

Taxonomy, phylogeny and population biology of *Mycosphaerella* species occurring on *Eucalyptus*

By

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Declaration

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Philosophiae Doctor to the University of Pretoria contains my own independent work. This work has hitherto not been submitted for any degree at any other University

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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

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.

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.