

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

BY

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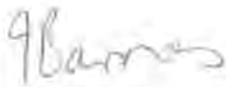
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DECLARATION

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.



Irene Barnes

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I dedicate this thesis to my loving mother

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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 gasheerreëks 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 teenwoordig 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 vermelding 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 geïsoleer. 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 nuwe 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.

CHAPTER 1



**A review of *C. fimbriata* and
other species of *Ceratocystis***

INTRODUCTION

Ceratocystis sensu lato includes a group of ascomycetous fungi that are plant pathogens primarily associated with sap staining of wood, and which are distinctly adapted to insect dispersal. This broader group of fungi, more commonly known as the ophiostomatoid fungi, includes over 100 species in the genera *Ceratocystis* Ellis & Halst., *Ceratocystiopsis* Upad. & Kendr., *Ophiostoma* H. & P. Syd. (De Hoog & Scheffer 1984, Wingfield *et al.* 1993) and *Gondwanamyces* Marais & M. J. Wingf. (Marais *et al.* 1998).

The oldest genus of ophiostomatoid fungi is *Ceratocystis*, which was first established in the late eighteenth century when *Ceratocystis fimbriata* Ellis & Halst. was described as the cause of a black rot of sweet potato (*Ipomoea batatas* [L.] Lam.) (Halsted & Fairchild 1891). Members of *Ceratocystis* are typified by perithecia with globose bases and long necks, emitting ascospores in droplets from their apices (Halsted & Fairchild 1891, Hunt 1956, Upadhyay 1981).

The taxonomy of *Ceratocystis* spp. was controversial from an early stage. Confusion first emerged when the ascocarps or perithecia of the type species, *C. fimbriata*, were mistaken for pycnidia and similarly, the ascospores for conidia (Halsted & Fairchild 1891). Since then, the genus has been subjected to many name changes, as attempts have been made to resolve the confusion generated by this misinterpretation. Substantial confusion has also arisen due to the general morphological similarities of *Ophiostoma* and *Ceratocystis* spp., and the fact that species of these two genera have similar biologies (Upadhyay 1993, Wingfield *et al.* 1993). The taxonomic history of *Ceratocystis sensu lato* has been eloquently reviewed numerous times (De Hoog 1974, Upadhyay 1981, De Hoog & Scheffer 1984) and will, therefore, not be repeated here. An extract taken from the presentation of Seifert & Okada (1993), however, summarizes the name changes and taxonomic history of the type species of *Ceratocystis* and is repeated here as follows:

Ceratocystis (*ceratos* = horn and *cyst* = pouch or sac)

Ceratocystis fimbriata Ellis & Halsted, N.J. Agric. Exp. Sta. Bull. 76:14. 1890.

= *Sphaeronaema fimbriatum* (Ellis & Halsted) Sacc., Syll. Fung. 10:125. 1892.

= *Ceratostomella fimbriata* (Ellis & Halsted) Elliot, Phytopathology 13:56. 1923.

- = *Ophiostoma fimbriatum* (Ellis & Halsted) Nannf., Svenska Skogsfor. Tidskr. 32:408. 1934.
- = *Endoconidiophora fimbriata* (Ellis & Halsted) Davidson, J. Agric. Res. 50:800. 1935.
- = *Rostrella coffeae* Zimmermann, Medded. s'Lands Plantentuin 37:24. 1900.
- = *Endoconidiophora variospora* Davidson, Mycologia 36:303. 1944.
- = *Ceratocystis variospora* (Davidson) Moreau, Rev. Mycol. (Paris) Suppl. Col. 17:22. 1952.
- = *Ophiostoma variosporum* (Davidson) von Arx, Antonie van Leeuwenhoek 18:212. 1952.

The taxonomy of *Ceratocystis* was substantially complicated when *Ophiostoma* was described by Sydow & Sydow in 1919. This important genus also has globose perithecia emitting ascospores at the apices of long tubular necks. This similarity in morphology with species of *Ceratocystis* and *Ophiostoma* (Upadhyay 1993, Wingfield *et al.* 1993), led to considerable confusion between these two genera and as a result, the two genera have tended to be treated collectively (Elliott 1925, Hunt 1956, Upadhyay 1981). During the course of the last three decades, many studies have clearly shown that significant differences, both morphologically and genetically, exist to warrant the separation of these two genera (De Hoog 1974, Harrington 1981, De Hoog & Scheffer 1984, Wingfield *et al.* 1993). A third ophiostomatoid genus, *Ceratocystiopsis* Upadh. & Kendr., was easily distinguished from these two genera on the basis of sheathed, elongated to flaccate ascospores (Upadhyay 1981). Some authors, however, believe that *Ceratocystiopsis* should be reduced to synonymy with *Ophiostoma* (Hausner *et al.* 1993).

Morphologically, the most convincing characters separating *Ophiostoma* and *Ceratocystis* are elements of their associated anamorphs. *Ceratocystis* spp. have *Chalara* (Corda) Rabenh. anamorphs with long tubular conidiophores with chains of enteroblastically produced conidia (ring wall building development) (De Hoog 1974, Minter *et al.* 1983, Mouton *et al.* 1994). In contrast, *Ophiostoma* spp. have anamorphs in *Graphium* Corda, *Leptographium* Lag. & Melin and *Sporothrix* Hekt. & Perkins. The conidia in these cases are produced holoblastically (apical wall building development) (De Hoog 1974, Wingfield *et al.* 1993). *Ceratocystis* spp. also lack cellulose and rhamnose in their cell walls, while species of *Ophiostoma* contain both these polysaccharides in their cell walls (Rosinski & Campana 1964, Spencer & Gorin 1971, Jewell 1974, Weijman & De Hoog 1975). Biochemically, species in *Ceratocystis* are sensitive to the antibiotic cycloheximide

in culture, while *Ophiostoma* spp., have the ability to tolerate high concentrations of this antibiotic (Harrington 1981, De Hoog & Scheffer 1984).

The use of molecular techniques has provided the most convincing evidence justifying the separation between *Ceratocystis* and *Ophiostoma*. Phylogenetic studies based on DNA sequence have indicated that *Ophiostoma* spp. are most closely related to species in the Diaporthales and *Ceratocystis* spp. to taxa belonging to the Hypocreales (Spatafora & Blackwell 1994, Wingfield *et al.* 1999). These phylogenetic data have reaffirmed the taxonomic value of various phenotypic characteristics thought to separate *Ceratocystis* and *Ophiostoma* (Hausner *et al.* 1993, Spatafora & Blackwell 1994). It is now generally accepted that *Ophiostoma* and *Ceratocystis* are distinct and phylogenetically unrelated genera (Hausner *et al.* 1993, Spatafora & Blackwell 1994.). Their respective type species are *Ophiostoma piliferum* (Fr.) H. & P. Syd. and *Ceratocystis fimbriata*.

The objective of this review is twofold. Firstly, details of the biology of *Ceratocystis* spp., with particular reference to the type species, *C. fimbriata* is considered. A brief summary of the morphology, distribution, host-range, pathogenicity and reproduction of the pathogen is given. Secondly, the review deals with the molecular aspects used to characterise fungal taxa and populations, especially *Ceratocystis* spp.

MORPHOLOGY

The primary and most basic taxonomic tool used for the identification of fungi is morphology (Hunt 1956, Upadhyay 1981). *C. fimbriata* has a relatively simple holomorph morphology consisting of teleomorph and anamorph stages. A general description of *C. fimbriata* is given below, although it should be noted that isolates from different geographic locations tend to differ in colony type, growth rate and conidial states (Webster & Butler 1967a, b). Morphological descriptions are mostly made from isolates grown on artificial media.

Cultural characteristics: Cultures are hyaline when young becoming light brown and darker with age. The superficial, aerial mycelium is normally lighter in colour than the submerged mycelium, which is brownish to dark greenish (Hunt 1956, Upadhyay 1981).

The reverse side of cultures, therefore, normally provides a better indication of colony colour. Submerged mycelium also darkens as perithecia develop and the margins of the cultures normally consist of fine, radiating fibrils. Colonies are generally described as slow growing (approx. 21 mm in 10 days) (Hunt 1956, Upadhyay 1981). A certain level of variability in culture characteristics between different isolates of *C. fimbriata*, has been reported (Webster & Butler 1967a, b).

Teleomorph: The bases of the perithecia of *C. fimbriata* are dark brown to black, and globose (Hunt 1956, Upadhyay 1981, Grylls & Seifert 1993). The perithecial bases are sometimes ornamented with hyphae. Perithecial necks are long and slender, black at the base but hyaline towards the apex. The necks also taper slightly towards the tip. Hyaline ostiolar hyphae extend from the outer layers of the neck cells and are mostly convergent (Hunt 1956, Upadhyay 1981). Perithecia are produced singly or in clusters, superficially or are partly embedded in agar (Hunt 1956, Upadhyay 1981, Grylls & Seifert 1993).

Ascospores are produced in great numbers in the perithecial bases. These are hyaline, single-celled, and are characteristically hat-shaped (Hunt 1956, Upadhyay 1981, Grylls & Seifert 1993). Asci are evanescent and, therefore, rarely seen. During the formation of the ascospores, the cells lining the interior of the perithecial cavity disintegrate forming a gelatinous mass, which envelops the ascospores. Hydrostatic pressure develops inside the perithecia as it absorbs water pushing the oily gelatinous masses of ascospores up the perithecial necks and out of the ostioles as a white to yellowish droplet (Hunt 1956, Upadhyay 1981).

Anamorph: *Ceratocystis fimbriata* has a *Chalara* anamorph producing two types of conidia. The hyphae making up the mycelium are hyaline to pale brown, smooth and septate. Most of the hyphae terminate as conidiophores. Conidiogenous cells, at the end of the conidiophores, produce conidia through ring wall building (Minter *et al.* 1983). These enteroblastically formed conidia are either barrel-shaped or cylindrical with truncate ends. Both types of conidia are smooth-walled, non-septate, hyaline and borne singly or in chains. Thick-walled, oval chlamydospores are also characteristic. These are olive-brown in colour, normally found embedded in the agar and are formed singly or in short chains (Pontis 1951, Hunt 1956, Upadhyay 1981, Grylls & Seifert 1993).

Physiological characteristics: *Ceratocystis fimbriata* grows well on artificial media, especially 2 % malt extract agar (MEA). To obtain a higher ratio of perithecial to mycelial growth, any media used should, however, be supplemented with thiamin (Barnett & Lilly 1947). Perithecial formation occurs only when the ratio of thiamine to other nutrients in the medium is relatively high (Barnett & Lilly 1947). Calcium has also been shown to be favourable for the production of perithecia, while excess nitrogen prevents perithecial formation in *C. fimbriata* (Campbell 1960). To keep cultures from deteriorating and losing viability, masses of ascospores should be transferred. If only mycelium is repeatedly transferred, vitality can be lost, resulting in sterile cultures (Hunt 1956, Upadhyay 1981). Culture media appears to favour rapid mycelial growth at the expense of reproductive structures (Upadhyay 1981). The pH determined for optimal vegetative growth and formation of perithecia is between 5-7, with pH 6 the most optimal (Kile 1993, Upadhyay 1981). The optimum temperature for growth in culture is generally between the range 22-26 °C (Kile 1993) although with differing effects. For example, the conidial or vegetative growth forms abundantly at 18 °C, but with reduced perithecial growth. At 25 °C, however, more perithecial growth is observed (Barnett & Lilly 1947).

DISTRIBUTION AND HOST RANGE

Ceratocystis fimbriata was first discovered more than 100 years ago where it was causing severe rot of exotic *Ipomoea batatas* in New Jersey, U.S.A. (Halsted & Fairchild 1891). Since then, the fungus has been recorded from most parts of the world where it causes diseases on a wide range of plants (Kile 1993) (Table 1). *C. fimbriata* is thus an important plant pathogen, often resulting in large scale losses in agriculture and forestry (Kile 1993).

Most reports of *C. fimbriata* have been from Latin America (McCracken & Burkhardt 1977), where the fungus infects a wide range of native and exotic host plant species (Beeley 1929, Costa & Krug 1935, Sharples 1936, Iton 1959, Ribeiro *et al.* 1985) (Table 1). In areas such as Colombia, Venezuela and Brazil, *C. fimbriata* causes major losses in coffee (*Coffea arabica* L.) production (Pontis 1951). Other agricultural crops in these countries include *Citrus* spp., *Crotalaria* sp., *Inga* spp., *Mangifera indica* L. and *Theobroma cacao* L. (Rincon 1983, Nieto 1991, Buritica 1994, Mourichon 1994). Woody hosts which have been infected include *Acacia decurrens* (Wendl.) Willd. (Ribeiro *et al.*

1985), *Gmelina arborea* Roxb. (Muchovej *et al.* 1978) and *Hevea braziliensis* Muell.-Arg. (Sharples 1936).

Ceratocystis fimbriata is also present in Asia and Europe. At one stage, infections of *Ipomoea batatas* were a serious problem in Japan until effective control strategies were implemented to contain the disease (Clark & Moyer 1988). In Europe and U.S.A., the most important disease has been infection of *Platanus* spp. by *C. fimbriata* f.sp. *platani*. This pathogen causes severe cankers on trees (Walter *et al.* 1952, Panconesi 1981). Other hosts infected by *C. fimbriata* in these countries are listed in Table 1.

New reports of *C. fimbriata* infecting previously unknown hosts in new areas are continuously made. The presence of *C. fimbriata* in Australia was first recorded in 1987 (Walker *et al.* 1988) on rotting *Syngonium* spp. in nurseries. The pathogen is thought to have been introduced into the country via infected *Syngonium* material (Walker *et al.* 1988, Vogelzang & Scott 1991). The presence of *C. fimbriata* on the African continent has only recently been recognised (Roux *et al.* 1999) when severe wilting of *Eucalyptus* clones in plantations in the Republic of Congo were caused by this pathogen. The pathogen has subsequently also been discovered infecting *E. grandis* in Uganda (Roux *et al.* 2001a).

DISEASE DEVELOPMENT

Ceratocystis fimbriata has been found on a variety of substrates including root and fruit crops, ornamentals and forest trees (Halsted & Fairchild 1891, Pontis 1951, Walter *et al.* 1952, Gremmen & De Kam 1977, Kile 1993, Panconesi 1999, Roux *et al.* 2001a,) (Table 1). The disease symptoms produced by this pathogen vary according to the type of host it infects. Three main categories of symptoms are recognized. These include vascular stains, cankers, root and stem rots (Table 1).

Vascular stains normally occur on hardwood species (Kile 1993). After infection, for which a wound is required, mycelium grows in the medullary rays and axial parenchyma (Olchowecki & Reid 1973). The movement of *C. fimbriata* in the vascular system is, however, partially restricted to the area of infection. This is mainly due to plant host defense reactions against infection, such as the production of tyloses, gels and vessel wall

thickening (Clerivet & El Modafar 1994). The fungus then stains the sapwood and causes extensive discolouration. When the bark of the tree is removed, dark brown radial patches or streaks are usually observed (Pontis 1951, Kile 1993). This disease can be severe and has the potential to kill vigorously growing trees within a few months (Gibbs 1993).

The most common symptoms of infection by *C. fimbriata* are cankers (Table 1). Symptoms first appear as circular, necrotic areas surrounding pruning wounds or other mechanical injuries (Pontis 1951). Discolouration and death of the bark and wood occurs, resulting in stem or branch deformation or death of the affected plant part (Wood & French 1963, Kile 1993). Penetration of the wood by the fungus is slight. Infection, and therefore staining, is restricted to areas of the cambium and inner bark tissue, resulting in various types of lesions (Kile 1993, Hinds 1972). These cankers are formed by the mycelium continually invading the cambium during the dormant season, killing new zones of tissue. Continuous concentric layers of callus can be formed, producing cankers that appear target-shaped (Hinds 1972, Gremmen & De Kam 1977). Ceratocystis cankers are perennial and enlarge slowly with vertical growth exceeding horizontal growth (Pontis 1951, Hinds 1972). The development of cankers is, however, dependent on the virulence of the isolates, fungal-host interaction and the location of infection (Kile 1993).

Root and stem rots normally occur on agricultural crops. *C. fimbriata* has been reported to cause black rot of sweet potato, characterised by dark greenish circular spots on the tubers (Halsted & Fairchild 1891). It also causes mouldy rot of tapping panels on rubber (*Hevea* sp.) and rot disease of *Crotalaria* sp. (Pontis 1951) (Table 1). *C. fimbriata* infects the non-lignified or lightly lignified tissues and results in the blackening or browning of the infected areas of the host, causing chlorosis or wilting and stunting (Kile 1993).

DISSEMINATION

Many species of *Ceratocystis* are well-known for their association with certain insect genera (Crone & Bachelder 1961, Moller & DeVay 1968, Upadhyay 1981). In general, *Ceratocystis* spp. are vectored non-specifically by casual insects such as flies (Diptera) and beetles (Coleoptera: Nitidulidae) (Crone & Bachelder 1961, Moller & DeVay 1968, Hinds 1972, Juzwik & French 1983). There are, however, a small number of *Ceratocystis* spp.

such as *C. polonica* (Siemaszko) C. Moreau, *C. laricicola* Redfern & Minter, and *C. rufipenni* Wingfield, Harrington & Solheim, that are specifically associated with bark beetles that infest conifers (Siemaszko 1939, Jewell 1956, Redfern *et al.* 1987, Wingfield *et al.* 1997).

Ceratocystis fimbriata does not have close associations with any specific insect and is not reliant on insects for survival. Insects do, however, play an important role in the dispersal of this fungus (Moller & DeVay 1968, Upadhyay 1981). In an experiment to determine possible vectors for *C. fimbriata*, Hinds (1972), baited wounds on *Populus tremuloides* Michx. trees in Colorado with *C. fimbriata*. After catching insects visiting these sights, it was observed that *C. fimbriata* was not selective to a particular vector, but was carried by several orders and families including beetles, flies, aphids and moths. The primary vectors were beetles of the families Nitidulidae, Staphylinidae and Rhizophagidae. Crone & Bacheldor (1961), performed insect transmission studies on susceptible *Platanus* spp., and found that *Coleopterous* insects, especially *Nitidulids* were the main vectors distributing *C. fimbriata*. Eight different insect species and mites have also been reported to be vectors of *C. fimbriata* on stone fruits (Moller & DeVay 1968).

As with other *Ceratocystis* species, *C. fimbriata* is morphologically adapted for dispersal by insects. It has a long perithecial neck from which sticky ascospores are released. The characteristic fragrant, fruity aroma emitted from *C. fimbriata* serves as an additional attraction for insects (Moller & DeVay 1968, Kile 1993). When the insects forage on the fungi, the sticky masses of ascospores adhere to the body of the insects. As the insects move to neighbouring trees, the fungus is also transmitted to these new trees (Crone & Bacheldor 1961). *C. fimbriata*, however, requires fresh wounds to infect its hosts (Moller & DeVay 1968, Hinds 1972, Teviotdale & Harper 1991, Kile 1993). These wounds can be caused by insect damage or by mechanical or environmental factors such as hail and wind breakage (Leather 1966, Moller & DeVay 1968).

The principle factor responsible for the spread of *C. fimbriata* is man. There has been no indication that major economic losses on crops have occurred when the fungus was dependent on natural agencies for dispersal (Walter *et al.* 1952). It seems, especially on woody hosts, that the primary means of fungal transmission is mechanical, by way of infected pruning tools and related equipment (Walter *et al.* 1952, DeVay *et al.* 1968,

Teviotdale & Harper 1991). Walter *et al.* (1952) noted that in numerous small outbreaks of canker stain of *Platanus* sp., a very high proportion of the primary infections started at pruning wounds. Pontis (1951), also noted that most of the cankers observed on coffee trees were at sites where pruning had taken place.

PATHOGENICITY

Many studies pertaining to the pathogenicity of *C. fimbriata* have confirmed that different strains of the pathogen have developed strict host specificity (DeVay *et al.* 1963, Leather 1966, Kojima & Uritani 1974, Kile 1993, Kojima 1993). Although morphologically indistinguishable from the type species of *C. fimbriata* from sweet potato (*Ipomoea batatas*), most strains differ in their pathogenicity to different hosts. For example, coffee trees inoculated with a sweet potato strain showed no signs of infection, although severe cankers were produced from inoculations with the coffee (*Coffea* sp.) strain (Pontis 1951). Similarly, inoculations of sweet potato, cacao (*Theobroma cacao*) and coffee with an isolate of *C. fimbriata* from pimento (*Pimenta officinalis*) gave negative results (Leather 1966). Vogelzang & Scott (1991), carried out studies using an isolate of *C. fimbriata* from a goose foot plant (*Synгонium*) to determine the extent of its pathogenicity. They found that the isolate was not pathogenic on plum (*Prunus domestica* L.), almond (*P. dulcis* [Miller] D. Webb), apricot (*P. armeniaca* L.) or mango (*M. indica* L.) and only slightly pathogenic on taro (*Colocasia esculenta* [L.] Schott). Although the taro plants initially showed signs of infection, they were able to recover over time (Vogelzang & Scott 1991). Olson & Martin (1949), also found that isolates from rubber (*Hevea*) in Mexico were not pathogenic to sweet potato and likewise, isolates from sweet potato (U.S.A.), were not pathogenic to rubber plants.

In hybridisation studies, Olson & Martin (1949), showed that, although rubber tree isolates and sweet potato isolates were not pathogenic on their respective hosts, hybridisation between the two isolates was possible. Similarly, Feazell & Martin (1950), showed that sweet potato and sycamore isolates, although not pathogenic on their respective hosts, were able to hybridise with each other and produce fertile cultures. Webster & Butler (1967a), in similar but more detailed studies, identified that differences exist among *C. fimbriata* isolates from different hosts and origins. The ability of different strains of *C. fimbriata* to

hybridise and form fertile progeny increases the potential for new host-pathogen combinations to emerge (Kile 1993). Such hybridisations also give the fungus a greater ability to rapidly evolve and adapt to new hosts when introduced to a new environment (Brasier 1993).

Several studies based on pathogen-host interactions have identified certain factors within host plants that are linked to host specificity in certain strains of *C. fimbriata* (Kojima & Uritani 1974, Kojima & Uritani 1978, Kojima *et al.* 1982, Kawakita & Kojima 1983, El Modafar *et al.* 1995). In studies carried out by Kojima *et al.* (1993), potato roots and taro tubers were used as hosts, and seven strains of *C. fimbriata* isolated from sweet potato, coffee, prune, cacao, oak, taro and almond as the infection agents. Cross inoculations of these hosts with the different strains showed a variety of host-interactions and demonstrated that certain phytoalexins, spore agglutinating factors, divalent cations (Ca^{2+} and Mg^{2+}), and inhibitory proteins on germinated spores are all involved in determining host-pathogen specificity. These studies have provided much support for the hypothesis that *C. fimbriata* represents a species complex rather than a discrete taxon.

ORIGIN

A form of *C. fimbriata* similar to that infecting sweet potato, but different in host specificity, is considered native to North America (Walter *et al.* 1952, Anonymous 1986). This fungus, *Ceratocystis fimbriata* f.sp. *platani*, was found in low incidence infecting native *Platanus* sp. in areas not exposed to human activity (Walter *et al.* 1952). Spread of this fungus to other *Platanus* spp. and areas of the U.S.A. is believed to have started in New Jersey as early as 1929 (Jackson & Sleeth 1935). Subsequently, *C. fimbriata* f.sp. *platani* was reported causing wide-spread and severe canker stain disease to ornamental *Platanus* spp. throughout Europe, in countries such as Italy, France and Switzerland (Walter *et al.* 1952, Vigouroux 1979, Panconesi 1981). The pathogen is thought to have spread from the U.S.A. into Europe at several southern European ports at the end of the second world war (Anonymous 1986).

REPRODUCTION

Chalara, which relies solely on the asexual reproduction and production of conidia via mitosis, is the anamorph state of *Ceratocystis*. *Ceratocystis*, on the other hand, reproduces ascospores sexually via meiosis. Within *Ceratocystis*, species are either heterothallic such as *C. paradoxa* Dade, *C. radicola* Bliss, *C. fagacearum* (Bretz) Hunt and *C. eucalypti* Yuan & Kile (Hepting *et al.* 1952, Kile *et al.* 1996) or homothallic as in *C. fimbriata*, *C. pinicola* and *C. virescens* (Davids.) C. Moreau (Olson 1949, Webster & Butler 1967a, b, c, Harrington & McNew 1997).

Two opposite mating type genes, MAT-1 (self-sterile strains) and MAT-2 (self-fertile strains), are involved in the sexual reproduction in *Ceratocystis* spp. In heterothallic *C. eucalypti*, both mating type genes are required for sexual reproduction. Single ascospore progeny of crosses are then either of the MAT-1 or MAT-2 mating type. In homothallic *C. fimbriata*, sexual reproduction occurs by means of selfing. Single ascospore transfers from a selfing event (i.e. MAT-2 strain) produces an equal ratio of self-sterile and self-fertile progeny (Hansen & Snyder 1952, Webster & Butler 1967b, c). This phenomenon, whereby the MAT-2 gene is deleted, allowing for expression of the MAT-1 gene, has been attributed to uni-directional mating type switching (Harrington & McNew 1997, Witthuhn *et al.* 2000a). *C. fimbriata* has the ability, therefore, to outcross in nature with the potential to form new genotypes. The frequency and occurrence of this event, however, has not been determined.

MOLECULAR BIOLOGY

Since the advent of the polymerase chain reaction (PCR), molecular methods have become invaluable in studies of fungal phylogeny and population biology (Foster *et al.* 1993, Glass & Donaldson 1995). The ability to differentiate between fungi at the DNA level has provided additional characters with which to classify species. This has been particularly valuable where traditional phenotypic characteristics are limiting. Molecular techniques including studies of various molecules such as DNA, RNA, enzymes and other proteins have many applications. They can be used for taxonomic purposes to classify genera, species, and strains of fungi, to delimit individuals or species based on their genealogical or

phylogenetic relationships, to determine anamorph and teleomorph connections and to identify individuals or clones within fungal populations (Michelmore & Hulbert 1987, Foster *et al.* 1993, Maclean *et al.* 1993, Taylor *et al.* 1999a, b).

Sequences with useful phylogenetic inference come from genes that have the same function in all taxa, evolve at approximately the same rate, are present only once in a genome or behave like a single copy region (Glass & Donaldson 1995). One of the sets of genes that fulfils these criteria is the ribosomal RNA genes from the nuclear and mitochondrial genomes (Bruns *et al.* 1991). Fungal taxonomy and phylogenetic inferences have, therefore, been largely based on molecular sequence data obtained from the ribosomal gene cluster. It comprises three structural regions coding for the 5.8S, 18S (small subunit) and 28S (large subunit) ribosomal RNA genes (Mitchell *et al.* 1995). Interspersed between the genes are highly variable internal transcribed spacer regions ITS1 and ITS2 (Mitchell *et al.* 1995).

Sequences coding for the structural genes in the ribosomal operon are conserved due to their functional nature (Jukes 1987, Raue *et al.* 1988). There is limited sequence divergence within species making their use in the characterisation of organisms above the species level favourable. The ITS regions, on the other hand, are highly variable (Nazar *et al.* 1987, Mitchell *et al.* 1995). These regions evolve at different rates among fungi and thus, because of their high resolution, can be used to discriminate between taxa at different levels (Bruns *et al.* 1991, Messner *et al.* 1995, Mitchell *et al.* 1995). The ITS regions are routinely used to study closely related species and populations of fungi.

MOLECULAR GENETICS DEFINING *CERATOCYSTIS*

The ophiostomatoid fungi are thought to have similar morphologies due to convergent evolution (Wingfield *et al.* 1993). The use of morphological data alone has, therefore, proven insufficient to categorise taxa into distinct lineages. Orders and genera within the ascomycetes, including those belonging to the Ophiostomatales have, however, been successfully classified using ribosomal DNA sequences of the small subunit (18s) rDNA (Berbee & Taylor 1992, Spatafora & Blackwell 1993, 1994, Okada *et al.* 1997, 1998).

Various genes and enzymes have been used to address questions pertaining to the taxonomy of the species in the genus *Ceratocystis*. Paulin & Harrington (2000), used portions of the 18S and 28S rDNA domains to determine which *Chalara* anamorphs have phylogenetic affinities and, therefore, associations with teleomorphs belonging to the genus *Ceratocystis*. Wingfield *et al.* (1996), with the aid of sequence data from the ITS regions, showed that *Ceratocystis* isolates, causing severe disease on *Acacia mearnsii* in South Africa, and morphologically similar to *C. fimbriata*, represented a distinct, new species now named *C. albofundus* Wingfield, De Beer & Morris. Hausner *et al.* (1993), demonstrated the effectiveness of using restriction enzyme analysis on ribosomal and mitochondrial DNA to group species and strains within *Ceratocystis*. An easy and reliable identification method whereby species of *Ceratocystis* could clearly be distinguished using PCR-based restriction fragment length polymorphisms (RFLP), was developed by Witthuhn *et al.* (1999). In the same study, phylogenetic relationships between various *Ceratocystis* species were determined with the use of sequence data obtained from the large subunit of the rRNA gene.

Comprehensive studies on the *C. coerulescens* (Münch) Bakshi, complex using electrophoretic phenotypes based on isozyme analysis, showed that *C. coerulescens* represents a complex of five different species (Harrington *et al.* 1996). These morphologically indistinguishable variants were previously identified based solely on their different conifer hosts and insect vectors. DNA sequence data using ITS1, ITS2 and MAT-2 confirmed the delineation of the complex into five species and indicated that a strong monophyletic group exists for all *Ceratocystis* species occurring primarily on conifers (Witthuhn *et al.* 1998, Witthuhn *et al.* 2000b). The complex now consists of eleven species, highlighting the importance of host range in the evolution of *Ceratocystis* species.

In some cases, instead of showing divergence of taxa into different species, molecular tools have shown conspecificity of taxa. For example, in the *C. coerulescens* complex, *C. polonica* (Siemaszko) Moreau, and *C. laricicola* Redfern & Minter are morphologically identical but differ in their respective hosts and associated vectors. *C. polonica* infects spruce and is associated with *Ips typographus* L. (Siemaszko 1939) and *C. laricicola* attacks larch and is associated with the bark beetle *Ips cembrae* (Redfern *et al.* 1987). These two species differ in only one isozyme locus and it was suggested that they should be synonymized (Harrington *et al.* 1996). This suggestion was supported by identical ITS

sequences (Witthuhn *et al.* 1999). Further investigations using sequence data from the MAT-2 HMG box, however, showed that *C. laricicola* and *C. polonica*, could be differentiated from each other although separation was relatively weak (Witthuhn *et al.* 2000b). This is a clear example where analysis of multiple genealogical sequence data could be used to resolve an important taxonomic question.

Limited studies have been carried out to investigate the phylogenetic relationships between different host specific strains of *C. fimbriata*. After finding a virulent strain of this fungus causing severe wilting in *Eucalyptus* trees in the Republic of Congo, Roux *et al.* (1999), performed initial analysis to determine the possible origin of the strain. Phylogenetic data based on sequence from the ITS region, grouped the *C. fimbriata* isolates from Congo closest to those from South America and South Africa, but separate from isolates originating in North America. Further studies using ITS and MAT-2 sequence data on various isolates of *C. fimbriata* showed that they cluster into geographic and host-associated lineages (Baker & Harrington 2000, Baker *et al.* 2001). Isolates were separated into a North American clade, Latin American clade and an Asian clade (Baker & Harrington 2000). Within the Latin American clade, three specific host lineages, namely sweet potato, sycamore and cacao, were recognized. Results of the above studies suggest host specialization to be a major factor driving the speciation of specific lineages (Baker & Harrington 2000). They also indicate that *C. fimbriata* represents a complex of host specific strains or species, with a wide geographic distribution.

POPULATION GENETICS

Populations of pathogens and not individual strains generally cause disease. It is thus necessary for plant pathologists to study the genetics of pathogens at the population level. Population genetics, specifically in plant pathogenic fungi, is a rapidly emerging field of study providing valuable information for the development of management strategies to reduce the impact of pathogens (McDermott & McDonald 1993, Wolf & McDermott 1994 Brygoo *et al.* 1998). By studying levels of genetic diversity amongst and within populations, deductions can be made regarding the possible origin, structure, migration, mode of reproduction and evolutionary relationships of pathogens. Populations with higher diversities normally have an increased capacity to adapt to environmental changes via

natural selection. They are, therefore, less affected by introduced management strategies than populations with no or little diversity. If the origin, and subsequently, migration patterns of the pathogens are known, efficient quarantine strategies can be implemented to either contain a pathogen within a country or minimise introduction into other countries.

Genetic diversity studies in Ceratocystis

Using RFLP fingerprinting studies, Harrington *et al.* (1998), found a correlation between the type of reproduction of a fungus and the resulting genetic diversity that is observed within a native population. Radioactively labelled microsatellite probes (CAT)₅ and (CAC)₅ were hybridised to total genomic DNA digested with *Pst*I and the nuclear DNA diversity was determined. Similarly, the mitochondrial DNA diversity was calculated from *Hae*III-restricted mitochondrial DNA. *C. eucalypti* Yuan & Kile, an obligately outcrossing fungus, had a higher genetic diversity compared to *C. virescens* (Davids.) C. Moreau, which is homothallic. The asexual fungus, *Chalara australis* Walker & Kile was the least diverse (Harrington *et al.* 1998). Roux *et al.* (2001b), used the same techniques to determine the genetic diversity within a population of *C. albofundus* from South Africa. A high level of both nuclear and mitochondrial gene diversity was observed for this homothallic fungus (Roux *et al.* 2001b). This high gene diversity, its association with native *Protea* spp. and its limited geographic distribution, led the authors to hypothesise that *C. albofundus* is native to South Africa.

There has only been a single population study conducted on *C. fimbriata*. In an attempt to determine the genetic diversity and possible origin of *C. fimbriata* f.sp. *platani* in Europe, Santini & Capretti (2000), used RAPD and direct amplification of minisatellite-region DNA (DAMD) PCR on a population from Italy and isolates of *C. fimbriata* on different hosts from North America. Their investigation supported the view that *C. fimbriata* f.sp. *platani* was introduced into Europe from North America. The population in Italy was clonal, sharing an identical genotype with some of the isolates from North America.

Microsatellites as population genetic tools

Molecular methods are increasingly used to study populations of organisms and a variety of techniques have been developed to facilitate such studies. These include Amplified Fragment Length Polymorphisms (AFLP) (Majer *et al.* 1996), Randomly Amplified Polymorphic DNA (RAPDs) (Santini & Capretti 2000), Randomly Amplified

Microsatellites (RAMS) (Hantula *et al.* 1998), RFLP's (Kuninaga & Yokosawa 1992) and nuclear DNA fingerprinting markers using (CAT)₅ (DeScenzo & Harrington 1994, Roux *et al.* 2001b). Co-dominant markers such as microsatellites are, however, preferred for population studies as they can identify different alleles within a single locus in individuals.

The increased use of microsatellite markers for population studies is based on a number of factors. Microsatellites consist of runs of short repeating sequences that can be highly variable in number between individuals (Jarne & Lagoda 1996). This feature provides an attractive source of polymorphisms between isolates, which can easily be studied and compared between individuals by designing DNA primers flanking these repeating regions (Schlötterer & Pemberton 1998). Furthermore, microsatellites are co-dominant and inherited in a mendelian fashion, abundant in eucaryotic genomes, easy to use and score, and they are selectively neutral with a high mutation rate (Tautz 1989, Rafalski *et al.* 1996). Excellent reviews on design, function and applications of microsatellites can be found in '*Microsatellites, Evolution and Applications*', edited by Goldstein & Schlötterer (1998).

The success of using microsatellites, also known as simple sequence repeats (SSR) (Tautz 1989) or short tandem repeats (STRs) (Edwards *et al.* 1991), is evident in the number of population studies that have recently been carried out on fungi. For example, microsatellites have been used to determine gene flow between two ectomycorrhizal populations (Zhou *et al.* 2001), for the detection, quantification, and diversity of grass endophytes (Groppe *et al.* 1995, Moon *et al.* 1999) and host and pathogen population variations (Delmotte *et al.* 1999), population diversity studies (Longato & Bonfante 1997, Meng & Chen 2001), population structure studies (Burgess *et al.* 2001, Burcheli *et al.* 2001) and phylogenetic studies (Fisher *et al.* 2000).

CONCLUSIONS

The taxonomy of the genus *Ceratocystis* has been reviewed many times, and its placement within the Ascomycetes is well established. Within the genus, the phylogenetic placement of the different species has recently received much attention and continues to be investigated. Studies of different variants and forms found within species are, however,

seriously lacking. This is with the exception of *C. coerulescence*, where investigations on the different variants resulted in the description of five new species.

Ceratocystis fimbriata is thought to consist of a complex of morphologically similar species. This hypothesis has emerged from evidence based on host specialisation, pathogenicity and molecular differences observed between isolates from different hosts and different geographical locations. The phylogenetic relationships between the different strains within *C. fimbriata* are, however, poorly understood. Although the biology of this species has been well documented, there is also a general lack of knowledge regarding the origin, genetics, population dynamics and movement of isolates comprising this species.

Detailed studies on different populations of *C. fimbriata* from different hosts and countries is likely to yield valuable information pertaining to population diversities and structure of this pathogen. Since microsatellite markers are associated with the highest level of polymorphism, due to the high evolution rate at these loci, they are potentially the most informative class of markers. This makes them the prime choice for the use in studying populations. Data obtained using microsatellite markers will not only be valuable in determining the area of origin of *C. fimbriata*, but will also aid in the development of management and quarantine strategies against certain strains of *C. fimbriata*. Studies to collect such data form an important part of this thesis.

REFERENCES

- Anonymous. (1986) *Ceratocystis fimbriata* (Ell. & Halst.) f.sp. *platani* (Walter). *Bulletin OEPP/EPPO Bulletin* **16** : 21-24.
- Baker, C.J., Alfenas, A.C. & Harrington, T.C. (2001) Host specialization and cryptic species within *Ceratocystis fimbriata*. *Joint Meeting of the American Phytopathological Society, Mycological Society of America and Society of Nematologists* August 25-29, 2001. Salt Lake City, Utah, USA
- Baker, C.J. & Harrington, T. (2000) Host-specialized *Ceratocystis fimbriata* on cocoa, sweet potato and sycamore (Abstract). *Inoculum* **51**.
- Barnett, H.L. & Lilly, V.G. (1947) The relation of thiamin to the production of perithecia by *Ceratostomella fimbriata*. *Mycologia* **39** : 699-708.
- Batista, A.C. (1960) *Ceratocystis fimbriata* Ell. & Halst. sobre *Mangifera indica* L. Universidade do Recife, *Instituto de Micologia Publicaçã* no. 244.
- Beeley, F. (1929) Fungi and diseases of the tapping panel. *Quarterly Journal of Rubber Research Institute of Malaya* **4** : 250-272.
- Berbee, M.L. & Taylor, J.W. (1992) Convergence in ascospore discharge mechanism among *Pyrenomyce* fungi based on 18S ribosomal RNA gene sequence. *Molecular Phylogenetics and Evolution* **1** : 59-71.
- Brasier, C.M. (1993) The genetic system as a fungal taxonomic tool: gene flow, molecular variation and sibling species in the '*Ophiostoma piceae* – *Ophiostoma ulmi*' complex and its taxonomic and ecological significance. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert, & J.A. Webber, eds): 77-92. APS Press, St. Paul, Minnesota.
- Bruns, T.D., White, T.J. & Taylor, J.W. (1991) Fungal molecular systematics. *Annual Review of Ecological Systematics* **22** : 525-564.
- Brygoo, Y., Caffier, V., Carlier, J., Fabre, J-V., Fernandez, D., Giraud, T., Mourichon, X., Neema, C., Nottoghem, J-L., Pope, C., Tharreau, D. & Lebrun, M-H. (1998) Reproduction and population structure in phytopathogenic fungi. In *Molecular variability of fungal pathogens* (P. Bridge, Y. Couteaudier & J. Clarkson, eds): 133-148. CAB International, Wallingford, Oxon, U.K.
- Burcheli, E., Gautschi, B. & Shykoff, J.A. (2001) Differences in population structure of the anther smut fungus *Microbotryum violaceum* on two closely related host species, *Silene latifolia* and *S. dioica*. *Molecular Ecology* **10** : 285-294.

- Burgess, T., Wingfield, M.J. & Wingfield, B.D. (2001) Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied Environmental Microbiology* **67** : 354-362.
- Buritica, P. (1994) Directorio de Patógenos y Enfermedades de las plantas de importancia económica en Colombia. *En Prensa* 635 pp.
- Campbell, R.N. (1960) Some sap-stain fungi found in Minnesota. *Plant Disease Reporter* **44** : 625-628.
- ChunYee, L., KerChung, K., Cy, L. & Kc, K. (1997) Black rot of sun hemp caused by *Ceratocystis fimbriata*. *Plant Pathology Bulletin* **6** : 191-194.
- Clark, C.A. & Moyer, J.W. (1988) Field and storage diseases: Black rot. In *Compendium of sweet potato disease* : 18-20. APS Press, St. Paul, Minnesota.
- Clerivet, A. & El Modafar, C. (1994) Vascular modifications in *Platanus acerifolia* seedlings inoculated with *Ceratocystis fimbriata* f. sp. *platani*. *European Journal of Forest Pathology* **24** : 1-10.
- Costa, A.S. & Krug, H.P. (1935) Eine durch *Ceratostomella* hervorgerufene Welkekrankheit der *Crotalaria juncea* in Brasilien. *Phytopathology Zeitschrift* **8** : 507-513.
- Crone, L.J. & Bachelder, S. (1961) Insect transmission of canker stain fungus, *Ceratocystis fimbriata* f.sp. *platani*. *Phytopathology* **51** : 576.
- De Hoog, G.S. (1974) The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium* and *Calcarisporiella* gen. nov. *Studies in Mycology* **7** : 1-88.
- De Hoog, G.S. & Scheffer, R.J. (1984) *Ceratocystis* Versus *Ophiostoma*: A Reappraisal. *Mycologia* **76** : 292-299.
- DeVay, J.E., Lukezic, F.L., English, W.H. & Trujillo, E.E. (1963) *Ceratocystis* canker of stone fruit trees. *Phytopathology* **53** : 873.
- DeVay, J.E., Lukezic, F.L., English, W.H., Trujillo, E.E. & Moller, W.J. (1968) *Ceratocystis* canker of deciduous fruit trees. *Phytopathology* **58** : 949-954.
- Delmotte, F., Bucheli, E. & Scykoff, J.A. (1999) Host and parasite population structure in a natural plant-pathogen system. *Heredity* **82** : 300-308.
- DeScenzo, R.A. & Harrington, T.C. (1994) Use of (CAT)₅ as a DNA fingerprinting probe for fungi. *The American Phytopathological Society* **84** : 534-540.
- Echandi, E. (1955) Trunk and stem cancer of coffee trees. In *Revista de Biología Tropical*. Volume **3** : 237-241.

- Edwards, A., Civitello, A., Hammond, H.A. & Caskey, C.T. (1991) DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *American Journal of Human Genetics* **49** : 746-756.
- El Modafar, C., Clerivet, A., Vigouroux, A. & Macheix, J.J. (1995) Accumulation of phytoalexins in leaves of plane tree (*Platanus* spp.) expressing susceptibility or resistance to *Ceratocystis fimbriata* f.sp. *platani*. *European Journal of Plant Pathology* **101** : 503-509.
- Elliott, J.A. (1925) A cytological study of *Ceratostomella fimbriata* (E. & H.) Elliott. *Phytopathology* **15** : 417-424.
- Feazell, G.D. & Martin, W.J. (1950) Studies on *Ceratostomella fimbriata* from sweet potato and sycamore. *Phytopathology*. **40** : 787.
- Fisher, M.C., Koenig, G., White, T.J & Taylor, J.W. (2000) A test for concordance between the multilocus genealogies of genes and microsatellites in the pathogenic fungus *Coccidioides immitis*. *Molecular and Biological Evolution* **17** : 1164-1174.
- Foster, L.M., Kozak, K.R., Loftus, M.G., Stevens, J.J. & Ross, I. (1993) The polymerase chain reaction and its application to filamentous fungi. *Mycological Research* **97** : 769-781.
- Gibbs, J.N. (1993) The biology of Ophiostomatoid Fungi causing sapstain in trees and freshly cut logs. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.A. Webber, eds): 153-160. APS Press, St. Paul, Minnesota.
- Glass, N.L. & Donaldson, G.C. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61** : 1323-1330.
- Goldstein, D.B. & Shlötterer, C. (1998) *Microsatellites: Evolution and Applications*. Oxford University Press, Oxford, New York.
- Gremmen, J. & De Kam, M. (1977) *Ceratocystis fimbriata*, a fungus associated with poplar canker in Poland. *European Journal of Forest Pathology* **7** : 44-47.
- Groppe, K., Sanders, I., Wiemken, A. & Boller, T. (1995) A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichloe* spp.) in grasses. *Applied and Environmental Microbiology* **61** : 3943-3949.
- Grylls, B.T. & Seifert, K.A. (1993) A synoptic key to species of *Ophiostoma*, *Ceratocystis* and *Ceratocystiopsis*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and*

- Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.A. Webber, eds): 261-268. APS Press, St. Paul, Minnesota.
- Halsted, B.D. (1890) Some fungus diseases of the sweet potato. *New Jersey Agricultural College Experiment Station Bulletin* **76** : 7-14
- Halsted, B.D. & Fairchild, D.G. (1891) Sweet-potato black rot. *Journal of Mycology* **7** : 1-11.
- Hansen, H.N. & Snyder, W.C. (1952) Inheritance of the sexes and compatibilities in fungi. *Phytopathology* **42** : 479-480.
- Hantula, J., Niemi, E.M., Kaitera, J., Jalkanen, R. & Kurkela, T. (1998) Genetic variation of the resin top fungus in Finland as determined by random amplified microsatellites (RAMS). *European Journal for Forest Pathology* **28** : 361-372.
- Harrington, T.C. (1981) Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* **73** : 1123-1129.
- Harrington, T.C. & McNew, D.L. (1997) Self-fertility and uni-directional mating type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* **32** : 52-59
- Harrington, T.C., Steimel, J. & Kile, G. (1998) Genetic variation in three *Ceratocystis* species with outcrossing, selfing and asexual reproductive strategies. *European Journal of Forest Pathology* **28** : 217-226.
- Harrington, T.C., Steimel, J., Wingfield, M.J. & Kile, G. (1996) Isozyme variation and species delimitation in the *Ceratocystis coerulescens* complex. *Mycologia* **88** : 104-113.
- Hausner, G., Reid, J. & Klassen, G.R. (1993) Grouping of isolates and species of *Ceratocystis sensu lato* on the basis of molecular and morphological characters. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.A. Webber, eds): 93-104. APS Press, St. Paul, Minnesota.
- Hepting, G.H., Toole, E.R. & Boyce, J.S. (1952) Sexuality in the oak wilt fungus. *Phytopathology* **42** : 438-442.
- Hinds, T.E. (1972) *Ceratocystis* canker of Aspen. *Phytopathology* **62** : 213-220.
- Hunt, J. (1956) Taxonomy of the genus *Ceratocystis*. *Lloydia* **19** : 1-59.
- Iton, E.F. (1959) Studies on a wilt disease of cacao at River Estate. *Report on Cacao Research 1957-1958* : 55-64. St. Augustine, Trinidad: Regional Research Centre, Imperial College of Tropical Agriculture, University of the West Indies.

- Jackson, L.W.R. & Sleeth, H.B (1935) Disease killing *Platanus orientalis* in the eastern United States. *Phytopathology* **25** : 22.
- Jarne, P. & Lagoda, P.J.A. (1996) Microsatellites, from molecules to populations and back again. *Tree* **11** : 424-429.
- Jewell, T.R. (1956) Insect transmission of oak wilt fungus. *Phytopathology* **46** : 244-257
- Jewell, T.R. (1974) A qualitative study of cellulose distribution in *Ceratocystis* and *Europhium*. *Mycologia* **66** : 139-146
- Jukes, T.H. (1987) Transitions, transversions, and the molecular evolutionary clock. *Journal of Molecular Evolution* **26** : 87-98.
- Juzwik, J & French, D.W. (1983) *Ceratocystis fagacearum* and *C. oiceae* on the surface of free-flying and fungus-mat-unhabiting nitidulids. *Phytopathology* **73** : 1164-1168.
- Kawakita, K. & Kojima, M. (1983) The isolation and properties of a factor in taro tuber that agglutinates spores of *Ceratocystis fimbriata*, black rot fungus. *Plant and Cell Physiology* **24** : 41-49.
- Kile, G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.A. Webber, eds): 173-183. APS Press, St. Paul, Minnesota.
- Kile, G.A., Harrington, T.C., Yuan, Z.Q., Dudzinski, M.J. & Old, K.M. (1996) *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100** : 571-579.
- Kojima, M. (1993) Phytoalexins and other biochemical factors associated with infection by *Ceratocystis fimbriata*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.A. Webber, eds): 243-251. APS Press, St. Paul, Minnesota.
- Kojima, M. & Uritani, I. (1974) The possible involvement of a spore agglutinating factor(s) in various plants in establishing host specificity by various strains of black rot fungus, *Ceratocystis fimbriata*. *Plant & Cell Physiology* **15** : 733-737.
- Kojima, M. & Uritani, I. (1978) Studies on factors in sweet potato root which induce spore germination of *Ceratocystis fimbriata*. *Plant & Cell Physiology* **19** : 91-97.
- Kojima, M., Kawakita, K. & Uritani, I. (1982) Studies on a factor in sweet potato root which agglutinates spores of *Ceratocystis fimbriata*, black rot fungus. *Plant Physiology* **69** : 474-478.

- Kuninaga, S. & Yokosawa, R. (1992) Genetic diversity of *Fusarium oxysporum* f. sp. *Lycopersici* in restriction fragment length polymorphism of mitochondrial DNA. *Transaction of the Mycological Society of Japan* **33** : 449-459.
- Laia, M.L., Alfenas, A.C. & Harrington, T.C. (1999) Isolation, detection in soil, and inoculation of *Ceratocystis fimbriata*, causal agent of wilting, die-back and canker in *Eucalyptus*. *Australasian Plant Pathology Society*, 12th Biennial Conference Canberra.
- Leather, R.I. (1966) A canker and wilt disease of pimento (*Pimento officinalis*) caused by *Ceratocystis fimbriata* in Jamaica. *Transactions British Mycological Society* **49** : 213-218.
- Longato, S. & Bofante, P. (1997) Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. *Mycological Research* **101** : 425-432.
- Maclean, D.J., Braithwaite, K.S. Manners, J.M. & Irwin, J.A.G. (1993) How do we identify and classify fungal plant pathogens in the era of DNA analysis? *Advances in Plant Pathology* **10** : 207-244.
- Majer, D., Mithen, R., Lewis, B.G., Vos, P. & Oliver, R.P. (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* **100** : 1107-1111.
- Manion, P.D. & French, D.W. (1967) *Nectria galligena* and *Ceratocystis fimbriata* cankers of aspen in Minnesota. *Forest Science* **13** : 23-28.
- Marais, G.J., Wingfield, M.J., Viljoen, C.D. & Wingfield, B.D. (1998) A new ophiostomatoid genus from *Protea* infrutescences. *Mycologia* **90** : 136-141.
- McCracken, F.I. & Burkhardt, E.C. (1977) Destruction of sycamore by canker stain in the midsouth. *Plant Disease Reporter* **61** : 984-986.
- McDermott, J.M. & McDonald, B.A. (1993) Gene flow in plant pathosystems. *Annual Review of Phytopathology* **31** : 353-373.
- Meng, X. & Chen, W. (2001) Applications of AFLP and ISSR techniques in detecting genetic diversity in the soybean brown stem rot pathogen *Phialophora gregata*. *Mycological Research* **105** : 936-940.
- Messner, R. (1995) Sequences of ribosomal genes and internal transcribed spacers move three plant parasitic fungi, *Eremothecium ashbyi*, *Ashbya gossypii*, and *Nematospora coryli*, towards *Saccharomyces cerevisiae*. *Journal of General and Applied Microbiology* **41** : 31-42.

- Michelmore, R.W. & Hulbert, S.H. (1987) Molecular markers for genetic analysis of phytopathogenic fungi. *Annual Review of Phytopathology* **25** : 383-404.
- Minter, D.W., Kirk, P.M. & Sutton, B.C. (1983) Thallic phialides. *Transactions of the British Mycological Society* **80** : 39-66.
- Mitchell, J.I., Roberts, P.J & Moss, S.T. (1995) Sequence or structure? A short review of the application of nucleic acid sequence information to fungal taxonomy. *Mycologist* **9** : 67-75.
- Moller, W.J. & DeVay, J.E. (1968) Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58** : 1499-1507.
- Moon, C.D., Tapper, B.A. & Scott, B. (1999) Identification of *Epichloe* endophytes in planta by a microsatellite-based PCR fingerprinting assay with automated analysis. *Applied and Environmental Microbiology* **65** : 1268-1279.
- Mourichon, X. (1994) Serious citrus dieback in Colombia caused by *Ceratocystis fimbriata*. *Fruits* **49** : 5-6.
- Mouton, M., Wingfield, M.J. & Van Wyk, P.S. (1994) Conidium development in anamorphs of *Ceratocystis sensu lato*: a review. *South African Journal of Science* **90** : 293-298.
- Muchovej, J.J., Albuquerque, F.C. & Ribeiro, G.T. (1978) *Gmelina arborea* – a new host of *Ceratocystis fimbriata*. *Plant Disease Reporter* **62** : 717-719.
- Nazar, R.N., Weng, W.M. & Abrahamson, J.L.A (1987) Nucleotide sequence of the 18S-25S ribosomal RNA intergenic region from a thermophile, *Thermomyces lanuginosus*. *Journal of Bacteriological Chemistry*. **262** : 7523-7527.
- Nieto, I.L. (1991) Problemas patológicos en mango. En: *Memorias XII Congreso Ascolfi, Manizales* : 10-12.
- Okada, G., Seifert, K.A., Takematsu, A., Yamaoka, Y., Miyazaki, S. & Tubaki, K. (1998) A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. *Canadian Journal of Botany* **76** : 1495-1506.
- Okada, G., Takematsu, A. & Takamura, Y. (1997) Phylogenetic relationships of the hyphomycete genera *Chaetopsina* and *Kiionochaete* based on 18S rDNA sequences. *Mycoscience* **38** : 409-420.
- Olchowecki, A. & Reid, J. (1973) Taxonomy of the genus *Ceratocystis* in Manitoba. *Canadian Journal of Botany* **52** : 1675-1711.
- Olson, E.O. (1949) Genetics of *Ceratostomella*. I. strains in *Ceratostomella fimbriata* (Ell. & Halst.) Elliott from sweet potatoes. *Phytopathology* **39** : 548-561.

- Olsen, E.O. & Martin, W.J. (1949) Relationship of *Ceratostomella fimbriata* from *Hevea* rubber tree and sweet potato. *Phytopathology* **39** : 17.
- Panconesi, A. (1981) *Ceratocystis fimbriata* of plane trees in Italy; biological aspects and control possibility. *European Journal of Forest Pathology* **11** : 385-395
- Panconesi, A. (1999) Canker stain of plane trees: A serious danger to urban plantings in Europe. *Journal of Plant Pathology* **81** : 3-15.
- Paulin, A.E. & Harrington, T.C. (2000) Phylogenetic placement of anamorphic species of *Chalara* among *Ceratocystis* species and other ascomycetes. *Studies in Mycology* **45** : 209-222.
- Pontis, R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **41** : 179-184.
- Rafalski, J.A., Vogel, J.M., Morgante, M., Powell, W., Andre, C. & Tingey, S.V. (1996) Generating and using DNA markers in plants. In *Nonmammalian Genomic Analysis: A practical guide* : 75-133. Academic Press.
- Rauè, H.A., Klootwijk, J. & Musters, W. (1988) Evolutionary conservation of structure and function of high molecular weight ribosomal RNA. *Progress in Biophysics and Molecular Biology*. **51** : 77-129.
- Redfern, D.B., Stoakley, J.T., Steel, H. & Minter, M. (1987) Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36** : 467-480.
- Ribeiro, I.J.A., Ito, M.F., Filho, P. & De Castro, J.L. (1985) Gummosis of *Acacia decurrens* WILLD. caused by *Ceratocystis fimbriata* Ell. & Halst. *Summa Phytopathologica* **11** : 7.
- Rincon, O. (1983) El culivo del caucho en Colombia. En: *Revista ESSO Agricola* **2** : 5-14.
- Rosinski, M.A. & Campana, R.J. (1964) Chemical analysis of the cell wall of *Ceratocystis ulmi*. *Mycologia* **56** : 738-744.
- Roux, J., Coutinho, T.A., Mujuni Byabashaija, D. & Wingfield, M.J. (2001a) Diseases of plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97** : 16-18.
- Roux, J., Harrington, T.A., Steimel, J.P. & Wingfield, M.J. (2001b) Genetic variation in the wattle wilt pathogen *Ceratocystis albofundus*. *Mycoscience* **42** : 327-332.
- Roux, J., Wingfield, M.J., Wingfield, B.D., Bouillett, J-P. & Alfenas, A.C. (1999) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30** : 175-184.

- Santini, A. & Capretti, P. (2000) Analysis of the Italian population of *Ceratocystis fimbriata* f.sp. *platani* using RAPD and minisatellite markers. *Plant Pathology* **49** : 461-467.
- Schlötterer, C. & Pemberton, J. (1998) The use of microsatellites for genetic analyses of natural populations – a critical review. In *Molecular approaches to individuals, populations and species*, (R. DeSalle & B. Schierwater, eds): 71-86. Birkhäuser, Basel.
- Seifert, K.A. & Okada, G. (1993) *Graphium* anamorphs of *Ophiostoma* species and similar anamorphs of other ascomycetes. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.A. Webber, eds): 27-41. APS Press, St. Paul, Minnesota, USA.
- Sharples, A. (1936) Diseases and pests of the rubber tree. Pages 208-229. MacMillan, London.
- Siemaszko, W. (1939) Fungi associated with bark beetles in Poland. *Planta Polonica* **7** : 1-54.
- Spatafora, J.W. & Blackwell, M. (1993) Molecular systematics of unitunicate perithecial ascomycetes: The Clavicipitales-Hypocreales connection. *Mycologia* **85** : 912-922.
- Spatafora, J.W. & Blackwell, M. (1994) The polyphyletic origins of ophiostomatoid fungi. *Mycological Research* **98** : 1-9.
- Spencer, J.F.T. & Gorin, P.A.J. (1971) Systematics of the genera *Ceratocystis* and *Graphium*. Proton magnetic resonance spectra of the mannose-containing polysaccharides as an aid in classification. *Mycologia* **63** : 387-402
- Sydow, H. & Sydow, P. (1919) Mykologische mitteilungen. *Annales Mycologici* **17** : 33-47.
- Szkolnik, M. (1951) Coffee trunk and stem canker in Guatemala. *Plant Disease Reporter* **35** : 500-501.
- Taubenhaus, J. (1913) The black rots of the sweet potato. *Phytopathology*. **3** : 159-166.
- Tautz, D. (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research* **17** : 6463-6471.
- Taylor, J.W., Geiser, D.M., Burt, A. & Koufopanou, V. (1999a) The evolutionary biology and population genetics underlying fungal stain typing. *Clinical Microbiology Reviews* **12** : 126-146.

- Taylor, J.W., Jacobson, D.J. & Fisher, M.C. (1999b) The evolution of asexual fungi: reproduction, speciation and classification. *Annual Review of Phytopathology* **37** : 197-246.
- Teviotdale, B.L. & Harper, D.H. (1991) Infection of pruning and small bark wounds in almond by *Ceratocystis fimbriata*. *Plant Disease* **75** : 1026-1030.
- Upadhyay, H. P. (1981) A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens.
- Upadhyay, H.P. (1993) Classification of the Ophiostomatoid Fungi. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.A. Webber, eds): 7-14. APS Press, St. Paul, Minnesota.
- Vigouroux, A. (1979) Les 'dépérissements' des platanes: causes, importance, mesures envisageables. *Revue Forestière Française* **31** : 28-39.
- Vogelzang, B.K. & Scott, E.S. (1991) *Ceratocystis fimbriata*, causal agent of basal rot of *Syngonium* cultivars, and host range studies of isolates of *C. fimbriata* in Australia. *Australasian Plant Pathology* **19** : 82-89.
- Walker, J., Tesoriero, L., Pascoe, I. & Forsberg, L.I. (1988) Basal rot of *Syngonium* cultivars and the first record of *Ceratocystis fimbriata* from Australia. *Australasian Plant Pathology* **17** : 22-23.
- Walter, J.M., Rex, E.G. & Schreiber, R. (1952) The rate of progress and destructiveness of canker stain of planetrees. *Phytopathology* **42** : 236-239.
- Webster, R.K. (1967b) The inheritance of sexuality, colour and colony type in *Ceratocystis fimbriata*. *Mycologia* **59** : 222-233.
- Webster, R.K. & Butler, E.E. (1967a) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45** : 1457-1468.
- Webster, R.K. & Butler, E.E. (1967c) The origin of self-sterile, cross-fertile strains and culture sterility in *Ceratocystis fimbriata*. *Mycologia* **59** : 212-221.
- Weijman, A.C.M & De Hoog, G.S. (1975) On the subdivision of the genus *Ceratocystis*. *Antonie van Leeuwenhoek* **41** : 353-360.
- Wingfield, B.D., Viljoen, C.D. & Wingfield, M.J. (1999) Phylogenetic relationships of ophiostomatoid fungi associated with *Protea* infrutescences in South Africa. *Mycological Research* **103** : 1616-1620.
- Wingfield, M.J., De Beer, C., Visser, C. & Wingfield, B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *System Applied Microbiology* **19** : 191-202.

- Wingfield, M.J., Harrington, T.C. & Solheim, H. (1997) Two new species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75** : 827-834.
- Wingfield, M.J., Seifert, K.A. & Webber, J.A. (1993) *Ceratocystis* and *Ophiostoma*: *Taxonomy, Ecology and Pathogenicity*. APS Press, St. Paul, Minnesota.
- Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., Wolfaardt, M. & Harrington, T.C. (1998) Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90** : 96-101.
- Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J. & Harrington, T.C. (1999) PCR-based identification and phylogeny of species of *Ceratocystis* sensu stricto. *Mycological Research* **103** : 743-749.
- Witthuhn, R.C., Harrington, T.C., Wingfield, B.D., Steimel, J.P. & Wingfield, M.J. (2000a) Deletion of the *MAT-2* mating type gene during uni-directional mating type switching in *Ceratocystis*. *Current Genetics* **38** : 48-52.
- Witthuhn, R.C., Harrington, T.C., Steimel, J.P., Wingfield, B.D & Wingfield, M.J. (2000b) Comparison of isozymes, rDNA spacer regions, and *MAT-2* DNA sequences as Phylogenetic characters in the analysis of the *Ceratocystis coerulescens* complex. *Mycologia* **92**, 447-452.
- Wolf, S.M. & McDermott, J.M. (1994) Population genetics of plant pathogen interactions: The example of the *Erysiphe graminis-Hordeum vulgare* pathosystem. *Annual Review of Phytopathology* **32** : 89-113.
- Wood, F.A. & French, D.W. (1963) *Ceratocystis fimbriata*, the cause of a stem canker of quaking aspen. *Forestry of Science* **9** : 232-235.
- Zalasky, H. (1965) Morphology of *Ceratocystis fimbriata* in Aspen. *Canadian Journal of Botany* **43** : 625-627.
- Zhou, Z., Miwa, M. & Hogetsu, T. (2001) Polymorphism of simple sequence repeats reveals gene flow within and between ectomycorrhizal *Suillus grevillei* populations. *New Phytologist* **149** : 339-348.
- Zimmerman, A. (1900) Ueber den Krebs von *Coffea arabica*, verursacht *Rostrella coffeae* gen. et sp. n. *Bul. Institute of Botanical Gardens Buitenzorg* **4** : 19-22.

Table 1: Geographical distribution, host ranges and some of the diseases caused by *Ceratocystis fimbriata* reported in literature.

Disease	Host	Country	Reference	
Cankers	<i>Populus</i> spp.	Europe, Canada, U.S.A.	Wood & French 1963, Zalasky 1965, Manion & French 1967, Hinds 1972, Gremmen & De Kam 1977	
	<i>Pimenta officinalis</i>	Jamaica	Leather 1966	
	<i>Hevea brasiliensis</i>	Brazil	Beeley 1929, Sharples 1936	
	<i>Prunus</i> spp.	U.S.A.	DeVay 1968, Teviotdale & Harper 1991	
	<i>Citrus</i> spp	Colombia	Mourichon 1994	
	<i>Colocasia esculenta</i>	Western Samoa	Walker <i>et al.</i> 1988	
	<i>Xanthosoma sagittifolium</i>	Fiji	Walker <i>et al.</i> 1988	
	<i>Acacia decurrens</i>	Brazil	Ribeiro 1985	
	Wilts and canker stain	<i>Gmelina arborea</i>	Brazil	Muchovej <i>et al.</i> 1978
		<i>Eucalyptus</i> spp.	Brazil	Laia <i>et al.</i> 1999
		<i>Platanus</i> spp.	USA, France, Italy, Spain, Switzerland,	Walter <i>et al.</i> 1952, Vigouroux 1979, Panconesi 1981
		<i>Eucalyptus</i> hybrids	Republic of Congo	Roux <i>et al.</i> 1999
		<i>Eucalyptus</i> spp.	Uganda	Roux <i>et al.</i> 2001a
<i>Coffea arabica</i>		Central and South America, i.e. Indonesia, Colombia, Venezuela, Brazil	Zimmermann 1900, Pontis 1951, Szkolnik 1951, Echandi 1955	
<i>Theobroma cacao</i>		Central and South America	Iton 1959	
<i>Mangifera indica</i>		Brazil, Colombia	Batista 1960	
Root and stem rot		<i>Crotalaria juncea</i>	Brazil, Colombia, Taiwan	Costa 1935, ChunYee <i>et al.</i> 1997
		<i>Syngonium</i> cultivars	Australia	Walker <i>et al.</i> 1988
	<i>Ipomoea batatas</i>	U.S.A., South East Asia, Oceania, Papua New Guinea, World-wide	Halsted 1980, Clark & Moyer 1988, Taubenhau 1913	



CHAPTER 2

Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt and canker pathogen, *Ceratocystis fimbriata*

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ABSTRACT

Ceratocystis fimbriata is a serious wilt and canker stain pathogen of many economically important agricultural and forestry crops. It has a wide geographic distribution and host range that includes both woody and herbaceous plants. Previous studies using hybridisation, have shown that differences exist in isolates of *C. fimbriata* from different hosts and origins. These isolates also differ in colony morphology, pathogenicity, growth rate as well as conidial states. Therefore, it has been suggested that distinct strains or special forms are encompassed in *C. fimbriata*, and that these could be linked to host or geographical origin. The aim of this study was to develop PCR based microsatellite markers for population studies on *C. fimbriata*. ISSR-PCR was used to target specific microsatellite regions of genomic DNA from two isolates of *C. fimbriata*. These amplified products were cloned and sequenced. Primer pairs were designed from these sequences to flank the microsatellite regions. From 24 primer pairs, 11 polymorphic primers were selected. These primers were tested on a number of *C. fimbriata* isolates representing a wide host and geographic range. Cluster analyses of the results indicate that these microsatellite markers clearly distinguish between different geographical as well as host specific populations of *C. fimbriata*. The results are concordant with sequence data from the internal transcribed spacer (ITS) region of the rDNA operon of the same isolates. These markers will be useful in future studies of population structures and diversity in *C. fimbriata*.

INTRODUCTION

The fungal pathogen, *Ceratocystis fimbriata* Ell. & Halst., causes serious wilt and canker-stain diseases on a wide range of plants world-wide. Some of the economically important agricultural and tree crops damaged by this pathogen include sweet potato (Halsted & Fairchild 1891), coffee (Pontis 1951), cacao (Kile 1993), stone fruit trees (DeVay *et al.* 1963), poplar (Gremmen & De Kam 1977), rubber (Olsen & Martin 1949) as well as *Eucalyptus* spp. (Roux *et al.* 1998). Although *C. fimbriata* has predominantly been reported from Central and South America (McCracken & Burkhardt 1977), its occurrence is geographically widespread in temperate and tropical regions of the world.

Identification of *C. fimbriata* in disease reports and general taxonomic treatments has been based, for the most part, on morphological and cultural characteristics (Upadhyay 1981). Isolates from different hosts and geographic areas, however, have been shown to differ not only in colony morphology but also in growth rate and conidial states (Webster & Butler 1967). Pathogenicity tests have shown that some isolates tend to be host specific. For example, inoculation studies carried out on cacao, coffee and sweet potato with an isolate of *C. fimbriata* from pimento gave negative results (Leather 1966). Pontis (1951), inoculated coffee trees with coffee and sweet potato isolates and showed that only the coffee isolate could infect coffee.

The name, *C. fimbriata* f.sp. *platani*, has been assigned to *C. fimbriata* isolates that specifically infect plane trees (*Platanus* spp.). Although morphologically indistinguishable from the type species of *C. fimbriata* from sweet potato, the plane fungus is specifically pathogenic to its hosts. Furthermore, Webster & Butler (1967), in hybridisation studies, showed that differences exist in *C. fimbriata* isolates from different hosts and origins. Based on this finding, they suggested that *C. fimbriata* is comprised of distinct host specific strains. Host specialisation in morphologically indistinguishable isolates illustrates the taxonomic limits of morphology. It also emphasises the advantages of contemporary molecular techniques that might facilitate differentiation between strains or cryptic forms within fungal species (Fisher *et al.* 2000).

Many different DNA fingerprinting techniques have been used to define fungal populations, including Randomly Amplified Polymorphic DNA (RAPDs) (Santini &

Capretti 2000), Randomly Amplified Microsatellites (RAMS) (Hantula *et al.* 1998), Restriction Fragment Length Polymorphisms (RFLP's) (Kuninaga & Yokosawa 1992), nuclear DNA fingerprinting markers using (CAT)₅ (Roux *et al.* 2001) and Amplified Fragment Length Polymorphisms (AFLP) (Majer *et al.* 1996). There is, however, an increasing interest in using co-dominant markers in population studies. These markers allow for the detection and characterisation of multiple alleles at a given locus. Microsatellite regions provide an attractive source of polymorphisms between isolates and many properties favour their use as genetic markers. They are abundant in Eucaryotic genomes, have a co-dominant inheritance, are highly polymorphic, easy to use and score, and they are selectively neutral with a high mutation rate (Tautz 1989, Rafalski *et al.* 1996). Various studies employing microsatellite markers have made it possible to define population relationships, parentage and kinship, gene mapping and forensics (Queller *et al.* 1993, Engel *et al.* 1996, Jarne & Lagoda 1996).

In this study, we report on the development and characterisation of PCR based microsatellite markers for *C. fimbriata*. These primers were tested on isolates collected from a wide geographic and host range. Results based on microsatellites were also compared with those from sequence data obtained from the internal transcribed spacer region (ITS), and phylogenetic relationships between isolates were considered.

MATERIALS AND METHODS

Fungal isolates

Ceratocystis fimbriata isolates were selected to represent a wide variety of hosts and geographical origins (Table 1). Isolates were grown on potato dextrose agar (PDA, Biolab) containing chloramphenicol (100 mg/liter, Centaur Laboratories). A drop of ascospores from a single perithecium of each isolate was suspended in 50 µl mineral oil, spread onto PDA plates and incubated at 25 °C. Hyphal tips from germinating ascospores were isolated and incubated for 2 weeks at 25 °C in Erlenmeyer flasks containing 50 ml malt extract broth (2 %, Biolab). The mycelium was collected by filtration (Whatman No. 1) and lyophilized. The freeze-dried cultures were pulverized using liquid nitrogen. DNA was extracted for each isolate using the method previously described by Barnes *et al.* (2001).

ISSR- PCR

DNA from isolates CMW 4822 and CMW 4835 was randomly amplified using ISSR primers 5' DHB(CGA)₅, 5'HV(GT)₅G, 5' DDB(CCA)₅, 5'DBD(CAC)₅, 5' HVH(GTG)₅, 5'NDB(CA)₇C and 5'NDV(CT)₈ (Lieckfeldt *et al.* 1993, Buscot *et al.* 1996). The polymerase chain reaction (PCR) mixture consisted of a final concentration of 2 ng DNA, 200 µM of each dNTP, 600 nM primer, 3.5 U *Taq* DNA polymerase and 1x buffer with MgCl₂ (Roche Molecular Biochemicals, Alameda, CA). The reaction volume was adjusted to 50 µl using Sabax water. PCR conditions consisted of a 2 min denaturation step at 95 °C followed by 40 cycles of 30 s at 95 °C, 45 s at 48 °C and 2 min at 72 °C. A final step at 72 °C for 10 min ensured completion of the elongation process. Products were visualised on a 1.5% agarose gel containing ethidium bromide, under UV illumination and purified using the Magic PCR Preps Purification System (Promega Corp., Madison, WI) before cloning.

Cloning

The PCR products produced from each ISSR primer were cloned using the pGEM®-T Easy Vector System (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturers instructions. Bacterial colonies containing recombinant plasmids were selected and grown in test tubes containing 2 ml Luria-Bertani (LB) broth supplemented with 100 µg/ml ampicillin (Sigma Chemicals Co, USA). The cells were incubated at 37 °C for 8 hours after which plasmid DNA was recovered using alkaline lysis (Sambrook *et al.* 1989). This DNA was re-suspended in 20 µl water containing RNase (10 mg/ml, Roche Molecular Biochemicals). Restriction digests with *Eco*RI (Roche Molecular Biochemicals) were performed on the plasmid DNA. The sizes of the cloned fragments were determined using Agarose gel electrophoreses.

Primer design

For each ISSR reaction, cloned fragments of different sizes were sequenced using an ABI PRISM™ 377 Autosequencer (Perkin-Elmer Applied Biosystems, Inc., Foster City, Calif). Sequence reactions were carried out with universal plasmid primers T7 and SP6 using an ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems). Sequence electropherograms were analyzed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems). The fragments sequenced were screened for microsatellite regions. Primer pairs were designed to flank these microsatellite regions and to amplify a DNA fragment in the range of between 150 to

500 bp so that each primer pair produced a different sized fragment to facilitate multiplexing. Primers were also designed to have a length of between 18 to 22 bases, annealing temperatures ranging from 58 °C to 64 °C and a GC content of around 50% (Table 2). Genome walking was performed as described by Burgess *et al.* (2001) and Siebert *et al.* (1995), on sequences that contained microsatellite regions at either the beginning or end of the sequence. The DNA from isolate CMW 4829 was used in the restriction digestions with enzymes *EcoRV*, *Scal*, *HaeIII* and *RsaI* (Roche Molecular Biochemicals). Specific primers were designed for each of these sequences and used in subsequent reactions involved in genome walking.

Primer Testing

All the primers designed were used in PCR reactions to test for amplification. DNA from five isolates (CMW 2218, CMW 2242, CMW 4791, CMW 4829, and CMW 5328) with different geographic locations was selected for this purpose. PCR reactions were performed in 25 µl reaction volumes, consisting of 1 ng DNA, 200 µM of each dNTP, 300 nM of each primer, 0.35 U Expand High Fidelity enzyme (Roche Molecular Biochemicals) and 1.2 x Expand HF buffer containing 1.5 mM MgCl₂ (supplied with the enzyme). The PCR programme consisted of a 2 min denaturation step at 96 °C followed by 10 cycles of 20 s at 94 °C, 48 s at the specified annealing temperature for each primer and 45 s at 72 °C. A further 25 cycles were carried out with a 5 s extension after each cycle with the annealing time altered to 40 s. A final elongation step was carried out for 10 min at 72 °C. Samples were separated on a PAGE gel (6% acrylamide in 50 mM Tris-borate-EDTA buffer, 7 h at 140V) and subsequently silver stained (Blum 1987) to visualise amplification products. One primer from each primer pair that yielded a single polymorphic PCR product for all isolates, was re-synthesised and labelled with either a TET or FAM phosphoramidite fluorescent dye (MWG, Ebersberg, Germany) (Table 3). The labelled primers were then used in subsequent PCR reactions to amplify all *C. fimbriata* isolates in this study (Table 1).

Polymorphisms of loci

Fluorescent-labeled PCR products (0.5 µl containing 1.5 ng DNA) were combined with 0.5 µl of the internal standard GENESCAN –TAMRA (Perkin-Elmer Corp) and 1.5 µl of loading buffer. The samples were fractionated by PAGE (4.25 %) on an ABI Prism 377 DNA sequencer. The size of DNA fragments was determined using a combination of the

GeneScan® 2.1 analysis software (Perkin Elmer Corp.) and Genotyper® 3.0 (Perkin Elmer Corp.). A few isolates were sequenced to determine whether the observed polymorphisms resulted from mutations specifically within the microsatellite regions. Sequence reactions were carried out with an ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems) with protocols recommended by the manufacturers. Sequence electropherograms were analyzed with Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems).

ITS PCR and sequencing

Primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.* 1990) were used to sequence the ITS1, 5.8S and ITS2 ribosomal RNA operon regions. The PCR reaction mix consisted of 2–10 ng DNA, Expand High Fidelity PCR System enzyme mix (1.75 U) (Roche Molecular Biochemicals), 1 x Expand HF buffer containing 1.5 mM MgCl₂ (supplied with the enzyme), 200 nM of each primer and 200 μM of each dNTP. The same PCR programme was used as described above for the microsatellites. The annealing temperature was set to 55 °C. PCR amplicons were purified using the Magic PCR Preps Purification System (Promega, Madison, WI) and sequenced in both directions. Sequence electropherograms were analyzed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems).

Data analysis

For each isolate, the presence or absence of an allele at a specific locus was scored for each microsatellite primer and the data compiled in a matrix. Both the microsatellite matrix and the ITS sequences were analyzed using PAUP (Phylogenetic Analysis Using Parsimony [*and other methods]) (Swofford, 1998). The heuristic search option (based on parsimony) with random stepwise addition and tree bisection reconnection (TBR) was used. All characters were given equal weight and the Mulpar option was in effect. Length distribution of 1000 trees and confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis albofundus* de Beer, Wingfield & Morris, (CMW 2475, accession number AF043605), was used as the outgroup for the ITS sequence data set and treated as a monophyletic sister group with respect to the ingroup. All trees generated from the microsatellite matrix were rooted using the Midpoint rooting option. The microsatellite

data matrix was also analyzed using cluster analysis of Bray-Curtis coefficient of similarity and Euclidean distance using the program PRIMER (Clarke & Warwick 1994).

RESULTS

ISSR- PCR

All the RAMS primers produced between 7 to 10 bands, each ranging in size from 200 to 600 bp. For each primer, only the clones containing different sized inserts with lengths smaller than 1000 bp were selected for sequencing.

Primer design

A total of 52 cloned fragments were sequenced. From these, 18 primer pairs were designed to flank microsatellite regions. Six sequences from the cloned fragments had microsatellite regions at the beginning of the sequence. Genome walking was performed on these sequences to obtain the full microsatellite sequence with flanking regions suitable for primer design (Table 2). The microsatellite regions, around which primers were designed, included those rich in di-, tri-, and tetra-nucleotide repeats as well as regions rich in thymine repeats.

Primer testing

All the designed primers were tested on a number of *C. fimbriata* isolates to ensure effective amplification. Four primer pairs failed to amplify in PCR reactions at different annealing temperatures and one primer pair produced multiple bands. These five primers were discarded. Fractionation on PAGE gels revealed that of the 19 remaining primer pairs, eight were monomorphic and 11 polymorphic. The 11 polymorphic primer pairs were amplified at different annealing temperatures in order to obtain the highest optimal conditions to produce single, clean bands (Table 2). The eight putative monomorphic primers were further tested on a number of different *C. fimbriata* isolates in order to confirm their monomorphic nature.

Polymorphisms of loci

The 11 polymorphic primers successfully amplified all 21 isolates of *C. fimbriata* selected for testing. The only exception was primer CF 13/14 that produced null alleles for isolates

CMW 1547, CMW 2901, CMW 2911, CMW 2913 and CMW 3264. Genescan analysis showed that the polymorphic markers produced a total of 65 alleles across the 11 loci analyzed (Table 3). The smallest and largest number of alleles per locus was 3 and 10 respectively.

Alleles ranged from 154 to 415 bp in length. Of the 11 loci, two or more alleles from 6 loci were sequenced to determine the source of polymorphisms. Within the locus amplified by primer AG 17/18, the polymorphisms observed between the different alleles were due to indels of CTT repeats. The differences in alleles observed from the loci amplified by primers CF 5/6, CF 15/15 and CF 17/18 were due to indels of repeating units of CTG, CT and AG respectively. The polymorphisms observed in the alleles from primer CF 21/22 were due to a difference in the number of T repeats (Figure 2). There were a number of transitions and transversions between the different sequences within the same locus. This, however, did not alter the size of the allele. Although most of the polymorphisms observed were due to differences in the microsatellite regions, there were some insertions and deletions found within the flanking sequences.

Some alleles obtained in the 11 loci were unique to certain isolates or groups of isolates. Primers AG 7/8, AG 17/18, CF 11/12, CF 13/14, CF 15/16, CF 17/18 and CF 21/22 all amplified unique alleles for some of the isolates. Primer AG 7/8, a (TC)₂₁ repeat, produced the greatest number of unique alleles (5), CF 17/18, a (CA)₁₅ repeat, produced three unique alleles, while the others produced only one or two unique alleles from the 21 isolates studied.

Data analysis

A total of 65 aligned characters were obtained from analysis of the 11 microsatellite loci. The heuristic search option based on parsimony showed one of these characters to be constant, 23 parsimony un-informative and 41 parsimony informative. A total of four most parsimonious trees were generated with tree lengths of 96. The consistency index (CI), retention index (RI) and g₁ values were 0.67, 0.74 and -0.73 respectively for all trees. The dendograms produced from the analysis using the Bray-Curtis coefficient of similarity and the Euclidean distance were similar to the phylogenetic trees produced using PAUP. The dendogram based on similarity analysis was selected for presentation (Figure 1) and bootstrap values based on parsimony were included.

The manual alignment of the ITS sequences produced a total of 555 characters. Of these, 319 were constant, 89 parsimony un-informative and 147 parsimony informative. A total of four trees were produced with a length of 389. The CI, RI and gI values were 0.82, 0.85 and -1.57 respectively. One tree (Figure 1), with similar topology and minor differences in branch lengths from the other 11 trees was chosen for presentation.

The phylogenetic trees generated from the microsatellite and ITS data showed that isolates of *C. fimbriata* included in this study, reside in several sub-groups supported by high bootstrap values. All the *Platanus* isolates from France, Switzerland and Italy grouped together, forming a European sub-group with strong (100 %) bootstrap in both the microsatellite and the ITS tree. *C. fimbriata* isolates from Congo and Uganda grouped together with 62 % confidence in the microsatellite and 51 % in the ITS tree. African isolates were all collected from diseased *Eucalyptus* species and formed a separate central African clade. Another distinct sub-group encompassed all isolates from North America. These included isolates from Canada and the USA, with a bootstrap value of 70 % in the microsatellite and 100 % in the ITS tree. The Colombian isolates from *Coffea* sp. and *Citrus* sp. reside in two distinct sub-groups. Bootstrap values for both these groups were high. For the one group, these were 98 % in the microsatellite and 100 % in the ITS tree. Bootstrap values for the other sub-group were 72 % and 100 % respectively for the same trees.

DISCUSSION

In this study, 11 microsatellite primers that display strong polymorphisms in a wide variety of *C. fimbriata* isolates were produced. The markers clearly elucidate relationships between the different isolates based on host and geographic origin. These relationships were supported by analysis of sequence data from the ITS region of the rDNA operon. The results also support those of previous studies based on hybridisation (Webster & Butler 1967) and pathogenicity (Pontis 1951, Walter *et al.* 1952, Leather 1966), showing that *C. fimbriata* encompasses a wide variety of strains.

The phylogenetic and similarity trees generated from the microsatellite and ITS data clearly resolved isolates of *C. fimbriata* into distinct sub-groups based on hosts. Within

these host-based sub-groups, it was also possible to recognise further groupings linked to geographical origin of isolates. For example, all isolates from *Eucalyptus* grouped collectively in a Central African sub-group, but this could be further sub-divided to separate isolates from Congo and Uganda. The same was true for the isolates from North America, where those infecting *Populus* spp. were distinct from those from *Prunus*. Within the *Populus* sub-group, isolates from Canada and the USA were distinct. An intriguing observation was that isolates from Colombia reside in two strongly resolved clades, although most are found on a single host. We believe that the Colombian groups are genetically isolated and further studies using the microsatellite primers and a greater number of isolates are needed to test this hypothesis.

Santini & Capretti (2000), used RAPD and direct amplification of minisatellite-region DNA (DAMD) PCR to examine the genetic variability in a population of *C. fimbriata* f.sp. *platani* from Italy and some isolates of *C. fimbriata* from North America. Their study showed that all Italian isolates have the same genotype, thus representing a clone. Although they suggested the entire European population might be clonal and the result of a single introduction, the results of our study based on microsatellites show otherwise. Isolates from Switzerland, France and Italy had the same genotype, but differed from a second isolate from France. Although the numbers of isolates from Europe were few in our study, there is evidence for at least two different genotypes in Europe. We believe that this difference is due to the fact that Santini & Capretti (2000), used dominant markers, which have lower resolution than the co-dominant markers used in the present study.

We have shown that isolates from *Populus* in North America reside in a group that is distinct from the *Prunus* isolate. Similarly, our isolate from sweet potato (*Ipomoea batatas*) was different from all the other isolates. These results are similar to those of Santini & Capretti (2000), and strongly support the view that isolates of *C. fimbriata* can be divided into groups based on host. These data also support those from hybridisation experiments (Webster & Butler 1967), host specificity (Kojima *et al.* 1982), and pathogenicity assays (Pontis 1951, Walter *et al.* 1952, Leather 1966), suggesting the host represents an important factor separating isolates of *C. fimbriata*. Furthermore, the hypothesis that host range is an important factor in the evolution of *Ceratocystis* spp. (Witthuhn *et al.* 1998) is supported by the results of this study.

The two most common methods to develop microsatellite markers are through screening genomic DNA libraries for microsatellite- rich regions and through searching public DNA databases (Yu *et al.* 1999). The production of DNA libraries and their subsequent screening is time consuming and expensive. Although computer databases have refined the process of selecting microsatellite regions for primer design, large numbers of DNA sequences are required. These are not available for most fungi and therefore, we have followed the approach of Burgess *et al.* (2001) in choosing ISSR-PCR or RAMS- PCR to develop microsatellite markers for *C. fimbriata*. Of 52 clones that were sequenced, 22 primers were designed, and of these, 11 were polymorphic. The success rate using this method was 21 % for clones sequenced. Burgess *et al.* 2001, who also used this method, had a success rate of 35 %. This is compared to the less than 10 % success rate that is more commonly attained when using genomic libraries (Brady *et al.* 1996).

Sequences of PCR products amplified using the designed microsatellite primers revealed that the main source of polymorphisms was from mutations within the repetitive microsatellite regions. Although it has been suggested that most microsatellites in fungi represent AT repeats (Groppe *et al.* 1995), many of those in this study were of a CT repeat. Insertions and deletions also contributed to the polymorphisms observed between isolates. For example, the sequence data generated from the PCR amplicon using primer CF15/16 in isolate CMW 2901 from Canada, showed there was an insertion of 12 bp which was absent in all the other alleles sequenced. However, elsewhere in the allele sequence of this isolate, there was a deletion of 13 bp. Thus, a size difference of only one base pair was observed, despite the fact that the polymorphism was considerably larger. This indicates that microsatellite data might underestimate genetic diversity.

Some alleles present in the 11 loci were unique to certain isolates or groups of isolates. Goodwin *et al.* (1992) termed these types of alleles 'private alleles'. In the North American sub-group for example, three alleles in different loci were unique to this group and not found in any other isolate. The same was true with the Central African sub-group that had one of the Colombian sub-groups five unique alleles, not shared by any other isolates. Private alleles provide evidence for reproductive isolation over a long period of time (Fisher *et al.* 2000), and the fact that these groups had unique alleles further defines the sub-groups.

Detailed studies of the frequencies of specific alleles, particularly private alleles, in populations of *C. fimbriata* from different geographically diverse origins will make it possible to determine gene-flow between populations, as well as random mating mechanisms within the populations. Allelic differences at these polymorphic loci can also be used to determine evolutionary history and relationships between populations of *C. fimbriata* from different countries. Determining the area of origin of *C. fimbriata* will also aid in the development of management and quarantine strategies against certain strains of *C. fimbriata*.

REFERENCES

- Barnes, I., Roux, J., Coetzee, M.P.A., Wingfield, B.D. & Wingfield, M.J. (2001) Characterisation of *Seiridium* spp. associated with Cypress Canker based on β -tubulin and histone sequences. *Plant Disease* **85** : 317-321.
- Blum, H., Beier, H. & Gross, H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8** : 93-99.
- Brady, J.L., Scott, N.S. & Thomas, M.R. (1996) DNA typing of hops (*Humulus lupulus*) through application of RAPD and microsatellite marker sequences converted to sequence tagged sites (STS). *Euphytica* **91** : 277-284.
- Burgess, T., Wingfield, M.J. & Wingfield, B.D. (2001) Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology* **67** : 354-362.
- Buscot, F., Wipf, D., Battista, C.D., Munch, J.C., Botton, B. & Martin, F. (1996) DNA polymorphisms in morels: PCR/RFLP analysis of the ribosomal DNA spacers and microsatellite-primed PCR. *Mycological Research* **100** : 63-71.
- Clarke, K.R. & Warwick, R.M. (1994) Measures of similarity of species abundance/biomass between samples. In *Change in Marine Communities: An approach to statistical analysis and interpretation* Chapter 2: 1-8. Plymouth Marine Laboratory, Natural Environment Council, UK.
- DeVay, J.E., Lukezic, F.L., English, W.H. & Trujillo, E.E. (1963) *Ceratocystis* canker of stone fruit trees. *Phytopathology* **53** : 873.
- Engel, S.R., Linn, R.A., Taylor, J.F. & Davis, S.K. (1996) Conservation of microsatellite loci across species of *Artiodactyls*: Implications for population studies. *Journal of Mammalogy* **77** : 504-518.
- Fisher, M.C., Koenig, G., White, T.J & Taylor, J.W. (2000) A test for concordance between the multilocus genealogies of genes and microsatellites in the pathogenic fungus *Coccidioides immitis*. *Molecular and Biological Evolution* **17** : 1164-1174.
- Goodwin, S.B., Spielman, L.J., Matuszak, J.M., Bergeron, S.N. & Fry, W.E. (1992) Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in northern and central Mexico. *Phytopathology* **82** : 955-961.
- Gremmen, J. & De Kam, M. (1977) *Ceratocystis fimbriata*, a fungus associated with poplar canker in Poland. *European Journal for Forestry Pathology* **7** : 44-47.

- Groppe, K., Sanders, I., Wiemken, A. & Boller, T. (1995) A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichloë* spp.) in grasses. *Applied and Environmental Microbiology* **61** : 3943-3949.
- Halsted, B.D. & Fairchild, D.G. (1891) Sweet-potato black rot. *Journal of Mycology* **7** : 1-11.
- Hantula, J., Niemi, E.M., Kaitera, J., Jalkanen, R. & Kurkela, T. (1998) Genetic variation of the resin top fungus in Finland as determined by random amplified microsatellites (RAMS). *European Journal for Forestry Pathology* **28** : 361-372.
- Jarne, P. & Lagoda, P.J.A. (1996) Microsatellites, from molecules to populations and back again. *Tree* **11** : 424-429.
- Kile, G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.A. Webber, eds): 173-183. APS Press: St. Paul, Minnesota.
- Kojima, M., Kawakita, K. & Uritani, I. (1982) Studies on a factor in sweet potato root which agglutinates spores of *Ceratocystis fimbriata*, Black Rot Fungus. *Plant Physiology* **69** : 474-478.
- Kuninaga, S. & Yokosawa, R. (1992) Genetic diversity of *Fusarium oxysporum* f.sp. *lycopersici* in restriction fragment length polymorphisms of mitochondrial DNA. *Transactions of the Mycological Society of Japan* **33** : 449-459.
- Leather, R.I. (1966) A canker and wilt disease of pimento (*Pimenta officinalis*) caused by *Ceratocystis fimbriata* in Jamaica. *Transaction of the British Society* **42** : 213-218.
- Lieckfeldt, E., Meyer, W. & Börner, T. (1993) Rapid identification and differentiation of yeasts by DNA and PCR fingerprinting. *Journal of Basic Microbiology* **33** : 413-426.
- Majer, D., Mithen, R., Lewis, B.G., Vos, P. & Oliver, R.P. (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* **100** : 1107-1111.
- McCracken, F.I. & Burkhardt, E.C. (1977) Destruction of sycamore by canker stain in the midsouth. *Plant Disease Reporter* **61** : 984-986.
- Olsen, E.O. & Martin, W.J. (1949) Relationship of *Ceratostomella fimbriata* from *Hevea* rubber tree and sweet potato. *Phytopathology* **39** : 17.
- Pontis, R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **41** : 179-184.

- Queller, D.C., Strassmann, J.E. & Hughes, C.R. (1993) Microsatellites and Kinship. *Tree* **8**: 285-288.
- Rafalski, J.A., Vogel, J.M., Morgante, M., Powell, W., Andre, C. & Tingey, S.V. (1996) Generating and using DNA markers in plants. In *Nonmammalian Genomic Analysis: A practical guide*. : 75-133. Academic Press.
- Roux, J., Harrington, T.A., Steimel, J.P. & Wingfield, M.J. (2001). Genetic variation in the wattle wilt pathogen *Ceratocystis albofundus*. *Mycoscience* **42** : 327-332.
- Roux, J., Wingfield, M.J., Bouillette, J.P., Wingfield, B.D. & Alfenas, A.C. (1998) A serious new wilt disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30** : 175-184.
- Sambrook, J., Fritsch, T. & Maniatis, T. (1989) Molecular Cloning – A laboratory manual. Cold Spring Harbour Laboratory Press: New York.
- Santini, A. & Capretti, P. (2000) Analysis of the Italian population of *Ceratocystis fimbriata* f.sp. *platani* using RAPD and minisatellite markers. *Plant Pathology* **49** : 461-467.
- Siebert, P.D., Chenchik, A., Kellogg, D.E., Lykhanov, A. & Lukyanov, S.A. (1995) An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Research* **23** : 1087-1088.
- Swofford, D.L. (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods). Vers:4. Sinaur Associates: Sunderland, Massachusetts.
- Tautz, D. (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research* **17** : 6463-6471.
- Upadhyay, H.P. (1981) A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press: Athens.
- Walter, J.M., Rex, E.G. & Schreiber, R. (1952) The rate of progress and destructiveness of canker stain of plane trees. *Phytopathology* **42** : 236-239.
- Webster, R.K., & Butler, E.E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45** : 1457-1468.
- White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A sequencing Guide to Methods and Applications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White, eds): 315-322. Academic Press: San Diego, USA.

- Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., Wolfaardt, M. & Harrington, T.C. (1998) Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* 90 : 96-101.
- Yu, K., Park, S.J. & Poysa, V. (1999) Abundance and variation of microsatellite DNA sequences in beans (*Phaseolus* and *Vigna*). *Genome* 42 : 27-34.

Table 1: Isolates of *Ceratocystis fimbriata* used for the development and testing of microsatellite markers and their respective microsatellite profile.

Isolate no ¹	Host	Country	Collector	Microsatellite profile ²	ITS Genbank Accession No.
CMW 1547	<i>Ipomoea batatas</i>	Papua New Guinea (PNG)	E.H.C McKenzie	4 1 225306211	AF264904
CMW 1894	<i>Platanus</i> sp.	Switzerland	O. Petrini	4 7 217673513	AF395682
CMW 1896	"	"	"	4 7 217673513	AF395681
CMW 2218	"	France	C. Grosclaude	4 8 217663513	AF395680
CMW 2219	"	"	"	4 7 217673513	AF395679
CMW 2242	"	Italy	A. Panconesi	4 7 217673513	AF264903
CMW 2901	<i>Populus tremuloides</i>	Canada	G. Smalley	3 2 251101233	AF395696
CMW 3264	"	"	"	3 4 342401823	No sequence
CMW 2911	<i>Prunus</i> sp.	U.S.A	R. Bostock	2 2 252201133	AF395693
CMW 2913	<i>Populus tremuloides</i>	"	T. Hinds	3 2 252201233	AF395694
CMW 4791	<i>Eucalyptus</i> clone	Rep. of Congo	J. Roux	2 5 213242312	AF395685
CMW 4793	"	"	"	3 2 216432513	AF395684
CMW 4829	<i>Citrus</i> sp.	Colombia	B. L. Castro	3 2 216432513	AF395688
CMW 4835	<i>Coffea</i> sp.	"	"	2(10)239553724	AF395689
CMW 4824	"	"	"	2(10)332553724	AF395692
CMW 4844	"	"	"	1 9 216225312	AF395691
CMW 4845	"	"	"	1 4 214274312	AF395690
CMW 4903	<i>Eucalyptus</i> sp.	Brazil	A. Alfenas	1 6 214215312	AF395683
CMW 5312	<i>Eucalyptus grandis</i>	Uganda	J. Roux	3 2 117332513	AF395687
CMW 5328	"	"	"	3 3 218432413	AF395686

¹ All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

² The microsatellite profile represents the multilocus genotype observed for each isolate. Each different allele in a loci is allocated a number. All eleven loci are scored in this manner and when combined, a profile is created for each isolate. Isolates with the same microsatellite profile have the same genotype.

Table 2: Primers designed from inserts of plasmid clones.

Primer	Primer sequence	T _m ¹	T _a ²	Banding pattern
AG1	CGG GGA TGC TGT TGT CTC G	58.8	58	Polymorphic
AG2	GTT TCT CGA CTT CCA GGC CC	61.4		
AG3	CGT CGC ACG CTC ACA TCT AC	61.4	58	No amplification
AG4	GAC AGA GTC TCT GGA CAG	61		
AG5	CCT CGT TGA CTA GCA CGT CC	61.4	58	No amplification
AG6	CAC CTC AGC GTA ACG CGA G	59.4		
AG7	GGG GCG GTG GTG CAA TTG TC	61	56	Polymorphic
AG8	CGA GAC AGC AAC ACA AGC CC	61.4		
AG9	GAG CAG TTA AGC GGC GAT TC	59.4	55	Stutter bands
AG10	CGT CCG ATA GTA ACA GTC GTG	59.8		
AG11	GCA TCT CTT TGC CAA GCA AGC	59.8	55	Monomorphic
AG12	CCA TTA ATG CCA GCG GCA AC	59.4		
AG13	GGA GCA GCA ACT GGA GCA AC	61.4	58	Monomorphic
AG14	CCC CCT AGA CAG CAT ATC C	58.8		
AG15	GGA TAG CAG CGA CAA GGA CC	61.4	62	Polymorphic
AG16	CTT GAC CGA CCT GCC GAT TG	61.4		
AG17	CGG CCC TGC CAA CGG ATG	62.8	62	Polymorphic
AG18	GTC GGT GGT GGA GAC GGT C	63.1		
AG19	GGC GGC GCG CAA GAT CGC	65.1	60	Monomorphic
AG20	GCA GCC CGG GCC ACT TGC	65.1		
AG21	CGT GGC CGG AGT AAA TGA CTG	61.8	56	No amplification
AG22	GTC GGA AAC GGG TCA GTC AG	61.4		
AG23	GCT GAC ATG TCC GCA CTG C	61	56	Monomorphic
AG24	GCA TTC TGG CGC GTT GAG G	61		
AG27	CTA TTG CGG TCA CGT TCA CG	59.4	58	Monomorphic
AG28	CTC ATG GAA GGA AAG ACC GTG	59.8		
CF1	GGT GCA TGC GTG TGC ATA TGC	61.8	56	No amplification
CF2	CGC CAA GCT ATT TAG GTG ACA C	60.3		
CF5	CAT GGG CAT GCC TAG CCT TG	61.4	62	Polymorphic
CF6	GAC CAA AGA TGG TGG CGA GC	61.4		
CF7	CCG TGT AGG TCA CTA GGG TC	59.4	55	Monomorphic
CF8	CTA GCA AGC TGA CTG ACT GG	59.4		
CF9	GAT GTC GGT TAG GAC GGG	58.2	52	Monomorphic
CF10	CCC ACG GGA TCA ACT TGG	58.2		
CF11*	GGG ACG AAA CTG GAG CGT CT	61.4	60	Polymorphic
CF12	CTC CCA AAC TCC ATG CTC TTG	59.8		
CF13*	GAT CGA TCG GCA GAC CGA TAC	61.8	64	Polymorphic
CF14	GGA ACT CTG ATG CCT CCA GTG	61.8		
CF15*	CAG GGA CTA GGG TCT GCC AG	63.5	60	Polymorphic
CF16	CGT TTG CAA GGC AAG GCA GC	61.4		
CF17*	CGA GCC AAG ACG TTC ATT GAA G	60.3	64	Polymorphic
CF18	GAA ACC GAG AGT CAT CGT CC	59.4		
CF19*	GCA GCT CTA GTC GTC AGA TAC	59.8	55	Monomorphic
CF20	CTA GAG GCC AGC TCT TGT GC	61.4		
CF21*	GCA CTA CGA GAA TAG AAT GCA G	58.4	60	Polymorphic
CF22	GCG TTG AAA GAT GTG GCG TG	59.4		
CF23	CAG GGA ATT CCC GAT GGC AG	61.4	60	Polymorphic
CF24	CAT GAT CGA CAA GGG CGC TG	61.4		

¹ T_m (° C) = melting temperature

² T_a (° C) = annealing temperature

* Primers designed from genome walking.

Table 3: Core sequences and allelic properties of polymorphic primers designed for *C. fimbriata*.

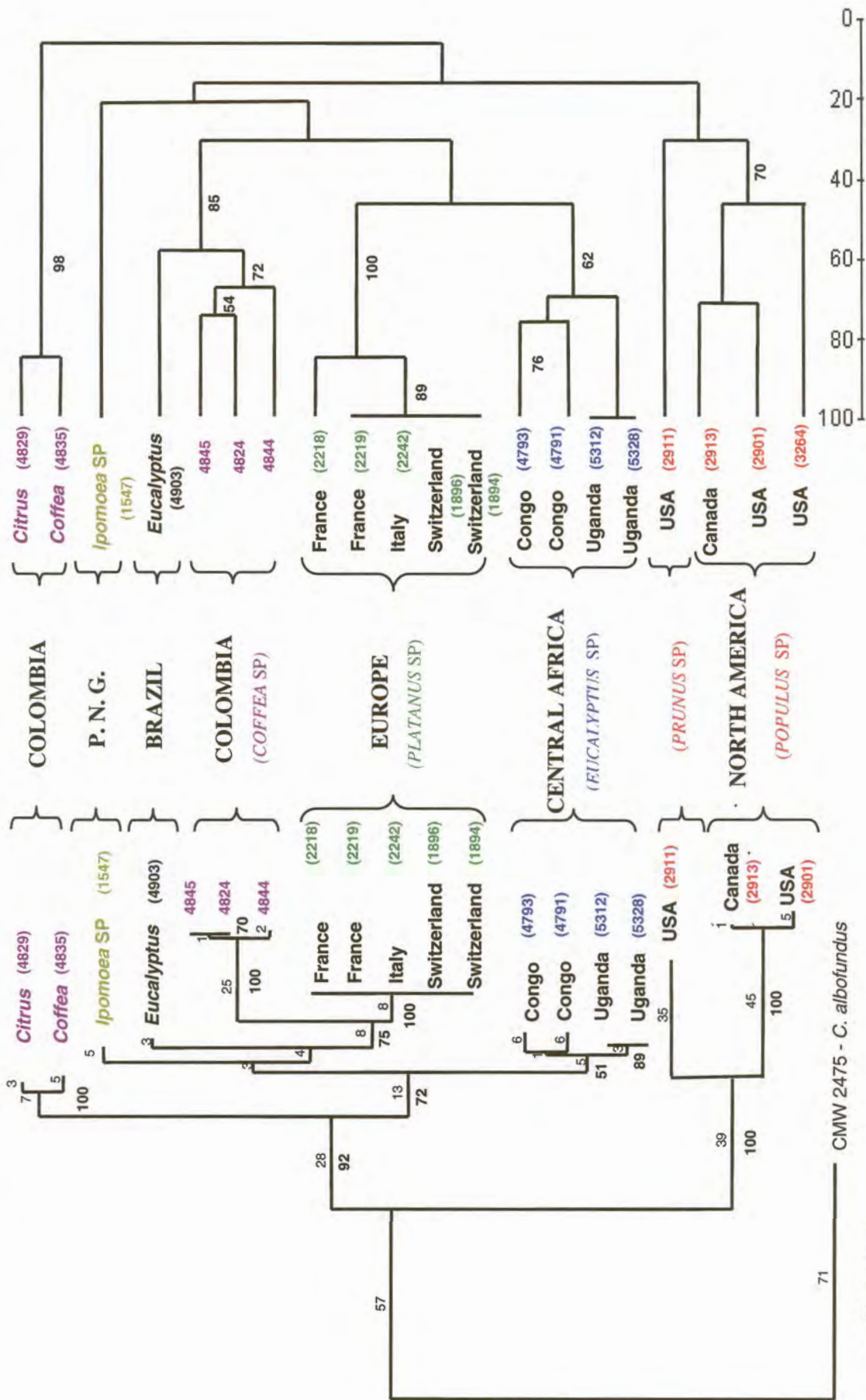
Primer	Floures-cent label	Core sequence	Allelic range	No of alleles	Alleles
AG 1/2	FAM	(T) ₇ C(T) ₂ CGC(T) ₄ (CTTT) ₂ GC(T) ₄ C(T) ₃ C (T) ₂ G(T) ₄ (CTT) ₂	255 - 266	4	255,263,265,266
AG 7/8	TET	(TC) ₂₁ (TTC) ₂	284 - 304	10	284,285,286,288,289,290,298,299,301,304
AG 15/16	FAM	Regions rich in A interrupted by C and G	272 - 276	3	272,274,276
AG 17/18	FAM	(T) ₅ (C) ₂ (CT) ₂ T(CTT) ₆ (T) ₂ (C) ₃ TC(T) ₃	304 - 309	5	304,305,307,308,309
CF 5/6	TET	(TGC) ₁₁	359 - 385	9	359,365,366,368,369,371,377,380,385
CF 11/12	TET	CA(AC) ₇ GC(AC) ₂ (N) _x (G) ₈	216 - 230	6	216,217,218,219,222,230
CF 13/14	TET	(T) ₅ (N) _x (A) ₇ (N) _x (C) ₁₁ (N) _x (AGCAC) ₅	402 - 415	7	402,403,405,406,410,414,415
CF 15/16	FAM	(CT) ₅ (N) _x (CT) ₃ (N) _x (CT) ₃ sequence rich in T	218 - 267	6	218,240,248,250,254,267
CF 17/18	TET	(CA) ₁₅ sequence rich in GT and T	266 - 292	8	266,267,268,271,272,277,279,292
CF 21/22	TET	(T) ₈ (N) _x (T) ₆ (N) _x (C) ₂ (T) ₃ C(CT) ₂ (CCTT) ₂ C (T) ₃ C(T) ₂ C(T) ₄	250 - 259	3	250,255,259
CF 23/24	TET	TGCA(TG) ₁₅	154 - 168	4	154,156,160,168

Figure 1: Comparison of a dendrogram for *Ceratocystis fimbriata* isolates produced using 11 polymorphic microsatellite loci with a phylogram produced from the ITS sequences of the same isolates. The dendrogram was produced using cluster analysis of Bray-Curtis coefficient of similarity in PRIMER and the phylogram using the Heuristic search option with parsimony in PAUP 4. Bootstrap values are indicated below the branches while the number of base substitutions is indicated above the branches.



ITS Tree

Microsatellite Tree



CMW 2475 - C. albifundus

Figure 2: Sequences of *Ceratocystis fimbriata* isolates produced from primers AG 7/8, AG 17/18, CF 5/6, CF 15/16, CF 17/18 and CF 21/22.

Figure 3: Sequence alignments (555bp) of the ITS regions of *Ceratocystis fimbriata* isolates used in this study. Dots represent bases identical to *C. fimbriata* from Papua New Guinea (CMW 1547), N indicates unknown bases and dashes represent gaps introduced to maximise alignment.

		10	20	30	40	50	60	70
Papua New Guinea	(CMW 1547)	CCATGTGTGA	ACGTACC-TA	TCTT-GTAGT	GA-GAT-GAA	T-GCTGTTTT	GGTGGT----	AGGG-----
France	(CMW 2219)
France	(CMW 2218)
Italy	(CMW 2242)
Switzerland	(CMW 1896)
Switzerland	(CMW 1894)
Brazil	(CMW 4903)C.....
Congo	(CMW 4793)
Congo	(CMW 4791)A.....T.....
Uganda	(CMW 5328)
Uganda	(CMW 5312)
Colombia	(CMW 4829)A.....C.....T---
Colombia	(CMW 4835)A.T.C.....A.....T---
Colombia	(CMW 4845)
Colombia	(CMW 4844)
Colombia	(CMW 4824)
U.S.A	(CMW 2911)A.....CAT-C..A..A..-A-.....C...TTT-GGAA--
U.S.A.	(CMW 2913)	NNNNNNNNNN	NNNNNNNN--C..TA-C...-C...C...TTTG
Canada	(CMW 2901)A.....C--C..TA-C...-C...C...TTTG

		80	90	100	110	120	130	140
Papua New Guinea	(CMW 1547)	CCC--TTCT-	-----GAAGG	GAGGG-----	-----CA	CC--GCTGCC	AGCAG--TAT	TAGTCT-CAC
France	(CMW 2219)G.
France	(CMW 2218)G.
Italy	(CMW 2242)G.
Switzerland	(CMW 1896)G.
Switzerland	(CMW 1894)G.
Brazil	(CMW 4903)AT.G.
Congo	(CMW 4793)AG.
Congo	(CMW 4791)A
Uganda	(CMW 5328)AT.G.
Uganda	(CMW 5312)AT.G.
Colombia	(CMW 4829)A..G...G-A.....T...
Colombia	(CMW 4835)A..G...G-A.....T...
Colombia	(CMW 4845)	G.....G.
Colombia	(CMW 4844)T-----A.....G.
Colombia	(CMW 4824)	G.....G.
U.S.A	(CMW 2911)A--C	CTAT-A..AT	.GA..A----	-----CTTT.T	--AA...A..	.A.....T...
U.S.A.	(CMW 2913)	...CG...C	CTGTTA..AA	.G.AAGAAGG	GGGGCAAA.T	.TAA.....	G...CA--T...
Canada	(CMW 2901)	...CG...C	CTGTTA..AA	.G.AAGAAGG	GGGGCAAA.T	.TAA.....	G...CA--T...

		150	160	170	180	190	200	210
Papua New Guinea	(CMW 1547)	CACTATAAA-	----CTCTTT	T--ATTATTT	T---CTAGA-	-TTTTT----	----CATT-G	CTGAGTGGCA
France	(CMW 2219)	...G....-	-----T-.C.-.-	---.T--	-----
France	(CMW 2218)	...G....-	-----T-.C.-.-	---.T--	-----
Italy	(CMW 2242)	...G....-	-----T-.C.-.-	---.T--	-----
Switzerland	(CMW 1896)	...G....-	-----T-.C.-.-	---.T--	-----
Switzerland	(CMW 1894)	...G....-	-----T-.C.-.-	---.T--	-----
Brazil	(CMW 4903)	...G....-	-----	T-	-----	-----
Congo	(CMW 4793)	...G....A	AAAA.....	..C..	-----	-----
Congo	(CMW 4791)	...G....-	-----	T-	-----	-----
Uganda	(CMW 5328)	...G....A	-----	T-	-----	-----
Uganda	(CMW 5312)	...G....A	-----	T-	-----	-----
Colombia	(CMW 4829)	...G....A	-----	T-.AT...TA.	-----	-----
Colombia	(CMW 4835)	...GG...A	-----	TT .AT...	-----	-----	-----
Colombia	(CMW 4845)	...G....-	-----	T-----	-----	...CTTAA	TTTC...T.
Colombia	(CMW 4844)	...G....-	-----	T-----	-----	...CTTAA	TTTC...T.
Colombia	(CMW 4824)	...G....-	-----	T-----	-----	...CTTAA	TTTC...T.
U.S.A	(CMW 2911)	T..-...T	T--...T.	---...T...	.TTA....A	A...---GA	TTT-...-
U.S.A.	(CMW 2913)	..-...A	-----	...T...	.T-A....A	A...---GA	TTT-...-
Canada	(CMW 2901)	..-...A	-----	...T...	.TTA....A	A...---GA	TTT-...-

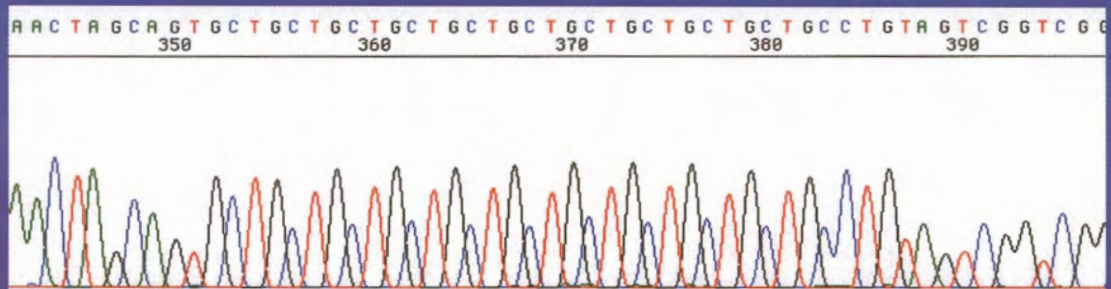
		220	230	240	250	260	270	280
Papua New Guinea	(CMW 1547)	T-AACTATAA	AAAAA--GTT	AAAAC TTTC A	ACAACGGATC	TCTTGGTCT	AGCATCGATG	AAGAACGCAG
France	(CMW 2219)	-----	-----	-----
France	(CMW 2218)	-----	-----	-----
Italy	(CMW 2242)	-----	-----	-----
Switzerland	(CMW 1896)	-----	-----	-----
Switzerland	(CMW 1894)	-----	-----	-----
Brazil	(CMW 4903)	-----	-----	-----
Congo	(CMW 4793)	-----	-----	-----
Congo	(CMW 4791)	-----	-----	-----
Uganda	(CMW 5328)	-----	-----	-----
Uganda	(CMW 5312)	-----	-----	-----
Colombia	(CMW 4829)	A-	-----	-----
Colombia	(CMW 4835)	AA	-----	-----
Colombia	(CMW 4845)	A-	-----	-----
Colombia	(CMW 4844)	A-	-----	-----
Colombia	(CMW 4824)	A-	-----	-----
U.S.A	(CMW 2911)	-----	-----	-----
U.S.A.	(CMW 2913)	.A.....	-----	-----	-----
Canada	(CMW 2901)	.A.....	-----	-----	-----

		290	300	310	320	330	340	350
Papua New Guinea	(CMW 1547)	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTTCAGTGAA	TCATCGAATC	TTTGAACGCA	CATTGCGCCT
France	(CMW 2219)
France	(CMW 2218)
Italy	(CMW 2242)
Switzerland	(CMW 1896)
Switzerland	(CMW 1894)
Brazil	(CMW 4903)
Congo	(CMW 4793)
Congo	(CMW 4791)
Uganda	(CMW 5328)
Uganda	(CMW 5312)
Colombia	(CMW 4829)
Colombia	(CMW 4835)
Colombia	(CMW 4845)
Colombia	(CMW 4844)
Colombia	(CMW 4824)
U.S.A	(CMW 2911)
U.S.A.	(CMW 2913)
Canada	(CMW 2901)

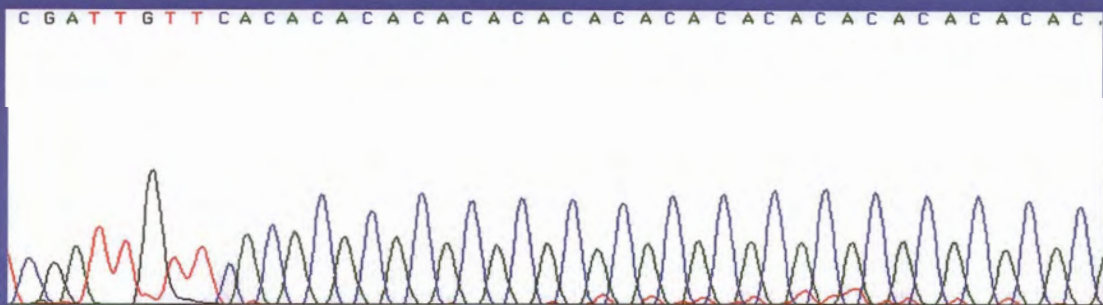
		360	370	380	390	400	410	420
Papua New Guinea	(CMW 1547)	GGCAGTATTC	TGCCAGGCAT	GCCTGTCCGA	GCGTCATTTT	ACCACTCAA-	GA-TT---AT	TCT-----
France	(CMW 2219)G	..C.CC---	.T-----
France	(CMW 2218)G	..C.CC---	.T-----
Italy	(CMW 2242)G	..C.CC---	.T-----
Switzerland	(CMW 1896)G	..C.CC---	.T-----
Switzerland	(CMW 1894)G	..C.CC---	.T-----
Brazil	(CMW 4903)-	..C.C---	.T-----
Congo	(CMW 4793)-TT-----
Congo	(CMW 4791)-TT-----
Uganda	(CMW 5328)-TT-----
Uganda	(CMW 5312)-TT-----
Colombia	(CMW 4829)-	...-CC----	..TTT-----
Colombia	(CMW 4835)-	...-CC----	..TTT-----
Colombia	(CMW 4845)-	..C.CC----	..TT-----
Colombia	(CMW 4844)-	..C.CC----	..TT-----
Colombia	(CMW 4824)-	..C.CC----	..TT-----
U.S.A	(CMW 2911)-	...AAC---	.T.TT-----
U.S.A.	(CMW 2913)-	..AA.-AC--	.T.TTTTTTT
Canada	(CMW 2901)-	..AA.-AC--	.T.TTATTTT

		430	440	450	460	470	480	490
Papua New Guinea	(CMW 1547)	--TTCTTGGC	GTTGGAGGTC	CTGTTCT---	-----CC	CCTGAACAGG	CCGCCGAAAT	GCATCGGCTG
France	(CMW 2219)	-G.....	-----	-----T.....
France	(CMW 2218)	-G.....	-----	-----T.....
Italy	(CMW 2242)	-G.....	-----	-----T.....
Switzerland	(CMW 1896)	-G.....	-----	-----T.....
Switzerland	(CMW 1894)	-G.....	-----	-----T.....
Brazil	(CMW 4903)	-G.....	-----	-----T.....
Congo	(CMW 4793)	-C.....	-----	-----T.....
Congo	(CMW 4791)	-C.....	-----	-----T.....
Uganda	(CMW 5328)	-C.....	-----	-----
Uganda	(CMW 5312)	-C.....	-----	-----
Colombia	(CMW 4829)	-G.....	-----	-----T.....
Colombia	(CMW 4835)	-G.....	-----	-----T.....
Colombia	(CMW 4845)	-G.....	-----	-----C.T.....
Colombia	(CMW 4844)	-G.....	-----	-----C.T.....
Colombia	(CMW 4824)	-G.....	-----	-----C.T.....
U.S.A	(CMW 2911)	--C.T---.TGTT	TTTTTTTTAT	A.....T.....
U.S.A.	(CMW 2913)	TTC.T---.TTTA	-----G.....T.....
Canada	(CMW 2901)	TTC.T---.TTTA	-----G.....T.....
		500	510	520	530	540	550	
Papua New Guinea	(CMW 1547)	TTA----TAC	TTGCC-AACT	CCCCTGTGTA	GTATAAAA-T	TTCT-AATT	TTTACACTTT	GAAGT
France	(CMW 2219)
France	(CMW 2218)
Italy	(CMW 2242)
Switzerland	(CMW 1896)
Switzerland	(CMW 1894)
Brazil	(CMW 4903)
Congo	(CMW 4793)
Congo	(CMW 4791)
Uganda	(CMW 5328)
Uganda	(CMW 5312)
Colombia	(CMW 4829)C.
Colombia	(CMW 4835)C.
Colombia	(CMW 4845)
Colombia	(CMW 4844)
Colombia	(CMW 4824)
U.S.A	(CMW 2911)T..A.A.TT---C.
U.S.A.	(CMW 2913)CT..A.A.TT---C.
Canada	(CMW 2901)CT..A.A.TTTT.--C.

CHAPTER 3



Population structure of
Ceratocystis fimbriata from
 Congo, Colombia and
 Uruguay determined using
 microsatellite markers



ABSTRACT

Ceratocystis fimbriata is a haploid ascomycete, pathogenic on many different hosts. The fungus has a worldwide distribution, including all continents except Antarctica. Although *C. fimbriata* has been known for over a century, very little is known regarding the population biology or origin of this important pathogen. In this study, we used 11 polymorphic PCR-based microsatellite markers to examine the population structure and genetic diversity for three different populations of *C. fimbriata*. Populations consisting of 32 isolates from each of Congo and Colombia and 22 from Uruguay were studied. High genetic diversities for all populations were observed. Colombia had the most diverse population including 82 % of all alleles and 53 % of the unique alleles. Genetic differentiation between the three populations was great ($G_{ST} = 0.39$) and minimal gene flow was observed ($Nm = 0.77$). I_A , PTLPT, and linkage disequilibrium tests to determine mode of reproduction, showed little evidence for recombination within populations. *C. fimbriata* appears to reproduce primarily without outcrossing. UPGMA dendrograms based on microsatellite distance showed that the Colombian population was more distantly related to the Congo and Uruguay populations than the latter were to each other. Some of the groups in the Colombian and Uruguay populations were genetically similar to those for isolates from the Congo. Results suggest that the African isolates of *C. fimbriata* originated in Latin America. Moreover, evidence indicates that Latin America is most likely a center of origin for *C. fimbriata*.

INTRODUCTION

Ceratocystis fimbriata Ellis & Halst., is an ascomycete that is an important pathogen on a wide range of plants, often resulting in large scale losses due to disease (Kile 1993). Hosts include agricultural crops such as sweet potato tubers (*Ipomoea batatas*) and woody plants including *Eucalyptus* and *Acacia* trees (Halsted & Fairchild 1891, Ribeiro 1985, Roux *et al.* 2001). In Colombia, for example, this pathogen causes major losses to the production of coffee (*Coffea arabica*) (Pontis 1951). Other agricultural crops in this country such as *Citrus* spp., *Crotalaria* sp., *Inga* spp., *Mangifera indica* and *Theobroma cacao* are also severely affected by *C. fimbriata* (Rincon 1983, Nieto 1991, Buritica 1994, Mourichon 1994). The disease symptoms produced by *C. fimbriata* vary, depending on host, and include canker-stain, vascular wilts and root/tuber rots (Pontis 1951, Hinds 1972, Kile 1993).

Although *C. fimbriata* has been known for more than a century (Halsted & Fairchild 1891), very little is known regarding the population biology or origin of this pathogen. *Ceratocystis fimbriata* f.sp. *platani* is thought to be native to North America (Walter *et al.* 1952) and to have spread to Europe during the Second World War (Anonymous 1986) causing serious outbreaks of canker stain disease on *Platanus* spp. (Panconesi 1999). The presence of *C. fimbriata* in Asia was first noted in 1900 when the fungus caused a serious disease of coffee, in Java (Zimmerman 1900). Subsequently, the same disease of coffee was reported from Colombia in 1932 (Muchovej 1978, Pontis 1951). Despite its worldwide distribution, *C. fimbriata* has predominantly been reported from Central and South America (McCracken & Burkhardt 1977) and is especially common on a wide variety of hosts in different parts of Latin America (Beeley 1929, Costa 1935, Sharples 1936, Iton 1959, Ribeiro 1985, Barnes Chapter 5). The pathogen was discovered in Australia for the first time in 1987 on rotting *Syngonium* spp. in nurseries but is not recognized as a serious pathogen in that country (Walker *et al.* 1988, Vogelzang & Scott 1990). *C. fimbriata* was not reported from Africa until 1998, when it was discovered causing severe wilt disease on *Eucalyptus* clones in the Republic of Congo (Roux *et al.* 1999). Since then, it has also been reported from Uganda on *E. grandis* (Roux *et al.* 2001).

Although *C. fimbriata* was first described from infected sweet potato in the United States of America (U.S.A.) (Halsted & Fairchild 1891), it is not known whether the pathogen is a

native to the U.S.A. Since its occurrence is so widespread, especially in Latin America, and on hosts not present in the U.S.A, Latin America appears as a more likely area of origin. Studying migration patterns and levels of diversities within *C. fimbriata* populations, it would be possible to gain insight regarding the probable areas of origin and spread of this important pathogen.

Molecular markers, especially microsatellites, or simple sequence repeats, represent ideal tools for studying population genetics of fungi (Tenzer *et al.* 1999, Santini & Capretti 2000, Bucheli *et al.* 2001, Burgess *et al.* 2001). These co-dominant markers allow for the identification of multiple polymorphic alleles at any given locus. By comparing multilocus genotypes, identification of individuals within a morphologically identical population is possible. The ability to accurately identify genotypes thus, makes it possible to study relationships between different populations of the same species.

A set of PCR-based microsatellite markers, specifically designed for population studies, has recently been developed for *C. fimbriata* (Barnes *et al.* 2001a, Chapter 2). The aim of the present investigation was to compare genetic diversities in three different populations of *C. fimbriata* from the Republic of Congo (Africa), Colombia and Uruguay (Latin America), using these microsatellite markers. The genetic diversities, mode of reproduction, population structure, gene flow, as well as the relationships between the populations were thus considered.

MATERIALS AND METHODS

Fungal isolates

In this study we utilised populations of *C. fimbriata* from three different countries. The isolates representing the Colombian population were collected from infected soil and coffee plants in coffee plantations. An additional two isolates were from citrus plants surrounding these coffee plantations (Table 1). The Republic of Congo population was comprised of isolates from individual trees in two different *Eucalyptus* plantations. Fifteen isolates were collected from diseased coppice stems of hybrid *Eucalyptus urophylla* S.T. Blake X *E. pellita* F. Muell. (UP) trees in the Kissoko plantation and seventeen from *E. grandis* Hill ex. Maid. X *E. tereticornis* Sm. (GT) trees in the Tchittanga plantation (Roux

et al. 1999) (Table 1). The Uruguay population was comprised of 22 isolates collected from individual diseased *E. grandis*, in a single plantation, near the city of Rivera (Chapter 5) (Table 1).

All isolates were grown on 2 % malt extract agar (MEA, Biolab) supplemented with thiamine (100 mg/liter) (Centaur Laboratories). For DNA extraction, an ascospore mass from a single perithecium of each culture was incubated for 2 weeks at 25 °C in an Erlenmeyer flask containing 50 ml malt extract broth (2 %, Biolab). After sufficient growth, the mycelium was collected by filtration, freeze-dried, crushed and the DNA was extracted using the method previously described by Barnes *et al.* (2001b).

Microsatellite PCR amplification

Eleven microsatellite primers, designed for *C. fimbriata* (Barnes *et al.* 2001a, Chapter 2), were used in PCR reactions, for all isolates. PCR reaction mixtures, and primer conditions for these isolates were the same as those described previously (Barnes *et al.* 2001a, Chapter 2). The reverse primer, for primer pair CF 15/16, was replaced with a second primer, CF16.b 5'(CGT TTG CAA GGC AAG GCA GC) 3'. The expected size of the PCR amplicon, therefore, changed from 250 to 480 bp. This was done in order to increase the efficiency of multiplexing, during genescan analysis (Table 2). PCR products were not purified.

Genescan analysis

To facilitate multiplexing of the genescan analysis, PCR products were arranged according to the approximate size of the PCR product and type of fluorescent label attached to the primer (Table 2). Samples were prepared and analysed in the same manner as previously described (Chapter 2) using GeneScan® 2.1 analysis software (Perkin Elmer Corp.) and Genotyper® 3.0 (Perkin Elmer Corp.). Samples that showed ambiguity in results were re-amplified and re-analysed.

Population analysis

Genetic variation

Two measures of genetic variation were calculated. Allele frequencies (Table 3), were determined for each locus and subsequently used to determine the gene diversity of the

whole population according to the formulae, $H = 1 - \sum_k x_k^2$, where x_k is the frequency of the k^{th} genotype (Nei 1973) (Table 5). Values tending towards 1 indicate diverse populations and 0, uniform populations. Each allele at a locus was assigned an alphabetical character according to its size (Table 3). The alphabetical character for each of the eleven loci was combined to produce the multilocus genotype (Table 4). The multilocus genotype was used to calculate the genotypic diversity of the populations using the formulae $G = 1 / \sum [f_x (x/n)^2]$, where n is the sample size, and f_x is the number of genotypes occurring x times in the sample (Stoddart & Taylor 1988). To compare the genotypic diversities between populations, the maximum percentage of diversity, obtained in each population was calculated $\hat{G} = G/N * 100$ where N is the population size (MacDonald *et al.* 1994). A t-test, with a significance level of $P \geq 0.05$, was used to determine if the difference between the percentages of maximum diversity between populations was significant (Chen *et al.* 1994).

Genetic distance and population structure

The genetic distance of the three populations was calculated using total nucleotide length for each allele. A distance matrix, using the measure $\delta\mu^2$ (Goldstein *et al.* 1995), was constructed in the program MICROSAT (<http://human.stanford.edu/microsat>). A UPGMA (Unweighted Pair-Group Method with Arithmetic mean) dendrogram based on the distance matrix was constructed in MEGA version 2.1 (Kumar *et al.* 2001). Each individual population was analyzed using all the available isolates. Two dendrograms for the combined populations were constructed. The first of these was to show genetic distance between all the isolates studied. In this case, all the duplicate genotypes were removed. The second dendrogram was constructed to reflect the genetic distance between the three populations.

The amount of genetic differentiation (G_{ST}) (Nei 1973), between populations was calculated in POPGENE version 1.31 (<http://www.ualberta.ca/~fyeh/fyeh>). This measure reflects the amount of sub-structuring occurring between populations. In general, a value in the range of 0 to 0.05 indicates little, 0.05 to 0.15 moderate, 0.15 to 0.25 great, and above 0.25 indicates very great, genetic differentiation (Wright 1978).

The amount of gene flow (Nm) between the populations was calculated from the estimate of G_{ST} (Slatkin & Barton 1989) where $Nm = 0.5(1 - G_{ST})/G_{ST}$ (McDermott & McDonald

1993). Populations that are completely genetically isolated will have N_m values of zero, and G_{ST} values of one (Hartl & Clark 1989).

Reproduction

The mode of reproduction for each population was determined using the index of association (I_A) (Taylor *et al.* 1999a) and the parsimony tree length permutation test (PTLPT) (Burt *et al.* 1996). For each isolate, the output data obtained from the genescan analysis were organised into a spreadsheet according to the presence or absence of each allele at all eleven loci. The resulting matrix, including multilocus genotypes, was used in further computations. The PTLPT was calculated from PAUP version 4 (Phylogenetic Analysis Using Parsimony [*and other methods]) (Swofford 1998), and the I_A using Multilocus (<http://www.bio.ic.ac.uk/evolve/software/multilocus/>). Each population was analysed separately. For these analyses, data for 1000 randomly recombining populations for the I_A test, and 500 for the PTLPT test, was simulated and a distribution range for these recombining populations, produced. The value of the observed data (actual population being studied), was compared to this distribution range to determine whether recombination is occurring (Taylor *et al.* 1999a).

The amount of non-random association amongst polymorphic loci or the gametic disequilibrium was also calculated (Hartl & Clark 1989). The two-locus LD option in POPGENE was used to calculate the estimated gametic equilibria between all pairs of polymorphic loci (Weir 1979) (Table 5). Probability values were set at $P < 0.05$.

RESULTS

Genescan analysis

The eleven microsatellite markers produced a total of 58 alleles across all 86 isolates in the three populations studied. Individually, Colombia had the greatest number of alleles (47) followed by Congo (33) and Uruguay (22). Most alleles present in the Congo and Uruguay population (82 %), were also present in the Colombian population (Table 3). Monomorphic alleles were only present in the Uruguay population at locus A15/16, allele size 247 and at locus C21/22, allele size 250 (Table 3). Unique, or “private”, alleles were found in all three

populations. Congo had 2 unique alleles over 2 loci, Colombia had 9 over 4 loci, and Uruguay had 6 unique alleles over 5 loci (Table 4).

Population analysis

Genetic variation

In most cases, one allele dominated in frequency compared to the other alleles at the same locus in the populations studied. Of these, Congo and Uruguay shared the same dominant allele at a specific locus, 27 % of the time. Congo and Colombia shared the same dominant allele in only 9 % of the cases (Table 3). Based on allele frequencies, all three populations had a high level of gene diversity. Colombia had the highest gene diversity (0.48), followed by Congo (0.33) and Uruguay (0.31) (Table 5). From a total of 86 isolates used in this study, there were 51 different genotypes for *C. fimbriata*. Although there were isolates with the same genotype (clones) within a population, none of the same genotypes were found between populations. The level of genotypic diversity amongst the populations was relatively high (Table 5), with the Colombian population having the highest value. However, if this was corrected for sample size, Uruguay had the greatest maximum level of genotypic diversity at 47.83 % while Colombia had 44 % and Congo, 23.88 % (Table 5).

Genetic distance and population structure

UPGMA dendrograms based on microsatellite distance separated the Congo populations into two lineages. These lineages were clearly defined by means of geographical location and host range. All the isolates collected from the Tchittanga plantation were distinct from the isolates collected at Kissoko plantation (Figure 1). The Colombian population could also be separated into two groups (Group 1 and Group 2), with substantial genetic distance between them (Figure 2). The same was true for the Uruguay population, which was highly structured with isolates residing in three groups (Figure 3). G_{ST} values confirmed this high genetic differentiation with values as high as 0.4 between Colombia and Uruguay (Table 5).

When all genotypes of all the isolates of *C. fimbriata* were pooled and analyzed collectively, the dendrogram was structured into two major groups. Most of the groups formed in the individual population analysis were retained in the combined analysis

(Figure 4). The most obvious retention of isolates was Group 1 of the Colombian population. This group was unique and comprised essentially the entire second group within the dendrogram, with the exception of one isolate from Congo. The second Colombian group (Group 2), was mainly interspersed amongst the isolates from Congo, with very little genetic difference. Group 1 of the Uruguay population was also separated from all other isolates. Group 2 and 3, although distinct, were not as strongly isolated and had closest associations with isolates from Congo (Figure 4).

The *C. fimbriata* isolates considered in this study had a high level of substructure with a G_{ST} value of 0.39 (Table 5). When only the distances between populations were analysed, Congo and Uruguay were most similar, and distantly separated from the Colombian population (Figure 5). The amount of gene flow (Nm), between populations (overall $Nm = 0.77$), was relatively low (Table 5).

Reproduction

In both the I_A and PTLPT tests, all the observed values for the Congo, Colombian and Uruguay populations fell outside the distribution range of a recombining population ($P < 0.001$ for I_A tests; $P = 0.002$ for PTLPT tests) (Figure 6). The hypothesis that the fungus is predominantly clonal is thus supported in the case of *C. fimbriata*. This observation is validated from results obtained from the linkage disequilibrium analysis. The amount of fixation of polymorphic loci (LD) in the populations ranged from 20 to 27 %, which is relatively high. A much lower level of linkage disequilibrium is expected for populations that are randomly mating.

The Colombian population had the greatest level of linkage disequilibrium, yet it also had the highest gene diversity. The I_A and LD tests were, therefore, calculated separately for each of Group 1 and Group 2 in the Colombian population. Results provided evidence for recombination in both smaller groups in I_A analyses (Figure 7) and in the amount of linkage disequilibrium (Group 1 = 2.22 %, Group 2 = 3.4 %). The G_{ST} value between the sub-populations was high (0.5), indicating a large amount of genetic differentiation between the two populations. The populations are also genetically isolated with little or no gene flow between them ($Nm = 0.48$)

DISCUSSION

To the best of our knowledge, this study is the first to consider population diversity in *C. fimbriata* using co-dominant DNA-based markers. High levels of genetic diversity were observed within populations of *C. fimbriata* from the three countries studied. Furthermore, the populations were highly structured with little evidence of gene flow.

Our results showed that the Colombian population of *C. fimbriata* was the most genetically diverse. This could be attributed to the population containing almost all the alleles present in the other populations and also containing the highest number of genotypes. The two very distinct groups observed within this population using microsatellites, were previously observed in analyses of sequence data from the ITS region of the ribosomal DNA operon (Barnes *et al.* 2001a). These two groups appear to be reproductively isolated with little, or no gene flow between them. There was, however, evidence for recombination within the two groups based on I_A and linkage disequilibrium analyses. Based on large genetic distances and reproductive isolation, we believe that these two distinct lineages of *C. fimbriata* from Colombia, constitute discrete populations that have undergone recent speciation or are, at best ‘sibling species’. In his definition of the term “sibling species”, Brasier (1993), defined these species as “more likely to be identified by population, genetical and molecular methods than by traditional morphological taxonomy”. In most cases, these species show no morphological difference, but exhibit strong reproductive isolation.

Recent studies with other fungi have shown that cryptic species, which are not morphologically distinguishable, can be differentiated using multiple gene genealogies (Geiser *et al.* 1998, Taylor *et al.* 1999a, Taylor *et al.* 1999b, Koufopanou *et al.* 2001). A population study of *Aspergillus flavus* from Australia, for example, signified the existence of a species barrier in the population (Geiser *et al.* 1998). The use of five genes in a population of *Coccidioides immitis* (Koufopanou *et al.* 2001) provided evidence for outcrossing in what is believed to be a clonal pathogen and showed subdivision of the population into two reproductively isolated groups. Although we have strong evidence to support the view that the two populations of *C. fimbriata* from Colombia probably represent discrete taxa, formal descriptions of these taxa awaits completion of multiple gene genealogies to confirm this hypothesis.

Of the three populations of *C. fimbriata* studied, the Uruguay population had the second highest number of unique alleles. The genotypic diversity value adjusted for sample size (maximum genotypic diversity), however, indicated this population to have a higher level of genetic diversity than the Colombian population. It is possible that the level of genetic diversity within this population was underestimated due to lower sample sizes compared to the other populations. Although the population was highly structured into three clonal lineages, a larger sample of isolates would be required to determine whether these groups are reproductively isolated.

Very little differentiation was found within the Republic of Congo population of *C. fimbriata*. The sensitivity of the markers, however, made it possible to distinguish between isolates collected from the two plantations sampled, dividing the population into two lineages. Genetic distance analysis showed close relationships between some of the Congo isolates with those from both Colombia and Uruguay. This suggests a Latin American origin for *C. fimbriata* isolates in Africa. A similar study on *Fusarium cicinatum* Nirenberg & O'Donnell indicated a southeastern U.S.A. population as the source of origin for the populations in California and Japan based on common genotypes producing patterns of relatedness (Milgroom 1994, Wikler *et al.* 2000). A single introduction of a large number of genotypes or multiple introductions of genotypes into the Republic of Congo, might account for the high level of genetic diversity within this essentially clonal population. The level of gene flow (Nm), between the Congo population and those in South and Central America was, however, relatively low (0.77), but is not surprising considering the geographical barrier between populations.

Host specialization appears to be an important factor influencing the evolution of *C. fimbriata*. This view emerges from the fact that the Congo and Uruguay populations from *Eucalyptus* spp., were genetically more similar to each other than they were to the Colombia population from *Coffea* sp. This is despite the fact that Colombia and Uruguay are geographically closer to each other than they are to Congo. Host specialization in *C. fimbriata* has been suggested in a number of previous studies based on pathogenicity (Pontis 1951, Walter *et al.* 1952, Webster & Butler 1967, Leather 1966, Kojima *et al.* 1982) and phylogenetic relationships (Baker *et al.* 2000, Santini & Capretti 2000, Barnes *et al.* 2001a). Our findings add substantially to the view that *C. fimbriata* probably represents a number of taxa, and that host specialisation is a major factor defining these units.

Results of this study show that all the populations of *C. fimbriata* have high levels of gene and genotypic diversity, but simultaneously have many loci that are in gametic disequilibrium. These high levels of genetic diversity in the populations indicate that sexual outcrossing is occurring within them. In contrast, large numbers of loci in gametic disequilibrium suggests clonal reproduction in the populations. This result might appear contradictory but has also emerged from studies with other fungal populations. For example, a population of *Erysiphe graminis* f.sp. *hordei* (Egh) from France, was shown to be highly diverse (65 - 83 % maximum diversity) while 20 % of the loci were in linkage disequilibrium, indicating that the population is clonal (Brygoo *et al.* 1998). *C. fimbriata* is a homothallic fungus, thus capable of producing progeny, after meiosis, that are genetically identical to parent strains. It is also possible that asexual propagules contribute to spread, although the relative importance of sexual versus asexual reproduction in nature is not known. It is, therefore, not surprising that most tests in this study show evidence of clonality. A limited level of recombination was, however, observed within populations, particularly from Colombia. New genotypes are formed in this way, apparently increasing the overall genetic diversity of the pathogen population.

In a study of the population diversity of *C. fimbriata* f.sp. *platani* from Italy, Santini & Capretti (2000) showed that the fungus was clonal, sharing identical genotypes with isolates from the USA. This led the authors to conclude that *C. fimbriata* f.sp. *platani* in Europe originated in the USA and has subsequently reproduced clonally. Results of the present study indicate a Latin American origin for the African (Congo) population of *C. fimbriata*. Moreover, the high levels of genetic diversity for Latin American populations of the fungus suggest that the center of origin for *C. fimbriata* might be in Latin America. In this regard, it would be interesting to compare allele frequencies and diversities for a USA population of *C. fimbriata* with those from Latin America. This is especially since Walter *et al.* (1952) suggested that *C. fimbriata* f.sp. *platani* is native to the USA.

REFERENCES

- Anonymous. (1986) *Ceratocystis fimbriata* (Ell. & Halsted) f.sp. *platani* (Walter). *Bulletin OEPP/EPPO Bulletin* **16** : 21- 24.
- Baker, C.J. & Harrington, T. (2000) Host-specialized *Ceratocystis fimbriata* on cocoa, sweetpotato and sycamore (Abstract). *Inoculum* **51** (3).
- Barnes, I., Gaur, A., Burgess, T., Roux, J., Wingfield, B.D. & Wingfield, M.J. (2001a) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular Plant Pathology* **2** : 319-325.
- Barnes, I., Roux, J., Coetzee, M.P.A., Wingfield, B.D. & Wingfield, M.J. (2001b) Characterisation of *Seiridium* spp. associated with Cypress Canker based on β -tubulin and histone sequences. *Plant Disease* **85** : 317-321.
- Beeley, F. (1929) Fungi and diseases of the tapping panel. *Quarterly Journal of Rubber Research Institute of Malaya* **4** : 250-272.
- Brasier, C.M. (1993) The genetic system as a fungal taxonomic tool: gene flow, molecular variation and sibling species in the '*Ophiostoma piceae* – *Ophiostoma ulmi*' complex and its taxonomic and ecological significance. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield, M.J., Seifert, K.A. and Webber, J.A. eds.). APS Press, St. Paul, Minnesota. pp. 77-92.
- Brygoo, Y., Caffier, V., Carlier, J., Fabre, J-V., Fernandez, D., Giraud, T., Mourichon, X., Neema, C., Nottoghem, J-L., Pope, C., Tharreau, D. & Lebrun, M-H. (1998) Reproduction and population structure in phytopathogenic fungi. In *Molecular variability of fungal pathogens* (Bridge, P., Couteaudier, Y. & Clarkson, J. eds.): 133-148. CAB International, Wallingford, Oxon, U.K.
- Bucheli, E., Gautschi, B. & Shykoff, J.A. (2001) Differences in population structure of the anther smut fungus *Microbotryum violaceum* on two closely related host species, *Silene latifolia* and *S. dioica*. *Molecular Ecology* **10** : 285-294.
- Buritica, P. (1994) Directorio de Patógenos y Enfermedades de las plantas de importancia económica en Colombia. *En Prensa*
- Burgess, T., Wingfield, M.J. & Wingfield, B.D. (2001) Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology* **67** : 354-362.

- Burt, A., Carter, D.A., Koenig, G.L., White, T.J. & Taylor, J.W. (1996) Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. *Proceedings of the National Academy of Science U.S.A.* **93** : 770-773.
- Chen, R.S., Boeger, J.M. & McDonald, B.A. (1994) Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology* **3** : 209-218.
- Costa, A.S. & Krug, H.P. (1935) Eine durch *Ceratostomella* hervorgerufene Welkekrankheit der *Crotalaria juncea* in Brasilien. *Phytopathology Z.* **8** : 507-513.
- Geiser, D.M., Pitt, J. & Taylor, J.W. (1998) Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proceedings of the National Academy of Science U.S.A.* **95** : 388-393.
- Goldstein, D.M., Pitt, J.I. & Taylor, J.W. (1995) An evaluation of genetic differences for use with microsatellite loci. *Proceedings of the National Academy of Science U.S.A.* **92** : 6723-6727.
- Halsted, B.D. & Fairchild, D.G. (1891) Sweet-potato black rot. *Journal of Mycology* **7** : 1-11.
- Hartl, D.L. & Clark, A.G. (1989) Principles of population genetics. Sinauer Associates Inc., Sunderland, Mass.
- Hinds, T.E. (1972) *Ceratocystis* canker of Aspen. *Phytopathology* **62** : 213-220.
- Iton, E.F. (1959) Studies on a wilt disease of cacao at River Estate. *Report on Cacao Research 1957-8*. St. Augustine, Trinidad: Regional Research Centre, Imperial College of Tropical Agriculture, University of the West Indies, 55-64.
- Kile, G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield, M.J., Seifert, K.A. & Webber, J.A., eds): 173-183. APS Press, St. Paul, Minnesota.
- Kojima, M., Kawakita, K. & Uritani, I. (1982) Studies on a factor in sweet potato root which agglutinates spores of *Ceratocystis fimbriata*, Black Rot Fungus. *Plant Physiology* **69** : 474-478.
- Koufopanou, V., Burt, A., Szaro, T. & Taylor, J.W. (2001) Gene genealogies, cryptic species, and molecular evolution in the human pathogen *Coccidioides immitis* and relatives (Ascomycota, Onygenales). *Molecular Biology and Evolution* **18** : 1246-1258.
- Kumar, S., Tamura, K., Jakobsen, I.B. & Nei, M. (2001) MEGA2: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA.

- Leather, R.I. (1966) A canker and wilt disease of pimento (*Pimenta officinalis*) caused by *Ceratocystis fimbriata* in Jamaica. *Transaction of the British Society* **42** : 213-218.
- McCracken, F.I. & Burkhardt, E.C. (1977) Destruction of sycamore by canker stain in the midsouth. *Plant Disease Reporter* **61** : 984-986.
- McDermott, J.M. & McDonald, B.A. (1993) Gene flow in plant pathosystems. *Annual Review of Phytopathology* **31** : 353-373.
- McDonald, B.A., Miles, J., Nelson, L.R. & Pettway, R.E. (1994) Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum*. *Phytopathology* **84** : 250-255.
- Milgroom, M.G. (1994) Population biology of the chestnut blight fungus, *Cryphonectria parasitica*. *Canadian Journal of Botany* **73** : S311-S319.
- Mourichon, X. (1994) Serious citrus dieback in Colombia caused by *Ceratocystis fimbriata*. *Fruits* **49** : 5-6.
- Muchovej, J.J., Albuquerque, F.C. & Ribeiro, G.T. (1978) *Gmelina arborea* – a new host of *Ceratocystis fimbriata*. *Plant Disease Reporter* **62** : 717-719.
- Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences U.S.A.* **70** : 3321-3323.
- Nieto, I.L. (1991) Problemas patológicos en mango. En: *Memorias XII Congreso Ascolfi, Manizales* : 10-12.
- Panconesi, A. (1999) Canker stain of plane trees: A serious danger to urban plantings in Europe. *Journal of Plant Pathology* **81** : 3-15.
- Pontis, R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **41** : 179-184.
- Ribeiro, I.J.A., Ito, M.F., Filho, P. & De Castro, J.L. (1985) Gummosis of *Acacia decurrens* Willd. Caused by *Ceratocystis fimbriata* ELL. & HALST. *Summa Phytopathologica* **11** : 7.
- Rincon, O. (1983) El culivo del caucho en Colombia. En: *Revista ESSO Agricola* **2** : 5-14.
- Roux, J., Coutinho, T.A., Mujuni Byabashaija, D. & Wingfield, M.J. (2001) Diseases of plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97** : 16-18.
- Roux, J., Wingfield, M.J., Wingfield, B.D., Bouillett, J-P. & Alfenas, A.C. (1999) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30** : 175-184.

- Santini, A. & Capretti, P. (2000) Analysis of the Italian population of *Ceratocystis fimbriata* f.sp. *platani* using RAPD and minisatellite markers. *Plant Pathology* **49** : 461-467.
- Sharples, A. (1936) Diseases and pests of the rubber tree, pp. 208-229. MacMillan, London.
- Slatkin, M. & Barton, N.H. (1989) A comparison of three indirect methods for estimating average levels of gene flow. *Evolution*, **43** : 1349-1368.
- Stoddart, J.A. & Taylor, J.F. (1988) Genotypic diversity: estimation and prediction in samples. *Genetics* **118** : 705-711.
- Swofford, D.L. (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods). Vers:4. Sinaur Associates: Sunderland, Massachusetts.
- Taylor, J.W., Geiser, D.M., Burt, A. & Koufopanou, V. (1999a) The evolutionary biology and population genetics underlying fungal strain typing. *Clinical Microbiology Reviews* **12** : 126-146.
- Taylor, J.W., Jacobson, D.J. & Fisher, M.C. (1999b) The Evolution of Asexual Fungi: Reproduction, Speciation and Classification. *Annual Review of Phytopathology* **37** : 197-246.
- Tenzer, I., Ivanissevich, S.D., Morgante, M. & Gessler, C. (1999) Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. *The American Phytopathological Society* **89** : 748-753.
- Vogelzang, B. K. & Scott, E. S. (1990) *Ceratocystis fimbriata*, causal agent of basal rot of *Syngonium* cultivars, and host range studies of isolates of *C. fimbriata* in Australia. *Australasian Plant Pathology* **19** : 82-89.
- Walker, J., Tesoriero, L., Pascoe, I. & Forsberg, L.I. (1988) Basal rot of *Syngonium* cultivars and the first record of *Ceratocystis fimbriata* from Australia. *Australasian Plant Pathology* **17** : 22-23.
- Walter, J.M., Rex, E.G. & Schreiber, R. (1952) The rate of progress and destructiveness of canker stain of plane trees. *Phytopathology* **42** : 236-239.
- Webster, R.K., & Butler, E.E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45** : 1457-1468.
- Weir, B.S. (1979) Inferences about linkage disequilibrium. *Biometrics* **35** : 235-254.
- Wikler, K. & Gordon, T.R. (2000) An initial assessment of genetic relationships among populations of *Fusarium circinatum* in different parts of the world. *Canadian Journal of Botany* **78** : 709-717.

Wright, S. (1978) Variability within and among natural populations. Vol. 4. The Univ. of Chicago Press, Chicago.

Zimmerman, A. (1900) Ueber den Krebs von *Coffea arabica*, verursacht *Rostrella coffeae* gen. et sp. n. Bul. *Institute of Botanical Gardens Buitenzorg* 4 : 19-22.

Table 1: Isolates of *Ceratocystis fimbriata* from Congo, Colombia and Uruguay used in this study.

Isolate numbers*	Country of origin	Host
CMW 4748	Congo	<i>Eucalyptus</i> (UP)**
CMW 4781 - 4782	"	"
CMW 4785 - 4788	"	"
CMW 4794 - 4810	"	"
CMW 4769	"	<i>Eucalyptus</i> (GT)***
CMW 4783 - 4784	"	"
CMW 4789 - 4793	"	"
CMW 4811 - 4713	Colombia	<i>Coffea arabica</i>
CMW 4817 - 4818	"	"
CMW 4820	"	"
CMW 4822	"	"
CMW 4824 - 4825	"	"
CMW 4827 - 4828	"	"
CMW 4830 - 4836	"	"
CMW 4838 - 4841	"	"
CMW 4843	"	"
CMW 4821	"	Coffee soil
CMW 4823	"	"
CMW 4837	"	"
CMW 4842	"	"
CMW 4844 - 4846	"	"
CMW 4826	"	<i>Citrus</i>
CMW 4829	"	"
CMW 7381	Uruguay	<i>Eucalyptus grandis</i>
CMW 7383	"	"
CMW 7385	"	"
CMW 7387	"	"
CMW 7389	"	"
CMW 7508	"	"
CMW 7510	"	"
CMW 7512	"	"
CMW 7514	"	"
CMW 7516	"	"
CMW 7518	"	"
CMW 7520	"	"
CMW 7522	"	"
CMW 7524	"	"
CMW 7764 - 7771	"	"

* Isolates of *C. fimbriata* are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

** *Eucalyptus urophylla* X *E. pellita* (UP) from Kissoko plantation

*** *E. grandis* X *E. terricornis* (GT) from Tchittanga plantation

Table 2: Organization of PCR products for each isolate amplified with eleven microsatellite makers for Genescan analysis. Three lanes on a polyacrylamide gel were used for each individual isolate screened in the population.

	Fluorescent label	Allele size (bp)
Lane 1		
CF 21/22	TET	250 - 259
AG 17/18	FAM	304 - 309
CF 5/6	TET	359 - 385
Lane 2		
CF 23/24	TET	154 - 168
CF 11/12	TET	216 - 230
CF 17/18	TET	304 - 309
CF 13/14	TET	402 - 415
Lane 3		
AG 1/2	FAM	255 - 266
AG 15/16	FAM	272 - 276
AG 7/8	TET	284 - 304
CF 15/16b	FAM	480 - 500

Table 3: Alleles, genotype configuration for each allele and allele frequencies for each isolate present at eleven microsatellite loci.

Locus	*Allele	**Genotype configuration	***Allele frequencies		
			Congo	Colombia	Uruguay
A1/2	255	A	-	0.13	-
	263	B	0.53	0.81	0.05
	264	C	0.13	-	0.91
	266	D	0.03	-	-
	265	E	0.31	0.06	-
	268	F	-	-	0.05
A7/8	285	A	0.25	0.03	-
	288	B	-	0.03	0.41
	289	C	0.19	0.03	-
	290	D	0.53	0.09	0.32
	300	E	-	0.31	-
	301	F	-	0.06	-
	304	G	-	0.41	-
	305	H	0.03	0.03	-
	287	I	-	-	0.27
A15/16	274	A	0.97	0.53	1.00
	276	B	0.03	0.47	-
A17/18	304	A	0.71	0.25	0.82
	305	B	0.19	0.03	-
	307	C	0.03	0.69	0.14
	308	D	-	0.03	-
	311	E	0.03	-	0.05
	313	F	0.03	-	-
CF 5/6	365	A	0.72	0.13	-
	368	B	-	0.06	0.77
	369	C	-	0.03	-
	370	D	-	0.03	-
	371	E	0.25	0.03	-
	382	F	-	0.41	-
	384	G	-	0.03	-
	385	H	0.03	0.28	-
	367	I	-	-	0.23
C11/12	217	A	0.72	0.22	0.68
	219	B	0.25	0.03	-
	222	C	0.03	0.75	-
	220	D	-	-	0.32
C13/14	402	A	-	0.03	0.18
	403	B	-	0.06	-
	405	C	0.97	0.13	-
	410	D	0.03	0.53	-
	413	E	-	0.09	-
	414	F	-	0.06	-
	415	G	-	0.09	-
	400	H	-	-	0.77
	407	I	-	-	0.05
C15.2	477	A	0.94	0.13	0.14
	480	B	0.06	0.78	-
	487	C	-	0.09	0.86
C17/18	268	A	0.72	0.22	0.36
	271	B	0.25	0.03	0.64
	279	C	0.03	0.75	-
C21/22	250	A	0.97	0.25	1.00
	255	B	0.03	0.72	-
	256	C	-	0.03	-
C23/24	156	A	0.72	0.22	0.36
	160	B	0.25	0.03	0.64
	168	C	0.03	0.75	-

* Alleles observed at each locus for all three populations of *C. fimbriata* from Congo, Colombia and Uruguay.

** Each allele in the locus is scored with an alphabetical character. The combination of these characters for each isolate across all loci will give the multilocus genotype (Table 4).

*** Allele frequencies were calculated by taking the number of times the allele was present in the population and dividing it by the total number of isolates in the population.

Table 4: Multilocus genotypes and unique alleles present in *C. fimbriata* populations from Congo, Colombia and Uruguay.

Congo population		Colombian population		Uruguay population	
Isolates	Multilocus genotype	Isolates	Multilocus genotype	Isolates	Multilocus genotype
4748	B D A A A A C A A A A	4811	B F A C F C E B C B C	7381	C I A A B A H C B A B
4769	E A A A E B C A B A B	4812	B E B C F C D B C B C	7383	C I A A B A H C B A B
4781	E D A E A A C A A A A	4813	B E B C F C D B C B C	7385	F B A A B A H C B A B
4782	B D A B A A C A A A A	4817	B G B C H C D B C B C	7387	C I A A B A H C B A B
4783	E A A A E B C A B A B	4818	B D A A A A C A A A A	7389	C I A A B A H C B A B
4784	E A A A E B C A B A B	4820	B G B C H C D B C B C	7508	B I A A B A I C B A B
4785	B D A A A A C B A A A	4821	B G B C F C D B C B C	7510	C D A A B A H C A A A
4786	B D A B A A C A A A A	4822	B G B C H C D B C B C	7512	C B A C B D A C B A B
4787	B C A A A A C A A A A	4823	B G B C F C D B C B C	7514	C B A C B D A C B A B
4788	B C A A A A C A A A A	4824	A F A A E A B C A A A	7516	C B A A B D H C B A B
4789	E A A A E B C A B A B	4825	B G A B H C D B C B C	7518	C B A A B D H C B A B
4790	E A A A E B C A B A B	4826	B G A C H C D B C B C	7520	C B A A B D H C B A B
4791	E A A A E B C A B A B	4827	B E A C F C G B C B C	7522	C D A A B A H C A A A
4792	E A A B E B C A B A B	4828	E D A A A A C A A A A	7524	C B A E B D A C B A B
4793	E A A A E B C A B A B	4829	B G A C H C D B C B C	7764	C B A A B A H C B A B
4794	C C A A A A C A A A A	4830	B G A C H C D B C B C	7765	C B A A B A H C A A A
4795	B D A B A A C A A A A	4831	A C A A A A C A A A A	7766	C D A A I A H A A A A
4796	B D A A A A C A A A A	4832	B E A A C A B C A A A	7767	C D A C I D H C A A A
4797	B D A A A A C A A A A	4833	B G A C F C G B C B C	7768	C D A A I A H A A A A
4798	B D A A A A C A A A A	4834	B G A C H C D B C B C	7769	C D A A I A H C A A A
4799	B D A A A A C A A A A	4835	B G A C A C D B C B C	7770	C I A A I A A C B A B
4800	B D A A A A C A A A A	4836	B E A C F C F B C B C	7771	C D A A B A H A A A A
4801	B D A A A A C A A A A	4837	B E A C F C F B C B C		
4802	B H B C A A C A A A A	4838	B H A D F C D B C C C		
4803	C D A B A A C A A A A	4839	B E A C F C D B C B C		
4804	C D A A A A C A A A A	4840	B E A C F C E B C B C		
4805	B D A A H C D B C B C	4841	B G A C H C D B C B C		
4806	B C A A A A C A A A A	4842	B E A C F C E B C B C		
4807	B C A A A A C A A A A	4843	B E A C G C D B C B C		
4808	C C A B A A C A A A A	4844	A B A A B A G B A A A		
4809	E D A A A C A A A A A	4845	A D A A B A A C A A A		
4810	D D A F A A C A A A A	4846	E A A A D B C A B A B		

(.) Dots represent isolates that failed to amplify with a specific primer. The shaded areas represent alleles that are unique to a particular population. In the Congo population three unique alleles were observed in three different loci. The multilocus genotype for each isolate is the combination of the alphabetical letters across all eleven loci. For example, the multilocus genotype for the Congo isolate CMW 4748 is BDAAAACAAAA.

Table 5: Population diversity parameters calculated for *C. fimbriata* isolates from Congo, Colombia and Uruguay.

	Congo	Colombia	Uruguay	Total
Population size	32	32	22	86
Number of alleles	33	47	24	58
No of unique alleles	2	9	6	17
Gene diversity ^a	0.33	0.48	0.31	0.63
Genotypic diversity ^b	7.64	14.22	10.52	
Max. genotypic diversity ^c	23.88%	44%	47.83%	
Linkage disequilibrium ^d	20,4 %	27%	19.56%	
	Congo/Colombia Colombia/Uruguay Congo/Uruguay			
Gst ^e	0.307	0.359	0.307	0.390
Nm ^f	1.128	0.892	1.126	0.770

^a Nei's (1973) formula, $H = 1 - \sum_k x_k^2$, where x_k is the frequency of the k^{th} genotype.

^b $G = 1 / \sum [f_x (x/n)^2]$, where n is the sample size, and f_x is the number of genotypes occurring x times in the sample (Stoddart & Taylor 1988).

^c \hat{G} To calculate maximum diversity, G is divided by the sample size.

^d The two-locus LD option in POPGENE estimates gametic equilibria between pairs of loci (Weir 1979). The number of significant linkage disequilibria obtained (Congo-108, Colombia-298, Uruguay-54) is divided by $[x(x-1)/2]$, where x = the number of alleles. This value is multiplied by 100 to obtain percentages.

^e G_{ST} = Genetic differentiation coefficient (Nei 1973)

^f Nm = geneflow, calculated from the estimate of G_{ST} . E.g., $Nm = 0.5(1 - G_{ST}) / G_{ST}$; (McDermott & McDonald 1993, Slatkin & Barton 1989).

Figure 1: UPGMA dendrogram of the Congo population based on genetic distance calculated using Delta mu squared ($\delta\mu^2$) on total nucleotide length. The 32 isolates making up this population are sub-structured into two groups. The GT *Eucalyptus* clones from Tchittanga plantation are clearly differentiated from the UP *Eucalyptus* clones collected from Kissoko plantation.

Congo population

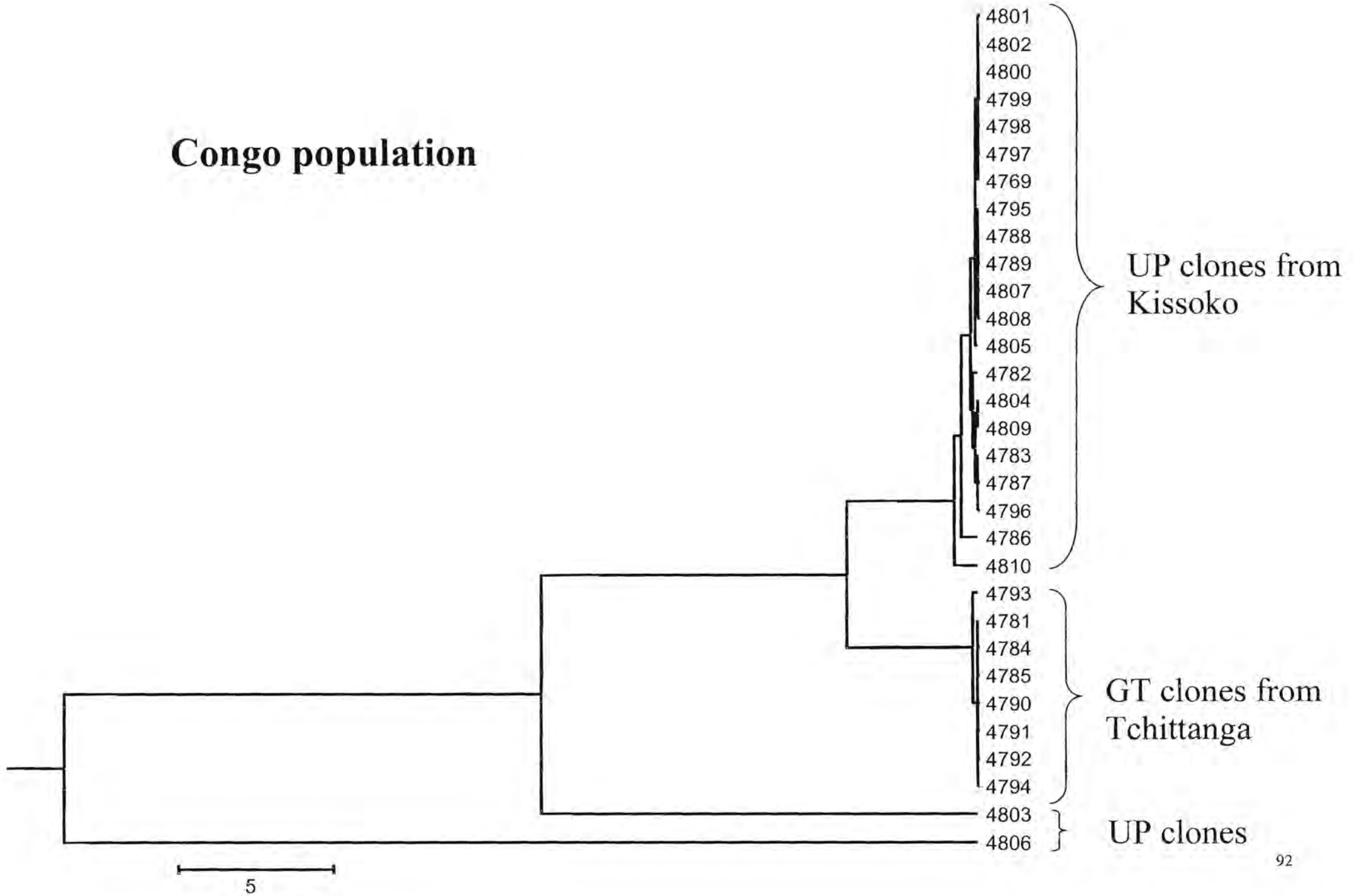


Figure 2: UPGMA dendrogram of the Colombian population. Genetic distance was based on total nucleotide length of each allele using the algorithm, $\delta\mu^2$. The 32 isolates making up this population are sub-structured into two main groups.

Colombian population

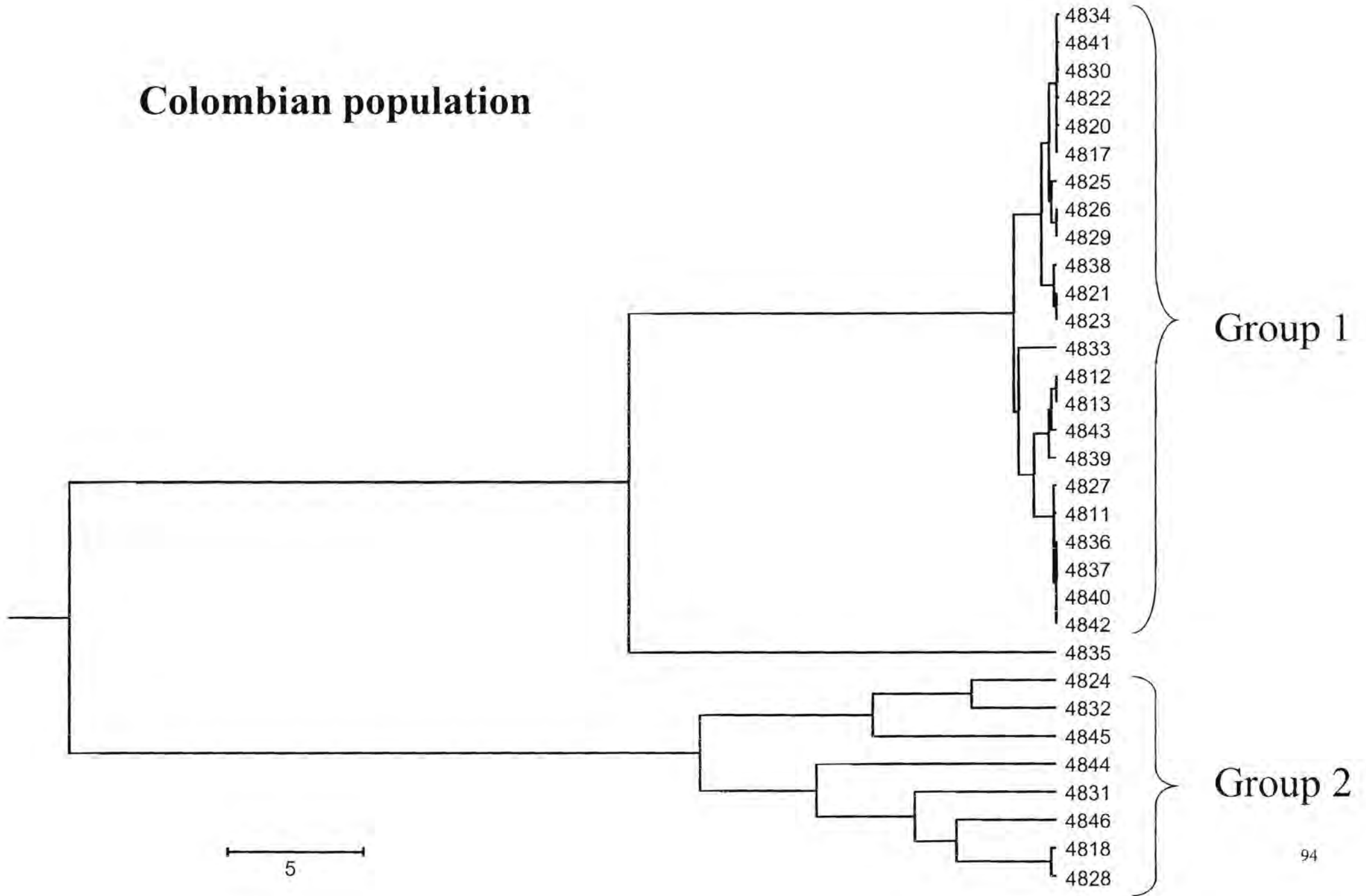


Figure 3: UPGMA dendrogram of the Uruguay population based on genetic distance calculated using Delta mu squared ($\delta\mu^2$) on total nucleotide length. The population, consisting of 22 isolates, is separated into three different lineages.

Uruguay population

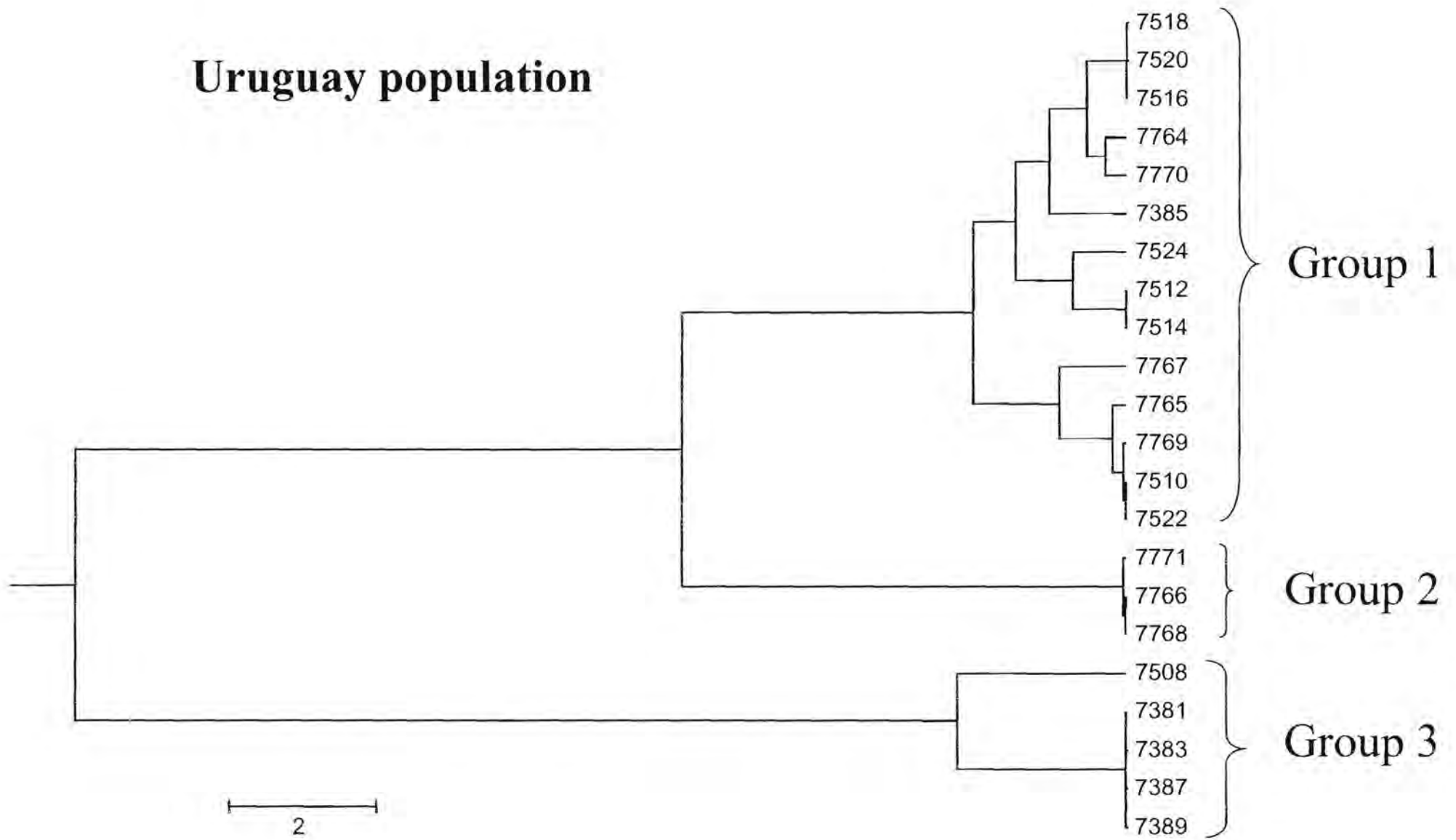


Figure 4: UPGMA dendrogram of the combined populations of *C. fimbriata* from Congo, Colombia and Uruguay. Genetic distances were calculated using $\delta\mu^2$ on the total nucleotide length of the alleles. Duplicate genotypes found within a population were removed in the analysis. The combined populations of *C. fimbriata* show substantial substructure, with a G_{ST} value of 0.39. The Colombian, Group 1 and the Uruguay Group 1 were genetically differentiated from the other isolates and were thus labelled ‘unique’. The remaining groups from these two populations were intermixed, especially with the isolates from Congo. ‘Col’ refers to isolates from Colombia, ‘Con’ from the Republic of Congo and ‘Uru’ from Uruguay.

Combined populations of *C. fimbriata* from Congo, Colombia and Uruguay

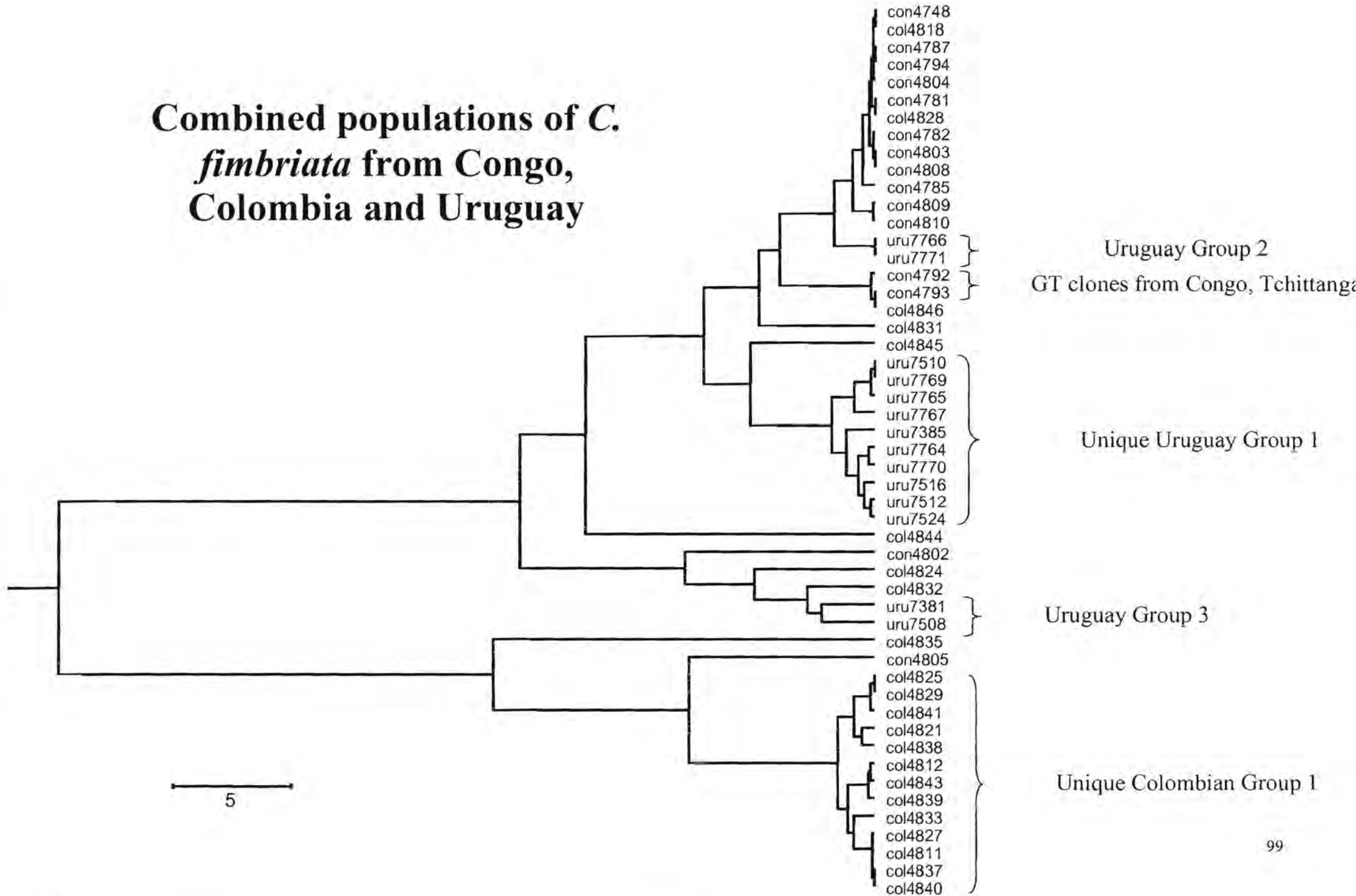


Figure 5: UPGMA dendrogram of *C. fimbriata* from Congo, Colombia and Uruguay. Congo and Uruguay have the least genetic distance compared with the Colombian population. Both the populations from Congo and Uruguay are from *Eucalyptus* spp. while the Colombian population is from *Coffea* spp.

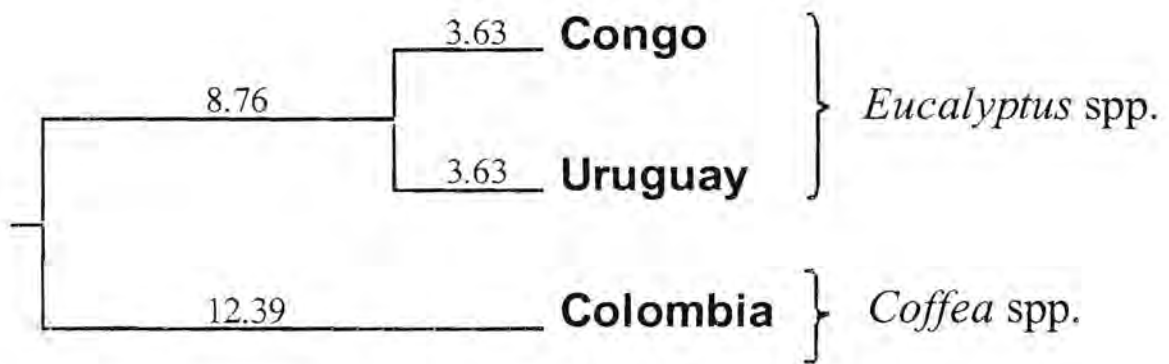


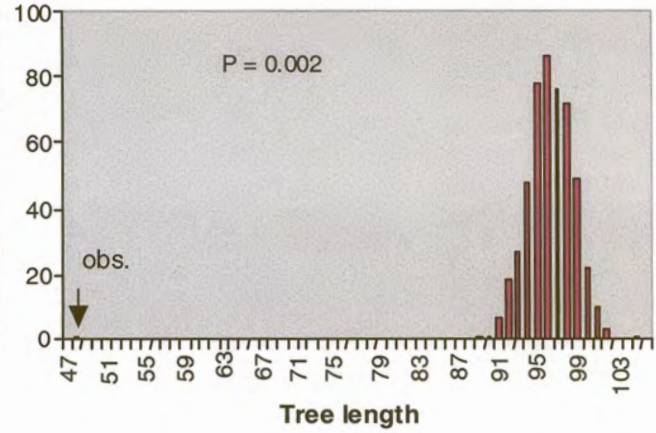
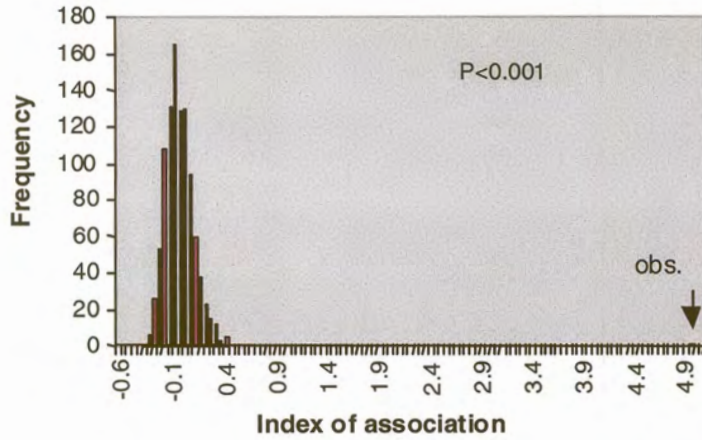
Figure 6: Histograms representing the distribution range of randomly recombining populations using a) the index of association (I_A) with 1000 randomized datasets b) parsimony tree lengths (PTLPT) based on 500 randomisation events. All three populations of *C. fimbriata* were analysed. The observed values for (I_A) in all three populations falls well beyond the distribution range for the recombining population, indicating significant correlation of alleles across loci ($P < 0.001$). Similarly, the observed tree length was significantly shorter ($P = 0.002$) than that for the randomised data set based on permutation tests. Both tests indicate a clonal reproductive mode for the populations of *C. fimbriata* from Congo, Colombia and Uruguay.



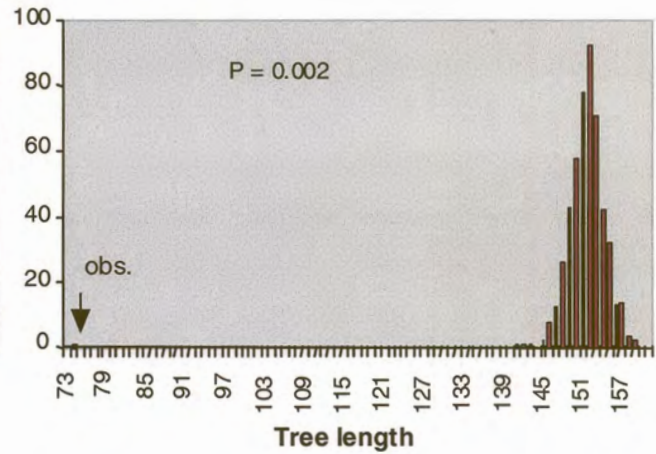
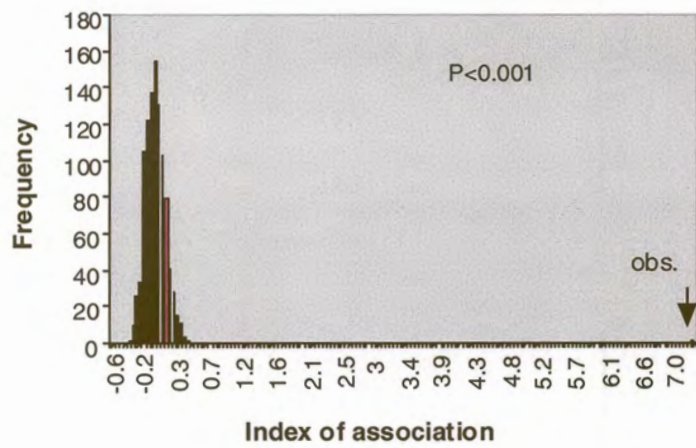
a) I_A TESTS

b) PTLPT

Congo population



Colombian population



Uruguay population

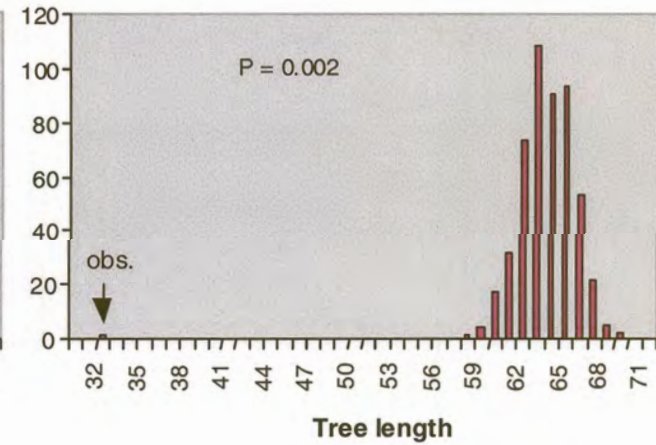
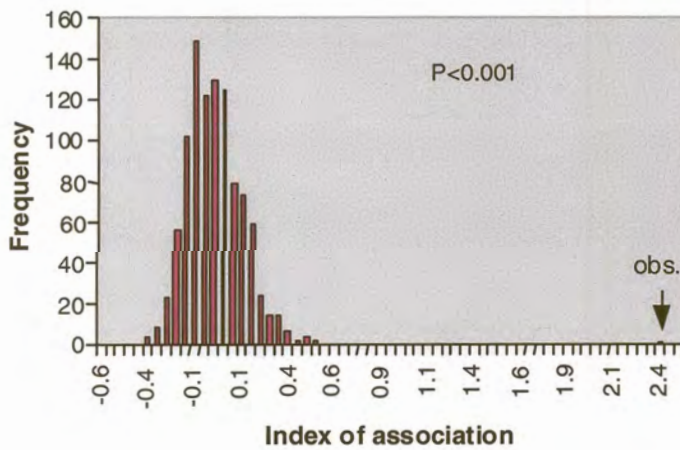
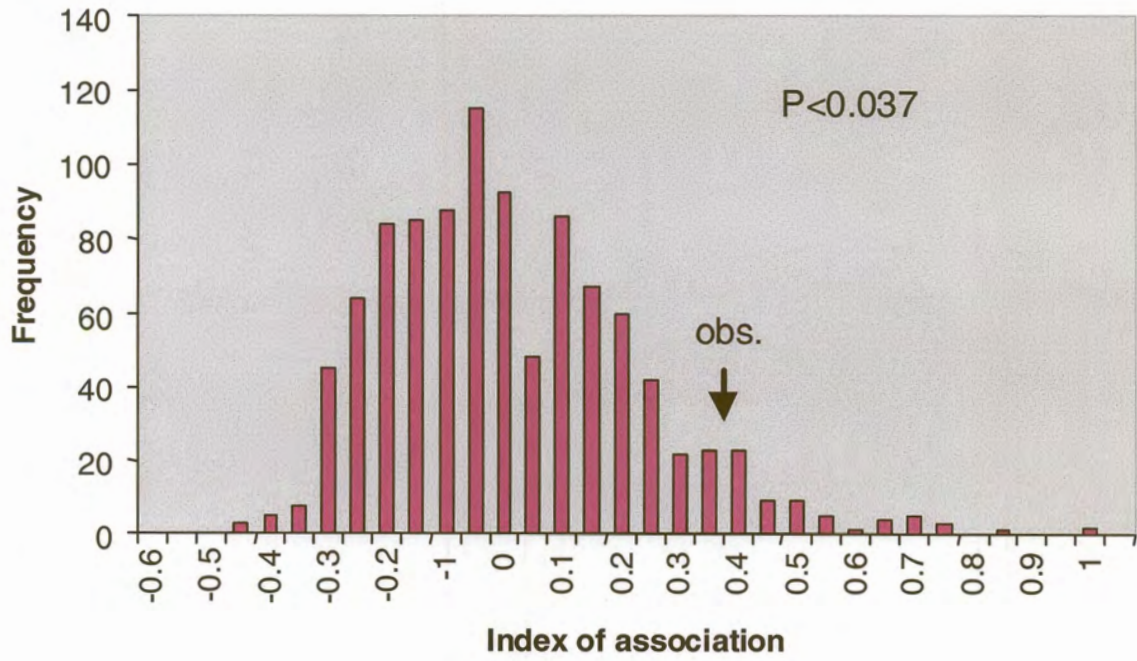


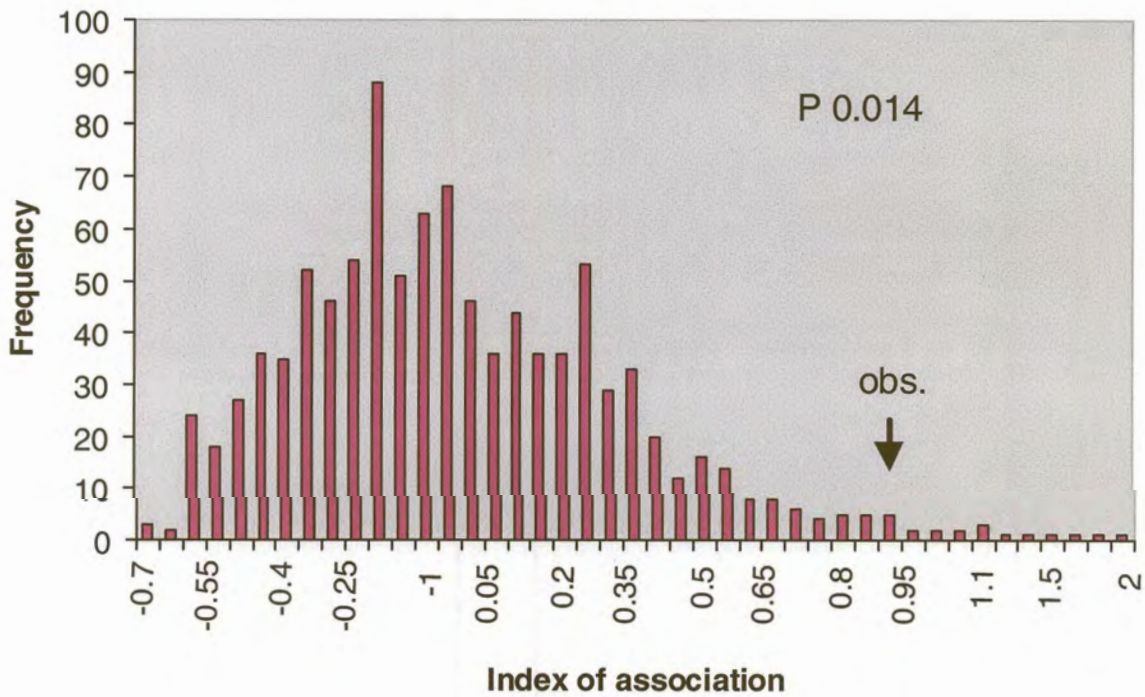
Figure 7: Histograms representing the frequency distribution range of 1000 randomly recombining populations using the index of association. Observed values were calculated for the two distinct groups differentiated in the UPGMA microsatellite distance analysis in the Colombian population. a) Colombian Group 1 and b) Colombian Group 2. In both cases, the observed value falls within the distribution range of the simulated recombining population. The null hypothesis that recombination is occurring, is supported.



Colombia Group 1



Colombia Group 2





CHAPTER 4

**Microsatellite markers reveal
population structure and
diversity of the wilt pathogen
*Ceratocystis albofundus***

ABSTRACT

Ceratocystis albobundus causes a serious wilt and canker disease on *Acacia mearnsii* trees in South Africa and Uganda. In a previous study using an oligonucleotide probe, a high level of gene diversity was demonstrated in a South African population of *C. albobundus*. This, and the association of the pathogen with native South African *Protea* species, has led to the hypothesis that *C. albobundus* is probably native in South Africa. The aim of this study was to use co-dominant microsatellite markers developed for the closely related *C. fimbriata*, to determine gene and genotypic diversity as well as population structure for *C. albobundus*. The primary mode of reproduction of a South African population consisting of 40 isolates was calculated using the index of association (I_A) and the parsimony tree length permutation test (PTLPT). Eight of the eleven microsatellite markers developed for *C. fimbriata*, successfully amplified microsatellite regions in *C. albobundus*, confirming the close relatedness of these fungi. A gene diversity value of 0.432 was obtained with the microsatellite markers compared to the 0.213 gene diversity value previously found using a single oligonucleotide. The I_A and PTLPT tests indicated that the population is predominantly clonal, suggesting that, although sexual structures are the dominant reproductive form, *C. albobundus* relies largely on homothallic reproduction. The higher gene diversity value obtained with microsatellite markers illustrates the greater power of resolution of co-dominant markers, and provides a more accurate reflection of population structure and diversity in *C. albobundus*. Our results add further support to the view that *C. albobundus* is native to South Africa. This, coupled with the high gene diversity emphasizes the need for a robust *A. mearnsii* breeding program to reduce the impact of *Ceratocystis* wilt.

INTRODUCTION

The genus *Ceratocystis* Ell. & Halst., includes many plant pathogenic species (Kile 1993). *Ceratocystis fimbriata* Ell. & Halst., is well known to be one of the most destructive plant pathogens in this group. This fungus has a cosmopolitan distribution, and causes diseases on numerous woody and herbaceous hosts that are important, both in forestry and agriculture (Halsted & Fairchild 1891, Pontis 1951, Walter *et al.* 1952, Gremmen & De Kam 1977, Kile 1993, Panconesi 1999, Roux *et al.* 1999b).

Ceratocystis albofundus Wingfield, De Beer and Morris, shows a close association with *C. fimbriata* based both on morphology and DNA sequence data. *C. albofundus* was first described in 1996, after it was found infecting commercial plantations of *Acacia mearnsii* de Wild. (black wattle), in the Mkomasi river valley, South Africa (Morris *et al.* 1993, Wingfield *et al.* 1996). Although black wattle is considered an invasive weed in most parts of South Africa, it is grown commercially in the KwaZulu-Natal and southern Mpumalanga Provinces. The bark is a valuable source of high quality tannins and other extracts used in the manufacture of wood adhesives, flotation agents and the tanning of soft leather (Anonymous 2001). The wood is popular in the pulp and paper industry, and is also used for fuel and building material (Bakshi 1976, Anonymous 2001).

When it was first discovered causing *Ceratocystis* wilt of black wattle, *C. albofundus* was initially identified as *C. fimbriata* (Morris *et al.* 1993). Obvious morphological differences, of which the most notable was the colour of the perithecial base, however, promoted detailed taxonomic studies on this pathogen. Morphologically, *C. albofundus* is distinguished from *C. fimbriata* based on its light coloured perithecial bases and dark necks, compared with the typically black bases and necks in *C. fimbriata*. The perithecia of *C. albofundus* also have divergent ostiolar hyphae, in contrast to the typically convergent ostiolar hyphae of *C. fimbriata* (Wingfield *et al.* 1996). Molecular evidence has provided unequivocal proof that *C. albofundus* represents a distinct species. Sequence data from the internal transcribed spacer (ITS) region of the rDNA operon and part of the large sub-unit ribosomal RNA gene, grouped

C. albofundus apart from all other known *Ceratocystis* species, although closest to *C. fimbriata* (Wingfield *et al.* 1996, Witthuhn *et al.* 1999).

Ceratocystis albofundus has a narrow host range and geographical distribution. Previous reports of *C. fimbriata* occurring in South Africa on two native *Protea* species (Gorter 1977), were re-evaluated, showing that these isolates represent *C. albofundus* (Wingfield *et al.* 1996). To date, *C. albofundus* has, therefore, been reported only from the two native *Protea* species and a number of Australian *Acacia* spp. including *A. mearnsii*, *A. dealbata* and *A. decurrens* (Gorter 1977, Morris *et al.* 1993). Until very recently, the fungus was known only from South Africa, but it has now also been discovered in Uganda (Roux *et al.* 2001).

Roux *et al.* (2001) determined the nuclear and mitochondrial gene diversities of *C. albofundus*. To determine the nuclear DNA diversity, these authors used the radio-actively labelled microsatellite probe (CAT)₅, after digesting total genomic DNA with the restriction enzyme *Pst*I. For the mitochondrial DNA diversity, mitochondrial DNA was digested with the restriction enzyme *Hae*III and the resulting RFLP fingerprint scored to obtain a diversity value. Because of a lack of other *C. albofundus* populations, they compared their results with published data for three other *Ceratocystis* spp., thought to be native in their respective areas of origin (Harrington *et al.* 1998). As a result, they found a high nuclear and mitochondrial gene diversity for *C. albofundus* (Roux *et al.* 2001). This, the association of *C. albofundus* with native South African *Protea* spp. and a restricted geographic distribution, led these authors to suggest that *C. albofundus* is probably native to South Africa.

Recently, Barnes *et al.* (2001) developed 11 PCR-based microsatellite markers for *C. fimbriata* (Chapter 2). These markers proved to be highly effective in defining the population structure and genetic relatedness between *C. fimbriata* isolates from different geographic and host origins (Chapter 2). Because of the close relationship between *C. albofundus* and *C. fimbriata*, it was decided to test the markers developed for *C. fimbriata* on *C. albofundus*. Those markers that were effective for the latter fungus, were subsequently used to determine the population diversity and structure of a South African population of *C. albofundus*. To enable appropriate comparison, the same isolates used by Roux *et al.* (2001) were used. The

possible reproductive mode of the South African population of *C. albobundus* was also considered.

MATERIALS AND METHODS

Fungal isolates and DNA extraction

All 40 isolates of *C. albobundus* used in this study were obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). These cultures were isolated from diseased and dying *A. mearnsii* trees in various plantations and naturally regenerated stands in South Africa (Figure 1). Isolates were grown on malt extract agar (MEA) (2 %, Biolab, Auckland, NZ) at 25 °C until sporulation was observed. Single spore masses were lifted from the apex of perithecial necks with sterilized toothpicks and transferred into Erlenmeyer flasks containing 50 ml malt extract broth (2 %, Biolab). Flasks were incubated at 25 °C until a thick mat of mycelium had formed on the surface of the broth. The mycelium was collected, lyophilized and DNA was isolated as previously described by Roux *et al.* (2001). A phenol:chloroform (1:1) step was included to further purify the DNA. RNase (10 mg/ml, Roche Molecular Biochemicals) was added to digest the RNA after DNA isolation. DNA samples were run on a 1.5 % agarose gel stained with ethidium bromide to determine the presence and quality of the DNA. The DNA concentration was estimated visually by comparing the intensity of the DNA bands with those of a lambda DNA marker of known concentration.

Microsatellite PCR amplification

Eleven microsatellite primers, shown to be polymorphic for isolates of *C. fimbriata* (Chapter 2), were tested on five *C. albobundus* isolates (CMW 4059, CMW 4061, CMW 4062, CMW 4071, CMW 4082) to determine whether they amplified the desired PCR product. PCR reactions were specific for each primer, as determined by its optimum annealing temperature (Chapter 2, Table 2). The final concentrations for the reagents used in the PCR reactions were 2 ng DNA, 1.2 x Expand High Fidelity Buffer containing 1.5 mM MgCl₂ (Roche Molecular Biochemicals), 200 μM of each dNTP, 300 nM of the forward and reverse primer (Chapter 2)

and 0.35 U Expand HF enzyme. The reactions were carried out in a total volume of 25 μ l with a PCR program consisting of a 2 min denaturation step at 96 °C, 10 cycles of 20 s at 94 °C, 48 s at the annealing temperature specified for each primer and 45 s at 72 °C. A further 25 cycles were included, with a 5 s extension after each cycle. A final elongation step was carried out for 10 min at 72 °C.

Primer sets that gave positive results for each of the five test isolates were used for analysis of the remaining 35 isolates of *C. albobundus*. For the primer sets that did not amplify the desired PCR product, a range of annealing temperatures and DNA concentrations were tested. Where primers failed to produce products after these manipulations, they were discarded. The DNA concentrations of the PCR products were measured visually against the intensity of a 100 bp marker (Roche Diagnostics, Mannheim, Germany) on a 2 % Agarose gel stained with ethidium bromide, exposed to UV illumination.

Genescan analysis

All the PCR products for each isolate amplified using a different primer, were combined into three Eppendorf tubes (Table 2). Each tube contained PCR fragments with non-overlapping size ranges. Where the fragment sizes overlapped slightly, care was taken that they were labeled with a different phosphoramidite fluorescent dye (TET or FAM). The amount of DNA added to each tube was calculated such that in a final volume of 100 μ l, the concentration of each product was 1.5 ng. Each sample fractionated by PAGE (Polyacrylamide Gel Electrophoresis) (4.25 %) on an ABI Prism 377TM DNA sequencer, consisted of 0.5 μ l DNA from one of the tubes containing the combined PCR products, 0.5 μ l internal standard (GENESCAN –TAMRA, Perkin-Elmer Corp) and 1.5 μ l of loading buffer. The combination of different PCR amplicons in a single sample allowed for analysis of these samples within one lane on a PAGE gel. The sizes of the DNA fragments were determined using a combination of the GeneScan® 2.1 analysis software (Perkin Elmer Corp.) and Genotyper® 3.0 (Perkin Elmer Corp.).

Genetic distance and population structure

The genetic distance of the South African *C. albofundus* population was calculated based on the number of repeats within the microsatellite regions. The number of microsatellite repeats in each allele was estimated according to the length of the allele. The program MICROSAT (<http://human.stanford.edu/microsat>), constructed a distance matrix based on the absolute distance (D_{AD}), using the number of repeats estimated for each allele. The matrix was then analyzed in MEGA version 2.1 (Kumar *et al.* 2001), to obtain a dendrogram based on neighbor-joining.

Statistical analysis

Gene diversity

Each isolate was scored according to the presence or absence of an allele at a specific locus and the data compiled in a matrix (Table 3). The frequency of each allele was calculated by taking the number of times that the allele was present in the population and dividing it by the total number of isolates in the population. The allele frequencies were used to calculate the gene diversity (Nei 1973), $H = 1 - \sum_k x_k^2$, where x_k is the frequency of the k^{th} genotype (Table 4).

Genotypic diversity

The number of multilocus genotypes present in the population was determined by compiling a microsatellite profile for each isolate (Table 3). This was based on the size of the allele observed for each isolate at each of the eight loci. Isolates with the same microsatellite profile had the same genotype and were thus assigned the same genotype number. The genotypic diversity was then calculated using the formula, $\hat{G} = 1 / \sum [f_x (x/n)^2]$, where n is the sample size, and f_x is the number of genotypes occurring x times in the sample (Stoddart & Taylor 1988).

Reproductive mode

To determine the mode of reproduction of *C. albofundus*, the index of association (I_A) (Taylor *et al.* 1999), and the parsimony tree length permutation test (PTLPT) (Burt *et al.* 1996), was calculated. The I_A was calculated using the program Multilocus

(<http://www.bio.ic.ac.uk/evolve/software/multilocus/>) with 1000 randomisations and the PTLPT test with PAUP (Phylogenetic Analysis Using Parsimony) (Swofford 1998). Both tests use the multilocus genotype in a data matrix, to test the association among loci. They thus distinguish between the null hypothesis that recombination is occurring and the alternative hypothesis that the fungus is predominantly clonal. The observed data are compared with those from a simulated randomly recombining population. Where the observed data fall within the distribution range of the recombined data, the hypothesis that recombination is occurring, is supported. If not, the hypothesis that recombination in the population is occurring, can be rejected. The I_A was calculated for the entire South African population as well as for sub-populations consisting of isolates from Bloemendal, Dalton and Vryheid. Sub-populations from East London, Cintsa and Umkomaas were not included due to low numbers of isolates available for those areas (Figure 1).

RESULTS

Microsatellite PCR amplification

Eight of the 11 microsatellite primers designed for *C. fimbriata* (Barnes *et al.* 2001) gave amplification products for *C. albofundus*. Primers CF11/12 and CF13/14 gave no amplification products while primer AG1/2 produced multiple bands.

Genescan analysis

A total of 24 alleles were produced from eight loci ranging in size from 166 to 387 bp (Table 3). Four of the loci produced two alleles each. The remaining loci, AG7/8, CF21/22 and CF17/18 produced nine, four and three alleles respectively. Only one locus, (CF15/16.2), produced a monomorphic allele of 260 bp. AG7/8 was the only locus that produced private alleles. From a total of four private alleles, three were observed in isolates from Vryheid and one from Bloemendal.

Genetic distance and population structure

The dendrogram produced from the distance matrix using Neighbor-joining, clearly showed that there is no grouping of isolates according to geographic origin (Figure 2). In three cases, isolates from different geographic locations had the same genotype and, therefore, the same genetic distance from all the other isolates of *C. albobundus*. These included CMW 4110 from Bloemendal, CMW 4095 from East London and CMW 4097 from Cintsa; CMW 4069 from Vryheid, CMW 4059, CMW 4063 and CMW 4064 from Bloemendal and lastly, CMW 4091 from Umkomaas and CMW 4109 from Bloemendal (Figure 2). Different genotypes were distributed evenly throughout the entire population.

Statistical analysis

Gene diversity

The gene diversity values were calculated using allele frequencies. Since locus AG 7/8 had the highest variable number of allele frequencies, it also had the highest gene diversity value of 0.752. Locus CF 16/17 had one allele and, therefore, had an allele frequency of one. Because this allele was monomorphic, it did not contribute to the calculation of the overall gene diversity. The gene diversities calculated for each allele were pooled and divided by the total number of loci, to give a gene diversity value of 0.432 for the whole population (Table 4).

Genotypic diversity

The South African *C. albobundus* population considered in this study had a total of 28 different genotypes. Of these, 21 occurred once in the population. Three of the genotypes occurred three times, three occurred twice and one occurred four times (Table 3). These values were used to calculate the genotypic diversity value of 0.20, which was then divided by the number of isolates to give a maximum genotypic diversity value of 50 %.

Reproductive mode

The observed I_A for the entire *C. albobundus* population was indicated well beyond the distribution for the randomized datasets, at a value of 0.992 ($P < 0.001$) (Figure 3A). The PTLPT gave the same result with the observed tree length value of 62, which is much shorter than the lengths of 500 randomized trees ($P = 0.002$) (Figure 3B). The significant P values show

a strong correlation between the alleles at the 8 different loci and, thus, indicate that the alleles are in linkage disequilibrium. Both these tests reject the null hypothesis that recombination is active in the population. Instead, they suggest that clonal reproduction is predominant. When the *C. albobundus* population is divided into sub-populations based on collection areas, the observed I_A values are contradictory (Table 5). Both the P-values for Dalton and Bloemendal were highly significant, indicating strong linkage between the alleles even though there is some indication of recombination taking place. The P-value for Vryheid was not significant and the position of the observed value is placed well within the histogram based on 1000 randomizations (Table 5). These results indicate that there is no linkage of alleles in this sub-population and there is strong evidence for recombination. The high gene diversity value obtained for this sub-population is, therefore, expected.

DISCUSSION

In this study, we have shown that the PCR-based microsatellite markers developed to study the population and evolutionary biology of *Ceratocystis fimbriata*, can be used equally effectively for *C. albobundus*. This is perhaps not surprising as *C. albobundus*, and *C. fimbriata* are known to be closely related species (Morris *et al.* 1993, Wingfield *et al.* 1996, Witthuhn *et al.* 1999). Our results, therefore, serve to confirm the very close relationship between these two important plant pathogens.

Population diversity results obtained in this study of *C. albobundus* correspond well with those published previously using alternative fingerprinting methods (Roux *et al.* 2001). Roux *et al.* (2001) compared the nuclear and mitochondrial gene diversity of a population of *C. albobundus* with data previously published (Harrington *et al.* 1998), for three other native *Ceratocystis* spp. The nuclear and mitochondrial gene diversity for *C. albobundus* was relatively high, compared with that of the obligately outcrossing *C. eucalypti*. Our results using PCR-based microsatellite markers showed an even higher gene diversity value for the same population of *C. albobundus* previously considered by Roux *et al.* (2001). This adds further support to the view that *C. albobundus* is native to South Africa.

The co-dominant nature of the microsatellite markers has made it possible to gain a considerably deeper insight into the population biology of the pathogen which was not previously possible using dominant markers such as CAT₅ and isoenzymes (Roux *et al.* 2001). This is mainly due to the ability of microsatellite markers to expose information regarding alleles at individual loci. These polymorphic alleles are useful for determining biological aspects such as gene-flow among populations and reproductive strategy, which is not possible using dominant markers (Burt *et al.* 1996, Taylor *et al.* 1999). For example, in this study, it was possible to show that the *C. albofundus* population encompasses extensive diversity but more importantly, reproduction was occurring in a clonal manner.

Our results, showing a strong clonal nature for *C. albofundus* in South Africa, might be viewed as contradictory to the fact that the fungus appears to reproduce predominantly via sexual structures. However, previous studies have shown that perithecia in this pathogen predominately arise after unidirectional mating type switching and are thus generally homothallic (De Beer 1994, Witthuhn *et al.* 2000). In South Africa, *C. albofundus* is a major pathogen of an exotic forest plantation tree, but has also been found on native *Protea* species. This strongly suggests that the fungus is native in the country and has moved from native to exotic plants. The clonal nature of isolates of the fungus on exotic *A. mearnsii*, suggests that isolates from native plants that have evolved the capacity to infect *A. mearnsii*, are being maintained in the exotic population in strong clonal lineages. This is presumably reinforced by a general lack of sexual recombination in the fungus on *A. mearnsii*.

The clonal reproductive mode of *C. albofundus* further contradicts the unexpectedly high levels of genotypic diversities obtained in the population. Many of the genotypes within this population differed only at one allele at a specific locus, making the genetic distance between isolates slight. High mutation rates could account for this phenomenon. Since *C. albofundus* is haploid, any mutations that occur will automatically change the genotype and hence increase the diversity of the population. Another consideration is the time that the fungus has been in a particular area. The longer the population has been in an area, the greater the chance for mutational events to occur. Higher levels of genotypic diversity are, therefore, expected in native as opposed to introduced populations. Although recombination could also account for

high genotypic diversities observed, we have already established a lack of sexual recombination and strong clonal lineages within the population. There was, however, some evidence of sexual reproduction in some of the sub-populations, but in these cases, the values could be biased due to the low number of isolates analyzed. Mutational events are, therefore, considered the primary cause for the high genotypic diversity observed. These mutational events could also be linked to a large number of strains from native plants, adapting to infect *A. mearnsii*.

Ceratocystis albobundus is currently known only from native South African *Protea* species and exotic Australian *Acacia* species. In the future, it will be interesting to compare the population genetics of isolates from South Africa with those from Uganda, where the fungus has recently been discovered (Roux *et al.* 2001). Of equal interest would be to study a collection of isolates from native *Protea* species in South Africa. Although considerable effort has been made to collect the fungus from these plants, *C. albobundus* remains known from only two herbarium specimens on *Protea*. While the tools are now present to gain a thorough understanding of the population biology and origin of *C. albobundus*, further studies are limited by difficulties in obtaining isolates of this fungus.

REFERENCES

- Anonymous. (2001) South African wattle extract, a natural product. Wattle Industry Centre. Pietermaritzburg, South Africa. 8 pp.
- Bakshi, B.K. (1976) *Wattles-Acacia* spp. In *Forest Pathology: Principles and practice in forestry*. Pp. 191-194. F.K.I. Press, Forest Research Institute and Colleges, Dehra Dun, India.
- Barnes, I., Gaur, A., Burgess, T., Roux, J., Wingfield, B.D. & Wingfield, M.J. (2001) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular Plant Pathology* **2** : 319-325.
- Burt, A., Carter, D.A., Koenig, G.L., White, T.J. & Taylor, J.W. (1996) Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. *Proceedings of the National Academy of Science U.S.A.* **93** : 770-773.
- De Beer, C. (1994) *Ceratocystis fimbriata* with special reference to its occurrence as a pathogen of *Acacia mearnsii* in South Africa. M.Sc. thesis. University of the Orange Free State, Bloemfontein, South Africa.
- Gorter, G.J.M.A. (1977) *Index of plant pathogens and the diseases they cause in cultivated plants in South Africa*. Department of Agricultural Technical Services, Cape Town, South Africa. Science Bulletin No. 392.
- Gremmen, J. & De Kam, M. (1977) *Ceratocystis fimbriata*, a fungus associated with poplar canker in Poland. *European Journal of Forest Pathology* **7** : 44-47.
- Halsted, B.D. & Fairchild, D.G. (1891) Sweet-potato black rot. *Journal of Mycology* **7** : 1-11.
- Harrington, T.C., Steimel, J. & Kile, G. (1998) Genetic variation in three *Ceratocystis* species with outcrossing, selfing and asexual reproductive strategies. *European Journal of Forest Pathology* **28** : 217-226.
- Kile, G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield, M.J., Seifert, K.A. & Webber, J.A., eds): 173-183. APS Press, St. Paul, Minnesota.
- Kumar, S., Tamura, K., Jakobsen, I.B. & Nei, M. (2001) MEGA2: Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, Arizona, USA.

- Morris, M.J., Wingfield, M.J. & De Beer, C. (1993) Gummosis and wilt of *Acacia meurnsii* in South Africa caused by *Ceratocystis fimbriata*. *Plant Pathology* **42** : 814-817.
- Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences U.S.A.* **70** : 3321-3323.
- Panconesi, A. (1999) Canker stain of plane trees: A serious danger to urban plantings in Europe. *Journal of Plant Pathology* **81** : 3-15.
- Pontis, R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **41** : 179-184.
- Roux, J., Harrington, T.C., Steimel, J.P. & Wingfield, M.J. (2001) Genetic variation in the wattle wilt pathogen *Ceratocystis albofundus*. *Mycoscience* **42** : 327-332.
- Roux, J., Wingfield, M.J., Wingfield, B.D., Bouillett, J.P. & Alfenas, A.C. (1999b) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30** : 175-184.
- Stoddart, J.A. & Taylor, J.F. (1988) Genotypic diversity: estimation and prediction in samples. *Genetics* **118** : 705-711.
- Swofford, D.L. (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods). Vers:4. Sinaur Associates, Sunderland, Massachusetts.
- Taylor, J.W., Jacobson, D.J. & Fisher, M.C. (1999) The Evolution of Asexual Fungi: Reproduction, Speciation and Classification. *Annual Review of Phytopathology* **37** : 197-246.
- Walter, J.M., Rex, E.G. & Schreiber, R. (1952) The rate of progress and destructiveness of canker stain of plane trees. *Phytopathology* **42** : 236-239.
- Wingfield, M.J., De Beer, C., Visser, C. & Wingfield, B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematics and Applied Microbiology* **19** : 191-202.
- Witthuhn, R.C., Harrington, T.C., Wingfield, B.D., Steimel, J.P. & Wingfield, M.J. (2000) Deletion of the *MAT-2* mating type gene during uni-directional mating type switching in *Ceratocystis*. *Current Genetics* **38** : 48-52.
- Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J. & Harrington, T.C. (1999) PCR-based identification and phylogeny of species of *Ceratocystis sensu stricto*. *Mycological Research* **103** : 743-749.

Table 1: Isolates of *Ceratocystis albobundus* from South Africa used in this study.

Isolate No.	Origin	Collector
CMW 4059	BLOEMENDAL	J. Roux
CMW 4061	“	“
CMW 4062	“	“
CMW 4063	“	“
CMW 4064	“	“
CMW 4065	“	“
CMW 4066	“	“
CMW 4068	“	“
CMW 4109	“	“
CMW 4110	“	“
CMW 4097	CINTSA	T.C. Harrington
CMW 4079	DALTON	J. Roux
CMW 4080	“	“
CMW 4082	“	“
CMW 4083	“	“
CMW 4084	“	“
CMW 4085	“	“
CMW 4086	“	“
CMW 4087	“	“
CMW 4088	“	“
CMW 4089	“	“
CMW 4090	“	J. Roux
CMW 4103	“	“
CMW 4104	“	“
CMW 4092	EAST LONDON	T.C. Harrington
CMW 4093	“	“
CMW 4094	“	“
CMW 4095	“	“
CMW 4091	UMKOMAAS	J. Roux
CMW 4069	VRYHEID	“
CMW 4070	“	“
CMW 4071	“	“
CMW 4072	“	“
CMW 4073	“	“
CMW 4074	“	“
CMW 4075	“	“
CMW 4076	“	“
CMW 4077	“	“
CMW 4078	“	“
CMW 4107	“	“

Table 2: Organization of PCR products for each isolate into sample tubes for Genescan analysis.

Isolate			
Tube 1	Primer CF21/22 - TET (250-285 bp)*	Primer AG17/18 - FAM (304-313 bp)	Primer CF5/6 - TET (359-385 bp)
Tube 2	Primer CF23/24 - TET (154-168 bp)	Primer CF15/16.2 - FAM (218-267 bp)	Primer CF17/18 - TET (267-292 bp)
Tube 3	Primer AG15/16 - FAM (274-288 bp)	Primer AG7/8 - TET (284-323 bp)	

*The fragment sizes are based on those calculated from chapter 2.

Table 3: Allele sizes and genotypes recorded for each isolate based on Genescan analysis of eight microsatellite markers.

Isolate	Microsatellite profile	Genotype	A7/B										A15/16		A17/18		CF 5/6		C15/16.2		C17/18			C21/22			C23/24	
			309	322	323	325	326	327	331	332	334	288	293	310	311	380	387	260	288	291	292	283	284	285	166	168		
CMW4059	21111212	1		1										1		1		1		1						1		
CMW4061	91111212	2										1		1		1		1		1						1		
CMW4062	21111211	3		1										1		1		1		1				1				
CMW4063	21111212	1		1										1		1		1		1						1		
CMW4064	21111212	1		1										1		1		1		1						1		
CMW4065	31111312	4			1									1		1		1			1	1				1		
CMW4066	61111312	5								1				1		1		1			1	1				1		
CMW4068	21111232	6		1										1		1		1					1			1		
CMW4109	21111222	18		1										1		1		1		1			1			1		
CMW4110	62111322	7								1				1	1		1		1			1		1		1		
CMW4097	62111322	7								1				1	1		1		1			1		1		1		
CMW4079	61111212	8								1				1		1		1		1		1				1		
CMW4080	61111212	8								1				1		1		1		1		1				1		
CMW4082	51211222	9								1				1		1	1		1		1			1		1		
CMW4083	51211212	10								1				1		1	1		1		1			1		1		
CMW4084	61211222	11								1				1		1	1		1		1			1		1		
CMW4085	61211222	11								1				1		1	1		1		1			1		1		
CMW4086	61211222	11								1				1		1	1		1		1			1		1		
CMW4087	21111322	12		1										1		1		1			1		1			1		
CMW4088	61111212	8								1				1		1		1		1		1				1		
CMW4089	51211212	10								1				1		1	1		1		1		1			1		
CMW4090	61111222	13								1				1		1		1		1			1			1		
CMW4103	21211322	14		1										1		1	1		1			1		1		1		
CMW4104	21211212	15		1										1		1	1		1		1			1		1		
CMW4092	52211232	16								1				1		1	1		1		1			1		1		
CMW4093	62111332	17								1				1	1		1		1			1			1	1		
CMW4094	62111332	17								1				1	1		1		1			1			1	1		
CMW4095	62111322	7								1				1	1		1		1			1		1		1		
CMW4091	21111222	18		1										1		1		1		1			1			1		
CMW4069	21111212	1		1										1		1		1		1		1				1		
CMW4070	22111212	19		1										1		1		1		1		1				1		
CMW4071	41211222	20								1				1		1	1		1		1			1		1		
CMW4072	82121222	21										1		1	1		1		1		1			1		1		
CMW4073	71111121	22								1				1		1		1		1			1		1			
CMW4074	72121332	23								1				1	1		1		1			1		1		1		
CMW4075	72211121	24								1				1		1	1		1		1			1		1		
CMW4076	22111312	25		1										1	1		1		1			1	1			1		
CMW4077	32111322	26			1									1	1		1		1			1		1		1		
CMW4078	22111232	27		1										1	1		1		1		1			1		1		
CMW4107	12111131	28	1											1	1		1		1		1			1	1			

Table 4: Allele frequency and gene diversity values calculated for the South African population of *Ceratocystis albofundus*. Values for the sub-populations, Bloemendal, Dalton and Vryheid are also provided.

Locus	Allele	RSA		Bloemendal		Dalton		Vryheid	
		Allele freq. ¹	H ²	Allele freq.	H	Allele freq.	H	Allele freq.	H
A7/8	309	0.025		0.000		0.000		0.091	
	322	0.350		0.600		0.231		0.364	
	323	0.050		0.100		0.000		0.091	
	325	0.025		0.000		0.000		0.091	
	326	0.100		0.000		0.231		0.000	
	327	0.325		0.200		0.538		0.000	
	331	0.075		0.000		0.000		0.273	
	332	0.025		0.000		0.000		0.091	
	334	0.025	0.751	0.100	0.580	0.000	0.604	0.000	0.760
	A15/16	288	0.650		0.900		0.000		0.273
293		0.350	0.455	0.100	0.180	0.000	0.000	0.727	0.397
A17/18	310	0.725		0.000		0.385		0.818	
	311	0.275	0.399	0.000	0.000	0.615	0.473	0.182	0.298
CF 5/6	380	0.950		0.000		0.000		0.818	
	387	0.050	0.095	0.000	0.000	0.000	0.000	0.182	0.298
C17/18	288	0.075		0.000		0.000		0.273	
	291	0.625		0.700		0.846		0.455	
	292	0.300	0.514	0.300	0.420	0.154	0.260	0.273	0.645
C21/22	283	0.400		0.700		0.462		0.273	
	284	0.425		0.200		0.538		0.455	
	285	0.175	0.629	0.100	0.460		0.497	0.273	0.645
C23/24	166	0.100		0.100		0.000		0.273	
	168	0.900	0.180	0.900	0.180	0.000		0.727	0.397
			0.432		0.364		0.459		0.491

¹ Allele frequency was calculated by dividing the total number of isolates present in the population by the number of times that the allele was present in the population.

² Gene diversity was calculated according to Nei's 1973 formula. Primer C15/16.2 was monomorphic and therefore excluded from analysis.

Table 5: Index of association values for the South African population as well as the sub-populations based on geographic location.

	RSA	Bloemendal	Dalton	Vryheid
Linkage groups¹	23	13	9	20
Isolates	40	8	13	11
Gene diversity	0.432	0.364	0.459	0.491
P-values²	< 0.001	=0.004	=0.001	=0.076
Observed value of I_A	0.992	1.175	0.907	0.367
Min randomized value³	-0.246	-0.724	-0.336	-0.510
Max randomized value³	0.379	1.417	0.907	1.056
Reproductive mode	Clonal	Recombining	Recombining/Clonal	Recombining

¹ The linkage groups indicate the number of polymorphic alleles present in each population used for the calculation of linkage disequilibrium.

² The P-value gives an indication as to how significant the linkage disequilibrium event is. A P-value of below 0.01 is highly significant while a P-value of greater than 0.05 is not significant. Only Vryheid has a P-value that is not significant and therefore has no linkage of alleles.

³ The minimum and maximum values were obtained for 1000 randomization events. If the observed value falls within this range, the population is said to be recombining.

Figure 1: South African map showing the geographical locations of *C. albofundus* isolates collected from diseased *Acacia mearnsii* used in this study. Most of the isolates were collected from the Kwa-Zulu Natal province (shaded area).

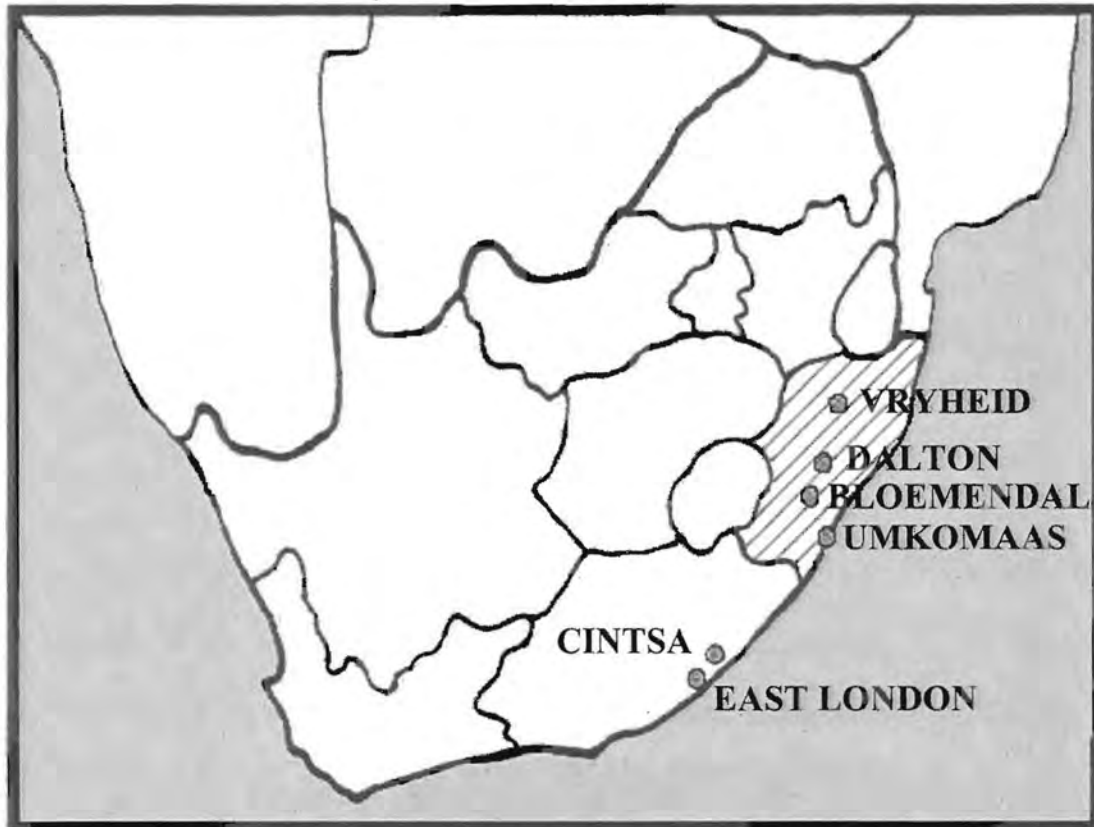


Figure 2: Neighbour-joining dendrogram showing genetic identities among 40 isolates from a South African population of *Ceratomyces albobundus*. Distance analysis was based on absolute distance, D_{AD} , using the number of microsatellite repeats observed at each allele. The isolates are identified by the geographical location they were collected from, followed by their respective CMW numbers. No structuring of the population into geographical location was observed.

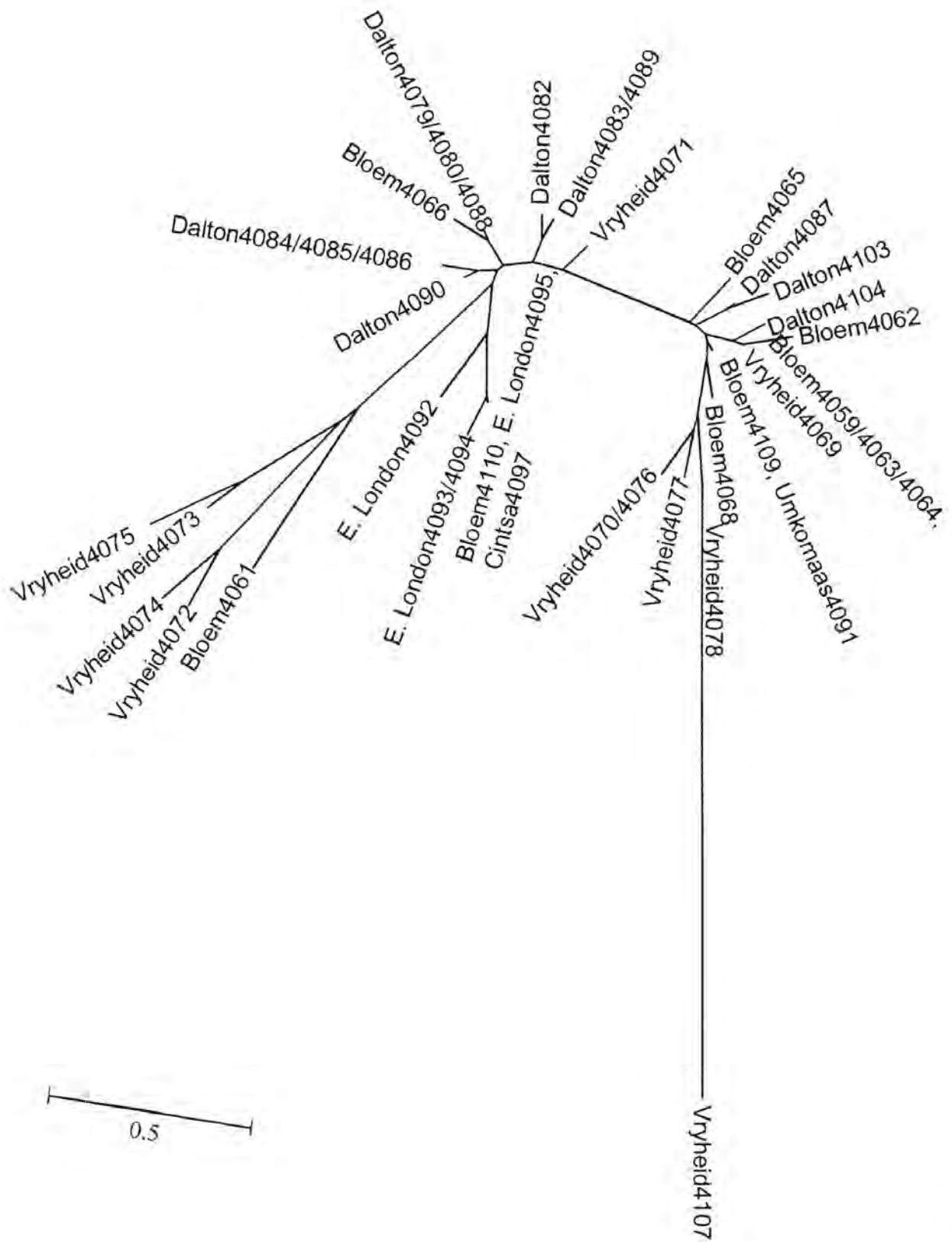


Figure 3: Histograms of randomisation tests where alleles have been randomly shuffled across isolates for each locus. a) The observed Index of association (I_A) across loci falls well beyond the distribution for the randomised datasets, indicating significant correlation of alleles across loci ($P < 0.001$). b) Parsimony Tree Length Permutation Tests (PTLPT) based on 500 randomisation events. The tree length for the observed data is significantly shorter than that for the randomised data set. Both tests indicate a clonal reproductive mode for the South African *C. albobundus* population.

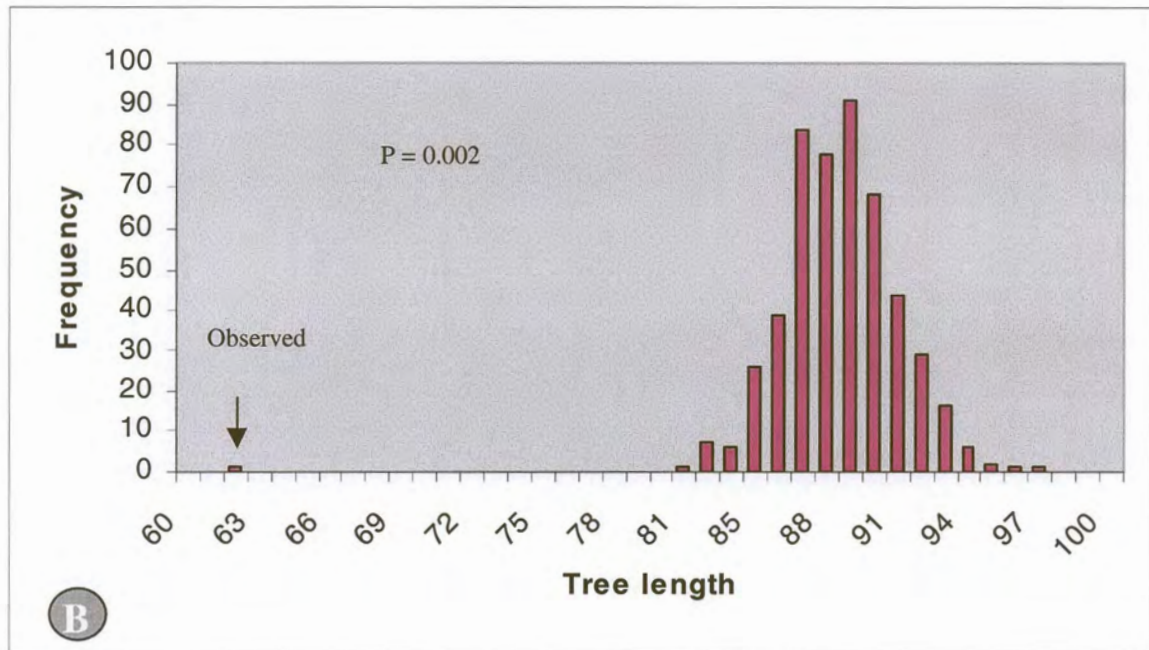
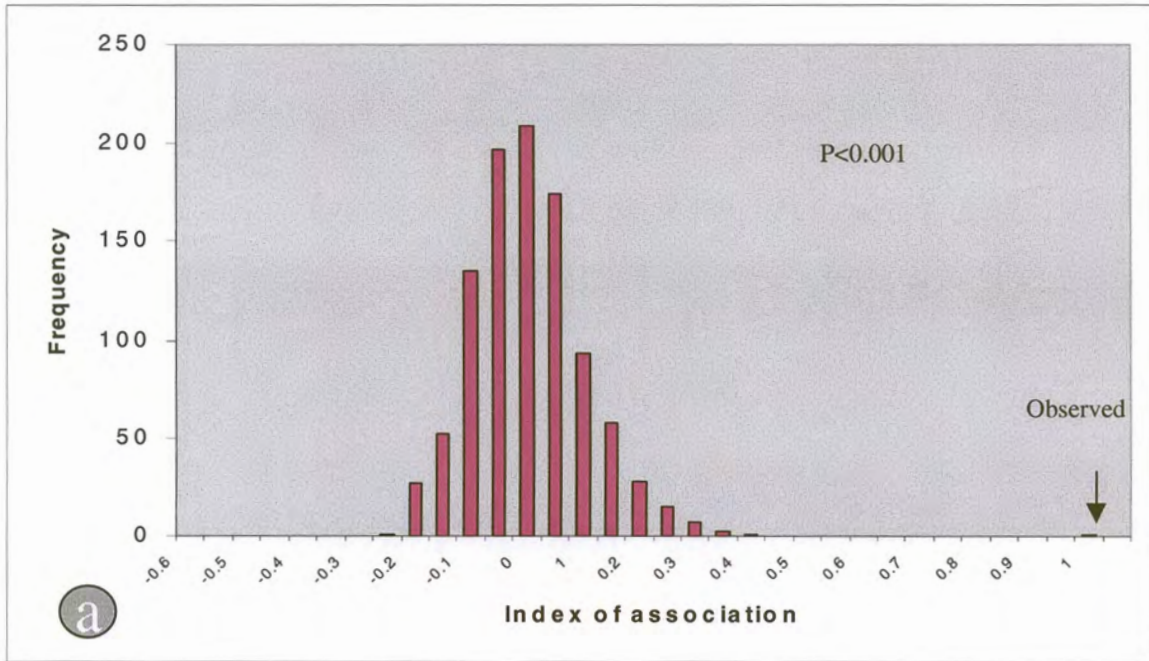
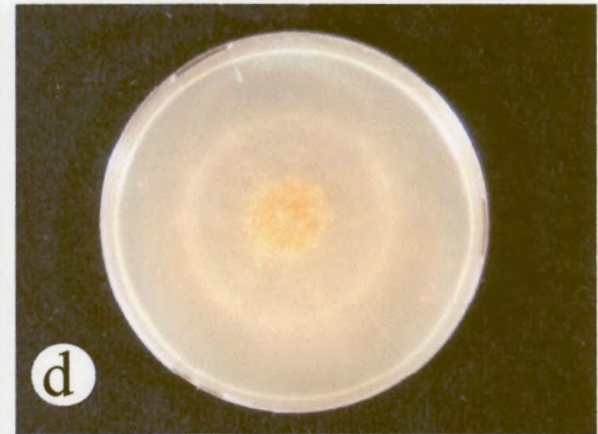
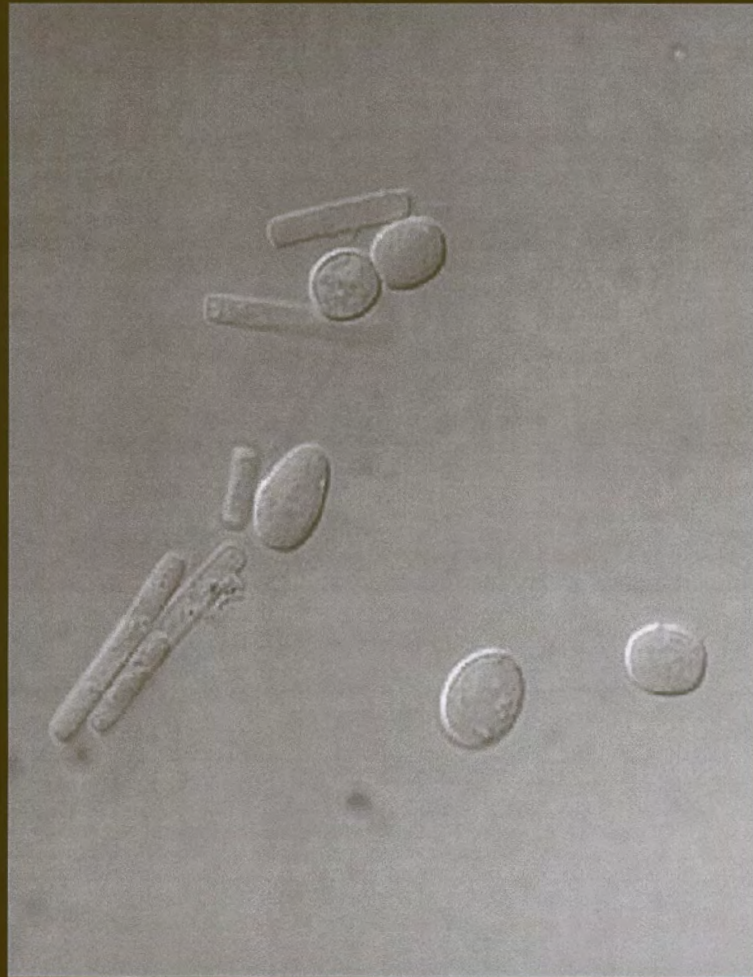


Figure 4: Disease symptoms and culture characteristics of *Ceratocystis albobundus*. a) Rapid wilting and die-back of the branches. Trees may die within a few weeks after infection. b) Cankers and typical gum formation on the bark of infected trees. c) Internal discolouration and streaking of the xylem. d) A white, creamy culture is produced when the pathogen is grown on 2 % malt extract agar. e) Light perithecial base, distinctive of *C. albobundus*. Sticky spore drops collect at tips of perithecial necks allowing for effective transmission.





CHAPTER 5



First report of
Ceratocystis fimbriata
from *Eucalyptus grandis*
in Uruguay

ABSTRACT

Uruguay has a rapidly growing forestry industry consisting mainly of exotic *Pinus* and *Eucalyptus* spp. *E. grandis* and *E. globulus* are used for the production of pulp, paper and timber, which are mainly exported as raw materials. Recently, there have been reports of individual, mature *E. grandis* trees wilting and dying rapidly in plantations. The aim of this investigation was to conduct a survey of dying *E. grandis* in the Rivera area of Uruguay, to determine the cause of Eucalyptus wilt. Typical sap staining symptoms were observed on recently pruned *E. grandis*. Pruning sites showing symptoms were cut from the stems of dying trees. Discs of discoloured wood were stored in a moist environment to induce fungal sporulation. Perithecia, typical of a *Ceratocystis* sp., were found covering the edges of the wood where streaking symptoms occurred. The internal transcribed spacer regions of the ribosomal RNA operon of the *Ceratocystis* sp. was amplified and sequenced using primers ITS1 and ITS4. Results showed that the fungus is morphologically identical to *Ceratocystis fimbriata*. Sequence data confirmed the placement of this fungus amongst other isolates of *C. fimbriata*. Furthermore, the sequence data showed that the Uruguay isolates are most closely related to those from diseased *Eucalyptus* spp. in Brazil, Congo and Uganda. This provides additional evidence to the hypothesis that strains of *C. fimbriata* reside in host specific and geographically isolated groups. *C. fimbriata* is a well known pathogen of many woody plants and appears to be a serious threat to intensively managed *E. grandis* in Uruguay, where the fungus was not previously known. The relationship between pruning and infection will, therefore, need to be evaluated.

INTRODUCTION

Uruguay has a rapidly growing forestry industry consisting mainly of exotic *Pinus* and *Eucalyptus* spp. A total of 4 % of the Country's land is allocated to forestry, and approximately 290 000 hectares, increasing in size by 2.9 % per annum, is used exclusively for growing *Eucalyptus* spp. (Bettucci *et al.* 1997, 1999). The two main species used for forestry are *E. grandis* W. Hill ex Maiden and *E. globulus* Labill.

Eucalyptus spp. are native to Australia, Indonesia and surrounding islands (Poynton 1979). Due to the excellent quality and versatility of its wood, *Eucalyptus* spp. have become one of the world's most widely planted forestry crops (Poynton 1979, Turnbull 1991, Wingfield *et al.* 2001). In Uruguay, *Eucalyptus* plantations were initially established from seeds obtained locally and also from Australia, Chile and South Africa. Although *Eucalyptus* spp. in Uruguay have largely been removed from their natural enemies, planting these trees in an exotic location will expose them to new diseases, for which they may lack natural resistance. It is also likely that pathogens from their areas of origin will be introduced and these could result in large-scale losses due to the relatively narrow genetic base in commercial plantations (Wingfield & Wingfield 1999, Wingfield 1999).

Recently, individual mature *E. grandis* trees have been noted to be wilting and dying rapidly, in plantations near Rivera in northern Uruguay. This sudden death of trees has given rise to considerable concern, especially due to the fact that it is mature trees that are dying. The aim of this investigation was to conduct a survey of *E. grandis* in Uruguay to determine the probable cause of the Eucalyptus wilt disease.

MATERIALS AND METHODS

Collection and isolation of fungi

During April of 2001, individual 1 to 5-year-old *E. grandis* trees were observed dying rapidly in the Rivera area of Northern Uruguay (Figure 1a). Sections of bark and wood were sampled

at approximately breast height from the stems of 21 dying trees. These sections were cut into discs 8 cm wide, wrapped in newspaper and placed in plastic bags for 6 days at room temperature to induce fungal sporulation. Discs were, thereafter, inspected using a dissection microscope.

Perithecia, typical of a *Ceratocystis* sp., were observed covering the edges of the wood where streaking symptoms occurred (Figure 1d, Figure 2a). Ascospore masses accumulating at the tips of the perithecial necks were transferred to 2 % MEA (malt extract agar, Biolab) using a sterile needle. Ascospore masses from structures in cultures were repeatedly transferred to clean plates, until pure cultures had been obtained. One pure culture was thus obtained for each of the 21 trees sampled.

Morphology

The morphology of the *Ceratocystis* sp. was studied on the original wood and on structures produced on 2 % MEA plates (Figure 2a, b, c). Samples were prepared by mounting fungal structures on microscope slides in lactophenol, amended with cotton blue. Identification of the fungus was based on the shape and size of perithecia, ascospores, conidia, as well as colour of perithecia and hyphal ornamentations on the perithecial bases. Colours were determined using the colour charts of Rayner (1970).

DNA extraction

For molecular analysis, single ascospore masses were transferred from pure cultures and grown in 50 ml malt broth for 12 days at 22 °C. Mycelial mats were freeze-dried and pulverized using liquid nitrogen. DNA was extracted using a phenol/chloroform protocol as previously described by Barnes *et al.* (2001b).

PCR amplification

Primers, specifically designed to amplify the internal transcribed spacer regions of the ribosomal RNA operon (White *et al.* 1990), were used to sequence the ITS1, 5.8S and ITS2 regions. The polymerase chain reaction (PCR) mixture, in a total volume of 50 µl, contained 2–10 ng DNA, 1.75 U enzyme (Expand H.F. PCR System, Roche Molecular Biochemicals), 1

x buffer containing 1.5 mM MgCl₂ (supplied with the enzyme), 200 nM of primers ITS1 and ITS4, and 200 μM of each dNTP. The PCR programme contained an initial denaturation step at 96 °C for 2 min, followed by 10 cycles of 20 s at 94 °C, 48 s at 55 °C, and 45 s at 72 °C. A 5 s extension after each cycle with the annealing time altered to 40 s, was carried out for a further 25 cycles. A 10 min elongation step at 72 °C ended the programme. All PCR amplicons were purified using the Magic PCR Preps Purification System (Promega, Madison, WI).

Sequencing

PCR products were amplified in both directions for sequencing, using an ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Inc., Foster City, Calif.), according to the manufacturers protocol. Primers ITS1 and ITS4 were used. Sequence reactions were run on PAGE (polyacrylamide gel electrophoresis) using an ABI PRISM™ 377 Autosequencer (Perkin-Elmer Applied Biosystems). Sequence electropherograms were analyzed using Sequence Navigator version 1.0.1 (Perkin-Elmer).

Phylogeny

Forward and reverse sequences were matched and analysed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, Calif.). Additional sequences for use in the analysis were obtained from Genbank (Table 1). Sequences were manually aligned by means of gap insertions. The phylogeny of the aligned sequences was determined using PAUP* 4.0 (Phylogenetic Analysis Using Parsimony [*and other methods]) (Swofford 1998). All characters were given equal weight and missing data were treated as a fifth character (new state). The heuristic search option with random stepwise addition and 'tree bisection reconnection' (TBR) was used as the swapping algorithm. The Mulpar option was in effect and branches collapsed if they equaled zero. Confidence levels of the branching points were determined using 1000 bootstrap replicates and length distribution of 1000 trees. *Petriella setifera* (Schmidt) Curzi (AFO43596), was used as the outgroup and treated as a paraphyletic sister group to the ingroup. All sequences derived in this study have been deposited in Genbank (Table 1).

RESULTS

Collection and isolation of fungi

Dead and dying *E. grandis* trees from which samples were taken, retained their leaves, suggesting that wilt had occurred rapidly (Figure 1a). Small cankers on the bark of dying trees were also observed. Removal of the bark revealed extensive kino production in the cambium (Figure 1b). Dissection of the stems showed a streaking pattern of discolouration of the wood, emerging specifically from recent pruning sites (Figure 1c).

Incubation of wood discs from dying trees resulted in the relatively rapid production of perithecia. Isolations were easily made from spore masses on the perithecial necks. A total of 21 isolates were obtained from the trees sampled.

Morphology

Cultures were olivaceous brown (21''K) in colour (Figure 2b) and produced a fruity aroma. Perithecia were produced on both wood pieces and agar plates (Figure 2a, c). The globose bases of the perithecia were black with long necks ending in ostiolar hyphae (Figure 2a, c). The ostiolar hyphae extended from the outer layer of the neck cells and were mostly divergent. Ascospores were typically hat-shaped in morphology (Figure 2d). Cylindrical and barrel shaped conidia were observed (Figure 2d). Morphologically, the fungus is identical to descriptions previously given for *Ceratocystis fimbriata* Ellis & Halst. (Hunt 1956, Upadhyay 1981).

Phylogeny

Sequencing of the ITS 1 and ITS2 regions resulted in a DNA sequence of 570 bp long. Manual alignment of the sequences for representative isolates used in this study (Figure 3) generated a total of 551 characters. Of these characters, 170 were constant, 127 parsimony-uninformative and 254 parsimony-informative. Three most parsimonious trees with a length of 800 were produced. The consistency index (CI), retention index (RI) and gi values were 0.76, 0.79 and -1.22 respectively for all trees. Trees had slight differences in topology where bootstrap values were smaller than 50 %. One tree was chosen for presentation (Figure 4).

The *Ceratocystis* sp. collected from symptomatic *E. grandis* trees in Uruguay resided in the clade typified by *C. fimbriata*, with a bootstrap value of 100% (Figure 4). Our results show that the Uruguay isolates are most closely related to isolates from Brazil, South America. In terms of host range, *C. fimbriata* isolates from *E. grandis* in Uruguay, grouped together in a larger clade encompassing all the isolates collected from *Eucalyptus* in Brazil, Republic of Congo and Uganda (Table 1, Figure 4).

DISCUSSION

This study represents the first report of the important wilt pathogen *C. fimbriata* from Uruguay. In this country, the fungus is associated with wood stain and rapid death of *E. grandis*. This is a serious disease that appears to result from infection of recent pruning wounds. Pruning is an important silvicultural practice where *Eucalyptus* is used to produce solid timber products. In future, infection of pruning wounds by *C. fimbriata* will need to be avoided.

Ceratocystis fimbriata is well-known for causing wilt diseases on many woody hosts (Kile 1993). However, the fungus has only recently been described as a pathogen on *Eucalyptus* spp. These reports have emerged from Brazil (Laia *et al.* 1999, Roux *et al.* 1999), Republic of Congo (Roux *et al.* 1999) and Uganda (Roux *et al.* 2001). The only other *Ceratocystis* spp. known to infect *Eucalyptus* trees is *C. eucalypti* Z. Q. Yuan & Kile (Kile *et al.* 1996) and *C. pirilliformis* Barnes & Wingfield (Chapter 6), both from Australia.

Pathogenicity tests conducted, using a range of *C. fimbriata* isolates, have shown that this fungus tends to be host specific (Pontis 1951, Leather 1966). Sequencing data from this study grouped the Uruguay isolates from *E. grandis* in a clade, together with other isolates from *Eucalyptus* spp. This provides added evidence for the presence of host specific groups in the pathogen (Barnes *et al.* 2001a). Furthermore, the Uruguay isolates in this study were most closely related to isolates from Brazil, which is geographically adjacent to Uruguay. These

results support the view that *C. fimbriata* represents a species complex (Webster & Butler 1967), based on host and geographic location (Barnes *et al.* 2001a).

Pruning of *E. grandis* is a common practice in forest plantations and is implemented twice, sometimes three times during the growing period of trees with a 15-16 year rotation. This practice decreases the branch knots, which increases the quality of the wood, making it more valuable (Beadle 1999). All evidence from this study suggests that infections by *C. fimbriata* have occurred shortly after pruning. Strategies will now need to be developed to avoid such infections.

Wounds are essential for infection by *C. fimbriata* (Moller & DeVay 1968, Kile *et al.* 1993). Opportunistic sap-feeding insects, mainly flies (Diptera) and picnic beetles (Nitidulidae) (Crone & Bachelder 1961, Moller & DeVay 1968, Hinds 1972) have been shown to be the main vectors of *C. fimbriata*, moving the fungus between freshly made wounds on trees. Although insects are also probably the primary agents of dispersal in Uruguay, other means of spread of this fungus are possible. Since the primary source of open wounds on *E. grandis* trees in Uruguay is due to pruning, infected pruning shears or other similar equipment (Teviotdale 1991), could contribute to the spread of the fungus from diseased to healthy trees.

REFERENCES

- Barnes, I., Gaur, A., Burgess, T., Roux, J., Wingfield, B.D. & Wingfield, M.J. (2001a) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular Plant Pathology* **2** : 319-325.
- Barnes, I., Roux, J., Coetzee, M.P.A., Wingfield, B.D. & Wingfield, M.J. (2001b) Characterisation of *Seiridium* spp. associated with cypress canker based on β -tubulin and histone Sequences. *Plant Disease* **85** : 317-321.
- Beadle, C. (1999) Pruning, a knotty problem for plantation timber. *CSIRO Media Release* 99/57.
- Bettucci, L., Alonso, R. & Fernández, L. (1997) A comparative study of fungal populations in healthy and symptomatic twigs and seedlings of *Eucalyptus globulus* in Uruguay. *Sydowia* **49** : 109-117.
- Bettucci, L., Alonso, R. & Tiscornia, S. (1999) *Endophytic mycobiota* of healthy twigs and the assemblage of species associated with twig lesions of *Eucalyptus globulus* and *E. grandis* in Uruguay. *Mycological Research* **103** : 468-472.
- Crone, L.J. & Bachelder, S. (1961) Insect transmission of the canker stain fungus, *Ceratocystis fimbriata* F. *platani*. *Phytopathology* **51** : 576.
- Hinds, T.E. (1972) *Ceratocystis* canker of Aspen. *Phytopathology* **62** : 213-220.
- Hunt, J. (1956) Taxonomy of the genus *Ceratocystis*. *Lloydia* **19** : 1-59.
- Kile, G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.A. Webber, eds):173-183. APS Press, St. Paul, Minnesota.
- Kile, G.A., Harrington, T.C., Yuan, Z.Q., Dudzinski, M.J. & Old, K.M. (1996) *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100** : 571-579.
- Laia, M.L., Alfenas, A.C. & Harrington, T.C. (1999) Isolation, detection in soil, and inoculation of *Ceratocystis fimbriata*, causal agent of wilting, die-back and canker in *Eucalyptus*. *Australasian Plant Pathology Society 12th Biennial Conference Canberra*.

- Leather, R.I. (1966) A canker and wilt disease of pimento (*Pimenta officinalis*) caused by *Ceratocystis fimbriata* in Jamaica. *Transaction of the British Society* **42** : 213-218.
- Moller, W.J. & DeVay, J.E. (1968) Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58** : 1499-1507.
- Pontis, R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **41** : 179-184.
- Poynton, R.J. (1979) *Tree Planting in Southern Africa*. Volume 2. The Eucalypts. Department of Forestry. Republic of South Africa.
- Rayner, R.W. (1970) A Mycological color chart. Commonwealth Mycological Institute and British Mycological Society, Kew, Surrey.
- Roux, J., Coutinho, T.A., Mujuni Byabashaija, D. & Wingfield, M.J. (2001) Diseases of plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97** : 16-18.
- Roux, J., Wingfield, M.J., Wingfield, B.D., Bouillett, J.P. & Alfenas, A.C. (1999) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30** : 175-184.
- Swofford, D.L. (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods). Vers 4. Sinaur Associates, Sunderland, Massachusetts.
- Teviotdale, B.L. (1991) Infection of pruning and small bark wounds in almond by *Ceratocystis fimbriata*. *Plant Disease* **75** : 1026-1030.
- Turnbull, J.W. (1991) Future use of *Eucalyptus*: opportunities and problems. *IUFRO Symposium. Intensive Forestry: The Role of Eucalypts*, Durban, 2-6 September, South African Institute of Forestry.
- Upadhyay, H.P. (1981) A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens.
- Webster, R.K. & Butler, E.E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45** : 1457-1468.
- Wingfield, M. (1999) Pathogens in exotic plantation forestry. *International Forestry Review* **1**: 163-168.
- Wingfield, M.J. & Wingfield, B.D. (1999) A positive prognosis for plantation species. Forestry and Logging, Research on plantation species. *Asian Timber* 16-19.

- Wingfield, M.J., Slippers, B., Roux, J. & Wingfield, B.D. (2001) Worldwide movement of exotic forest fungi, especially in the tropics and the Southern Hemisphere. *BioScience* **51** : 134-140.
- White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A sequencing Guide to Methods and Applications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White, eds): 315-322. Academic Press, San Diego, USA.

Table 1: *Ceratocystis* collections selected for sequence comparisons in this study.

Isolate	Country	ITS Genbank		Host	Collector
		Accession No.	Culture no*		
<i>C. fimbriata</i>	Italy	AF264903	CMW 2242	<i>Platanus</i> sp.	A. Panconesi
"	Switzerland	AF395681	CMW 1896	"	O. Petrini
"	Brazil	AF395683	CMW 4903	<i>Eucalyptus</i> sp.	A. Alfenas
"	Uruguay**	AF453438	CMW 7383	<i>Eucalyptus grandis</i>	M. J. Wingfield
"	"	AF453439	CMW 7387	"	"
"	"	AF453440	CMW 7389	"	"
"	Colombia	AF395688	CMW 4829	<i>Citrus</i> sp.	B. L. Castro
"	"	AF395689	CMW 4835	<i>Coffea</i> sp.	"
"	"	AF395691	CMW 4844	"	"
"	"	AF395692	CMW 4824	"	"
"	Congo	AF395684	CMW 4793	<i>Eucalyptus</i> clone	J. Roux
"	Uganda	AF395687	CMW 5312	<i>Eucalyptus grandis</i>	"
<i>C. albofundus</i>	South Africa	AF264910	CMW 2148	<i>Acacia mearnsii</i>	M.J. Morris
"	"	AF043605	CMW 2475	"	S. McLennan
<i>C. coerulescens</i>	Germany	U75615	CBS 140.37	<i>Picea abies</i>	T. Rohde
<i>C. fagacearum</i>	USA	AF043598	CMW 2651	<i>Quercus</i> sp.	F. Paula
<i>C. eucalypti</i>	Australia	U75627	CMW 3254	<i>Eucalyptus sieberi</i>	G. Kile
<i>C. virescens</i>	USA	AF043603	CMW 0460	<i>Quercus</i> sp.	-
<i>C. moniliformis</i>	South Africa	AF043597	CMW 3782	<i>Erythrina</i> sp.	G. Zhao
<i>Petriella setifera</i>	Kenya	AF043596	ATCC 26490	<i>Rock hyrax dung</i>	-

* CMW represents cultures from the culture collections of the Forestry and Agricultural Biotechnology Institute, ATCC from the American Type Culture Collection and CBS, Centraalbureau voor Schimmelcultures.

**All the Uruguay sequences were obtained during this study. Other sequences were obtained from Genbank.

Figure 1: Symptoms of infection of *E. grandis* with *C. fimbriata* in Uruguay. a) Single, mature trees dying with typical wilt symptoms. b) Kino pockets under the bark of *E. grandis*. c) Discolouration of the wood at site where side branch had been pruned. d) Cross section through trunk of *E. grandis* showing the streaking discolouration caused by *C. fimbriata*.



Figure 2: Morphological and cultural characteristics of *C. fimbriata* from *E. grandis* in Uruguay. a) Perithecia produced on the wood of *E. grandis*. b) Cultures of *C. fimbriata* on 2 % MEA have a brownish cultural colour (21°K). c) Black, globose perithecial base with spore droplets accumulating on long, black perithecial necks. d) Three different spore types produced by *C. fimbriata*: cylindrical and barrel-shaped conidia and hat-shaped ascospores.

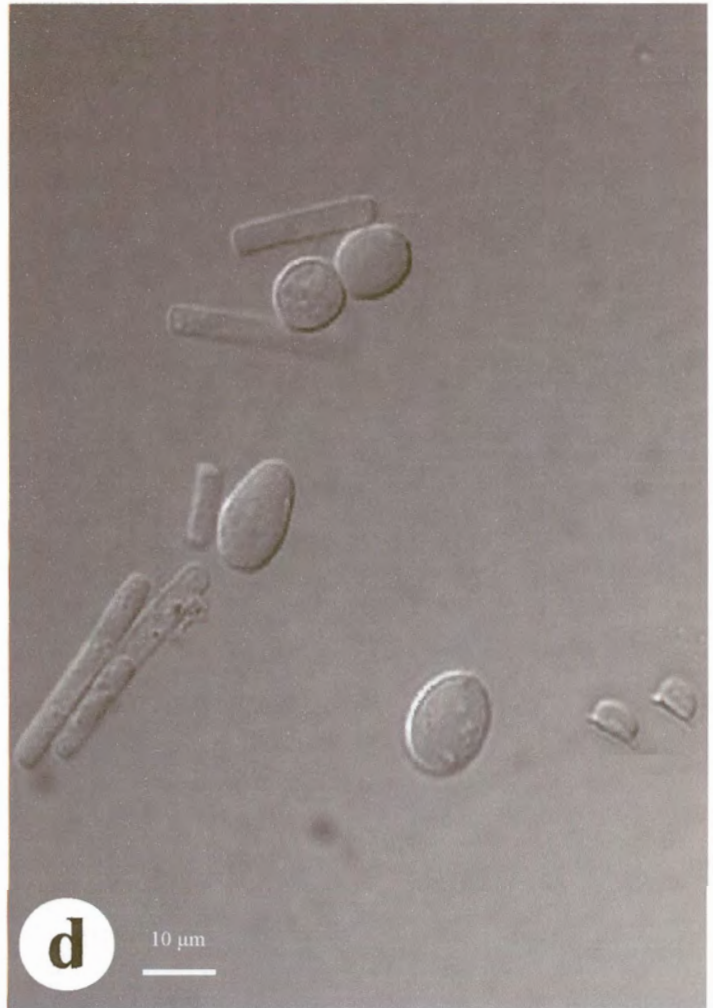
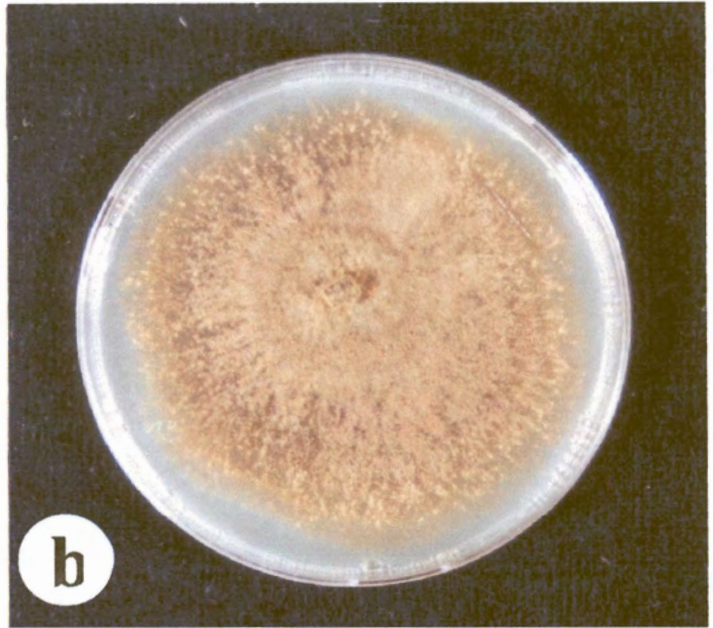
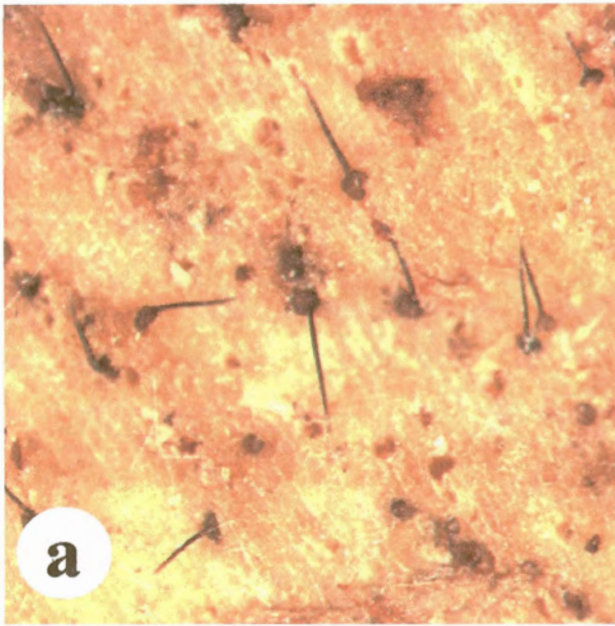


Figure 3: Sequence alignments of the ITS region (551 bp) for *C. fimbriata* from Uruguay with other *C. fimbriata* and *Ceratocystis* species. Sequences with homology to *C. fimbriata* from Italy (AF264903) (top line), are represented with dots. A dash represents gaps introduced to maximize alignments and N, missing bases

		10	20	30	40	50	60	70
<i>Italy</i>	(AF264903)	CCATGTGTGA	ACGTACC-TA	TCTT-GTAGT	GA-GATGAAT	GCTG-TTTTG	GTGGT-AGGG	-----CCC-
<i>Switzerland</i>	(AF395681)
<i>Brazil</i>	(AF395683)C.....
<i>Uruguay</i>	(AF453438)C.....
<i>Uruguay</i>	(AF453439)C.....
<i>Uruguay</i>	(AF453440)C.....
<i>Colombia</i>	(AF395688)A.....C.....T.....
<i>Colombia</i>	(AF395689)A.T.C.....A.....T.....
<i>Colombia</i>	(AF395691)
<i>Colombia</i>	(AF395692)
<i>Congo</i>	(AF395684)
<i>Uganda</i>	(AF395687)
<i>C. albobundus</i>	(AF264910)	G.TGCCT..G	TG.G----G	...T....	.GT.T..C	..C....	..AT..A..	-----
<i>C. albobundus</i>	(AF043605)	G.TGCCT..G	TG.G----G	...T....	.GT.T..C	..C....	..AT..A..	-----
<i>C. coerulescens</i>	(U75615)	...A.....	...A...C..	.T..T...-C....	..C....	-----
<i>C. fagacearum</i>	(AF043598)	...T.....	...A..A.CG.	.T..TT.TTC	TC----T..	A...C....	..C....	A-----
<i>C. eucalypti</i>	(U75627)	...A.....	...A..A.C.T	CT-----C....	..C....	-----
<i>C. virescens</i>	(AF043603)	...A.....	...A..A.C..	.T-----C....	..C....	-----
<i>C. moniliformis</i>	(AF043597)	...T.....	.TT..-CAC	AAACATCGAA	-----	CTGCGA..	..C---G..	-----
<i>Petriella setifera</i>	(AF043596)	..C.T.....	..C.TA.C..	.T---.TA-	--T.T.---	--GCC-.C.	..C---G...TT	---AG.C
		80	90	100	110	120	130	140
<i>Italy</i>	(AF264903)	-TTCTG----	-AAGGG----	-----CAC	C-----	GCTGCCAGCA	GTA-TAG--T	CT-C-GCCAC
<i>Switzerland</i>	(AF395681)
<i>Brazil</i>	(AF395683)A.A----	---GGG...T....	..T.....
<i>Uruguay</i>	(AF453438)A.A----	---GGG...T....	..T.....
<i>Uruguay</i>	(AF453439)A.A----	---GGG...T....	..T.....
<i>Uruguay</i>	(AF453440)A.A----	---GGG...T....	..T.....
<i>Colombia</i>	(AF395688)A.....	---GGGG...TA....	..T.-A....
<i>Colombia</i>	(AF395689)A.....	---GGGG...TA....	..T.-A....
<i>Colombia</i>	(AF395691)T---	---A...
<i>Colombia</i>	(AF395692)G...
<i>Congo</i>	(AF395684)A.A----	---GGG...
<i>Uganda</i>	(AF395687)A.A----	---GGG...T....	..T.....
<i>C. albobundus</i>	(AF264910)	-----	GGGCA.CCCA	CTACCGCT.G	.CA-----C.TACAAG.	..TTTA....
<i>C. albobundus</i>	(AF043605)	-----	GGGCA.CCCA	CTACCGCT.G	.CA-----C.TACAAG.	..TTTA....
<i>C. coerulescens</i>	(U75615)	C..GGTTTGA	A..AAACAAG	T-----G...	-----
<i>C. fagacearum</i>	(AF043598)	C..-CTTTCT	TC....GATG	TTT-----	-----
<i>C. eucalypti</i>	(U75627)	C..GGTAACA	--CAA.TCT-	-----G..-	-----	-----
<i>C. virescens</i>	(AF043603)	C..GGTAACA	--CAA-----	-----G	T....G..-	-----	-----
<i>C. moniliformis</i>	(AF043597)	..CTCCCCGC	CCG.CA-GT-	-----	-----	-----	-----A	..CTT-----
<i>Petriella setifera</i>	(AF043596)	CCC-----	---AAA--G	C-----TTCT	.CCGCCGG--	---CAGCA--	----CTAAA.	T-CTTAATTT

		150	160	170	180	190	200	210
<i>Italy</i>	(AF264903)	TGTAAA----	-CTCTT----	AT-ATTTTT-	CCAGA--TT	TTTT-----	-CATT-GCTG	AGTGGCAT--
<i>Switzerland</i>	(AF395681)
<i>Brazil</i>	(AF395683) TTT-	..T.....	.T.....
<i>Uruguay</i>	(AF453438) TTT-	..T.....	.T.....
<i>Uruguay</i>	(AF453439) TTT-	..T.....	.T.....
<i>Uruguay</i>	(AF453440) TTT-	..T.....	.T.....
<i>Colombia</i>	(AF395688) A-- TTT-TTA.....
<i>Colombia</i>	(AF395689)	..G..A-- TTTTT.....
<i>Colombia</i>	(AF395691) TTT-T.....	..C.TAATTT	C...T...
<i>Colombia</i>	(AF395692) TTT-T.....	..C.TAATTT	C...T...
<i>Congo</i>	(AF395684) AAAA	A..... TT--	..T.C.....	.T.....
<i>Uganda</i>	(AF395687) A-- TTT-	..T.....	.T.....
<i>C. albofundus</i>	(AF264910)	.A.....	..CT.CT--G	TAT.....T	--.A.A--	..AAAA--
<i>C. albofundus</i>	(AF043605)	.A.....	..CT.CT--G	TAT.....T	--.A.A--	..AAAA--
<i>C. coerulescens</i>	(U75615)	.A..T.AAAA	A.A..CTTT-	--A..A..-	.T...GAA..	-----ATTT	T....T-
<i>C. fagacearum</i>	(AF043598)	.A.TT.CAAA TTT-	--A.....	.T...GAA..	-----ATT-T...TT
<i>C. eucalypti</i>	(U75627)	.A.TT.CAAA TTT-T...GAA..	-----ATT-T-
<i>C. virescens</i>	(AF043603)	.A.TTT-AAA	A..... TTTT	T.....	.T.A.GAA..	-----ATT-T-
<i>C. moniliformis</i>	(AF043597)	-----GA	A...G.TTT-	--T..A----	-T.A.GAA..	-----ATT-A...TT
<i>Petriella setifera</i>	(AF043596)	.-AT.-GCG	GA.T-ATACT	T-----	.TGA.TACAA	..-AC-----

		220	230	240	250	260	270	280
<i>Italy</i>	(AF264903)	--AACTATAA	AAAA---G--	TTAAAACTTT	CAACAACGGA	TCTCTTGGCT	CTAGCATCGA	TGAAGAACGC
<i>Switzerland</i>	(AF395681)
<i>Brazil</i>	(AF395683) A--
<i>Uruguay</i>	(AF453438) A--
<i>Uruguay</i>	(AF453439) A--
<i>Uruguay</i>	(AF453440) A--
<i>Colombia</i>	(AF395688) AA--
<i>Colombia</i>	(AF395689) AAA-
<i>Colombia</i>	(AF395691) AA--
<i>Colombia</i>	(AF395692) AA--
<i>Congo</i>	(AF395684) A--
<i>Uganda</i>	(AF395687)
<i>C. albofundus</i>	(AF264910) A--
<i>C. albofundus</i>	(AF043605) A--
<i>C. coerulescens</i>	(U75615)ATA.T
<i>C. fagacearum</i>	(AF043598)A.A.T
<i>C. eucalypti</i>	(U75627)ATA.T
<i>C. virescens</i>	(AF043603)ATA.T
<i>C. moniliformis</i>	(AF043597)	T----ATA..	T-----TA
<i>Petriella setifera</i>	(AF043596)	AA...A.A--	-----	.A.....T..G.....

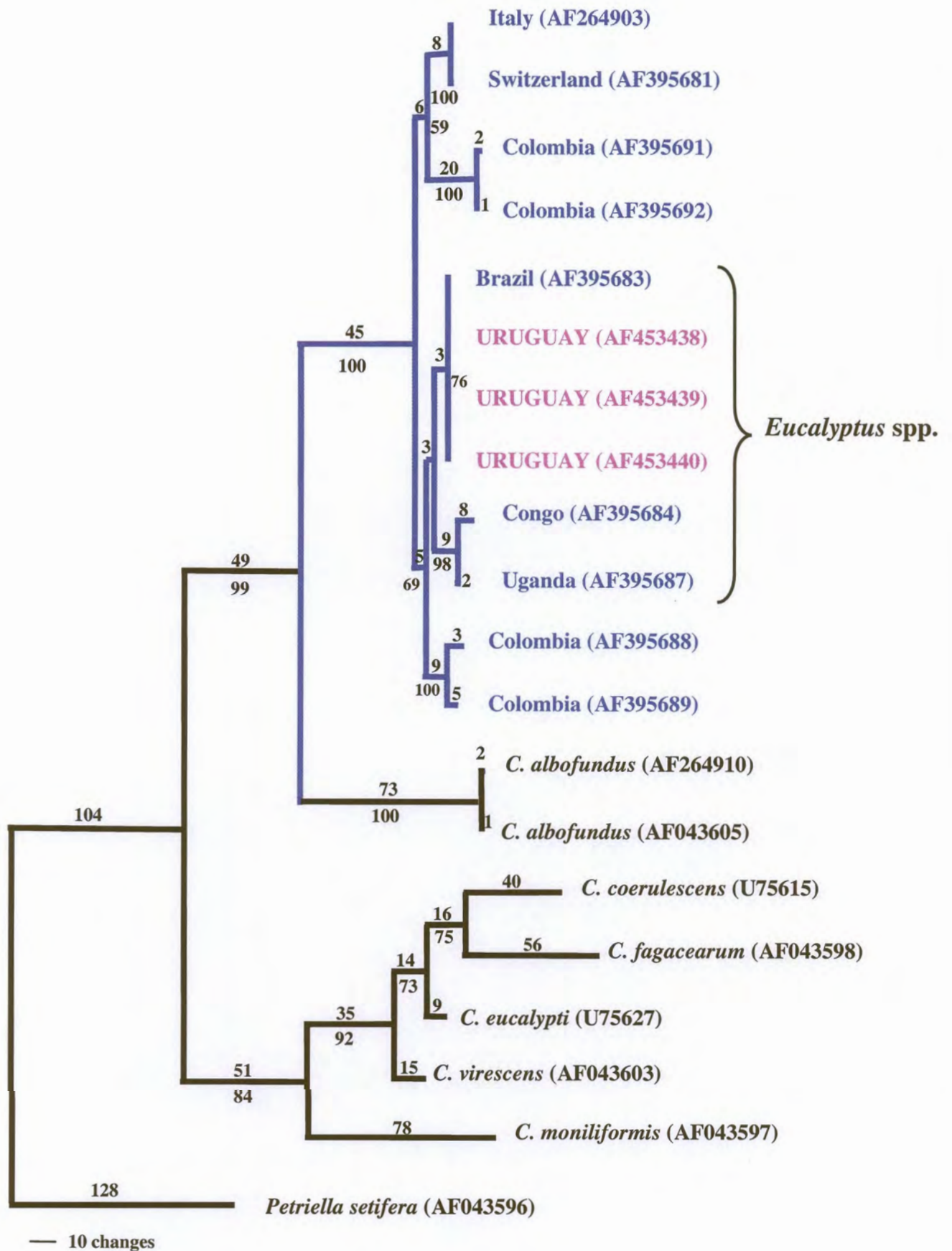
		290	300	310	320	330	340	350
<i>Italy</i>	(AF264903)	AGCGAAATGC	GATAAGTAAT	GTGAATTGCA	GAATTCAGTG	AATCATCGAA	TCTTTGAACG	CACATTGCGC
<i>Switzerland</i>	(AF395681)
<i>Brazil</i>	(AF395683)
<i>Uruguay</i>	(AF453438)
<i>Uruguay</i>	(AF453439)
<i>Uruguay</i>	(AF453440)
<i>Colombia</i>	(AF395688)
<i>Colombia</i>	(AF395689)
<i>Colombia</i>	(AF395691)
<i>Colombia</i>	(AF395692)
<i>Congo</i>	(AF395684)
<i>Uganda</i>	(AF395687)
<i>C. albofundus</i>	(AF264910)C.
<i>C. albofundus</i>	(AF043605)
<i>C. coerulescens</i>	(U75615)C.....GC.
<i>C. fagacearum</i>	(AF043598)
<i>C. eucalypti</i>	(U75627)C.....
<i>C. virescens</i>	(AF043603)C.....
<i>C. moniliformis</i>	(AF043597)
<i>Petriella setifera</i>	(AF043596)

		360	370	380	390	400	410	420
<i>Italy</i>	(AF264903)	CTGGCAGTAT	TCTGCCAGGC	ATGCCTGTCC	GAGCGTCATT	TCACCACTCA	AGGACTCC--	-TTT-GTT--
<i>Switzerland</i>	(AF395681)----
<i>Brazil</i>	(AF395683)--T...--
<i>Uruguay</i>	(AF453438)--T...--
<i>Uruguay</i>	(AF453439)--T...--
<i>Uruguay</i>	(AF453440)--T...--
<i>Colombia</i>	(AF395688)CT--T...--
<i>Colombia</i>	(AF395689)CT--T...--
<i>Colombia</i>	(AF395691)----
<i>Colombia</i>	(AF395692)----
<i>Congo</i>	(AF395684)TATT--
<i>Uganda</i>	(AF395687)TATT--
<i>C. albofundus</i>	(AF264910)T.....T--GA...TT
<i>C. albofundus</i>	(AF043605)T--GA...TT
<i>C. coerulescens</i>	(U75615)TG---
<i>C. fagacearum</i>	(AF043598)A.....T.....CTT-G--
<i>C. eucalypti</i>	(U75627)-G--
<i>C. virescens</i>	(AF043603)-G--
<i>C. moniliformis</i>	(AF043597)CA.....C.....TG.....T-G--
<i>Petriella setifera</i>	(AF043596)C.....A.....G.....CTCG.	G---.CTA--	----A...TT

		430	440	450	460	470	480	490
Italy	(AF264903)	CTTGCGCTTG	GAGGTCCTGT	TCT-----	-----CCCCT	GAACAGGCCG	CCGAAATGTA	TCGGCTGTTA
Switzerland	(AF395681)
Brazil	(AF395683)
Uruguay	(AF453438)
Uruguay	(AF453439)
Uruguay	(AF453440)
Colombia	(AF395688)
Colombia	(AF395689)
Colombia	(AF395691)	-----C
Colombia	(AF395692)	-----C
Congo	(AF395684)
Uganda	(AF395687)C
<i>C. albofundus</i>	(AF264910)	---.T...T-A---	---CC.TT..C
<i>C. albofundus</i>	(AF043605)	---.T...T-A---	---CC.TT.CC
<i>C. coerulescens</i>	(U75615)	...T...	...A..C.C	GTCITTTTTG	TTT-----	...G....C
<i>C. fagacearum</i>	(AF043598)	---.T...	...A..C-C	A-CTTGTCAC	AA-----	...G.C.A-CAG
<i>C. eucalypti</i>	(U75627)	---.T...	...A.TC.C	ATCTTA--TG	A-----	...G....C
<i>C. virescens</i>	(AF043603)	---.T...	...A....	.G.TT--TC	AA-----	...A....C
<i>C. moniliformis</i>	(AF043597)	---.TT...	...AG....	G..AT----	-----GC	...G...T	.T.....CGT
<i>Petriella setifera</i>	(AF043596)	T.AAA.T---	-GA--AGGA.	CGG----TG	TTGGGG.G..	AC.G--CGGT	T.TTCGGAGC	AGCTG.A---

		500	510	520	530	540	550
Italy	(AF264903)	----TACTTG	CC-AACTCCC	CTGTGTAGTA	TAAAA-TTTC	T-AATTTTTA	CACTTTGAAG T
Switzerland	(AF395681)	-----	-----	-----	-----	-----	-----
Brazil	(AF395683)	-----	-----	-----	-----	-----	-----
Uruguay	(AF453438)	-----	-----	-----	-----	-----	-----
Uruguay	(AF453439)	-----	-----	-----	-----	-----	-----
Uruguay	(AF453440)	-----	-----	-----	-----	-----	-----
Colombia	(AF395688)	-----C	-----	-----	-----	-----
Colombia	(AF395689)	-----C	-----	-----	-----	-----
Colombia	(AF395691)	-----	-----	-----	-----	-----	-----
Colombia	(AF395692)	-----	-----	-----	-----	-----	-----
Congo	(AF395684)	-----	-----	-----	-----	-----	-----
Uganda	(AF395687)	-----	-----	-----	-----	-----	-----
<i>C. albofundus</i>	(AF264910)	TTTT	-----	-----	C..G.T..T	A.....	.G....G..
<i>C. albofundus</i>	(AF043605)	TTTT	-----	-----	C..G.T..T	A.....	.G....G..
<i>C. coerulescens</i>	(U75615)	T---.T...	...G..T..	-----	A---TA..T	-----	.G.....A C
<i>C. fagacearum</i>	(AF043598)	T---.T...	...G..T..	...C....	A..C-T...-	GTG-....-	.G...C...A C
<i>C. eucalypti</i>	(U75627)	T---.T...	...G..T..	-----	A---TA..T	-----	.G.....A C
<i>C. virescens</i>	(AF043603)	T---.T...	...G..T..	-----	A---TA..-	CT-....-A C
<i>C. moniliformis</i>	(AF043597)	T---.A..	...GT..T..	-----	A..C-....-	---...G..GA C
<i>Petriella setifera</i>	(AF043596)	---G--G--	--CCCTG---	-----A.A..	---CAG.GG	CGGTCCC GCC	GCGGCGC NNN N

Figure 4: Phylogenetic tree based on sequence data from the ITS regions of the rRNA operon. The phylogram was obtained using the heuristic search option with random stepwise addition and tree bisection reconnection in PAUP. Branch lengths are indicated above the branches and bootstrap values below. *Petriella setifera* was used as the outgroup. *C. fimbriata* from Uruguay is placed within the larger clade typified by *C. fimbriata* (blue), closest to isolates from Brazil, Congo and Uganda. These isolates were all collected from diseased *Eucalyptus* species.

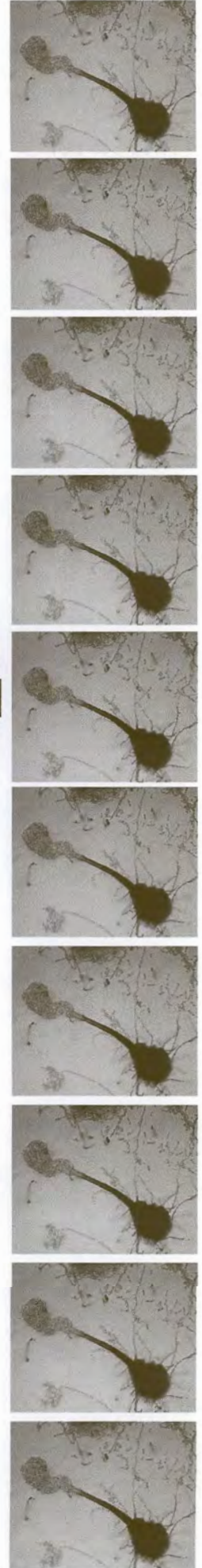
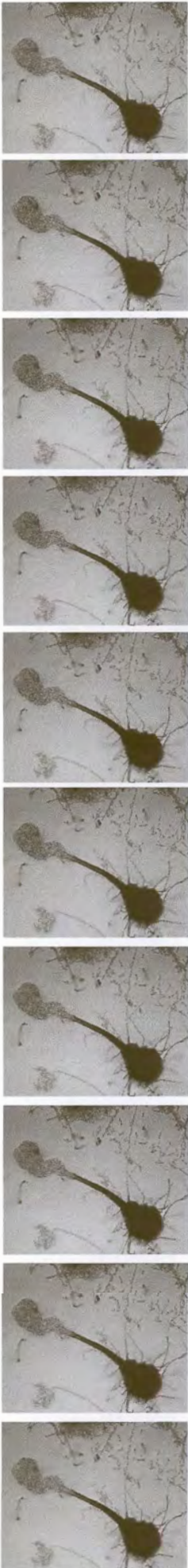




CHAPTER 6



*Ceratocystis
pirilliformis*, a new
species from
Eucalyptus nitens
in Australia



ABSTRACT

Only two species of *Ceratocystis* are known to occur on *Eucalyptus*. *Ceratocystis fimbriata* has recently been recognised as a serious pathogen in Uganda, Congo and Brazil, causing rapid wilting and death of these trees. *C. eucalypti* is considered native to Australia where it occurs in Tasmania and Victoria and appears not to be a pathogen. *Ceratocystis* species typically infect wounds on trees. This study was undertaken to determine whether *Ceratocystis* species, including *C. eucalypti*, would infect artificially induced wounds on *E. nitens* near Canberra (ACT) and *E. globulus* near Cann River (VIC), in Australia. Ten trees at each site were wounded in October 2000 and wounds were examined approximately one month later. Perithecia, characteristic of *Ceratocystis* species, were found covering the wounds and also in isolations using carrot baiting. The *Ceratocystis* species collected from *E. nitens* has hat-shaped ascospores similar to those of *C. fimbriata* and very different to those of *C. eucalypti* which was not collected during this baiting trial. The fungus collected, also differs from *C. fimbriata* and all other *Ceratocystis* spp. in its unique pear-shaped perithecial bases. DNA sequence data from the ITS and 5.8S rRNA operon confirmed that the fungus from *E. nitens* in Australia is unique. In this study, we describe it as a new species, *C. pirilliformis*.

INTRODUCTION

The genus *Ceratocystis* Ellis & Halst. was first described in 1891 (Halsted & Fairchild 1891), to provide a name for the causal agent of black rot of sweet potato (*Ipomoea batatas*). The genus was typified as having perithecia with globose bases and elongated necks giving rise to sticky drops of ascospores at their apices (Halsted & Fairchild 1891, Hunt 1956, Upadhyay 1981). For almost a Century subsequent to its description, the genus *Ceratocystis* had a taxonomically complex history. This was mainly because it was confused with *Ophiostoma* spp. that have ascocarps of similar morphology (Upadhyay 1993, Wingfield *et al.* 1993). In recent years, molecular evidence has clearly shown that *Ceratocystis* and *Ophiostoma* are phylogenetically distinct, with the former genus related to species in the Hypocreales and the latter the Diaporthales (Spatafora & Blackwell 1994, Wingfield *et al.* 1999).

Phylogenetic studies based on DNA sequence analysis have shown that certain morphological characteristics thought to separate *Ceratocystis* and *Ophiostoma* are taxonomically valuable (Hausner *et al.* 1993, Spatafora & Blackwell 1994). One of the most decisive characters defining *Ceratocystis* spp., is the presence of *Chalara* anamorphs with long tubular conidiophores producing chains of enteroblastic conidia (De Hoog 1974). In contrast, *Ophiostoma* spp. have anamorphs in *Graphium* Corda, *Leptographium* Lagerb. & Melin and *Sporothrix* Hektoen & Perkins, in which conidia are produced holoblastically (De Hoog 1974, Wingfield *et al.* 1993). Another taxonomically useful character used to separate species in these genera is the sensitivity of *Ceratocystis* spp. to cycloheximide and the ability of *Ophiostoma* spp. to tolerate high concentrations of this antibiotic (Harrington 1981, De Hoog & Scheffer 1984).

Ceratocystis spp. include important plant pathogens that differ in host range, pathogenicity, as well as morphological and biochemical characteristics (Kile 1993). The best known of these pathogens is *C. fimbriata* Ellis & Halst. that has a wide host range including root crops as well as fruit, ornamental and forest trees (Halsted & Fairchild 1891, Pontis 1951, Walter *et al.* 1952, Gremmen & De Kam 1977, Kile 1993, Panconesi 1999, Roux *et al.* 2001a). This species is thought to represent a complex of morphologically similar species (Webster &

Butler 1967, Barnes *et al.* 2001). Other important pathogens include *C. fagacearum* (Bretz.) Hunt., the causal agent of oak wilt in North America (Hunt 1956, Kile 1993), *C. albofundus* Wingfield, De Beer & Morris, that causes a serious disease on plantation grown black wattle (*A. mearnsii* de Wild.) in Africa (Morris *et al.* 1993, Roux *et al.* 2001b) and *C. paradoxa* (Dade) C. Moreau, that is the causal agent of stem bleeding disease of coconut in the tropics (Kile 1993).

Ceratocystis spp. are well known for their association with insects, and, in general, are vectored non-specifically by flies (Diptera) and beetles (Coleoptera: Nitidulidae) (Crone & Bachelder 1961, Moller & DeVay 1968b, Hinds 1972, Juzwik & French 1983). *Ceratocystis* spp. require wounds to infect their hosts (Kile 1993). Freshly cut wounds are attractive to sap feeding insects that carry the fungi to these substrates. Sticky spore droplets produced on the tips of the perithecia adhere to the bodies of the insects. These insects then frequent other wounded sites on trees and thus contribute to the dispersal of the fungus from diseased to healthy trees (Moller & DeVay 1968b, Upadhyay 1981). This insect-fungus relationship is facilitated by the fact that most *Ceratocystis* spp. produce fragrant aromatics that attract these insects (Kile 1993).

Kile *et al.* 1996, conducted a wounding trial on *Eucalyptus* spp. in Australia, which gave rise to a previously un-described species of *Ceratocystis*. This fungus, now known as *C. eucalypti* Z. Q. Yuan & Kile, appears to be native to Australia, where it was found in Tasmania and Victoria on *E. sieberi* L. Johnson and *E. globoidea* Blakely. *C. eucalypti* appears to be a non-pathogenic colonist of fresh wounds on *Eucalyptus* spp.

The only other *Ceratocystis* sp. known to infect *Eucalyptus* trees is *C. fimbriata*. It has been reported from Congo (Roux *et al.* 2001a), Brazil (Laia *et al.* 1999), Uganda (Roux *et al.* 2001b) and Uruguay (Barnes Chapter 5). *C. fimbriata* is considered to be a serious pathogen, especially in *Eucalyptus* plantations in Congo where it is causing rapid wilting and death of trees (Roux *et al.* 2001a, Roux *et al.* 2001b).

In an attempt to re-isolate *C. eucalypti* and to extend our knowledge of its host range and distribution, an artificial wounding trial was conducted on *E. nitens* in Canberra (ACT) and *E. globulus* near Cann River (VIC), in Australia. No *C. eucalypti* was isolated, but a previously undescribed species of *Ceratocystis* was abundant on the wounds. The aim of this investigation was to describe this new species.

MATERIALS AND METHODS

Fungal isolates

Artificial wounds were made on stems of *Eucalyptus nitens*, approximately 1.2 m above the ground near Canberra (ACT) and on *E. globulus* near Cann River (VIC), in Australia. A 10 cm wide brick-cutting chisel was used to remove blocks of bark approximately 10 x 10 cm from the stems to expose the cambium. Thereafter, a second wound approximately 2 cm deep was made at the center of the exposed cambium in order to expose the xylem. The wounds were made in early October 2000 on 10 trees at each site. Wounds were examined in early November 2000 and slices of wood were removed from their surfaces for laboratory examination.

Chips of wood, approximately 4 cm square and 5 mm thick, from all trees were incubated in Petri dishes containing moistened filter paper to induce the production of fungal structures. Duplicate isolations were made by wrapping pieces of wood tightly between two slices of surface disinfected carrot (~5 mm thick), which is an effective method of isolating *Ceratocystis* spp. from wood (Moller & DeVay 1968a). Perithecia, characteristic of *Ceratocystis* spp., were found covering the wood chips and the carrot baits collected from five trees at the Canberra site. This fungus was not found on samples from Cann River. Cultures were obtained by transferring masses of ascospores from the tips of perithecial necks onto 2 % malt extract agar (MEA, Biolab) and incubating them at 25 °C. All cultures are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Duplicate isolates have been deposited in the culture collection of the Centraalbureau voor Schimmelcultures, Delft, Netherlands.

Morphology

The growth rates of three isolates (CMW 6579, CMW 6670 and CMW 7569) of the *Ceratocystis* sp. were determined on 2 % MEA. Isolates were cultured for two weeks at 25 °C for growth rate studies and mycelial plugs were removed from the edges of actively growing cultures with a 4 mm cork borer. A single plug was placed at the centre of 60 mm Petri dishes containing 2 % MEA for each isolate. Four plates for each isolate were incubated at 15 °C, 20 °C, 25 °C and 30 °C respectively. Two diameter measurements at right angles to each other were made for each culture after 12 days of incubation. This resulted in eight measurements for each isolate at each temperature. Averages were computed for all growth measurements.

Morphological characteristics were described from pure cultures on 2 % MEA. Fungal structures were mounted in lactophenol blue. Fifty measurements for each taxonomically relevant structure were made and corresponding ranges, averages and standard deviations calculated. Colour descriptions were determined using the colour charts of Rayner (1970). Microscope observations were made using a Carl Zeiss microscope and photographic images were captured electronically with a Zeiss Axio Vision camera system.

DNA sequence comparisons

DNA extraction

Single ascospore drops were transferred from pure, sporulating cultures to 50 ml malt extract broth using sterile toothpicks. Cultures were incubated at 25 °C for two weeks to obtain thick mycelial mats. These were freeze-dried, crushed in liquid nitrogen and the DNA extracted according to the method described by Barnes *et al.* 2001.

PCR amplification

The two internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S gene of the ribosomal RNA operon were amplified using primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.* 1990). Polymerase chain reaction (PCR) mixtures consisted of 200 nM of each primer, 200 µM of each dNTP, Expand High Fidelity PCR System enzyme mix (1.75 U) (Roche Molecular Biochemicals), 1 x Expand HF buffer containing 1.5 mM MgCl₂ (supplied with the

enzyme) and 2 – 10 ng DNA. Reaction volumes were adjusted to 50 µl with Sabax water. The PCR programme was set at 96 °C for 2 min, followed by 10 cycles at 94 °C for 20 s, 55 °C for 48 s and 72 °C for 45 s. A further 25 cycles were included with a 5 s extension after each cycle with the annealing time altered to 40 s. A final step of 10 min at 72 °C completed the programme. PCR amplicons were purified using the Magic PCR Preps, Purification System (Promega, Madison, WI).

Sequencing

PCR amplicons were sequenced using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems), according to the manufacturers protocol. Primers ITS1 and ITS4 were used for the forward and reverse reactions. Sequence reactions were run on PAGE (polyacrylamide gel electrophoresis) using an ABI PRISM™ 377 Autosequencer (Perkin-Elmer Applied Biosystems). Sequence electropherograms were analysed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems).

The sequences obtained for the *Ceratocystis* sp. from *E. nitens* were compared to ITS sequences of known *Ceratocystis* spp. obtained from Genbank (Table 1). Sequences were manually aligned and analysed in PAUP version 4 (Phylogenetic Analysis Using Parsimony [*and other methods]) (Swofford 1998). All characters were given equal weight and the Mulpar option was in effect. The heuristic search based on parsimony with tree bisection reconnection was used to obtain the phylogram. Length distribution of 1000 trees and confidence intervals using 1000 bootstrap replicates were calculated. *Petriella setifera* (Schmidt) Curzi (accession number AF043596), a member of the Microascales, was used as the outgroup and treated as a paraphyletic sister group with respect to the ingroup. All sequences derived in this study have been deposited in Genbank (Table 1).

RESULTS

Morphology

Colonies were slow growing reaching a diameter of 22 mm in 12 days. Optimal temperature for growth was 25 °C. Although the cultures grew faster at 25 °C, a greater number of perithecia were produced at 20 °C. Cultures were white at first becoming darker with age. The submerged mycelium was olive green in colour (21''''b) with the aerial mycelium white to greyish (21''''d). Cultures had a fruity aroma, typical of *Ceratocystis* spp.

Perithecia, with black, pear-shaped bases, developed evenly across the cultures (Figure 2a). Ascospores were hat-shaped (Figure 2c). The anamorph state of the fungus was typical of *Chalara* spp. and two different morphological forms of conidia were observed. Conidia were either long and cylindrical or barrel-like in shape (Figure 2e, f). The fungus is very similar to *C. fimbriata*, but clearly differs with respect to the shape of the perithecial base. This difference suggested that this species might be distinct and, therefore, justified comparisons at the DNA level.

DNA sequence comparisons

Sequencing of the ITS and 5.8S region of the ribosomal rRNA operon of the *Ceratocystis* sp. from *E. nitens* in Australia, produced approximately 647 base pairs. A total of 574 characters were analysed after the manual alignment of the *Ceratocystis* sp. with other *Ceratocystis* spp. (Figure 3). Parsimony analysis indicated that 179 characters were constant, 85 were parsimony un-informative and 310 were parsimony informative. A single, most parsimonious tree (Figure 4), was produced with a length of 979. The CI, RI and g1 values were 0.705, 0.754 and -0.922 respectively.

The *Ceratocystis* spp. included in the analyses were separated into two distinct clades based on the aligned ITS and 5.8S sequences. The *Ceratocystis* sp. from *E. nitens*, grouped together within a single well defined clade including *C. fimbriata* and *C. albofundus*, separate from the other *Ceratocystis* spp. Although the *Ceratocystis* sp. from Australia was most closely related to *C. fimbriata* and *C. albofundus*, it was clearly separated from these two species, residing in

a well resolved sub-clade supported by a 100 % bootstrap value. Based on sequence data, this species clearly represents a unique taxon with *C. albofundus* as its closest relative.

The *Ceratocystis* from *E. nitens* in Australia is morphologically distinct from all other species of *Ceratocystis*. Sequence data also clearly define it as unique. The following description is, therefore, provided for it.

The species description presented here is for the purposes of this thesis only. A formal description of this species will be presented in the mycological literature at a later date.

Ceratocystis pirilliformis I. Barnes & M. J. Wingfield sp. nov.

Etym.: Shaped like a little pear; referring to the characteristic pear-shaped perithecia of this species.

Stat.conid.: *Chalara*

Coloniae tarde crescentiae, usque ad 22 mm diametro post 12 dies; effusae, mycelio aereo laete olivaceo-griseo, mycelio immerso griseo-olivaceo, pagina reversa coloniae groseo-olivacea. Perithecia nigra, basin versus globosa, 115.2 - 186.8 (-205.5) μm diametro, collo pyriformi, 35.8 - 76.5 (-86.8) x 23.9 - 42.4 (-75.2) μm , cum hyphis ornato. Colla peritheciarum erecta vel curvata, parietibus laevis vel crenulatis, basin versus 16.8 - 32.7 (-39.8) μm lata, apicem versus 11.9 - 21.0 (-25.3) μm lata; 372.4 - 683.0 (-777.5) μm longa, hyphis ostiolaribus inclusis. Ascosporae unicellulares, hyalinae, e supra visae ellipticae, 7.7 - 11.5 (-14.1) x 4.1 - 6.0 (-7.3) μm , vagina hyalina aspectu laterali petasiformi circumcincta, 2.9 - 5.2 (-6.5) x 2.0 - 3.8 (- 5.4) μm , margine 4.7 - 6.4 (-7.5) μm longa. Conidiophorae Chalariformes, erectae, ramosae vel non ramosae, hyalinae, multiseptatae, laeves, 62 - 147.2 (-216) μm longae, cellula terminali conidiogena inclusa. Cellulae conidiogena tubulares, determinatae, cylindricae vel lageniformes, 31.9 - 63.6 (-72.4) μm longae. Conidia enteroblastica phialidosa, formis duabus: I) doliiformia, basi truncata, parietibus laevis, non septata, mononemata, hyalina, 4.1 - 5.6 (-6.4) x 3.2 - 4.3 (-4.8) μm , solitarie vel in catenis portata, vel II) cylindrica, apicibus rotundatis, laevia, non septata, hyalina, 11.6 - 25.2 (-33.0) x 2.4 - 4.0 (-4.7) μm , in catenis portata.

Colonies slow growing with optimal growth at 25 °C on 2 % MEA, reaching 22 mm diameter in 12 days. No growth below 15 °C or above 30 °C. Colonies effuse, aerial mycelium 'pale olivaceous grey' (21''''d), submerged mycelium 'grey olivaceous' (21''''b) (Figure 1). Reverse side of colony 'grey olivaceous' (21''''b). Submerged mycelium darkens as perithecia develop, fine, radiating fibrils observed. Perithecial development in 8 days and maturing within 12 days. *Perithecia* produced superficially or partly embedded in agar, black (7''''k). Bases pyriform with globose part 115.2 - 186.8 (-205.5) µm in diameter and necks 35.8 - 76.5 (-86.8) x 23.9 - 42.4 (-75.2) µm, ornamented with hyphae (Figure 2a). *Perithecial necks* erect, occasionally curved, black at bases becoming sub-hyline towards apices, smooth to crenulate walled, necks taper slightly from 18.8 - 32.7 (-39.8) µm wide at base to 11.9 - 21 (-25.3) µm wide at apices, 372.4 - 683 (-777.5) µm long including ostiolar hyphae. *Ostiolar hyphae* extending from the outer layer of the neck cells, hyaline, strait or flexous, subulate, mostly convergent, non-septate (Figure 2b). *Asci* evanescent. *Ascospores* accumulating in gelatinous droplets at tips of perithecial necks, single celled, hyaline, elliptical in top view, 7.7 - 11.5 (-14.1) x 4.1 - 6 (-7.3) µm, surrounded by a hyaline gelatinous sheath appearing hat-shaped in side view, 2.9 - 5.2 (-6.5) x 2 - 3.8 (-5.4) µm, brim 4.7 - 6.4 (-7.5) µm long (Figure 2c). *Hyphae* hyaline to isabelline (17''i), smooth, septate, 2.6 - 5 (-5.7) µm diameter. *Conidiophores*, *Chalara*-like, erect, branched or unbranched, (Figure 2d), hyaline, multiseptate, smooth, 62 - 147.2 (-216) µm long including terminal conidiogenous cell. *Conidiogenous* cells tubular, determinate, cylindrical to lageniform, 31.9 - 63.6 (-77.4) µm long. *Conidia* enteroblastic phialidic of two types: I) Barrel-shaped, doliiform with truncate base, smooth walled, non septate, monomematous, hyaline, 4.1 - 5.6 (-6.4) x 3.2 - 4.3 (-4.8) µm, borne in chains (Figure 2e), II) Cylindrical with apices rounded, smooth, non-septate, hyaline, 11.6 - 25.2 (-33) x 2.4 - 4 (-4.7) µm, borne in chains (Figure 2f). *Chlamydospores* oval, thick walled, smooth, isabelline (17''i) to olivaceous (21''m), 7.8 - 12 (-13) x 5.4 - 8.3 (-9.5) µm, embedded in agar, formed singly or in short chains.

Holotype: PREM57323 (CMW 6579), isolated from wounds on *Eucalyptus nitens*, collected: M. J. Wingfield, Uriarra, near Canberra, Australia, November 2000. **Paratypes:** PREM57322 (CMW 6569), PREM57320 (CMW 6574), PREM57325 (CMW 6583), PREM57321 (CMW 6566), PREM57324 (CMW 6577) isolated from wounds of *Eucalyptus nitens*, collected: M. J. Wingfield, Uriarra, near Canberra, Australia, November 2000.

DISCUSSION

Ceratocystis pirilliformis represents a taxon that can easily be distinguished from all other species of *Ceratocystis*. Although morphologically most similar to *C. fimbriata* and *C. albofundus*, its distinctly pear shaped perithecial bases are unique. DNA sequence data, based on ITS rRNA, support the treatment of *C. pirilliformis* as a new species. This is only the third species of *Ceratocystis* known to occur on *Eucalyptus*.

Most morphological characteristics used to describe *Ceratocystis* spp. are highly variable and are not considered taxonomically valuable (Wingfield *et al.* 1996). Many of the measurements taken for specific structures of *C. pirilliformis* fall within the ranges observed for *C. fimbriata* and *C. albofundus*. *C. pirilliformis* is morphologically similar to *C. fimbriata* and *C. albofundus*, but there are some obvious differences between these three species. *C. albofundus* has light coloured perithecial bases, divergent ostiolar hyphae and lacks chlamydospores (Wingfield *et al.* 1996). *C. pirilliformis* and *C. fimbriata* are almost indistinguishable from each other. Both have dark perithecial bases, divergent ostiolar hyphae and chlamydospores. The only obvious difference between them is the shape of their perithecial bases. *C. fimbriata* has globular perithecial bases, whereas bases of *C. pirilliformis* are distinctly pear shaped.

Culture morphology of *C. fimbriata*, *C. albofundus* and *C. pirilliformis* also differs. Whereas the colony colour of *C. albofundus* is pale, almost creamy, *C. fimbriata*'s varies from greenish to brown. *C. pirilliformis* has aerial mycelium that is greyish and submerged mycelium that is green, sometimes turning brown in older cultures.

Ceratocystis pirilliformis and *C. eucalypti* are both found on *Eucalyptus* spp. in Australia. *C. eucalypti* is probably endemic to this area (Kile *et al.* 1996), and we suspect that the same is true of *C. pirilliformis*. They are, however, morphologically very different. The most obvious difference is the shape of the ascospores. *C. eucalypti* has elongated, sheathed ascospores whereas *C. pirilliformis* has hat-shaped ascospores. Only three other *Ceratocystis* spp. have hat-shaped ascospores. These are *C. moniliformis* (Hedge.) C. Moreau, *C. fimbriata* and *C.*

albofundus. *C. moniliformis*, however, differs from other species with hat-shaped ascospores in having short conical spines on the perithecial bases (Upadhyay 1981).

Phylogenetic analysis based on the ITS regions strongly support the morphological differences observed between the different *Ceratocystis* spp. in this study. Species within *Ceratocystis* can be divided into two major subgroups. The one well recognised sub-group in *Ceratocystis* is typified by *C. coerulescens* (Whitthuhn *et al.* 1999), and species which do not have hat shaped ascospores. *C. eucalypti* is most closely related to species in the *C. coerulescens* group, which is also very different to *C. pirilliformis*. This also confirms that ascospore shape is a reasonably strong indicator of phylogenetic relationships within *Ceratocystis*.

The second major subgroup in *Ceratocystis* is typified by *C. fimbriata*. It is not surprising, therefore, that due to its morphological similarities with *C. fimbriata*, *C. pirilliformis* resides in a strongly resolved clade, within this subgroup. *C. pirilliformis* is most closely related to *C. albofundus* which is presently known only from Africa (Morris *et al.* 1993, Roux *et al.* 2001b). The fact that it is morphologically more similar to *C. fimbriata*, supports the view that morphology does not necessarily reflect phylogenetic relatedness.

Eucalyptus nitens, also known as shining gum, occurs naturally in Australia where it is mainly restricted to the south-eastern parts (Poynton 1979). Thousands of hectares are increasingly being planted in New South Wales and especially in Tasmania (Beadle 1999). The tree is also widely planted in South Africa for pulp production and it is increasingly being used as a hybrid partner with other species in order to increase their cold tolerance. At the present time, we know nothing of the pathogenicity of *C. pirilliformis*, although it is certainly able to cause wood discolouration. Given the pathogenicity of many species in the *Ceratocystis* group, and the importance of *E. nitens*, it will be important to test this feature of *C. pirilliformis* in the future.

REFERENCES

- Beadle, C. (1999) Pruning a knotty problem for plantation timber. *CSIRO Media Release* 99/57.
- Barnes, I., Roux, J., Coetzee, M.P.A., Wingfield, B.D. & Wingfield, M.J. (2001) Characterisation of *Seiridium* spp. associated with Cypress Canker Based on β -tubulin and Histone Sequences. *Plant Disease* **85** : 317-321.
- Crone, L.J. & Bachelder, S. (1961) Insect transmission of canker stain fungus, *Ceratocystis fimbriata* F. *platani*. *Phytopathology* **51** : 576.
- De Hoog, G.S. (1974) The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium* and *Calcarisporiella* gen. nov. *Studies in Mycology* **7** : 1-84.
- De Hoog, G.S. & Scheffer, R.J. (1984) *Ceratocystis* Versus *Ophiostoma*: A Reappraisal. *Mycologia* **76** : 292-299.
- Gremmen, J. & De Kam, M. (1977) *Ceratocystis fimbriata*, a fungus associated with poplar canker in Poland. *European Journal of Forestry Pathology* **7** : 44-47.
- Halsted, B.D. & Fairchild, D.G. (1891) Sweet-potato black rot. *Journal of Mycology* **7** : 1-11.
- Harrington, T.C. (1981) Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* **73** : 1123-1129.
- Hausner, G., Reid, J. & Klassen, G.R. (1993) Grouping of isolates and species of *Ceratocystis sensu lato* on the basis of molecular and morphological characters. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield, M.J., Seifert, K.A. & Webber, J.A., eds): 93-104. APS Press, St. Paul, Minnesota.
- Hinds, T.E. (1972) *Ceratocystis* canker of Aspen. *Phytopathology* **62** : 213-220.
- Hunt, J. (1956) Taxonomy of the genus *Ceratocystis*. *Lloydia* **19** : 1-59.
- Juzwik, J. & French, D.W. (1983) *Ceratocystis fagacearum* and *C. piceae* on the surfaces of free-flying and fungus-mat-inhabiting nitidulids. *Phytopathology* **73** : 1164-1168.
- Kile, G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield, M.J., Seifert, K.A. & Webber, J.A., eds): 173-183. APS Press, St. Paul, Minnesota.

- Kile, G.A., Harrington, T.C., Yuan, Z.Q., Dudzinski, M.J. & Old, K.M. (1996) *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100** : 571-579.
- Laia, M.L., Alfenas, A.C. & Harrington, T.C. (1999). Isolation, detection in soil, and inoculation of *Ceratocystis fimbriata*, causal agent of wilting, die-back and canker in *Eucalyptus*. *Australasian Plant Pathology Society* 12th Biennial Conference Canberra.
- Moller, W.J. & DeVay, J.E. (1968a) Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. *Phytopathology* **58** : 123-126.
- Moller, W.J. & DeVay, J.E. (1968b) Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58** : 1499-1507.
- Morris, M.J., Wingfield, M.J. & De Beer, C. (1993) Gummosis and wilt of *Acacia mearnsii* in South Africa caused by *Ceratocystis fimbriata*. *Plant Pathology* **42** : 814-817.
- Panconesi, A. (1999) Canker stain of plane trees: A serious danger to urban plantings in Europe. *Journal of Plant Pathology* **81** : 3.
- Pontis, R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **41** : 179-184.
- Poynton, R.J. (1979) *Tree planting in Southern Africa. The Pines*. South African Forestry Research Institute. **1** : 576.
- Rayner, R.W. (1970) *A Mycological colour chart*. Commonwealth Mycological Institute and British Mycological Society, Kew, Surrey.
- Redfern, D.B., Stoakley, J.T., Steel, H. & Minter, M. (1987) Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36** : 467-480.
- Roux, J. & Wingfield, M.J. (2001b) First report of *Ceratocystis* wilt of *Acacia mearnsii* in Uganda. *Plant Disease* **85** : 1029.
- Roux, J., Coutinho, T.A., Mujuni Byabashaija, D. & Wingfield, M.J. (2001a) Diseases of plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97** : 16-18.
- Siemaszko, W. (1938) Zespolny Grybow Towarzyszacych Kornikom Polskim. *Planta Polonica* **7** : 1-52.
- Spatafora, J.W. & Blackwell, M. (1994) The polyphyletic origins of ophiostomatoid fungi. *Mycological Research* **98** : 1-9.

- Swofford, D.L. (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods). Vers 4. Sinaur Associates, Sunderland, Massachusetts.
- Upadhyay, H.P. (1981) *A monograph of Ceratocystis and Ceratocystiopsis*. University of Georgia Press, Athens.
- Upadhyay, H.P. (1993) Classification of the Ophiostomatoid Fungi. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield, M.J., Seifert, K.A. & Webber, J.A., eds): 7-14. APS Press, St. Paul, Minnesota.
- Walter, J.M., Rex, E.G. & Schreiber, R. (1952) The rate of progress and destructiveness of canker stain of plane trees. *Phytopathology* **42** : 236-239.
- Webster, R.K., & Butler, E.E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45** : 1457-1468.
- White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A sequencing Guide to Methods and Applications* (Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J., eds): 315-322. Academic Press, San Diego, USA.
- Wingfield, B.D., Viljoen, C.D. & Wingfield, M.J. (1999) Phylogenetic relationships of ophiostomatoid fungi associated with *Protea* infrutescences in South Africa. *Mycological Research* **103** : 1616-1620.
- Wingfield, M.J., De Beer, C., Visser, C. & Wingfield, B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematics and Applied Microbiology* **19** : 191-202.
- Wingfield, M.J., Harrington, T.C. & Solheim, H. (1997) Two new species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75** : 827-834.
- Wingfield, M.J., Seifert, K.A. & Webber, J.A. (1993) *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. APS Press, St. Paul, Minnesota.
- Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J. & Harrington, T.C. (1999) PCR-based identification and phylogeny of species of *Ceratocystis sensu stricto*. *Mycological Research* **103** : 743-749.

Table 1: Isolates of *Ceratocystis* spp. used in ITS sequence comparisons.

Isolate	Genbank no.*	Isolate no.#	Origin	Host
<i>C. adiposa</i>	AF275545	2575	Japan	<i>Prunus persica</i>
<i>C. albofunda</i>	AF264910	2148	South Africa	<i>Acacia mearnsii</i>
"	AF043605	2475	"	"
<i>C. coerulescens</i>	U75615	-	Germany	<i>Picea abies</i>
<i>C. eucalypti</i>	U75627	3254	Australia	<i>Eucalyptus sieberi</i>
<i>C. fagacearum</i>	AF043598	2651	USA	<i>Quercus</i> sp.
<i>C. fimbriata</i>	AF395679	2219	France	<i>Platanus</i> sp.
"	AF453438	7383	Uruguay	<i>Eucalyptus grandis</i>
<i>C. moniliformis</i>	AF043597	3782	South Africa	<i>Erythrina</i> sp.
<i>C. paradoxa</i>	U75630	1546	New Zealand	<i>Musa</i> sp. x <i>paradisiaca</i>
<i>C. radicicola</i>	AF043599	3191	USA	<i>Phoenix dactylifera</i>
<i>C. resinifera</i>	U75618	3263	Norway	<i>Picea abies</i>
<i>C. virescens</i>	AF043603	460	USA	<i>Quercus</i> sp.
" <i>C. pirilliformans</i>	AF427104	6569	Australia	<i>Eucalyptus nitens</i>
"	AF427106	6574	"	"
"	AF427105	6579	"	"
<i>Petriella setifera</i>	AF043596	-	Kenya	<i>Rock hyrax</i> dung

*ITS sequences can be retrieved from Genbank at <http://www.ncbi.nlm.nih.gov/Taxonomy/>.

** Isolates sequenced in this study.

All isolates, (CMW) are available from the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002.



Table 2: Comparison of morphological characteristics of *Ceratocystis pirilliformis* from Australia with published descriptions of *C. fimbriata* and *C. albofundus*.

Character	<i>C. fimbriata</i>	<i>C. albofundus</i>			<i>C. pirilliformis</i>
	Upadhyay 1981	Wingfield <i>et al.</i> 1996			
		PREM 51643	PREM 51641	PREM 51645	PREM 57323
Perithecial base width	121-255	88-200 (157)	104-200 (158)	160-368 (245)	115.2-205.5 (161.7)
Neck length	950-1200	480-736 (628)	208-840 (510)	440-1000 (685)	372.4-777.5 (577.1)
Neck width, base	18-40	24-40 (31)	20-32 (27)	32-64 (44)	18.8-39.8 (28.3)
Neck width, tip	10-18	16-20 (17)	16-24 (17)	16-32 (25)	11.9-25.3 (17.7)
Ascospore length	3.5-8	5-7 (5.8)	4-6.5 (5.2)	5-6 (5.6)	2.9-6.5 (4.5)
Ascospore width	2-2.5	3-5 (4)	3.5-5 (4.2)	3-5 (3.7)	2-5.4 (3.2)
Cylindrical conidia, length	9-21	9-23 (14.3)	8-24 (14.6)	7-20 (13.8)	11.6-33 (20.2)
Cylindrical conidia, width	2-6.5	3-5 (3.9)	3-4 (3.5)	3-4 (3.3)	2.4-4.7 (3.6)
Barrel conidia, length	9-17	5-13 (8)	6-10 (7)	6-12 (9)	4.1-6.4 (5.1)
Barrel conidia, width	5-13	4-9 (8)	4-8 (6)	4-10 (7)	3.2-4.8 (3.9)
Hyphal diameter	1.5-5	2-3	2-5	2.3-3.1	2.6-5.7 (4.2)
Perithecial colour	brown-dark brown/black	black	yellowish with black neck	yellowish with black neck	black
Colony colour	hyaline-subhyaline, greyish brown – dark brown	greenish brown	creamy – light brown	creamy – light brown	greyish – greenish
Hyphal colour	hyaline - pale brown	hyaline	hyaline	hyaline	hyaline - pale brown
Ostiolar hyphae arrangement	straight / flexuous	convergent	divergent	divergent	convergent
Odour	-	banana oil	fruit-like	fruit-like	fruit-like

For each isolate, the ranges of measurements are given in μm , followed by the mean value of 50 measurements in brackets. PREM numbers refer to specimens deposited in the mycological herbarium of the National Herbarium of South Africa, Department of Agricultural Technical Services, Pretoria.

Figure 1: Cultural characteristics of *Ceratocystis* species most closely related to *C. pirilliformis* a) *C. albofundus* from *Acacia mearnsii* (CMW 2475) in South Africa, has a clear, creamy cultural colour (21''f), b) *C. pirilliformis* (CMW 6579) has light grey aerial mycelium (21''''d), with greenish to brownish submerged mycelium (21''''b) and c) *C. fimbriata* from *Eucalyptus grandis* in Uruguay (CMW 7383), has a brownish cultural colour (21''K). All cultures were grown on 2 % MEA at 25 °C for approximately three weeks.



a) CMW 2475



b) CMW 6579



c) CMW 7383



Figure 2: Morphological characteristics of *Ceratocystis pirilliformis* (PREM 57323). a) Pear-shaped perithecia with hyphal ornamentation on the perithecial base; b) Ostiolar hyphae with ascospores emerging through the opening of the perithecial neck; c) Hat-shaped ascospores in side and top view; d) Conidiophores with cylindrical conidia being released from the conidiogenous cells; e) Barrel-shaped conidia; f) Cylindrical conidia; g) Chlamyospore (all scale bars = 10 μm).

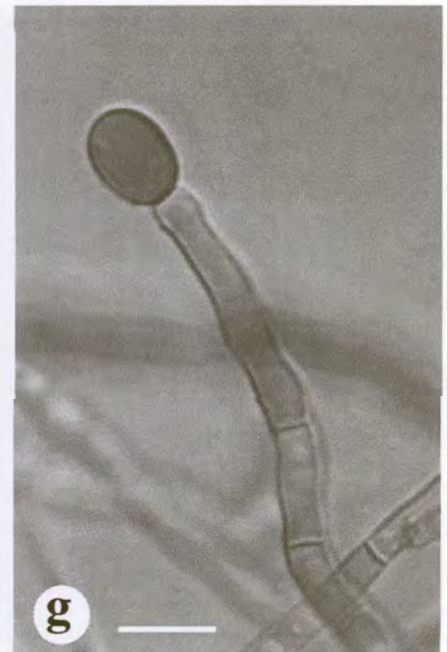
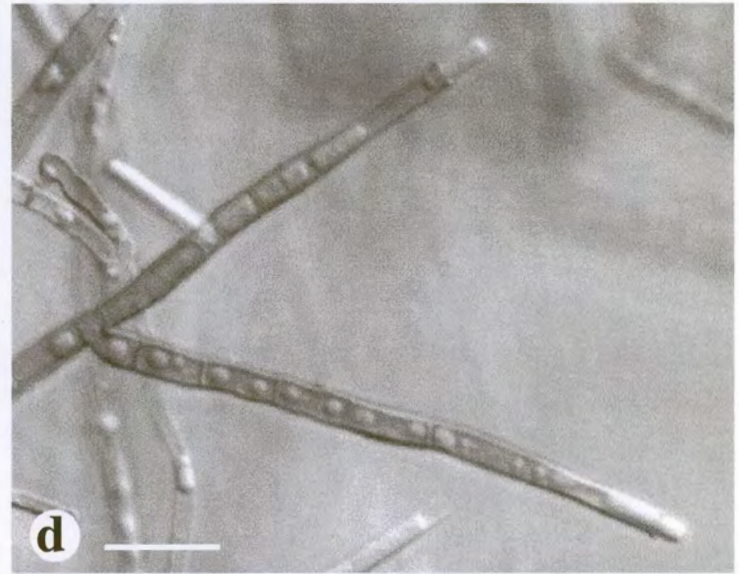
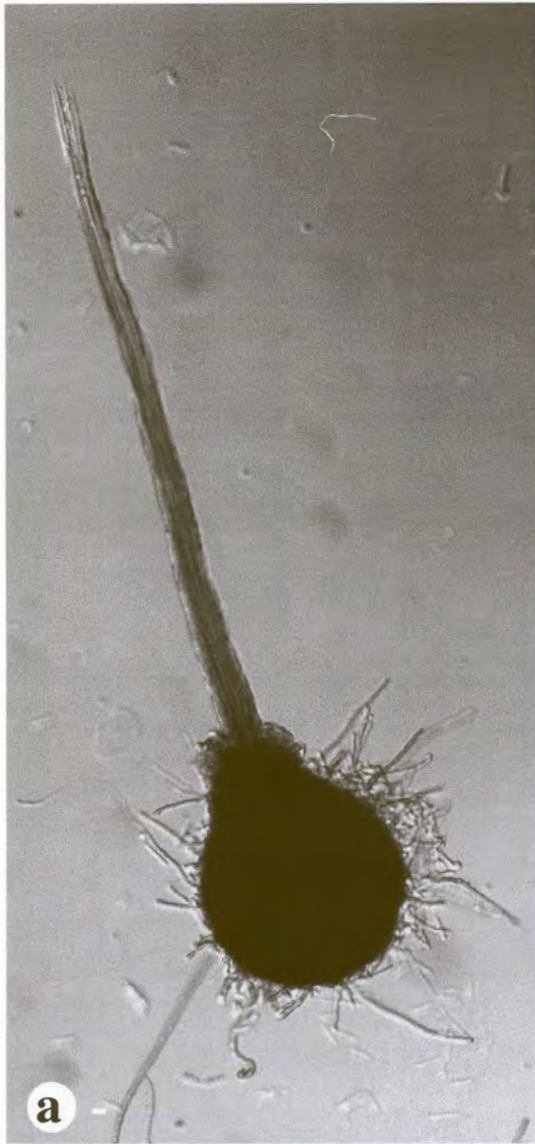


Figure 3: Sequence alignment (574bp) of the ITS region of *Ceratocystis pirilliformis* and other *Ceratocystis* species. Dots represent bases identical to *C. adiposa* (top line), N indicates unknown bases and dashes represent gaps introduced to maximise alignment.

		10	20	30	40	50	60	70
<i>C. adiposa</i>	(AF275545)	CCATTTGTGA	ACAT-ACCTA	TCTTTA----	-----	-----	CTGC-TTTGG	CGTGGT-CCT
<i>C. coerulescens</i>	(U75615)A.....-...T	.T.----AG-	-----	-----AG.----
<i>C. eucalypti</i>	(U75627)A.....-...T	CT-----AG-	-----	-----AG.----
<i>C. fagacearum</i>	(AF043598)G.	.T...TTTTC	TC-----	-----TAATAAG.--GA..
<i>C. moniliformis</i>	(AF043597)TT.-C.AC.	AACA.CGAA-	-----	-----GA.....	..G.T----
<i>C. paradoxa</i>	(U75630)T.-...T	CT-----GG-	-----	-----AG.T----
<i>C. ra dicicola</i>	(AF043599)T.-...T	.T.----AG-	-----	-----AG-T.A...
<i>C. resinifera</i>	(U75618)A.....-...T	CT-----AG-	-----	-----AG.----
<i>C. virescens</i>	(AF043603)A.....-...T	.T-----AG-	-----	-----AG.----
<i>C. albobundus</i>	(AF264910)	G.TGCCCT..G	TGGG----GGTAGT	GGTGTTAAC-	-----	..CTT...AT	-----A
<i>C. albobundus</i>	(AF043605)	G.TGCCCT..G	TGGG----GGTAGT	GGTGTTAAC-	-----	..CTT...AT	-----A
<i>C. fimbriata</i>	(AF395679)G.....	..G.-.....-GTAGT	GA-GATGAAT	-----G	..T-.....	T.G-----.
<i>C. fimbriata</i>	(AF453438)G.....	..G.AC....-GTAGT	GAGATGA--	-----ATG	..T-.....	T.G-----.
<i>C. pirilliformis</i>	(AF427104)G.T.....-GTACT	GA-GATGAAT	AAACAATATGTAG-----.
<i>C. pirilliformis</i>	(AF427105)G.T.....-GTACT	GA-GATGAAT	AAACAATATGTAG-----.
<i>C. pirilliformis</i>	(AF427106)G.T.....-GTACT	GA-GATGAAT	AAACAATATGTAG-----.
<i>Petriella setifera</i>	(AF043596)	..C.....	..C.T....-GTTA-	-----TGTTG	--CC-T--CG	-GCGGGG.T-	--A.-----

		80	90	100	110	120	130	140
<i>C. adiposa</i>	(AF275545)	TGGGGATTG-	-----	-----	-----	-----	T	GCCGGTAGTA
<i>C. coerulescens</i>	(U75615)	.G.TTTGAAA	AAAACAAGT-	-----	-----	-----	C.C....
<i>C. eucalypti</i>	(U75627)	.G.TA.CA--	CAAGTCT--	-----	-----	-----	-----
<i>C. fagacearum</i>	(AF043598)	..TTTC..CA	GGGGATGTTT	-----	-----	-----	C.	...A.....
<i>C. moniliformis</i>	(AF043597)	C.CC.CCC.G	CA-GT-----	-----	-----	-----	AC.	CTT-----
<i>C. paradoxa</i>	(U75630)	-----	-----	-----	-----	-----C.
<i>C. radicola</i>	(AF043599)GAA--	-----	-----	-----	-----	CC.	...A.C....
<i>C. resinifera</i>	(U75618)	.G.TA.AA--	CAA-----	-----	GTCTG-----	-----	-----C.
<i>C. virescens</i>	(AF043603)	.G.TA.CA--	CAA-----	-----	GTCTG-----	-----	-----
<i>C. albobundus</i>	(AF264910)	AG...GCA.C	CCACTACCGC	TAGCCACCAG	CAGCATACAA	GTCTTTTACC	ACTATAAACC	TT.T...-..
<i>C. albobundus</i>	(AF043605)	AG...GCA.C	CCACTACCGC	TAGCCACCAG	CAGCATACAA	GTCTTTTACC	ACTATAAACC	TT.T...-..
<i>C. fimbriata</i>	(AF395679)	-----	--AGGG----	CCCTTCT--G	AAGGG-----	CA---CCGCT	GCCAGCAGTA	TA-----
<i>C. fimbriata</i>	(AF453438)	-----	--AGGG----	CCCTTCT--G	AAGAGAGGG-	CA---CCGCT	GCCAGCAGTA	TTA-----
<i>C. pirilliformis</i>	(AF427104)	.G...GGG.G	G-AGAGCTCC	GCCTTGTGTG	AAG--TTTTT	TTCTTTTCCA	CTACCAGCAG	TATAAA....
<i>C. pirilliformis</i>	(AF427105)	.G...GGG.G	--AGAGCTCC	GCCTTGTGTG	AAG--TTTTT	TTCTTTTCCA	CTACCAGCAG	TATAAA....
<i>C. pirilliformis</i>	(AF427106)	.G...GGG.G	--AGAGCTCC	GCCTTGTGTG	AAG--TTTTT	TTCTTTTCCA	CTACCAGCAG	TATAAA....
<i>Petriella setifera</i>	(AF043596)	-.CCCC----	--AAA-----	GC-----	-----TTCTC	CCGCCGG--	----CAGCA-	-----

		150	160	170	180	190	200	210
<i>C. adiposa</i>	(AF275545)	TTTAC-----	-----AAACTCTT	TT-----A-A	TTT---CTAG	AGAATT--AT	T--CATTGCT	
<i>C. coerulescens</i>	(U75615)	.A.-----	-----AAA	AA.C.....	-----A.T.	-----T-	.TT.....	
<i>C. eucalypti</i>	(U75627)	-----	..T-----	-----	-----	-----	
<i>C. fagacearum</i>	(AF043598)	-----	..T-----	-----	-----	-----	
<i>C. moniliformis</i>	(AF043597)	-----	-----G....G.	..TT--.T.	-----A	-----T-	-----	
<i>C. paradoxa</i>	(U75630)	CAA.....	-----	-----T.	-----	-----	-----	
<i>C. radiculicola</i>	(AF043599)	.AA.-----	-----C.....	..T-----	-----	-----T-	-----	
<i>C. resinifera</i>	(U75618)	-----AA.....	-----T.	-----	-----	-----	
<i>C. virescens</i>	(AF043603)	..T-----	-----A.....	..TTTT--	-----A	-----	-----	
<i>C. albofundus</i>	(AF264910)	..TTT----	-----A.....	..--AA.A.	-----	-----	-----	
<i>C. albofundus</i>	(AF043605)	..TTT----	-----A.....	..--AA.A.	-----	-----	-----	
<i>C. fimbriata</i>	(AF395679)	-G.CT-C---	GCCACTGTAA	A-----	-----T.	..TT-.C..	TTT..TC..	--GC----
<i>C. fimbriata</i>	(AF453438)	-G.CTTC---	GCCACTGTAA	A-----	..T----.TT	A..TT-....	TTT..-C..	--GC----
<i>C. pirilliformis</i>	(AF427104)	G..TTCT-CT	TTACCACTAA	AA.....	-----A.T.	..TTA-...	.A.T.-G..	.T-.....
<i>C. pirilliformis</i>	(AF427105)	G..TTCT-CT	TTACCACTAA	AA.....	-----A.T.	..TTA-...	.A.T.-G..	.T-.....
<i>C. pirilliformis</i>	(AF427106)	G..TTCT-CT	TTACCACTAA	AA.....	-----A.T.	..TTA-...	.A.T.-G..	.T-.....
<i>Petriella setifera</i>	(AF043596)	-----CT--	-----AA.TT--	-----A.	..TA-....	C.G.....	---AC....	

		220	230	240	250	260	270	280
<i>C. adiposa</i>	(AF275545)	GAGTTG--CA	TTTAACA-AA	-TA-----	-G--TTAAAA	CTTTCAACAA	CGGATCTCTT	GGCTCTAGCA
<i>C. coerulescens</i>	(U75615)G.---T.---	..A-----	-----	-----	-----	-----
<i>C. eucalypti</i>	(U75627)G.---T.---	..A-----	-----	-----	-----	-----
<i>C. fagacearum</i>	(AF043598)A.---	-----	-----	-----	-----	-----
<i>C. moniliformis</i>	(AF043597)A.---T-.T.	A.-----	-.TA.....	-----	-----	-----
<i>C. paradoxa</i>	(U75630)G.----T.	A..A-----	-----	-----	-----	-----
<i>C. radiculicola</i>	(AF043599)G.----T.	A..A-----	-----	-----	-----	-----
<i>C. resinifera</i>	(U75618)G.---T.---	..A-----	-----	-----	-----	-----
<i>C. virescens</i>	(AF043603)G.---T.---	..A-----	-----	-----	-----	-----
<i>C. albofundus</i>	(AF264910)G.----T.---	..AAAAAA-	-----	-----	-----	-----
<i>C. albofundus</i>	(AF043605)G.----T.---	..AAAAAA-	-----	-----	-----	-----
<i>C. fimbriata</i>	(AF395679)G.----T.---	..AAAAAA-	-----	-----	-----	-----
<i>C. fimbriata</i>	(AF453438)G.----T.---	..AAAAAA-	-----	-----	-----	-----
<i>C. pirilliformis</i>	(AF427104)GAAG..	..-A..-T.	..AA-----	-----	-----	-----	-----
<i>C. pirilliformis</i>	(AF427105)GAAG..	..-A..-T.	..AA-----	-----	-----	-----	-----
<i>C. pirilliformis</i>	(AF427106)GAAG..	..-A..-T.	..AA-----	-----	-----	-----	-----
<i>Petriella setifera</i>	(AF043596)	..A.-A..	A.---	---AAAC---	-AAA.A....	-----	-----	..T..G..

		290	300	310	320	330	340	350
<i>C. adiposa</i>	(AF275545)	TCGATGAAGA	ACGCAGCGAA	ATGCGATAAG	TAATGTGAAT	TGCAGAATTC	AGTGAATCAT	CGAATCTTTG
<i>C. coerulescens</i>	(U75615)C.
<i>C. eucalypti</i>	(U75627)C.
<i>C. fagacearum</i>	(AF043598)
<i>C. moniliformis</i>	(AF043597)
<i>C. paradoxa</i>	(U75630)C.C.
<i>C. radiculicola</i>	(AF043599)C.C.
<i>C. resinifera</i>	(U75618)C.
<i>C. virescens</i>	(AF043603)C.
<i>C. albobundus</i>	(AF264910)
<i>C. albobundus</i>	(AF043605)
<i>C. fimbriata</i>	(AF395679)
<i>C. fimbriata</i>	(AF453438)
<i>C. pirilliformis</i>	(AF427104)
<i>C. pirilliformis</i>	(AF427105)
<i>C. pirilliformis</i>	(AF427106)
<i>Petriella setifera</i>	(AF043596)

		360	370	380	390	400	410	420
<i>C. adiposa</i>	(AF275545)	AACGCACATT	GCGCCTAGCA	GTATTCTGCT	AGGCATGCCT	GTCCGAGCGT	CATTTCACCA	CTCAAGCTTC
<i>C. coerulescens</i>	(U75615)	GC...G...CCT
<i>C. eucalypti</i>	(U75627)G...CC.
<i>C. fagacearum</i>	(AF043597)C.T
<i>C. moniliformis</i>	(CMW3782)C...	...C...	G.....CT
<i>C. paradoxa</i>	(U75630)	A...G...	...C...	...T...CT
<i>C. radiculicola</i>	(AF043599)G...	...C...CT
<i>C. resinifera</i>	(U75618)G...	...C...CT
<i>C. virescens</i>	(AF043603)G...	...C...C.
<i>C. albobundus</i>	(AF264910)G...	...C...AC.T
<i>C. albobundus</i>	(AF043605)C...G.T.	...C...AC.T
<i>C. fimbriata</i>	(AF395679)G...	...C...GACT
<i>C. fimbriata</i>	(AF453438)G...	...C...AC-T
<i>C. pirilliformis</i>	(AF427104)G...	...C...CAACT
<i>C. pirilliformis</i>	(AF427105)G...	...C...CAACT
<i>C. pirilliformis</i>	(AF427106)G...	...C...CAACT
<i>Petriella setifera</i>	(AF043596)CG...	...A...C	G.....C	TCG.GC...AA

		430	440	450	460	470	480	490
<i>C. adiposa</i>	(AF275545)	GCTT-----	---GGTGTG	GAGGACCCGC	TG-----	-TCAA--GCG	GGCCG---CC	GAAATGCATC
<i>C. coerulescens</i>	(U75615)	GTCTTTTTTG	T.T-----
<i>C. eucalypti</i>	(U75627)T...	ATCTTA----	-.G.T-----
<i>C. fagacearum</i>	(AF043598)	A-CTTGTC--	-A...-----	.C.A-----
<i>C. moniliformis</i>	(AF043597)-GTT	-----	...AG..T.T	GCTAT-----	-----GC...-TCT--
<i>C. paradoxa</i>	(U75630)C....	--GTTT-----	-----A...
<i>C. radiculicola</i>	(AF043599)	GTTTTCTTTT	-----A...
<i>C. resinifera</i>	(U75618)	ATCTT-----	-----A---
<i>C. virescens</i>	(AF043603)T.T	.TTT-----	-----AA---
<i>C. albofundus</i>	(AF264910)T-AGTT	-TT.....T..T.T	.CTTACCCTT	CCG...--A
<i>C. albofundus</i>	(AF043605)T-AGTT	-TT.....T..T.T	.CTTACCCTT	C.G...--A
<i>C. fimbriata</i>	(AF395679)	C...T--GTT	CTT..C....T..T.T	.CT--CCCC-	-.G...--AT...
<i>C. fimbriata</i>	(AF453438)	CT..T--GTT	CTT..C....T..T.T	.CT--CCCC-	-.G...--AT...
<i>C. pirilliformis</i>	(AF427104)	AT..TTT---	CTT.....T..T.T	.CTT-C----	-.G...--AT...
<i>C. pirilliformis</i>	(AF427105)	AT..TTT---	CTT.....T..T.T	.CTT-C----	-.G...--AT...
<i>C. pirilliformis</i>	(AF427106)	AT..TTT---	CTT.....T..T.T	.CTT-C----	-.G...--AT...
<i>Petriella setifera</i>	(AF043596)	.T..TTTAAA	CT-----	A.GATCGG--	-.GTTGG.GC	.CTACAG--.	.GTTCTTCGG

		500	510	520	530	540	550	560
<i>C. adiposa</i>	(AF275545)	GGCTGTAGT-	---ATTTGC-	-AGCTTCCCT	GCGTAG--TA	AAAC-----	-----TTT-	-GTG-TT---
<i>C. coerulescens</i>	(U75615)TA.-	-----T.....	-----	-----A.T	T---A..TT-
<i>C. eucalypti</i>	(U75627)TA.-	---C....T.....	-----	-----A.T	T---A..T--
<i>C. fagacearum</i>	(AF043598)	-----	-----T
<i>C. moniliformis</i>	(AF043597)GT.-	---CA...	---T....	.T.....	-----	-----	-----A..GTT
<i>C. paradoxa</i>	(U75630)TA.-	---C....	-----	-----T
<i>C. radiculicola</i>	(AF043599)TA.-	---C....	-----	-----C.T
<i>C. resinifera</i>	(U75618)TA.-	---C....T.....	-----	-----A.T
<i>C. virescens</i>	(AF043603)TA.-	---C....T.....	-----	-----A.-
<i>C. albofundus</i>	(AF264910)TA.T	TTT.C...C	-A..C...	.T...TA--	---AAGA--	---TTT...	---AAA..TTT
<i>C. albofundus</i>	(AF043605)TA.T	TTT.C...C	-A..C...	.T...TA--	---AAGA--	---TTT...	---AAA..TTT
<i>C. fimbriata</i>	(AF395679)TA.-	---C...C	-A..C...	.T...TA..	-----	-----	---C.AA..TTT
<i>C. fimbriata</i>	(AF453438)TA.-	---C...C	-A..C...	.T...TA..	-----	-----	---C.AA..TTT
<i>C. pirilliformis</i>	(AF427104)TAA-	---C...C	-A..C...	.T...TA..	..GGAATAAT	TTTTTT...	---CCAA..TTT
<i>C. pirilliformis</i>	(AF427105)TAA-	---C...C	-A..C...	.T...TA..	..GGAATAAT	TTTTTT...	---CCAA..TTT
<i>C. pirilliformis</i>	(AF427106)TAA-	---C...C	-A..C...	.T...TA..	..GGAATAAT	TTTTTT...	---CCAA..TTT
<i>Petriella setifera</i>	(AF043596)	A..A.CT..A	-----	G---C..TG	---A.ATA--	-----	-----CAG	T.GCGG.CCC

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<i>C. adiposa</i>	(AF275545)	--ACGCTTTG AACC
<i>C. coerulescens</i>	(U75615)	--.....A.
<i>C. eucalypti</i>	(U75627)	--.....A.
<i>C. fagacearum</i>	(AF043598)	--.....C..A.
<i>C. moniliformis</i>	(AF043597)	--G.A.....A.
<i>C. paradoxa</i>	(U75630)	--.....A.
<i>C. radicicola</i>	(AF043599)	--.....A.
<i>C. resinifera</i>	(U75618)	--.....A.
<i>C. virescens</i>	(AF043603)	--..A.....A.
<i>C. albofundus</i>	(AF264910)	--.....G.GT
<i>C. albofundus</i>	(AF043605)	--.....G.GT
<i>C. fimbriata</i>	(AF395679)	--..A.....GT
<i>C. fimbriata</i>	(AF453438)	--..A.....GT
<i>C. pirilliformis</i>	(AF427104)	T-..AT.....GT
<i>C. pirilliformis</i>	(AF427105)	T-..AT.....GT
<i>C. pirilliformis</i>	(AF427106)	T-..AT.....GT
<i>Petriella setifera</i>	(AF043596)	GCCGCGGCGC NNNN

Figure 4: Phylogram obtained with sequence alignments of the ITS 1, 5.8s and the ITS 2 region of the rRNA operon using PAUP. Relationships between different *Ceratocystis* spp. are presented with special emphasis showing the unique clade of *Ceratocystis pirilliformis* from Australia. *Petriella setifera* was used as the outgroup. Tree lengths are indicated above the branches and bootstrap confidence values are indicated below the branches.

