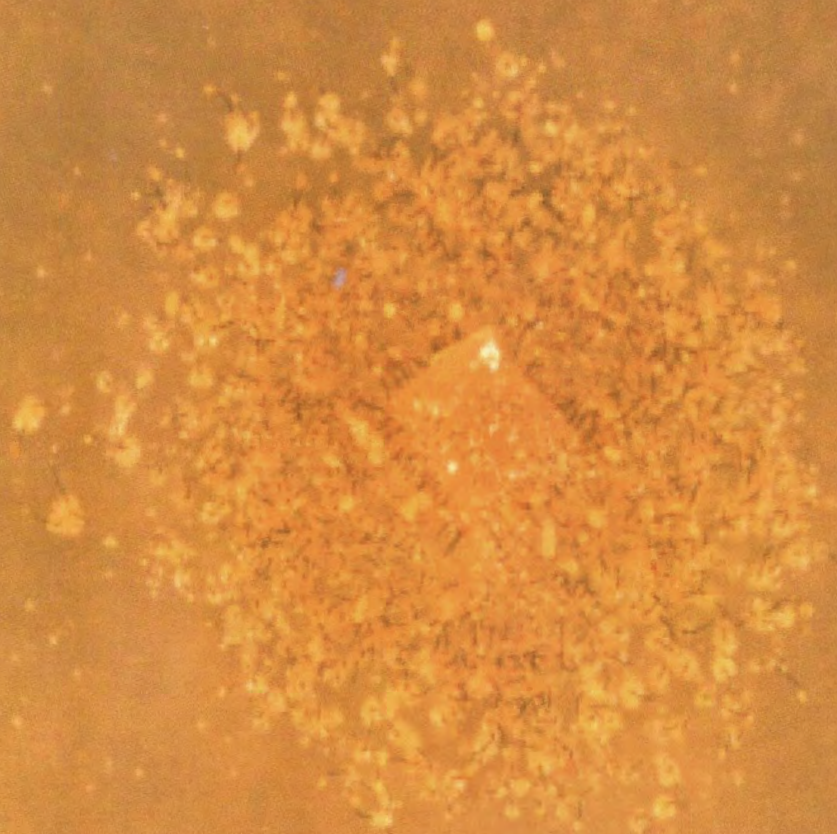
CHAPTER 4



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

ABSTRACT

Ceratocystis albofundus 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. albofundus*. This, and the association of the pathogen with native South African *Protea* species, has led to the hypothesis that *C. albofundus* 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. albofundus*. 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. albofundus*, 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. albofundus* 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. albofundus*. Our results add further support to the view that *C. albofundus* 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. albofundus* was also considered.

MATERIALS AND METHODS

Fungal isolates and DNA extraction

All 40 isolates of *C. albofundus* 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. albofundus* 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 $MgCl_2$ (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 µl 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. albofundus*. 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 µl, the concentration of each product was 1.5 ng. Each sample fractionated by PAGE (Polyacrylamide Gel Electrophoresis) (4.25 %) on an ABI Prism 377™ DNA sequencer, consisted of 0.5 µl DNA from one of the tubes containing the combined PCR products, 0.5 µl internal standard (GENESCAN –TAMRA, Perkin-Elmer Corp) and 1.5 µl 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 dendogram 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, $\hat{G} = 1/\sum[fx\ (x/n)^2]$, 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 dendogram 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. albofundus*. 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. albofundus* population considered in thus 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. albofundus* 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. albofundus* 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 Vryhied 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. albofundus*. This is perhaps not surprising as *C. albofundus*, 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. albofundus* 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. albofundus* with data previously published (Harrington *et al.* 1998), for three other native *Ceratocystis* spp. The nuclear and mitochondrial gene diversity for *C. albofundus* 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. albofundus* previously considered by Roux *et al.* (2001). This adds further support to the view that *C. albofundus* 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 CAT_5 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 albofundus 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. albofundus* 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. albofundus*, 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 mearnsii* 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 albofundus* 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.

			A7/8									A15/16		A17/18		CF 5/6		C15/16.2	C17/18			C21/22			C23/24	
Isolate	Microsatellite profile	Genotype	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		1				1
CMW4061	91111212	2									1	1		1		1		1		1		1				1
CMW4062	21111211	3		1								1		1		1		1		1		1			1	
CMW4063	21111212	1		1								1		1		1		1		1		1				1
CMW4064	21111212	1		1								1		1		1		1		1		1				1
CMW4065	31111312	4			1							1		1		1		1			1	1				1
CMW4066	61111312	5						1				1		1		1		1			1	1				1
CMW4068	21111232	6		1								1		1		1		1		1				1		1
CMW4109	21111222	18		1								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				1
CMW4080	61111212	8						1				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			1
CMW4088	61111212	8						1				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			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			1
CMW4069	21111212	1		1								1		1		1		1		1		1				1
CMW4070	22111212	19		1									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		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.

		RSA		Bloemendal		Dalton		Vryheid	
Locus	Allele	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 groups[1]	23	13	9	20
Isolates	40	8	13	11
Gene diversity	0.432	0.364	0.459	0.491
P-values[2]	< 0.001	=0.004	=0.001	=0.076
Observed value of I_A	**0.992**	**1.175**	**0.907**	**0.367**
Min randomized value[3]	-0.246	-0.724	-0.336	-0.510
Max randomized value[3]	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).

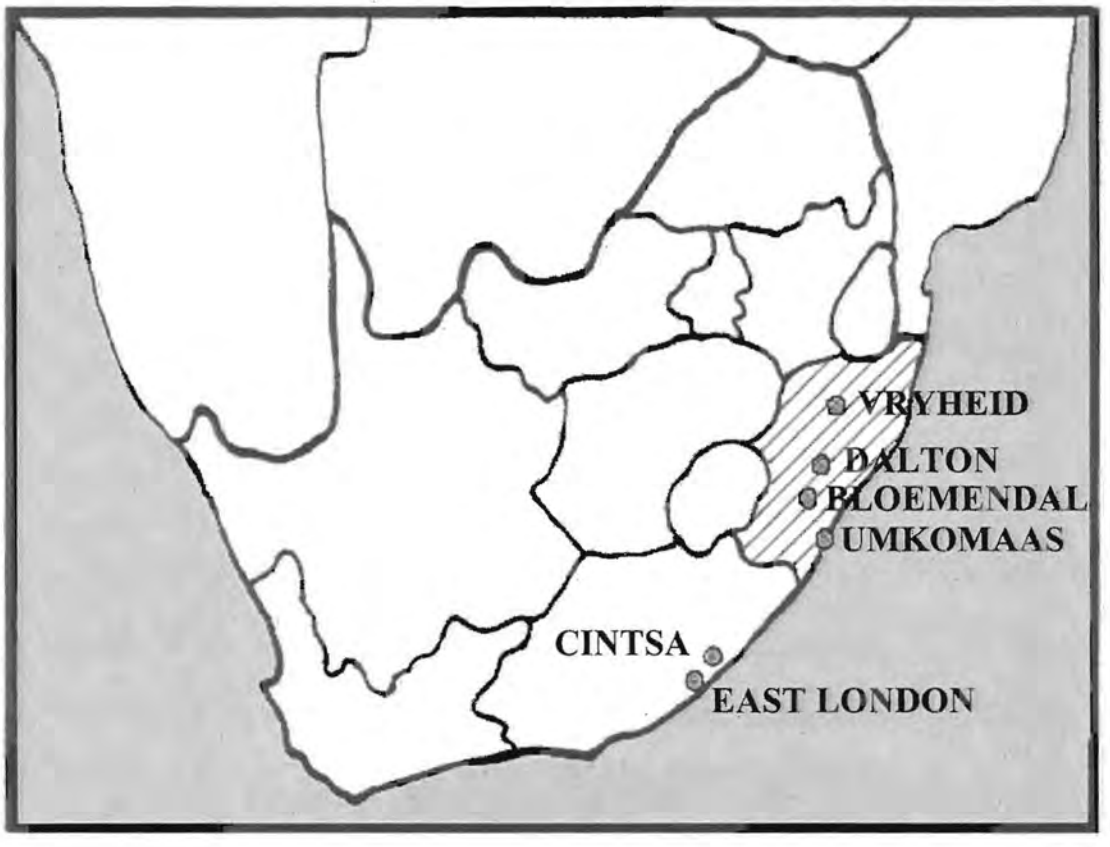

VRYHEID
DALTON
BLOEMENDAL
UMKOMAAS
CINTSA
EAST LONDON

Figure 2: Neighbour-joining dendogram showing genetic identities among 40 isolates from a South African population of *Ceratocystis albofundus*. 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.

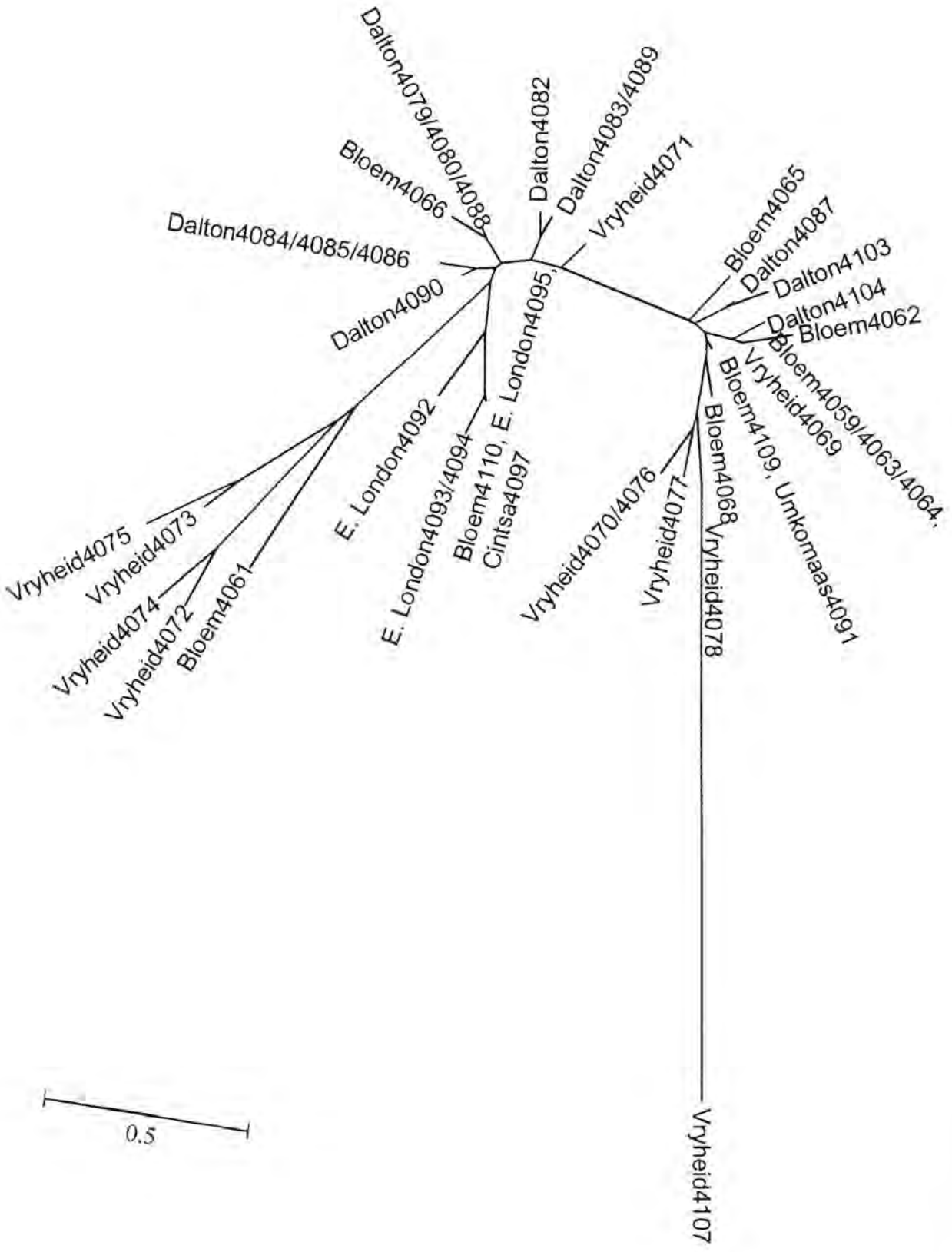
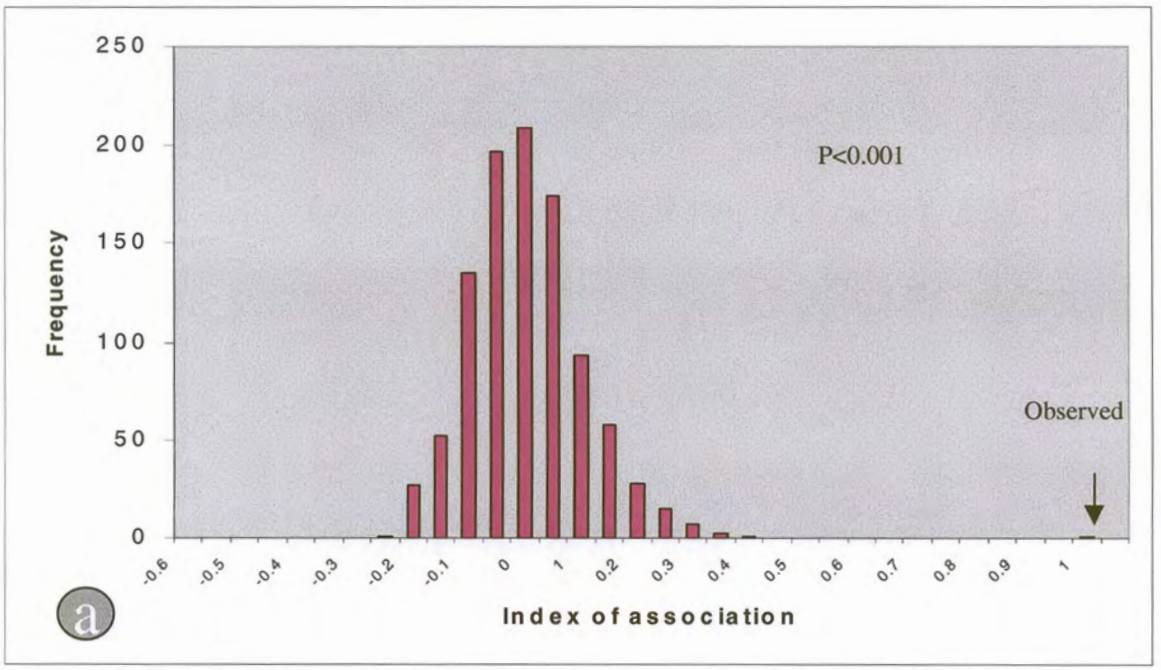

Dalton4079/4080/4088
Dalton4082
Dalton4083/4089
Bloem4066
Vryheid4071
Dalton4084/4085/4086
Bloem4065
Dalton4087
Dalton4103
Dalton4104
Bloem4062
Dalton4090
E. London4095
Vryheid4069
Bloem4059/4063/4064
Bloem4109, Umkomaas4091
E. London4092
E. London4093/4094
Bloem4110, E. London4097
Cintsa4097
Vryheid4070/4076
Vryheid4077
Bloem4068
Vryheid4078
Vryheid4075
Vryheid4073
Vryheid4074
Vryheid4072
Bloem4061
Vryheid4107
0.5

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. albofundus* population.


250
200
150
100
50
0
Frequency
P<0.001
Observed
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0
1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Index of association
a

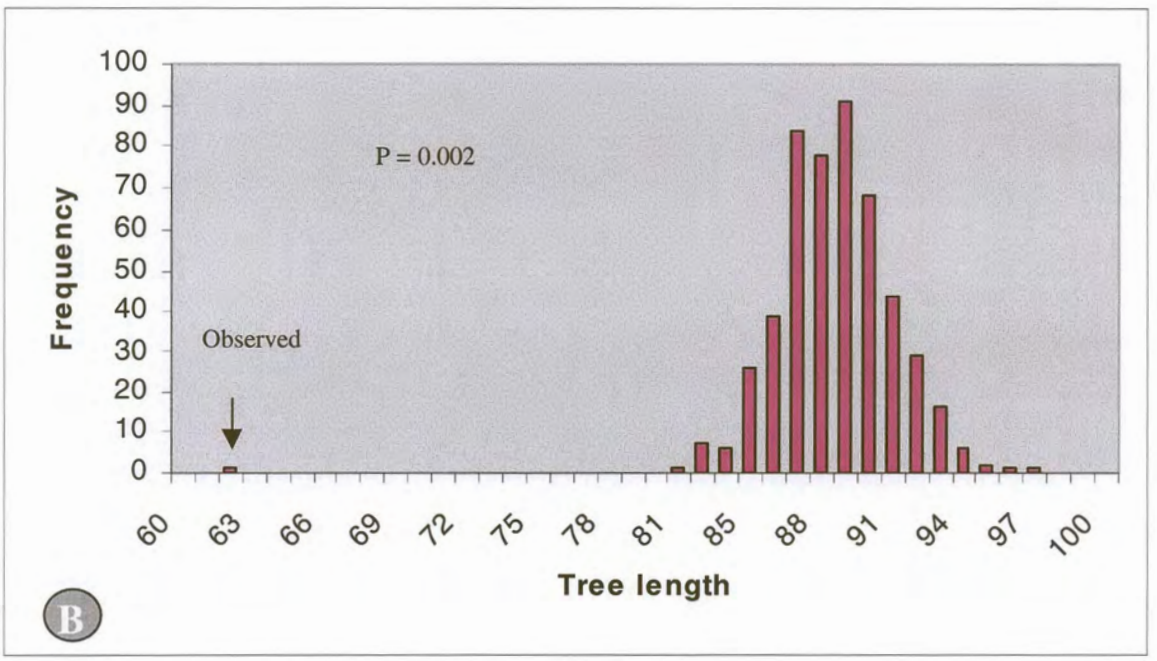
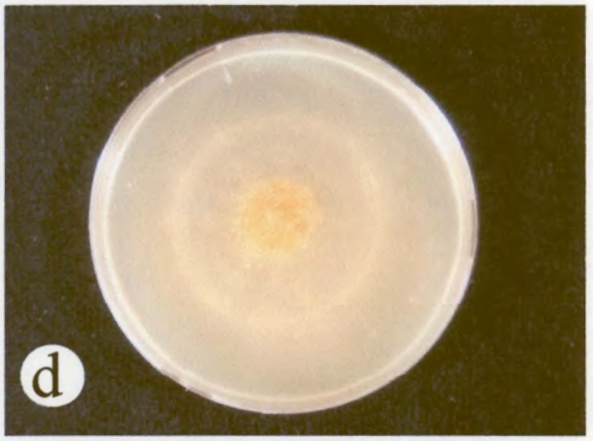
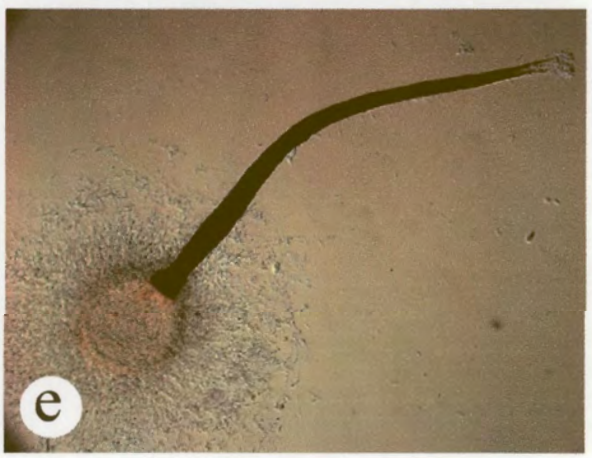
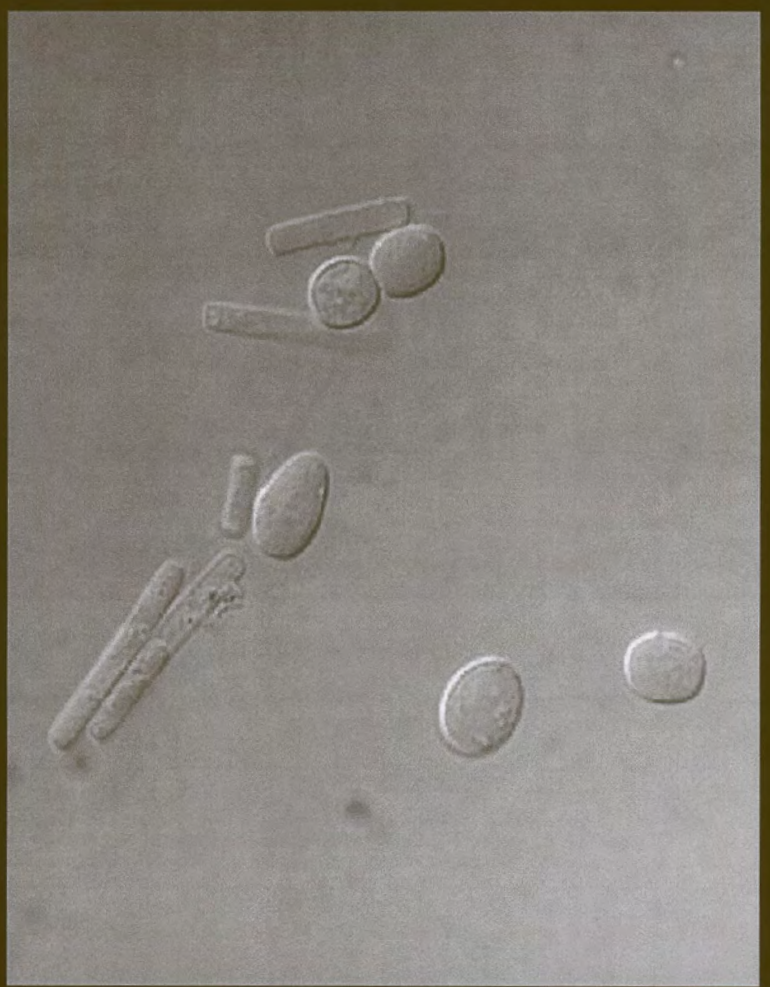

100
90
80
70
60
50
40
30
20
10
0
Frequency
P = 0.002
Observed
60
63
66
69
72
75
78
81
85
88
91
94
97
100
Tree length
B

Figure 4: Disease symptoms and culture characteristics of *Ceratocystis albofundus*. 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. albofundus*. 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 $MgCl_2$ (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 olivacious 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 g1 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 Accession No.	Culture no*	Host	Collector
C. fimbriata	Italy	AF264903	CMW 2242	*Platanus* sp.	A. Panconesi
"	Switzerland	AF395681	CMW 1896	"	O. Petrini
"	Brazil	AF395683	CMW 4903	*Eucalyptus* sp.	A. Alfenas
"	Uruguay**	AF453438	CMW 7383	*Eucalyptus grandis*	M. J. Wingfield
"	"	AF453439	CMW 7387	"	"
"	"	AF453440	CMW 7389	"	"
"	Colombia	AF395688	CMW 4829	*Citrus* sp.	B. L. Castro
"	"	AF395689	CMW 4835	*Coffea* sp.	"
"	"	AF395691	CMW 4844	"	"
"	"	AF395692	CMW 4824	"	"
"	Congo	AF395684	CMW 4793	*Eucalyptus* clone	J. Roux
"	Uganda	AF395687	CMW 5312	*Eucalyptus grandis*	"
C. albofundus	South Africa	AF264910	CMW 2148	*Acacia mearnsii*	M.J. Morris
"	"	AF043605	CMW 2475	"	S. McLennan
C. coerulescens	Germany	U75615	CBS 140.37	*Picea abies*	T. Rohde
C. fagacearum	USA	AF043598	CMW 2651	*Quercus sp.*	F. Paula
C. eucalypti	Australia	U75627	CMW 3254	*Eucalyptus sieberi*	G. Kile
C. virescens	USA	AF043603	CMW 0460	*Quercus sp.*	-
C. moniliformis	South Africa	AF043597	CMW 3782	*Erythrina sp.*	G. Zhao
Petriella setifera	Kenya	AF043596	ATCC 26490	*Rock hyrax* dung	-

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

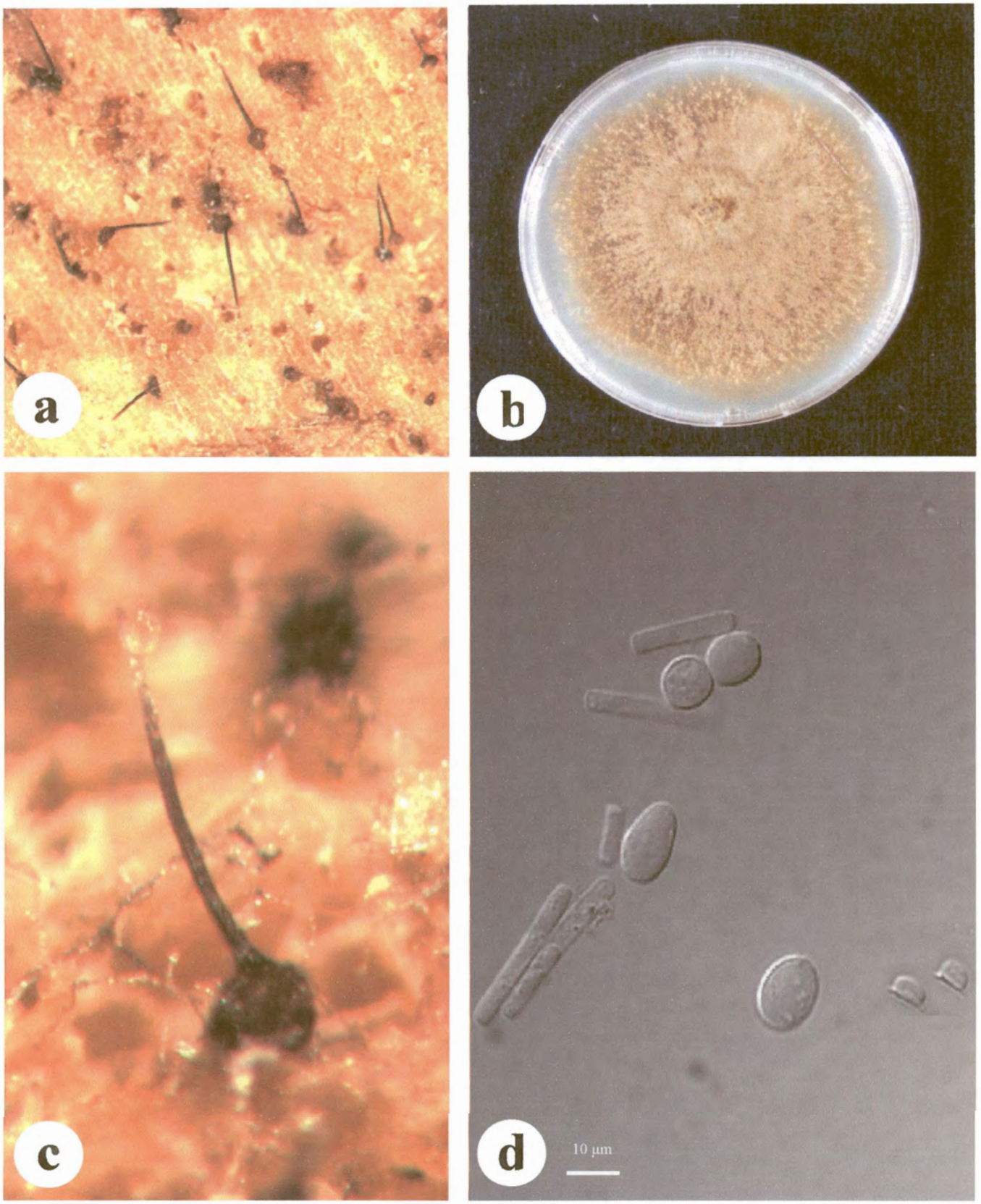


Figure 3: Sequence alignments of the ITS region (551 bp) for *C. fimbriata* from Uruguay with other *C. fimbriata* and *Ceratocystis* species. Sequences with homology to *C. fimbriata* from Italy (AF264903) (top line), are represented with dots. A dash represents gaps introduced to maximize alignments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

```
                                  10         20         30         40         50         60         70
Italy             (AF264903)  CCATGTGTGA ACGTACC-TA TCTT-GTAGT GA-GATGAAT GCTG-TTTTG GTGGT-AGGG ------CCC-
Switzerland       (AF395681)  .......... .......-.. ....-..... ..-....... ....-..... .....-.... ------...-
Brazil            (AF395683)  .......... .......C.. ....-..... ..-....... ....-..... .....-.... ------...-
Uruguay           (AF453438)  .......... .......C.. ....-..... ..-....... ....-..... .....-.... ------...-
Uruguay           (AF453439)  .......... .......C.. ....-..... ..-....... ....-..... .....-.... ------...-
Uruguay           (AF453440)  .......... .......C.. ....-..... ..-....... ....-..... .....-.... ------...-
Colombia          (AF395688)  .......... ..A....C.. ....-..... ..-....... ....-..... .....T.... ------...-
Colombia          (AF395689)  .......... ..A.T..C.. ....-..... ..A....... ....-..... .....T.... ------...-
Colombia          (AF395691)  .......... .......-.. ....-..... ..-....... ....-..... .....-.... ------...-
Colombia          (AF395692)  .......... .......-.. ....-..... ..-....... ....-..... .....-.... ------...-
Congo             (AF395684)  .......... .......-.. ....-..... ..-....... ....-..... .....-.... ------...-
Uganda            (AF395687)  .......... .......-.. ....-..... ..-....... ....-..... .....-.... ------...-
C. albofundus     (AF264910)  G.TGCCT..G TG.G----.G ..--T..... .GT.T.-..C -..-C....- -.AT--.A.. ----------
C. albofundus     (AF043605)  G.TGCCT..G TG.G----.G ..--T..... .GT.T.-..C -..-C....- -.AT--.A.. ----------
C. coerulescens   (U75615)    ....A..... ..A.--.C-- .T..T--..- ---------- -...C...-. .C----...- ----------
C. fagacearum     (AF043598)  ....T..... ..A.-A.CG. .T..TT.TTC TC----T... A...C...-. .C----.... A---------
C. eucalypti      (U75627)    ....A..... ..A.-A.C.T CT-----..- ---------- -...C...-. .C----...- ----------
C. virescens      (AF043603)  ....A..... ..A.-A.C.. .T-----..- ---------- -...C...-. .C----...- ----------
C. moniliformis   (AF043597)  ....T..... .TT.--.CAC AAACATCGAA ---------- CTGCGA..-. .C----G..- ----------
Petriella setifera (AF043596) ..C.T..... ..C.TA.C-. .T---..TA- --T.T.---- --GCC--.C. .C----G... TT----AG.C

                                  80         90        100        110        120        130        140
Italy             (AF264903)  -TTCTG---- -AAGGG---- -------CAC C--------- GCTGCCAGCA GTA-TAG--T CT-C-GCCAC
Switzerland       (AF395681)  -.....---- -.....---- -------... .--------- .......... ...-...--. ..-.-.....
Brazil            (AF395683)  -.....---- -...A.A--- ----GGG... .--------- .......... ...T...--. ..T.-.....
Uruguay           (AF453438)  -.....---- -...A.A--- ----GGG... .--------- .......... ...T...--. ..T.-.....
Uruguay           (AF453439)  -.....---- -...A.A--- ----GGG... .--------- .......... ...T...--. ..T.-.....
Uruguay           (AF453440)  -.....---- -...A.A--- ----GGG... .--------- .......... ...T...--. ..T.-.....
Colombia          (AF395688)  -.....---- A.....---- ---GGGG... .--------- .......... ...TA..--. ..T.-A....
Colombia          (AF395689)  -.....---- A.....---- ---GGGG... .--------- .......... ...TA..--. ..T.-A....
Colombia          (AF395691)  -.....---- -.....T--- -------A.. .--------- .......... ...-...--. ..-.-.....
Colombia          (AF395692)  -.....---- -.....---- -------... .--------- ......G... ...-...--. ..-.-.....
Congo             (AF395684)  -.....---- -...A.A--- ----GGG... .--------- .......... ...-...--. ..-.-.....
Uganda            (AF395687)  -.....---- -...A.A--- ----GGG... .--------- .......... ...T...--. ..T.-.....
C. albofundus     (AF264910)  ---------- GGGCA.CCCA CTACCGCT.G .CA------- ----...... .C.TACAAG. ..TTTA....
C. albofundus     (AF043605)  ---------- GGGCA.CCCA CTACCGCT.G .CA------- ----...... .C.TACAAG. ..TTTA....
C. coerulescens   (U75615)    C..GGTTTGA A..AAACAAG T--------- ---------- -.....G... .--------- ----------
C. fagacearum     (AF043598)  C..-CTTTCT TC....GATG TTT------- ---------- -.......-- ----...--- ----------
C. eucalypti      (U75627)    C..GGTAACA --CAA.TCT- ---------- ---------- --....G.-- ----...--- ----------
C. virescens      (AF043603)  C..GGTAACA --CAA----- ---------- ---------G T.....G.-- ----...--- ----------
C. moniliformis   (AF043597)  -.CTCCCCGC CCG.CA-GT- ---------- ---------- ---------- ---------A ..CTT-----
Petriella setifera (AF043596) CCC------- ----AAA--G C-----TTCT .CCGCCGG-- ---CAGCA-- ----CTAAA. T-CTTAATTT
```

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

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

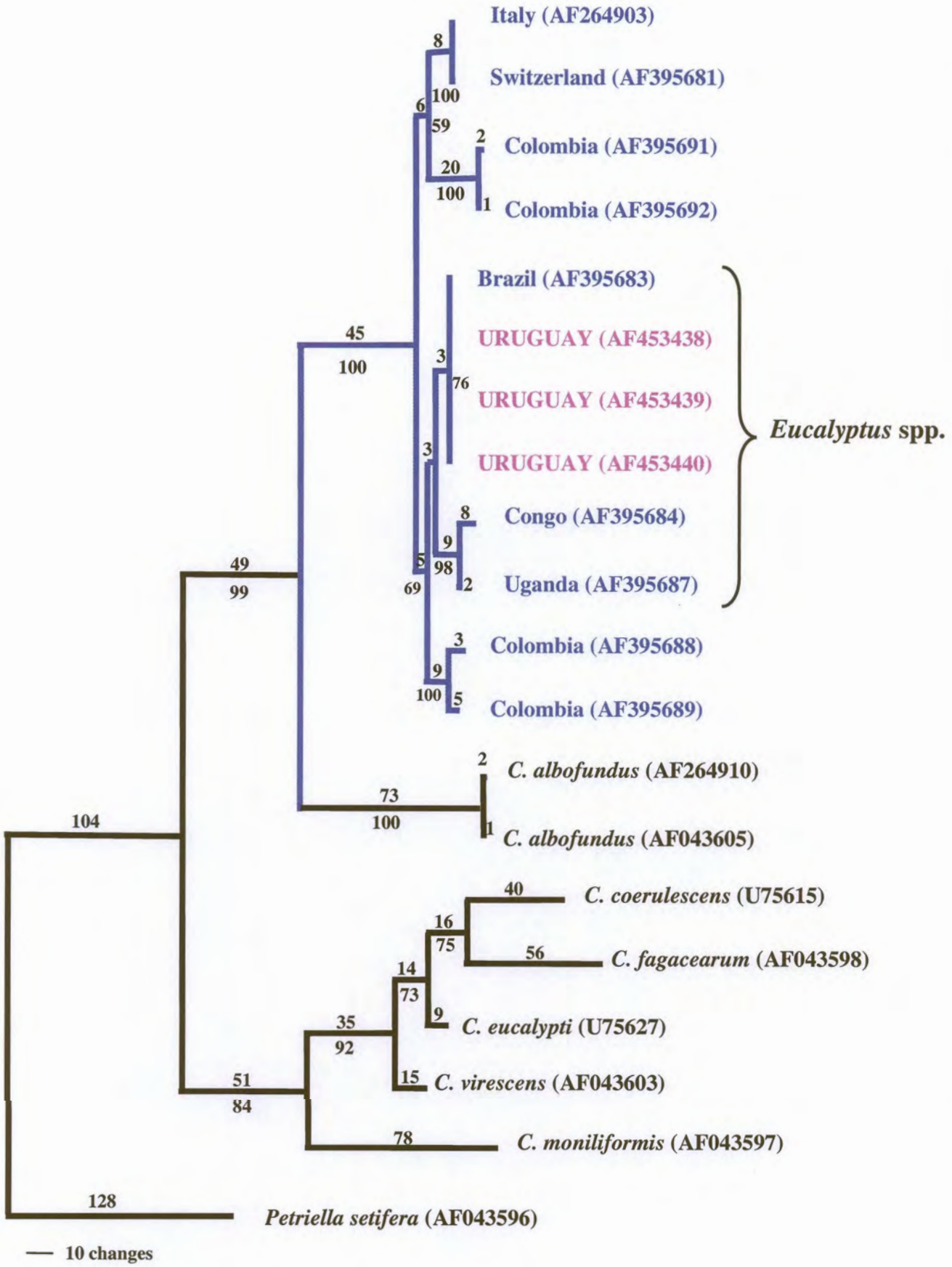
```
                                290        300        310        320        330        340        350
Italy             (AF264903)    AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
Switzerland       (AF395681)    .......... .......... .......... .......... ..........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

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

```
                                  430         440         450         460         470         480         490
Italy             (AF264903)  CTTGGCGTTG 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. ..........
C. albofundus     (AF264910)  ---..T.... .......... ...T-A---- ---CC.TT.. .......... ........C. ..........
C. albofundus     (AF043605)  ---..T.... .......... ...T-A---- ---CC.TT.C .......... ........C. ..........
C. coerulescens   (U75615)    .....T.... ....A..C.C GTCTTTTTTG TTT------- .--.G..... ........C. ..........
C. fagacearum     (AF043598)  ---..T.... ....A..C-C A-CTTGTCAC AA-------- .--.G.C.A- ........C. ........AG
C. eucalypti      (U75627)    ---..T.... ....A.TC.C ATCTTA--TG A--------. .--.G..... ........C. ..........
C. virescens      (AF043603)  ---..T.... ....A..... .G.TT---TC AA-------- ---......A ........C. ..........
C. moniliformis   (AF043597)  ---.TT.... ...AG..... G..AT----- --------GC .--.G...T .T......C. ........GT
Petriella setifera (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)  ----...... ..-....... .......... .....-.... .-........ .......... .
C. albofundus     (AF264910)  TTTT...... ..-....... .......... C..G.T...T .A........ .G.....G.. .
C. albofundus     (AF043605)  TTTT...... ..-....... .......... C..G.T...T .A........ .G.....G.. .
C. coerulescens   (U75615)    T----.T... .--.G..T.. .......... A----TA..T ---......-. .G.......A C
C. fagacearum     (AF043598)  T----.T... .--.G..T.. ...C...... A..C-T...- GTG-..---. .G...C...A C
C. eucalypti      (U75627)    T----..... .--.G..T.. .......... A----TA..T ---......-. .G.......A C
C. virescens      (AF043603)  T----..... .--.G..T.. .......... A----TA.-- CT-....--. .........A C
C. moniliformis   (AF043597)  T----..A.. .--.GT.T.. .......... A..C--...- ---...G..G .........A C
Petriella setifera (AF043596) ----G--G-- --CCCTG--- -----A.A.. ----CAG.GG CGGTCCCGCC GCGGCGCNNN 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 $MgCl_2$ (supplied with the

enzyme) and 2 – 10 ng DNA. Reaction volumes were adjusted to 50 ul 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 AFO43596), 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 aerio 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 conidiogenae 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 otiolar 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 hyphe and chalamydospores. 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 Reasearch 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) Zespoly 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 ophiotomatoid 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
C. adiposa	AF275545	2575	Japan	*Prunus persica*
C. albofunda	AF264910	2148	South Africa	*Acacia mearnsii*
"	AF043605	2475	"	"
C. coerulescens	U75615	-	Germany	*Picea abies*
C. eucalypti	U75627	3254	Australia	*Eucalyptus sieberi*
C. fagacearum	AF043598	2651	USA	*Quercus* sp.
C. fimbriata	AF395679	2219	France	*Platanus* sp.
"	AF453438	7383	Uruguay	*Eucalyptus grandis*
C. moniliformis	AF043597	3782	South Africa	*Erythrina* sp.
C. paradoxa	U75630	1546	New Zealand	*Musa* sp. *x paradisiaca*
C. radicicola	AF043599	3191	USA	*Phoenix dactylifera*
C. resinifera	U75618	3263	Norway	*Picea abies*
C. virescens	AF043603	460	USA	*Quercus* sp.
"C. pirilliformans	AF427104	6569	Australia	*Eucalyptus nitens*
"	AF427106	6574	"	"
"	AF427105	6579	"	"
Petriella setifera	AF043596	-	Kenya	*Rock hyrax* 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	*C. fimbriata* Upadhyay 1981	*C. albofundus* Wingfield *et al.* 1996			*C. pirilliformis*
		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.

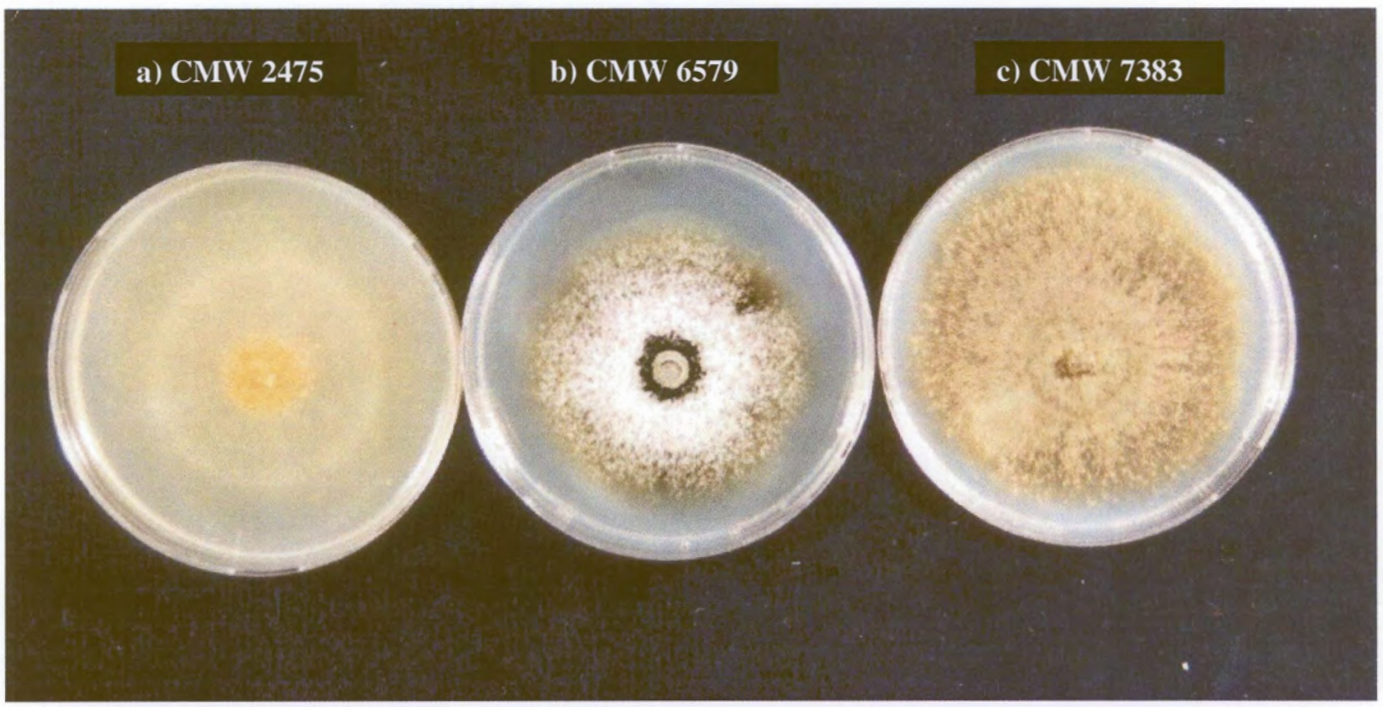

a) CMW 2475
b) CMW 6579
c) CMW 7383

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) Chlamydospore (all scale bars = 10 µm).

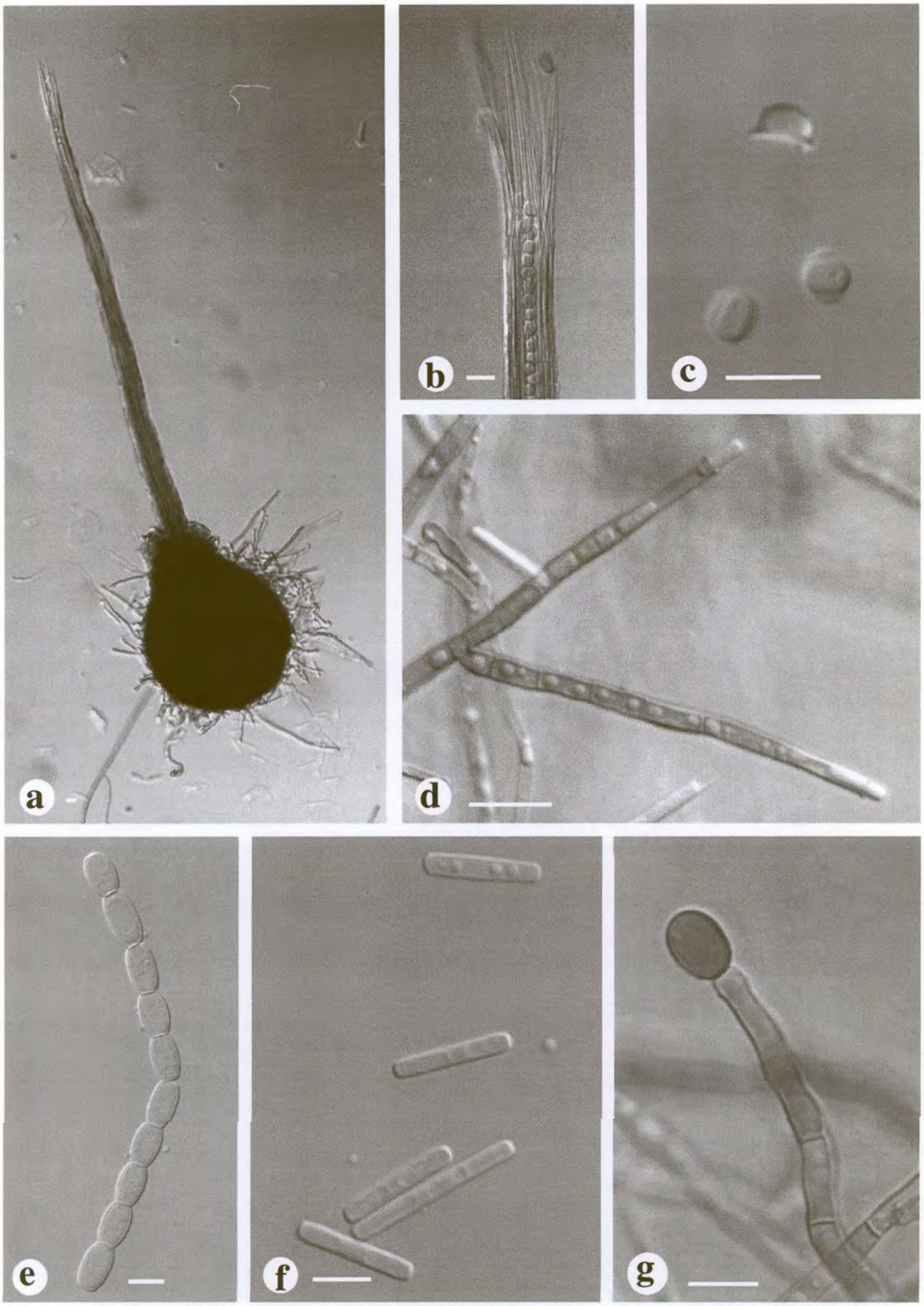


Figure 3: Sequence alignment (574bp) of the ITS region of *Ceratocystis pirilliformis* and other *Ceratocystis* species. Dots represent bases identical to *C. adiposa* (top line), N indicates unknown bases and dashes represent gaps introduced to maximise alignment.

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

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

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

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

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

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

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

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

```
                                  570
C. adiposa          (AF275545)    --ACGCTTTG AACC
C. coerulescens     (U75615)      --........ ..A.
C. eucalypti        (U75627)      --........ ..A.
C. fagacearum       (AF043598)    --......C. ..A.
C. moniliformis     (AF043597)    --G.A..... ..A.
C. paradoxa         (U75630)      --........ ..A.
C. radicicola       (AF043599)    --........ ..A.
C. resinifera       (U75618)      --........ ..A.
C. virescens        (AF043603)    --..A..... ..A.
C. albofundus       (AF264910)    --........ G.GT
C. albofundus       (AF043605)    --........ G.GT
C. fimbriata        (AF395679)    --..A..... ..GT
C. fimbriata        (AF453438)    --..A..... ..GT
C. pirilliformis    (AF427104)    T-..AT.... ..GT
C. pirilliformis    (AF427105)    T-..AT.... ..GT
C. pirilliformis    (AF427106)    T-..AT.... ..GT
Petriella setifera  (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.