Population genetic analyses suggest that the *Eucalyptus* fungal pathogen *Ceratocystis fimbriata* has been introduced into South Africa

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Ceratocystis fimbriata **is one of the most important pathogens of woody plants in the world. This fungus is also a serious pathogen of *Eucalyptus* species in various countries and has recently been reported from wounds on these trees in South Africa. Inoculation studies have shown that the fungus can cause disease on *Eucalyptus* spp. in South Africa, although trees dying in the field due to infection have never been encountered. The origin of *C. fimbriata* in South Africa is unknown, but there is some phylogenetic evidence that the fungus has a Latin American origin. In this study, microsatellite markers specifically designed for *C. fimbriata* were used to analyse the population diversity of 80 *C. fimbriata* isolates from *Eucalyptus* trees in South Africa. Analyses showed a low gene diversity (H = 0.36) and the population was found to be predominantly clonal (I_A = 0.95, P < 0.0001). The genotypic diversity was high (G_{ST} = 0.99) and the maximum genotypic diversity was very low (1.23%). These data provide good evidence that *C. fimbriata* has been introduced into South Africa.**

Introduction

Eucalyptus species are widely grown in commercial plantations and by subsistence farmers in the southern hemisphere, including South Africa.[1,2] In this country, forest plantations cover approximately 1.4 million ha, with *Eucalyptus* comprising about 40% of the total area.[3] This is an important crop that sustains large pulp, sawn timber and construction industries. Sustainable forestry with exotic *Eucalyptus* spp. in plantations such as those in South Africa are, however, threatened by fungal pathogens.[4–6]

Ceratocystis fimbriata Ellis & Halst. is one of the most important pathogens of trees and other woody crops in the world, causing vascular wilt or canker stain diseases.[7,8] The fungus is the best-known species in the genus, first having been described by Halsted[9] as the causal agent of black rot of sweet potato. *Ceratocystis fimbriata* infects trees through wounds[10,11] and is able to survive in the soil for many years.[12,13]

Ceratocystis fimbriata was first found associated with dying *Eucalyptus* spp. in the Republic of Congo and in Brazil in the late 1990s.[14] In both situations the fungus caused rapid wilt and death of these trees. It was subsequently reported from Uruguay, resulting in tree death after pruning[15] and from *E. grandis* trees showing signs of wilt in Uganda.[16] More recently, *C. fimbriata* has been found on *E. grandis* in South Africa in an artificial wounding study conducted to consider the presence of *Ceratocystis* spp. in the country.[17] Since its discovery in South Africa, *C. fimbriata* has commonly been isolated from artificially inflicted wounds on trees, naturally occurring wounds and on stumps of recently felled trees. Although South African isolates have been shown as pathogenic in inoculation studies,[17] the fungus has not been directly connected to disease outbreaks on *Eucalyptus* trees in the field.

The use of phylogeny to distinguish between isolates from different hosts and geographic areas has been used reasonably extensively for *C. fimbriata*.[15,18–20] The gene region most successfully used to characterize isolates is the Internal Transcribed Spacer (ITS) 1 and 2 regions including the 5.8S gene of the rDNA operon. Based on sequences for these regions, three major sub-clades can be distinguished for *C. fimbriata* and these reflect collections of isolates from Asia, Latin America and North America.[21]

Microsatellite markers are ideal tools to study the population biology of fungi.[22,23] These markers are co-dominant, and allow for the identification of multiple polymorphic alleles at any given locus. These different alleles can then be used to define the genotypes of individuals, allowing for their distinction within the same species. Microsatellite markers have been used effectively in population-orientated studies of *C. fimbriata*.[18,24] In these studies, they have provided valuable information regarding the possible origin of this important pathogen.

Various studies have shown that *C. fimbriata* represents a species complex.[25] Thus, isolates initially treated as a single species have been shown to represent distinct taxa. The first clear evidence of this trend emerged with the discovery of the wattle wilt pathogen, *C. albifundus*. The causal agent of this disease was initially identified as *C. fimbriata*[26] but it was later shown to represent a distinct species.[27] More recent studies have led to the description of numerous species in the *C. fimbriata sensu lato* complex.[20,28–30]

The fact that *C. fimbriata* occurs on *Eucalyptus* spp. in South America suggests that it could have originated in that part of the world and then introduced into Africa. This has been suggested on the basis of various phylogenetic studies,[14,16] although there has been no specific consideration of the origin of *C. fimbriata* in South Africa. The aim of the study reported here was to consider the genetic diversity of a collection of isolates of *C. fimbriata* from South Africa, using 27 microsatellite markers specifically developed for this fungus in two previous studies.

Materials and methods

Fungal isolates and DNA extractions

Isolates of *C. fimbriata* were collected from *Eucalyptus* trees (pure species and hybrids) growing in plantations in four areas of South Africa (Table 1). To obtain isolates, wounds similar to those described by Barnes *et al.*[28] and used by Roux *et al.*[17] to obtain *C. fimbriata* isolates, were made on approximately five-year-old *E. grandis* trees in the Hazyview/Wilgeboom (Mpumalanga province), Paulpietersburg and KwaMbonambi (KwaZulu-Natal province) areas of the country. Isolates were also collected from the surfaces of freshly cut stumps or logs of recently harvested trees in the Tzaneen area (Limpopo province).

Ceratocystis fimbriata was isolated either directly from fruiting structures on the cambium and/or inner bark or by using carrot baiting.[31] Ascospore masses were transferred to 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with streptomycin sulphate (0.001 g/l, Sigma, Steinheim, Germany) and incubated at 25°C. All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria.

Isolates of *C. fimbriata* were incubated at 25°C for two weeks prior to DNA extraction. Fungal biomass was scraped into microcentrifuge tubes using a sterile scalpel. The microcentrifuge tubes containing fungal samples were lyophilized for 24 h and the mycelium ground to a fine

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Table 1. Isolates of *Ceratocystis fimbriata* obtained in South Africa, and used in this study.

Isolate number	Origin	Collector(s)
CMW 9997–10000	Hazyview/Wilgeboom	M. van Wyk
CMW 10002	"	"
CMW 10131–10133	"	"
CMW 10139	"	"
CMW 10166–10167	"	"
CMW 10312–10314	"	"
CMW 10431	"	"
CMW 10751–10753	"	"
CMW 10755–10758	"	"
CMW 10760–10762	"	"
CMW 11177–11179	"	"
CMW 12276–12278	"	"
CMW 12282	"	"
CMW 11531	KwaMbonambi	"
CMW 11536–11537	"	"
CMW 11543	"	"
CMW 13029–13031	"	J. Roux
CMW 13041	"	"
CMW 11700–11701	Paulpietersburg	H. Hatting
CMW 12663–12668	"	"
CMW 12670	"	"
CMW 13019	"	J. Roux
CMW 13022–13028	"	"
CMW 13040	"	"
CMW 15054–15064	Tzaneen	G. Kamgan Nkuekam
CMW 15066–15076	"	"

powder with a glass rod in the presence of liquid nitrogen. A total of 800 μl DNA extraction buffer (DEB) — 250 mM Tris-HCl [pH 8], 250 mM NaCl, 25 mM EDTA [pH 8], 0.5% SDS — was added to the powdered mycelium, mixed using a vortex mixer and incubated in a heating block at 37°C for 30 min. Phenol:chloroform (5:3) extractions were performed until a clear interphase was obtained. DNA was precipitated by adding 3 M sodium acetate (0.1 volumes) [pH 5.5] and 2 volumes of 100% ethanol. Samples were incubated at 4°C overnight. The mixture was subjected to 30 min centrifugation at 10 000 rpm, the supernatant was discarded, and the pellets washed twice with 70% ethanol. The nucleic acid was subsequently air-dried for 10 min and resuspended in 50 μl sterile water. The DNA concentration was determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, Delaware).

Phylogenetic analysis

The ITS 1 and 2 regions including the 5.8S gene of the rDNA operon of all isolates were amplified using the primer pair ITS1 and ITS4.[32] The PCR reactions consisted of 2 ng DNA, 1 × Fast Start *Taq* Buffer containing $MgCl_2$ (Roche Molecular Biochemicals), 0.38 mM $MgCl_2$ (Roche Molecular Biochemicals), 200 μM of each dNTP, 300 nM of each of the forward and reverse primers and 2 U Fast Start *Taq* enzyme. The final volume was adjusted to 25 μl with sterile water. PCR reactions were performed in a Mastercycler gradient thermal cycler (Eppendorf, Germany) under the following conditions: 2 min at 96°C, followed by 10 cycles of 20 s at 94°C, 40 s at 55°C and 45 s at 72°C. The last three temperature intervals were repeated for another 30 cycles with a 5 s increase per cycle for the annealing step at 55°C, followed by an elongation step at 72°C. A final step of 10 min at 72°C completed the reaction cycles. Products were resolved by electrophoresis in a 2% agarose gel (Roche Diagnostics, Mannheim), stained with ethidium bromide. The PCR products were purified using 6% Sephadex columns (Sigma, Steinheim, Germany). For sequencing, the same primers were used as for the generation of the PCR products. Sequences were determined using an ABI Prism™ 3100 Autosequencer (Applied BioSystems, Foster City, California) and sequence data were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems).

Sequences for the ITS region of *C. fimbriata* isolates from *Eucalyptus* trees were compared with those of morphologically similar *Ceratocystis* spp. obtained from GenBank (Table 2). Sequences were aligned manually and analysed using PAUP version 4.0b10*.[33] The heuristic search was performed with 100 random addition sequence replications. Gaps were treated as a fifth character. Confidence intervals of branching points were determined using 1000 bootstrap replicates. The out-group used was *C. virescens* and it was rooted as a monophyletic sister group.

Table 2. Species of *Ceratocystis* used to determine the phylogeny of *Ceratocystis fimbriata* from *Eucalyptus* in South Africa.

Ceratocystis sp.	CMW no.	GenBank no.	Host	Country
C. fimbriata	CMW 15049	DQ 520629	*Ipomea batatas*	U.S.A.
C. fimbriata	CMW 1547	AF 264904	*I. batatas*	Papua New Guinea
C. platani	CMW 14802	DQ 520630	*Platnaus* sp.	U.S.A.
C. platani	CMW 1896	AF 395680	*Platnaus* sp	France
C. cacaofunesta	CMW 15051	DQ 520636	*Theobroma cacao*	Brazil
C. cacaofunesta	CMW 14809	DQ 520637	*T. cacao*	Ecuador
C. fimbriata	CMW 5312	AY 395687	*Eucalyptus* sp.	Uganda
C. fimbriata	CMW 5328	AF 395686	*Eucalyptus* sp.	Uganda
C. fimbriata	CMW 7383	AF 453438	*Eucalyptus* sp.	Uruguay
C. fimbriata	CMW 4903	AF 395683	*Eucalyptus* sp.	Brazil
C. fimbriata	CMW 7765	DQ 520635	*Eucalyptus* sp.	Uruguay
C. fimbriata	CMW 10000	DQ 520631	*Eucalyptus* sp.	South Africa
C. fimbriata	CMW 13019	DQ 520632	*Eucalyptus* sp.	South Africa
C. fimbriata	CMW 13041	DQ 520633	*Eucalyptus* sp.	South Africa
C. fimbriata	CMW 15154	DQ 520634	*Eucalyptus* sp.	South Africa
C. pirilliformis	CMW 6569	AF 427104	*Eucalyptus* sp.	Australia
C. pirilliformis	CMW 6579	AF 427105	*Eucalyptus* sp.	Australia
C. polychroma	CMW 11424	AY 528970	*Sygyzium aromaticum*	Sulawesi
C. polychroma	CMW 11436	AY 528971	*S. aromaticum*	Sulawesi
C. albifundus	CMW 4068	DQ 520638	*Acacia mearnsii*	South Africa
C. albifundus	CMW 5329	AF 388947	*A. mearnsii*	South Africa
C. variospora	C 1965	AY 907045	*Prunus* sp.	U.S.A.
C. variospora	C 1707	AY 907044	*Betula* sp.	Japan
C. smalleyi	C 1828	AY 907032	*Carya* sp.	U.S.A.
C. smalleyi	C 1410	AY 907031	*Carya* sp.	U.S.A.
C. caryae	C 1829	AY 907035	*Carya* sp.	U.S.A.
C. caryae	C 1827	AY 907034	*Carya* sp.	U.S.A.
C. populicola	C 995	AY 907029	*Populus* sp.	Poland
C. populicola	C 685	AY 907028	*Populus* sp.	Canada
C. virescens	CMW 11164	DQ 520639	*Fagus americanum*	U.S.A.

CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.
C = From the collection of T.C. Harrington.

Polymorphic marker amplification

To amplify putative polymorphic loci, 27 microsatellite marker pairs were used. Eleven of these had previously been developed by Barnes *et al.*[18] and 16 by Steimel *et al.*,[24] specifically for *C. fimbriata*. The primers were relabelled with the G5 labelling kit (Applied Biosystems, Warrington, U.K.) and amplified products were run on an ABI Prism™ 3100 DNA sequencer. Consistency of marker amplification across instruments was tested by analysing 10 isolates on both an ABI 377 and an ABI 3100 DNA sequencer. The optimum annealing temperature for all primer sets was as described previously,[18,24] but some isolates required annealing temperatures during the amplification that were different from those previously reported. The PCR reactions consisted of 2 ng DNA, 1 × Fast Start *Taq* Buffer containing $MgCl_2$ (Roche Molecular Biochemicals), 0.38 mM $MgCl_2$ (Roche Molecular Biochemicals), 200 μM of each dNTP, 300 nM of each of the forward and reverse primers and 2 U Fast Start *Taq* enzyme. The final volume was adjusted to 15 μl with sterile water. The PCR reactions for all primer sets were as described by Barnes *et al.*[18] The PCR products were analysed on a 2% agarose gel stained with ethidium bromide, exposed to UV light. A 100-bp marker (Roche Molecular Biochemicals) was included as the size standard. The PCR products were multiplexed according to their size and fluorescent label attached to the primer. Each sample contained 1.5 ng PCR product in total, 1.1 μl 1 × loading buffer and 0.4 μl internal standard GeneScan-500 LIZ® (Applied Biosystems, Warrington). Thereafter, the samples were separated on a 4.25% PAGE gel using an ABI Prism™ 3100 sequencer. Analysis was done with the statistical software Genescan 2.1 and Genotyper 3.0 (Applied Biosystems).

Statistical analyses

The allelic frequencies were determined with the statistical program Multilocus.[34] This was achieved by dividing the number of times that the alleles occur by the population size. Each allele that was different in size was assigned a different number according to the number of alleles present at that locus. With the aid of this numbering system, it was possible to define a multilocus profile and a genotype for each isolate. Allelic frequencies were then used to determine the fixation of specific loci and to consider the relative occurrence of different alleles.

Genescan analysis was performed following the manufacturer's instructions (Perkin Elmer). Gene diversity for the population was determined from allelic frequencies using the formula $H = 1 - \sum_k x_k^2$, where x_k is the frequency of the k genotype,[35] and with the statistical program Popgene 1.31.[36] Maximum diversity was obtained when H approached 1. In addition, genotypic diversity (G_{ST}) was calculated using the method described by Stoddart and Taylor[37] and the formula

$$G_{ST} = \frac{1}{\sum_{x=0}^{N} f_x \left(\frac{x}{N}\right)^2},$$

where N is the sample size, and f_x is the number of genotypes occurring x times in the sample.

The software program Multilocus[34] was used to model G_{ST} against the number of loci, with 1000 resampling repetitions. The resultant sigmoidal graph, drawn from G_{ST} vs number of loci, was used to determine whether the data matrix was sufficiently large to support statistical significance. The maximum genotypic diversity was determined by the formula $\hat{G} = G/N \times 100$, where N is the sample size.[38] The mode of reproduction of the fungus was tested using the Index of Association (I_A) (10 000 repetitions) with the program Multilocus.[34]

Results

Fungal isolates and PCR reactions

Eighty isolates were obtained from the four sampling localities in South Africa. These were all from different trees and included 8, 18, 32 and 22 isolates from KwaMbonambi, Paulpietersburg, Hazyview/Wilgeboom and Tzaneen, respectively. These isolates were analysed as a single population defined on the basis of host and not geographical origin. The isolates grown in pure culture were identified as *C. fimbriata* based on morphological characteristics typical for this species.

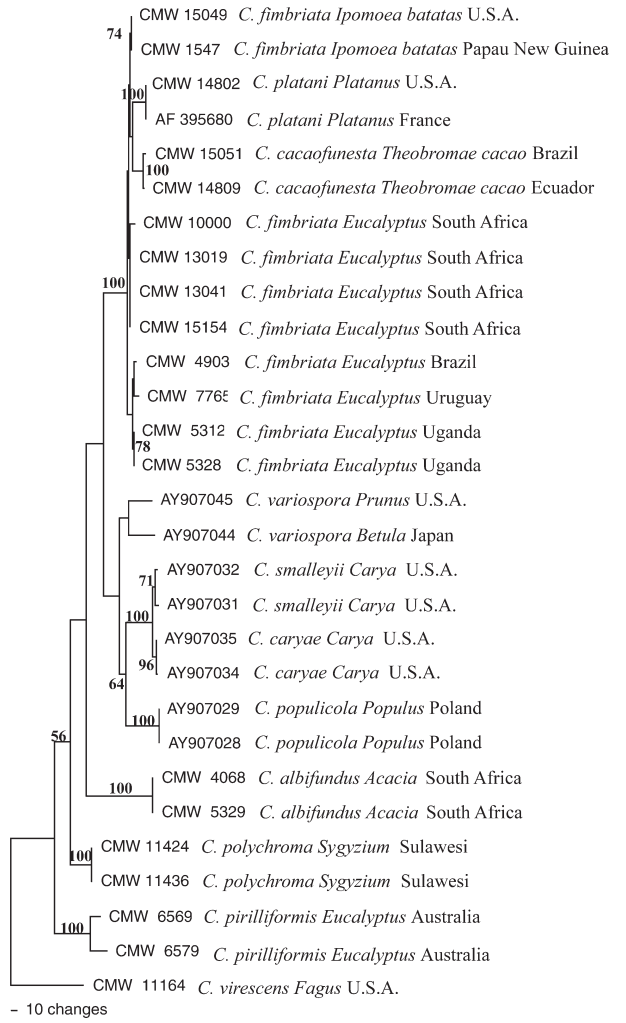

Fig. 1. Phylogenetic tree based on the ITS region, including the 5.8S rDNA operon of *Ceratocystis fimbriata sensu lato*. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated in bold above the branches. *Ceratocystis virescens* is used as the out-group taxon.

Phylogenetic analysis

From phylogenetic analyses, the fungus isolated from *Eucalyptus* trees in South Africa grouped together with *C. fimbriata* isolates from other hosts and geographic regions. Twenty-four most parsimonious trees were obtained and one was selected for presentation (Fig. 1). There were 584 characters of which 249 were constant, 84 were parsimony-uninformative and 251 were parsimony-informative. The tree consisted of 789 steps, the consistency index (CI) = 0.702, the homoplasy index (HI) = 0.298, the retention index (RI) = 0.810 and rescaled index (RC) = 0.569. Phylogenetic analysis showed that the isolates from South Africa grouped together in a single sub-clade together with isolates previously identified as *C. fimbriata* from *Eucalyptus* trees, within the larger *C. fimbriata sensu lato* clade (Fig. 1). The bootstrap values on the tree showed clearly that *C. fimbriata* isolates from *Eucalyptus* trees in South Africa form a distinct sub-clade together with isolates of this fungus from *Eucalyptus* trees elsewhere in the world.

Population genetic analyses

Ten loci were amplified using ten of the eleven primer pairs developed by Barnes *et al.*[18] One primer pair, AG 1/AG 2, from the same study did not amplify the expected locus. Sixteen loci were amplified using the primer pairs developed by Steimel *et al.*[24]

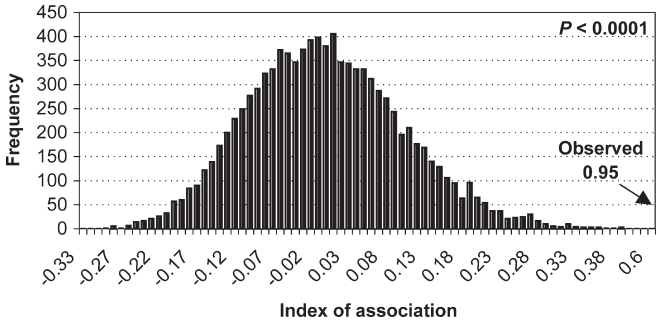

Fig. 2. Index of association (I_a) in relation to frequency, showing that the *Ceratocystis fimbriata* population obtained from *Eucalyptus* trees in South Africa reproduces asexually.

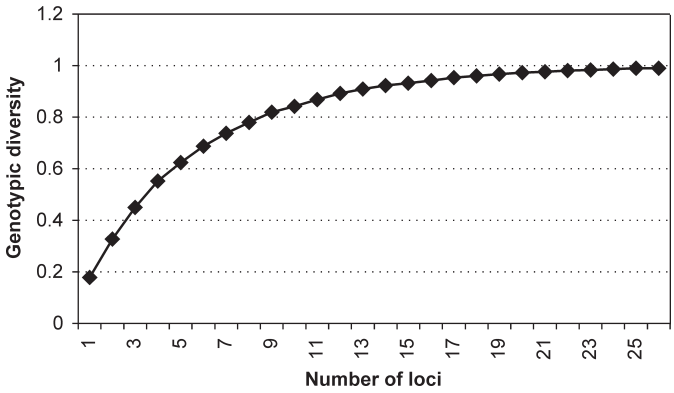

Fig. 3. Genotypic diversity versus number of loci. This result indicates that the population chosen is a good representative of the true population because a plateau was reached.

Thus, a total of 26 microsatellite primer pairs were available for this study.

The same allelic range was observed for all isolates in the analysis using both the ABI 377 and the ABI 3100 DNA sequencers. Six primer pairs had monomorphic loci and thus a total of 19 polymorphic loci was used. The average number of alleles for all loci was 2.88, while the effective number of alleles was 1.23. Where more than one allele was obtained, in most cases there was one that had a very high frequency. However, there were cases where the distribution was more even among the alleles.

In total, 46 genotypes were obtained with the most common genotype occurring 12 times. The gene diversity was low (H = 0.36) for the 80 *C. fimbriata* isolates included in this study. The genotypic diversity for the population was 0.99 and the maximum genotypic diversity was very low (1.23%). The Index of Association (I_A) was 0.95 (Fig. 2). The observed value for I_A was outside of the bell curve and so the population is apparently clonal. The genotypic diversity vs the number of loci had a high level of significance, showing that an adequate number of isolates and loci had been used in the analyses (Fig. 3).

Separate analyses were conducted for isolates from the four different geographic regions to ascertain whether alleles specific to the areas could be detected. There were, however, no alleles specific to any of the four regions sampled and so the isolates were treated as a single population.

Discussion

In this study, we have considered the population diversity of a collection of isolates of *C. fimbriata* from *Eucalyptus* spp. in South Africa. Various studies have previously been undertaken to consider the identity of the fungus in Africa.[14,16,17] Ours is, however, the first investigation where a large number of primer pairs has been used to consider the diversity and possible origin of the pathogen in South Africa. In this regard, our data showed very low gene diversity for a large number of isolates, and this suggests strongly that *C. fimbriata* was introduced into the country. Its nature as an introduced pathogen on *Eucalyptus* trees also corresponds with its recent discovery on this host in South Africa.[17]

The gene diversity observed for the South African population of *C. fimbriata* was low in comparison with previous studies of fungal pathogens. Low gene diversity can be attributed to several factors, including a founder effect after introduction into a new environment, lack of sexual outcrossing resulting in loss of gametic equilibrium and genetic drift, and rapid natural selection of specific genotypes that are capable of colonizing the host. In the case of *C. fimbriata* in South Africa, we believe that all of these forces might be acting to shape the population. Because this fungus is exclusively found on a non-native host in South Africa, and that it has only recently been discovered,[14] we postulate that it has been introduced and has not been in the country for long.

Simulation of genotypic diversity against number of loci (1000 repetitions per locus) resulted in a graph demonstrating equilibrium being reached at *c.* 22 loci, out of a possible 24 (Fig. 3). This indicates that the data matrix used for population genetic analysis was saturated. The data matrix is a representative sample of the true population, and addition of more data points would not improve the reliability of statistical analyses. The percentage of maximum genotypic diversity obtained (1.23%) provides good evidence that the population is not diverse and likely propagates asexually. Barnes *et al.*[43] studied genetic diversity in two populations of *C. albifundus*, and showed that the maximum percentage of genotypic diversity was 62% and 44% for the South African and Ugandan populations, respectively. This was interpreted as providing evidence that *C. albifundus* is native to the African continent. In contrast, data gathered during the present study suggest that *C. fimbriata* in South Africa is probably an introduced pathogen, based on its very low level of diversity and apparently clonal reproductive strategy.

The Index of Association provides an indication of the mode of a population's reproduction.[33] Results of this study, showing an observed value outside of the bell-shaped curve, indicate that *C. fimbriata* from *Eucalyptus* trees in South Africa has a predominantly clonal reproductive strategy. High levels of clonality might be offset by sexual reproduction in a fungus.[38,42] Although the fungus produces viable progeny from sexual structures in South Africa, there appears to have been little outcrossing amongst the different genotypes that were apparently introduced into the country. This is consistent with the fact that the fungus is homothallic[25] and able to produce perithecia from single ascospores, without necessarily undergoing outcrossing. This low level of outcrossing of *C. fimbriata* from *Eucalyptus* in South Africa supports the view that it has been introduced and has probably not been in the country for long.

If *C. fimbriata* has been introduced into South Africa as our results suggest, it is important to know how it came about. It might have occurred via contaminated germ plasm from South America. We believe that this is highly unlikely, however, as *Eucalyptus* trees have been introduced into the country primarily through seed from Australia. A more likely route of entry is via infected timber used as packaging, which represents an important mode of accidental entry for many pests and pathogens.[44] Alternatively, these fungi are known to be carried by casual insects such as nitidulid beetles,[31,45] which could have entered South Africa carrying spores of *C. fimbriata.* From a first point of entry, the fungus could easily have spread rapidly to wounds on trees that are typically visited by these insects.

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