Cortinas *et al*. (2006a) emended the description of *Colletogloeopsis* to also accommodate pycnidial anamorphs. Since the description of *Colletogloeopsis*, several new *Colletogloeopsis* spp. have been identified from *Eucalyptus* leaves including *Colletogloeopsis stellenboschiana* Crous and the *Colletogloeopsis* anamorph of *Mycosphaerella pseudocryptica* Crous (Crous *et al*. 2006). In total, six *Mycosphaerella* spp. occurring on *Eucalyptus* are known to have *Colletogloeopsis* anamorphs, namely *Mycosphaerella* sp. (*C. stellenboschiana*), *Mycosphaerella* sp. [*C. zuluense* (M.J. Wingf., Crous & T.A. Coutinho) M.N. Cortinas, M.J. Wingf., & Crous], *Mycosphaerella* sp. (*C. gauchensis* M.N. Cortinas, Crous & M.J. Wingf.), *M. cryptica* (*C. nubilosum*), *M. molleriana* (*C. molleriana*) and *M. pseudocryptica* (*Colletogloeopsis* sp.) (Crous 1998, Cortinas *et al*. 2006a, b, Crous *et al*. 2006).

5.2 *Coniothyrium*

Sutton (1975) described *Coniothyrium kallangurense* B. Sutton & Alcorn from leaves of *Eucalyptus microcordyis* in Queensland, Australia. He noted that two other *Coniothyrium* spp. from *Eucalyptus* (*C. eucalypticola* B. Sutton and *C. ahmadii* B. Sutton) differed from *C. kalangurense* in having thick-walled, ornamented conidia. Crous *et al*. (1988) identified *C. ovatum* on *E. cladocalyx* and *Eucalyptus lehmannii* in South Africa. *C. ovatum* was responsible for leaf spots on these species and produced substomatal black pycnidia that formed long cirri with slightly verrucose dark brown conidia that are obovate with truncate bases (Crous *et al*. 1989a). They further stated that *C. ovatum* had a limited distribution and as such posed little threat due to the ability of *Eucalyptus* trees to outgrow the pathogen (Crous *et al*. 1988, Crous *et al*. 1989a). Milgate *et al*. (2001) isolated *M. vespa* from *Eucalyptus* leaves in Tasmania and found it to produce an anamorph resembling *C. ovatum*.

Coniothyrium zuluense M.J. Wingf., Crous & T.A. Coutinho, known to cause stem lesions on *Eucalyptus*, was thought to reside within the *Pleosporales* of the *Leptosphaeriaceae*. However, by employing DNA sequence data from the 18S and ITS gene regions of the rRNA operon, Cortinas *et al*. (2006a) found that *C. zuluense* does not group within the *Pleosporales* and rather in *Mycosphaerella* close to *Mycosphaerella* spp. producing *Colletogloeopsis* anamorphs. Therefore, *C. zuluense* was transferred to *Colletogloeopsis* as *Colletogloeopsis zuluense* and the generic circumscription of *Colletogloeopsis* was emended to include *Coniothyrium*-like species that produce pycnidia (Cortinas *et al*. 2006a).

5.3 *Davisoniella*

Swart (1988) described the coelomycete genus *Davisoniella* H.J. Swart that is characterised by the production of stromatic, subepidermal conidiomata and holoblastic, percurrent conidiogenous cells that arise from the inner walls of the conidiomatal locule and conidia that are oval, brown, verruculose, rounded apices, truncate bases with marginal frills. Swart (1988) further described one species, namely *Davisoniella eucalypti* H.J. Swart, from leaves of *Eucalyptus marginata* to be accommodated within this genus. Only one *Mycosphaerella* species known from *Eucalyptus* has been identified as having a *Davisoniella* anamorphic state. *Mycosphaerella davisoniellae* Crous was described from *Eucalyptus marginata* leaves in western Australia (Crous *et al*. 2006). The anamorph of *M. davisioniellae*, *Davisoniella eucalypti*, produces subcylindrical, ampulliform or doliiform conidiogenous cells that proliferate percurrently (Swart 1988, Crous *et al*. 2006). Conidia of *D. eucalypti* are known to be solitary, aseptate, verruculose, thick-walled, oval with truncate to subtruncate bases and

distinct basal frills (Crous *et al*. 2006). Phylogenetically and morphologically, *Davisoniella* spp. are related to *Colletogloeopsis* spp., but can be distinguished from the latter by producing unilocular to multilocular conidiomata on the leaf surfaces (Crous *et al*. 2006).

5.4 *Dissoconium*

De Hoog *et al*. (1983) established the genus *Dissoconium* de Hoog, Oorschot & Hijwegen based on the type species *Dissoconium aciculare* de Hoog, Oorschot & Hijwegen. This fungus produces conidia that are sub-hyaline, thin-walled, continuous or with a median septum, has one-celled conidia which are obovoidal, two-celled conidia which are constricted at the septum, rounded apical cells and inflated and often broader basal cells. De Hoog *et al*. (1991) described the new species *Dissoconium dekkeri* de Hoog & Hijwegen, which is a presumed mycoparasite on *Erysiphaceae* spp. Crous *et al*. (1999) sequenced the ITS region of species of *Mycosphaerella* and *Dissoconium* and found that *M. lateralis* (anamorph: *Uwebraunia lateralis* Crous & M.J. Wingf.) grouped in a clade with *Dissoconium* spp., and reduced *U. lateralis* to synonymy under *Dissoconium dekkeri*. Other *Mycosphaerella* spp. occurring on *Eucalyptus* known to produce *Dissoconium* anamorphs include *Mycosphaerella communis* Crous & J.P. Mansilla (anamorph: *Dissoconium commune* Crous & J.P. Mansilla) (Crous *et al*. 2004a). The status of *Uwebraunia*, which is morphologically indistinguishable from *Dissoconium*, is presently unclear.

5.5 *Mycovellosiella*

Species of *Mycovellosiella* produce superficial hyphae on the host plant, with intercalary conidiogenous cells with lateral nodes, conspicuous conidial scars and conidiophores that are produced terminally and laterally with conidia that are either formed singly or in chains (von Arx 1983, Crous & Braun 2003). Braun (1993, 1995) transferred some species of *Ramularia*, *Cerocosporella* Sacc. and *Cercospora* to *Mycovellosiella* due to their formation of superficial mycelia, coloured conidiophores and conidia and the production of secondary mycelium. *Mycovellosiella tasmaniensis* Crous & M.J. Wingf. (teleomorph: *M. tasmaniensis*) was described from leaves of *E. nitens* in Tasmania and represented the only species of *Mycovellosiella* to be described from *Eucalyptus* (Crous *et al*. 1998). *Mycovellosiella tasmaniensis* is characterised by septate conidiophores that arise from superficial mycelia with terminal, mono to polyblastic conidiogenous cells that proliferate sympodially and

forming catenate conidia that occur in chains and conidia that have flattened, darkened, refractive, thickened loci (Crous *et al*. 1998).

Upon further evaluation of *Mycovellosiella* spp., Crous & Braun (2003) found that the development of secondary superficial mycelia with solitary conidiophores is a variable character and stated that the development of creeping superficial hyphae should not be used to distinguish between cercosporoid genera. Therefore, Crous & Braun (2003) found that there was no true character to separate *Mycovellosiella* from *Passalora* and synonomised *Mycovellosiella* under *Passalora sensu lato*.

5.6 *Passalora*

Passalora is a cercosporoid hyphomycete anamorph genus of *Mycosphaerella* and has the type species *Passalora bacilligera* Fr. & Mont. This fungus is characterised by coloured conidiophores and ellipsoidal to fusiform, obclavate to subcylindrical, 1–3-septate, pigmented conidia that are formed singly (Crous & Braun 2003). Conidia in *Passalora* are ellipsoid-ovoid, broadly fusiform, clavate, obclavate, subcylindrical, colourless to pigmented, broad with few septa (Braun 1995). The conidiophores are loosely fasciculate (Braun 1995). Stewart *et al*. (1999) found that from sequence data analysis, isolates of *Passalora* grouped separate from *Cercospora* and *Pseudocercospora*, thus agreeing with the generic circumscription of *Passalora*. Crous & Braun (2003) reduced *Berteromyces* Cif. and *Oreophyllum* Cif. to synonymy with *Passalora*. Two species of *Passalora* are known from *Eucalyptus*, namely *Passalora zambiae* Crous & T. Coutinho (teleomorph: *Mycosphaerella* sp.) and *Passalora eucalypti* (Crous & Alfenas) Crous & U. Braun (teleomorph: *Mycosphaerella* sp.) (Crous *et al*. 2004a). *Passalora zambiae* produces medium brown, smooth, branched or unbranched, 0–2-septate conidiophores (Crous *et al*. 2004a). Furthermore, conidiogenous cells are terminal and intercalary, tapering to truncate apices and proliferating sympodially to produce catenulate conidia (Crous *et al*. 2004a).

5.7 *Phaeophleospora*

Phaeophleospora is a coelomycete genus of *Mycosphaerella*. There have been many revisions of the taxonomy of this genus and its close morphological similarity to other coelomycete genera. Due to this similarity, many species have in the past been incorrectly accommodated in various other coelomycete genera. *Phaeoseptoria eucalypti* Hansf. was described by

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Hansford (1956) from *E. grandis* in Sydney, Australia. However, Walker (1962) studied other collections of *P. eucalypti* and found that Hansfords' (1956) original description made no mention of the characteristic annellations seen around the conidiogenous loci. Walker (1962), therefore, amended Hansfords' (1956) description and included morphological characters such as annellations and roughened conidia. Dick (1982) recorded *P. eucalypti* from *Eucalyptus saligna* in New Zealand where it is responsible for causing purple leaf lesions on *Eucalyptus* spp. *Phaeoseptoria eucalypti* was subsequently also collected in South Africa from several *Eucalyptus* spp. (Crous *et al*. 1988). Walker (1962), noted that *Phaeoseptoria* resembles another coelomycete genus *Hendersonia* Berk., and Swart & Walker (1988), considered *Hendersonia grandispora* McAlpine to be congeneric with *P. eucalypti*.

Walker *et al*. (1992) examined the generic circumscription of *P. eucalypti* and found that *P. eucalypti* was congeneric with another well-known *Eucalyptus* pathogen, *Cercospora epicoccoides* Cooke & Massee. The type of *H. grandispora* was also examined and found that it too was incorrectly identified as a species of *Hendersonia*, and, instead was morphologically identical to several collections of *P. eucalypti*. Walker *et al*. (1992), also examined the type species of another *Phaeoseptoria* species from *Eucalyptus*, namely *Phaeoseptoria luzonensis* T. Koboyashi and found that this taxon also did not represent a distinct species and that it too was congeneric with *P. eucalypti*. Therefore, *C. epicoccoides*, *H. grandispora* and *P. luzonensis* were all found to be congeneric with *P. eucalypti* (Walker *et al*. 1992).

Walker *et al*. (1992), also found that several other *Eucalyptus* leaf fungi [*Septoria pulcherrima* Gadgil & M.A. Dick, *S. normae* Heatler, *S. ceuthosporoides* (Cooke & Harkn.) Sacc., *S. mortolensis* Penz. & Sacc. and *S. eucalypti* G. Winter & Roum.] were morphologically identical to *Cercospora eucalypti* Cooke & Massee apud Cooke. They further erected a new genus *Kirramyces* J. Walker, B. Sutton & Pascoe in which they placed *Kirramyces epicoccoides* (Cooke & Massee) J. Walker, B. Sutton & Pascoe (= *Cercospora epicoccoides*, *Hendersonia grandispora*, *Phaeoseptoria eucalypti*, *Phaeoseptoria luzonensis*) and *Kirramyces eucalypti* (Cooke & Massee) J. Walker, B. Sutton & Pascoe [= *Cercospora eucalypti*, *Pseudocercospora eucalypti* (Cooke & Massee) Gou & Liu, *Septoria pulcherrima*, *Stagonospora pulcherrima* (Gadgil & M.A. Dick) H.J. Swart]. Wingfield *et al*. (1996) described a new species of *Kirramyces*, *Kirramyces destructans* M.J. Wingf. & Crous causing a serious leaf disease on *Eucalyptus* in Indonesia. Crous *et al*. (1997), found that *Phaeophleospora* is morphologically similar to *Kirramyces*, and that *Kirramyces* only differed from *Phaeophleospora* by the production of more conidial septa. Crous *et al*. (1997),

therefore synonomised *Kirramyces* under the older name of *Phaeophleospora*. Genera that are morphologically similar to *Phaeophleospora* include *Microsphaeropsis* Höhn., *Colletogloeopsis*, *Readeriella* Syd. and *Coniothyrium* (Maxwell *et al*. 2003). Several species of *Mycosphaerella* occurring on *Eucalyptus* are known to have *Phaeophleospora* anamorphs namely *M. suttonii* (anamorph: *P. epicoccoides*), *M. toledana* Crous & G. Bills (anamorph: *P. toledana* Crous & G. Bills) *Mycosphaerella* spp. [anamorphs: *P. eucalypti*, *P. destructans* (M.J. Wingf. & Crous) Crous, F.A. Ferreira & B. Sutton] (Crous 1998, Crous *et al.* 2004a).

5.8 *Pseudocercospora*

Pseudocercospora is a morphologically variable genus (Crous *et al*. 2000). This genus accommodates synnematal counterparts of *Cercospora* that are characterised by unthickened conidial scars and pigmented conidia with percurrent and sympodial conidiogenous cell growth (von Arx 1983, Crous *et al*. 1989d, Sutton & Hennebert 1994, Crous *et al*. 1999, Crous *et al*. 2000). Sutton & Hennebert (1994) stated that *Pseudocercospora* is closer to the annellidic coelomycetes than to the sympodial coelomycetes due to the unthickened conidial scars observed for *Pseudocercospora* spp.

There are several cercosporoid genera that are closely allied to *Pseudocercospora*. Sutton & Hennebert (1994) noted that due to the variation in conidiomatal structure of certain species, *Pseudocercospora* is also closely related to *Phleospora*. By sequencing the ITS region of species of *Pseudocercospora sensu stricto* and *Paracercospora* Deighton, Stewart *et al*. (1999) found that *Paracercospora* isolates grouped with isolates from *Pseudocercospora* and, therefore, suggested that *Paracercospora* should be reduced to synonymy with *Pseudocercospora*. *Pseudocercosporella* is also closely allied to *Pseudocercospora*. *Pseudocercosporella* is characterised by the production of hyaline conidia and hyaline or almost hyaline conidiogenous cells. Beilharz *et al*. (2002) described *Pseudocercospora warcupii* Beilharz, Pascoe & Parbery, which represents a species intermediate between *Pseudocercospora* and *Pseudocercosporella* in producing both pigmented structures characteristic of *Pseudocercospora* and hyaline structures characteristic of *Pseudocercosporella*. The hyphomycete genus *Cercostigmina* U. Braun is closely allied to *Pseudocercospora* and it has been found through DNA sequencing studies that some species of *Cercostigmina* cluster with species of *Pseudocercospora*, suggesting that *Cercostigmina* should be reduced to synonymy with *Pseudocercospora* (Crous & Braun 2003). Crous *et al*. (2006) also showed that *Stigmina* Sacc. and *Phaeoisariopsis* Ferraris are synonyms of

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Pseudocercospora, and thus reduced them to synonymy under *Pseudocercospora*, which was especially conserved for this purpose.

It has been hypothesized that *Pseudocercospora* represents a monophyletic lineage within *Mycosphaerella* (Stewart *et al*. 1999, Ávila *et al*. 2005). However, by employing DNA sequence data from four nuclear gene regions, Hunter *et al*. (2006) found that *Pseudocercospora* is not monophyletic and instead is polyphyletic within *Mycosphaerella*. This has been substantiated by the observation that *Pseudocercospora* has evolved more than once within *Mycosphaerella* (Crous & Braun 2003, Ayala-Escobar *et al*. 2006, Crous *et al*. 2006). Furthermore it appears that species of *Pseudocercospora* have speciated relatively recently due to the short branch lengths of *Pseudocercospora* spp. observed in phylogenetic studies (Ávila *et al*. 2005).

There are currently many *Mycosphaerella* spp. known from *Eucalyptus* leaves that are known to form *Pseudocercospora* anamorphs (Crous 1998, Crous *et al*. 2004a, 2006). Perhaps the most pathogenic *Pseudocercospora* species is *P. eucalyptorum*, which is a cosmopolitan cercosporoid fungus occurring on *Eucalyptus* leaves. This species has a wide *Eucalyptus* host range in South Africa, but is most prevalent on *E. nitens* (Crous *et al.* 1989a, d).

5.9 *Pseudocercosporella*

Von Arx (1983) stated that the conidiogenous structures of *Pseudocercosporella* and *Cercoseptoria* Petr. were similar, and that the type species of *Pseudocercosporella*, *P. ipomoeae* Sawada ex Deighton, should be classified in *Cercoseptoria*. Braun (1990) disagreed with von Arx (1983) and preferred to retain *Pseudocercosporella* apart from *Cercoseptoria* because the two genera possessed species with pigmented and colourless structures. *Pseudocercosporella* is characterised by unthickened, inconspicuous conidial scars and solitary conidia (Braun 1990). Braun (1992) transferred species of *Ramularia* to *Pseudocercosporella* due to the presence of solitary conidia and conidial scars that are inconspicuous and unthickened. Two species of *Mycosphaerella* occurring on *Eucalyptus* leaves are known to produce *Pseudocercosporella* anamorphs, namely *Mycosphaerella endophytica* (anamorph: *P. endophytica* Crous & H. Smith) and *Mycosphaerella pseudoendophytica* Crous & G.C. Hunter (anamorph: *Pseudocercosporella* sp.) (Crous 1998, Crous *et al*. 2006).

5.10 *Readeriella*

Readeriella is a coelomycete genus characterized by the formation of spherical, dark brown immersed pycnidia with annellidic, lagenifrom to ampulliform, aseptate, hyaline, unbranched, 1–3 annelate conidiogenous cells formed on the inner pycnidial walls and conidia that are holoblastic, pale brown, smooth-walled, aseptate, truncate having a basal marginal frill and projecting laterally with 3 rounded points (Sutton 1971). *Readeriella* has the type species *Readeriella mirabilis* Syd. & P. Syd., which was described from leaves of *Eucalyptus capitellata* and *E. regnans* (Sutton 1971). *Readeriella mirabilis* has also been identified from leaves of *Eucalyptus cinerea* and *Eucalyptus nicholii* that are used for ornamental foliage in Australia (Barber *et al*. 2003). *Readeriella readeriellophora* Crous & J.P. Mansilla (teleomorph: *M. readeriellophora* Crous & J.P. Mansilla) was described from *E. globulus* leaves in Spain (Crous *et al*. 2004a). Another *Readeriella* sp., *Readeriella novozelandiae* Crous was described from *Eucalyptus botryoides* leaves in New Zealand (Crous *et al*. 2004a). *Readeriella novozelandiae* was found to be morphologically similar to *R. mirabilis* but could be distinguished from the latter species by having smaller conidia (Crous *et al*. 2004a). All *Readeriella* species that are currently known have been described from *Eucalyptus*.

5.11 *Sonderhenia*

Sonderhenia accommodates pycnidial anamorphs of *Mycosphaerella* and is characterised by percurrently proliferating conidiogenous cells and distoseptate conidia (Verkley & Priest 2000, Crous & Braun 2003). In a study of leaf inhabiting fungi from Australia, Swart & Walker (1988) noted that two species of *Hendersonia*, *Hendersonia eucalypticola* A.R. Davis and *Hendersonia eucalyptorum* Hansf. were very similar to species of *Phaeoseptoria* Speg. in their conidiogenesis. However, *P. eucalypti* differed in having larger pycnidia and longer conidia. Swart & Walker (1988) further compared the two species of *Hendersonia* to the type species of *Phaeoseptoria*, and found that it produced smooth-walled euseptate conidia that were different to the rough-walled distoseptate conidia of the *Hendersonia* spp. A new genus, *Sonderhenia* was, therefore, established to accommodate the two species of *Hendersonia*. The *Hendersonia* spp. were transferred to *Sonderhenia* as *S. eucalyptorum* (Hansf.) H.J. Swart & J. Walker and *S. eucalypticola* (A.R. Davis) H.J. Swart & J. Walker. *Sonderhenia* spp. are distinguished from *Phaeophleospora* by having distoseptate conidia (Crous *et al*. 2001a). Both *S. eucalypticola* and *S. eucalyptorum* have been isolated from *Eucalyptus* plantations in

Tasmania and Victoria, Australia, where they appear to be of minor importance (Park & Keane 1984, Carnegie *et al*. 1998, Milgate *et al*.2001).

5.12 *Stenella*

Stenella is based on the type species *Stenella araguata* Syd., which is characterised by the formation of superficial, verruculose, external secondary mycelium, solitary conidiophores that arise laterally or terminally from superficial hyphae, conspicuous conidiogenous loci and conidia that have slightly thickened and darkened conidial hila (Crous *et al*. 2000, Crous & Braun 2003). The production of verrucose superficial hyphae separates *Stenella* from *Mycovellosiella* and *Phaeoramularia* Munt.-Cvetk. (Crous *et al*. 2000, Crous *et al*. 2001a). Four *Stenella* spp. are known to occur on *Eucalyptus* leaves, namely *S. parkii* Crous & Alfenas (*M. parkii*), *Stenella* sp. (*M. scytalidii* Crous & M.J. Wingf.), *Stenella pseudoparkii* Crous & M.J. Wingf. (*Mycosphaerella* sp.) and *Stenella xenoparkii* Crous & M.J. Wingf. (*Mycosphaerella* sp.) (Crous 1998, Crous *et al*. 2006).

5.13 *Trimmatostroma*

Sutton & Ganapathi (1978) described *Trimmatostroma excentricum* B. Sutton & Ganap. from leaves of *E. delegatensis* in New Zealand, and noted that the conidia of *T. excentricum* were regularly asymmetric, which differed to other species of *Trimmatostroma* Corda in which the conidia are irregular and variable in shape and septation. They also found that conidial development of *T. excentricum* was unique within *Trimmatostroma*. Dick (1982) identified *Trimmatostroma bifarium* Gadgil & Dick and *T. excentricum* from *Eucalyptus* leaves in New Zealand where they were responsible for the production of leaf spots on various *Eucalyptus* spp. Dick (1982) stated that the disease was most notable in the lower crown and was of no economic significance. Park & Keane (1982a) identified *T. excentricum* from mature foliage of *E. globulus* in Victoria, Australia where it produced four-celled conidia in chains from conidiophores that are aggregated into a sporodochium. Recently, *Mycosphaerella pseudosuberosa* Crous & M.J. Wingf. was described from *Eucalyptus* leaves in Uruguay and was found to produce a *Trimmatostroma* anamorph in culture (Crous *et al*. 2006).

Trimmatostroma has been further established as an anamorph of *Mycosphaerella* through DNA-based phylogenetic studies. Taylor *et al*. (2003) used DNA sequence data from the ITS gene region to show that *Mycosphaerella microspora* (Joanne E. Taylor & Crous)

Joanne E. Taylor & Crous (anamorph: *Trimmatostroma microspora* Joanne E. Taylor & Crous), a leaf pathogen of *Protea* spp., groups within *Mycosphaerella*. Furthermore, Crous *et al*. (2006) used ITS sequence data to shown that *M. pseudosuberosa* (anamorph: *Trimmatostroma* sp.) also grouped within *Mycosphaerella*. This is the first *Trimmatostroma* sp. to be linked as an anamorph to those *Mycosphaerella* spp. occurring on *Eucalyptus*.

5.14 *Uwebraunia*

The hyphomycete genus *Uwebraunia* Crous & M.J. Wingf. is characterised by having smooth, olivaceous, obclavate, 1-septate conidia with unthickened hila, produced on pale medium brown conidiogenous cells with several percurrent proliferations (Crous & Wingfield 1996, Crous 1998). This genus was established for three species of *Mycosphaerella* occurring on *Eucalyptus*, *Mycosphaerella juvenis* Crous & M.J. Wingf. (*Uwebraunia juvenis* Crous & M.J. Wingf.), *Mycosphaerella ellipsoidea* Crous & M.J. Wingf. (*Uwebraunia ellipsoidea* Crous & M.J. Wingf.) and *M. lateralis* (*Uwebraunia lateralis* = *Dissoconium dekkeri*) (Crous & Wingfield 1996). As stated above, the current status of *Uwebraunia* is uncertain.

6.0 IDENTIFICATION TECHNIQUES

Identification of *Mycosphaerella* spp. is difficult, as species frequently occur without their presumed anamorphs. Therefore, morphological characteristics of the teleomorph were initially used for the identification of *Mycosphaerella* spp. However, the teleomorph morphology is relatively conserved, and only a few characters can be used for species identification (Crous *et al*. 2000). Thus, subsequent studies on *Mycosphaerella* spp. have concentrated on morphological characters of the many anamorph genera known to be associated with this genus (Crous 1998, Crous *et al*. 2004a, Crous *et al*. 2006). Furthermore, the advent of DNA sequence-based technologies has provided several techniques that can be applied to fungal species identification. This is particularly true for *Mycosphaerella* spp., where current identifications rely on both morphological characteristics and DNA-based techniques.

Ascospore germination patterns can be helpful for the initial identification of *Mycosphaerella* spp. Following discharge and germination, ascospores of *Mycosphaerella* spp. exhibit various morphologies. Park & Keane (1982a) first used ascospore germination patterns while investigating the taxonomy of *M. nubilosa*, *M. cryptica* and *M. parva* R.F. Park

& Keane occurring on *E. globulus* subsp. *globulus* in Victoria, Australia. Crous (1998) subsequently described 14 different ascospore germination patterns (Type A–N) for those species of *Mycosphaerella* occurring on *Eucalyptus* spp. Ascospores can germinate and form germ tubes that grow parallel to the long axis of the spore (Type B, C, F), perpendicular to the long axis of the spore (Type, A, L, M, N) and ascospores may change in colour and become verruculose (Type E, G, H, L, N) (Crous 1998). These characters are useful for placing unidentified *Mycosphaerella* spp. into more specific groups, thus narrowing down the possible identity of the *Mycosphaerella* isolate.

The use of Randomly Amplified Polymorphic DNA (RAPDs) has been effective in distinguishing between morphologically closely related *Mycosphaerella* spp. Carnegie *et al.* (2001) employed RAPDs to effectively distinguish between *M. cryptica*, *M. gregaria* Carnegie & Keane, *M. nubilosa* and *M. marksii* Carnegie & Keane. This technique was also applied to separate four banana leaf pathogens, *M. fijiensis* M. Morelet, *M. musicola* R. Leach ex J.L. Mulder, *M. musae* (Speg.) Syd. & P. Syd. and *M. minima* Stahel (Johanson *et al.* 1994). DNA-based methods such as RAPDs that may distinguish between species based on differences in DNA sequence are particularly advantageous. Reliability and standardisation of RAPDs across laboratories is, however, problematic and a disadvantage of this technique.

Species-specific DNA primers have proven effective for distinguishing between morphologically similar *Mycosphaerella* species. Johanson & Jeger (1993), developed species-specific primers for the banana pathogens, *M. fijiensis* and *M. musicola*. The development of these primers was based on differences in the DNA sequence of these two pathogens within the ITS regions of the rDNA operon. These primers showed high specificity for their particular *Mycosphaerella* sp. and could amplify the target *Mycosphaerella* DNA from leaf material either at an early or late necrotic stage of infection. Species-specific primers have also been developed for species of *Mycosphaerella* occurring on *Eucalyptus*. Kularatne *et al*. (2004) developed a PCR-RFLP technique for the effective identification of *M. nubilosa* and *M. cryptica*. This technique also allowed for these species to be detected directly from infected *Eucalyptus* leaf material. Furthermore, Maxwell *et al* (2005) developed species-specific primers for *M. cryptica*, *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva*. All of these species are known to occur in Australia and several other countries where they cause MLD (Crous 1998). The developed primers were also able to amplify DNA from the selected *Mycosphaerella* spp. from infected *Eucalyptus* leaves. Such a technique is particularly applicable to quarantine facilities, seed banks and nurseries where the presence of specific *Mycosphaerella* spp. can be detected *in planta*.

Genomic, or PCR based Restriction Fragment Length Polymorphisms (RFLP`s) have been applied to the identification *Mycosphaerella* spp. Carlier *et al*. (1994), developed genomic RFLP probes to study the genetic relatedness of *M. fijiensis* isolates and found that these probes showed a high level of intraspecific polymorphism between isolates of *M. fijiensis*. Hunter (2002) employed the restriction enzyme *Hae*III to distinguish between species of *Mycosphaerella* occurring on *Eucalyptus* leaves and found that this enzyme was effective in grouping species into smaller groups based on their RFLP banding profile.

Sequencing of various DNA gene regions has become the commonly chosen technique used for the identification of *Mycosphaerella* spp. occurring on *Eucalyptus* trees. DNA sequence data from the ITS region has become the traditional gene region used for *Mycosphaerella* spp. identification (Crous *et al*. 2000, 2001a, b, Hunter *et al*. 2004a, b). However, the ITS gene region does not always provide sufficient resolution to distinguish between *Mycosphaerella* spp. and their anamorphs (Verkley & Starink-Willemse 2004). Therefore, several other nuclear gene regions such as the Large Subunit (LSU), Actin (ACT), Translation Elongation Factor 1-alpha (EF-1α) and Beta tubulin (Bt) gene regions have also recently been used to examine species boundaries and cryptic taxa within *Mycosphaerella* (Crous *et al*. 2006, Hunter *et al*. 2006). Undoubtedly, the generation of more DNA sequence datasets from various nuclear and mitochondrial gene regions and the combination of these datasets will contribute to the understanding of species concepts within *Mycosphaerella* in the future and give an indication of teleomorph and anamorph morphologies that are phylogenetically informative.

The application of several DNA-based molecular techniques to the identification of *Mycosphaerella* spp. has become markedly easier. Furthermore, identifications based on DNA data are more conclusive and trustworthy than initial identifications that were solely based on morphological characteristics. It is important though to link results from DNA-based identification methods to sexual or asexual *Mycosphaerella* morphologies to obtain features of these states that are phylogenetically reliable and informative.

7.0 PHYLOGENY

With the advent of molecular biology and DNA-based studies of fungal genomes, it has become possible to study evolutionary relationships among many fungi. In *Mycosphaerella* the ITS regions have been commonly used to answer questions regarding speciation and phylogeny. However, recently, DNA sequence data from other gene regions have been

incorporated into existing DNA sequence datasets to increase resolution among *Mycosphaerella* spp. and elucidate phylogenetic species concepts (Ávila *et al*. 2005, Hunter *et al*. 2006).

Surveys to identify *Mycosphaerella* spp. infecting *Eucalyptus* spp. have become common with the increased importance of plantation forestry in the Southern Hemisphere, especially in Australia and South Africa. This has led to the identification of many previously undescribed *Mycosphaerella* spp. From surveys in South Africa during the 1990`s, Crous & Wingfield (1996) identified several new *Mycosphaerella* spp. From this study it was hypothesized that *Mycosphaerella* was a heterogeneous group of fungi that were polyphyletic with a diverse group of monophyletic anamorph genera (Crous & Wingfield 1996, Crous 1998). Crous *et al*. (2000) noted that it was unclear whether *Mycosphaerella* is a monophyletic group or if the morphology was derived. They further stated that if the morphology was derived then *Mycosphaerella* would be paraphyletic or polyphyletic. Monophyletic groups within *Mycosphaerella* could then be characterised according to their anamorphs. This hypothesis was contradicted by Goodwin *et al*. (2001) and Crous *et al*. (2001b) who found *Mycosphaerella* to be largely monophyletic with polyphyletic anamorph genera. It was also noted that *Mycosphaerella* was of ancient origin and that the morphology of the teleomorph has been retained through natural selection, but that the anamorph characteristics seem to be highly mutable and thus anamorphs should not be used to delimit phylogenetic relationships.

Crous *et al*. (2000) sequenced the ITS region of approximately 46 *Mycosphaerella* spp. and found that three major clades could be resolved. These included a major monophyletic *Mycosphaerella* clade, a second clade corresponding to *Dissoconium* anamorphs and a third representing isolates of *Ramulispora* Miura. Crous *et al*. (1999, 2001a) were also able to prove that several isolates of *Mycosphaerella* spp., corresponding to various anamorph genera, grouped at various places within a larger *Mycosphaerella* clade suggesting that several anamorph genera within *Mycosphaerella* have evolved more than once within the genus. This was supported by Verkley *et al*. (2004b) and Verkley & Starink-Willemse (2004) who showed that *Septoria* was polyphyletic within *Mycosphaerella* and that it has evolved more that once within *Mycosphaerella*. Maxwell (2003) also showed that *Mycosphaerella* is composed of polyphyletic anamorph genera, with different lineages of anamorph genera grouping on separate branches within the *Mycosphaerella* morphology.

8.0 SPECIES COMPLEXES

DNA sequencing has increased the genetic resolution that is now available to study species boundaries and species concepts. This is particularly true for species of *Mycosphaerella*. Early studies into *Mycosphaerella* spp. on *Eucalyptus* relied predominantly on morphological descriptions (Crous 1998). However, by employing DNA sequence results from various gene regions it has become evident that several *Mycosphaerella* spp. represent a complex of morphologically similar yet phylogenetically distinct taxa (Crous *et al*. 2004a, 2006, Hunter *et al*. 2006). Several species complexes have now been identified from those *Mycosphaerella* spp. occurring on *Eucalyptus* (Crous *et al*. 2004a, 2006).

Mycosphaerella heimii, *M. crystallina* Crous & M.J. Wingf., *M. irregulariramosa* and *M. heimioides* are all species that are known from *Eucalyptus* (Crous 1998). All of these species have *Pseudocercospora* anamorphs and produce pale brown, smooth to finely verruculose, obclavate to subcylindrical conidia, with internal or external mycelia, small, dense fascicles on brown stromata. They all cluster together in a well supported phylogenetic clade when sequenced and as such are considered to all represent varieties of *M. heimii* and are referred to as the *M. heimii* complex (Crous *et al*. 2000, Crous *et al*. 2001a). This view was supported by Maxwell (2003) who found that these species grouped together in a separate clade based on ITS sequence data.

Pseudocercospora eucalyptorum has a wide geographic and eucalypt host range. From a phylogenetic analysis of *Mycosphaerella* spp. occurring on *Eucalyptus*, Crous *et al*. (2004a) showed that isolates of *P. eucalyptorum* collected from several locations did not form a monophyletic clade, but, instead were dispersed within a larger *Pseudocercospora* clade. Thus, isolates of *P. eucalyptorum* actually represent a species complex.

Many isolates of *Mycosphaerella* from several different hosts may be morphologically indistinguishable but phylogenetically may represent distinct entities and eventual phylogenetic species (e.g. *Cercospora apii* complex) (Crous & Braun 2003, Groenewald *et al*. 2005, 2006). By sequencing other loci of the *Mycosphaerella* genomes we may be able to achieve increased resolution between *Mycosphaerella* spp., thereby identifying new cryptic morphological species that were once thought of as well defined morphological species (Crous *et al*. 2004a). Maxwell (2003) found that there was a high level of intra-specific variation between certain species of *Mycosphaerella* based on an ITS phylogeny and suggested that this variation may be due to the fact that some species may represent species complexes.

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By employing ITS DNA sequence data from more that 295 *Mycosphaerella* isolates from *Eucalyptus*, Crous *et al*. (2006) identified many new *Mycosphaerella* species complexes. These species complexes included the *Colletogloeopsis zuluensis*, *M. molleriana*, *M. cryptica*, *M. suttonii*, *M. suberosa*, *M. marksii*, *M. heimii*, *M. flexuosa*, *M. parva* and *M. endophytica* species complexes. Employing more *Mycosphaerella* isolates in phylogenetic studies, therefore, allows for the clearer elucidation of species complexes (Hunter *et al*. 2006).

9.0 POPULATION BIOLOGY OF *MYCOSPHAERELLA* SPECIES

Many *Mycosphaerella* spp. are important pathogens of economically important crops. In the past, most studies of *Mycosphaerella* spp. have focussed on their taxonomy, phylogeny, epidemiology and host associations. Recently, however, the study of population dynamics of fungal pathogens, and particularly *Mycosphaerella* spp., has helped to understand the population structure of many important *Mycosphaerella* spp. Limited population biology research has, however, been conducted on those *Mycosphaerella* spp. occurring on *Eucalyptus* leaves. In contrast, extensive research into the population biology of several other *Mycosphaerella* spp. such as *Mycosphaerella graminicola* (Fuckel) J. Schröt (Linde *et al*. 2002, Zhan *et al*. 2003), *M. fijiensis* (Carlier *et al*. 1996, Hayden *et al*. 2003a) and *M. musicola* (Hayden *et al*. 2003b) have been published in recent years. Results from these studies have led to and increased understanding of aspects of population dynamics within *Mycosphaerella*, such as population structure, distribution of genetic diversity, gene flow, centres of origin and mating strategies. Results of population biology studies from other *Mycosphaerella* pathosystems provides knowledge that can be used for future population biology studies of *Mycosphaerella* spp. occurring on *Eucalyptus* spp.

9.1 CENTRE OF ORIGIN

It is important to determine the centre of origin and diversity of a particular fungal pathogen. This is because these centres of origin and diversity would be a primary location in which to search for resistant or tolerant plant host genotypes. It is generally accepted that centres of origin for species would be those geographic areas that have the greatest gene diversity (McDonald 1997).

Putative centres of origin have been determined for certain *Mycosphaerella* spp. such as *M. fijiensis*, *M. graminicola* and *M. nubilosa* that are well-characterised pathogens of

banana, wheat and *Eucalyptus* respectively. Carlier *et al*. (1996) determined that populations of *M. fijiensis* from south-east Asia had greater allelic and gene diversity than *M. fijiensis* populations from Africa, the Pacific Islands and Latin America. Here, more than 88 % of the alleles detected in the African, Pacific Islands and Latin American populations were also found in the south-east Asian *M. fijiensis* population, indicating that south-east Asia most likely represents the centre of origin for *M. fijiensis*.

Zhan *et al*. (2003) investigated the global structure of the wheat pathogen *M. graminicola* by employing data generated from genomic RFLPs and found that the *M. graminicola* populations from the Middle East (Israel and Syria) exhibited higher gene diversity values than *M. graminicola* populations from America, Australia, Europe or North America. It was, therefore, suggested that *M. graminicola* originates from the Middle East where wheat was also first domesticated (Zhan *et al*. 2003).

9.2 DISTRIBUTION OF GENETIC DIVERSITY

A question that is often asked in population biology studies is how the genetic diversity of the pathogen population is distributed and on what scale is this diversity perpetuated (McDonald 1997). Answers to this question have important implications on control strategies and host resistance breeding. Distribution of genetic diversity has been effectively determined for a number of *Mycosphaerella* pathosystems.

It has been shown that the majority of genetic diversity (> 90 %) for populations of *M. fijiensis* is distributed within banana plantations (Rivas *et al*. 2004). A similar pattern of genetic distribution has also been found for *M. graminicola* where more than 79–93 % of the genetic diversity of a *M. graminicola* population is observed to occur within plots of a wheat field (Boeger *et al*. 1993, Zhan *et al*. 2003). The wheat glume blotch pathogen, *Phaeosphaeria nodorum* (E. Müll.) Hedjar. (anamorph: *Stagonospora nodorum* (Berk.) E. Castell. & Germano), also exhibits most of its gene diversity within wheat fields, and it has been found that 96 % of the gene diversity of *P. nodorum* populations from Texas, Oregon and Switzerland is found on a local level within a wheat field (Keller *et al.* 1997).

The scale of genetic diversity can be very small, such as within single lesions. Several different genotypes of *M. graminicola* have been found occupying a single lesion on a wheat leaf (Schneider *et al*. 2001, Linde *et al*. 2002, Zhan *et al*. 2003). From these studies it appears that the genetic distribution of certain *Mycosphaerella* spp. occurs at a limited spatial scale with several unique genotypes occupying a single plot or plant within a plantation or field.

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9.3 MATING STRATEGIES

Fungi are typically obligately outcrossing (heterothallic) or interbreeding (homothallic). Fungal reproductive modes play an important role in the biology and population dynamics of fungal pathogens. This is also true for *Mycosphaerella* spp. where the majority of these pathogens tend to be heterothallic. Through heterothallism and sexual reproduction novel alleles are introduced into a population that allows a fungal pathogen to overcome host resistance (Milgroom 1996, Zhan *et al*. 2001, Zhan & McDonald 2004). In contrast, homothallism generally results in progeny that are identical to the parent. Through homothallism, specific fungal clones are perpetuated through time and space. It is important, therefore, to determine the reproductive strategy of fungal pathogens as it influences the population structure and ability of these pathogens to overcome resistant host genotypes. Reproductive modes of several *Mycosphaerella* spp. have been determined by investigating the number and frequency of genotypes, through determining the indices of multilocus disequilibria or by the amplification and sequencing of mating type genes.

Many *Mycosphaerella* spp. exhibit a heterothallic mating strategy. Much research has been conducted on the wheat blotch pathogen *M. graminicola*. Zhan *et al*. (2003), investigated the global diversity of *M. graminicola* and found there to be a high level of genotypic diversity in *M. graminicola* populations from America, Europe, Australia, the Middle East and North Africa. It was also found that there was random association of alleles among 14 RFLP loci, suggesting that sexual reproduction occurs within *M. graminicola*. Sexual reproduction in *M. graminicola* was further substantiated by (Zhan *et al*. 2002) who found both mating type genes (*MAT* 1-1 and *MAT* 1-2) present in equal frequency in populations of *M. graminicola* collected from 12 different countries.

Heterothallism is known to occur in the banana pathogen *M. fijiensis*. Carlier *et al.* (1996) showed that there was a high level of gametic disequilibrium between RFLP loci of populations of *M. fijiensis* from south-east Asia and Africa suggesting that these were randomly mating heterothallic populations. Hayden *et al.* (2003a) studied *M. fijiensis* populations from Papua New Guinea and several islands of the Torres Strait and found through the Fischer exact test and the index of association tests that there was no significant gametic disequilibrium among these *M. fijiensis* populations, also indicating that it is heterothallic.

Although the majority of *Mycosphaerella* spp. are known to be heterothallic, there are examples of *Mycosphaerella* spp. that exhibit a homothallic mating strategy. Milgate *et al.* (2005) showed that there was significant linkage disequilibrium in a population of the *Eucalyptus* leaf pathogen, *M. cryptica*, from Tasmania. This indicates that there is a lack of recombination and suggests that this is not a strictly heterothallic fungus.

9.4 GENE FLOW

Gene flow is an important aspect when considering the population dynamics of pathogens. Gene flow can be defined as the change in a population due to the movement of gametes, individuals or groups of individuals from one place to another (Slatkin 1987). Gene flow can serve to homogenise distant pathogen populations and prevent such populations from evolving into different species (Slatkin 1987, Zhan & McDonald 2004). The determination of gene flow within *Mycosphaerella* pathosystems is important and gives an indication of the similarity that exists between distant *Mycosphaerella* populations.

Gene flow has been determined for several populations of *Mycosphaerella* spp. The most important method of gene flow between populations of *Mycosphaerella* spp. is through airborne ascospores, and it has been shown that gene flow is one of the evolutionary forces acting to maintain within-population variation (Boeger *et al*. 1993, Zhan & McDonald 2004). Furthermore, alternative hosts may also facilitate gene flow of *Mycosphaerella* spp. between their primary hosts (Boeger *et al*. 1993). Keller *et al*. (1997) showed that populations of *Phaeosphaeria nodorum*, separated by 2000–7000 km, were not significantly differentiated, and showed that this was due to extensive gene flow. Gene flow has also served to homogenise populations of the maize pathogen *Cercospora zeae-maydis* Tehon & E.Y. Daniels from Uganda, Kenya and Rwanda (Okori *et al*. 2003).

10.0 SPREAD AND CONTROL OF *MYCOSPHAERELLA* SPECIES

The movement and spread of fungal pathogens is of primary importance when considering management strategies for their control. This is particularly true for the many species of *Mycosphaerella* that are known to be important pathogens of economically important crop plants. *Mycosphaerella* spp. can be spread to new areas in several ways and thus begin new infections in native and exotic environments.

Mycosphaerella spp. are known to infect many woody or herbaceous hosts. Many *Mycosphaerella* spp. are primarily pathogens of a single plant host. However, *Mycosphaerella* spp. may also be infecting alternative hosts, apart from their preferred host. This has been hypothesized for *M. graminicola*, causing leaf spot on wheat (Boeger *et al*. 1993). It is known that *M. graminicola* can also infect annual blue grass and if this alternative host also occurs between wheat fields it may act as a source for continued ascospore inoculum (Boeger *et al*. 1993). It has also been hypothesised that species of *Mycosphaerella* occurring on *Eucalyptus* may infect alternative hosts in attempts to move to their primary *Eucalyptus* hosts. This scenario has been termed the "pogo stick hypothesis" and is evident through the identification of fungal pathogens on non-native hosts in low levels (Crous & Groenewald 2005). Recently, isolates of *M. citri*, primarily a pathogen of citrus, have been found to infect and cause lesions on *Acacia mangium* from Thailand (Crous *et al*. 2004b).

Infected seed may serve as a vehicle for the movement of *Mycosphaerella* spp. into non-native environments (Maxwell *et al*. 2003). Seed that are transferred between countries or breeding programs should thus be tested for pathogen propagules (Boeger *et al*. 1993). The movement of seed has also been suggested as a possible method of movement of *Colletogloeopsis zuluensis* around the world to countries where *Eucalyptus* spp. are commercially grown (Cortinas *et al*. 2006b). Infected seed may also be tested for the presence of *Mycosphaerella* spp. by using species-specific primers that have been developed for several *Mycosphaerella* spp. that infect *Eucalyptus* leaves (Kularatne *et al*. 2004, Maxwell *et al*. 2005).

Control of MLD is difficult and largely depends on a combination of several factors. Hybrid tree species are known to be more tolerant to various *Mycosphaerella* spp. when compared to certain pure tree species. The use of hybrid poplar tree species has been suggested for the control and management of *M. populorum* (anamorph: *Septoria musiva* Peck), the causal agent of leaf spot and cankers of various *Populus* spp. (Feau *et al*. 2005). However, certain *Eucalyptus* hybrids have been found to be more susceptible to MLD than parental *Eucalyptus* species. Dungey *et al*. (1997) and Carnegie & Ades (2002) found that F1 *E. globulus* × *E. nitens* hybrids were more susceptible to *M. cryptica* and *M. nubilosa* than any of the parent *Eucalyptus* species. Carnegie & Ades (2002) further suggested that *Eucalyptus* hybrids not be used in environments where MLD is severe. Thus, hybrid tree species should be propagated in areas where MLD is known to be of lesser importance and such hybrid *Eucalyptus* spp. combined with a diversity of *Eucalyptus* spp. would provide the

most opportunity to combat MLD on *Eucalyptus* (Purnell & Lundquist 1986, Wingfield *et al.* 2001).

It is also known that pure host species do exist that show elevated levels of tolerance to *Mycosphaerella* infection (Patton & Spear 1983). For example, Carnegie *et al.* (1998) found significant variation in MLD disease incidence between 13 *Eucalyptus* species and that *Eucalyptus cypellocarpa* L. Johnson, *E. nitens* and *E. globulus* were most susceptible to MLD while *Eucalyptus elata* Dehnh. and *Eucalyptus oreades* R.T. Bak. were more resistant to MLD.

The use of various *Eucalyptus* species provenances may also provide a means to combat the development of MLD. Initial studies of *E. nitens* provenances in South Africa for commercial forestry resulted in the finding that New South Wales provenances of *E. nitens* were generally more resistant to MLD than Victorian *E. nitens* provenances (Purnell & Lundquist 1986). This has been further substantiated by Hood *et al.* (2002) who, during an *E. nitens* provenance trial in New Zealand, found that leaf retention of New South Wales provenances of *E. nitens* was higher than that of Victorian provenances when exposed to MLD.

Variation in susceptibility to MLD has also been determined at the *Eucalyptus* sub-species level. Carnegie & Ades (2005) found significant differences in MLD disease severity of mature foliage between the four sub-species of *E. globulus* namely *E. globulus* ssp. *bicostata* (Maiden *et al.*) Kirkpatr., *E. globulus* ssp. globulus Labill., *E. globulus* ssp. *maidenii* (F. Muell.) Kirkpatr. and *E. globulus* ssp. *pseudoglobulus* (Naudin ex Maiden) Kirkpatr. Here, it was found that *E. globulus* ssp. *bicostata* was more susceptible to *M. cryptica* than any of the other *E. globulus* sub-species while *E. globulus* ssp. *maidenii* exhibited the highest level of tolerance to *M. cryptica*.

Considering that Australia represents the origin of *Eucalyptus* spp. and most likely the centre of diversity for *Mycosphaerella* spp. that occur on these trees, it seems reasonable to expect that co-evolution has occurred between *Mycosphaerella* spp. and their *Eucalyptus* hosts in this country. Therefore, resistant *Eucalyptus* species and genotypes should be sourced from Australia, alternatively, natural *Eucalyptus* land races may also serve as source to *Mycosphaerella* resistance.

Available moisture levels play an important part in the development of *Mycosphaerella* ascomata and ascospores. Knowing the optimal amount of leaf moisture necessary for the development and maturation of ascomata and ascospores of *Mycosphaerella* spp., the frequency and length of watering in nursery systems could be adjusted to decrease

the levels of inoculum (Mondal & Timmer 2002). Furthermore, any dead or decomposing leaf material present in a nursery or plantation acts as an inoculum source, as *Mycosphaerella* pseudothecia are still capable of development in such material (Park & Keane 1987). Therefore, it would be beneficial to remove any such material from a nursery system. Overhead irrigation mechanisms should also be avoided in nurseries as water will accumulate on leaf surfaces and stimulate the production of ascospores. Alternatively drip irrigation may be used for watering nursery stock, thereby avoiding the high humidity levels and the periods that water may accumulate on leaf surfaces.

Fungicide applications may provide a means to control the development of *Mycosphaerella* spp. In a nursery environment this method may be feasible due to the smaller size of the *Eucalyptus* seedlings and the growth tunnels in which they are housed. Dick (1982) suggested that the use of fungicides should be considered in nurseries for the control of *M. cryptica*. Furthermore, it has been suggested that the cost of fungicide applications may be reduced by spraying during the vegetative period of the host (Park 1988b). Carnegie & Ades (2003) showed that the spraying of both a protectant and systemic fungicide significantly reduced the development of MLD on both juvenile and adult foliage of *E. globulus* during a fungicide spray trial in Victoria, Australia. Disease forecasting systems may also be used to determine the most appropriate time for fungicide application (Jacome *et. al*. 1991). However, once deployed into the field on a plantation level, the use of fungicide applications would be economically unviable for forestry companies.

The movement of infected plant material between countries and continents appears to be on the increase (Wingfield *et al*. 2001). Therefore, many fungal pathogens will most likely be introduced into new environments (Wingfield 1999). Quarantine measures should consequently be strictly implemented and updated to reduce the risk of fungal pathogens being introduced into non-native environments. This is particularly true for *Mycosphaerella* spp. occurring on *Eucalyptus*. There are approximately 100 *Mycosphaerella* spp. known from *Eucalyptus* occurring in many countries (Crous 1998, Crous *et al*. 2004a, Crous *et al*. 2006) and it is important that these *Mycosphaerella* spp. be incorporated into quarantine regulations and actionable lists.

11.0 CONCLUSION

From published literature, it is clear that extensive research has been conducted on species of *Mycosphaerella*. This knowledge has increased our understanding into various aspects of their taxonomy, epidemiology, host associations, host susceptibility and phylogeny.

The taxonomy of *Mycosphaerella* species is particularly complex. It has become apparent that the teleomorph stage is morphologically conserved and does not offer sufficient morphological characteristics to discriminate between *Mycosphaerella* species. This has resulted in the fact that morphological characteristics of the anamorph state has become important in *Mycosphaerella* species identification. Many different genera representing the coelomycetes and hyphomycetes are now recognised as anamorph genera of *Mycosphaerella*. It is clear though that the generic circumscription of several anamorph genera is debatable, and should be re-evaluated in terms of their morphological similarities to other anamorph genera, that are newly linked to *Mycosphaerella*. This may lead to the synonymy of several anamorph genera and to an overall decrease in the number of anamorph genera associated with *Mycosphaerella*.

Many morphological and DNA-based methods have been used to identify and characterise species of *Mycosphaerella*. The use of DNA sequencing though has now become the definitive technique that is used in species identification. This has allowed for the realisation that one morphological species does not always represent a single monophyletic taxon. Instead, from such studies, it has become apparent that many species complexes exist within *Mycosphaerella*. The use of DNA sequence data from several nuclear gene regions has thus allowed for increased resolution both at the species level and deeper nodes within *Mycosphaerella* as a whole. Undoubtedly, further studies employing DNA sequence results from a broader range of gene regions will increase out discriminatory power within *Mycosphaerella* and allow the effective identification of species but also link DNA sequences to morphologies that are phylogenetically informative.

Population biology studies on species of *Mycosphaerella* have been limited to a few well-known plant pathogens. These studies have increased our knowledge into the structure of *Mycosphaerella* populations, their diversity and movement. However, existing population biology research on *Mycosphaerella* has focussed on species occurring on various other hosts other than *Eucalyptus*. Therefore, further population biology research should be conducted into *Mycosphaerella* species occurring on this host. This research should incorporate results of population biology studies of other *Mycosphaerella* pathosystems and combine that

information generated from those *Mycosphaerella* species occurring on *Eucalyptus*. Such information will be important from an academic standpoint but also from a forestry and quarantine perspective. Population biology knowledge acquired of *Mycosphaerella* species occurring on *Eucalyptus* will aid in the establishment effective *Eucalyptus* breeding programmes and will be added to quarantine action lists to prohibit the introduction of novel more virulent *Mycosphaerella* species and genotypes.

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2

A multi-gene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves

Abstract: Species of the ascomycete genus *Mycosphaerella* are regarded as some of the most destructive leaf pathogens of a large number of economically important crop plants. Amongst these, approximately 60 *Mycosphaerella* spp. have been identified from various *Eucalyptus* spp. where they cause leaf diseases collectively known as Mycosphaerella Leaf Disease (MLD). Species concepts for this group of fungi remain confused, and hence their species identification is notoriously difficult. Thus, the introduction of DNA sequence comparisons has become the definitive characteristic used to distinguish species of *Mycosphaerella*. Sequences of the Internal Transcribed Spacer (ITS) region of the ribosomal RNA operon have most commonly been used to consider species boundaries in *Mycosphaerella*. However, sequences for this gene region do not always provide sufficient resolution for cryptic taxa. The aim of this study was, therefore, to use DNA sequences for three loci, ITS, Translation Elongation Factor 1-alpha (EF-1α) and Actin (ACT) to reconsider species boundaries for *Mycosphaerella* spp. from *Eucalyptus*. A further aim was to study the anamorph concepts and resolve the deeper nodes of *Mycosphaerella*, for which part of the Large Subunit (LSU) of the nuclear rRNA operon was sequenced. The ITS and EF-1α gene regions were found to be useful, but the ACT gene region did not provide species-level resolution in *Mycosphaerella*. A phylogeny of the combined DNA datasets showed that species of *Mycosphaerella* from *Eucalyptus* cluster in two distinct groups, which might ultimately represent discrete genera.

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INTRODUCTION

Species of *Eucalyptus* are native to Australia with isolated pockets of native *Eucalyptus* forests also occurring in Papua New Guinea, Indonesia and the Philippines (Turnbull 2000). Many *Eucalyptus* spp. have been removed from these centres of origin to new environments where they are typically propagated in plantations for the production of paper, pulp and other wood products (Wingfield 1999, Turnbull 2000, Wingfield *et al*. 2001). In these non-native environments, *Eucalyptus* trees are susceptible to many pests and diseases including those known in their areas of origin and others that have undergone host shifts (Wingfield 2003, Slippers *et al*. 2005). These pests and diseases cause significant annual losses to *Eucalyptus* plantations resulting in decreased revenue for commercial forestry companies.

Mycosphaerella Johanson is one of the largest genera of the ascomycetes, accommodating more than 3000 species. Approximately 60 *Mycosphaerella* spp. have been associated with leaf diseases of many *Eucalyptus* spp., and these are collectively referred to as Mycosphaerella Leaf Disease (MLD) (Crous 1998, Maxwell *et al*. 2003, Crous *et al*. 2004a). The disease is particularly prevalent on the juvenile leaves and shoots of *Eucalyptus* trees, where infection results in premature defoliation, twig cankers and stunting of tree growth (Lundquist & Purnell 1987, Crous 1998, Park *et al*. 2000, Carnegie & Ades 2003). However, several *Mycosphaerella* spp. can also infect adult *Eucalyptus* foliage, and this has been attributed to their ability to produce a proto-appressorium that enables direct cuticle penetration (Ganapathi 1979, Park & Keane 1982b). In some situations, trees may thus be subjected to infection by a suite of different *Mycosphaerella* spp.

Identification of *Mycosphaerella* spp. based on morphology is known to be difficult. This is because these fungi tend to produce very small fruiting structures with highly conserved morphology, and they are host-specific pathogens that grow poorly in culture. Traditionally, morphological characters of the teleomorph and anamorph have been used in species delimitation (Crous 1998). Park & Keane (1982a) introduced ascospore germination patterns as an additional characteristic to identify *Mycosphaerella* spp., and Crous (1998) subsequently identified 14 different ascospore germination patterns for the *Mycosphaerella* spp. occurring on *Eucalyptus*. Crous (1998) and Crous *et al*. (2000) also introduced features of these fungi growing in culture and especially anamorph morphology as important and useful characteristics on which to base species delimitation. DNA-based methods such as RAPDs and species-specific primers have also been employed to distinguish between *Mycosphaerella* species occurring on *Eucalyptus* (Carnegie *et al*. 2001, Maxwell *et al*. 2005).

Comparisons of DNA sequence data have emerged as the most reliable technique to identify *Mycosphaerella* spp. The majority of studies employing DNA sequence data for species identification have relied on sequence data from the Internal Transcribed Spacer (ITS) region of the ribosomal RNA operon (Crous *et al*. 1999, 2001, 2004a, b, Hunter *et al*. 2004a, b). Although comparisons of gene sequences for this region have been useful, the resolution provided by this region is not uniformly adequate to discriminate between individuals of a species complex or to effectively detect cryptic species (Crous *et al*. 2004b). Thus, recent studies have shown the importance of employing Multi-Locus Sequence Typing (MLST) to effectively identify cryptic fungal species and to study species concepts (Taylor & Fischer 2003).

A single morphological species does not always reflect a single phylogenetic unit (Taylor *et al*. 2000). Within *Mycosphaerella*, teleomorph morphology is conserved and the anamorph morphology provides additional characteristics to discriminate between taxa (Crous *et al*. 2000). Yet the collective teleomorph and anamorph morphology is often not congruent with phylogenetic data. Thus, recent phylogenetic studies have led to the recognition of several species complexes within *Mycosphaerella* (Crous *et al*. 2001, 2004b, Braun *et al*. 2003). Most of these studies have been based on comparisons of sequences for the ITS regions of the ribosomal DNA operon. Given the important data that have emerged from them, it is well recognised that greater phylogenetic resolution will be required for future taxonomic studies on *Mycosphaerella* species.

The aim of this study was to use MLST to consider species and anamorph concepts in *Mycosphaerella* spp. occurring on *Eucalyptus*. This was achieved by sequencing four nuclear gene regions, namely part of the Large Subunit (D1–D3 of LSU) and ITS region of the nuclear rRNA operon, and a portion of the Actin (ACT) and Translation Elongation Factor 1-alpha (EF-1α) gene regions.

MATERIALS AND METHODS

Mycosphaerella isolates

For this study, an attempt was made to obtain cultures of as many *Mycosphaerella* spp. known to infect *Eucalyptus* leaves as possible. All cultures used in this investigation where already in existence and are housed in culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1). All cultures were grown

on 2 % (wt/v) malt extract agar (MEA) (Biolab, South Africa), at 25 ºC for approximately 2–3 mo to obtain sufficient mycelial growth for DNA extraction.

DNA isolation

Mycelium from actively growing cultures was scraped from the surface of cultures, freeze-dried for 24 h and then ground to a fine powder using liquid nitrogen. DNA was isolated using the phenol : chloroform (1 : 1) extraction protocol as described in Hunter *et al.* (2004a, b). DNA was precipitated by the addition of absolute ethanol (98 % EtOH). Isolated DNA was cleaned by washing with 70 % ethanol (70 % EtOH) and dried under vacuum. SABAX water was used to resuspend the isolated DNA. RNaseA (10 µg/µL) was added to the resuspended DNA and incubated at 37 ºC for approximately 2 h to digest any residual RNA. Isolated DNA was visualised in a 1 % agarose gel (wt/v) (Roche Diagnostics, Mannheim), stained with ethidium bromide and visualised under ultra-violet light.

PCR amplification and purification

DNA (*ca*. 20 ng) isolated from the *Mycosphaerella* spp. used in this study was used as a template for amplification using the Polymerase Chain Reaction (PCR). All PCR reactions were mixed in a total volume of 25 µL containing 10 × PCR Buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP) (Roche Diagnostics, South Africa), 0.2 µM of forward and reverse primers (Inqaba Biotech, South Africa) and 1.25 U Taq DNA Polymerase (Roche Diagnostics, South Africa) and DNA (20 ng/µL). Sterilised distilled water was added to obtain a final volume of 25 µL.

The ITS-1, ITS-2 and the 5.8 S gene regions of the ITS region of the rRNA operon were amplified using primers ITS-1 (5`–TCC GTA GGT GAA CCT GCG G–3`) and LR-1 (5`–GGT TGG TTT CTT TTC CT–3`) (White *et al*. 1990, Vilgalys & Hester 1990). Reaction conditions for the ITS gene regions followed those of Crous *et al*. (2004a) and Hunter *et al*. (2004a, b).

A portion of the LSU (including domains D1–D3) of the rRNA operon was amplified using primers LR0R (5'–ACC CGC TGA ACT TAA GC–3') (Moncalvo *et al*. 1995) and LR7 (5'–TAC TAC CAC CAA GAT CT–3') (Vilgalys & Hester 1990). PCR cycling conditions were as follows: an initial denaturation step of 96 ºC for 2 min, followed by 35

cycles of denaturation at 94 °C for 30 s, primer annealing at 62 °C for 30 s, primer extension at 72 °C for 1 min and a final elongation step at 72 °C for 7 min.

A portion of the EF-1α was amplified using the primers EF1-728F (5'–CAT CGA GAA GTT CGA GAA GG–3') and EF1-986R (5'–TAC TTG AAG GAA CCC TTA CC–3') (Carbone & Kohn 1999). Reaction conditions were: an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s and primer extension at 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 7 min.

A portion of the ACT gene was amplified using the primers ACT-512F (5'–ATG TGC AAG GCC GGT TTC GC–3') and ACT-783R (5'–TAC GAG TCC TTC TGG CCC AT–3') (Carbone & Kohn 1999). PCR reaction conditions were: an initial denaturation step at 96 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s and extension at 72 °C for 45 s. This was followed by 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C and elongation at 72 °C for 45 s with an increase of 5 s per cycle. The reaction was completed with a final elongation step at 72 °C for 7 min.

All PCR products were visualised in 1.5 % agarose gels (wt/v) stained with ethidium bromide and viewed under ultra-violet light. Sizes of PCR amplicons were estimated by comparison against a 100 bp molecular weight marker (O' RangeRuler™ 100 bp DNA ladder) (Fermentas Life Sciences, U.S.A.). Prior to DNA sequencing, PCR products were purified through Centri-sep spin columns (Princeton Separations, Adelphia, NJ) containing Sephadex G-50 (Sigma Aldrich, St. Louis, MO) as outlined by the manufacturer.

DNA sequencing and phylogenetic analysis

Purified PCR products were used as template DNA for sequencing reactions on an ABI PRISM™ 3100 Automated DNA sequencer (Applied Biosystems, Foster City, CA). The ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) was used for sequencing reactions following the manufacturer's instructions. Most sequencing reactions were performed with the same primers used for PCR reactions. Exceptions were in the case of the ITS region where two internal primers ITS-2 (5'–GCT GCG TTC TTC ATC GAT GC–3') and ITS-3 (5'–GCA TCG ATG AAG AAC GCA GC–3') (White *et al.* 1990) were included for the sequencing reactions. Similarly, for the LSU region two internal primers LR3R (5'–GTC TTG AAA CAC GGA CC–3') and LR-16 (5'-TTC CAC CCA AAC ACT CG-3') were used for the sequencing reactions.

All resulting sequences were analysed with Sequence Navigator v. 1.0.1 (Applied Biosystems, Foster City, CA). Sequence alignments were done using MAFFT (Multiple alignment program for amino acid or nucleotide sequences) v. 5.667 (Katoh *et al*. 2005) and manually adjusted where necessary. Phylogenetic analyses and most parsimonious trees were generated in PAUP v. 4.0b10 (Swofford 2002) by heuristic searches with starting trees obtained through stepwise addition with simple addition sequence and with the MULPAR function enabled. Tree Bisection Reconnection (TBR) was employed as the swapping algorithm. All gaps were coded as missing data and characters were assigned equal weight. Branch support for nodes was obtained by performing 1000 bootstrap replicates of the aligned sequences. For parsimony analyses, measures that were calculated include tree length (TL), retention index (RI), consistency index (CI), rescaled consistency index (RC) and homoplasy index (HI). *Botryosphaeria ribis* Grossenb. & Duggar was used as the outgroup to root all trees.

A Partition Homogeneity Test (Farris *et al*. 1994), of all possible combinations, consisting of 1000 replicates on all informative characters was conducted in PAUP to determine if the LSU, ITS and EF-1α datasets were combinable. All sequences of *Mycosphaerella* spp. used in this study have been deposited in GenBank (Table. 1). Sequence alignments and trees of the LSU, ITS, EF-1α and ACT have been deposited in TreeBASE (accession numbers: LSU = SN2535, ITS = SN2534, EF-1α = SN2536, ACT = SN2537).

Parsimony and distance analyses of combined DNA sequence alignments were conducted in PAUP. Parsimony analyses of all DNA sequence alignments were identical to those described earlier. For distance analyses, Modeltest v. 3.04 (Posada & Crandall 1998) was used to determine the best evolutionary model to fit the combined DNA sequence alignment. A neighbour-joining analysis with an evolutionary model was conducted in PAUP. Here, the distance measure was a general time-reversible (GTR) and the proportion of sites assumed to be invariable (I) was 0.4919, identical sites were removed proportionally to base frequencies estimated from all sites, rates of variable sites assumed to follow a gamma distribution (G) with shape parameter of 0.6198. Ties (if encountered) were broken randomly.

RESULTS

DNA sequencing and phylogenetic analysis

Large Subunit (LSU) phylogeny: The LSU alignment had a total length of 1714 characters. An indel of 383 bp present in *Mycosphaerella ohnowa* Crous & M.J. Wingf. (CBS 112973) and *Mycosphaerella mexicana* Crous (CBS 110502) was excluded from the analyses. In the LSU dataset, 1075 characters were constant while 77 characters were parsimony-uninformative and 179 characters were parsimony-informative. Parsimony analysis of the LSU dataset resulted in the retention of thirty most parsimonious trees (TL = 663, CI = 0.519, RI = 0.878, RC = 0.456). One of these trees (Fig. 1) could be resolved into two clades (Clades 1–2). Clade 1, supported with a bootstrap value of 70 %, included *Mycosphaerella* isolates characterised by *Phaeophleospora* Rangel (*M. ambiphylla* A. Maxwell, *M. suttoniae* Crous & M.J. Wingf.), *Colletogloeopsis* Crous & M.J. Wingf. [*M. molleriana* (Thüm.) Lindau, *M. vespa* Carnegie & Keane, *M. cryptica* (Cooke) Hansf.], *Uwebraunia* Crous & M.J. Wingf. [*M. nubilosa* (Cooke) Hansf.], *M. ohnowa*, *Readeriella* Syd. & P. Syd. (*M. readeriellophora* Crous & J.P. Mansilla), and *Passalora* Fr. (*M. tasmaniensis* Crous & M.J. Wingf.) anamorphs.

The second major clade (Clade 2) resolved in the LSU tree was well-supported with a bootstrap value of 98 %. *Mycosphaerella* species in this clade also grouped strongly following their anamorph associations. Here *Mycosphaerella* isolates could be resolved into several sub-clades also characterised by their anamorph associations. These were *Sonderhenia* H.J. Swart & J. Walker (*M. walkeri* R.F. Park & Keane.), *Pseudocercospora* Speg. [*M. heimioides* Crous & M.J. Wingf., *M. heimii* Crous, *M. crystallina* Crous & M.J. Wingf., *M. irregulariramosa* Crous & M.J. Wingf., *M. colombiensis* Crous & M.J. Wingf., *M. gracilis* Crous & Alfenas, *Pseudocercospora robusta* Crous & M.J. Wingf., *Ps. natalensis* Crous & T. Coutinho, *M. fori* G.C. Hunter, Crous & M.J. Wingf., *Ps. basitruncata* Crous, *Ps. pseudoeucalyptorum* Crous, *Ps. eucalyptorum* Crous, M.J. Wingf., Marasas & B. Sutton., *Ps. paraguayensis* (Koboyashi) Crous, *Ps. basiramifera* Crous] *Passalora* [*Pass. eucalypti* (Crous & Alfenas) Crous & U. Braun, *Pass. zambiae* Crous & T. Coutinho], and *Dissoconium* de Hoog, Oorschot & Hijwegen (*M. lateralis* Crous & M.J. Wingf., *M. communis* Crous & J.P. Mansilla).

Internal Transcribed Spacer (ITS) phylogeny: The ITS sequence alignment consisted of a total of 793 characters. Of these, 499 characters were constant, 62 characters were variable and parsimony-uninformative and 232 characters were parsimony-informative. A 185 bp indel was observed in isolates of *M. gregaria* Carnegie & Keane (CBS 110501), *M.*

endophytica Crous & H. Smith (CBS 111519) and *M. endophytica* (CMW 5225) and was excluded in the phylogenetic analysis.

A heuristic search of the ITS dataset resulted in the retention of four most parsimonious trees (TL = 871, RI = 0.782, CI = 0.358, RC = 0.280). One of these phylogenetic trees (Fig. 2) generated by parsimony analysis of the ITS alignment could be resolved into two monophyletic clades (Clades 1–2). Clade 1 was only weakly supported with a bootstrap value of 50 % after 1000 bootstrap replicates. Clade 1 could be further resolved into several smaller sub-clades where isolates grouped strongly based on their anamorph affiliations. These included *Sonderhenia*, *Pseudocercospora*, *Passalora*, *Uwebraunia*/*Pseudocercosporella* Deighton, *Stenella* Syd., *Readeriella*, *Phaeophleospora* and *Colletogloeopsis*. The second monophyletic clade (Clade 2) grouped sister to the first larger monophyletic clade and contained isolates of *M. lateralis* and *M. communis* (*Dissoconium* anamorphs). This clade was well-supported with a bootstrap value of 100 % after 1000 bootstrap replicates.

Translation Elongation Factor 1-alpha (EF -1α) phylogeny: The EF-1α alignment contained 373 characters. Of these, 41 characters were constant, 23 characters were variable and parsimony-uninformative and 309 characters were parsimony-informative. Heuristic searches resulted in the retention of six most parsimonious trees (TL = 3194, RI = 0.777, CI = 0.345, RC = 0.268), one of which is shown (Fig. 3). Species of *Mycosphaerella* could be resolved into three clades (Clades 1–3).

Clade 1 was weakly supported with a bootstrap value of 67 %. This clade contained *Mycosphaerella* isolates represented by *Pseudocercospora*, *Sonderhenia*, *Phaeophleospora*, *Colletogloeopsis*, *Uwebraunia*, *Readeriella* and *Passalora* anamorphs. Clade 2 was sister to Clade 1 and had a higher bootstrap support of 80 %. Within this clade, *Mycosphaerella* isolates could be separated into three sub-clades that were well-supported. These three sub-clades contained species of *Mycosphaerella* that produced *Pseudocercosporella*, *Uwebraunia*, *Pseudocercospora*, *Passalora* and *Stenella* anamorphs. Clade 3 with bootstrap support of 80 % included isolates of *M. lateralis* and *M. communis* and was basal to Clades 1 and 2.

Actin (ACT) phylogeny: The aligned ACT sequence dataset contained a total of 294 characters. Of these, 135 characters were constant, 30 characters were variable and parsimony-uninformative and 129 characters were parsimony-informative. Heuristic searches of the aligned ACT dataset resulted in the retention of six most parsimonious trees (TL = 1007, RI = 0.682, CI = 0.235, RC = 0.160). One of these trees, shown in Fig. 4, was very

poorly resolved and all deeper nodes were present in a basal polytomy. However, certain smaller clades were resolved and these included a clade including *M. fori*, *M. gracilis*, *Ps. eucalyptorum*, *Ps. pseudoeucalyptorum*, *Ps. robusta*, *Ps. basitruncata*, *Ps. natalensis*, *Ps. basiramifera* and *Ps. paraguayensis*. This clade was supported with a bootstrap value of only 67 %. Another clade supported with a bootstrap value of 100 % contained isolates of *M. ellipsoidea* Crous & M.J. Wingf., *M. endophytica* and *M. gregaria*. Isolates of *M. ambiphylla*, *M. molleriana* and *M. vespa* also clustered together with 100 % bootstrap support. Isolates of *M. intermedia* M.A. Dick & Dobbie, *M. marksii* Carnegie & Keane and *Pseudocercospora epispermogonia* Crous & M.J. Wingf. grouped together in a clade that was supported with a bootstrap value of 84 %. Isolates of *M. flexuosa* Crous & M.J. Wingf., *M. lateralis* and *M. communis* were also accommodated in a well-supported clade with a bootstrap value of 99 %. Isolates of *M. grandis* Carnegie & Keane and *M. parva* R.F. Park & Keane were also resolved into a clade with a bootstrap value of 99 %.

Phylogeny of combined dataset: A partition homogeneity test of the combined LSU, ITS and EF-1α alignment conducted in PAUP resulted in a P-value of 0.001 for all possible combinations of the LSU, ITS and EF-1α DNA alignments. This value is less than the conventionally accepted P-value of $P > 0.05$ required to combine data. However, several studies have accepted a P-value of 0.001 or greater and have further stated that the conventional P-value of 0.05 is inordinately conservative (Cunningham 1997, Darlu & Lecointre 2002, Dettman *et al*. 2003). Thus, the LSU, ITS and EF-1α DNA sequence datasets were combined. The ACT dataset was omitted from the combined dataset due to the lack of resolution among species of *Mycosphaerella*. Therefore, the combined LSU, ITS and EF-1α dataset had a total length of 2880 characters. Of these, 1459 were constant, 150 were variable and parsimony-uninformative and 701 characters were parsimony-informative. An indel of 382 bp was excluded for *M. ohnowa* CBS 112973 and *M. mexicana* CBS 110502 and another indel of 186 bp was excluded for *M. gregaria* CBS 110501 and *M. endophytica* CMW 5225 and CBS 111519. A parsimony analysis resulted in the retention of ten most parsimonious trees (TL = 1677, CI = 0.384, RI = 0.817, RC = 0.314, HI = 0.616). One of these trees (Fig. 5) exhibited a similar topology to that obtained from the LSU alignment. From the analysis of the combined dataset, isolates of *Mycosphaerella* could again be resolved into two clades (Clades 1–2) (Fig. 5). Clade 1 was poorly supported with a bootstrap value of only 66 % and the same isolates were contained in this clade as in the LSU clade 1 (Fig. 1). Clade 2 of the combined phylogenetic tree was well-supported with a bootstrap value of 81 %. This clade

could be further resolved into several smaller well-supported sub-clades containing *Mycosphaerella* isolates that grouped according to their anamorph associations (Fig. 5). Neighbour-joining analysis yielded a phylogenetic tree with the same topology as the most parsimonious trees (data not shown). Here, all *Mycosphaerella* spp. could be resolved into two main clades (Clade 1–2), similar to those of the parsimony analysis (Fig. 5). *Mycosphaerella* spp. could be further sub-divided into several sub-clades corresponding to their anamorph associations, similar to those observed for the parsimony analysis.

DISCUSSION

Results of this study represent the first attempt to employ DNA sequence data from a relatively large number of nuclear gene regions in order to consider the phylogenetic relationships for *Mycosphaerella* spp. occurring on *Eucalyptus* leaves. Other similar studies have relied entirely on sequence data of the ITS region (Crous *et al*. 1999, 2001, 2004a, 2006, Hunter *et al*. 2004b). Although the ITS region offers sufficient resolution to distinguish most taxa, it has not been adequate to separate cryptic taxa in *Mycosphaerella* (Crous *et al*. 2004b). Results of the present study showed that combined DNA sequence data from the LSU, ITS and EF-1α gene regions offer increased genetic resolution to study species concepts in *Mycosphaerella*. However, genes such as the ACT, did not support data emerging from the other loci sequenced, and indicated variation within some clades that were well supported by sequences of other loci and morphological characteristics. These observations led us to exclude ACT data from the final analyses. A similar finding has also emerged from other studies including greater numbers of *Mycosphaerella* species (Crous & Groenewald, unpubl. data).

Mycosphaerella ambiphylla, *M. molleriana* and *M. vespa* grouped together in a well-supported clade in the phylogeny emerging from the combined alignment. This was also true for the ITS, EF-1α and ACT phylogenies where these isolates grouped in a distinct clade with a 100 % bootstrap support. *Mycosphaerella molleriana* and *M. vespa* both have *Colletogloeopsis* anamorphs, however, *M. ambiphylla* produces a *Phaeophleospora* anamorph (Crous & Wingfield 1997a, Maxwell *et al*. 2003). Interestingly, the *Phaeophleospora* anamorph of *M. ambiphylla* was differentiated from *Colletogloeopsis* only by the fact that conidia are produced in a pycnidium as opposed to an acervulus (Maxwell *et al*. 2003). Application of conidiomatal structure to differentiate anamorphs of *Mycosphaerella* has previously been viewed with circumspection especially because *Mycosphaerella* anamorphs

can produce different conidiomatal forms under differing environmental conditions (Crous *et al*. 2000, Cortinas *et al*. 2006). Therefore, the placement of the *M. ambiphylla* anamorph in *Phaeophleospora* is questioned and it should be re-evaluated in terms of its morphological similarities to *Colletogloeopsis*.

Ascospore germination patterns of *M. ambiphylla*, *M. molleriana* and *M. vespa* are all similar, with germ tubes that grow parallel to the long axis of the spore and ascospores with a slight constriction at the median septum, typical of a type C ascospore germination pattern (Crous 1998, Carnegie & Keane 1998, Maxwell *et al*. 2003). Furthermore, overlap is seen in ascospore dimensions of the three species where those of *M. molleriana* are (11–)12–14(–17) × (2.5–)3.5–4(–4.5) µm, those of *M. vespa* 9.5–16.5 × 2.5–4 µm and those of *M. ambiphylla* are (12–)14–15(–22) ×(3.5–)4.5–5(–6) µm (Crous 1998, Carnegie & Keane 1998, Maxwell *et al*. 2003). Leaf lesions of the three species are also similar, pale brown to dark red-brown with lesions of *M. vespa* and *M. ambiphylla* often producing a red margin that was, however, not observed in *M. molleriana* (Crous 1998, Carnegie & Keane 1998, Maxwell *et al*. 2003). Morphological features of *M. ambiphylla*, *M. molleriana* and *M. vespa* are also very similar. This is supported in the DNA phylogeny of the present study where these three species appear to represent a single taxon and therefore suggest that *M. ambiphylla*, *M. molleriana* and *M. vespa* should be synonomised under *M. molleriana*, which is the oldest epithet. We therefore reduce *M. ambiphylla* and *M. vespa* to synonymy with *M. molleriana* as follows:

Mycosphaerella molleriana (Thüm) Lindau, Natürliche Pfanzenfamilie, 1: 424. 1897.

≡ *Sphaerella molleriana* Thüm., Revista Inst. Sci. Lit. Coimbra 28: 31. 1881.

= *Mycosphaerella vespa* Carnegie & Keane, Mycol. Res. 102: 1275. 1998.

= *Mycosphaerella ambiphylla* A. Maxwell, Mycol. Res. 107: 354. 2003.

Anamorph: *Colletogloeopsis molleriana* Crous & M. J. Wingf., Canad. J. Bot. 75: 670. 1997.

Mycosphaerella flexuosa has no known anamorph (Crous 1998). An isolate of this fungus included in the present study grouped together with isolates of *M. ohnowa* in the LSU, ITS, EF-1α and combined dataset with high bootstrap support. This similarity was also observed in a recent study of *Mycosphaerella* spp. on *Eucalyptus* based on ITS sequence data (Crous *et al*. 2004a). *Mycosphaerella ohnowa* is also not known to produce an anamorph (Crous *et al*. 2004a). Although these two species are phylogenetically similar, they can be distinguished from one another based on different ascus and ascospore dimensions, ascospore germination patterns and cultural characteristics (Crous 1998, Crous *et al*. 2004a). Although

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morphologically distinct, it is interesting that these two taxa are phylogenetically so closely related and might suggest a recent speciation event.

Isolates of *M. grandis* and *M. parva* consistently grouped together in a separate clade in all of the DNA sequence datasets in this study. This has also been shown by Crous *et al*. (2004a), where isolates of these two species grouped together in a distinct clade based on ITS DNA sequences. *Mycosphaerella grandis* was originally described from *E. grandis* in Australia, and recognised as a distinct species of *Mycosphaerella* due to its lesion characteristics, and ascospore morphology (Carnegie & Keane 1994). However, Crous (1998) examined the type of *M. grandis* and *M. parva* and found the two species to be congeneric, and reduced them to synonymy under *M. parva*. Results from the present study support the synonymy.

Mycosphaerella lateralis and *M. communis*, both known to have *Dissoconium* anamorphs, showed various phylogenetic placements in this study. From the LSU phylogeny, *M. lateralis* and *M. communis* were situated within a large *Mycosphaerella* clade sister to a *Pseudocercospora* sub-clade. However, in the ITS and EF-1α phylogenies the *Dissoconium* clade was situated basal to the larger *Mycosphaerella* clade. This is consistent with findings of Crous *et al*. (1999, 2000) where the *Dissoconium* clade also resided outside the larger monophyletic *Mycosphaerella* clade. The LSU gene region is well-known to be conserved and to show less nucleotide differences than the ITS and EF-1α gene regions. Although the house-keeping genes investigated here lead to the conclusion that *Dissoconium* could be different from *Mycosphaerella s. str*., this proved not to be the case when LSU data were considered. *Dissoconium* is morphologically identical to *Uwebraunia*, and the separation of these two genera no longer seems tenable. Only two species, *M. ellipsoidea* and *M. nubilosa,* have *Uwebraunia* anamorphs (Crous *et al*. 2004a). However, cultures of both species produced these anamorphs only upon initial isolation, and those that are currently available are sterile. In contrast, strains with *Dissoconium* anamorphs readily produce those in culture, and they usually sporulate profusely. It appears that the status of *Uwebraunia* will only be resolved once fresh, sporulating collections of either *M. ellipsoidea* or *M. nubilosa* can be obtained.

Mycosphaerella spp. with *Pseudocercospora* anamorphs grouped into three clades in all of the phylogenies generated in this study. Species in the *Pseudocercospora* clades have short branch lengths arising from a common internode, suggesting that they have speciated relatively recently from a common ancestor (Ávila *et al*. 2005) and most likely have co-evolved with their *Eucalyptus* hosts as suggested by Crous *et al*. (2000). Ávila *et al*. (2005)

suggested that *Pseudocercospora* may represent a monophyletic lineage. But, results of this and other studies (Ayala-Escobar *et al*. 2006) have shown that *Pseudocercospora* is paraphyletic in *Mycosphaerella* and has evolved more than once in the genus. The availability of new DNA datasets for several gene regions are likely to resolve cryptic species and species complexes within *Pseudocercospora,* as has already been shown for the *M. heimii* and the *P. eucalyptorum* species complexes (Crous *et al*. 2000, 2004a).

Mycosphaerella heimioides, *M. heimii*, *M. crystallina* and *M. irregulariramosa* are all morphologically similar and are regarded as members of the *M. heimii* species complex (Crous & Wingfield 1997b, Crous *et al*. 2001). Previous studies based on ITS DNA sequence data have demonstrated the phylogenetic relatedness of these four species (Crous *et al*. 2001, Crous *et al*. 2004a). However, bootstrap support for their phylogenetic placement was low (Crous *et al*. 2004a). The phylogeny of combined DNA sequence data in this study showed that the four species in the *M. heimii* complex reside in a well-supported clade (bootstrap support 97 %). Furthermore, there is concordance across all gene regions for the node from which all four species branch, however, there is discord at nodes internal to that node. The short branch lengths indicate that the four species have also recently diverged from a common ancestor.

In the phylogeny based on the combined sequence datasets, *M. gracilis* grouped in a well-supported *Pseudocercospora* clade that also included isolates of *Ps. robusta*, *M. fori*, *Ps. pseudoeucalyptorum*, *Ps. eucalyptorum*, *Ps. basitruncata*, *Ps. natalensis*, *Ps. paraguayensis* and *Ps. basiramifera*. This is the first study in which DNA sequence data for *M. gracilis* have been incorporated into a phylogeny. In the ITS, EF-1α and ACT phylogenies, *M. gracilis* was phylogenetically most closely related to *Ps. pseudoeucalyptorum*. However, *M. gracilis* (anamorph: *Pseudocercospora gracilis* Crous & Alfenas) can be distinguished from *Ps. pseudoeucalyptorum* by its single conidiophores arising exclusively from secondary mycelium, which is different to *Ps. pseudoeucalyptorum* in which conidiophores arise from loose or dense fascicles of a stroma (Crous 1998, Crous *et al*. 2004a). Furthermore, conidia of *Ps. gracilis* are more septate, longer, and more uniformly cylindrical in shape than those of *Ps. pseudoeucalyptorum* (Crous 1998, Crous *et al*. 2004a). Results of the present study clearly emphasise the fact that species which are morphologically distinct, can be very closely related.

An interesting result emerging from the phylogenetic analyses in this study was the placement of *Pseudocercospora epispermogonia* in relation to *Mycosphaerella marksii* and *Mycosphaerella intermedia*. Sequences for all but the ACT gene region showed that these

three taxa represent the same phylogenetic species. Although it has previously been suggested that *M. marksii* should have a *Stenella* anamorph because of its proximity to *Mycosphaerella parkii* Crous, M.J. Wingfield, F.A. Ferreira & Alfenas (Crous *et al*. 2001), the current data suggest that this anamorph could be *Ps. epispermogonia*. Crous & Wingfield (1996) described *Ps. epispermogonia* from spermatogonia on lesions colonised by *M. marksii*, but failed to link the two states because single-ascospore cultures did not form an anamorph in culture.

Mycosphaerella intermedia is morphologically similar to *M. marksii*. Both *M. marksii* and *M. intermedia* ascospores germinate in a typical Type B ascospore germination pattern with germ tubes growing parallel to the long axis of the ascospore with no distortion, darkening or constriction of the ascospore occurring (Carnegie & Keane 1994, Crous 1998, Dick & Dobbie 2001). Furthermore, overlap is seen in the ascospore dimensions of *M. marksii* and *M. intermedia* with those of *M. marksii* being 12.5–22.5(17.9) × 2.5–5.0(3.1) µm and those of *M. intermedia* 12–16 × 2–4 µm (Carnegie & Keane 1994, Dick & Dobbie 2001). Leaf lesions of these two species are also similar with those of *M. marksii* being grey on the adaxial leaf surface and yellow to red-brown on the abaxial leaf surface and surrounded by a red-brown margin while lesions of *M. intermedia* are pale on the abaxial surface and rust-brown with a slightly raised dark-brown margin surrounded by a red-purple zone on the adaxial leaf surface (Carnegie & Keane 1994, Dick & Dobbie 2001). Due to the phylogenetic and morphological similarity, we reduce *M. intermedia* to synonymy with *M. marksii* as follows:

Mycosphaerella marksii Carnegie & Keane, Mycol. Res. 98: 413–416. 1994.

= *Mycosphaerella intermedia* M. A. Dick & Dobbie, New Zealand. J. Bot. 39: 270. 2001.

Anamorph: *Pseudocercospora epispermogonia* Crous & M. J. Wingf., Mycologia 88: 456. 1996.

Mycosphaerella africana Crous & M.J. Wingf., *M. aurantia* A. Maxwell and *M. keniensis* Crous & T. Coutinho have no known anamorphs. Previous studies based on ITS sequence data have suggested that *M. africana* and *M. keniensis* grouped close to *Mycosphaerella* spp. with *Passalora* anamorphs. It has thus been assumed that *M. africana* and *M. keniensis* would have *Passalora* anamorphs if they were to be found (Crous *et al.* 2000). However, the phylogenies emerging from LSU, ITS and EF-1α sequences and the combined data for the three regions showed that *M. africana*, *M. keniensis* and *M. aurantia* consistently group separately from the *Passalora* anamorphs, close to a clade of isolates with

Uwebraunia and *Pseudocercosporella* anamorphs. The association of these three taxa to *Passalora* is thus doubted. Furthermore, the clade containing *M. africana*, *M. aurantia* and *M. keniensis* is also well-supported and seems to represent a single evolving lineage.

Moreover, results of the present study show that *M. aurantia* and *M. africana* represent a single phylogenetic species. These two species consistently grouped together in all phylogenies with *M. keniensis* grouping as a sister. *Mycosphaerella aurantia* was described from leaves of *E. globulus* in south-western Australia and is known only from this location (Maxwell *et al*. 2003). Morphologically, *M. aurantia* produces asci and ascospores that are similar in size and morphology to *M. africana.* However, the ascospores of *M. aurantia* are not constricted at the median septum whereas those of *M. africana* had such constrictions, and ascospores of *M. aurantia* are longer (9–)11–12(–15) µm than those of *M. africana* (7–)8–10(–11) µm (Crous 1998, Maxwell *et al*. 2003). Furthermore, *M. aurantia* produces lateral hyaline germ tubes that grow parallel to the long axis of the ascospore and become slightly verrucose to produce lateral branches upon prolonged incubation (Maxwell *et al.* 2003). This is in contrast to ascospores of *M. africana* that germinate in an irregular fashion producing distinctly dark verrucose germ tubes from different positions of the distorted ascospore (Crous 1998). It is intriguing that these two species, which are morphologically quite distinct, would represent a single phylogenetic species. Additional isolates of these species are required to determine whether they represent two distinct taxa or are conspecific.

Mycosphaerella gregaria was described from leaves of *E. grandis* in Victoria, Australia (Carnegie & Keane 1997). No anamorph has been observed for this species (Carnegie & Keane 1997, Crous 1998). An isolate of *M. gregaria*, collected from *E. globulus* in Australia, consistently grouped in a clade with isolates of *M. endophytica* and *M. ellipsoidea*. *Mycosphaerella endophytica* and *M. ellipsoidea* are known to have *Pseudocercosporella* and *Uwebraunia* anamorphs, respectively (Crous 1998). Based on previous studies employing ITS sequence data, isolates of *M. endophytica* grouped sister to isolates of *M. aurantia*, *M. ellipsoidea* and *M. africana* (Crous *et al*. 2004a). However, based on sequence data from the four gene regions employed in this study, isolates of *M. endophytica* grouped in a distinct well-supported clade with *M. ellipsoidea*. This is interesting because *M. ellipsoidea* has an *Uwebraunia* anamorph (Crous & Wingfield 1996). *Mycosphaerella endophytica* and *M. pseudoendophytica* Crous & G.C. Hunter are the only *Mycosphaerella* spp. occurring on *Eucalyptus* that are known to have *Pseudocercosporella* anamorphs (Crous 1998, Crous *et al*. 2006).

Phylogenies emerging from analyses of sequences for the four gene regions considered in this study suggest that *Mycosphaerella* constitutes heterogenous groups of which only a few are closely linked to certain anamorph genera. It is evident that for the larger part the evolution of the anamorph genera within *Mycosphaerella* has been polyphyletic, and not monophyletic as previously suggested. This can be seen by the multiple evolution of anamorph genera such as *Passalora, Pseudocercospora*, *Phaeophleospora* and *Stenella* within *Mycosphaerella* (Crous *et al*. 2006). It would thus not be advisable to predict anamorph relationships based on the phylogenetic position within *Mycosphaerella*. Not only has the same morphology evolved more than once in the group, but disjunct anamorph morphologies also frequently cluster together (Crous *et al*. 2000, 2004a, 2006). This makes the interpretation difficult, and predictions based on position in clades unreliable.

The production of four nucleotide sequence datasets for species of *Mycosphaerella* occurring on *Eucalyptus* leaves should serve as a framework for the more accurate taxonomic placement of these fungi in future. The importance of species complexes in *Mycosphaerella* has become more evident in this genus in recent years (Crous *et al*. 2004a, b, 2006). To study species complexes, variable gene regions must be studied and the generation of greater numbers of datasets should allow for increased resolution at the species level. This in turn will aid in the resolution of species complexes and cryptic speciation. Studies of the deeper branches for groups in *Mycosphaerella* can in future utilise sequence data for the LSU region that have not previously been available. These should provide a more lucid indication and support for phenotypic characters that are phylogenetically informative.

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Table 1. Isolates of *Mycosphaerella* used in this study for DNA sequencing and phylogenetic analysis.

Teleomorph	Anamorph	Isolate No.			Host	Country	Collector	GenBank Accession No.			
		CMW	CBS	STEU				LSU	ITS	ACT	EF-1α
M. africana	Unknown	3026	116155	795	*E. viminalis*	South Africa	P.W. Crous	DQ246258	DQ267577	DQ147608	DQ235098
		4945	116154	794	*E. viminalis*	South Africa	P.W. Crous	DQ246257	AF309602	DQ147609	DQ235099
M. ambiphylla	*Phaeophleospora* sp.	14180	110499	N/A	*E. globulus*	Australia	A. Maxwell	DQ246219	AY725530	DQ147669	DQ235103
M. aurantia	Unknown	14460	110500	N/A	*E. globulus*	Australia	A. Maxwell	DQ246256	AY725531	DQ147610	DQ235097
M. colombiensis	*Pseudocercospora colombiensis*	4944	110969	1106	*E. urophylla*	Colombia	M.J. Wingfield	DQ204744	AY752149	DQ147639	DQ211660
		11255	110967	1104	*E. urophylla*	Colombia	M.J. Wingfield	DQ204745	AY752147	DQ147640	DQ211661
M. communis	*Dissoconium commune*	14672	114238	10440	*E. globulus*	Spain	J.P. Mansilla	DQ246262	AY725541	DQ147655	DQ235141
		14673	110976	849	*E. cladocalyx*	South Africa	P.W. Crous	DQ246261	AY725537	DQ147654	DQ235140
M. cryptica	*Colletogloeopsis nubilosum*	3279	110975	936	*E. globulus*	Australia	A.J. Carnegie	DQ246222	AF309623	DQ147674	DQ235119
		2732	N/A	355	*Eucalyptus* sp.	Chile	M.J. Wingfield	N/A	AF309622	N/A	N/A
M. crystallina	*Pseudocercospora crystallina*	3042	N/A	800	*E. bicostata*	South Africa	M.J. Wingfield	DQ204746	DQ267578	DQ147637	DQ211662
		3033	681.95	802	*E. bicostata*	South Africa	M.J. Wingfield	DQ204747	AY490757	DQ147636	DQ211663
M. ellipsoidea	*Uwebraunia ellipsoidea*	4934	N/A	1224	*Eucalyptus* sp.	South Africa	Unknown	DQ246253	AF309592	DQ147647	DQ235129
		5166	N/A	1225	*Eucalyptus* sp.	South Africa	Unknown	DQ246254	AF309593	DQ147648	DQ235127
M. endophytica	*Pseudocercosporella endophytica*	14912	111519	1191	*Eucalyptus* sp.	South Africa	P.W. Crous	DQ246255	DQ267579	DQ147646	DQ235131
		5225	N/A	1192	*Eucalyptus* sp.	South Africa	P.W. Crous	DQ246252	DQ267580	DQ147649	DQ235128
M. flexuosa	Unknown	5224	111012	1109	*E. globulus*	Colombia	M.J. Wingfield	DQ246232	AF309603	DQ147653	DQ235126
M. fori	*Pseudocercospora* sp.	9095	N/A	N/A	*E. grandis*	South Africa	G.C. Hunter	DQ204748	AF468869	DQ147618	DQ211664
		9096	N/A	N/A	*E. grandis*	South Africa	G.C. Hunter	DQ204749	DQ267581	DQ147619	DQ211665

Teleomorph	Anamorph	Isolate No.			Host	Country	Collector	GenBank Accession No.			
		CMW	CBS	STEU				LSU	ITS	ACT	EF-1α
M. gracilis	*Pseudocercospora gracilis*	14455	243.94	730	*E. urophylla*	Indonesia	A.C. Alfenas	DQ204750	DQ267582	DQ147616	DQ211666
M. grandis	Unknown	8557	N/A	N/A	*E. globulus*	Chile	A. Rotella	DQ246241	DQ267583	DQ147644	DQ235108
		8554	N/A	N/A	*E. globulus*	Chile	M.J. Wingfield	DQ246240	DQ267584	DQ147643	DQ235107
M. gregaria	Unknown	14462	110501	N/A	*E. globulus*	Australia	A. Maxwell	DQ246251	DQ267585	DQ147650	DQ235130
M. heimii	*Pseudocercospora heimii*	4942	110682	760	*Eucalyptus* sp.	Madagascar	P.W. Crous	DQ204751	AF309606	DQ147638	DQ211667
M. heimioides	*Pseudocercospora heimioides*	14776	111364	N/A	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	DQ204752	DQ267586	DQ147632	DQ211668
		3046	111190	1312	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	DQ204753	AF309609	DQ147633	DQ211669
M. intermedia	Unknown	7163	114356	10902	*E. saligna*	New Zealand	K. Dobbie	DQ246247	AY725546	N/A	N/A
		7164	114415	10922	*E. saligna*	New Zealand	K. Dobbie	DQ246248	AY725547	DQ147627	DQ235132
M. irregulariramosa	*Pseudocercospora irregulariramosa*	4943	114774	1360	*E. saligna*	South Africa	M.J. Wingfield	DQ204754	AF309607	DQ147634	DQ211670
		5223	N/A	1362	*E. saligna*	South Africa	M.J. Wingfield	DQ204755	AF309608	DQ147635	DQ211671
M. ohnowa	Unknown	4937	112896	1004	*E. grandis*	South Africa	M.J. Wingfield	N/A	AF309604	DQ147662	DQ235125
		4936	112973	1005	*E. grandis*	South Africa	M.J. Wingfield	DQ246231	AF309605	DQ147661	DQ235124
M. keniensis	Unknown	5147	111001	1084	*E. grandis*	Kenya	T. Coutinho	DQ246259	AF309601	DQ147611	DQ235100
M. lateralis	*Uwebraunia lateralis*	14906	110748	825	*E. grandis* × *E. saligna*	South Africa	G. Kemp	DQ204768	AF173315	DQ147651	DQ211684
		5164	111169	1232	*E. globulus*	Zambia	T. Coutinho	DQ246260	AY25550	DQ147652	DQ235139
M. madeirae	*Pseudocercospora* sp	14458	112895	3745	*E. globulus*	Madeira	S. Denman	DQ204756	AY725553	DQ147641	DQ211672
M. marksii	Unknown	14781	682.95	842	*E. grandis*	South Africa	G. Kemp	DQ246249	DQ267587	DQ147624	DQ235133
		5150	110920	935	*E. botryoides*	Australia	A.J. Carnegie	DQ246250	AF309588	DQ147625	DQ235134

Teleomorph	Anamorph	Isolate No.			Host	Country	Collector	GenBank Accession No.			
		CMW	CBS	STEU				LSU	ITS	ACT	EF-1α
		5230	N/A	782	*E. botryoides*	Australia	A.J. Carnegie	DQ246246	DQ267588	DQ147626	DQ235135
M. mexicana	Unknown	14461	110502	N/A	*E. globulus*	Australia	A. Maxwell	DQ246237	AY725558	DQ147660	DQ235123
M. readeriellophora	*Readeriella readeriellophora*	14233	114240	10375	*E. globulus*	Spain	J.P. Mansilla	DQ246238	AY725577	DQ147658	DQ235117
M. molleriana	*Colletogloeopsis molleriana*	4940	111164	1214	*E. globulus*	Portugal	S. McCrae	DQ246220	AF309620	DQ147671	DQ235104
		2734	111132	784	*E. globulus*	U. S. A.	M.J. Wingfield	DQ246223	AF309619	DQ147670	DQ235105
M. nubilosa	*Uwebraunia juvenis*	3282	116005	937	*E. globulus*	Australia	A.J. Carnegie	DQ246228	AF309618	DQ147666	DQ235111
		9003	114708	N/A	*E. nitens*	South Africa	G.C. Hunter	DQ246229	AF449099	DQ147667	DQ235112
M. parkii	*Stenella parkii*	14775	387.92	353	*E. grandis*	Brazil	M.J. Wingfield	DQ246245	AY626979	DQ147612	DQ235137
M. parva	Unknown	14459	110503	N/A	*E. globulus*	Australia	A. Maxwell	DQ246243	AY626980	DQ147645	DQ235110
		14917	116289	10935	*Eucalyptus* sp.	South Africa	P.W. Crous	DQ246242	AY725576	DQ147642	DQ235109
M. suberosa	Unknown	5226	436.92	515	*E. dunnii*	Brazil	M.J. Wingfield	DQ246235	AY626985	DQ147656	DQ235101
		7165	N/A	N/A	*E. muelleriana*	New Zealand	Unknown	DQ246236	DQ267589	DQ147657	DQ235102
M. suttoniae	*Phaeophleospora epicoccoides*	5348	N/A	1346	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	DQ246227	AF309621	DQ147673	DQ235116
M. vespa	*Colletogloeopsis* sp.	11558	117924	N/A	*E. globulus*	Tasmania	Unknown	DQ246221	DQ267590	DQ147668	DQ235106
M. tasmaniensis	*Passalora tasmaniensis*	14780	111687	1555	*E. nitens*	Tasmania	M.J. Wingfield	DQ246233	DQ267591	DQ147676	DQ235121
		14663	114556	N/A	*E. nitens*	Tasmania	M.J. Wingfield	DQ246234	DQ267592	DQ147677	DQ235122
M. toledana	*Phaeophleospora toledana*	14457	113313	N/A	*Eucalyptus* sp.	Spain	P.W. Crous	DQ246230	AY725580	DQ147672	DQ235120
M. walkerii	*Sonderhenia eucalypticola*	20333	N/A	N/A	*E. globulus*	Chile	M.J. Wingfield	DQ267574	DQ267593	DQ147630	DQ235095
		20334	N/A	N/A	*E. globulus*	Chile	M.J. Wingfield	DQ267575	DQ267594	DQ147631	DQ235096
Unknown	*Passalora eucalypti*	14907	111306	1457	*E. saligna*	Brazil	P.W. Crous	DQ246244	AF309617	DQ147678	DQ235138
Unknown	*Passalora zambiae*	14782	112971	1227	*E. globulus*	Zambia	T. Coutinho	DQ246264	AF725523	DQ147675	DQ235136

Teleomorph	Anamorph	Isolate No.			Host	Country	Collector	GenBank Accession No.			
		CMW	CBS	STEU				LSU	ITS	ACT	EF-1α
Unknown	*Pseudocercospora epispermogonia*	14778	110750	822	*E. grandis* × *E. saligna*	South Africa	G. Kemp	DQ204757	DQ267596	DQ147629	DQ211673
		14786	110693	823	*E. grandis* × *E. saligna*	South Africa	G. Kemp	DQ204758	DQ267597	DQ147628	DQ211674
Unknown	*Phaeophleospora eucalypti*	11687	113992	N/A	*E. nitens*	New Zealand	M. Dick	DQ246225	DQ267598	DQ147664	DQ235115
		14910	111692	1582	*Eucalyptus* sp.	New Zealand	M.J. Wingfield	DQ246224	DQ267599	DQ147663	DQ235114
Unknown	*Pseudocercospora basitruncata*	14914	114664	1202	*E. grandis*	Colombia	M.J. Wingfield	DQ204759	DQ267600	DQ147622	DQ211675
		14785	111280	1203	*E. grandis*	Colombia	M.J. Wingfield	DQ204760	DQ267601	DQ147621	DQ211676
Unknown	*Pseudocercospora basiramifera*	5148	N/A	N/A	*E. pellita*	Thailand	M.J. Wingfield	DQ204761	AF309595	DQ147607	DQ211677
Unknown	*Pseudocercospora eucalyptorum*	5228	110777	16	*E. nitens*	South Africa	P.W. Crous	DQ204762	AF309598	DQ147614	DQ211678
Unknown	*Pseudocercospora natalensis*	14777	111069	1263	*E. nitens*	South Africa	T. Coutinho	DQ267576	N/A	DQ147620	N/A
		14784	111070	1264	*E. nitens*	South Africa	T. Coutinho	DQ204763	AF309594	DQ147623	DQ211679
Unknown	*Pseudocercospora paraguayensis*	14779	111286	1459	*E. nitens*	Brazil	P.W. Crous	DQ204764	DQ267602	DQ147606	DQ211680
Unknown	*Pseudocercospora pseudoeucalyptorum*	14908	114242	10390	*E. globulus*	Spain	J.P. Mansilla	DQ204765	AY725526	DQ147613	DQ211681
		14911	114243	10500	*E. nitens*	New Zealand	W. Gams	DQ204766	AY725527	DQ147615	DQ211682
Unknown	*Pseudocercospora robusta*	5151	111175	1269	*E. robusta*	Malaysia	M.J. Wingfield	DQ204767	AF309597	DQ147617	DQ211683

Teleomorph	Anamorph	Isolate No.			Host	Country	Collector	GenBank Accession No.			
		CMW	CBS	STEU				LSU	ITS	ACT	EF-1α
Unknown	*Readeriella novaezelandiae*	14913	114357	10895	*E. botryoides*	New Zealand	M.A. Dick	DQ246239	DQ267603	DQ147659	DQ235118
Botryosphaeria ribis	*Fusicoccum ribis*	7773	N/A	N/A	*Ribus* sp.	U. S. A.	G. Hudler.	DQ246263	DQ267604	DQ267605	DQ235142

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

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

STEU: Culture collection of Stellenbosch University, South Africa. Isolate numbers from Crous (1998).

N/A: Not available

Figure 1. Phylogram obtained from the Large Subunit (LSU) rDNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing two well-supported main clades (Clades 1–2). Tree length = 663, CI = 0.519, RI = 0.878, RC = 0.456. Bootstrap values based on 1000 replicates are indicated above branches. Anamorph affinities are indicated next to the vertical lines.

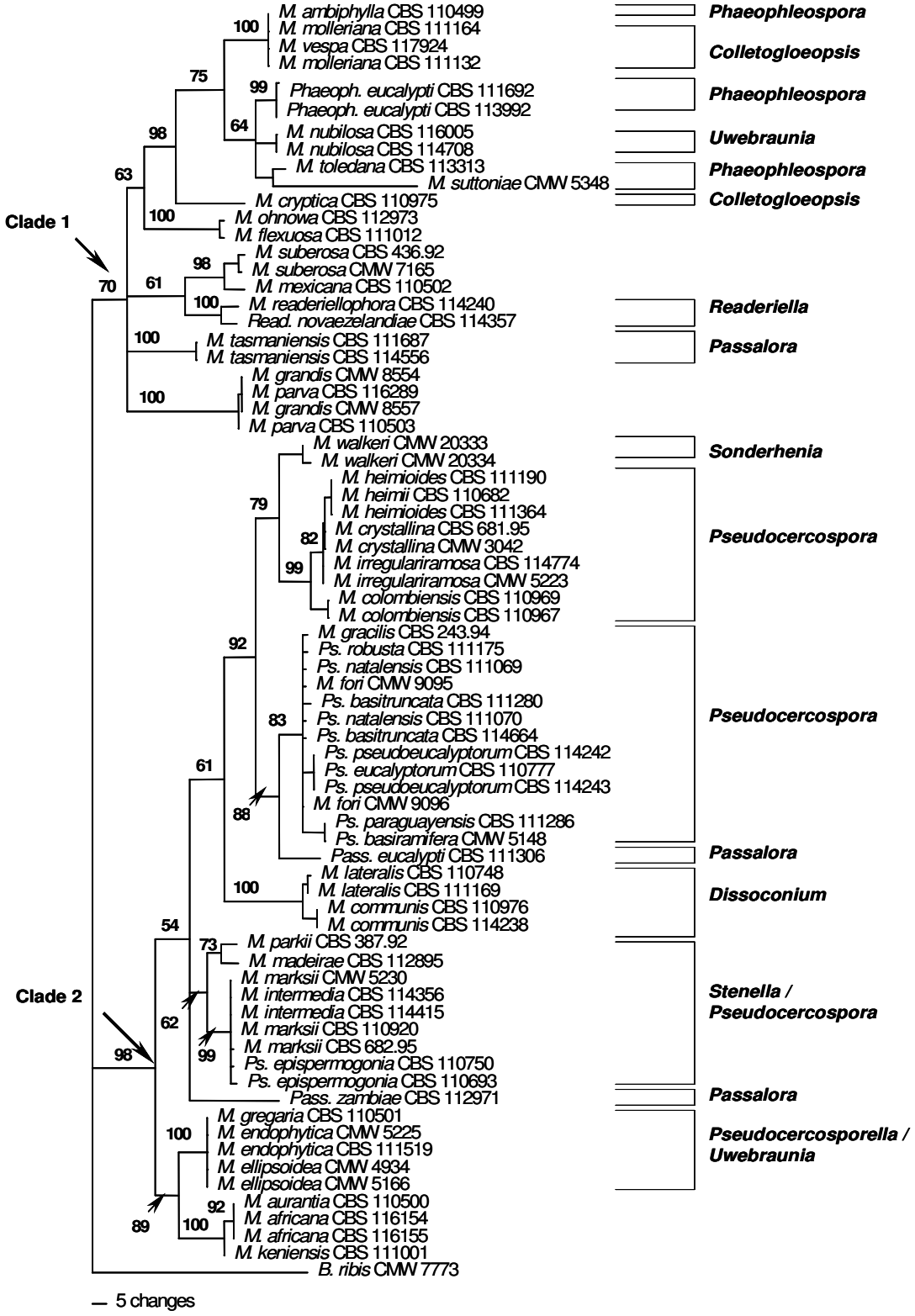
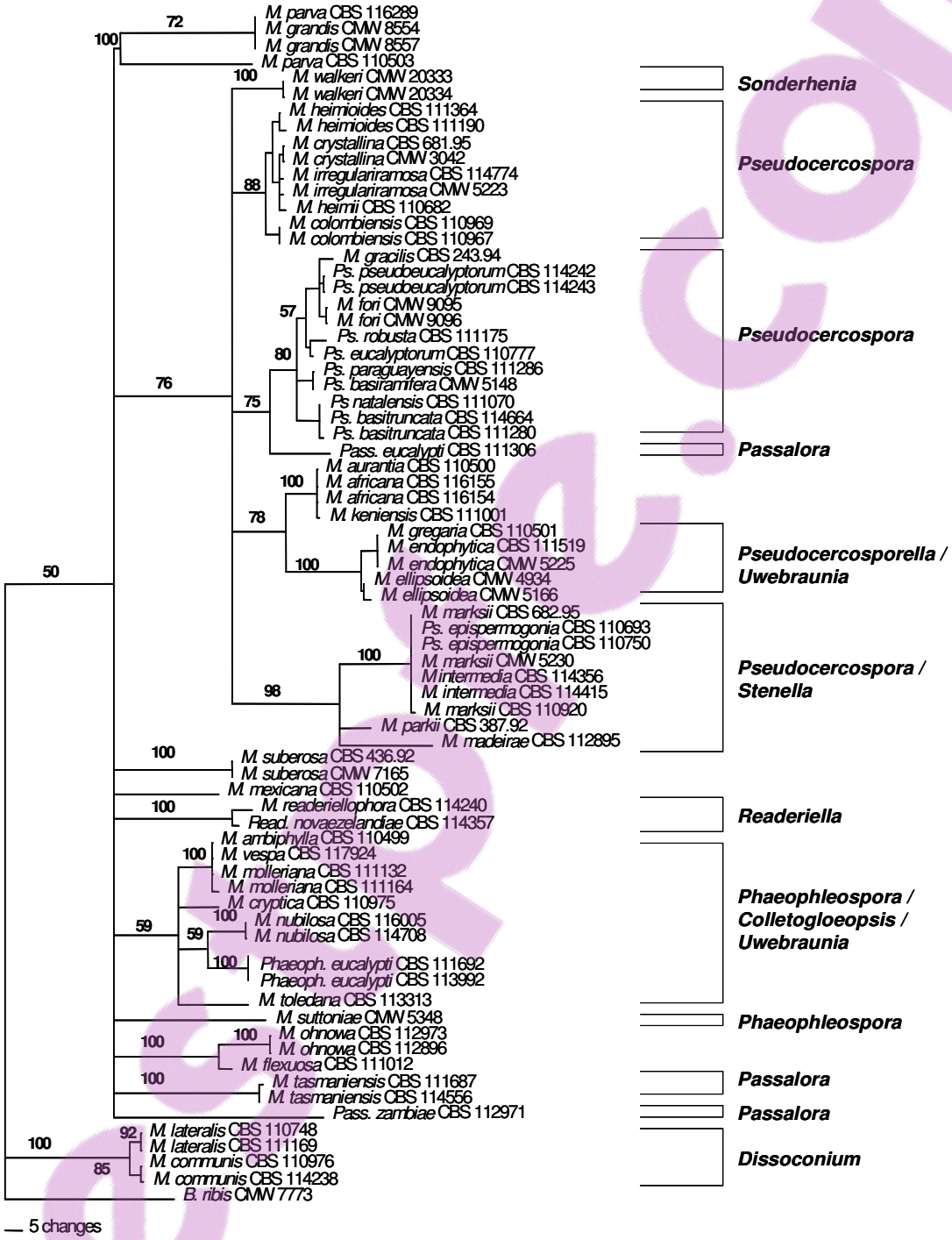

M. ambiphylla CBS 110499
M. molleriana CBS 111164
M. vespa CBS 117924
M. molleriana CBS 111132
Phaeoph. eucalypti CBS 111692
Phaeoph. eucalypti CBS 113992
M. nubilosa CBS 116005
M. nubilosa CBS 114708
M. toledana CBS 113313
M. suttoniae CMW 5348
M. cryptica CBS 110975
M. ohnowa CBS 112973
M. flexuosa CBS 111012
M. suberosa CBS 436.92
M. suberosa CMW 7165
M. mexicana CBS 110502
M. readeriellophora CBS 114240
Read. novaezelandiae CBS 114357
M. tasmaniensis CBS 111687
M. tasmaniensis CBS 114556
M. grandis CMW 8554
M. parva CBS 116289
M. grandis CMW 8557
M. parva CBS 110503
M. walkeri CMW 20333
M. walkeri CMW 20334
M. heimioides CBS 111190
M. heimii CBS 110682
M. heimioides CBS 111364
M. crystallina CBS 681.95
M. crystallina CMW 3042
M. irregulariramosa CBS 114774
M. irregulariramosa CMW 5223
M. colombiensis CBS 110969
M. colombiensis CBS 110967
M. gracilis CBS 243.94
Ps. robusta CBS 111175
Ps. natalensis CBS 111069
M. fori CMW 9095
Ps. basitruncata CBS 111280
Ps. natalensis CBS 111070
Ps. basitruncata CBS 114664
Ps. pseudoeucalyptorum CBS 114242
Ps. eucalyptorum CBS 110777
Ps. pseudoeucalyptorum CBS 114243
M. fori CMW 9096
Ps. paraguayensis CBS 111286
Ps. basiramifera CMW 5148
Pass. eucalypti CBS 111306
M. lateralis CBS 110748
M. lateralis CBS 111169
M. communis CBS 110976
M. communis CBS 114238
M. parkii CBS 387.92
M. madeirae CBS 112895
M. marksii CMW 5230
M. intermedia CBS 114356
M. intermedia CBS 114415
M. marksii CBS 110920
M. marksii CBS 682.95
Ps. epispermogonia CBS 110750
Ps. epispermogonia CBS 110693
Pass. zambiae CBS 112971
M. gregaria CBS 110501
M. endophytica CMW 5225
M. endophytica CBS 111519
M. ellipsoidea CMW 4934
M. ellipsoidea CMW 5166
M. aurantia CBS 110500
M. africana CBS 116154
M. africana CBS 116155
M. keniensis CBS 111001
B. ribis CMW 7773
5 changes
Clade 1
Clade 2
Phaeophleospora
Colletogloeopsis
Phaeophleospora
Uwebraunia
Phaeophleospora
Colletogloeopsis
Readeriella
Passalora
Sonderhenia
Pseudocercospora
Pseudocercospora
Passalora
Dissoconium
Stenella / Pseudocercospora
Passalora
Pseudocercosporella / Uwebraunia

Figure 2. Phylogram obtained from the Internal Transcribed Spacer (ITS) DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves indicating two monophyletic clades (Clades 1–2). Tree length = 871, CI = 0.358, RI = 0.782, RC = 0.280.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M. heimioides CBS 111364
M. heimioides CBS 111190
M. crystallina CBS 681.95
M. crystallina CMW 3042
M. irregulariramosa CBS 114774
M. irregulariramosa CMW 5223
M. heimii CBS 110682
M. colombiensis CBS 110969
M. colombiensis CBS 110967
M. gracilis CBS 243.94
Ps. pseudobeucalyptorum CBS 114242
Ps. pseudobeucalyptorum CBS 114243
M. fori CMW 9095
M. fori CMW 9096
Ps. robusta CBS 111175
Ps. eucalyptorum CBS 110777
Ps. paraguayensis CBS 111286
Ps. basiramifera CMW 5148
Ps natalensis CBS 111070
Ps. basitruncata CBS 114664
Ps. basitruncata CBS 111280
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M. aurantia CBS 110500
M. africana CBS 116155
M. africana CBS 116154
M. keniensis CBS 111001
M. gregaria CBS 110501
M. endophytica CBS 111519
M. endophytica CMW 5225
M. ellipsoidea CMW 4934
M. ellipsoidea CMW 5166
M. marksii CBS 682.95
Ps. epispermogonia CBS 110693
Ps. epispermogonia CBS 110750
M. marksii CMW 5230
M intermedia CBS 114356
M. intermedia CBS 114415
M. marksii CBS 110920
M. parkii CBS 387.92
M. madeirae CBS 112895
M. suberosa CBS 436.92
M. suberosa CMW 7165
M. mexicana CBS 110502
M. readeriellophora CBS 114240
Read. novaezelandiae CBS 114357
M. ambiphylla CBS 110499
M. vespa CBS 117924
M. molleriana CBS 111132
M. molleriana CBS 111164
M. cryptica CBS 110975
M. nubilosa CBS 116005
M. nubilosa CBS 114708
Phaeoph. eucalypti CBS 111692
Phaeoph. eucalypti CBS 113992
M. toledana CBS 113313
M. suttoniae CMW 5348
M. ohnowa CBS 112973
M. ohnowa CBS 112896
M. flexuosa CBS 111012
M. tasmaniensis CBS 111687
M. tasmaniensis CBS 114556
Pass. zambiae CBS 112971
M. lateralis CBS 110748
M. lateralis CBS 111169
M. communis CBS 110976
M. communis CBS 114238
B. ribis CMW 7773
5 changes
Sonderhenia
Pseudocercospora
Pseudocercospora
Passalora
Pseudocercosporella / Uwebraunia
Pseudocercospora / Stenella
Readeriella
Phaeophleospora / Colletogloeopsis / Uwebraunia
Phaeophleospora
Passalora
Passalora
Dissoconium

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Figure 3. Phylogram obtained from the Translation Elongation Factor 1-alpha (EF-1α) DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing three main clades. Tree length = 3194, CI = 0.345, RI = 0.777, RC = 0.268.

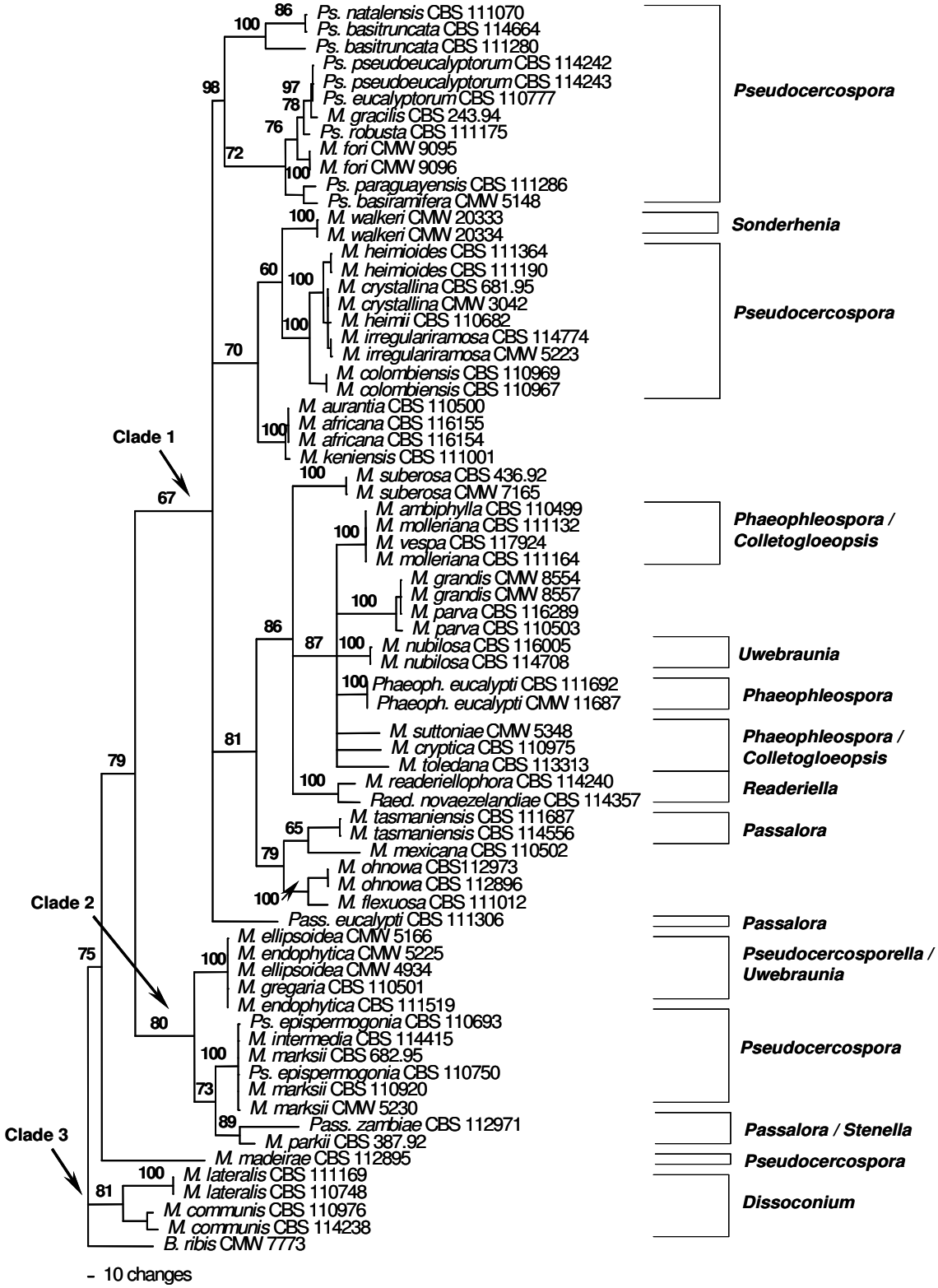

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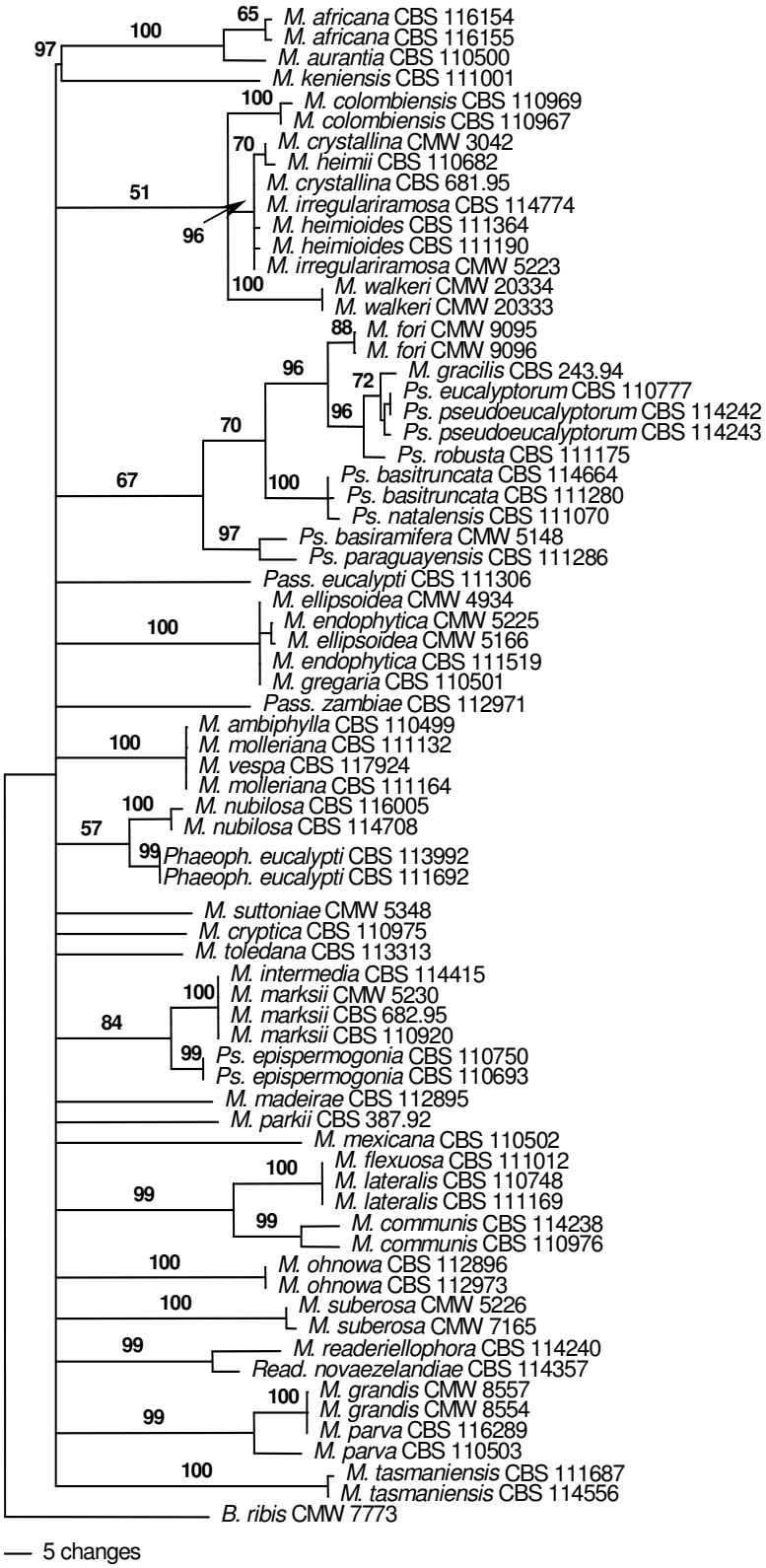
Figure 4. Phylogram obtained from the Actin (ACT) DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves. Tree length = 1007, CI = 0.235, RI = 0.682, RC = 0.160.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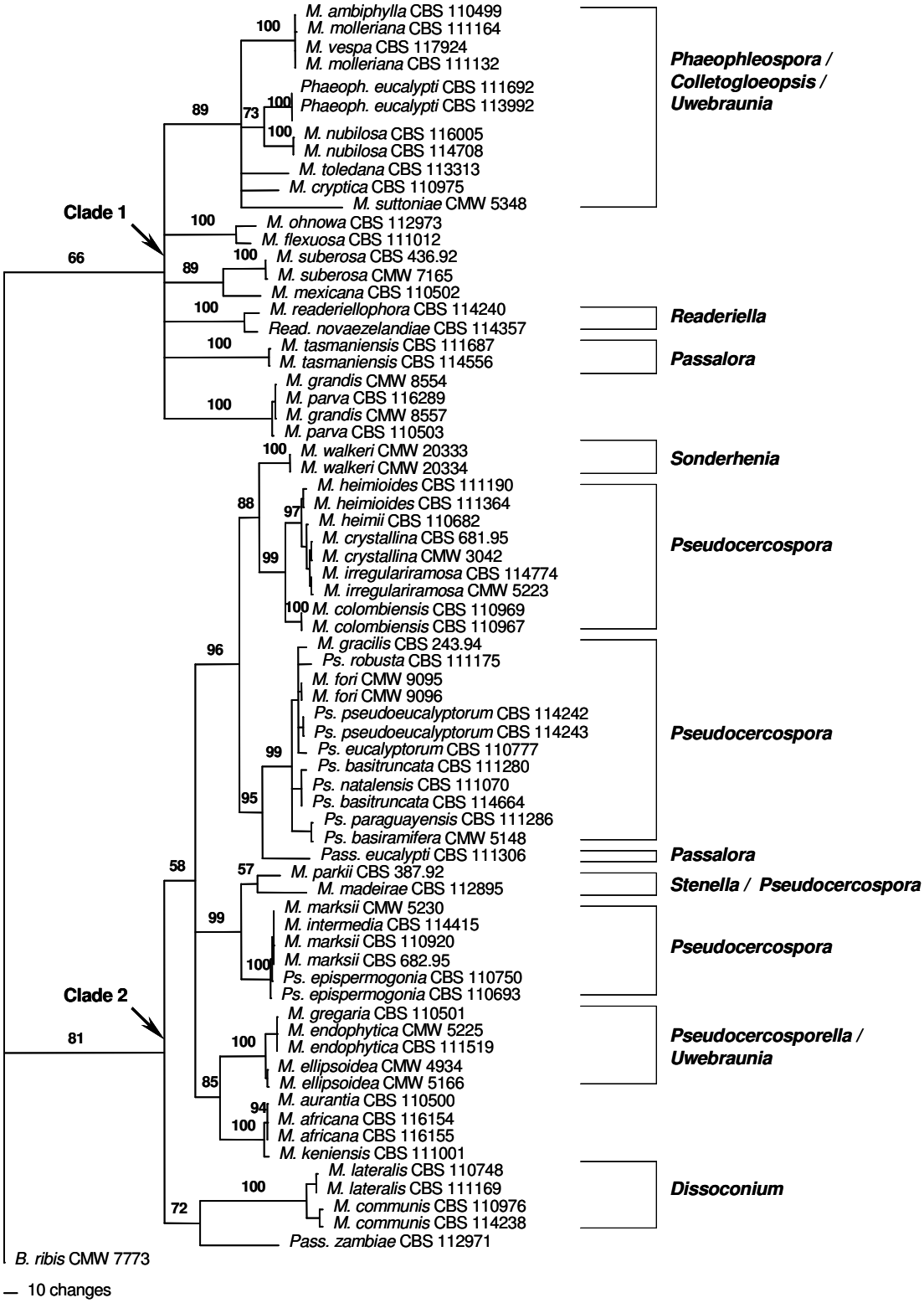
M. africana CBS 116155
M. aurantia CBS 110500
M. keniensis CBS 111001
M. colombiensis CBS 110969
M. colombiensis CBS 110967
M. crystallina CMW 3042
M. heimii CBS 110682
M. crystallina CBS 681.95
M. irregulariramosa CBS 114774
M. heimioides CBS 111364
M. heimioides CBS 111190
M. irregulariramosa CMW 5223
M. walkeri CMW 20334
M. walkeri CMW 20333
M. fori CMW 9095
M. fori CMW 9096
M. gracilis CBS 243.94
Ps. eucalyptorum CBS 110777
Ps. pseudoeucalyptorum CBS 114242
Ps. pseudoeucalyptorum CBS 114243
Ps. robusta CBS 111175
Ps. basitruncata CBS 114664
Ps. basitruncata CBS 111280
Ps. natalensis CBS 111070
Ps. basiramifera CMW 5148
Ps. paraguayensis CBS 111286
Pass. eucalypti CBS 111306
M. ellipsoidea CMW 4934
M. endophytica CMW 5225
M. ellipsoidea CMW 5166
M. endophytica CBS 111519
M. gregaria CBS 110501
Pass. zambiae CBS 112971
M. ambiphylla CBS 110499
M. molleriana CBS 111132
M. vespa CBS 117924
M. molleriana CBS 111164
M. nubilosa CBS 116005
M. nubilosa CBS 114708
Phaeoph. eucalypti CBS 113992
Phaeoph. eucalypti CBS 111692
M. suttoniae CMW 5348
M. cryptica CBS 110975
M. toledana CBS 113313
M. intermedia CBS 114415
M. marksii CMW 5230
M. marksii CBS 682.95
M. marksii CBS 110920
Ps. epispermogonia CBS 110750
Ps. epispermogonia CBS 110693
M. madeirae CBS 112895
M. parkii CBS 387.92
M. mexicana CBS 110502
M. flexuosa CBS 111012
M. lateralis CBS 110748
M. lateralis CBS 111169
M. communis CBS 114238
M. communis CBS 110976
M. ohnowa CBS 112896
M. ohnowa CBS 112973
M. suberosa CMW 5226
M. suberosa CMW 7165
M. readeriellophora CBS 114240
Read. novaezelandiae CBS 114357
M. grandis CMW 8557
M. grandis CMW 8554
M. parva CBS 116289
M. parva CBS 110503
M. tasmaniensis CBS 111687
M. tasmaniensis CBS 114556
B. ribis CMW 7773
5 changes

Figure 5. Phylogram obtained from the combined LSU, ITS and EF-1α DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing two main clades. Tree length = 1677, CI = 0.384, RI = 0.817, RC = 0.314.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M. molleriana CBS 111164
M. vespa CBS 117924
M. molleriana CBS 111132
Phaeoph. eucalypti CBS 111692
Phaeoph. eucalypti CBS 113992
M. nubilosa CBS 116005
M. nubilosa CBS 114708
M. toledana CBS 113313
M. cryptica CBS 110975
M. suttoniae CMW 5348
Phaeophleospora / Colletogloeopsis / Uwebraunia
Clade 1
M. ohnowa CBS 112973
M. flexuosa CBS 111012
M. suberosa CBS 436.92
M. suberosa CMW 7165
M. mexicana CBS 110502
M. readeriellophora CBS 114240
Read. novaezelandiae CBS 114357
Readeriella
M. tasmaniensis CBS 111687
M. tasmaniensis CBS 114556
Passalora
M. grandis CMW 8554
M. parva CBS 116289
M. grandis CMW 8557
M. parva CBS 110503
M. walkeri CMW 20333
M. walkeri CMW 20334
Sonderhenia
M. heimioides CBS 111190
M. heimioides CBS 111364
M. heimii CBS 110682
M. crystallina CBS 681.95
M. crystallina CMW 3042
M. irregulariramosa CBS 114774
M. irregulariramosa CMW 5223
M. colombiensis CBS 110969
M. colombiensis CBS 110967
Pseudocercospora
M. gracilis CBS 243.94
Ps. robusta CBS 111175
M. fori CMW 9095
M. fori CMW 9096
Ps. pseudoeucalyptorum CBS 114242
Ps. pseudoeucalyptorum CBS 114243
Ps. eucalyptorum CBS 110777
Ps. basitruncata CBS 111280
Ps. natalensis CBS 111070
Ps. basitruncata CBS 114664
Ps. paraguayensis CBS 111286
Ps. basiramifera CMW 5148
Pseudocercospora
Pass. eucalypti CBS 111306
Passalora
M. parkii CBS 387.92
M. madeirae CBS 112895
Stenella / Pseudocercospora
M. marksii CMW 5230
M. intermedia CBS 114415
M. marksii CBS 110920
M. marksii CBS 682.95
Ps. epispermogonia CBS 110750
Ps. epispermogonia CBS 110693
Pseudocercospora
Clade 2
M. gregaria CBS 110501
M. endophytica CMW 5225
M. endophytica CBS 111519
M. ellipsoidea CMW 4934
M. ellipsoidea CMW 5166
M. aurantia CBS 110500
M. africana CBS 116154
M. africana CBS 116155
M. keniensis CBS 111001
Pseudocercosporella / Uwebraunia
M. lateralis CBS 110748
M. lateralis CBS 111169
M. communis CBS 110976
M. communis CBS 114238
Pass. zambiae CBS 112971
Dissoconium
B. ribis CMW 7773
10 changes

3

Pseudocercospora flavomarginata sp. nov., from *Eucalyptus* leaves in Thailand

Abstract: *Mycosphaerella* represents one of the largest ascomycete genera accommodating more than 3000 names. Approximately 60 *Mycosphaerella* species have been linked to leaf diseases on *Eucalyptus* species, collectively known as Mycosphaerella Leaf Disease (MLD). Many hyphomycete and coelomycete anamorph genera are linked to *Mycosphaerella* and several species of the hyphomycete genus *Pseudocercospora* are associated with MLD symptoms on various *Eucalyptus* species. *Eucalyptus* trees in Vietnam and Thailand, particularly those of *E. camaldulensis* and hybrids of this species, commonly have a leaf spot disease caused by a species of *Pseudocercospora*. Lesions associated with this disease are very characteristic, with chlorotic margins and masses of brown conidiophores occurring predominantly on the abaxial lesion surface. The aim of this study was to characterise the *Pseudocercospora* species associated with this disease. This was achieved through studying the morphology of the fungus and via DNA sequence analysis from four nuclear gene regions. Results showed that the fungus represents an undescribed species of *Pseudocercospora*, that is formally described here as *Pseudocercospora flavomarginata*.

Published as: Hunter GC, Crous PW, Wingfield BD, Pongpanich K, Wingfield MJ (2006). *Pseudocercospora flavomarginata* sp. nov., from *Eucalyptus* leaves in Thailand. *Fungal Diversity* **22**: 71–90.

INTRODUCTION

Species of *Eucalyptus* are currently some of the most popular tree species for commercial forestry, with plantations expanding more rapidly than for any other tree species (Turnbull 2000). This is largely due to their favourable wood qualities, relatively rapid growth and their adaptability to a very wide range of different environments (Turnbull 2000). *Eucalyptus* spp. are thus propagated in commercially productive plantations in many tropical and sub-tropical countries, where timber is used primarily for the paper and pulp industry. In south-east Asia, the forestry industry is expanding rapidly, and it has been estimated that there are approximately 2 million ha of *Eucalyptus* plantations in the area (Old *et al*. 2003).

The genus *Mycosphaerella* Johanson is one of the largest genera of *Ascomycetes*, accommodating more than 3000 names (Corlett 1991, 1995, Crous 1998, Aptroot 2006). Approximately 60 *Mycosphaerella* species have been associated with diseases on *Eucalyptus* leaves, collectively known as Mycosphaerella Leaf Disease (MLD) (Crous 1998, Maxwell *et al*. 2003, Crous *et al*. 2004, 2006, Hunter *et al*. 2004, 2006). Symptoms of MLD include leaf spots, defoliation, twig cankers and in severe cases stunting of tree growth (Park & Keane 1982, Carnegie & Keane 1994, Crous 1998).

The teleomorph state, represented by abundant small pseudothecia, is most commonly associated with MLD on *Eucalyptus* leaves (Crous 1998). *Mycosphaerella* is, however, linked to approximately 23 anamorph genera including both coelomycetes and hyphomycetes (Crous *et al*. 2000, Crous & Braun 2003, Kirschner *et al*. 2004, Schubert & Braun 2005). Recent surveys of *Eucalyptus* foliage in many parts of the world have led to a significant increase in the number of *Mycosphaerella* spp. found on these trees (Crous *et al*. 2004, 2006).

The anamorphic genus *Pseudocercospora* Speg. is large and morphologically diverse (Crous *et al*. 2000). Species of *Pseudocercospora* occur on many plant hosts where they cause leaf spots (Crous & Braun 1996, Crous *et al*. 1997, Crous & Braun 2001, Braun & Dick 2002). These fungi typically have dematiaceous conidiophores and scolecosporous conidia with inconspicuous conidial scars and conidiogenous cells that proliferate sympodially and percurrently (Crous & Wingfield 1997, Crous *et al*. 2000, Crous & Braun 2003).

A very distinct leaf spot disease especially on *Eucalyptus camaldulensis* and hybrids of this and other species is well known in Thailand and Vietnam (Old *et al*. 2003). The symptoms of this disease are very obvious and not easily confused with other leaf spots. Based on the occurrence of abundant conidiophores that are found on the lesion, it is well recognised that the disease is caused by a species of *Pseudocercospora* (Old *et al*. 2003). The

fungus has, however, never been critically compared with other *Mycosphaerella* spp. occurring on *Eucalyptus*, nor has it been formally named. The aim of this study was, therefore, to characterise the fungus and to provide a name for it. This was achieved through critical study of its morphological characteristics by comparison to other *Pseudocercospora* spp. known to occur on *Eucalyptus* (Crous 1998, Braun & Dick 2002) and via comparisons of DNA sequences for the Large Subunit (LSU) and Internal Transcribed Spacer (ITS) region of the rRNA operon, the Actin (ACT) and Translation Elongation Factor 1-alpha (EF-1α) gene regions.

MATERIALS AND METHODS

Sample collection and fungal isolations

Leaf spots on *Eucalyptus camaldulensis* and hybrids of this species were collected from trees growing in plantations in various parts of Thailand. Diseased leaves showing the typical lesions with very distinct chlorotic margins and bearing conidiophores of a *Pseudocercospora* sp. were collected for subsequent laboratory study.

Leaf lesions were examined under a dissection microscope for the presence of *Pseudocercospora* conidiophores. A sterile inoculation needle was used to scrape conidia from the lesions and these were spread onto 2 % MEA agar plates (wt/v) (malt extract agar) (Biolab, South Africa) and incubated at 25 °C. Subsequently, single germinating conidia were lifted from the plates and transferred to fresh 2 % MEA agar plates. Agar plates were incubated at 25 °C in the dark for 21 d to allow for culture growth. Isolates of *Mycosphaerella* species occurring on *Eucalyptus* and known to have *Pseudocercospora* anamorphs were also included in this study for comparative purposes (Table 1). These isolates were all grown on 2 % MEA agar plates for approximately 1 mo to ensure sufficient mycelial growth. All cultures used during this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria, Pretoria, South Africa. Representative cultures have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1).

DNA extraction

Mycelia were scraped from actively growing cultures, freeze dried for 24 h, and then ground into a fine powder using liquid nitrogen. DNA was isolated according to the method of Hunter

et al. (2004). A 1 : 1 phenol : chloroform extraction was used. DNA was precipitated by the addition of absolute ethanol (98 % EtOH). Isolated DNA was cleaned by the addition of 70 % ethanol (70 % EtOH) and dried under vacuum. Sterile water was used to resuspend the isolated DNA. RNaseA (10 µg/µL) was added to the resuspended DNA and incubated at 37 °C for approximately 2 h to digest any residual RNA. Isolated DNA was visualised in a 1 % agarose gel (wt/v) (Roche Diagnostics, Germany) stained with ethidium bromide.

PCR amplification and purification

DNA (*ca*. 20 ng) isolated from the unknown *Pseudocercospora* sp. and representative *Mycosphaerella* isolates that were used for comparative purposes, was used as a template for amplification with the polymerase chain reaction (PCR). For the purposes of this study, four nuclear gene regions were chosen for amplification and subsequent DNA sequencing. These included the internal transcribed spacer (ITS) region and the large subunit (LSU) of the rRNA operon, a portion of the translation elongation factor 1-alpha gene (EF-1α) and a portion of the actin (ACT) gene regions.

All PCR reaction mixtures for the four gene regions were performed in a total volume of 25 µL containing 10 × PCR Buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP) (Roche Diagnostics, South Africa), 0.2 µM of forward and reverse primers (Inqaba Biotech, South Africa), 1.25 U Taq DNA Polymerase (Roche Diagnostics, South Africa) and DNA (20 ng/µL). Sterilised distilled water was added to achieve a final volume of 25 µL.

The ITS-1, ITS-2 and the 5.8 S gene regions of the internal transcribed spacer (ITS) region of the rRNA operon were amplified using primers ITS-1 (5`–TCC GTA GGT GAA CCT GCG G–3`) and LR-1 (5`–GGT TGG TTT CTT TTC CT–3`) (Vilgalys & Hester 1990, White *et al*. 1990). PCR reaction conditions for the ITS followed those of Crous *et al*. (2004) and Hunter *et al*. (2004). A portion of the large subunit (containing domain D1–D3) of the nuclear rRNA operon was amplified using primers LROR (5'–ACC CGC TGA ACT TAA GC–3') (Moncalvo *et al*. 1995) and LR7 (5'–TAC TAC CAC CAA GAT CT–3') (Vilgalys & Hester 1990). PCR reaction conditions were as follows: an initial denaturation temperature of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 62 °C for 30 s, primer extension at 72 °C for 1 min and a final elongation step at 72 °C for 7 min.

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A portion of the translation elongation factor 1-alpha gene (EF-1α) was amplified using the primers EF1-728F (5'–CAT CGA GAA GTT CGA GAA GG–3') and EF1-986R (5'–TAC TTG AAG GAA CCC TTA CC–3') (Carbone & Kohn 1999). Reaction conditions were as follows: an initial denaturation temperature of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s and primer extension at 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 7 min.

A portion of the actin (ACT) gene was amplified using the primers ACT-512F (5'–ATG TGC AAG GCC GGT TTC GC–3') and ACT-783R (5'–TAC GAG TCC TTC TGG CCC AT–3') (Carbone & Kohn 1999). PCR reaction conditions were as follows: an initial denaturation step at 96 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s and extension at 72 °C for 45 s. This was followed by 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s and elongation at 72 °C for 45 s with an increase of 5 s per cycle for elongation. The reaction was completed with a final elongation step at 72 °C for 7 min.

All PCR products were visualised in 1.5 % agarose gels (wt/v) stained with ethidium bromide, under ultra-violet light. Sizes of PCR amplicons were estimated by comparison against a 100 bp molecular weight marker (O' RangeRuler^{TM} 100bp DNA ladder) (Fermentas Life Sciences, U.S.A.). For further DNA sequencing, PCR products were purified through Centri-sep spin columns (Princeton separations, Adelphia, NJ) containing Sephadex G-50 (Sigma Aldrich, St. Louis, MO) as outlined by the manufacturer.

DNA sequencing and phylogenetic analysis

The purified PCR products were used as template DNA for cycle sequencing reactions using the ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) following the manufacturers instructions. The same primers as used for the PCR reactions were also used for sequencing reactions. However, additional internal primers were used for both the ITS and LSU regions. These were ITS-2 (5'–GCT GCG TTC TTC ATC GAT GC–3') and ITS-3 (5'–GCA TCG ATG AAG AAC GCA GC–3') (White *et al.* 1990) in the case of the ITS and LR3R (5'–GTC TTG AAA CAC GGA CC–3') and LR-16 (5'–TTC CAC CCA AAC ACT CG–3') in the case of LSU. The precipitated sequencing reactions were then run on an ABI PRISM^{TM} 3100 Automated DNA sequencer (Applied Biosystems, Foster City, CA).

All resulting sequences were analysed with Sequence Navigator v. 1.0.1 (Applied Biosystems, Foster City, CA). Sequence alignments were done using MAFFT v. 5.667 (Katoh *et al*. 2005) incorporating the E-INS-i alignment strategy. A partition homogeneity test (Farris *et al*. 1994), on all possible combinations, of 1000 replicates on all informative characters was conducted in PAUP v. 4.10b (Swofford 2002) to determine if the DNA datasets from the four gene regions were combinable.

For phylogenetic analyses, both parsimony and distance analyses were conducted. For parsimony analysis, most parsimonious trees were generated by heuristic searches with starting trees obtained through stepwise addition with the MULPAR function enabled. Tree bisection reconnection (TBR) was employed as the branch-swapping algorithm. All gaps were coded as missing data and characters were assigned equal weight. Statistic support for nodes was obtained by performing 1000 bootstrap replicates.

Modeltest v. 3.04 (Posada & Crandal 1998) was used to determine the most appropriate nucleotide substitution model to be applied to the combined DNA sequence alignment and GTR + I + G was chosen from the Akaike Information Criterion (AIC) (base frequencies: π_A = 0.2387, π_C = 0.2588, π_G = 0.2780, π_T = 0.2245; substitution rates: A/C = 1.4579, A/G = 1.8851, A/T = 1.7352, C/G = 1.2094, C/T = 4.2937, G/T = 1.0000; proportion of invariable sites (I) = 0.5562; gamma shape distribution parameter = 0.5825). Following this, a neighbour-joining (NJ) analysis using the GTR + I + G substitution model was conducted in PAUP. Here, identical sites were removed proportionally to base frequencies estimated from all sites, rates of invariable sites assumed to follow a gamma distribution and ties were broken if encountered. *Mycosphaerella lateralis* Crous & M.J. Wingf. (anamorph: *Dissoconium dekkeri* de Hoog & Hijwegen) was used as an outgroup to root all trees.

Morphological studies

Conidia and conidiophores of the undescribed *Pseudocercospora* sp. were mounted in lactic acid or bromophenol blue on microscope slides. Leaf tissue was mounted in Jung Tissue Freezing Medium (Leica Microsystems AG, Wetzlar, Germany) and sections (10 µm) were cut using a Leica CM 100 Freezing microtome (Leica Microsystems AG, Wetzlar, Germany). Both cross sections and structures taken from the surface of lesions were examined under a Zeiss Axioskop light microscope (Carl Zeiss, Jena, Germany) using differential interference contrast. Fifty measurements of all taxonomically relevant structures were made at × 1000 magnification. Morphological characteristics of the unidentified *Pseudocercospora* sp. from

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Thailand were compared with other *Pseudocercospora* spp. known to occur on *Eucalyptus* (Crous 1998, Braun & Dick 2002). Representative herbarium specimens of the Thailand *Pseudocercospora* sp. have been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Growth characteristics of the undescribed *Pseudocercospora* sp. on agar medium were also defined. Plugs (5 mm diam) of agar were cut from the actively growing margins of pure cultures of ex-type isolates and transferred to the centres of 90 mm 2 % MEA plates. Agar plates were incubated in the dark at temperatures between 0 °C and 35 °C at five degree intervals. Colony diameters were determined every 7 d for one mo. Three plates were used per isolate at each temperature, and the experiment was repeated once. Colony colours and morphologies were described after one mo using the using colour charts of Rayner (1970).

RESULTS

PCR Amplification and analysis of sequence data

Amplification of the LSU, ITS, EF-1α and ACT gene regions for all the isolates used in this study resulted in amplification products of approximately 600 bp for ITS, 300 bp for EF-1α, 250 bp for ACT and 1500 bp for LSU.

Results of the partition homogeneity test using 1000 replicates resulted in a P-value > 0.001 for all possible combinations of the LSU, ITS, EF-1α and ACT DNA sequence alignments. Therefore, DNA sequence alignments of the ITS, EF-1α, ACT and LSU gene regions were combined. For parsimony analysis, heuristic searches of the combined DNA sequence alignment resulted in the retention of six most parsimonious trees, one of which is shown here (Fig. 1). The tree had a length of 1417 steps (CI = 0.709, RI = 0.8961, HI = 0.291). A total of 2665 characters were evaluated of which 1998 were constant, 153 were parsimony uninformative and 514 were parsimony informative. Bootstrap searches of the combined dataset produced a tree with the same topology of the most parsimonious tree. Isolates of *Mycosphaerella* spp. and isolates of the unknown *Pseudocercospora* sp. could be resolved into two well-supported monophyletic clades (Clades I–II) with bootstrap values of 83 % and 96 % respectively (Fig. 1).

Clade I could be further resolved into four well-supported sub-clades (sub-clades 1–4) (Fig. 1). Clade 1 included isolates of *P. flavomarginata*, *P. paraguayensis* (Kobayashi) Crous and *P. basiramifera* Crous (Bootstrap = 100 %), Clade 2 contained isolates of

Mycosphaerella fori G.C. Hunter, Crous & M.J. Wingf., *M. gracilis* Crous & Alfenas, *P. eucalyptorum* Crous, M.J. Wingf., Marasas & B. Sutton, *P. pseudoeucalyptorum* Crous (Bootstrap = 98 %). Clade 3 contained isolates of *P. basitruncata* Crous and *P. natalensis* Crous & T. Coutinho (Bootstrap = 100 %) and clade 4 contained isolates of *M. colombiensis* Crous & M.J. Wingf., *M. crystallina* Crous & M.J. Wingf., *M. irregulariramosa* Crous & M.J. Wingf., *M. heimii* Crous and *M. heimioides* Crous & M.J. Wingf. (Bootstrap = 100 %).

For distance analysis, neighbour-joining analyses yielded a phylogenetic tree with the same topology as the most parsimonious trees generated by parsimony analyses (Fig. 2). Here isolates of *Mycosphaerella* and *Pseudocercospora* were resolved into two well-supported monophyletic clades containing the same isolates as those in the parsimony analysis (Fig. 1). Furthermore, for the distance analysis, isolates of *P. flavomarginata* grouped into a well-supported clade of their own, sister to *P. paraguayensis* and *P. basiramifera.*

Morphology

One of the most obvious distinguishing features of the *Pseudocercospora* sp. on *E. camaldulensis* found in Thailand and Vietnam is the symptoms associated with the taxon (Fig. 3). These are very distinct angular chlorotic spots that later become necrotic lesions on the leaves, typically surrounded by halos of chlorotic tissue. Lesions typically bear dense clusters of dark brown conidiophores on the abaxial surface but these are occasionally also found on the adaxial surfaces of lesions. Lesions can be very common, mostly on leaves on the lower branches, and susceptible *Eucalyptus* clones can be seriously defoliated.

Cultures of the *Pseudocercospora* sp. grew relatively rapidly on 2 % MEA. Results from growth comparisons at different temperatures showed that the optimal temperature for growth was 25 °C. At 25 °C, cultures had a growth rate of 29 mm in one mo on MEA. Minimum and maximum temperatures were between 5–10 °C and 30–35 °C, respectively. Cultures were pale olivaceous-grey on the surface, and greenish-black in reverse. Cultures exhibited irregular margins and produced profuse aerial mycelium while colony borders were generally darker (greenish-black) than the colony centre that tended to become paler as the culture aged.

Taxonomy

DNA sequence comparison of the *Pseudocercospora* sp. considered in the study has shown that the species is different from all other *Mycosphaerella* spp. considered during this study. The symptoms associated with this fungus are also very obvious and unique. In addition, the

morphology of the fungus is unlike that of any other *Pseudocercospora* sp. known on *Eucalyptus*. We therefore describe it as a new species as follows:

Pseudocercospora flavomarginata G.C. Hunter, Crous & M.J. Wingf., **sp. nov.** (Figs. 4, 5)
MycoBank No.: MB500513

Etymology: Named for the characteristic chlorotic borders surrounding the angular necrotic lesions on *Eucalyptus camaldulensis* leaves.

Conidiophorae dense fasciculatae, brunneae, apicem versus pallidiores, non ramosae, parietibus crassis, laeves, 0–4-septatae, subcylindricae, rectae ver curvatae, e cellulis stromatis bene evoluti exorientes, (18–)32–36(–53) × (2–)3–4(–5) µm. *Conidia* solitaria, recta vel subfalcata, pallide brunnea, laeves, parietibus crassis, guttulata, acicularia vel obclavata, apice obtusa, basi rotundata vel longe obconico-truncata, 2–7-septata, (28–)46–54(–90) × (2–)3(–4) µm. *Teleomorpha* ignota.

***Holotype*:** Thailand: Chachoengsao Province near Pratchin Buri: on leaves of *Eucalyptus camaldulensis* 2004, M.J. Wingfield (PREM 58952– holotypus; cultura viva ex-types CBS 118841, 118823, 118824).

Additional material examined (paratypes): Thailand, Chachoengsao Province near Pratchin Buri on leaves of *Eucalyptus camaldulensis*, 2004, M.J. Wingfield (CMW 17703, PREM 58953); Thailand, Chachoengsao Province near Pratchin Buri on leaves of *Eucalyptus camaldulensis*, 2004 M.J. Wingfield (CMW 17707, PREM 58954); Thailand, Chachoengsao Province near Pratchin Buri on leaves of *Eucalyptus camaldulensis*, 2004 M.J. Wingfield (CMW17708, PREM 58955).

Leaf spots appear as chlorotic spots, distinct, scattered over leaves, amphigenous, circular to angular, 3–20 mm diam (Fig. 3), pale to dark brown becoming necrotic and darker with age, definite chlorotic margin on abaxial and adaxial leaf surfaces but more obvious on adaxial leaf surfaces. *Mycelium* internal and external, pale brown, septate, branched, thick-walled, smooth to finely verruculose, 2–4 µm diam. *Caespituli* amphigenous, predominantly epiphyllous, evenly distributed over lesion, brown to black on leaves, 74 µm wide and 90 µm high (Figs. 4, 5). *Conidiophores* fasciculate, grouped in dense fascicles, conidiophores brown becoming paler towards apex, unbranched, thick-walled, smooth, 0–4-septate, subcylindrical, straight to curved, arising from cells of a well developed stroma (18–)32–36(–53) × (2–)3–4(–5) µm. *Stromata* well-developed, prominent, immersed becoming erumpent, brown, 56 µm wide and 47 µm high. *Conidiogenous cells* terminal, smooth, thick-walled, pale brown, unbranched, tapering to a rounded apex, proliferating sympodially or 1–2 times percurrently,

(6–)14–17(–25) × (2–)3(–5) µm. *Conidial scars*, unthickened and not darkened. *Conidia* solitary, straight or slightly curved, pale brown, smooth, thick-walled, guttulate, acicular to obclavate, apex obtuse, base rounded to long obconic-truncate, 2–7-septate, (28–)46–54(–90) × (2–)3(–4) µm. *Hilum* unthickened, not darkened (Figs. 4, 5). *Spermagonium* present in lesions, well developed, prominent, immersed becoming erumpent 64 µm wide and 57 µm high. Spermatia not observed. Teleomorph unknown.

Cultures: Cultures 29 mm diam on MEA after 1 mo at 25 °C in the dark. Colonies pale olivaceous-grey 21’’’’’b (surface) and greenish-black 33’’’’’i (reverse). Margins regular to irregular, aerial mycelium profuse. Border darker (greenish-black) than colony centers, which become paler with age. Colony not sectored and folding absent.

Cardinal temperatures: Minimum 5–10 °C, optimum 25 °C, maximum 30–35 °C.

Hosts: *Eucalyptus camaldulensis*, *E. camaldulensis* hybrids.

Distribution: Thailand, Vietnam.

Notes: *Pseudocercospora flavomarginata* is morphologically and phylogenetically similar to *Pseudocercospora paraguayensis*. However, *P. flavomarginata* can be distinguished from *P. paraguayensis* by the very distinct chlorotic borders around the typically irregular lesions. Conidiophores are longer and have more septa than those of *P. paraguayensis*. Furthermore, conidium development is sympodial and percurrent in *P. flavomarginata,* but exclusively sympodial in *P. paraguayensis*.

DISCUSSION

Pseudocercospora flavomarginata is a fungal pathogen of *E. camaldulensis* that is very well-known in Thailand and Vietnam where it causes leaf spots typically on the lower leaves of young *E. camaldulensis* trees (Old *et al*. 2003). It is thus unfortunate that it has not previously been formally named. Perhaps this is to some extent due to the fact that there are various *Pseudocercospora* spp. on *Eucalyptus* leaves and DNA sequence data have not previously been available to reinforce the fact that it represents a novel taxon.

Although *Pseudocercospora basiramifera* is known to occur in Thailand on *E. camaldulensis* and *E. pellita* (Crous 1998), it was not encountered during the present study. *Pseudocercospora flavomarginata* can be distinguished from *P. basiramifera* by the presence of red lesion margins in *P. basiramifera* as opposed to the very characteristic chlorotic margins found around lesions of *P. flavomarginata*. Furthermore, *P. flavomarginata* has

smaller conidia that are 2–7-septate and (28–)46–54(–90) × (2–)3(–4) µm in contrast to conidia of *P. basiramifera,* which are 3–10-septate and (35–)50–70(–80) × 2(–3) µm (Crous 1998).

Various *Pseudocercospora* spp. other than *P. flavomarginata* are known to occur in south-east Asia on *Eucalyptus*. These include *Pseudocercospora eucalyptorum*, *P. gracilis* Crous & Alfenas, and *P. heimioides* Crous & M.J. Wingf. (Crous *et al*. 1989, Crous & Alfenas 1995, Crous & Wingfield 1997). *Pseudocercospora flavomarginata* is common in Thailand and Vietnam and can be distinguished from *P. eucalyptorum*, *P. gracilis* and *P. heimioides* by characteristic leaf lesions with prominent yellow borders. This in contrast to pale to grey-brown lesions with purple borders produced by *P. eucalyptorum*, pale brown lesions with red to brown borders formed by *P. gracilis,* and brown to absent leaf spots of *P. heimioides* (Crous *et al*. 1989, Crous & Alfenas 1995, Crous & Wingfield 1997, Crous 1998).

Phylogenetically, *P. flavomarginata* is closely related to *P. paraguayensis*, which is known from eucalypts in South America (Crous 1998). *Pseudocercospora flavomarginata* can be distinguished from *P. paraguayensis* by the prominent chlorotic borders around lesions, conidiophores that are more numerously septate and longer than those of *P. paraguayensis*, conidia that are more numerously septate than those of *P. paraguayensis* and conidium development that is sympodial and percurrent, in contrast to the exclusively sympodial development in *P. paraguayensis*.

DNA sequence comparisons in this study resolved *Pseudocercospora* spp. into two well-supported monophyletic clades, supporting results of Crous *et al*. (2000), and the recent finding of Ayala-Escobar *et al*. (2006) that *Pseudocercospora* is paraphyletic within *Mycosphaerella*. While these groups are very clearly delineated, it is not possible to link them to any distinct morphological characteristics. However, larger sequence datasets that consider additional isolates of *Cercospora* spp. and specifically *Pseudocercospora* spp. might in future resolve specific lineages that could be linked to phylogenetically informative morphological characteristics.

Pseudocercospora crystallina Crous & M.J. Wingf., *P. irregulariramosa* Crous & M.J. Wingf., *P. heimii* Crous and *P. heimioides* are morphologically similar species that have been shown, based on ITS sequence data, to group in a single clade (Crous *et al*. 2000, 2001). These fungi are regarded as a species complex known as the *Mycosphaerella heimii* complex (Crous *et al*. 2000). Based on DNA sequence results from the four gene regions in this study, species within the *M. heimii* complex clustered together, supporting previous findings. Based on the distance of the *M. heimii* complex from its most recent ancestor, it is evident that the

divergence from this node is relatively recent. This suggests that speciation amongst members of the *M. heimii* complex has occurred recently.

Eucalyptus spp. are native to Australia, Papua New Guinea, Indonesia and the Philippines (Turnbull 2000). It is possible that *P. flavomarginata* may be present in Australia and other areas where *Eucalyptus* spp. are native. Further surveys of both natural and commercially propagated *Eucalyptus* spp. could result in collections of *P. flavomarginata* and increase the known host range of this species.

Eucalyptus camaldulensis is one of the species that is most commonly grown in south-east Asia. Considering that many commercially propagated eucalypts are clones or hybrids (Old *et al*. 2003), it will be important to consider the host distribution of *P. flavomarginata* in this area. It is also well-known that some clones can be particularly susceptible to pathogens while others, even those relatively closely related, can be resistant. Therefore, future surveys and pathogenicity studies would aid in selecting and propagating genotypes of *Eucalyptus* that are tolerant to pathogens such as *P. flavomarginata*.

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Table 1. Isolates of *Mycosphaerella* and *Pseudocercospora* used for DNA sequencing and phylogenetic analysis.

Isolate No.			Teleomorph (*Mycosphaerella*)	Anamorph	Origin	GenBank Accession No.			
CMW	**CPC**	**CBS**				**LSU**	**ITS**	**ACT**	**EF-1α**
4944	1106	110969	***M. colombiensis***	***P. colombiensis***	*E. urophylla*, Colombia	DQ204744	AY752149	DQ147639	DQ211660
11255	1104	110967	*M. colombiensis*	*P. colombiensis*	*E. urophylla*, Columbia	DQ204745	AY752147	DQ147640	DQ211661
3042	800	N/A	*M. crystallina*	*P. crystallina*	*E. bicostata*, South Africa	DQ204746	DQ267578	DQ147637	DQ211662
3033	802	681.95	*M. crystallina*	*P. crystallina*	*E. bicostata*, South Africa	DQ204747	AY490757	DQ147636	DQ211663
9095	N/A	N/A	*M. fori*	*Pseudocercospora* sp.	*E. grandis*, South Africa	DQ204748	AF468869	DQ147618	DQ211664
9096	N/A	N/A	*M. fori*	*Pseudocercospora* sp.	*E. grandis*, South Africa	DQ204749	DQ267581	DQ147619	DQ211665
14455	730	243.94	*M. gracilis*	***P. gracilis***	*E. urophylla*, Indonesia	DQ204750	DQ267582	DQ147616	DQ211666
4942	760	110682	*M. heimii*	*P. heimii*	*Eucalyptus* sp., Madagascar	DQ204751	AF309606	DQ147638	DQ211667
14776	N/A	111364	*M. heimioides*	*P. heimioides*	*Eucalyptus* sp., Indonesia	DQ204752	DQ267586	DQ147632	DQ211668
3046	1312	111190	*M. heimioides*	*P. heimioides*	*Eucalyptus* sp., Indonesia	DQ204753	AF309609	DQ147633	DQ211669
4943	1360	114774	***M. irregulariramosa***	***P. irregulariramosa***	*E. saligna*, South Africa	DQ204754	AF309607	DQ147634	DQ211670
5223	1362	N/A	*M. irregulariramosa*	*P. irregulariramosa*	*E. saligna*, South Africa	DQ204755	AF309608	DQ147635	DQ211671
14458	3745	112895	*M. madeirae*	*Pseudocercospora* sp.	*E. globulus*, Madeira	DQ204756	AY725553	DQ147641	DQ211672
14778	822	110750	***M. marksii***	***P. epispermogonia***	*E grandis*×*saligna*, South Africa	DQ204757	DQ267596	DQ147629	DQ211673
14786	823	110693	***M. marksii***	***P. epispermogonia***	*E grandis*×*saligna*, South Africa	DQ204758	DQ267597	DQ147628	DQ211674
14914	1202	114664	Unknown	*P. basitruncata*	*E. grandis*, Colombia	DQ204759	DQ267600	DQ147622	DQ211675
14785	1203	111280	Unknown	*P. basitruncata*	*E. grandis*, Colombia	DQ204760	DQ267601	DQ147621	DQ211676
5148	N/A	N/A	Unknown	*P. basiramifera*	*E. pellita*, Thailand	DQ204761	AF309595	DQ147607	DQ211677
5228	16	110777	Unknown	*P. eucalyptorum*	*E. nitens*, South Africa	DQ204762	AF309598	DQ147614	DQ211678
14784	1264	111070	Unknown	*P. natalensis*	*E. nitens*, South Africa	DQ204763	AF309594	DQ147623	DQ211679
14779	1459	111286	Unknown	*P. paraguayensis*	*E. nitens*, Brazil	DQ204764	DQ267602	DQ147606	DQ211680
14908	10390	114242	Unknown	*P. pseudoeucalyptorum*	*E. globulus*, Spain	DQ204765	AY725526	DQ147613	DQ211681
14911	10500	114243	Unknown	*P. pseudoeucalyptorum*	*E. nitens*, New Zealand	DQ204766	AY725527	DQ147615	DQ211682
5151	1269	111175	Unknown	*P. robusta*	*E. robusta*, Malaysia	DQ204767	AF309597	DQ147617	DQ211683
13586	N/A	118841	Unknown	*P. flavomarginata*	*E. camaldulensis*, Thailand	DQ153306	DQ155657	DQ166513	DQ156548

Isolate No.			Teleomorph (*Mycosphaerella*)	Anamorph	Origin	GenBank Accession No.			
CMW	**CPC**	**CBS**				**LSU**	**ITS**	**ACT**	**EF-1α**
13594	N/A	118824	Unknown	***P. flavomarginata***	***E. camaldulensis*****,** Thailand	DQ153308	DQ155658	DQ166514	DQ156549
13590	N/A	118823	Unknown	*P. flavomarginata*	*E. camaldulensis*, Thailand	DQ153307	DQ155659	DQ166515	DQ156550
14906	825	110748	***M. lateralis***	*Dissoconium dekkeri*	*E. grandis*×*saligna*, South Africa	DQ204768	AF173315	DQ147651	DQ211684

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

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

CPC: Working collection of Pedro Crous, formerly in STE-U, now housed at CBS. Isolate numbers from Crous (1998).

N/A: Not available.

Figure 1. Phylogram of *Mycosphaerella* and *Pseudocercospora* spp. occurring on *Eucalyptus* indicating the phylogenetic placement of *Pseudocercospora flavomarginata*. One of six most parsimonious trees generated from a heuristic search of combined DNA alignments of LSU, ITS, ACT and EF-1α data in PAUP v. 4.0b10 (Length = 1417, CI = 0.79, RI = 0.8961, HI = 0.291). Bootstrap values of 1000 replicates are indicated above branches. The tree was rooted to *Mycosphaerella lateralis*.

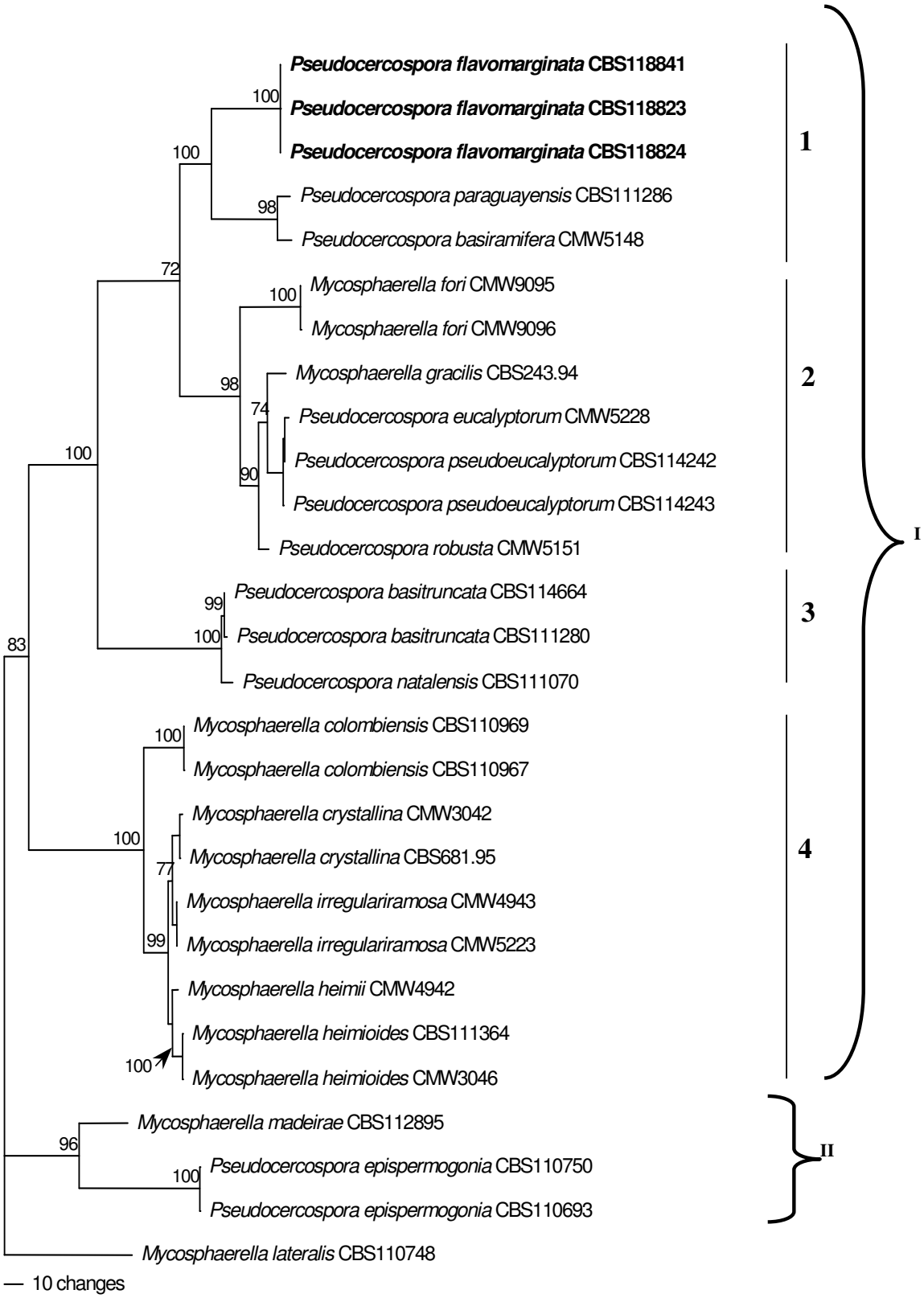
Pseudocercospora flavomarginata CBS118841
Pseudocercospora flavomarginata CBS118823
Pseudocercospora flavomarginata CBS118824
Pseudocercospora paraguayensis CBS111286
Pseudocercospora basiramifera CMW5148
Mycosphaerella fori CMW9095
Mycosphaerella fori CMW9096
Mycosphaerella gracilis CBS243.94
Pseudocercospora eucalyptorum CMW5228
Pseudocercospora pseudoeucalyptorum CBS114242
Pseudocercospora pseudoeucalyptorum CBS114243
Pseudocercospora robusta CMW5151
Pseudocercospora basitruncata CBS114664
Pseudocercospora basitruncata CBS111280
Pseudocercospora natalensis CBS111070
Mycosphaerella colombiensis CBS110969
Mycosphaerella colombiensis CBS110967
Mycosphaerella crystallina CMW3042
Mycosphaerella crystallina CBS681.95
Mycosphaerella irregulariramosa CMW4943
Mycosphaerella irregulariramosa CMW5223
Mycosphaerella heimii CMW4942
Mycosphaerella heimioides CBS111364
Mycosphaerella heimioides CMW3046
Mycosphaerella madeirae CBS112895
Pseudocercospora epispermogonia CBS110750
Pseudocercospora epispermogonia CBS110693
Mycosphaerella lateralis CBS110748
10 changes
1
2
3
4
I
II

Figure 2. Neighbour-joining (NJ) tree of *Mycosphaerella* and *Pseudocercospora* spp. obtained from distance analysis of combined LSU, ITS, ACT and EF-1α DNA sequence alignments using the GTR + I + G DNA substitution model. Bootstrap values after 1000 replicates are indicated above branches. The tree was rooted to *Mycosphaerella lateralis*.

NJ

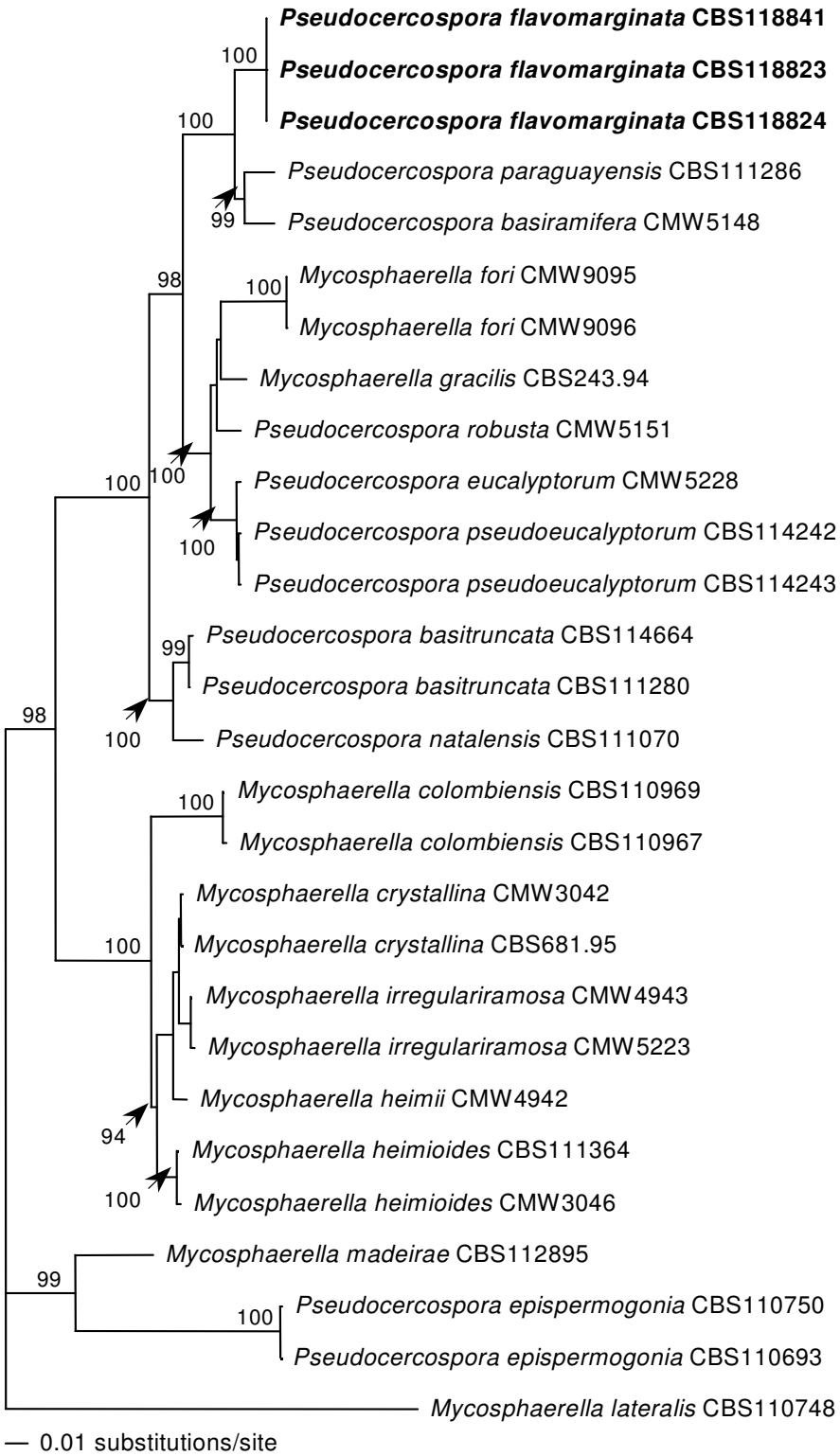
Pseudocercospora flavomarginata CBS118841
Pseudocercospora flavomarginata CBS118823
Pseudocercospora flavomarginata CBS118824
Pseudocercospora paraguayensis CBS111286
Pseudocercospora basiramifera CMW5148
Mycosphaerella fori CMW9095
Mycosphaerella fori CMW9096
Mycosphaerella gracilis CBS243.94
Pseudocercospora robusta CMW5151
Pseudocercospora eucalyptorum CMW5228
Pseudocercospora pseudoeucalyptorum CBS114242
Pseudocercospora pseudoeucalyptorum CBS114243
Pseudocercospora basitruncata CBS114664
Pseudocercospora basitruncata CBS111280
Pseudocercospora natalensis CBS111070
Mycosphaerella colombiensis CBS110969
Mycosphaerella colombiensis CBS110967
Mycosphaerella crystallina CMW3042
Mycosphaerella crystallina CBS681.95
Mycosphaerella irregulariramosa CMW4943
Mycosphaerella irregulariramosa CMW5223
Mycosphaerella heimii CMW4942
Mycosphaerella heimioides CBS111364
Mycosphaerella heimioides CMW3046
Mycosphaerella madeirae CBS112895
Pseudocercospora epispermogonia CBS110750
Pseudocercospora epispermogonia CBS110693
Mycosphaerella lateralis CBS110748
0.01 substitutions/site

Figure 3. Symptoms of *Pseudocercospora flavomarginata* on leaves of *Eucalyptus camaldulensis*. *Pseudocercospora flavomarginata* produces necrotic lesions surrounded by characteristic chlorotic tissue. Lesions bear clusters of dark brown conidiophores on the abaxial and adaxial lesion surfaces.

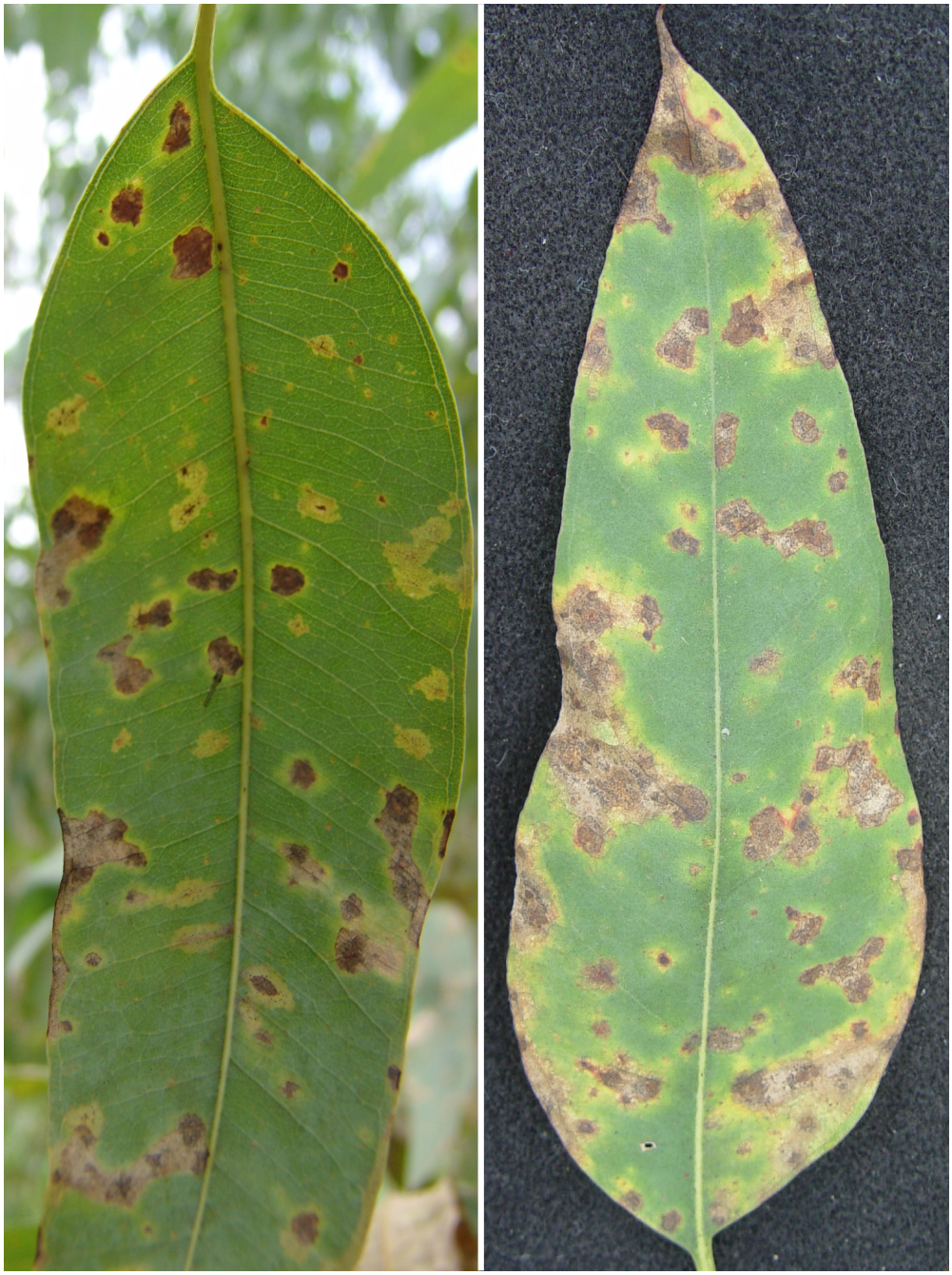

Figure 4. Morphological characteristics of *Pseudocercospora flavomarginata* (PREM 58952, MycoBank 500513). **A–D.** Straight or slightly curved, guttulate, acicular to obclavate conidia with obtuse apex and rounded to long obconic-truncate base. **E–F.** Fasciculate conidiophores that are unbranched, thick walled, subcylindrical, straight to curved. Bar = 10 µm.

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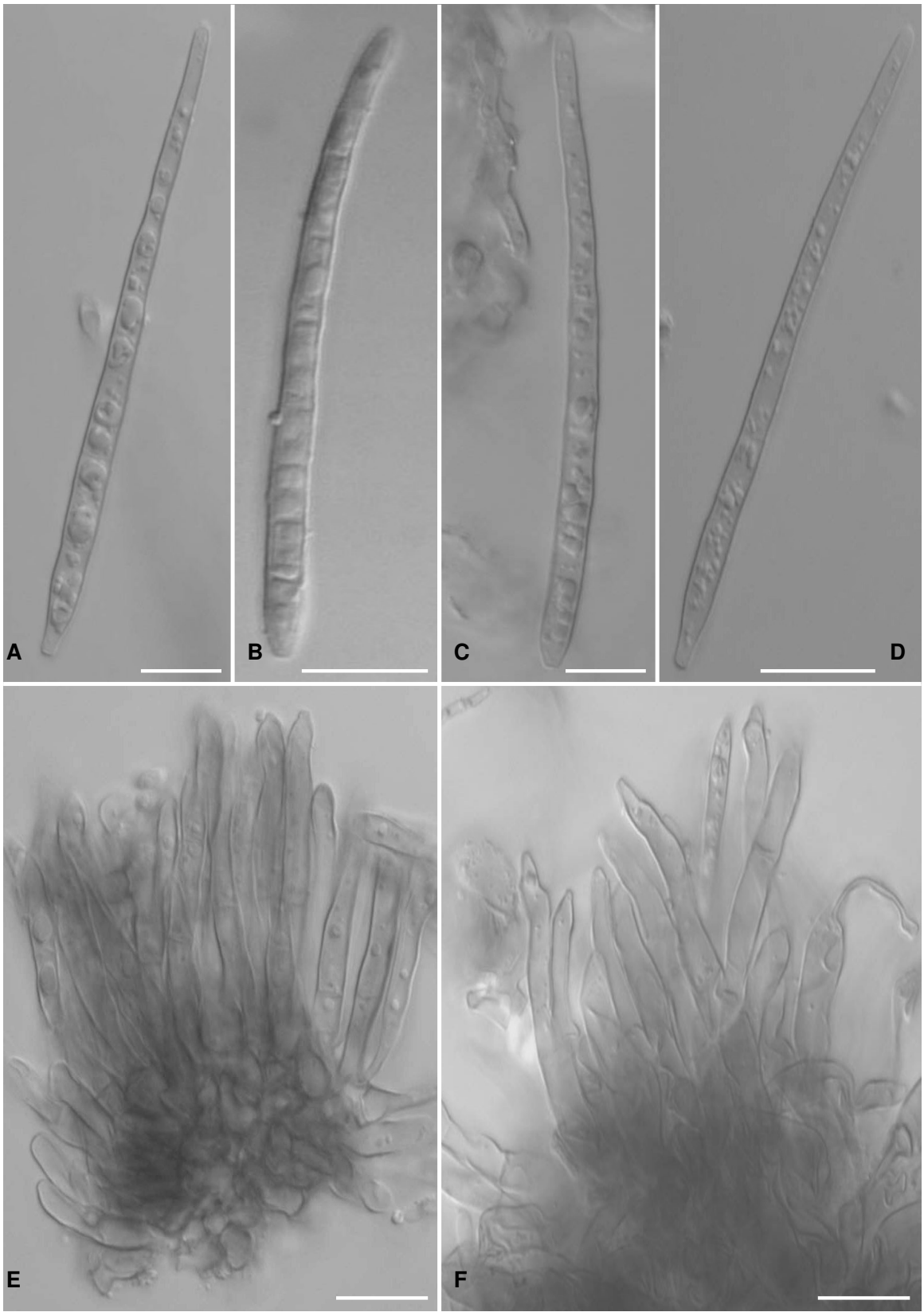
A
B
C
D
E
F

Figure 5. Line drawings of *Pseudocercospora flavomarginata*. **A.** Conidia. **B.** Well-developed, immersed young spermagonium with developed conidiophores that become lighter towards the apex. **C.** Septate, branched, thick walled, smooth, external mycelium. **D.** Fascicle of conidiophores. Bar = 10 µm.

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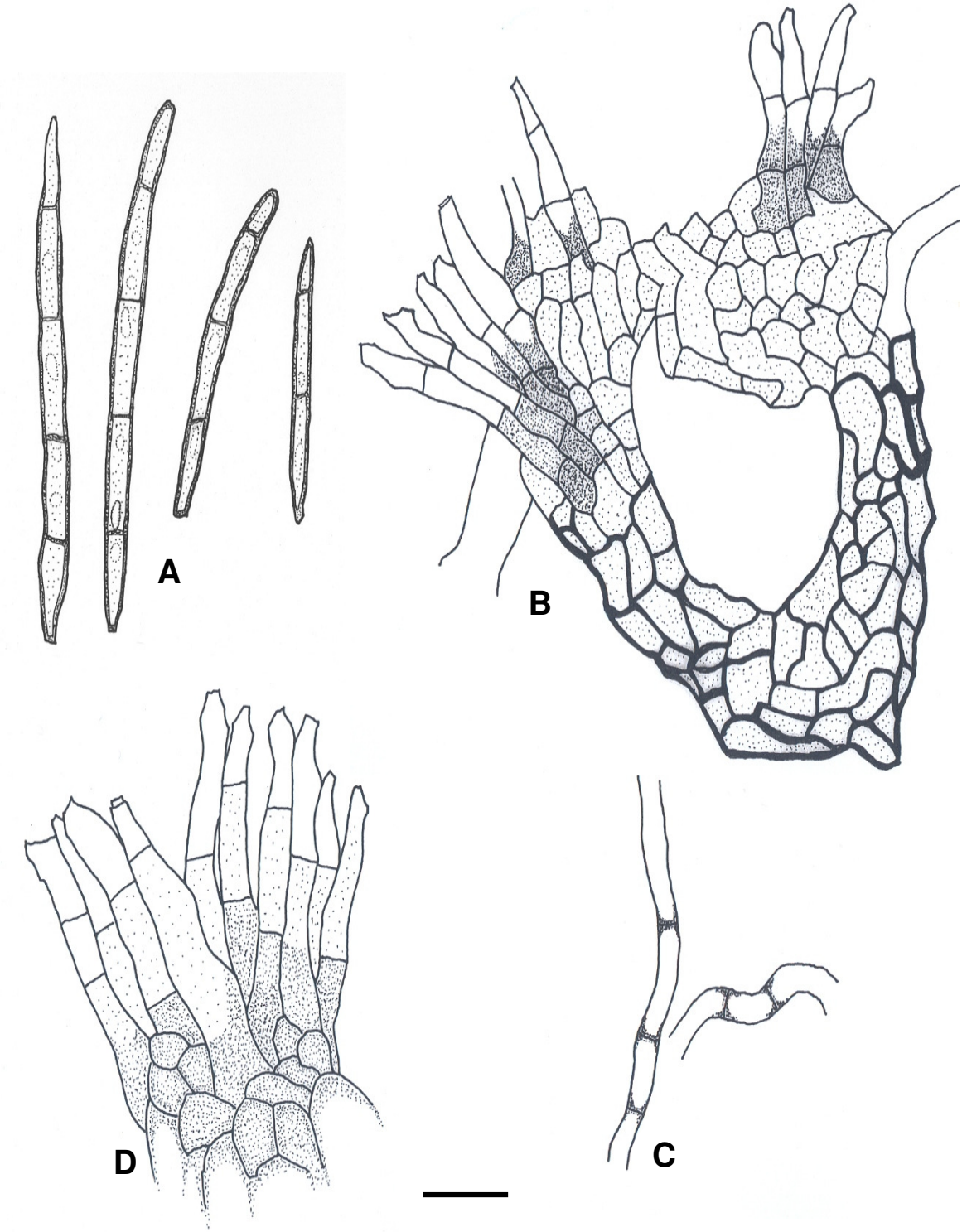
A
B
C
D

4

Development of polymorphic microsatellite markers for the *Eucalyptus* leaf pathogen *Mycosphaerella nubilosa*

Abstract: *Mycosphaerella nubilosa* is one of the most important *Eucalyptus* leaf pathogens, causing premature defoliation and stunting of growth. The aim of this study was to develop polymorphic microsatellite markers for *M. nubilosa*. Fifteen primer sets were developed and evaluated for polymorphism. Two primers were monomorphic, three primers did not amplify the desired region and 10 primer pairs were polymorphic. These microsatellite markers will be applied to population biology studies of *M. nubilosa* collections from several countries. These studies will promote an understanding of the genetics and the global movement of *M. nubilosa* that is severely limiting plantation development.

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INTRODUCTION

Species of the ascomycete fungal genus *Mycosphaerella* Johanson are amongst the most serious leaf pathogens of agricultural and forestry crops. More than 60 *Mycosphaerella* spp. are known from *Eucalyptus* spp. alone, and many of these result in serious diseases such as Mycosphaerella Leaf Disease (MLD) (Crous 1998, Crous *et al*. 2004, Hunter *et al*. 2004a). Infection results in leaf spots and twig cankers causing premature defoliation and stunting of tree growth (Crous 1998, Park *et al*. 2000).

Mycosphaerella nubilosa (Cooke) Hansf. is one of the most important *Mycosphaerella* spp. causing MLD in South Africa (Crous *et al*. 2004, Hunter *et al*. 2004b). *Eucalyptus nitens*, the most widely planted cold tolerant species of *Eucalyptus* in the country, is particularly susceptible to MLD. This is especially evident during the first two years of tree growth when juvenile leaves become severely infected resulting in defoliation and growth loss (Purnell & Lundquist 1986, Lundquist & Purnell 1987). Despite the importance of MLD, very little is known regarding the genetic structure, population dynamics or reproductive strategies of *M. nubilosa*. The aim of this study was, therefore, to develop polymorphic microsatellite markers for *M. nubilosa*.

Polymorphic microsatellite markers were developed using a single isolate of *M. nubilosa* (CBS 114708). This isolate was collected from *E. nitens* in the KwaZulu-Natal Province of South Africa during the course of a survey of *Mycosphaerella* spp. on *Eucalyptus* (Hunter *et al*. 2004b). DNA was collected from this isolate using the 1 : 1 phenol : chloroform isolation method as described by Hunter *et al*. (2004a, b). Isolates of *M. nubilosa* used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

The fast isolation by AFLP of sequences containing repeats (FIASCO) method for microsatellite isolation (Zane *et al*. 2002) with modifications (Cortinas *et al*. 2006) was used for microsatellite development. Briefly, DNA of *M. nubilosa* (1 µg) was digested with *Mse*I and ligated to an adaptor in the presence of a high concentration of ligase enzyme. This mixture was incubated overnight at 37 °C and the reaction was terminated by incubation at 65 °C for 20 min. A 1 : 10 dilution was prepared of the digestion-ligation mixture and 5 µL used for subsequent polymerase chain reaction (PCR) amplification following the methods of Zane *et al*. (2002). Following amplification, PCR products were hybridised to $(ATCC)_5$, $(GATA)_6$,

$(AG)_{10}$, $(GT)_{17}$, $(TC)_{15}$ and $(CA)_{15}$ biotinylated probes. DNA-probe complexes were subsequently isolated through magnetic bead capture. *Mycosphaerella nubilosa* DNA-containing repeats were cloned into the TOPO TA Cloning Kit (Invitrogen) following the manufacturer`s instructions and Nakabonge *et al*. (2005).

Bacterial clones were selected and diluted in 25 µL sterile water. This suspension was incubated at 96 °C for 7 min and 1 µL was removed for further colony PCR reactions. Colony PCR reactions were carried out in 50 µL reaction volumes containing 10 × PCR buffer, 1.5 mM $MgCl_2$, 300 mM each of TOPO M13 primers (5`–GTAAAACGACGGCCAG–3`/5`–CAGGAAACAGCTATGAC–3`), 5.0 µM dNTPs, 5.0 U Taq DNA polymerase (Roche Diagnostics, South Africa) and sterile distilled water to achieve a final volume of 50 µL (Cortinas *et al*. 2006). Colony PCR reactions included an initial denaturation at 96 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 53 °C for 30 s, elongation at 72 °C for 1 min and a final elongation to complete the reaction at 72 °C for 7 min. PCR products between 100 and 500 bp were subsequently selected and purified through Sephadex G-50 (Sigma Aldrich, St. Louis, MO) in Centri-sep spin columns (Princeton separations, Adelphia, NJ) following the manufacturer`s instructions. The purified PCR products were used as template DNA for cycle sequencing reactions using the ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) following the manufacturer`s instructions. The same primers used for the PCR reactions were also used for sequencing reactions. Precipitated PCR products were run on an ABI PRISM™ 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA) and evaluated for the presence of microsatellites. Following these protocols, a total of 126 clones were sequenced and 15 potential microsatellite regions were identified from the selected *M. nubilosa* isolate.

Primers for the 15 potential microsatellite regions were developed using the primer development software PRIMER 3 (Rozen & Skaletsky 2000) and used to test for polymorphism in nine isolates of *M. nubilosa* from various locations including Spain (CMW 12569, CMW 12568), Tanzania (CMW 18616, CMW 18617), Australia (CMW 18619, CMW 18618, CMW 18620, CMW 18621) and South Africa (CBS 114708). DNA from these *M. nubilosa* isolates was used as a template for PCR reactions in an Eppendorf Mastercycler Personal PCR machine (Eppendorf AG, Germany) with the designed primers. PCR reactions included an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C or 60 °C for 30 s, extension at 72 °C for 1 min and a final

extension step at 72 °C for 10 min. DNA was amplified in a 25 µL reaction volume containing PCR Buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 5.0 mM dNTP's 0.2 µM of each forward and reverse primer, 1.25 U Taq Polymerase (Roche Diagnostics, South Africa) and sterile water was added to achieve a final volume of 25 µL. Amplified DNA was visualised on 2 % agarose gels stained with ethidium bromide and viewed under ultra violet light. PCR products were purified using Sephadex G-50 (Sigma Aldrich, St. Louis, MO) in Centri-sep spin columns (Princeton separations, Adelphia, NJ). PCR products were sequenced as described earlier and evaluated for sequence polymorphism between the nine isolates of *M. nubilosa*. Following sequence evaluation using the 15 primer pairs, it was found that two primer pairs amplified a region monomorphic for all *M. nubilosa* isolates, three primer pairs did not amplify the desired region and 10 primer pairs were polymorphic for the *M. nubilosa* isolates.

Primer pairs that exhibited polymorphism were flourescently labelled (Applied Biosystems, South Africa) (Table 1) and used for further analysis on 18 *M. nubilosa* isolates (CMW 18616, CMW 18617, CBS 114708, CMW 12569, CMW 12568, CMW 12546, CMW 12562, CMW 12598, CMW 12600, CMW 12574, CMW 12551, CMW 12556, CMW 12557, CMW 12549, CMW 18619, CMW 18618, CMW 18620, CMW 18621). All forward primers were labelled with fluorescent dyes, except for locus MN-2 in which the reverse primer was flourescently labelled (Table 1). Allele sizes of amplified PCR products were determined by electrophoresis on an ABI PRISM™ 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA) and compared against a GeneScan 500-LIZ size standard (Applied Biosystems, Warrington, U.K.). Data analysis was conducted with GENESCAN and GENEMAPPER software (Applied Biosystems, Foster City, CA). A total of 32 alleles were obtained across the 10 loci for the 18 isolates of *M. nubilosa*. The most polymorphic locus was MN-8, which exhibited six alleles for the *M. nubilosa* isolates tested. Loci were tested for linkage disequilibrium using the program MULTILOCUS (Agapow & Burt 2001). From this test an observed $\bar{r}_s$ value of -0.02 (P = 0.728) was obtained, indicating that there is random association of alleles in the test population.

The polymorphic primer sets that were developed for *M. nubilosa* were also tested for cross amplification on six other *Mycosphaerella* spp. that are phylogenetically closely related to *M. nubilosa*. These were, *M. ohnowa* Crous & M.J. Wingf., *M. molleriana* (Thüm) Lindau, *M. vespa* Carnegie & Keane, *M. ambiphylla* A. Maxwell, *M. toledana* Crous & G. Bills and *M. cryptica* (Cooke) Hansf. PCR amplification with primers for locus MN-1 resulted in a

single band of the predicted size for *M. molleriana*, *M. vespa* and *M. ambiphylla* (data not shown). Amplification with all other polymorphic primer pairs resulted in multiple fragments or no amplification for the *Mycosphaerella* spp. tested.

The polymorphic microsatellite markers developed for *M. nubilosa* in this study will be applied to populations of this species from several countries. This will promote a better understanding of the genetic structure and the reproductive mechanisms of this important pathogen. This knowledge will contribute to improved breeding strategies and longer-term durability of resistance in trees chosen for plantation development.

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Table 1. Characteristics of 10 polymorphic microsatellite markers developed for *Mycosphaerella nubilosa* (MN). **H**: Gene Diversity (Nei 1973), calculated using MULTILOCUS (Agapow & Burt 2001).

Locus name	Fluorescent label	Primer Sequence (5`–3`)	Tm (°C)	No. of alleles	H	PCR product size (bp)	Core sequence	Individuals typed per locus	GenBank Accession no.
MN-1	NED	TCCTGAAATGAGTGCAGACG TCCTCATCCTCTGTGGAACC	60	2	0.20	257–271	$(AG)_{10}(TG)_{10}$	18	DQ096633
MN-2	6-FAM	CATTGCTTCGGCGGTTATAG ATGCACGAAGTCGTTGTTTG	60	3	0.20	182–266	$(ACT)_8$.59bp.$(AC)_{11}$	18	DQ096634
MN-3	VIC	GACTCAACCGTCGTCGAAAC CGAACTGAATCCGCTGTGTA	60	3	0.30	306–320	$(AC)_{13}$	18	DQ096635
MN-4	NED	TGTCACAAGACTTTGGATTGC CCACCACAATCTCCTCACAA	60	4	0.44	137–165	$(ATTGTGG)_{10}$	18	DQ096636
MN-7	6-FAM	CGCCTCACAGTTACACATGG CGAAAGGCTGAGGCTGAA	60	2	0.20	377–395	$(TGTA)_6$	18	DQ096637
MN-8	PET	TTCTATATACTATATTCTATTTAGG ATATACTATATCTAAAAGAGGTAG	53	6	0.51	202–322	$(CTCTCTATA)_{20}$	18	DQ096638
MN-9	NED	CGAATGGGCTATCAGAAACG ACAGGGCAAGGACCTCGTAT	60	4	0.38	211–221	$(CT)_{20}$	18	DQ096639
MN-10	PET	ACACCTCGAAATCGCTCATC TAGCTCTGTGCTGCCTTTGA	60	2	0.20	136–144	$(TC)_{11}$	18	DQ096640
MN-11	VIC	CTCACCAGTGCCGTCTAGGT GGAAATCCTGCCCTAACCTC	60	3	0.44	193–223	$(TTGGTG)_5$	18	DQ096641
MN-14	6-FAM	TCGACTACCGTAGGGGACTACT ATGCACGAAGTCGTTGTTTG	60	3	0.20	100–112	$(AC)_{13}$	18	DQ096642

5

Global movement and population biology of the *Eucalyptus* leaf pathogen *Mycosphaerella nubilosa*

Abstract: Approximately 80 species of the ascomycete genus *Mycosphaerella* are responsible for leaf diseases on *Eucalyptus*. One of these species, *M. nubilosa*, is highly pathogenic to cold-tolerant *Eucalyptus* spp. which are commonly propagated for commercial forestry operations. Infection by *M. nubilosa* causes a disease known as Mycosphaerella Leaf Blotch (MLB) causing premature leaf abscission and in severe cases, stunting of tree growth. Although the taxonomy of *M. nubilosa* has been treated relatively closely, little is known regarding its population biology. Using 10 polymorphic DNA-based microsatellite markers, the genetic diversity of *M. nubilosa* populations from several different countries was considered. Results of these studies show that *M. nubilosa* from eastern Australia (New South Wales) has a higher gene and genotypic diversity than all introduced populations, supporting the view that this represents the origin of the pathogen. It was also evident that *M. nubilosa* populations from Europe and Tanzania are clonal, with the same multilocus haplotypes occurring in South Africa, but being absent in Australia. This suggests that *M. nubilosa* may have been introduced into Europe via Africa. Based on these results, we propose a pathway of gene flow of *M. nubilosa* from Australia to South Africa, into Africa and finally to Europe.

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INTRODUCTION

The ascomycete fungal genus *Mycosphaerella* Johanson includes more than 80 species of fungi that have been associated with leaf diseases of *Eucalyptus* spp. (Crous 1998, Crous *et al*. 2004, 2006). Infection by *Mycosphaerella* spp. can result in a decreased capacity of young *Eucalyptus* leaves to undergo photosynthesis, resulting in premature leaf defoliation, leading to reduced growth of trees (Park & Keane 1982b, Lundquist & Purnell 1987, Carnegie & Ades 2003, Milgate *et al*. 2005a, Pinkard & Mohammed 2006). Recognition that there can be a large number of *Mycosphaerella* spp. known from *Eucalyptus* leaves has led to the understanding that *Eucalyptus* trees are susceptible to different *Mycosphaerella* spp. throughout their growth phase (Carnegie & Ades 2005).

Mycosphaerella nubilosa (Cooke) Hansf. is one of the most important *Mycosphaerella* spp. on *Eucalyptus* and it causes the disease commonly referred to as Mycosphaerella Leaf Blotch (MLB) particularly on cold-tolerant *Eucalyptus* spp. such as *E. globulus* and *E. nitens* (Park & Keane 1987, Carnegie *et al*. 1998, Carnegie & Ades 2002, Hunter *et al*. 2004a). This fungus typically infects juvenile leaves and susceptible trees can be severely defoliated (Park & Keane 1982b, Lundquist & Purnell 1987, Milgate *et al*. 2005a). In severe cases, trees do not enter the adult leaf stage and plantation establishment can fail (Lundquist & Purnell 1987).

Mycosphaerella nubilosa was first described from *Eucalyptus* leaves in Victoria, Australia (Cooke 1891). Subsequent to its description, the fungus has been recognized as a severe *Eucalyptus* leaf pathogen in Tasmania, Victoria, New South Wales, South and Western Australia (Hansford 1956, Park & Keane 1982 a, b, Park 1988, Maxwell *et al*. 2001, Milgate *et al*. 2001, 2005a). Furthermore, *M. nubilosa* is also known from the North Island of New Zealand (Dick 1982). In Africa, *M. nubilosa* has been identified from several countries including Ethiopia, Kenya, South Africa, Tanzania and Zambia (Crous *et al*. 2004, 2006, Hunter *et al*. 2004a, Alemu *et al*. 2006). Likewise, *M. nubilosa* has been accidentally introduced into commercial plantations of *E. globulus*, where it has caused serious damage in Spain and Portugal (Crous *et al*. 2004).

Mycosphaerella nubilosa infects several species of *Eucalyptus*, including *E. bridgesiana*, *E. cypellocarpa*, *E. grandis*, *E. globulus*, *E. nitens*, *E. quadrangulata* (Dick 1982, Crous 1998, Crous *et al*. 2004, Hunter *et al*. 2004 a, b, Milgate *et al*. 2005a). Due to their rapid growth, frost tolerance and favourable wood qualities, *E. nitens* and *E. globulus* are amongst the most popular *Eucalyptus* spp. propagated for commercial forestry in countries

such as Australia, Spain, Portugal and several African countries. These *Eucalyptus* spp. are particularly susceptible to *M. nubilosa*. Thus, MLB caused by *M. nubilosa* can be a serious impediment to the sustainability of *E. nitens* and *E. globulus* plantations.

Mycosphaerella nubilosa is a haploid ascomycete that in nature has been seen only in its sexual state. This is represented by many small black pseudothecia, containing eight-spored asci, that are amphigenous but predominantly hypophyllous on lesion surfaces (Park & Keane 1982a, Crous 1998, Crous *et al*. 2004). Upon sufficient wetting, ascospores are released from pseudothecia and act as primary inoculum that is predominantly wind-dispersed (Park & Keane 1982b, Park 1988). Ascospores germinate on *Eucalyptus* leaves where they infect via stomata and after successive developmental stages result in the formation of mature ascocarps on diseased leaf tissue (Park & Keane 1982b). Although the anamorph state *Uwebraunia juvenis* Crous & M.J. Wingf. has been linked to *M. nubilosa*, this connection has not been reconfirmed (Crous *et al*. 2004) and remains doubtful. Recognising the presence of an anamorph state is important because it represents a source of secondary inoculum in nature, responsible for short distance dispersal and infections of the same plant. This has for example been shown for *Septoria tritici* Desm. (teleomorph: *Mycosphaerella graminicola* (Fuckel) J. Schröt.), where conidia are splash-dispersed resulting in short distance dispersal and infection within the same plant or neighbouring plants (McDonald & Martinez 1990, Boeger *et al*. 1993, Linde *et al*. 2002).

The only research conducted on the population biology of *Mycosphaerella* spp. occurring on *Eucalyptus* has been by Milgate *et al.* (2005b), who considered the important pathogen *Mycosphaerella cryptica* (Cooke) Hansf. In contrast, extensive research has been conducted on other pathosystems involving *Mycosphaerella* spp. and their anamorphs on a wide range of important agronomic crops. These include *M. graminicola* (anamorph: *S. tritici*) that causes leaf blotch disease on wheat as well as *Mycosphaerella fijiensis* M. Morelet and *Mycosphaerella musicola* R. Leach ex Mulder, causing leaf diseases of banana (Carlier *et al*. 1996, Linde *et al*. 2002, Hayden *et al*. 2003a, b). These population biology studies have promoted an increased knowledge of population structure, distribution of genetic diversity, gene flow, centres of origin, reproductive modes and the contribution of mating strategy to population structure (Carlier *et al*. 1996, Linde *et al*. 2002, Hayden *et al*. 2003a, b, Zhan *et al.* 2003).

Most research conducted on *M. nubilosa* has focussed on its epidemiology, host susceptibility, taxonomy and phylogenetic placement with respect to other species of *Mycosphaerella*. However, little is known regarding the population biology of this important

Eucalyptus leaf pathogen. The aim of this study was, therefore, to investigate the population biology of *M. nubilosa* from several countries by employing allele size data for 10 polymorphic microsatellite loci. There were four primary objectives in this study that included a consideration of the genetic diversity of *M. nubilosa*, examination of variation in genetic diversity between different countries, determination of the likely origin and global movement of *M. nubilosa* and to determine the likely mating strategy of the pathogen.

MATERIALS AND METHODS

Isolation and isolates

Diseased *Eucalyptus* leaves infected with *M. nubilosa* were collected from plantations in five countries on three continents including, Australia, Portugal, South Africa, Spain and Tanzania (Table 1). A hierarchical sampling strategy was followed for South Africa where one diseased tree at the centre of a plantation was selected for sampling and many diseased leaves were collected from this single tree (–1T). One diseased leaf was then taken from 40–60 trees randomly sampled throughout the plantation around the central tree, extending outwards (–MT). In all other locations, only the second level of hierarchy was sampled, where individual leaves were taken from individual trees in a single plantation (–MT). Furthermore, a third level of hierarchy was incorporated where one lesion from one leaf off one tree was sampled and several isolates were collected from this lesion (–1L).

Two populations of *M. nubilosa* were collected in Australia. From eastern Australia, one leaf was randomly collected from 40 different *E. globulus* trees in one plantation near Bonalbo, north-eastern New South Wales (EA-MT). Similarly, in Western Australia one leaf was randomly collected from 32 different *E. globulus* trees in several different plantations in the south of the state. For the purposes of this study, the samples collected from Western Australia were grouped together into one population and analysed as at the plantation level of hierarchy (WA-MT). One plantation of *E. globulus* was sampled in northern Spain and one leaf was selected from 55 different trees (S-MT). Likewise, one plantation of *E. globulus* was sampled in central Portugal where 42 leaves were taken from an equal number of different *E. globulus* trees (P-MT).

Isolates of *M. nubilosa* were collected from two countries in Africa, namely South Africa and Tanzania. In Njombe, Tanzania, 56 different *E. globulus* trees showing MLB symptoms were randomly sampled, and one leaf was collected from each tree (T-MT). In

South Africa, three plantations of *E. nitens* were sampled in the Mpumalanga Province, namely the Issabelladale, Wynton and Rooihoogte plantations. From the Rooihoogte plantation, 48 leaves displaying spots were randomly collected from a single tree in the centre of the plantation (R-1T). At this plantation, a single lesion from a single leaf was sampled and 32 isolates were collected from this (R-1L). In addition, a single leaf was randomly collected from 56 different trees within the Roooihoogte plantation (R-MT). From the Wynton plantation, 64 leaves exhibiting MLB symptoms were collected from a single tree at the centre of the plantation (W-1T). Furthermore, 64 trees showing MLB symptoms in this plantation were randomly selected and one diseased leaf was taken from each tree (W-MT). From the Issabelladale plantation, 40 leaves showing MLB lesions were randomly collected from one tree at the centre of the plantation (I-1T). In addition, 60 trees within the same plantation were randomly selected and one leaf was collected from each of these trees (I-MT).

Isolation, culture growth and DNA extraction

Isolations of *Mycosphaerella nubilosa* from diseased *Eucalyptus* leaves followed the protocol of Crous (1998). One lesion containing pseudothecia of *M. nubilosa* was excised from each *Eucalyptus* leaf collected from the various locations and placed in water for approximately 2 h. Lesions were then dried and attached to the undersides of Petri dish lids with adhesive tape with pseudothecia facing downward over 2 % malt extract agar (MEA) (Biolab, South Africa). Petri dishes, containing lesions, were incubated in the dark at room temperature for 24 h to allow for ascospore discharge from pseudothecia and germination on agar medium. After 24 h, Petri dishes were evaluated for the presence of germinating ascospores. One ascospore per lesion, exhibiting the type F ascospore germination pattern (Crous 1998, Crous *et al*. 2004), was cut from the agar surface and transferred to fresh 2 % MEA and incubated at 25 °C for approximately 2–3 mo to ensure sufficient mycelial growth. All isolates of *M. nubilosa* used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Mycelium from actively growing single-spore cultures of *M. nubilosa* was scraped from the surface of cultures, freeze dried for 24 h and ground to a fine powder using liquid nitrogen. DNA was isolated using the phenol : chloroform (1 : 1) extraction protocol as described in Hunter *et al*. (2004a, b). DNA was precipitated by the addition of absolute ethanol (98 % EtOH). Isolated DNA was cleaned by washing with 70 % ethanol and dried under vacuum. Triple distilled water was used to resuspend the isolated DNA. RNaseA (10 µg/µL) was added to the resuspended DNA and incubated at 37 °C for approximately 2 h to

digest any residual RNA. Isolated DNA was separated by electrophoresis in 1 % agarose gels (wt/v) (Roche Diagnostics, Mannheim), stained with ethidium bromide and visualised under ultra-violet light.

PCR amplification and allele size determination

DNA from isolates of *M. nubilosa* served as template DNA for polymerase chain reactions (PCR) in an Eppendorf Mastercycler PCR machine (Eppendorf AG, Germany). Flourescently labelled primers used for PCR reactions were the 10 polymorphic microsatellite primers developed by Hunter *et al*. (2006). The PCR reaction mixture and conditions were the same as those described previously (Hunter *et al*. 2006). Amplified DNA was visualised in 2 % agarose gels stained with ethidium bromide and viewed under ultra violet light. Sizes of PCR amplicons were estimated by comparison against a 100 bp molecular weight marker (O' RangeRuler^{TM} 100bp DNA ladder) (Fermentas Life Sciences, U.S.A.). PCR products were purified using Sephadex G-50 (Sigma Aldrich, St. Louis, MO) in Centri-sep spin columns (Princeton separations, Adelphia, NJ) as outlined by the manufacturer.

Allele sizes of amplified PCR products were determined by electrophoresis on an ABI PRISM^{TM} 3100 Automated DNA sequencer (Applied Biosystems, Foster City, CA) and compared against a GENESCAN –500 LIZ (Applied Biosystems, Warrington, U.K.) internal size standard. Allele sizes were analysed with GENESCAN and GENEMAPPER software (Applied Biosystems, Foster City, CA). Allele size data from GENESCAN and GENEMAPPER were collected in spreadsheets and the number of alleles per locus and the total number of multilocus haplotypes (MLH's) (hereafter referred to as haplotypes) across all the *M. nubilosa* populations were determined.

Gene and genotypic diversity

The frequency of alleles at each locus was calculated for every *M. nubilosa* population. Allele diversity was also determined using the software program POPGENE (Yeh *et al*. 1999) and the gene diversity (*H*) of Nei (1973) was calculated at each locus and also averaged over all loci ($H = 1 - \sum x_k^2$), where x_k is the frequency of the k^{th} allele. Chi square tests for differences in allele frequencies between the *M. nubilosa* populations were also calculated from clone corrected datasets (Workman & Niswander 1970).

Every *M. nubilosa* isolate was assigned a haplotype code according to the observed allele sizes across the 10 microsatellite loci. Genotypic diversity (*G*) was calculated using the

equation of Stoddard & Taylor (1988) ($G = 1 \Big/ \sum_{x=0}^{N} \left[f_X \left(X/N \right)^2 \right]$), where N is the sample size and f_x is the observed frequency of the x^{th} genotype. To compare genotypic diversities between the various *M. nubilosa* populations, the maximum percentage of genotypic diversity ($\hat{G}$) was calculated ($\hat{G} = G/N \times 100$), where G is the observed genotypic diversity and N is the sample size (Chen *et al.* 1994). Genotypic diversity values were also compared between *M. nubilosa* populations at the plantation level for significant differences using a *t*-test (Chen *et al*. 1994).

Population differentiation and gene flow

Clone corrected populations of *M. nubilosa* were used to calculate population differentiation (θ) between the South African and Australian *M. nubilosa* populations using the computer software program Multilocus (Agapow & Burt 2001), using 1000 randomised datasets. The population differentiation value (θ), which is Weir's formulation of Wrights' $F_{ST,}$ was calculated ($Q = \Sigma Q_2 \text{-} \Sigma Q_3 / \Sigma \left(1\text{-}Q_3 \right)$), where Q_2 is the probability that two alleles from the same population are the same and Q_3 is the probability that two alleles from differing populations are the same and for multiple loci Q_2 andQ_3 are summed across the evaluated loci (Weir 1996, Agapow & Burt 2001). Furthermore, gene flow (M) was calculated from theta (θ) using the equation of Cockerham & Weir (1993) ($M = \left[\left(1/\theta \right) - 1 \right] / 2$).

Mode of reproduction

Multilocus linkage disequilibrium between the 10 microsatellite loci was tested for each South African *M. nubilosa* population at the plantation level separately (R-MT, I-MT, W-MT) and the two Australian (WA-MT, EA-MT) populations and also the combined South African *M. nubilosa* populations (R-MT + I-MT + W-MT) at the plantation level by determining the Index of Association (I_A) using the program Multilocus (Maynard Smith *et al*. 1993, Agapow & Burt 2001). The I_A value was determined for both clone corrected and uncorrected datasets. In Multilocus, the expected I_A data for 1000 randomisations were calculated and compared to the observed I_A value. The null hypothesis for the Index of Association test is random association of alleles indicating sexual reproduction and heterothallism. For this to be true the observed I_A value will fall within the distribution of the randomised values. However, if the I_A value falls outside of the distribution of randomised values, it is an indication of non-random association of alleles and homothallism.

RESULTS

Isolates

A total of 497 isolates of *M. nubilosa* were collected from diseased *Eucalyptus* leaves for this study (Table 1). These represented a wide range of geographic locations and also the different hierarchical levels. From Europe, a total of 97 *M. nubilosa* isolates were collected and these included 55 isolates from Spain and 42 isolates from Portugal. Fifty-six isolates were retrieved from the Tanzanian collection and 293 isolates from the three *E. nitens* plantations in South Africa. Nineteen isolates were from New South Wales in eastern Australia and 32 isolates were from Western Australia.

PCR amplification and allele size determination

A total of 66 different alleles were observed across all 10 loci for all the *M. nubilosa* populations (Table 2) with an average of 6.6 alleles per locus. The Australian *M. nubilosa* populations, WA-MT and EA-MT, exhibited the greatest number of alleles across the 10 loci, namely 22 and 44 respectively. Many unique alleles were present in the eastern Australian population (EA-MT), with 32 unique alleles for this population across all 10 loci. The Western Australian population (WA-MT) had seven unique alleles within loci MN-2, MN-7, MN-8 and MN-11.

South African *M. nubilosa* populations exhibited much lower numbers of alleles across the 10 loci than those observed from Australia. In total, 25 different alleles were observed across all of the South African *M. nubilosa* populations at the various hierarchical levels and the majority of these alleles were shared between the South African *M. nubilosa* populations (Table 2). South African populations R-MT and I-1T exhibited the most alleles for any of the South African populations with 18 alleles across the 10 loci. Allele numbers were similar for all of the South African *M. nubilosa* populations at the single tree or plantation hierarchical level and ranged from 16 alleles (I-MT, W-1T), 17 alleles (R-1T, W-MT) and 18 alleles (R-MT, I-1T) (Table 2). Interestingly, one *M. nubilosa* population (R-1L) collected from a single lesion on an *E. nitens* leaf from the Rooihoogte plantation in South Africa yielded a total of 13 different alleles for the 10 microsatellite loci across 32 *M. nubilosa* isolates (Table 2). Only three alleles were unique for the South African populations, namely allele 209 (locus MN-8) for population R-MT, allele 340 (locus MN-8) for population

W-1T and allele 192 (locus MN-11) for population R-1T (Table 2). For the Spain, Portugal and Tanzanian *M. nubilosa* populations, all loci were monomorphic.

When considering all the *M. nubilosa* populations together, all of the 10 microsatellite loci were polymorphic. However, among the South African *M. nubilosa* populations, loci MN-1, MN-2, MN-7 and MN-14 were monomorphic, thus only 60 % of the loci were polymorphic for the South African populations. In contrast, 60 % of the loci were polymorphic for the Western Australian (WA-MT) population and 80 % were polymorphic for the New South Wales (EA-MT) population.

Gene and genotypic diversity

The mean gene diversity (*H*) values across all loci were moderate to low across all of the *M. nubilosa* populations (Table 2). The South African *M. nubilosa* populations had a relatively low gene diversity, ranging from 0.149–0.250. The lowest gene diversity observed for South Africa was for population R-1L that had a gene diversity of 0.149, while the highest level of gene diversity observed in South Africa was for the W-MT population with a value of 0.250. The gene diversity for the Western Australia population (WA-MT) was comparable to those observed for the South African *M. nubilosa* populations with a value of 0.242. In contrast, a substantially higher gene diversity value of 0.506 was observed for the New South Wales population (EA-MT).

A total of 68 different haplotypes were observed across all *M. nubilosa* populations from the various locations considered. Almost half (49 %) of the haplotypes were unique and were detected only once. The South African populations had a total of 35 different haplotypes. The majority of these haplotypes were distributed among the various South African *M. nubilosa* populations. However, seven haplotypes were unique to certain South African populations namely, MNG-38 and MNG-39 were unique to population R-1T, MNG-22 and MNG-23 were unique to population I-1T, MNG-16 was unique to I-MT, MNG-36 was unique to population W-1T and MNG-29 was unique to population W-MT. Therefore, 9.7 % of the haplotypes that were observed for the South African populations were unique. Haplotype MNG-2 was observed a total of 48 times across the South African *M. nubilosa* populations and occurred in all South African populations except population R-1L. None of the haplotypes that were observed for the South African populations occurred in either the Western Australian population (WA-MT) or the New South Wales population (EA-MT). However, the Spanish and Portuguese *M. nubilosa* populations both had only one haplotype, namely MNG-1, that was also observed in the R-MT, W-1T and W-MT *M. nubilosa*

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populations of South Africa. Furthermore, the Tanzanian *M. nubilosa* population also had only one haplotype, namely MNG-2, that was also observed in the R-1T, R-MT, I-1T, I-MT, W-1T and W-MT South African *M. nubilosa* populations.

A total of 26 different haplotypes were observed across the two Australian *M. nubilosa* populations (WA-MT and EA-MT). Population WA-MT had 10 haplotypes that were unique to this population and did not occur in the EA-MT population. The most frequently observed haplotype from the WA-MT population was MNG-59 that occurred 10 times in this population. The EA-MT population had a total of 16 different haplotypes that were also unique to this population. The haplotypes of the EA-MT population either occurred only once, while only three haplotypes occurred twice namely, MNG-43, MNG-44 and MNG-45.

Genotypic diversity (G) varied between the various populations of *M. nubilosa* from the various locations (Table 3). The lowest maximum percentage of genotypic diversity ($\hat{G}$), 7.1 %, was observed for the South African *M. nubilosa* population from one lesion (R-1L). However, maximum percentage of genotypic diversity values for South African *M. nubilosa* populations at the plantation level were comparable with values of 30.14 %, 30.10 % and 30.20 % for R-MT, I-MT and W-MT respectively (Table 3). Interestingly, the Western Australian population (WA-MT) had the second lowest value of genotypic diversity of 15.20 % of the theoretical maximum value. This was lower than those for all the South African populations except for population R-1L. The highest genotypic diversity was observed for the New South Wales population (EA-MT) where a value of 76 % of the theoretical maximum was observed. No significant differences ($P < 0.05$) in genotypic diversities were observed between the *M. nubilosa* populations at the plantation level (R-MT, I-MT, W-MT, WA-MT, EA-MT) using a *t*-test (data not shown).

Population differentiation and gene flow

Based on contingency chi squared tests, there were no significant differences in allele frequencies at any of the loci when the three *M. nubilosa* sub-populations (R-1L, R-1T and R-MT) from the Rooihoogte *E. nitens* plantation in South Africa were considered (Table 4). Similarly, no significant differences between the allele frequencies at any of the loci from the Issabelladale *M. nubilosa* sub-populations (I-1T, I-MT) and the Wynton *M. nubilosa* sub-populations (W-1T, W-MT) were observed (Table 4). Three sub-populations at the plantation level from South Africa (R-MT, I-MT, W-MT) were also compared with each other and no significant differences were observed between the allele frequencies at any of the 10 loci

(Table 4). It was, therefore, decided to combine the three South African populations at the plantation level (R-MT, I-MT, W-MT) and to clone-correct this dataset, in order to compare it to the two Australian *M. nubilosa* populations from Western Australia and New South Wales, eastern Australia (WA-MT, EA-MT).

Based on contingency chi squared tests, significant differences ($P < 0.05$) in allele frequencies were observed at nine of the 10 polymorphic loci when the two Australian *M. nubilosa* populations (WA-MT, EA-MT) were compared against each other (Table 5). These differences were observed at loci MN-1, MN-4, MN-7, MN-8, MN-9, MN-10, MN-11 and MN-14. Significant differences ($P < 0.05$) in allele frequencies were also observed at five loci when the combined South African *M. nubilosa* population (RSA) was compared with the Western Australian population (WA-MT). These differences were observed at loci MN-3, MN-4, MN-7, MN-8 and MN-11. Significant differences in allele frequencies were detected for all 10 loci (MN-1 to MN-14) when the combined South African *M. nubilosa* population (RSA) was compared with the New South Wales *M. nubilosa* population (EA-MT) (Table 5).

Population differentiation values (θ) were not significantly different between any of the South African *M. nubilosa* populations at the plantation level (R-MT, I-MT, W-MT) (Table 6). Gene flow (M) between the three South African populations was high with the greatest number of migrants ($M = 82.47$) occurring between the W-MT and I-MT populations and the least number of migrants ($M = 9.63$) occurring between the I-MT and R-MT populations (Table 6). There were, however, significant differences ($P < 0.001$) between the three South African *M. nubilosa* populations at the plantation level (R-MT, I-MT, W-MT) and the two Australian *M. nubilosa* populations at the plantation level (WA-MT, EA-MT) based on the values of θ (Table 6). Similarly, θ values indicated a significant population differentiation ($P < 0.001$) between the two Australian (WA-MT, EA-MT) *M. nubilosa* populations (Table 6). Very little gene flow ($M = 0.52$) between the Western Australian and New South Wales *M. nubilosa* populations was observed. However, there was low gene flow ($M = 1.13–1.54$) between the Western Australian *M. nubilosa* population (WA-MT) and the three South African *M. nubilosa* populations (R-MT, I-MT, W-MT) and extremely limited gene flow ($M = 0.41–0.43$) between the New South Wales population and the three South African populations (Table 6).

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Mode of reproduction

The Index of association values (I_A) differed for the clone corrected and clone uncorrected datasets. For clone corrected datasets, the observed I_A values did not differ significantly from the recombined datasets of *M. nubilosa* for all of the populations at the plantation level from South Africa and Western Australia and when the three South African populations were combined (Table 7). However, using the clone corrected data for the New South Wales *M. nubilosa* population resulted in an observed I_A value that did significantly differ ($P < 0.001$) from the recombined dataset.

When *M. nubilosa* populations from South Africa, Western Australia and New South Wales were used in an uncorrected dataset, significant differences in the observed I_A values from the recombined datasets were observed (Table 7). This was true for the three South African *M. nubilosa* populations at the plantation level (R-MT, I-MT, W-MT). However, the level of significance was not high (Table 7). When the three South African *M. nubilosa* populations from the plantation level were combined into a single population (RSA), there was a high significance value ($P < 0.001$) supporting the difference of the observed I_A from the recombined datasets. This was also true for the Western Australian (WA-MT) and New South Wales (EA-MT) *M. nubilosa* populations (Table 7, Fig. 1). As *M. nubilosa* is a sexually reproducing fungus, this is indicative of a homothallic (selfing) mating system.

DISCUSSION

Mycosphaerella nubilosa is the most important foliar pathogens of *Eucalyptus nitens* and *E. globulus* and in turn, these trees represent two of the most important sources of wood fibre derived from plantations. South Africa has the oldest plantation forestry programme based on non-native species in the world (Burgess & Wingfield 2001) and *M. nubilosa* was the first pathogen of *Eucalyptus* to be reported from this country (Doidge 1950). It has subsequently appeared in many areas of the world where *E. nitens* and *E. globulus* are grown. Although it has been assumed that the pathogen originated in Australia, and that it was accidentally introduced into other parts of the world, this hypothesis has never been tested experimentally. Results of this study thus provide the first evidence that *M. nubilosa* has moved from Australia to other parts of the world, where it has become one of the most important constraints to the propagation of *E. nitens* and *E. globulus* in plantations.

Mycosphaerella nubilosa was originally identified from Victoria in eastern Australia (Cooke 1891). Since this initial identification, it has been hypothesized that eastern Australia

would represent the centre of origin for *M. nubilosa*. Gene diversity and maximum percentage of genotypic diversity for the New South Wales *M. nubilosa* population was the highest of all populations evaluated during this study. Furthermore, the New South Wales *M. nubilosa* population had a greater number of alleles when compared to the other *M. nubilosa* populations. It is known that older pathogen populations representing centres of origin have higher gene diversity values due to the accumulation of mutations over time (McDonald 1997). Results of this study, therefore, support the view that *M. nubilosa* originated from eastern Australia.

An interesting observation in this study was the presence of shared alleles between Western Australia and South Africa. This finding suggests that *M. nubilosa* could have been introduced into South Africa from Western Australia and not from eastern Australia. However, *M. nubilosa* was only detected in Western Australia in 1999 (Maxwell *et al*. 2001), despite previous surveys (Carnegie *et al*. 1997) suggesting that it is relatively newly introduced into that area. There are also no known hosts of *M. nubilosa* that naturally occur in Western Australia. Furthermore, commercial forestry with *E. globulus* (a species native to south-eastern Australia) only began in Western Australia during the 1980's. It thus seems unlikely that *M. nubilosa* was introduced into South Africa from Western Australia. We thus support the alternative hypothesis that *M. nubilosa* populations from eastern Australia, not sampled in this study, acted as a source population for both Western Australia and South Africa. The fact that *E. globulus* seed from eastern Australia was the first *Eucalyptus* planting stock to be planted in South Africa would provide additional support, albeit anecdotal, for this view.

Gene diversities derived in this study for the various *M. nubilosa* populations from South Africa were comparable with each other. These similar gene diversity values and lack of significant population differentiation, combined with the high level of gene flow between the South African *M. nubilosa* populations, indicate that these populations are highly homogenous. Similar findings have emerged for the related pathogens *Cercospora zeae-maydis* Tehon & E.Y. Daniels and *M. graminicola*, where high levels of gene flow have served to homogenise populations of these pathogens (Okori *et al*. 2003, Zhan & McDonald 2004).

In South Africa, the gene diversity of isolates from a single tree was as great as the gene diversity of isolates from a single plantation and gene flow between plantations was very high. Similar findings of genetic diversity have been found for *M. fijiensis* on banana where the majority of genetic diversity for that pathogen exists at the plant and plantation levels

(Rivas *et al*. 2004). Our results are also consistent with those for other *Mycosphaerella* pathosystems. For example, the majority of diversity for *S. tritici* has been observed within a single plot of a wheat field (McDonald & Martinez 1990, Zhan *et al*. 2003). Likewise in *Septoria musiva* Peck from north-eastern America where more than 90 % of the genetic diversity was distributed within a single tree (Feau *et al*. 2005).

Results of this study have shown that haplotypes of *M. nubilosa* are shared between South Africa, Tanzania and Europe. Thus, the single haplotype (MNG-1) found in both the Spanish and Portuguese *M. nubilosa* populations was also found in the Rooihoogte and Wynton *M. nubilosa* populations of South Africa, but not in any of the other *M. nubilosa* populations considered. Likewise, the single haplotype (MNG-2) in the Tanzanian *M. nubilosa* population was present in the Rooihoogte, Issabelladale and Wynton *M. nubilosa* populations from South Africa. These results indicate that gene flow has occurred between South Africa, Tanzania and Europe, resulting in the establishment and persistence of specific haplotypes in Europe and in Tanzania. Based on gene flow data between Australia and South Africa and the same haplotypes that are shared between South Africa, Tanzania and Europe, it seems likely that *M. nubilosa* was first introduced into South Africa from Australia and that it was subsequently moved from South Africa into other parts of Africa and into Europe. This route of movement is also consistent with the pattern of establishment of *Eucalyptus* plantation development in Africa and Europe.

Populations of *M. nubilosa* from South Africa, Western Australia and eastern Australia were significantly differentiated. No haplotypes were shared between the South African, Western Australian or New South Wales *M. nubilosa* populations, also indicating significant population differentiation. Furthermore, gene flow between South Africa and the two Australian *M. nubilosa* populations was low. This is in contrast to the genetic similarity observed for *M. graminicola* populations from different continents (Linde *et al*. 2002). Based on the significant differentiation of the South African, Western Australian and New South Wales populations, it is possible these three populations may represent distinct species. However, DNA sequence data from the Internal Transcribed Spacer (ITS), Translation Elongation Factor 1-alpha (EF-1α), Beta tubulin (Bt) and Calmodulin (CAL) gene regions of *M. nubilosa* representatives from the South African, Western and New South Wales populations, no nucleotide differences were observed (data not shown). Furthermore, considering the extensive distances between *M. nubilosa* populations from South Africa, Western Australia and New South Wales, it is evident that these populations are isolated through distance.

Prior to this study, there has been no knowledge regarding the mode of reproduction of *M. nubilosa*. Park & Keane (1982b) hypothesised that *M. nubilosa* would most likely be homothallic due to the ability of this species to produce pseudothecia containing viable ascospores once grown in agar medium supplemented with *Eucalyptus* leaves. Linkage disequilibrium analyses in this study showed that there is non-random association of alleles within *M. nubilosa.* This would be consistent with a homothallic life history and supports the earlier view of Park & Keane (1982b). Likewise, Milgate *et al*. (2005b) showed that a *M. cryptica* population in Tasmania, Australia, exhibited significant linkage disequilibrium indicating that this population was not strictly heterothallic. The fact that two of the most important *Mycosphaerella* pathogens on *Eucalyptus* most likely have a homothallic mating strategy is intriguing because most *Mycosphaerella* spp. known from other hosts that have been studied are heterothallic (Carlier *et al*. 1996, Linde *et al*. 2002, McDonald & Linde 2002).

Despite the fact that *M. nubilosa* appears to be homothallic, a relatively high number of haplotypes exist in the South African and Australian populations of the pathogen. This is not necessarily unusual as relatively high levels of diversity have been found in various other homothallic fungi. This diversity is typically accounted for by rare mitotic crossing over or mutation events that generate recombinant genotypes (Taylor *et al*. 1999). Furthermore, the presence of sexual structures of *M. nubilosa* in nature is an indication that potential exists for sexual reproduction where chance outcrossing events could generate novel genotypes (Milgroom 1996). Considering that several haplotypes of *M. nubilosa* occupy the same lesion, it is likely that mycelium of different haplotypes will come into contact, leading to outcrossing events. These unique haplotypes also may be introductions from outside of South Africa most likely introduced through *Eucalyptus* plant material.

Numerous haplotypes were found in the South African and Australian *M. nubilosa* populations. Considering the number of different haplotypes and alleles observed in these *M. nubilosa* populations it would be difficult to effectively breed *Eucalyptus* trees that are tolerant to *M. nubilosa* infection. Genetically diverse and large pathogen populations have a greater evolutionary potential than small populations (McDonald & Linde 2002). Due to the large population sizes of *M. nubilosa* in South Africa and Australia, these populations would have greater evolutionary potential to overcome new *Eucalyptus* genotypes that are deployed in commercial forestry. However, considering that eastern Australia appears to be the centre of origin of *M. nubilosa*, resistance genes present in *Eucalyptus* hosts would most likely be found within this area and could be used for breeding tolerant *Eucalyptus* genotypes. It has for

instance, already been shown that wide variation exists within *E. globulus* at the subspecies, provenance and family level in susceptibility to MLD (Carnegie *et al*. 1994, Carnegie & Ades 2005, Milgate *et al*. 2005a).

A high number of haplotypes was observed in the Australian and South African *M. nubilosa* populations but not in those from other parts of the world. This presents a strong case for ensuring that movement of *Eucalyptus* germplasm does not occur. This knowledge should be incorporated into quarantine regulations and actionable lists in Australia and countries in Africa and Europe. It is known that seedborne infections can lead to epidemics of crop foliage (Milgroom & Peever 2003) and it has been suggested, but not proven, that *Mycosphaerella* spp. may be transferred on seed material. It has also been suggested that gene flow of *M. graminicola* genotypes may occur on a global scale due to the movement of infected wheat seed (McDonald *et al*. 1996). The movement of *Eucalyptus* seed should, therefore, be monitored and tested for the presence of *Mycosphaerella* propagules. This could be achieved through the use of *Mycosphaerella* species-specific primers or PCR-RFLP based techniques to identify *M. nubilosa* (Kularatne *et al*. 2004, Maxwell *et al*. 2005).

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Table 1. Origin of *Mycosphaerella nubilosa* isolates used for this study. Number of isolates per hierarchical sampling level. Population code (**P.C**) indicates the population code assigned to each population and sub-population.

Country	Area	Plantation	Hierarchical Level	P.C	Host	Collector	Date	No. Isolates
South Africa	Mpumalanga	Rooihoogte	1 Lesion	R-1L	*E. nitens*	G.C. Hunter	2005	32
			1 Tree	R-1T	*E. nitens*	G.C. Hunter	2005	48
			Plantation	R-MT	*E. nitens*	G.C. Hunter	2005	56
		Issabelladale	1 Tree	I-1T	*E. nitens*	G.C. Hunter	2005	30
			Plantation	I-MT	*E. nitens*	G.C. Hunter	2005	29
		Wynton	1 Tree	W-1T	*E. nitens*	G.C. Hunter	2005	49
			Plantation	W-MT	*E. nitens*	G.C. Hunter	2005	49
Australia	Western Australia	Albany	Plantation	WA-MT	*E. globulus*	A. Maxwell	2002	32
	New South Wales	Bonalbo	Plantation	EA-MT	*E. globulus*	A.J. Carnegie	2004	19
Spain			Plantation	S-MT	*E. globulus*	J.P.M. Vasquez	2002	55
Portugal			Plantation	P-MT	*E. globulus*	M.J. Wingfield	2005	42
Tanzania			Plantation	T-MT	*E. globulus*	J. Roux	2004	56
							Total	497

Table 2. Allele size (bp) and allele frequency at 10 microsatellite loci (MN-1 – MN-14) determined using the software program POPGENE for populations of *Mycosphaerella nubilosa* collected from South Africa (Rooihoogte, Issabelladale and Wynton) and Australia (Western Australia and New South Wales).

			South Africa						Australia	
			Rooihoogte		Issabelladale		Wynton		Western	NSW
Locus	**Allele**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
MN-1	**267**	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-
	253	-	-	-	-	-	-	-	-	1.000
MN-2	**183**	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.906	0.053
	185	-	-	-	-	-	-	-	0.094	-
	192	-	-	-	-	-	-	-	-	0.053
	194	-	-	-	-	-	-	-	-	0.053
	214	-	-	-	-	-	-	-	-	0.158
	222	-	-	-	-	-	-	-	-	0.158
	234	-	-	-	-	-	-	-	-	0.105
	262	-	-	-	-	-	-	-	-	0.053
	264	-	-	-	-	-	-	-	-	0.053
	265	-	-	-	-	-	-	-	-	0.158
	272	-	-	-	-	-	-	-	-	0.158
MN-3	**307**	-	-	-	-	-	-	-	-	0.211
	309	1.000	0.500	0.446	0.300	0.241	0.469	0.469	1.000	0.800
	315	-	0.500	0.554	0.700	0.759	0.531	0.531	-	-

		South Africa							Australia	
			Rooihoogte		Issabelladale		Wynton		Western	NSW
Locus	**Allele**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
MN-4	**141**	-	-	-	-	-	-	-	-	0.421
	148	-	-	-	-	-	-	-	-	0.263
	155	0.500	0.458	0.518	0.433	0.483	0.388	0.571	1.000	0.053
	162	0.500	0.452	0.482	0.533	0.517	0.612	0.429	-	0.158
	169	-	-	-	0.033	-	-	-	-	0.105
MN-7	**376**	-	-	-	-	-	-	-	0.344	-
	380	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.656	-
	396	-	-	-	-	-	-	-	-	0.684
	400	-	-	-	-	-	-	-	-	0.316
MN-8	**193**	-	-	-	-	-	-	-	-	0.053
	202	-	-	-	-	-	-	-	0.031	-
	209		-	0.018	-	-	-	-	-	-
	229	-	-	-	-	-	-	-	-	0.053
	238	0.563	0.625	0.554	0.633	0.724	0.653	0.429	-	0.580
	242	-	-	0.018	-	-	-	-	-	-
	247	-	-	-	-	-	-	-	0.344	0.158
	265	-	-	-	0.633	-	-	-	0.156	-
	274	-	-	-	-	-	-	-	-	0.053
	283	-	-	-	-	-	-	-	0.031	-
	292	-	-	-	-	-	-	-	-	0.105
	301	-	-	-	-	-	-	-	0.375	-
	319	0.438	0.375	0.411	0.333	0.276	0.327	0.571	0.031	-

		South Africa							Australia	
			Rooihoogte		Issabelladale		Wynton		Western	NSW
Locus	**Allele**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
	340	-	-	-	-	-	0.020	-	-	-
	355	-	-	-	-	-	-	-	0.031	-
MN-9	**202**	-	-	-	-	-	-	-	-	0.053
	210	-	-	-	-	-	-	-	-	0.474
	214	-	-	-	-	-	-	-	-	0.105
	216	-	0.250	0.375	0.133	0.207	0.286	0.408	0.844	-
	218	1.000	0.750	0.607	0.833	0.793	0.714	0.571	0.156	-
	220	-	-	0.018	-	-	-	-	-	0.053
	222	-	-	-	0.033	-	-	0.020	-	0.053
	226	-	-	-	-	-	-	-	-	0.053
	230	-	-	-	-	-	-	-	-	0.158
	234	-	-	-	-	-	-	-	-	0.053
MN-10	**135**	-	0.021	-	-	-	-	0.020	-	-
	137	1.000	0.979	1.000	1.000	1.000	1.000	0.980	1.000	0.158
	141	-	-	-	-	-	-	-	-	0.053
	145	-	-	-	-	-	-	-	-	0.158
	147	-	-	-	-	-	-	-	-	0.527
	153	-	-	-	-	-	-	-	-	0.105
MN-11	**192**	-	0.021	-	-	-	-	-	-	-
	194	0.469	0.458	0.375	0.467	0.379	0.367	0.367	0.500	1.000
	200	-	-	-	-	-	-	-	0.156	-
	206	-	-	-	-	0.035	-	-	0.344	-

		South Africa							Australia	
			Rooihoogte		Issabelladale		Wynton		Western	NSW
Locus	**Allele**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
	224	0.531	0.521	0.625	0.533	0.586	0.633	0.633	-	-
MN-14	**98**	-	-	-	-	-	-		-	0.158
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.875	-
	102	-	-	-	-	-	-	-	0.125	0.421
	104	-	-	-	-	-	-	-	-	0.263
	106	-	-	-	-	-	-	-	-	0.158
N^A		32	48	56	30	29	49	49	32	19
N^B		4	24	24	16	17	22	23	10	16
Alleles		13	17	18	18	16	16	17	22	44
P		3	6	5	5	5	5	6	6	8
P (%)		30	60	50	50	50	50	60	60	80
H		0.149	0.240	0.248	0.222	0.211	0.231	0.250	0.242	0.506
H*		0.125	0.258	0.255	0.248	0.242	0.247	0.255	0.246	0.506

N^A: Number of isolates used in the uncorrected dataset.

N^B: Number of isolates used in the clone corrected dataset.

P: Number of polymorphic loci.

P (%): Percentage of polymorphic loci.

H: Gene diversity of the population (Nei 1973).

H**: Gene diversity of the clone corrected population.

Table 3. Multi Locus Haplotypes (MLH's) of *Mycosphaerella nubilosa* isolates compiled from 10 polymorphic microsatellite markers. Haplotypes from Spain (S-MT), Portugal (P-MT), Tanzania (T-MT) all at the plantation level. From three plantations in South Africa at different hierarchical levels namely, Rooihoogte plantation at the plantation level (R-MT), within one tree (R-1T), within one lesion (R-1L), Issabelladale plantation at the plantation level (I-MT), within one tree (I-1T) and the Wynton plantation at the plantation level (W-MT), within one tree (W-1T). Also two *M. nubilosa* populations both at the plantation level from Australia, Western Australia (WA-MT) and New South Wales (EA-MT).

				South Africa							Australia	
	Spain	Portugal	Tanzania	Rooihoogte			Issabelladale		Wynton		Western	NSW
MLH	S-MT	P-MT	T-MT	R-1L	R-1T	R-MT	I-1T	I-MT	W-1T	W-MT	WA-MT	EA-MT
MNG-1	55	42				1			1	3		
MNG-2			56		7	8	10	7	11	5		
MNG-3					2	2	1	5	3			
MNG-4					1	3	2	2	1	4		
MNG-5					1	2		1				
MNG-6				14	3	3	4	1	8	6		
MNG-7					1		1	1				
MNG-8				1	2	1	1	2		1		
MNG-9							1	1				
MNG-10							1	1	1			
MNG-11					3	4		1	1	1		
MNG-12				16	5	1		1	3	1		
MNG-13						1		1				
MNG-14						4		1	5	1		

				South Africa							Australia	
	Spain	Portugal	Tanzania	Rooihoogte			Issabelladale		Wynton		Western	NSW
MLH	S-MT	P-MT	T-MT	R-1L	R-1T	R-MT	I-1T	I-MT	W-1T	W-MT	WA-MT	EA-MT
MNG-15					1	3	1	1		6		
MNG-16								1				
MNG-17						1	1	1		1		
MNG-18					2	2	1	1	1			
MNG-19							1			1		
MNG-20					3	2	1		2			
MNG-21				1	2	3	2		1	2		
MNG-22							1					
MNG-23							1					
MNG-24					1					3		
MNG-25					2				1			
MNG-26					1				2	1		
MNG-27					3				1	2		
MNG-28						3				1		
MNG-29										1		
MNG-30					1	1			1	1		
MNG-31						3				2		
MNG-32					2				1	1		
MNG-33					1				1	1		
MNG-34						3			1	2		

					South Africa						Australia	
	Spain	Portugal	Tanzania		Rooihoogte		Issabelladale		Wynton		Western	NSW
MLH	S-MT	P-MT	T-MT	R-1L	R-1T	R-MT	I-1T	I-MT	W-1T	W-MT	WA-MT	EA-MT
MNG-35					1	2			1	2		
MNG-36									1			
MNG-37					1				1			
MNG-38					1							
MNG-39					1							
MNG-40						1						
MNG-41						1						
MNG-42						1						
MNG-43												2
MNG-44												2
MNG-45												2
MNG-46												1
MNG-47												1
MNG-48												1
MNG-49												1
MNG-50												1
MNG-51												1
MNG-52												1
MNG-53												1
MNG-54												1

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				South Africa							Australia	
	Spain	Portugal	Tanzania	Rooihoogte			Issabelladale		Wynton		Western	NSW
MLH	**S-MT**	**P-MT**	**T-MT**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
MNG-55												**1**
MNG-56												**1**
MNG-57												**1**
MNG-58												**1**
MNG-59											**10**	
MNG-60											**4**	
MNG-61											**1**	
MNG-62											**9**	
MNG-63											**1**	
MNG-64											**2**	
MNG-65											**1**	
MNG-66											**2**	
MNG-67											**1**	
MNG-68											**1**	
N	55	42	56	32	48	56	30	29	49	49	32	19
N (g)	1	1	1	4	24	24	16	17	22	23	10	16
G	N/A	N/A	N/A	2.26	15.78	16.88	6.63	8.73	9.61	14.82	4.86	14.49
$\hat{G}$	N/A	N/A	N/A	7.1	32.9	30.14	22.1	30.1	19.6	30.2	15.2	76.3

N: Number of *M. nubilosa* isolates.

N (g): Number of Multi Locus Haplotypes (MLH's).

G: Genotypic diversity (Stoddard & Taylor 1988).

$\hat{\boldsymbol{G}}$: Maximum percentage of genotypic diversity.

Table 4. Gene Diversity (*H*) and contingency χ^2 tests for differences in allele frequencies for the 10 microsatellite loci across the clone corrected populations of *M. nubilosa* from different hierarchical levels. **(A)** Within one lesion (R-1L), within one tree (R-1T), within one plantation (R-MT) of the Rooihoogte plantation. **(B)** Within one tree (I-1T), within one plantation (I-MT) of the Issabelladale plantation. **(C)** Within one tree (W-1T), within one plantation (W-MT) of the Wynton plantation. **(D)** Comparison between three South African plantations at the plantation level (R-MT, I-MT, W-MT). Determined χ^2 values not significant. **N** = Number of *M. nubilosa* isolates from the clone corrected dataset.

	Gene Diversity (*H*)							A		B		C		D	
	R-1L	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	χ^2	**d.f.**	χ^2	**d.f.**	χ^2	**d.f.**	χ^2	**d.f.**
MN-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-
MN-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-
MN-3	0.00	0.49	0.49	0.43	0.46	0.48	0.50	3.0	2	0.06	1	0.22	1	2.2	2
MN-4	0.38	0.50	0.49	0.55	0.50	0.48	0.50	0.9	2	1.22	2	0.22	1	0.7	2
MN-7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-
MN-8	0.38	0.50	0.57	0.54	0.48	0.52	0.48	3.3	6	1.10	2	1.07	2	3.6	6
MN-9	0.00	0.47	0.52	0.46	0.46	0.48	0.54	3.6	4	1.37	2	1.07	2	3.9	6
MN-10	0.00	0.08	0.00	0.00	0.00	0.00	0.08	1.2	2	-	-	0.98	1	1.8	2
MN-11	0.50	0.53	0.49	0.49	0.53	0.50	0.45	2.2	4	1.10	2	1.07	1	5.8	4
MN-14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-
N	4	24	24	16	17	22	23								
Mean	0.13	0.23	0.25	0.25	0.24	0.25	0.25								

Table 5. Gene Diversity (*H*) and contingency χ^2 tests for differences in allele frequencies for the 10 polymorphic microsatellite loci across the clone corrected *M. nubilosa* populations from (**A**) South Africa [RSA = (R-MT + I-MT + W-MT)] and Western Australia (WA-MT), (**B**) South Africa [RSA = (R-MT + I-MT + W-MT)] and New South Wales, eastern Australia (EA-MT), (**C**) Western Australia (WA-MT) and New South Wales (EA-MT).

	Gene Diversity (*H*)			A		B		C	
Locus	**RSA**	**WA-MT**	**EA-MT**	**χ^2**	**d.f.**	**χ^2**	**d.f.**	**χ^2**	**d.f.**
MN-1	0.00	0.00	0.00	-	-	51.0*	1	26.0*	1
MN-2	0.00	0.18	0.88	3.6	1	46.5*	9	22.2*	10
MN-3	0.50	0.00	0.30	7.8*	1	15.9*	2	2.1	1
MN-4	0.50	0.00	0.69	9.4*	1	38.2*	4	22.2*	4
MN-7	0.00	0.32	0.43	7.3*	1	51.0*	2	26.0*	3
MN-8	0.54	0.84	0.63	39.6*	9	22.6*	8	20.4*	10
MN-9	0.54	0.32	0.76	5.1	3	46.4*	9	26.0*	9
MN-10	0.06	0.00	0.63	0.3	1	42.2*	5	19.0*	4
MN-11	0.52	0.62	0.00	19.0*	3	15.0*	2	9.9*	2
MN-14	0.00	0.18	0.73	3.6	1	51.0*	4	22.4*	4
N	35	10	16						
Mean	0.27	0.25	0.51						

* Indicates significant χ^2 values ($p < 0.05$).

Table 6. Population differentiation values (θ), above the diagonal, calculated after 1000 randomisations using the program Multilocus. Gene Flow (Number of migrants) (M) indicated below the diagonal.

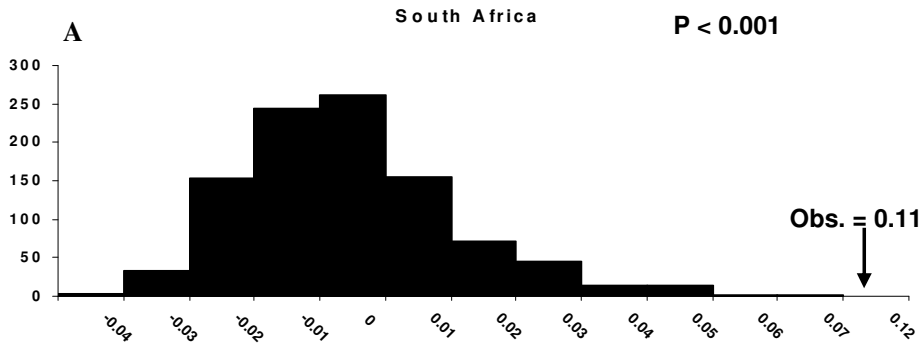
	R-MT	I-MT	W-MT	WA-MT	EA-MT
R-MT		-0.0054	-0.03034	0.25*	0.55*
I-MT	9.63		-0.0061	0.31*	0.54*
W-MT	16.93	82.47		0.29*	0.55*
WA-MT	1.54	1.13	1.24		0.49*
EA-MT	0.41	0.43	0.41	0.52	

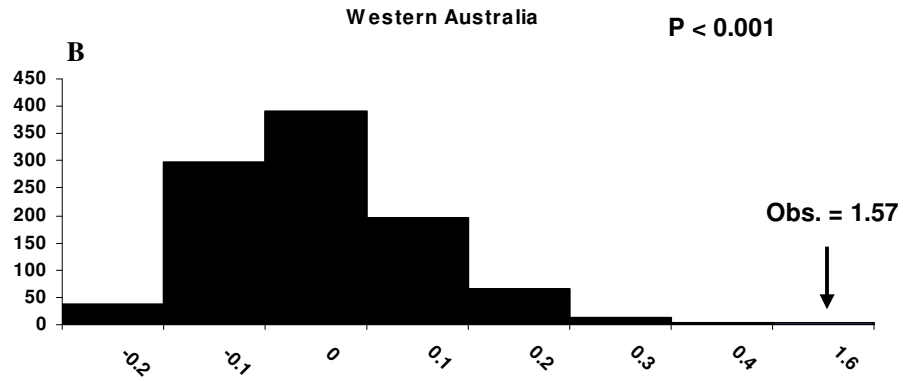
*: Indicates significant values ($p < 0.001$).

Table 7. Linkage disequilibrium (I_A) values for populations of *M. nubilosa* from South Africa at the plantation level (R-MT, I-MT, W-MT) and for the combined South African population (RSA) and for the two Australian *M. nubilosa* populations (WA-MT, EA-MT). Linkage disequilibrium values were determined for both (**A**) clone corrected and (**B**) uncorrected datasets using the program Multilocus with 1000 randomisations. N = Number of isolates, P = p-value.

	A			B		
	N	I_A	**P**	**N**	I_A	**P**
R-MT	24	-0.10	0.951	56	0.14	0.002
I-MT	17	-0.10	0.773	29	0.30	0.004
W-MT	23	-0.10	0.916	49	0.06	0.071
RSA	42	-0.12	1.0	134	0.11	< 0.001
WA-MT	10	0.66	0.031	32	1.57	< 0.001
EA-MT	16	1.30	< 0.001	19	1.38	< 0.001

Figure 1. Histograms of frequency distributions of Index of Association (I_A) values following 1000 randomisations in Multilocus of clone uncorrected *M. nubilosa* populations. (**A**) South African *M. nubilosa* populations combined at the plantation level (R-MT, I-MT, W-MT), (**B**) Western Australian *M. nubilosa* population (WA-MT), (**C**) New South Wales *M. nubilosa* population (EA-MT). Frequency indicated on Y-axis and Index of Association (I_A) indicated on X-axis.


A
South Africa
P < 0.001
300
250
200
150
100
50
0
Obs. = 0.11
-0.04
-0.03
-0.02
-0.01
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.12


B
Western Australia
P < 0.001
450
400
350
300
250
200
150
100
50
0
Obs. = 1.57
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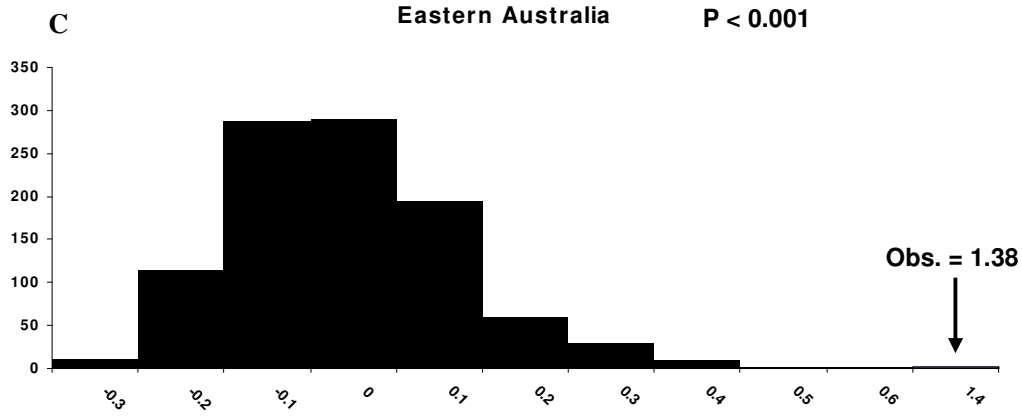
0
0.1
0.2
0.3
0.4
1.6


C
Eastern Australia
P < 0.001
350
300
250
200
150
100
50
0
Obs. = 1.38
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
1.4

6

Intra-specific variation in *Mycosphaerella nubilosa sensu lato*

Abstract: *Mycosphaerella nubilosa* is one of the most important pathogens of *Eucalyptus* leaves and causes premature defoliation and stunting of tree growth. Recent surveys of diseased *Eucalyptus* plantations have resulted in extensive collections of *M. nubilosa*. From comparisons of DNA sequences for the Internal Transcribed Spacer (ITS) region of the rRNA operon, it has become apparent that variation exists within this species. The aim of this study was to critically compare a large collection of isolates of *M. nubilosa* from different hosts and regions of the world. This was achieved by comparing isolates based on DNA sequence data from three nuclear gene regions, as well as analysis of microsatellite marker data. Results of this study indicate that intra-specific variation exists within the fungus known as *M. nubilosa*, which can be linked to host association and culture morphology. From DNA sequence data we designate two *M. nubilosa* ITS lineages indicative of host associations and geographic distribution. Although these may represent discrete taxa, current data are insufficient to describe the first lineage as a new species.

INTRODUCTION

Species of *Eucalyptus* are native to Australia, Papua New Guinea, Indonesia and the Philippines where they occur in large natural forests (Turnbull 2000). Many of these *Eucalyptus* spp. have been removed from their native ranges and propagated in commercially important plantations in various tropical and sub-tropical countries (Poynton 1979). In many non-native environments, *Eucalyptus* spp. have become second only to *Pinus* spp. in their use as a commercial forestry crop (Old *et al*. 2003). This is due to their favourable growth properties, relatively short rotation periods, climate adaptability and favoured wood properties (Poynton 1979, Turnbull 2000).

Mycosphaerella nubilosa (Cooke) Hansf. is one of the many species of *Mycosphaerella* Johanson that infect *Eucalyptus* leaves (Crous 1998, Crous *et al*. 2004, Crous *et al*. 2006). This species was originally identified from *Eucalyptus* leaves in Victoria, eastern Australia (Cooke 1891). Currently *M. nubilosa* is known from Australia, New Zealand and several countries from Africa and Europe (Dick 1982, Park & Keane 1982, Maxwell *et al*. 2001, Crous *et al*. 2004, Alemu *et al*. 2006). In these countries, *M. nubilosa* has become a serious impediment to the continued propagation of *E. globulus* and *E. nitens* (Park *et al*. 2000, Crous *et al*. 2004, Hunter *et al*. 2004). *M. nubilosa* infects juvenile *Eucalyptus* leaves causing reduced photosynthetic capability and premature defoliation of *Eucalyptus* trees (Park & Keane 1982, Pinkard & Mohammed 2006). Recently, however, *M. nubilosa* has also been identified from mature *Eucalyptus* foliage and in this regard it poses an increased threat to commercial *Eucalyptus* forestry.

DNA sequencing has become the definitive tool used for identification of *Mycosphaerella* species (Crous *et al*. 2006). The Internal Transcribed Spacer (ITS) region of the rRNA operon has been the preferred gene region used for phylogenetic analyses and identification of *Mycosphaerella* spp. (Crous *et al*. 2004, Hunter *et al*. 2004). Recently, however, DNA Multi Locus Sequence Typing (MLST) has resulted in a mutli-gene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves (Hunter *et al*. 2006b). Thus, existing DNA sequence data have clarified species concepts in *Mycosphaerella* and more particularly, have resulted in the identification of several species complexes (Crous *et al*. 2004, 2006). Analysis of sequences of the ITS region of the rRNA operon for more than 120 *Mycosphaerella* isolates from *Eucalyptus* enabled Crous *et al*. (2006) to identify 9 *Mycosphaerella* species complexes including several novel cryptic species of *Mycosphaerella*.

Crous *et al*. (2004, 2006) showed that *M. nubilosa* could be divided into two well-supported phylogenetic clades based on ITS sequence data. Recent surveys of diseased *Eucalyptus* plantations have resulted in many collections of *M. nubilosa* from several different countries where this pathogen is known to occur. These have made it possible to consider the phylogenetic relationships between *M. nubilosa* isolates more closely. The aim of this study was thus to use DNA sequence data to determine whether *M. nubilosa* represents a single monophyletic species, or a polyphyletic assemblage of more than one phylogenetic lineage. This was achieved by sequencing the ITS, Translation Elongation Factor 1-alpha (EF-1α) and Beta tubulin-2 (Bt-2) gene regions of several isolates of *M. nubilosa* from different geographical areas and *Eucalyptus* hosts. Furthermore, these sequence data were combined with culture studies and microsatellite amplification experiments of *M. nubilosa* isolates to determine if, and to what extent, variation exists within *M. nubilosa*.

MATERIALS AND METHODS

Isolation and isolates

Isolates of *M. nubilosa* were collected from several different geographic locations. In Australia, several areas were sampled including Western Australia, Tasmania, Victoria and New South Wales, constituting the major areas where *Eucalyptus* spp. are grown commercially. *Mycosphaerella nubilosa* isolates were also collected from New Zealand, Spain, Portugal, South Africa and Tanzania (Table 1).

Isolations of *M. nubilosa* from diseased *Eucalyptus* leaves followed the protocols of Crous (1998). Lesions bearing pseudothecia of *M. nubilosa* were excised from symptomatic *Eucalyptus* leaves and placed in water for 2 h. Lesions were then dried and attached to the undersides of Petri dish lids with adhesive tape having the pseudothecia facing downward over 2 % Malt Extract Agar (MEA) (Biolab, South Africa). Petri dishes were then incubated in the dark at room temperature for 24 h to allow for ascospore discharge, and germination on the agar medium. Petri dishes were then evaluated for the presence of germinating ascospores. Ascospores exhibiting the type F ascospore germination pattern (Crous 1998, Crous *et al.* 2004), were cut from the agar surface and transferred to fresh 2 % MEA agar plates and incubated at 25 °C for the establishment of pure cultures.

All *M. nubilosa* isolates used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (CMW), University of Pretoria, Pretoria, South Africa and the herbarium collection of Murdoch University (MURU), Perth,

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Western Australia, Australia (Table 1). Duplicates of the New South Wales collection are kept at the fungal collection of forestry New South Wales (NSWF), Sydney, Australia.

DNA extraction and PCR

Mycelium from actively growing single-ascospore cultures was scraped from the surface of agar plates, freeze dried for 24 h and ground to a fine powder using liquid nitrogen. DNA was isolated using a phenol : chloroform (1 : 1) extraction protocol as described in Hunter *et al.* (2004) and precipitated by the addition of absolute ethanol (98 % EtOH). Isolated DNA was cleaned by washing with 70 % ethanol and dried under vacuum. Triple distilled water was used to resuspend the isolated DNA. RNase A (10 µg/µL) was added to the resuspended DNA and incubated at 37 °C for approximately 2 h to digest residual RNA. Isolated DNA was separated by electrophoresis in 1 % agarose gels (wt/v) (Roche Diagnostics, Mannheim), stained with ethidium bromide and visualised under ultra-violet light.

DNA from *M. nubilosa* isolates was used as template DNA for amplification using the polymerase chain reaction (PCR). All PCR reactions were mixed in a total volume of 25 µL containing 10 × PCR Buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP) (Roche Diagnostics, South Africa), 0.2 µM of forward and reverse primers (Inqaba Biotech, South Africa) and 1.25 U Taq DNA Polymerase (Roche Diagnostics, South Africa) and DNA (20 ng/µL). Sterilised distilled water was added to obtain a final volume of 25 µL.

The ITS-1, ITS-2 and the 5.8 S gene regions of the ITS region of the rRNA operon were amplified using primers ITS-1 (5`–TCC GTA GGT GAA CCT GCG G–3`) and LR-1 (5`–GGT TGG TTT CTT TTC CT–3`) (White *et al.* 1990, Vilgalys & Hester 1990). A portion of the translation elongation factor 1-alpha (EF-1α) gene region was amplified using the primers EF1-728F (5'–CAT CGA GAA GTT CGA GAA GG–3') and EF1-986R (5'–TAC TTG AAG GAA CCC TTA CC–3') (Carbone & Kohn 1999). A portion of the Bt-2 gene region was amplified using primers Bt-2a (5'–GGT AAC CAA ATC GGT GCT GCT TTC–3') and Bt-2b (5'–ACC CTC AGT GTA GTG ACC CTT GGC–3') (Glass & Donaldson 1995). Reaction conditions for the ITS gene region followed those of Crous *et al.* (2004) and Hunter *et al.* (2004). Reaction conditions for the EF-1α gene region were the same as those used by Hunter *et al.* (2006b). The reaction conditions for the Bt-2 gene region were the same as those described for the ITS region (Hunter *et al.* 2004), however, the annealing temperature for the Bt-2 gene region was set to 55 °C.

All PCR products were visualised in 1.5 % agarose gels (wt/v) stained with ethidium bromide and viewed under ultra-violet light. Sizes of PCR amplicons were estimated by comparison against a 100 bp molecular weight marker (O' RangeRuler™ 100 bp DNA ladder) (Fermentas Life Sciences, U.S.A.). Prior to DNA sequencing, PCR products were purified through Centri-sep spin columns (Princeton Separations, Adelphia, NJ) containing Sephadex G-50 (Sigma Aldrich, St. Louis, MO) as outlined by the manufacturer.

DNA sequencing and phylogenetic analyses

Purified ITS, EF-1α and Bt-2 PCR products of *M. nubilosa* isolates were used as template DNA for sequencing reactions on an ABI PRISM™ 3100 Automated DNA sequencer (Applied Biosystems, Foster City, CA). The ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) was used for sequencing reactions following the manufacturer's instructions. Most sequencing reactions were performed with the same primers used for PCR reactions. Exceptions were in the case of the ITS region where two internal primers ITS-2 (5'–GCT GCG TTC TTC ATC GAT GC–3') and ITS-3 (5'–GCA TCG ATG AAG AAC GCA GC–3') (White *et al.* 1990) were included for the sequencing reactions.

ITS, EF-1α and Bt-2 sequences of *M. nubilosa* were also downloaded from GenBank and incorporated into phylogenetic analyses. All resulting sequences were analysed with Sequence Navigator v. 1.0.1 (Applied Biosystems, Foster City, CA). Sequence alignments were done using MAFFT (Multiple alignment program for amino acid or nucleotide sequences) v. 5.667 (Katoh *et al.* 2005) and manually adjusted where necessary. Phylogenetic distance and parsimony analyses of individual DNA sequence datasets and combined sequence datasets were conducted in PAUP v. 4.0b10 (Swofford 2002). For all distance and parsimony analyses that were conducted, isolates of *Mycosphaerella molleriana* (Thüm) Lindau and *Mycosphaerella cryptica* (Cooke) Hansf. were used to root phylogenetic trees.

For distance analyses, Modeltest v. 3.04 (Posada & Crandall 1998) was used to determine the best evolutionary model to fit the individual DNA sequence datasets and the combined DNA sequence alignment. A neighbour-joining analysis with the chosen evolutionary models was conducted in PAUP. For the ITS sequence dataset, the distance measure employed was the Hasegawa-Kishino-Yano (HKY) model. Here, the number of substitution types were two, transition/transversion ratio = 1.1144, base frequencies A = 0.21300, C = 0.3108, G = 0.25270 and T = 0.22350, assumed proportion of invariable sites (I)

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= 0.8567, the distribution of rates at variable sites was equal. Where they were encountered, ties were broken randomly.

For the Bt-2 DNA sequence dataset a General Time Reversible model (GTR + I) was selected from the Modeltest program. Here, the number of substitution types = 6, substitution rate matrix [A-C] =5.500, [A-G] = 2.7405, [A-T] = 0.000, [C-G] = 1.2594, [C-T] = 9.6068, [G-T] = 1.000, base frequencies A = 0.21220, C = 0.30270, G = 0.28620 and T = 0.19890, assumed proportion of invariable sites (I) = 0.6625, Distribution of rates at variable sites were equal and ties were broken randomly when they were encountered.

The Tamura-Nei (TrNef + I) evolutionary model was selected as the distance measure to be applied to the EF-1α DNA sequence alignment. Here, the number of substitution types = 6, substitution rate matrix [A-C] = 1.0000, [A-G] = 1.2098, [A-T] = 1.000, [C-G] = 1.000, [C-T] = 2.7318 and [G-T] = 1.000, base frequencies were equal, assumed proportion of invariable sites (I) = 0.4033, distribution of rates at variable sites were equal. Ties were broken randomly when they were encountered.

A Partition Homogeneity Test (PHT) (Farris *et al*. 1994), of all possible combinations, consisting of 1000 replicates on all informative characters was conducted in PAUP to determine if the ITS, Bt-2 and EF-1α sequence datasets could be combined. From the PHT, a P-value of 1.000 was obtained and therefore the ITS, Bt-2 and EF-1α sequence datasets were combined.

A modeltest of the combined DNA sequence dataset selected a transition model (TIM) to be applied to the combined DNA sequence dataset. For this distance analysis, the number of substitution types = 6, substitution rate matrix [A-C] = 1.0000, [A-G] = 1.0293, [A-T] = 0.5885, [C-G] = 0.5885, [C-T] = 2.5721, [G-T] = 1.0000, base frequencies A = 0.22090, C = 0.29600, G = 0.27040, T = 0.21270, assumed proportion of invariable sites (I) = 0.6478, distribution of rates at variable sites were equal. Where ties were encountered, they were broken randomly.

Parsimony analysis was also conducted on the combined ITS, Bt-2 and EF-1α sequence datasets. Here, heuristic searches were conducted in PAUP with starting trees obtained through stepwise addition with simple addition sequence and with the MULPAR function enabled. Tree Bisection Reconnection (TBR) was employed as the swapping algorithm. All gaps were coded as missing data and characters were assigned equal weight. Branch support for nodes was obtained by performing 1000 bootstrap replicates of the aligned sequences. For parsimony analyses, measures that were calculated included tree length (TL),

retention index (RI), consistency index (CI), rescaled consistency index (RC) and homoplasy index (HI). All sequences of *M. nubilosa* isolates used in this study have been deposited in GenBank (Table. 1).

Growth in culture

Growth characteristics of representative *M. nubilosa* isolates that grouped in different ITS clades were determined on MEA. Here, the ex-epitype isolate of *M. nubilosa* (CMW 3282, CBS 116005), that resided in ITS lineage 2 and *M. nubilosa* isolate CMW 6518, that was accommodated in ITS linage 1 were used for growth studies. Actively growing cultures were used, and mycelial plugs (3 mm diam) were cut from the margins of cultures and transferred to the centres of 90 mm MEA agar plates. Agar plates were incubated in the dark at temperatures between 0 °C and 35 °C at 5° intervals. Colony diameters were measured every 7 d for 1 mo. Two plates were used per isolate at each temperature, and the entire experiment was repeated once. Colony colours (Rayner 1970) and morphology was assessed after 1 mo of growth in culture.

Microsatellite amplification

DNA from three *M. nubilosa* isolates that resided in different ITS lineages were used for microsatellite amplification experiments. These isolates included the *M. nubilosa* ex-epitype isolate (CMW 3282, CBS 116005) which resided in ITS lineage 2 and two isolates of *M. nubilosa* (CMW 6518 and CMW 23911) from ITS lineage 1. DNA from these *M. nubilosa* isolates was used as template DNA for the amplification of 10 microsatellite regions.

Microsatellite primers developed by Hunter *et al*. (2006a) were used to amplify the desired microsatellite regions. PCR reactions included an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C (MN-8) or 60 °C for 30 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. DNA was amplified in 25 µL reaction volumes containing PCR Buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 5.0 mM dNTP's 0.2 µM of each forward and reverse primer, 1.25 U Taq Polymerase (Roche Diagnostics, South Africa) and sterile water was added to achieve a final volume of 25 µL. Amplified DNA was visualised in 1.5 % agarose gels stained with ethidium bromide and viewed under ultra violet light

RESULTS

DNA sequencing and phylogenetic analyses

ITS phylogeny: The total alignment of the ITS sequence dataset consisted of 341 characters and 90 taxa. Following neighbour-joining analyses of the aligned ITS dataset, two distinct ITS lineages were resolved, designated *M. nubilosa* ITS lineage 1 and *M. nubilosa* ITS lineage 2 (Fig. 1). *Mycosphaerella nubilosa* ITS lineage 1 was well supported with a bootstrap value of 76 % and included isolates of *M. nubilosa* from Victoria (eastern Australia, west of Melbourne), New Zealand, Tasmania and western Australia. *Mycosphaerella nubilosa* ITS lineage 2 was also well supported with a bootstrap value of 75 % and accommodated *M. nubilosa* isolates from Ethiopia, Kenya, Portugal, South Africa, Spain, Tanzania, western Australia, New South Wales and Victoria (eastern Australia, east of Melbourne). Lineage 2 also included the ex-epitype strain of *M. nubilosa* (CMW 3282) and isolates of *Mycosphaerella juvenis* Crous & M.J. Wingf. Lineage 1 could be distinguished from lineage 2 by four fixed base-pair polymorphisms, two transitions and two transversions, at nucleotide positions 13 (A/C), 37 (G/C), 255 (T/C) and 334 (C/T) (Table 2, Fig. 1).

Bt-2 phylogeny: The Bt-2 dataset consisted of a total of 348 characters and 56 taxa. Neighbour-joining analyses of the Bt-2 sequence data set resolved isolates of *M. nubilosa* into one main clade (Clade 1) supported with a bootstrap value of 99 %. However, isolates in clade 1 could be further separated into two sub-clades (Fig 2). *Mycosphaerella nubilosa* isolates accommodated in sub-clade 1 originated from New Zealand, Tasmania, western Australia, New South Wales and Victoria (eastern Australia). Sub-clade 1 was well supported with a bootstrap value of 84 % and the isolates residing in it could be differentiated from the *M. nubilosa* isolates in sub-clade 2 by a 3 base pair insertion at nucleotide position 191 (ACA/XXX) and a single base pair transition at position 215 (A/G) (Table 2, Fig. 2).

Interestingly, isolates of *M. nubilosa* from New South Wales (eastern Australia) (CMW 20469, CMW 23913, CMW 20468, CMW 32910, CMW 20467, CMW 23915) that were accommodated in *M. nubilosa* ITS lineage 2 of the ITS phylogeny, were accommodated in sub-clade 1 of the Bt-2 phylogeny along with isolates of *M. nubilosa* that were accommodated in *M. nubilosa* ITS lineage 1 of the ITS phylogeny (Fig. 1).

EF-1α phylogeny: A total of 291 characters and 55 taxa of the EF-1α sequence alignment were considered in the phylogenetic analysis. Following neighbour-joining analyses, the *M. nubilosa* isolates were not well resolved. All the *M. nubilosa* taxa were

accommodated in a single clade with a bootstrap value of 100 % (Fig. 3). All *M. nubilosa* isolates exhibited identical EF-1α sequences with no base pair polymorphisms (Table 2).

Combined phylogeny: Results from a Partition Homogeneity Tests (PHT), to determine if the ITS, Bt-2 and EF-1α datasets could be combined, gave a P-value of 1.000. Therefore the ITS, Bt-2 and EF-1α datasets were combined and a total of 980 characters and 53 taxa were considered for the phylogenetic analysis of this combined dataset. Following neighbour-joining analysis, the combined sequence data set could be resolved into two main clades (Fig. 4). Clade 1, supported with a bootstrap value of 93 % included *M. nubilosa* isolates from New Zealand, Tasmania, western Australia and Victoria (eastern Australia, west of Melbourne). Clade 2 was weakly supported with a bootstrap value of 59 % and contained *M. nubilosa* isolates from Spain, Tanzania, South Africa, Kenya, Portugal, western Australia, New South Wales and Victoria (eastern Australia, east of Melbourne) (Fig. 4).

For Parsimony analyses of the combined DNA sequence data set, 769 characters were constant, 159 characters were variable and parsimony uninformative and 52 characters were parsimony informative. Following heuristic searches of the combined DNA sequence dataset one most parsimonious tree was retained (Length = 53, CI = 0.981, RI = 0.990, RC = 0.972, HI = 0.019) (Fig. 5). This tree could be resolved into two main clades. Following 1000 bootstrap replicates, both clade 1 and clade 2 of the parsimony analyses were well supported with clade 1 having a support value of 75 % and contained *M. nubilosa* isolates from western Australia, New Zealand, Tasmania and Victoria (eastern Australia). Clade 2 was well supported with a bootstrap value of 83 % and contained the same *M. nubilosa* isolates as that for the neighbour-joining analyses. Both the neighbour-joining and parsimony phylogenetic trees had the same topologies which were also consistent with the topology of the ITS phylogram.

Growth in culture

Following 1 mo growth on MEA, isolates of *M. nubilosa* representing the two distinct ITS clades, ITS lineage 1 (*M. nubilosa* ex-epitype CMW 3282) and ITS lineage 2 (CMW 6518) had the same optimal growth temperature (Fig. 6). Isolate CMW 3282 had a colony diameter of 12 mm at 25 °C, which was the same as that of isolate CMW 6518. Isolate CMW 3282, did, however, exhibit more rapid growth at 15 and 20 °C than that of CMW 6518 (Fig. 6). At 15 and 20 °C, CMW 3282 had colony diameters of 8 mm and 11 mm respectively, while CMW 6518 had colonies of 6 and 9 mm at 15 and 20 °C degrees respectively.

Colonies of the *M. nubilosa* ex-epitype (CMW 3282) residing in ITS lineage 2 were circular with even to irregular margins; slight folding occurred with sectors of white mycelial growth, while aerial mycelium was sparse. Colonies pale greenish grey (surface) and olivaceous grey (reverse) (Rayner 1970). Colonies reaching a diameter of 12 mm after 1 mo growth on MEA (Fig. 7A).

In contrast, *M. nubilosa* isolate CMW 6518 residing in ITS lineage 1 had irregular colonies with uneven and irregular margins with folding and sectoring with predominant white mycelial tufts occurring as extensive aerial mycelium. Colonies pale greenish grey (surface) with prominent white mycelial tufts and pale olivaceous grey (reverse) (Rayner 1970). Colonies reaching a diameter of 12 mm after 1 mo growth on MEA (Fig. 7B). Older cultures of CMW 6518 do however change in their culture morphology and exhibit irregular margins with sparse aerial mycelium, colony centres are pale olivaceous grey (surface) and colony borders are darker than colony centres being olivaceous grey (surface) while being olivaceous grey (reverse) (Fig. 7C).

Another isolate (CMW 23911) residing in *M. nubilosa* ITS lineage 1, exhibited a slightly different culture morphology to that of CMW 6518 and CMW 3282. Here, CMW 23911 grew extremely slowly and only reaching a diameter of 2 mm on MEA at 25 °C after 1 mo. Older colonies of CMW 23911 had irregular margins, extensive folding and convolutions with sparse to no aerial mycelium, submerged mycelium. Colonies, olivaceous grey (surface) and iron grey (reverse) (Fig. 7D).

Microsatellite amplification

Amplification of the DNA for *M. nubilosa* ex-epitype isolate CMW 3282 (ITS lineage 2), with 10 microsatellite primers resulted in amplification products of the required size for all of the microsatellite regions (Fig. 8). However, amplification of DNA in *M. nubilosa* isolates CMW 6518 and CMW 23911 residing in ITS lineage 1, with the same primers resulted in the amplification of only eight (MN-1, MN-2, MN-3, MN-7, MN-9, MN-10, MN-11, MN-14) of the 10 microsatellite primers. Microsatellite primers MN-4 and MN-8 did not result in amplification for *M. nubilosa* isolates CMW 6518 and CMW 23911 from ITS lineage 1 (Fig. 8).

DISCUSSION

Results of this study have provided clear evidence that there is considerable variation amongst isolates of *M. nubilosa* from different parts of the world and from different hosts. This variation is best expressed in terms of two clear ITS lineages for the fungus. These lineages have definitive *Eucalyptus* host associations and geographic distributions. *Mycosphaerella nubilosa* ITS lineage 2 includes the ex-epitype isolate of the species, and by default should be treated as *M. nubilosa sensu stricto*. In contrast, isolates residing in the ITS lineage 1 might represent a discrete species but there are insufficient data presently available to describe it as a new taxon.

Isolates of *M. nubilosa* used in this study and representing the two ITS lineages exhibited slight differences in culture growth and morphology. Park & Keane (1982) described two types of culture morphology for *M. nubilosa*, one characterised by black, tightly packed, submerged mycelium and dense dark-green aerial hyphae and another characterised by diffuse submerged mycelium and white to olive-green aerial hyphae. Both of these culture morphologies were observed in the present study. The majority of *M. nubilosa* isolates in this study, however, exhibited white to olive green aerial hyphae. An isolate of *M. nubilosa* (CMW 23911) collected from Victoria, eastern Australia exhibited a different culture morphology, and produced dense dark-green cultures with sparse aerial mycelium, a morphology similar to that described by Park & Keane (1982).

Further evidence for the presence of intra-specific variation in *M. nubilosa* was shown through the amplification of microsatellite regions using primers developed for *M. nubilosa* (Hunter *et al*. 2006a). Results of this study indicate that the majority of the microsatellite primers developed for *M. nubilosa*, can also amplify their specific microsatellite region for isolates of *M. nubilosa* in ITS lineage 1. However, two microsatellite primers, MN-4 and MN-8, did not result in amplification products for *M. nubilosa* isolates from this ITS lineage. Furthermore, there appears to be size polymorphisms between the ex-epitype of *M. nubilosa* from ITS lineage 2 and two other *M. nubilosa* isolates from ITS lineage 1 in their amplification products with microsatellite primers MN-2, MN-7, MN-9. These microsatellite primers could, therefore, be used as a diagnostic tool to distinguish between *M. nubilosa* isolates residing in the two lineages defined in this study.

Phylogenies of *M. nubilosa* generated from the three gene regions used in this study were generally discordant with respect to each other. The ITS gene region contained the highest level of sequence polymorphisms for *M. nubilosa* isolates. The Bt-2 gene region had

fewer polymorphisms and, the EF-1α gene region exhibited no sequence polymorphisms in isolates of this fungus. The ITS phylogeny was consistent with results of Crous *et al.* (2004), who also showed the presence of two *M. nubilosa* clades. The present study, however, included a large number of isolates and thus provides additional support for two *M. nubilosa* lineages. From the combined phylogeny, it may seem that *M. nubilosa* contains two phylogenetic species. But, for this to be true, phylogenies from different loci would need to be concordant in tree topologies as suggested by Geiser *et al*. (1998) for species of *Fusarium* Link. Future studies should thus incorporate DNA sequence data from several more unlinked loci to determine if *M. nubilosa* ITS lineage 1 represents a cryptic taxon.

An important outcome of this study is the knowledge that the two *M. nubilosa* lineages defined in the ITS and combined phylogenies have unique hosts associations (Fig. 4). Isolates of *M. nubilosa* accommodated in ITS lineage 1 are known only to infect *E. globulus*. In contrast, *M. nubilosa* isolates from ITS Lineage 2 are known from *E. dunnii*, *E. globulus*, *E. maidenii*, *E. nitens* and several other *Eucalyptus* hosts. Such host specificity has been shown previously. For example *M. nubilosa* was isolated from *E. globulus* in Tasmania and not *E. nitens* (Dungey *et al*. 1997, Carnegie *et al*. 1998, Milgate *et al*. 2001, Carnegie & Ades 2002). This has also been observed in New Zealand where *M. nubilosa* was most commonly found on sub-species of *E. globulus* but not on *E. nitens* (Hood *et al*. 2002). Results of the present study, showing that isolates of *M. nubilosa* in ITS lineage 1 occur only on *E. globulus* and only in Tasmania and New Zealand, provide support for previous studies that suggested this association.

The distribution of the *M. nubilosa* ITS lineages observed in this study and their host associations may be explained in several ways. *Eucalyptus globulus* ssp. *globulus* is native to Tasmania, islands of the Bass Strait and isolated pockets in Victoria (Kirkpatrick 1974, Poynton 1979, Jordan *et al*. 1993). *M. nubilosa* ITS lineage 1 may originate in Tasmania on *E. globulus* ssp. *globulus*. This lineage may have been spread to New Zealand and Victoria, where it currently occurs on other *E. globulus* sub-species such as *E. globulus* ssp. *pseudoglobulus*, *E. globulus* ssp. *maidenii* and *E. globulus* ssp. *bicostata*. Isolates belonging to ITS lineage 1 may have further adapted to infect various other *Eucalyptus* spp such as *E. nitens*. As such, it could have evolved to form ITS lineage 2 that has now become common in Australia, Africa and Europe. Alternatively, two initial populations of *M. nubilosa* occurred, one that is native on *E. globulus* ssp. *globulus* in Tasmania and another that is able to infect several *Eucalyptus* spp. and native to Victoria and New South Wales. Over time, with the movement of *Eucalyptus* plant material from Tasmania to Victoria, sexual outcrossing

between these two populations could have occurred, yet retaining their close relationship. These hypotheses could be tested by population level studies employing microsatellite markers developed by Hunter *et al*. (2006a).

It is interesting that some isolates of *M. nubilosa* from New South Wales (eastern Australia) (CMW 20469, CMW 23913, CMW 20468, CMW 23910, CMW 20467, CMW 23915) that grouped in lineage 2 of the ITS phylogeny, grouped in sub-clade 1 of the Bt-2 phylogeny. This suggests that these isolates may represent a hybrid between isolates representing the *M. nubilosa* lineages. This would not be unusual as *M. nubilosa* ITS lineage 1 and ITS lineage 2 both infect *E. globulus*, which would provide an opportunity for mating and hybridisation to occur between the different groups.

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Table 1. Isolates of *Mycosphaerella nubilosa* used during this study for DNA sequencing analyses.

Isolate	Species	Host	Area	Collector	GenBank Accession No.		
					ITS	Bt-2	EF-1α
MURU 40	*M. nubilosa*	*E. globulus*	Tasmania, Australia	A. Maxwell	DQ923540	DQ992034	DQ974117
MURU 42	*M. nubilosa*	*E. globulus*	Tasmania, Australia	A. Maxwell	DQ923541	DQ992035	DQ974118
MURU 46	*M. nubilosa*	*E. globulus*	Tasmania, Australia	A. Maxwell	DQ923542	DQ992036	DQ974119
MURU 52	*M. nubilosa*	*E. globulus*	Victoria, eastern Australia	A. Maxwell	DQ923543	DQ992037	DQ974120
MURU 64	*M. nubilosa*	*E. globulus*	Victoria, eastern Australia	A. Maxwell	DQ923544	DQ992039	DQ974122
CMW 23911	*M. nubilosa*	*E. globulus*	Victoria, eastern Australia	A.J. Carnegie	DQ923552	DQ992049	DQ974151
CMW 3282	*M. nubilosa*	*E. globulus*	Victoria, eastern Australia	A.J. Carnegie	AF309618	DQ992068	DQ235111
CMW 4042	*M. nubilosa*	*E. globulus*	Western Australia	A. Maxwell	DQ923546	DQ992041	DQ974124
CMW 4053	*M. nubilosa*	*Eucalyptus* sp.	Tasmania, Australia	A.W. Milgate	DQ923545	DQ992040	DQ974123
CMW 6518	*M. nubilosa*	*E. globulus*	Dartmoon, Victoria, eastern Australia	P. Barber	DQ923551	DQ992051	DQ974145
CMW 14928	*M. nubilosa*	*Eucalyptus* sp.	New Zealand	M.J. Wingfield	DQ923548	DQ992043	DQ974126
CMW 14930	*M. nubilosa*	*Eucalyptus* sp.	New Zealand	M.J. Wingfield	DQ923547	DQ992042	DQ974125
CMW 12548	*M. nubilosa*	*E. globulus*	Spain	J.P.M. Vasquez	DQ923570	DQ992067	DQ974127
CMW 12594	*M. nubilosa*	*E. globulus*	Spain	J.P.M. Vasquez	DQ923568	DQ992058	DQ974128
CMW 12600	*M. nubilosa*	*E. globulus*	Spain	J.P.M. Vasquez	DQ923569	DQ992065	DQ974129
CMW 18702	*M. nubilosa*	*E. nitens*	South Africa	G.C. Hunter	DQ923572	DQ992060	DQ974130
CMW 18796	*M. nubilosa*	*E. globulus*	Portugal	M.J. Wingfield	DQ923565	DQ992055	DQ974131
CMW 18805	*M. nubilosa*	*E. globulus*	Portugal	M.J. Wingfield	DQ923567	DQ992053	DQ974132
CMW 18828	*M. nubilosa*	*E. globulus*	Portugal	M.J. Wingfield	DQ923566	DQ992061	DQ974133
CMW 20207	*M. nubilosa*	*E. nitens*	South Africa	G.C. Hunter	DQ923571	DQ992056	DQ974134

Isolate	Species	Host	Area	Collector	GenBank Accession No.		
					ITS	Bt-2	EF-1α
CMW 20467	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923549	DQ992045	DQ974139
CMW 20468	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923550	DQ992047	DQ974140
CMW 20469	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923577	DQ992044	DQ974141
CMW 21518	*M. nubilosa*	*E. nitens*	South Africa	G.C. Hunter	DQ923576	DQ992064	DQ974135
CMW 21603	*M. nubilosa*	*E. globulus*	Tanzania	J. Roux	DQ923563	DQ992070	DQ974136
CMW 21619	*M. nubilosa*	*E. globulus*	Tanzania	J. Roux	DQ923564	DQ992062	DQ974137
CMW 21633	*M. nubilosa*	*E. globulus*	Tanzania	J. Roux	DQ923562	DQ992066	DQ974138
CMW 22371	*M. nubilosa*	*E. globulus*	Western Australia	A. Maxwell	DQ923573	DQ992054	DQ974142
CMW 22381	*M. nubilosa*	*E. globulus*	Western Australia	A. Maxwell	DQ923574	DQ992057	DQ974143
CMW 22398	*M. nubilosa*	*E. globulus*	Western Australia	A. Maxwell	DQ923575	DQ992059	DQ974152
CMW 23908	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923556	DQ992063	N/A
CMW 23909	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923555	DQ992071	DQ974148
CMW 23910	*M. nubilosa*	*E. dunnii*	New South Wales, eastern Australia	A.J. Carnegie	DQ923558	DQ992046	DQ974154
CMW 23913	*M. nubilosa*	*E. dunnii*	New South Wales, eastern Australia	A.J. Carnegie	DQ923553	DQ992050	DQ974149
CMW 23912	*M. nubilosa*	*E. dunnii*	New South Wales, eastern Australia	A.J. Carnegie	DQ923554	N/A	N/A
CMW 23914	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923559	DQ992052	DQ974155
CMW 23915	*M. nubilosa*	*E. dunnii*	New South Wales, eastern Australia	A.J. Carnegie	DQ923560	DQ992048	DQ974146
CMW 23916	*M. nubilosa*	*E. nitens*	New South Wales, eastern Australia	A.J. Carnegie	DQ923561	DQ992069	DQ974147
CMW 23907	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923557	N/A	DQ974150

Table 2. Fixed polymorphisms observed between *Mycosphaerella nubilosa* isolates for the Internal Transcribed Spacer (ITS) and Beta-tubulin (Bt-2) gene regions. No polymorphisms were observed between *M. nubilosa* for the Translation Elongation Factor (EF-1α). X = deletion.

Locus	**Base pair fixed polymorphisms**			
ITS	**13**	**37**	**255**	**334**
	A/C	G/C	T/C	C/T
Bt-2	**191–201**	**215**	-	-
	ACA/XXX	A/G		
EF-1α	-	-	-	-

Figure 1. Neighbour-joining phylogram obtained from a distance analysis using the HKY substitution model on ITS sequence data of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are shown above branches. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups. Fixed sequence polymorphisms, separating clade 1 from clade 2, and their base pair positions are indicated at dotted line.

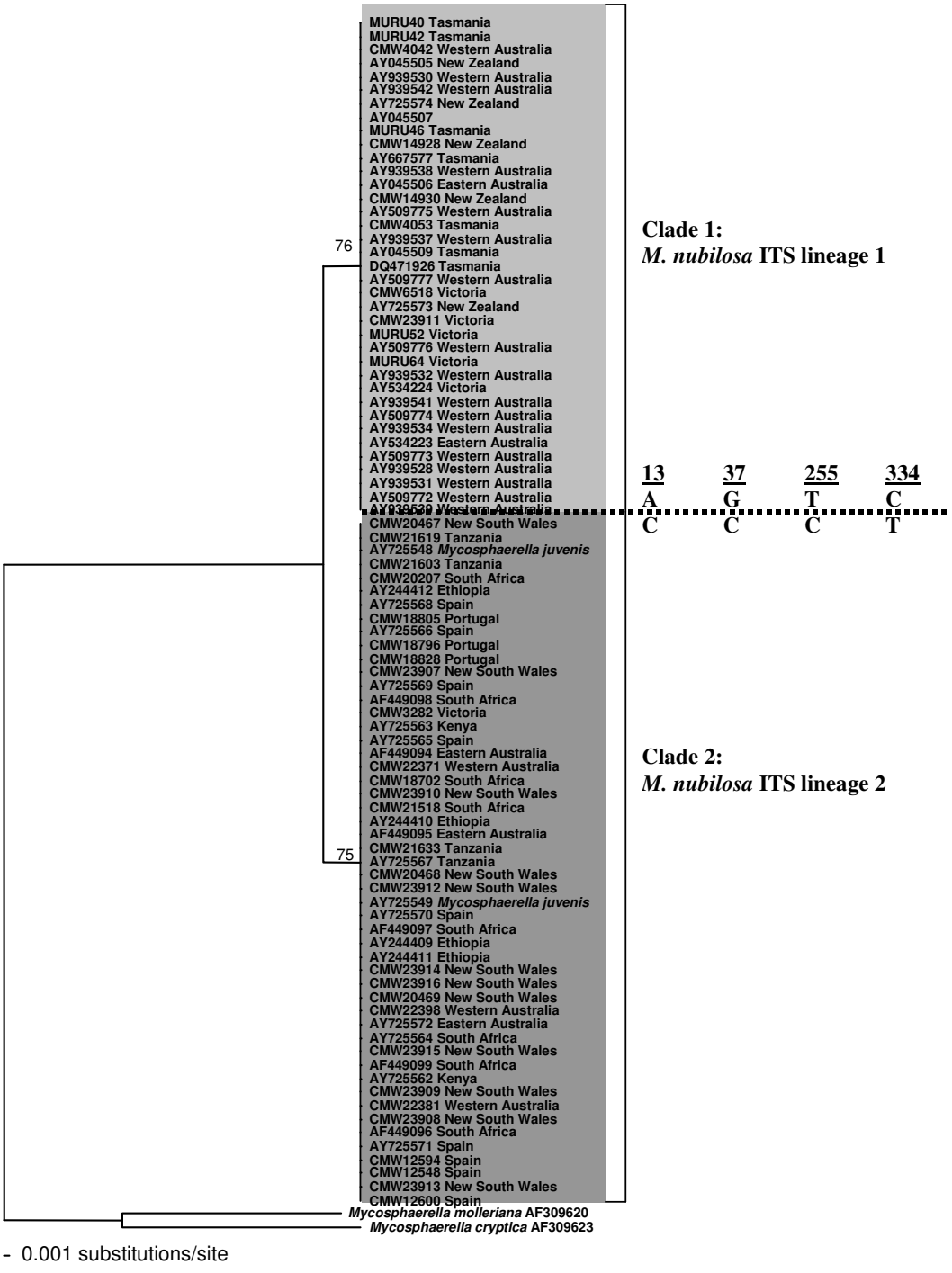
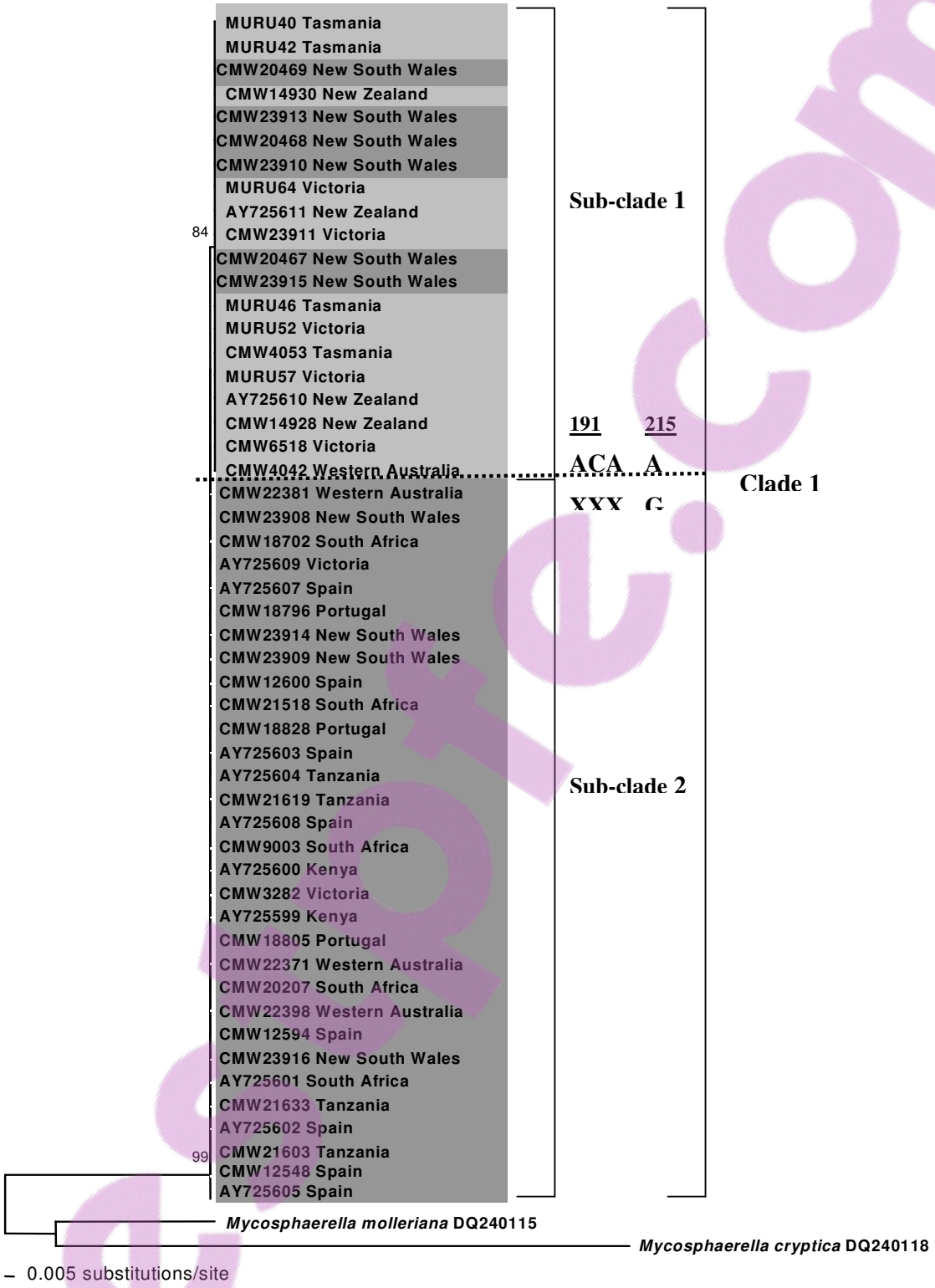

MURU40 Tasmania
MURU42 Tasmania
CMW4042 Western Australia
AY045505 New Zealand
AY939530 Western Australia
AY939542 Western Australia
AY725574 New Zealand
AY045507
MURU46 Tasmania
CMW14928 New Zealand
AY667577 Tasmania
AY939538 Western Australia
AY045506 Eastern Australia
CMW14930 New Zealand
AY509775 Western Australia
CMW4053 Tasmania
AY939537 Western Australia
76
AY045509 Tasmania
DQ471926 Tasmania
AY509777 Western Australia
CMW6518 Victoria
AY725573 New Zealand
CMW23911 Victoria
MURU52 Victoria
AY509776 Western Australia
MURU64 Victoria
AY939532 Western Australia
AY534224 Victoria
AY939541 Western Australia
AY509774 Western Australia
AY939534 Western Australia
AY534223 Eastern Australia
AY509773 Western Australia
AY939528 Western Australia
AY939531 Western Australia
AY509772 Western Australia
CMW20467 New South Wales
CMW21619 Tanzania
AY725548 Mycosphaerella juvenis
CMW21603 Tanzania
CMW20207 South Africa
AY244412 Ethiopia
AY725568 Spain
CMW18805 Portugal
AY725566 Spain
CMW18796 Portugal
CMW18828 Portugal
CMW23907 New South Wales
AY725569 Spain
AF449098 South Africa
CMW3282 Victoria
AY725563 Kenya
AY725565 Spain
AF449094 Eastern Australia
CMW22371 Western Australia
CMW18702 South Africa
CMW23910 New South Wales
CMW21518 South Africa
AY244410 Ethiopia
AF449095 Eastern Australia
75
CMW21633 Tanzania
AY725567 Tanzania
CMW20468 New South Wales
CMW23912 New South Wales
AY725549 Mycosphaerella juvenis
AY725570 Spain
AF449097 South Africa
AY244409 Ethiopia
AY244411 Ethiopia
CMW23914 New South Wales
CMW23916 New South Wales
CMW20469 New South Wales
CMW22398 Western Australia
AY725572 Eastern Australia
AY725564 South Africa
CMW23915 New South Wales
AF449099 South Africa
AY725562 Kenya
CMW23909 New South Wales
CMW22381 Western Australia
CMW23908 New South Wales
AF449096 South Africa
AY725571 Spain
CMW12594 Spain
CMW12548 Spain
CMW23913 New South Wales
CMW12600 Spain
Mycosphaerella molleriana AF309620
Mycosphaerella cryptica AF309623
- 0.001 substitutions/site
Clade 1:
M. nubilosa ITS lineage 1
13 37 255 334
A G T C
C C C T
Clade 2:
M. nubilosa ITS lineage 2

Figure 2. Neighbour-joining phylogram obtained from a distance analysis using the GTR + I substitution model on Bt-2 sequence data of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are shown above branches. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups. Fixed sequence polymorphisms, separating sub-clade 1 from clade 1, and their base pair positions are indicated at dotted line. **X** = deletion.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MURU42 Tasmania
CMW20469 New South Wales
CMW14930 New Zealand
CMW23913 New South Wales
CMW20468 New South Wales
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MURU64 Victoria
AY725611 New Zealand
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CMW23911 Victoria
CMW20467 New South Wales
CMW23915 New South Wales
MURU46 Tasmania
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CMW23908 New South Wales
CMW18702 South Africa
AY725609 Victoria
AY725607 Spain
CMW18796 Portugal
CMW23914 New South Wales
CMW23909 New South Wales
CMW12600 Spain
CMW21518 South Africa
CMW18828 Portugal
AY725603 Spain
AY725604 Tanzania
CMW21619 Tanzania
AY725608 Spain
CMW9003 South Africa
AY725600 Kenya
CMW3282 Victoria
AY725599 Kenya
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CMW22371 Western Australia
CMW20207 South Africa
CMW22398 Western Australia
CMW12594 Spain
CMW23916 New South Wales
AY725601 South Africa
CMW21633 Tanzania
AY725602 Spain
99
CMW21603 Tanzania
CMW12548 Spain
AY725605 Spain
Sub-clade 1
191
215
ACA A
XXX G
Clade 1
Sub-clade 2
Mycosphaerella molleriana DQ240115
Mycosphaerella cryptica DQ240118
0.005 substitutions/site

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Figure 3. Neighbour-joining phylogram obtained from a distance analysis using the TrNef + I substitution model on EF-1α sequence data of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are shown above branches. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups.

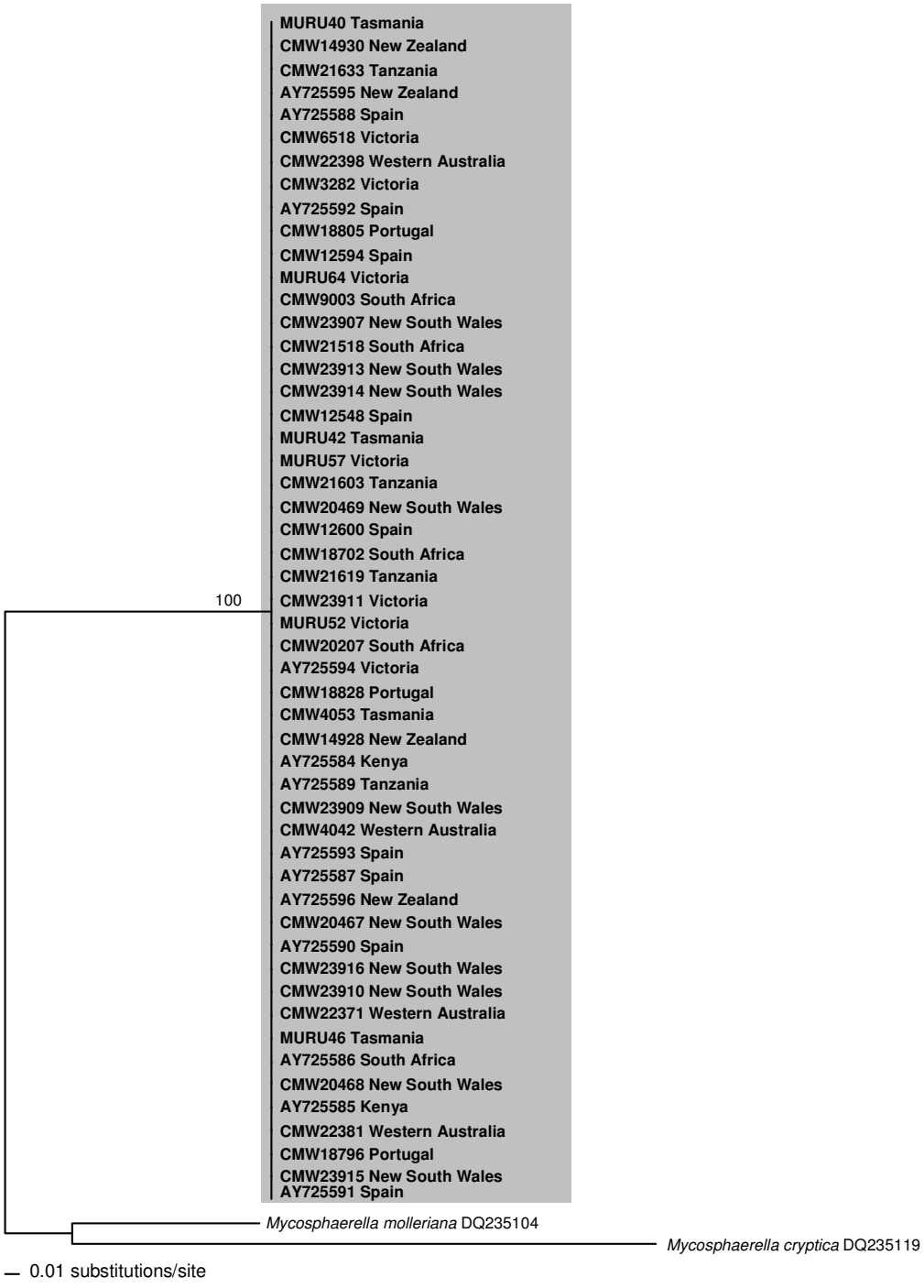

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Figure 4. Neighbour-joining phylogram obtained through distance analysis using the TIM substitution model on the combined ITS, Bt-2 and EF-1α DNA sequence datasets of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are indicated above branches or by arrows. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups. *Eucalyptus* host associations of the *M. nubilosa* isolates are indicated on the right of the phylogram.

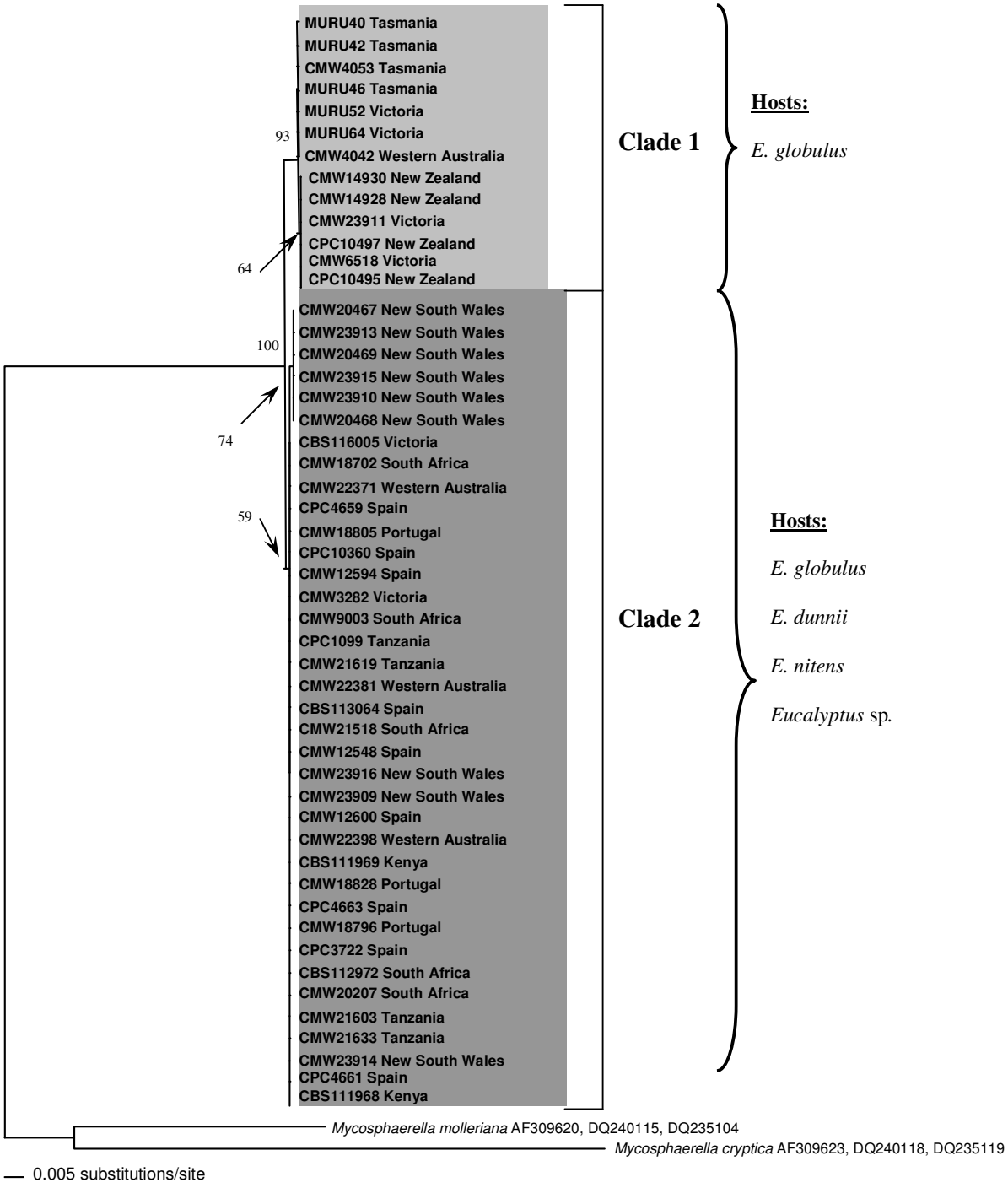

MURU40 Tasmania
MURU42 Tasmania
CMW4053 Tasmania
MURU46 Tasmania
MURU52 Victoria
MURU64 Victoria
CMW4042 Western Australia
CMW14930 New Zealand
CMW14928 New Zealand
CMW23911 Victoria
CPC10497 New Zealand
CMW6518 Victoria
CPC10495 New Zealand
CMW20467 New South Wales
CMW23913 New South Wales
CMW20469 New South Wales
CMW23915 New South Wales
CMW23910 New South Wales
CMW20468 New South Wales
CBS116005 Victoria
CMW18702 South Africa
CMW22371 Western Australia
CPC4659 Spain
CMW18805 Portugal
CPC10360 Spain
CMW12594 Spain
CMW3282 Victoria
CMW9003 South Africa
CPC1099 Tanzania
CMW21619 Tanzania
CMW22381 Western Australia
CBS113064 Spain
CMW21518 South Africa
CMW12548 Spain
CMW23916 New South Wales
CMW23909 New South Wales
CMW12600 Spain
CMW22398 Western Australia
CBS111969 Kenya
CMW18828 Portugal
CPC4663 Spain
CMW18796 Portugal
CPC3722 Spain
CBS112972 South Africa
CMW20207 South Africa
CMW21603 Tanzania
CMW21633 Tanzania
CMW23914 New South Wales
CPC4661 Spain
CBS111968 Kenya
93
64
100
74
59
Clade 1
Clade 2
Hosts:
E. globulus
Hosts:
E. globulus
E. dunnii
E. nitens
Eucalyptus sp.
Mycosphaerella molleriana AF309620, DQ240115, DQ235104
Mycosphaerella cryptica AF309623, DQ240118, DQ235119
0.005 substitutions/site

Figure 5. Parsimony phylogram of the combined ITS, Bt-2 and EF-1α sequence datasets of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are indicated below branches and branch lengths are indicated above branches. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups.

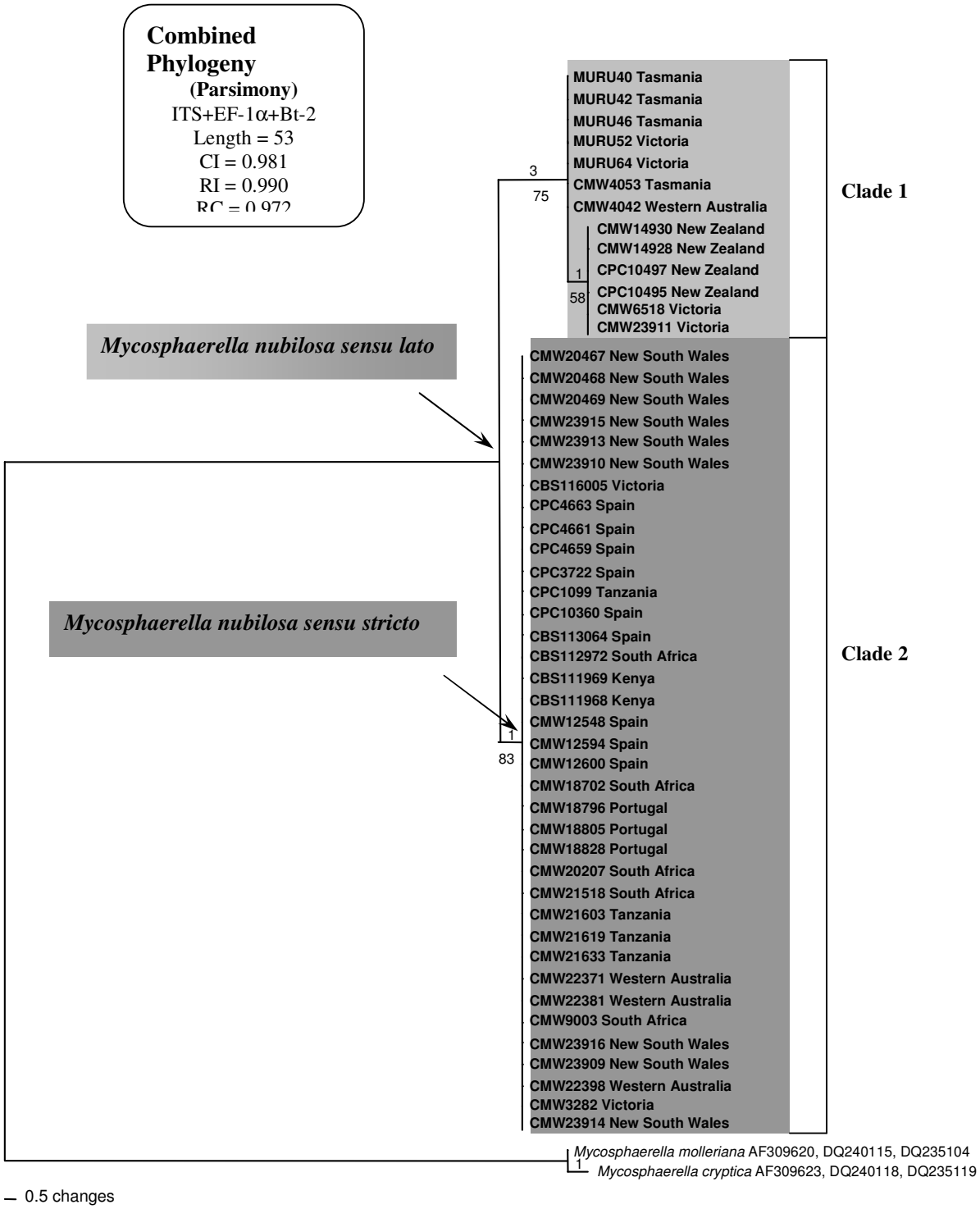
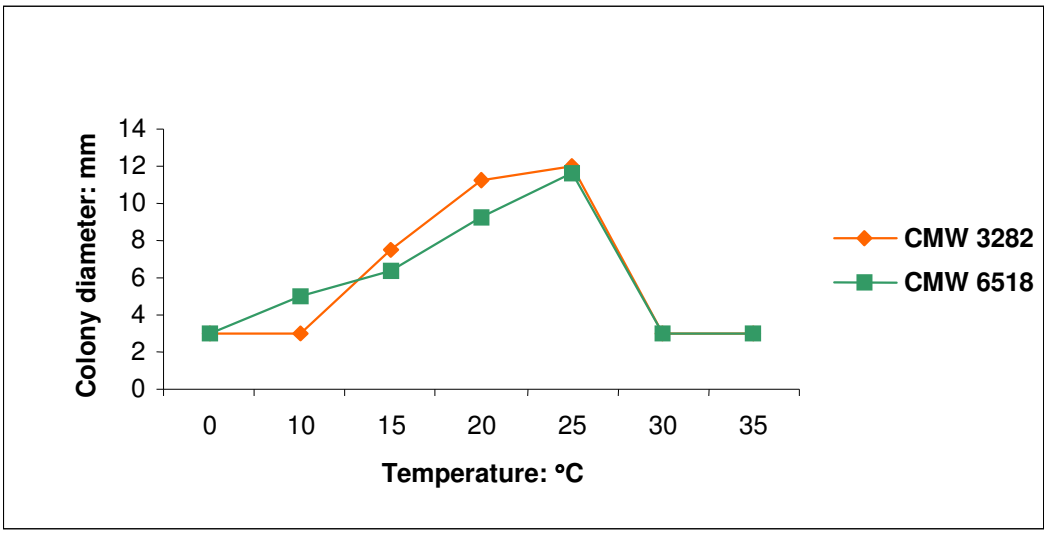

Combined Phylogeny
(Parsimony)
ITS+EF-1α+Bt-2
Length = 53
CI = 0.981
RI = 0.990
RC = 0.972
MURU40 Tasmania
MURU42 Tasmania
MURU46 Tasmania
MURU52 Victoria
MURU64 Victoria
CMW4053 Tasmania
CMW4042 Western Australia
CMW14930 New Zealand
CMW14928 New Zealand
CPC10497 New Zealand
CPC10495 New Zealand
CMW6518 Victoria
CMW23911 Victoria
3
75
1
58
Clade 1
Mycosphaerella nubilosa sensu lato
CMW20467 New South Wales
CMW20468 New South Wales
CMW20469 New South Wales
CMW23915 New South Wales
CMW23913 New South Wales
CMW23910 New South Wales
CBS116005 Victoria
CPC4663 Spain
CPC4661 Spain
CPC4659 Spain
CPC3722 Spain
CPC1099 Tanzania
CPC10360 Spain
CBS113064 Spain
CBS112972 South Africa
CBS111969 Kenya
CBS111968 Kenya
CMW12548 Spain
CMW12594 Spain
CMW12600 Spain
CMW18702 South Africa
CMW18796 Portugal
CMW18805 Portugal
CMW18828 Portugal
CMW20207 South Africa
CMW21518 South Africa
CMW21603 Tanzania
CMW21619 Tanzania
CMW21633 Tanzania
CMW22371 Western Australia
CMW22381 Western Australia
CMW9003 South Africa
CMW23916 New South Wales
CMW23909 New South Wales
CMW22398 Western Australia
CMW3282 Victoria
CMW23914 New South Wales
1
83
Clade 2
Mycosphaerella nubilosa sensu stricto
Mycosphaerella molleriana AF309620, DQ240115, DQ235104
1
Mycosphaerella cryptica AF309623, DQ240118, DQ235119
0.5 changes

Figure 6. Results of growth studies on *Mycosphaerella nubilosa* isolates (CMW 3282 ex-epitype, CMW 6518) after 1 mo growth on 2 % MEA at temperatures from 0 °C to 35 °C. CMW 3282 = ITS Lineage 2 and *M. nubilosa* ex-epitype, CMW 6518 = ITS Lineage 1.

Figure 7. Culture morphology of *Mycosphaerella nubilosa* isolates on 2 % MEA. (**A**) CMW 3282 following 1 mo growth on 2 % MEA, (**B**) CMW 6518 following 1 mo growth on 2 % MEA, (**C**) Older culture growth of CMW 6518 following 3 mo growth on 2 % MEA. (**D**) CMW 23911 following 3 mo growth on 2 % MEA.


Colony diameter: mm
14
12
10
8
6
4
2
0
0
10
15
20
25
30
35
Temperature: °C
CMW 3282
CMW 6518

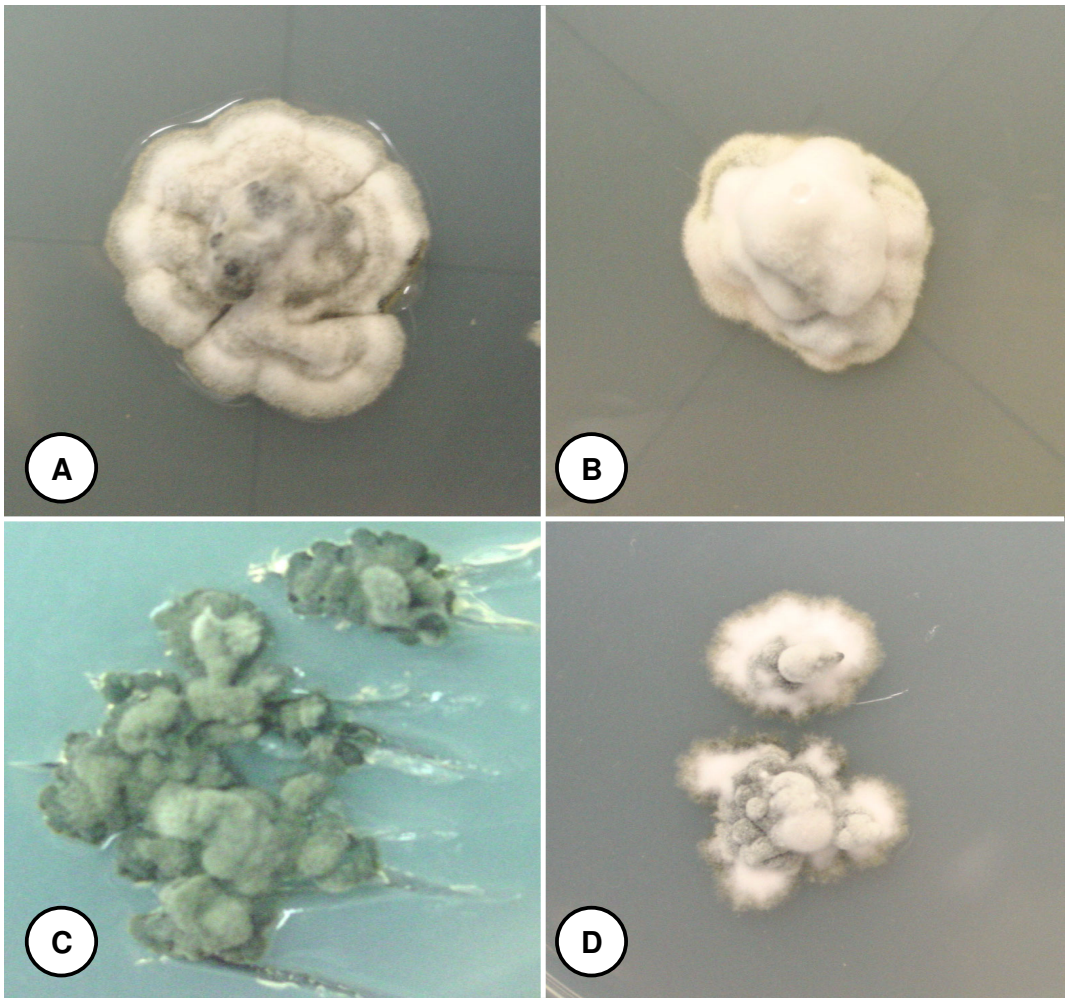
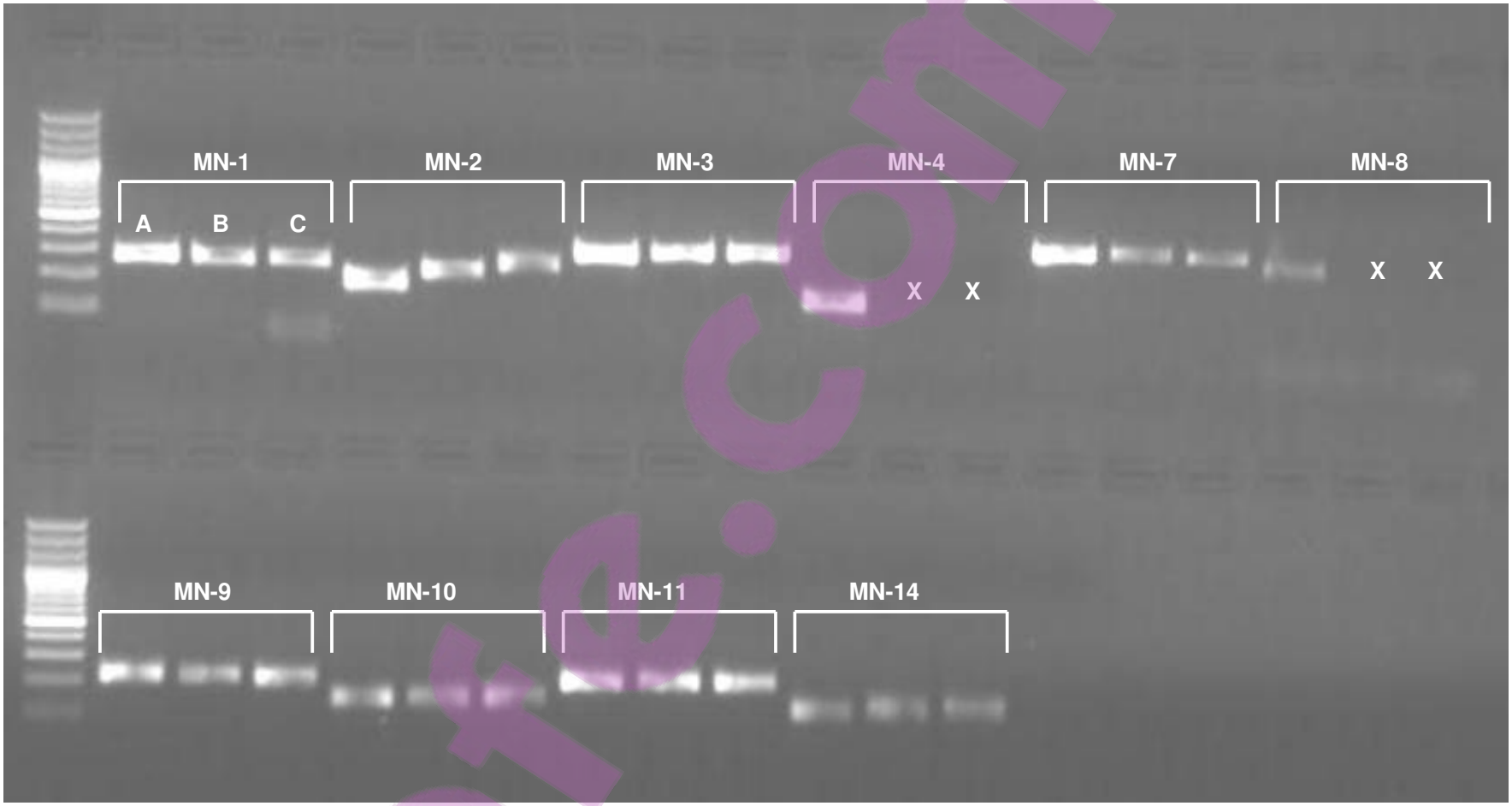

A
B
C
D

Figure 8. 1.5 % Agarose gel indicating the amplification of microsatellite regions for isolates of *Mycosphaerella nubilosa* using microsatellite primers developed by Hunter *et al*. (2006a). Lane **A** = CMW 3282, ITS Lineage 2 and *M. nubilosa* ex-epitype, Lane **B** = CMW 6518, ITS Lineage 1, Lane **C** = CMW 23911, ITS Lineage 1.


MN-1
A
B
C
MN-2
MN-3
MN-4
X
X
MN-7
MN-8
X
X
MN-9
MN-10
MN-11
MN-14