Taxonomy, phylogeny and population biology of *Ceratocystis* species with particular reference to *Ceratocystis fimbriata*

BY

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B.Sc. Hons. (U. P.)

Submitted in partial fulfillment of the requirements for the degree

Magister Scientiae

in the Faculty of Natural and Agricultural Sciences, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa

February 2002

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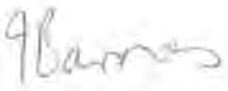
DECLARATION

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



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February 2002

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I dedicate this thesis to my loving mother

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to the following people:

My gratitude to Mike and Brenda Wingfield who welcomed me into their research programme, always had open doors and who have shown me how exciting and rewarding science can be.

I thank Dr Treena Burgess for introducing me into the world of microsatellites and population genetics. Thank you for always being enthusiastic and showing a keen interest in my work.

Dr Jolanda Roux for all your advice and guidance throughout this study and the many hours spent editing the manuscript.

To many members of the Fabulous Fabi Family, my friends, for sharing knowledge, giving inspiration and always providing me with moral support.

My husband, Kevin Barnes, who has unselfishly supported me throughout my entire degree, who has kept me company on many late nights and without whose love and understanding this thesis would not have been possible.

My sister, Karen Vincent, whose help in all the small matters have made an enormous difference. No one could ask for a better friend!

I would also like to thank the Tree Pathology Co-operative Program (TPCP), National Research Foundation (NRF) and University of Pretoria for providing financial support.

The Lord Jesus for giving me strength.

SUMMARY

Ceratocystis species represent some of the most important plant pathogens, attacking both agricultural and forestry crops. The research presented in this thesis focused on *C. fimbriata* and two closely related species, of which one is new to science. A summary of the literature regarding *Ceratocystis* spp., and specifically *C. fimbriata*, is presented in the first chapter. This chapter laid the foundation for the subsequent chapters by identifying various questions that need to be answered. For example, it has been suggested that *C. fimbriata* constitutes a species complex. Morphology has, however, not been sufficient to separate these species. The need to utilize more powerful tools to study the population structures within this taxon, and identify possible cryptic species, emerged from this review.

In the second chapter of this thesis, a study in which 11 polymorphic PCR-based microsatellite markers were designed for use on isolates of *C. fimbriata,* is presented. These markers amplified microsatellite regions within a number of different isolates from a wide geographical area and host range. These markers will, in future, be valuable for population studies in *C. fimbriata*. The same isolates used to develop and test the markers were sequenced for comparative purposes. The phylograms constructed from both data sets, separated isolates of *C. fimbriata* into distinct clades that correspond to geographic locations and host range. These data support the hypothesis that *C. fimbriata* constitutes a complex of morphologically similar species.

The 11 microsatellite markers were used to study three populations of *C. fimbriata,* in a study presented in chapter three. These populations originated from Congo, Colombia and Uruguay. All showed a high level of genetic diversity, with the Colombian population being the most diverse. The populations were also highly structured with minimal gene flow occurring between them. Tests to determine the mode of reproduction indicated little evidence for recombination within the homothallic *C. fimbriata* populations. Distance analysis showed that of the three populations, Congo and Uruguay, which were both from *Eucalyptus* spp., were the most closely related. Some isolates within the Congo population showed little genetic differentiation between those from Uruguay and Colombia. Based on a wide host range, high genetic diversity, and genetic similarity with isolates from Congo, Latin America is considered the likely center of origin for *C. fimbriata.*

Ceratocystis albofundus is morphologically and phylogenetically the species most closely related to *C. fimbriata*. A population of *C. albofundus* from South Africa was, therefore, chosen to test whether the microsatellite markers for *C. fimbriata* could be used across species boundaries. In chapter four, I show that eight of the markers successfully amplified regions within *C. albofundus*. Data from these markers indicated a very high genetic diversity within the population, despite its predominantly clonal mode of reproduction. The population also showed limited structure, with many of the same genotypes present within different geographical locations. The high diversity observed within the population, supports previous studies suggesting that *C. albofundus* is native to South Africa.

Recently, a serious wilt disease of pruned *Eucalyptus grandis* was reported in Uruguay. Studies in Chapter Five considered the cause of this disease. Morphological studies identified the causal agent as *Ceratocystis fimbriata*. Sequence comparisons of the ITS regions, with other *Ceratocystis* spp., placed the fungus from Uruguay amongst other isolates of *C. fimbriata*. This investigation presents a new report of *C. fimbriata* infecting *E. grandis* in Uruguay.

An artificial wounding trial was conducted on *E. nitens* in Australia to bait for *Ceratocystis* spp. The perithecia of the fungus collected from the wounds were typical of *Ceratocystis* spp. The fungus has hat-shaped ascospores, but has a unique pear-shaped perithecial base. Molecular evidence based on sequence data of the ITS regions, confirmed its unique nature. This fungus can, therefore, be distinguished from other *Ceratocystis* spp. based on morphological and DNA sequence data. In Chapter Six, this fungus is, therefore, designated as a new member of this genus and it is provided with the name *Ceratocystis pirilliformans*. It is phylogenetically most closely related to *C. fimbriata* and *C. albofundus*

OPSOMMING

Ceratocystis spesies sluit belangrike plant patogene in en veroorsaak siektes in beide die landbou- en bosbousindustrieë. Die navorsing in hierdie verhandeling fokus op die belangrike plant patogeen *C. fimbriata* en twee verwante spesies, waarvan een nuut is tot die wetenskap. Die verhandeling begin met 'n opsomming van die beskikbare literatuur oor *Ceratocystis* spesies in die algemeen, met 'n spesifieke fokus op *C. fimbriata*. Hierdie hoofstuk lê die grondslag vir die ander hoofstukke in die verhandeling, deur spesifieke leemtes en probleme in die algemene taksonomie en populasiebiologie van *C. fimbriata* aan te spreek. Van spesifieke belang hier is die moontlike bestaan van 'n kriptiese spesie binne *C. fimbriata*. Morfologiese data alleen kon nie hierdie spesie beskryf nie en die literatuurbespreking wys die belang van meer kragtige tegnieke om die populasies binne *C. fimbriata* te bestudeer uit. Hierdie molekulêre tegnieke sal ook van groot belang wees in die beskrywing van die kriptiese spesie binne *C. fimbriata*.

In Hoofstuk 2 word die ontwikkeling van 11 PKR-gebaseerde polimorfiese mikrosatellietmerkers vir *C. fimbriata* beskryf. Hierdie merkers amplifiseer mikrosatellietgebiede in verskillende *C. fimbriata* isolate. Dit is duidelik vanuit die navorsing in hierdie hoofstuk dat die mikrosatellietmerkers gebruik kan word vir populasiestudies vir 'n wye reeks van isolate. DNS - basispaaropeenvolging bepaling is gebruik om die resultate tussen die verskillende tegnieke te vergelyk. Resultate toon duidelik dat *C. fimbriata* isolate geskei kan word op grond van hulle geografiese oorsprong en gashere. Hierdie data ondersteun die hipotese dat *C. fimbriata* uit 'n kompleks van morfologies soortgelyke spesies bestaan.

Die 11 merkers, ontwikkel in Hoofstuk 2, is in die volgende hoofstuk gebruik om *C. fimbriata* populasies vanaf Kongo, Kolombië en Uruguay te vergelyk. Al drie populasies het 'n hoe hoeveelheid genetiese variasie getoon. Die isolate uit Kolombië toon die mees variasie. Die populasies was ook hoogs gestruktureerd, met minimale geenvloei tussen hulle. Analises om die voortplantingstrategieë van die populasies van die homotalliese *C. fimbriata* te ondersoek het min bewys vir rekombinasie getoon. Afstandsanalises het getoon dat die populasies vanaf Kongo en Uruguay, beide vanaf *Eucalyptus* spp., meer eenvormig is as aan die Kolombiaanse populasie. Sommige isolate in die Kongolese populasie het min verskille met die van Uruguay en Kolombië getoon, wat op 'n moontlike Suid-Amerikaanse oorsprong vir die fungus in Kongo dui. Die wye gasheerreeks en hoë

genetiese diversiteit binne Suid-Amerika dui sterk daarop dat *C. fimbriata* inheems aan daardie kontinent is.

Ceratocystis albofundus is geneties en morfologies die mees naasverwante spesie aan *C. fimbriata*. 'n Populasie van *C. albofundus* vanaf Suid-Afrika is in Hoofstuk 4 getoets om te bepaal of die mikrosatellietmerkers ontwikkel vir *C. fimbriata* oor spesiegrense kan werk. In hierdie hoofstuk is getoon dat 8 van die 11 merkers wat op *C. fimbriata* werk, doeltreffend mikrosatelliet areas in die *C. albofundus* genoom amplifiseer. Data verkry vanuit die populasiestudie met die 8 suksesvolle merkers toon dat daar 'n hoë diversiteit in die Suid-Afrikaanse populasie is ten spyte van die hoofsaaklik klonale voortplanting van *C. albofundus*. Die populasie het ook min struktuur getoon, met die meerderheid van die genotipes teenwoording binne al die verskillende geografiese gebiede waaruit die populasie versamel is. Die hoë diversiteit, soos bepaal met die mikrosatellietmerkers, bevestig vorige studies wat voorstel dat *C. albofundus* inheems aan Suid-Afrika is.

'n Ernstige verwelksiekte is onlangs op *E. grandis* in Uruguay aangemeld. In Hoofstuk 5 word die oorsaak van hierdie siekte ondersoek. Morfologiese studies van die geïsoleerde fungus het getoon dat die siekte deur *C. fimbriata* veroorsaak word. DNS-basispaarstudies van die 'ITS' gebied het die patogeen saam met *C. fimbriata* isolate vanaf ander gashere en geografiese gebiede geplaas. Hierdie studie verteenwoordig die eerste vernmelding van *C. fimbriata* as 'n patogeen op *Eucalyptus* spp. in Uruguay.

In die laaste hoofstuk van hierdie verhandeling word 'n wondingsproef op *E. nitens* in Australië bespreek. Die doel van hierdie eksperiment was om *Ceratocystis* spesies te isoleer. 'n Fungus met tipiese *Ceratocystis* perithesia en hoedvormige askospore is vanuit die wonde geisoleer. Die perithesia van hierdie *Ceratocystis* sp. het egter unieke peervormige basisse. Molekulêre studies van die 'ITS' gebiede van die RNS operon het bevestig dat die fungus 'n n uwe spesie binne *Ceratocystis* verteenwoordig. In Hoofstuk 6 word hierdie fungus as 'n nuwe spesie van *Ceratocystis*, naamlik *C. pirilliformis*, beskryf. Dit is naas verwant aan *C. fimbriata* en *C. albofundus*, beide op molekulêre en morfologiese vlakke.

PREFACE

The genus *Ceratocystis* forms part of the larger group of fungi broadly known as the as the Ophiostomatoid fungi. In contrast to biologically similar *Ophiostoma* spp., which resides in the Ophiostomatales, *Ceratocystis* is phylogenetically distinct and is related to members of the Microascales. *Ceratocystis* includes some of the most devastating plant and tree pathogens in the world and has been reported from all continents except Antarctica. In plantation forestry, *C. fimbriata* is a serious pathogen of *Eucalyptus* spp. and *C. albofundus* causes severe damage to *Acacia mearnsii*.

Ceratocystis fimbriata has been studied extensively over the past century, and research has focused on the biology, taxonomy, ecology, pathogenicity and toxin production of the pathogen. Evidence from these studies suggests that *C. fimbriata* comprises a number of host-specific strains representing a complex of different species. A small number of studies have been conducted using molecular sequence data to determine the phylogenetic placement of *C. fimbriata* amongst other *Ceratocystis* spp. However, very little research has been conducted in determining population structure, diversity and the relatedness of populations.

In the first chapter of this thesis, a broad review of the literature for *Ceratocystis* and particularly *C. fimbriata* is given. This review includes views on the taxonomy of *C. fimbriata* and various aspects of its biology. In addition, I provide details of molecular data to define the relationships amongst *Ceratocystis* species. This section clearly emphasizes the lack of studies pertaining to the recognition and identification of cryptic species and populations within *C. fimbriata*. Some of these questions are addressed in succeeding chapters.

Microsatellites are considered to be ideal markers for population investigations. This is because they are associated with a high level of polymorphism and can distinguish between individuals within a species. This makes microsatellites a preferred choice for the use in studying populations. The development of microsatellite markers for *C. fimbriata* is described in chapter two. These markers were tested on a number of different strains of *C. fimbriata* from different geographic areas. Relationships between isolates of *C. fimbriata* inferred using the microsatellite markers were further tested with sequence data.

Populations, as a unit, undergo evolution. It is thus necessary for plant pathologists to study the genetics of pathogens at the population level. The study of pathogen populations can provide valuable information for the development of management strategies, to reduce the impact of pathogens. By studying levels of genetic diversity amongst and within populations, deductions as to the possible origin, structure, migration, mode of reproduction and evolutionary relationships of pathogens can be made. In chapter three, the microsatellite markers developed in chapter two were used to infer population structures and diversities within and between three populations of *C. fimbriata* from Congo, Colombia and Uruguay.

Although microsatellite markers are constructed to be species specific, they can sometimes be used on populations of closely related species. Since *C. albofundus*, the wattle wilt pathogen, is the species most closely related to *C. fimbriata*, the versatility of the markers to work across species was tested on a population of *C. albofundus* from South Africa (chapter four). Further inferences on population structure, diversity and mode of reproduction in *C. albofundus* were also considered.

Subsequent to reports that individual, mature *Eucalyptus grandis* trees were wilting and dying rapidly in plantations in Uruguay, a survey was conducted to determine the cause of the disease. In chapter five, I consider the agent responsible for this disease using morphological and sequence data comparisons. This investigation gave rise to a new report of *C. fimbriata* infecting *E. grandis* in Uruguay.

A new species of *Ceratocystis* was discovered on *E. nitens* in Australia after a wounding trial was conducted on these trees. Chapter six deals with the characterization of this new species using morphology, and sequence data from the internal transcribed regions of the ribosomal genes. Comparisons of this fungus were made with other *Ceratocystis* spp. A new species of *Ceratocystis* causing disease on *E. nitens* trees that have been pruned, is described.