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Genetic diversity and interfertility among highly differentiated populations of *Ceratocystis fimbriata* in Brazil

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Mating studies showed that isolates of the insect-associated wilt pathogen *Ceratocystis fimbriata* from *Eucalyptus* spp., mango, fig, inhame (*Colocasia esculenta*), *Gmelina arborea* and sweet potato were interfertile, and progeny from those crosses showed normal segregation for microsatellite markers. Genetic diversity was compared among 13 populations of *C. fimbriata* collected from six states in Brazil using 15 highly polymorphic microsatellite markers. The gene diversity values of most eucalyptus and mango populations from Minas Gerais, Bahia, Rio de Janeiro and São Paulo states were similar to putatively native populations of *Ceratocystis platani* and *C. cacaofunesta*, two other species in the *C. fimbriata* complex that are homothallic. Index of association values indicated substantial asexual reproduction or selfing in populations on mango and eucalyptus. Most of these eucalyptus and mango populations were not highly differentiated from each other, and these populations and genotypes appeared to be more closely related to each other than to other populations by UPGMA analyses. By contrast, the *G. arborea* population from Pará and the fig and inhame populations from São Paulo had relatively low levels of diversity and were highly differentiated from each other and all other studied populations, suggesting that they were from different origins and had gone through genetic bottlenecks. One of the eucalyptus populations in Bahia consisted of a single genotype and may have been introduced to the site in infected cuttings from another Bahia location. Similarly, a mango population from Mato Grosso do Sul consisted of a single genotype, which was identical to one of the genotypes found on mango in São Paulo. Aside from introductions by humans, mating studies and genetic analyses suggest that limited dispersal distance and a high degree of selfing or asexual reproduction lead to local populations of *C. fimbriata* that have limited diversity but are highly differentiated from other populations.

Keywords: ceratocystis wilt, *Eucalyptus*, genetic diversity, introduced populations, mango, population genetics

Introduction

Ceratocystis fimbriata has been recognized to have a very broad host range (CAB International, 2005), but it is apparent that *C. fimbriata* is a complex of many cryptic species (Harrington, 2000; Engelbrecht & Harrington, 2005; Johnson *et al.*, 2005). In Brazil, *C. fimbriata* causes lethal, wilt-type diseases in woody hosts or rot of corms in many economically important hosts, but these hosts are not native to Brazil (CAB International, 2005). The first report of ceratocystis wilt in Brazil was in the state of São Paulo on *Crotalaria juncea* (Costa & Krug, 1935). Later, the disease was reported on *Mangifera indica* (mango) in the states of Pernambuco and São Paulo (Viégas, 1960). The pathogen also causes substantial mortality to mango in eastern Rio de Janeiro (Baker *et al.*, 2003; Silveira *et al.*, 2006). The fungus was first observed on *Eucalyptus* spp. (eucalyptus) in the south of Bahia (Ferreira *et al.*, 1999), but it is also common in the nearby state of Minas Gerais (Alfenas *et al.*, 2004), as well as in Maranhão and Mato Grosso do Sul (A.C. Alfenas, unpublished data). Substantial mortality of *Ficus carica* (edible fig) is caused by *C. fimbriata* in São Paulo, the only major fig-growing area in Brazil (Valarini & Tokeshi, 1980). *Ceratocystis fimbriata* and relatives cause a corm rot of *Colocasia esculenta* (inhame or taro) and related Araceae, and *C. fimbriata sensu stricto* is a common cause of black rot in corms of inhame in the state of São Paulo (Harrington *et al.*, 2005; Thorpe *et al.*, 2005). The pulpwood species *Gmelina arborea* was seriously affected in a major plantation project in the Amazonian state of Pará (Muchovej *et al.*, 1978; Fearnside, 1988). Ceratocystis wilt of *Theobroma cacao* (cacao/cocoa), caused by the related *Ceratocystis cacaofunesta*, is thought to be native to the upper Amazon Basin (Rondônia) and was

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introduced to Bahia on propagation material (Engelbrecht & Harrington, 2005; Engelbrecht *et al.*, 2007b). Aside from *C. cacaofunesta*, it is not known if there are other host-specialized, cryptic species within the *C. fimbriata* complex in Brazil.

Ceratocystis fimbriata is primarily a xylem pathogen (Harrington, 2000). On trees (mango, eucalyptus, fig, *G. arborea*, etc.), infection typically occurs through fresh wounds, often those made by contaminated cutting tools (Viégas, 1960; Rossetto & Ribeiro, 1990). However, the fungus is soilborne, and root infections are also common, at least in Brazil (Rossetto & Ribeiro, 1990; Laia *et al.*, 2000; CAB International, 2005). *Ceratocystis* species are strongly associated with insects (Harrington, 2009), although specific vectors of *C. fimbriata* in Latin America are not known. Trees with ceratocystis wilt are frequently attacked by ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae), and the fungus is readily dispersed by wind and rain as aleurioconidia (special, thick-walled conidia) in the insect frass that is expelled from the tree by the adult beetles (Iton & Conway, 1961; Baker *et al.*, 2003; Engelbrecht *et al.*, 2007b; Ocasio-Morales *et al.*, 2007). Infection may take place if the infested frass lands on a wound of a susceptible host or through root infection via soilborne aleurioconidia. Besides dispersal through aleurioconidia, sticky ascospore masses formed atop long-necked perithecia may be dispersed by insects such as flies and nitidulid beetles (Harrington, 2009). All members of the *C. fimbriata* complex are homothallic through unidirectional mating-type switching (Harrington & McNew, 1997; Witthuhn *et al.*, 2000), so even sexual reproduction usually occurs through selfing, which tends to limit genetic diversity in *Ceratocystis* populations (Harrington *et al.*, 1998). Limited dispersal of ascospores by insects or aleurioconidia in insect frass, along with a preponderance of selfing and asexual reproduction, are thought to result in isolated populations of the *C. fimbriata* complex that have relatively low genetic diversity and are highly differentiated from other populations (Roux *et al.*, 2001; Engelbrecht *et al.*, 2004, 2007b).

There is substantial genetic variation within the *C. fimbriata* complex (Baker *et al.*, 2003; Steimel *et al.*, 2004; Engelbrecht & Harrington, 2005; Johnson *et al.*, 2005; Thorpe *et al.*, 2005), and much of this variation is among clades and lineages associated with geographic regions or specific hosts. Three geographic clades are believed to be centered in North America, Asia and Latin America, respectively (Harrington, 2000; Johnson *et al.*, 2005). Isolates from Brazil have been placed in the Latin American clade of the *C. fimbriata* complex, along with the form of the pathogen from *Ipomoea batatas* (sweet potato), on which the species was first described (Engelbrecht & Harrington, 2005). Within the Latin American clade, inoculation studies and intersterility tests showed that the cause of ceratocystis wilt of cacao and ceratocystis wilt of *Platanus* spp. are distinct species, *C. cacaofunesta* and *C. platani*, respectively, each of which is monophyletic based on rDNA sequences (Baker *et al.*, 2003; Engelbrecht & Harrington, 2005; Engelbrecht *et al.*, 2007b). By contrast, phylogenetic analyses of rDNA indicated that Brazilian isolates of *C. fimbriata* from *G. arborea*, mango, eucalyptus, fig and inhame are each polyphyletic (Baker *et al.*, 2003; Thorpe, 2004; Thorpe *et al.*, 2005). Brazilian isolates of *C. fimbriata* have shown variation in aggressiveness to cultivated hosts, but there has been little evidence of host specialization, except that isolates from *G. arborea* were the most aggressive to seedlings of that species, and some eucalyptus isolates were particularly aggressive to eucalyptus hybrids (Baker *et al.*, 2003; Thorpe, 2004; Zauza *et al.*, 2004; Thorpe *et al.*, 2005).

Ceratocystis spp. can be readily introduced to new areas on solid wood packing material, contaminated tools and infected propagative material (Harrington, 2000; Baker *et al.*, 2003; CAB International, 2005; Engelbrecht *et al.*, 2007a; Ocasio-Morales *et al.*, 2007). Previous studies with members of the Latin American clade of *C. fimbriata* (Engelbrecht *et al.*, 2004, 2007b; van Wyk *et al.*, 2006; Ocasio-Morales *et al.*, 2007) and related *Ceratocystis* species (Roux *et al.*, 2001; Barnes *et al.*, 2005) were able to distinguish putatively native populations of the pathogen from introduced populations. Introduced populations showed signs of having gone through a severe genetic bottleneck, that is, there was substantially less diversity in putatively introduced populations than in putatively native populations, often with only a single genotype identified in introduced populations.

The first objective of this study was to determine the level of population differentiation of *C. fimbriata* in Brazil using highly polymorphic microsatellite markers. Secondly, the genetic diversity of individual populations was evaluated, with the aim of identifying what might be considered introduced and native populations. The third objective was to determine if there were barriers to sexual reproduction between Brazilian populations (i.e. if there were biological species of *C. fimbriata* in Brazil) and if Brazilian isolates were sexually compatible with *C. fimbriata sensu stricto*, *C. cacaofunesta* and *C. platani*.

Materials and methods

Fungal isolations

Isolates were collected from diseased trees in the field or, in the case of inhame, from corms with symptoms of black rot in grocery stores or markets. The fungus was baited from diseased wood or rotted corm tissue by placing pieces of discoloured tissue between two discs of carrot root. Ascospore masses from perithecia formed on the carrot discs were transferred to agar media for purification and then storage. Only one isolate per tree or corm was stored and used in genetic analyses. At each collection site, an attempt was made to collect samples from trees as far apart as possible. Pure cultures were stored at Iowa State University on malt agar media at −80°C on agar slants (isolates beginning with the letter C). The cultures stored at the Universidade Federal de Viçosa

Table 1 Number of genotypes and Nei's gene diversity (*H*) of populations of *Ceratocystis fimbriata, C. cacaofunesta* and *C. platani* based on 15 microsatellite loci

Species	Population	Host	Location	No. isolates	No. genotypes	Nei's gene diversity (*H*)	
						All isolates	Clone-corrected[a]
C. fimbriata	EucBA1	*Eucalyptus* spp.	Eunápolis, Bahia	26	14	0·2128	0·2476
	EucBA2a	*Eucalyptus* spp.	Caravelas, Bahia	6	1	0·0000	0·0000
	EucBA2b	*Eucalyptus* spp.	Caravelas, Bahia	6	4	0·2296	0·2583
	EucMG1	*Eucalyptus* spp.	Curvelo, Minas Gerais	18	14	0·2903	0·3083
	EucMG2	*Eucalyptus* spp.	Paracatu, Minas Gerais	6	6	0·2778	0·2778
	EucMG3	*Eucalyptus* spp.	João Pinheiro, Minas Gerais	6	5	0·2407	0·2502
	ManRJ1	*Mangifera indica*	Campus, Rio de Janeiro	14	10	0·3306	0·3813
	ManSP1	*M. indica*	São Paulo	8	5	0·3187	0·3627
	ManMS	*M. indica*	Aquidauana, Mato Grosso do Sul	5	1	0·0000	0·0000
	ManRJ2	*M. indica*	São Fidélis, Rio de Janeiro	19	6	0·1108	0·1889
	FicSP2	*Ficus carica*	Valinhos, São Paulo	20	2	0·0170	0·0333
	ColSP3	*Colocasia esculenta*	Piedade, São Paulo	12	5	0·1019	0·1600
	GmelPA	*Gmelina arborea*	Jari, Pará	5	1	0·0000	0·0000
	All Brazilian populations			151	74	0·6253	0·5526
	IpoWW	*Ipomoea batatas*	Worldwide	15	2	0·0083	0·0333
C. cacaofunesta[b]	CacBR	*Theobroma cacao*	Rondônia, Brazil	3	3	ND[c]	0·1979
		T. cacao	Bahia, Brazil	44	4	ND	0·0308
		T. cacao	Columbia	4	4	ND	0·0938
		T. cacao	Costa Rica	17	4	ND	0·0386
	CacEC	*T. cacao*	Ecuador[b]	9	5	ND	0·1020
C. platani[d]	PlaUS	*Platanus occidentalis*	Eastern USA[c]	33	11	ND	0·2178
		Platanus spp.	Europe	27	3	ND	0·0191
		Platanus spp.	California	7	4	ND	0·0625

[a]Clone correction removed isolates that had genotypes identical to other isolates from the same site.
[b]Data from Engelbrecht *et al.* (2007b) based on 16 loci.
[c]Not determined.
[d]Data from Engelbrecht *et al.* (2004) based on 16 loci.

(cultures with isolate numbers beginning with other letters) were stored at room temperature.

Fungal populations

Thirteen Brazilian populations (Table 1) of *C. fimbriata* were analysed, from five hosts in six states: Pará, Bahia, Minas Gerais, Rio de Janeiro, São Paulo and Mato Grosso do Sul (Fig. 1). Each population was from a single host species in an area usually less than 500 km^2. In the case of eucalyptus, each population was from a single plantation of a single eucalyptus clone. Gene diversity and other measures of the Brazilian populations of *C. fimbriata* were compared with previously published data using the same microsatellite markers applied to a nearly clonal, worldwide collection of *C. fimbriata* from sweet potato (Steimel *et al.*, 2004), native and introduced populations of *C. cacaofunesta* (Engelbrecht *et al.*, 2007b), and native and introduced populations of *C. platani* (Engelbrecht *et al.*, 2004).

Eucalyptus populations

Plantations of *Eucalyptus* species and hybrids were sampled in south Bahia and Minas Gerais. The EucBA1 plantation was north of Eunápolis and was heavily diseased when sampled in 2003. Plantation EucBA1 was heavily damaged in a windstorm in 2000, and in 2001 it was used as a source material for the production of rooted cuttings that were planted elsewhere in south Bahia. The site was a farm of unknown crops immediately prior to planting eucalyptus. The EucBA2b plantation was a hybrid of *E. grandis* × *E. urophylla* in its third rotation, with the first rotation started in 1989, but the vegetation prior to eucalyptus was not known. It was sampled in 2007. The EucBA2a population was sampled in 2007 from a second rotation of a different *E. grandis* × *E. urophylla* clone. Prior to the eucalyptus plantations, the EucBA2a site was under pasture.

Three eucalyptus plantations at three locations were sampled in Minas Gerais, and all sites were probably native Cerrado forest prior to eucalyptus cultivation. The EucMG1 population was an *E. grandis* × *E. urophylla* hybrid, but the vegetation prior to that was not known. The EucMG2 and EucMG3 populations were planted with the same eucalyptus clone, which differed from the clone used in EucMG1. Both EucMG2 and EucMG3 were in an agroforestry system, where agronomic plants were interplanted with eucalyptus. The EucMG2 plantation was sampled in 2007, and prior to the eucalyptus

Figure 1 Geographic distribution of the 13 studied *Ceratocystis fimbriata* populations in Brazil. The first three letters of each population name indicate the host (*Gmelina arborea*, *Eucalyptus* spp., *Mangifera indica*, *Colocasia esculenta* and *Ficus carica*), the last two letters indicate the state of origin, and if more than one population was sampled in that state, they were numbered.

agroforestry system the site was wild Cerrado vegetation. The EucMG3 population was also sampled in 2007. It had been first planted with eucalyptus seedlings 21 years earlier, but a neighbouring eucalyptus plantation was planted to mango 33 years earlier.

Mango populations

Isolates from mango were collected from dead or dying trees in experimental plantings, small farms, private gardens or along streets. The ManRJ1 population was sampled in 2003 from trees along streets or private gardens in southwestern Rio de Janeiro. The isolates from population ManRJ2 were collected from 1999 to 2003 in northeastern Rio de Janeiro from small farms near the town of São Fidélis. Three isolates of the ManSP1 population were collected from experimental mango plantings at the Instituto Agronômico de Campinas in 2000; two isolates were sampled from a commercial plantation in 2001; and three isolates was received from Instituto Biológico de São Paulo (M. Barreto Figueiredo), but the exact location of the diseased trees was unknown (Baker *et al.*, 2003; Thorpe, 2004). Isolates of the ManMS population were collected in 2008 from mature, diseased street trees in the city of Aquidauana, Mato Grosso do Sul, where the disease had recently been recognized.

Populations on other hosts

The GmePA population was collected in 1996 from a small number of planted trees of *G. arborea* in a large plantation project near the Jari River in the state of Pará, near the mouth of the Amazon River (Muchovej *et al.*, 1978; Fearnside, 1988). The 20 isolates of the fig population FicSP2 were sampled in 2001 and 2002 from commercial plantations in the vicinity of Valinhos, São Paulo (Valarini & Tokeshi, 1980), the primary area of commercial fig production in Brazil. The inhame population ColSP3 consisted of isolates obtained in 2002 from inhame corms with black rot symptoms purchased directly from farmers in distribution markets or a small grocery store near the major inhame-growing region of Piedade, São Paulo (Harrington *et al.*, 2005).

DNA extraction

Two methods were used to obtain DNA from cultures for use as template in polymerase chain reactions (PCR). Isolates were grown in 25 mL liquid medium of 2% malt extract and 0·2% yeast extract (MYE) at room temperature for 2 weeks, and DNA extraction followed the method of Engelbrecht *et al.* (2004) or a CTAB-based protocol (Murray & Thompson, 1980).

Microsatellite markers

The present study analysed 15 microsatellite loci (CfAAG8, CfAAG9, CfCAA9, CfCAA10, CfCAA15, CfCAA38, CfCAA80, CfCAT1, CfCAT3K, CfCAT1200, CfCAG5, CfCAG15, CfCAG900, CfGACA60 and CfGACA650) developed from the total genomic DNA of an isolate of *C. cacaofunesta* (Steimel *et al.*,

2004) and used in population studies on *C. cacaofunesta* (Engelbrecht *et al.*, 2007b) and *C. platani* (Engelbrecht *et al.*, 2004; Ocasio-Morales *et al.*, 2007). Of the 16 loci used in earlier studies, one (CfCAT9X) was not utilized because its alleles could not be consistently resolved with some of the Brazilian isolates.

For each primer pair specific to the flanking regions of the 15 simple sequence repeat regions, one of the primers was fluorescently labelled. PCR amplifications of all microsatellite loci were performed using a 96-well thermal cycler (PTC-100; MJ Research Inc.). Cycling conditions were a hot start at 85°C for 2 min and an initial denaturing step of 95°C for 95 s, then 35 cycles of 58°C for 60 s, 72°C for 72 s and 94°C for 30 s, followed by 58°C for 1 m, with a final extension at 72°C for 30 min. Each reaction (20 μL) contained 2 μL 10X reaction buffer (Promega Inc.), dNTPs at 200 μM each, 4 mM $MgCl_2$, 5·0 pmol each primer, 0·5 U *Taq* DNA polymerase (Promega Inc.) and 1–2 μL template DNA (50–300 ng μL^{-1}).

The PCR products were electrophoresed using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc.). Band sizes of the products were determined using marker standards and ABI GENESCAN analysis software v3.1.2 and GENOTYPER 2.0 software (Applied Biosystems Inc.). Each product length (within 1 bp) was considered a different allele. For most loci, alleles differed by increments of 3 bp. Earlier studies (Engelbrecht *et al.*, 2004, 2007b; Steimel *et al.*, 2004) used a different automatic scanning system that utilized polyacrylamide gel electrophoresis, and there were some differences in the band sizes (±2 bp) between alleles using the capillary system and the polyacrylamide system (Ocasio-Morales *et al.*, 2007). Select isolates were run on both the capillary and polyacrylamide systems to determine the necessary adjustment in allele sizes for each locus. Allele sizes of isolates from *C. platani* (southeastern USA), *C. cacaofunesta,* the sweet potato isolates and some eucalyptus isolates of *C. fimbriata* that were determined by the polyacrylamide system in earlier work (Engelbrecht *et al.*, 2004, 2007b; Steimel *et al.*, 2004) were converted to those of the capillary system for analyses.

Analyses

Nei's gene diversity for each population was calculated without and with clone-corrected data using POPGEN 1.32 software (Yeh & Boyle, 1997). Clone-corrected datasets were a subset of the population left after removing isolates that were genetically identical, that is, a genotype within a population was counted only once. Weir & Cockerham's (1984) theta (θ), which is comparable to Wright's F_{ST}, was calculated among pairs of populations using the program MULTILOCUS (Mac version 1.21). Nei's genetic distance between populations and UPGMA (unweighted pair group method with arithmetic mean) dendrograms were constructed using POPGEN 1.32. Bootstrap values for branches of the population trees were calculated from 100 replicates using SEQBOOT, GENDIST, NEIGHBOR and CONSENSE in PHYLIP version 3.6 (Felsenstein, 1993). Relationships among genotypes were also examined using genetic distance (Nei's) matrices, UPGMA trees, and 1000 bootstrap replications generated with PAUP* (Swofford, 1998).

Partition of total variance using analysis of molecular variance (AMOVA) on Euclidean distances was performed on mango and eucalyptus populations using ARLEQUIN 2.0 (Excoffier *et al.*, 2005). The significance of the variance components associated with different levels of genetic structure (geographic regions and populations) was tested using nonparametric permutations procedures (Barton & Slatkin, 1986).

To test for random mating within mango and eucalyptus populations, linkage disequilibrium was analysed using the index of association (I_A) statistic in MULTILOCUS. The populations were clone-corrected before analysis. Randomization procedures (1000 replications, without replacement) were used to test the significance of the departure of the observed I_A value from zero (randomly mating population).

Mating experiments

Representative tester isolates from the five Brazilian hosts (*G. arborea*, eucalyptus, mango, fig and inhame) and representative testers of an isolate of *C. fimbriata* from sweet potato, *C. cacaofunesta* and *C. platani* (Engelbrecht & Harrington, 2005) were used in mating experiments. Each pairing was attempted at least twice.

Isolates of *C. fimbriata* are of two mating types, MAT-1 and MAT-2, but MAT-2 isolates are capable of selfing through unidirectional mating-type switching (Harrington & McNew, 1997). Self-sterile, single-ascospore progeny (MAT-1) of isolates were recovered from selfings of MAT-2 field isolates. For MAT-2 testers, spontaneous, self-sterile mutants were recovered from sectors of otherwise self-fertile isolates. These MAT-2 sectors generally lacked protoperithecia and thus could not form perithecia through selfing and were only used as males in mating tests. To confirm that they still had the *MAT1-2* gene, genomic DNA of each sector was extracted, and PCR was performed to amplify a portion of the *MAT1-2* gene following the protocol of Engelbrecht & Harrington (2005) using primers CFM2-1 (5′-GCTACATTTTGT ATCGCAAAGAC-3′) and CFM2-2 (5′-TAGTGGGG ATATGTCAACATG-3′).

The MAT-1 testers (serving as females) were grown on MYEA (MYE plus 1·5% agar) for 7 days at room temperature and then spermatized by MAT-2 testers. The conidial suspension of the MAT-2 tester was prepared by flooding a 7-day-old MYEA plate with 10 mL sterilized deionized water (SDW), scraping the mycelium with a sterile spatula, and filtering the suspension through four layers of sterile cheesecloth. Then, 1 mL of inoculum was dispersed over the MAT-1 colony.

Cultures were observed for 4 weeks for the presence of perithecia and ascospore masses. Ascospore masses were examined microscopically (×400) to see if there were

abundant, normal-appearing ascospores (indicative of an interfertile cross) or if there were few or no ascospores, or if the ascospores were misshapen, which is typical of an interspecific cross or hybrid (Harrington & McNew, 1998; Harrington *et al.*, 2002). Also, ascospore masses from one or more perithecia of a cross were streaked onto fresh MYEA to observe whether the ascospores were viable and if the progeny had a uniform mycelial morphology, indicating an induced selfing had occurred, or if progeny showed the mycelial phenotypes of the two parents, indicating a successful cross (Harrington & McNew, 1998).

Single-ascospore progeny were recovered from selected pairings by dispersing an ascospore mass into Isopar M (a light, sulfur oil; Exxon Corp.) in a deep-glass slide and streaking onto MYEA (Harrington & McNew, 1997). Recovered single-ascospore progeny were tested for meiotic segregation of microsatellite markers. Genomic DNA from each of the progeny was extracted using the Prepman Ultra kit (Applied Biosystems) and PCR amplification was performed using fluorescently-labelled and unlabelled primers flanking each of three microsatellite loci (CAA80, CAT1200, and CAA38 or CAA9) described in Steimel *et al.* (2004). The sizes of PCR products (alleles) were determined as described earlier, and comparisons were made between progeny and their respective parents. Segregation of the three microsatellite markers among the progeny (deviation from 1:1:6, male-parental:female-parental:non-parental genotype) was analysed using a chi-squared test (SAS statistical software; SAS Institute).

Results

Population analyses

Of the 15 microsatellite loci, 14 were polymorphic among isolates of the 13 Brazilian populations of *C. fimbriata* (Table 2). When previous data from the sweet potato form of *C. fimbriata* and populations of *C. cacaofunesta* and *C. platani* were included, all loci were polymorphic, each locus had between two and 27 alleles, and there were 106 microsatellite genotypes identified from among the 278 isolates tested (Table 2). Only one microsatellite genotype was found in more than one population; the five isolates from population ManMS in Mato Grosso do Sul had an identical genotype, and this genotype was also found in one of the mango isolates from the São Paulo population (ManSP1).

Nei's gene diversity (H) was calculated for each of the 13 Brazilian populations of *C. fimbriata* (Table 1). A second calculation of gene diversity was made using only unique genotypes from each population (clone-corrected for population), and these values were compared to those of a worldwide collection of *C. fimbriata* isolates from sweet potato, populations of *C. cacaofunesta*, and three populations of *C. platani*. Relatively high levels of diversity were found in populations EucBA1, EucBA2b, EucMG1, EucMG2, EucMG3, ManRJ1 and ManSP1, as well as in the Rondônia population of *C. cacaofunesta* and in the eastern North American population of *C. platani*, which are putatively native populations (Table 1). A genetic diversity value of 0·0 (i.e. all isolates from the population had exactly the same genotype) was found in populations EucBA2a, ManMS and GmePA. Relatively low values of diversity were found in populations ManRJ2, FicSP2 and ColSP3, as well as the putatively introduced populations of the sweet potato population (IpoWW), the Ecuadorian, Costa Rican, Colombian and Bahian populations of *C. cacaofunesta*, and the California and European populations of *C. platani* (Table 1).

Weir & Cockerham's theta (θ) was used to estimate the degree of differentiation between populations. Theta varies from 0·0 (no differentiation among populations) to 1·0 (populations completely differentiated). Most of the eucalyptus and mango populations were similar to each other, with θ values ranging from 0·082 to 0·629, with the exception of the ManMS and ManRJ2 populations (Table 3). The ManMS population was represented by a single genotype, which was least differentiated from the ManSP1 population (θ = 0·356). The ManRJ2 population was differentiated from all other populations ($\theta \geq$ 0·754). Aside from the comparisons among populations from mango and eucalyptus, all other θ values were above 0·553, indicating substantial differentiation among the populations.

The UPGMA tree constructed using allele frequencies of populations showed that the 13 Brazilian populations, the population on sweet potato, two populations of *C. cacaofunesta* and the eastern USA population of *C. platani* were relatively distinct, with few populations connected by short branch lengths or with bootstrap support (Fig. 2). The branch connecting populations ManSP1 and ManMS had weak bootstrap support (66%), as did the branch connecting all the eucalyptus populations (46%) (Fig. 2).

The UPGMA trees constructed using genotypes separated the isolates into nine groups: PlaUS, CacBR, CacEC, FicSP2, ManRJ2, ColSP3, GmelPA, IpoWW and all the populations on eucalyptus and mango except ManRJ2 (Fig. 3). There was moderate support for branches grouping the genotypes from populations ColSP3 (82%), CacEC (51%) and FicSP2 (51%). The genotypes from ManRJ2 grouped separately from other mango genotypes. Aside from the ManRJ2 population, the genotypes from mango and all the genotypes from eucalyptus appeared to be related to each other (Fig. 3), and the eucalyptus isolates and mango isolates tended to be in two clusters, one dominated by genotypes from mango (except the ManRJ2 genotypes) and another dominated by genotypes from eucalyptus (Fig. 4). However, there was no bootstrap support for the branches separating the mango and eucalyptus clusters.

Because most of the populations from eucalyptus and mango had relatively high levels of diversity and appeared to form natural populations, they were grouped into geographic regions and AMOVA was utilized to determine the amount of variation attributable to regions,

Table 2 Microsatellite alleles found for 15 loci among isolates of 17 populations of *Ceratocystis fimbriata*, *C. cacaofunesta* and *C. platani*

	Locus														
Population	AAG8	AAG9	CAA9	CAA10	CAA15	CAA38	CAA80	CAT1	CAT1200	CAG5	CAG15	CAG900	GACA60	GACA650	CAT3K
EucBA1	C	C	B, C	A, B, C	B, C	A, H, L, M	C, D, G	D	B, C, D	C	A, B	A	A, B, C	C, E	A
EucBA2a	C	C	B	B	B	M	D	D	C	C	A	A	B	C	A
EucBA2b	B, C	C	A, B, I	A, B	A, B	E, I, M, N	D, F	D, E	C	C	A	A	B, C	C	A
EucMG1	C, D	B, C	A, B, G, J	A, B, C	B, C	F, M, N, P	A, F, H, I	C, D	B, C	C	A, C, D, E, F, H, J	A	B	C	A
EucMG2	C	C	A, B	B, C	A, B, C	M, N, O	A, D, F, H	D, E	C	C	E,F, I	A	C	C, D	A
EucMG3	C	C	B	A, C	B, C	M, N	A, F, G	D, E	C	B, C	A, E,F, I	A	C	C, D	A
ManRJ1	A, C, D	A, C	B	A, B, C, Null	B, C	A, B, M	D, G, H, K	B, D	B, C	A, C, F	A, I, K, M	A	B, C	C, E	A, F
ManSP1	A, C	C	B, E	A, B, C	B, C	C, I, J, M	E, F,H, J	C, D	A, B, C	C	B, E, I, L	A	B	C,E	C
ManMS	A	C	E	A	C	J	J	C	B	C	I	A	B	E	C
ManRJ2	E,F	A	H	A	D	D	D	A, D	A,D,E	D, E	G, I	A	B, E	D	B
FicSP2	C	D	D, E	B	D	G	H	B	F	G	M	A	B	A	F
ColSP3	C	D, H	F	A	B, C	K	A, E	A, D	A	E	F, H	A	B, D	B, C	C
GmePA	A	B	B	D	C	E	D	G	E	C	B	A	B	C	D
IpoWW	A, E	C	E	C	B	R	A	C	C	C	N	A	B	E	B
PlaUS	A	E	V, X, Y, Z, W, AA	E	H, E	C, Q	N, O, P	C	I, J	E	P, Q, R, S, T, U, V	A	B	F, G, H, I, J, K	B, D, F
CacBR	E, H, I	E	L, N, Q, R, S, T, U	A, B	B, C, F	L, N, S, T	B, E, M	C, H, I	C	H	E, F, G	A	B, F	A, D, F	D, E
CacEC	G	F, G	K, M, O, R	B	G	C	L	B	G, H	E	B, I, O	B	B	A	F
No. alleles	9	8	27	5	8	20	16	9	10	8	22	2	6	11	6
Size range (bp)	171–232	393–416	156–406	124–136	286–343	132–271	265–331	246–269	370–406	298–338	174–396	189–192	184–219	209–327	309–330

Different letters in the same column represent different alleles for that locus based on different sizes (base pairs) of the PCR product.

Table 3 Estimate of differentiation (theta, θ) among 17 populations of *Ceratocystis fimbriata*, *C. cacaofunesta* and *C. platani* using 15 microsatellite loci

Population	EucBA1	EucBA2a	EucBA2b	EucMG1	EucMG2	EucMG3	ManRJ1	ManSP1	ManMS	ManRJ2	FicSP2	ColSP3	GmePA	Ipo	CacBR	CacEC	PlaUS
EucBA1																	
EucBA2a	0·467																
EucBA2b	0·405	0·483															
EucMG1	0·313	0·298	0·264														
EucMG2	0·388	0·531	0·202	0·217													
EucMG3	0·459	0·581	0·202	0·194	0·082												
ManRJ1	0·406	0·504	0·466	0·324	0·384	0·422											
ManSP1	0·507	0·629	0·500	0·377	0·432	0·482	0·267										
ManMS	0·737	1·000	0·800	0·667	0·759	0·794	0·572	0·356									
ManRJ2	0·804	0·896	0·817	0·768	0·804	0·820	0·754	0·780	0·891								
FicSP2	0·828	0·981	0·908	0·792	0·888	0·908	0·775	0·836	0·983	0·923							
ColSP3	0·750	0·905	0·775	0·698	0·736	0·753	0·679	0·692	0·892	0·856	0·934						
GmePA	0·724	1·000	0·794	0·658	0·740	0·787	0·623	0·666	1·000	0·885	0·983	0·907					
IpoWW	0·792	0·989	0·872	0·722	0·847	0·863	0·746	0·761	0·987	0·917	0·984	0·941	0·991				
CacBR	0·778	0·823	0·758	0·730	0·769	0·769	0·759	0·751	0·811	0·808	0·852	0·811	0·830	0·816			
CacEC	0·778	0·901	0·776	0·733	0·781	0·805	0·691	0·727	0·899	0·867	0·914	0·869	0·897	0·936	0·766		
PlaUS	0·756	0·805	0·802	0·712	0·748	0·758	0·697	0·685	0·766	0·787	0·842	0·791	0·784	0·810	0·762	0·827	

Shaded values (θ < 0·5) indicate relatively low differentiation between populations.

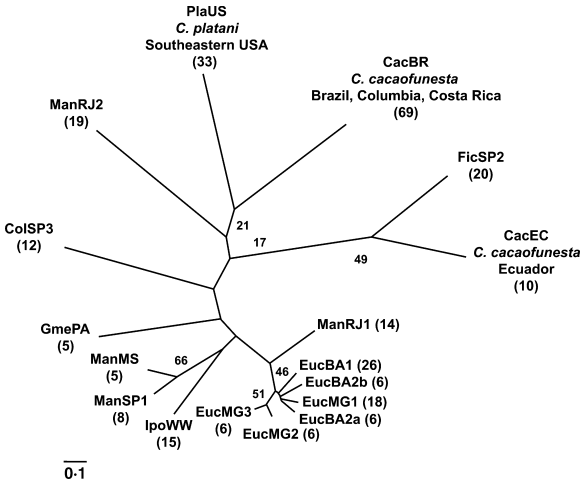

Figure 2 Dendrogram of *Ceratocystis fimbriata*, *C. cacaofunesta* and *C. platani* populations generated by UPGMA (unweighted pair group method, arithmetic mean) based on allele frequencies of 15 microsatellite loci. Bootstrap values are shown alongside the branches. The number of isolates sampled from each population is in parentheses. SP, São Paulo; BA, Bahia; RJ, Rio de Janeiro; MG, Minas Gerais; MS, Mato Grosso do Sul, PA, Pará; WW, worldwide.

among populations within regions, and within populations (Table 4). The single-genotype populations ManMS and EucBA2a were considered atypical and were excluded from the analysis. The results showed that most (48%) of the variation was attributable to variation among regions, 41% to the diversity within populations, and only 11% to variation among populations within regions.

The index of association (I_A) was used to test for random mating (using clone-corrected data) in the ManRJ1, ManRJ2, ManSP1, EucBA1/EucBA2b and EucMG1/EucMG2/EucMG3 regions (Table 5). The value of I_A is expected to be near zero in a randomly mating population, and 1000 randomized replicates were performed to assess whether the value obtained was significantly different from zero. Considering all populations together, the I_A value obtained showed evidence for non-random mating or asexual reproduction (I_A = 2·7, P < 0·001). The populations in the five regions each showed significant departures from the values expected for random mating, with the three EucMG populations showing the lowest value of I_A and the São Paulo mango population showing the highest value I_A.

Mating studies

Testers from isolates of each of the five Brazilian host groups successfully crossed with the majority of the other Brazilian testers of opposite mating type (Table 6). In most of these crosses, there were many more than 25 perithecia with normal ascospore masses per plate. In successful crosses, perithecia usually developed within a week and produced thick, creamy ascospore masses at the tips of perithecial necks.

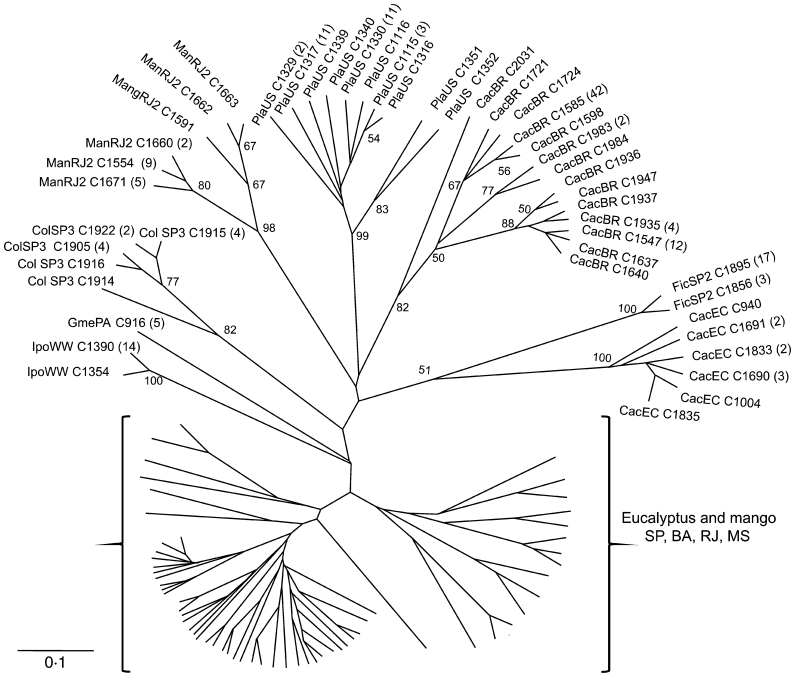

Figure 3 Dendrogram of *Ceratocystis fimbriata, C. cacaofunesta* and *C. platani* genotypes generated by UPGMA (unweighted pair group method, arithmetic mean) based on Nei's genetic distance. Bootstrap values greater than 50 are shown alongside the branches. If two or more isolates shared a genotype, only one isolate number is listed, but the number of isolates with that genotype is in parentheses. SP, São Paulo; BA, Bahia; RJ, Rio de Janeiro; MS, Mato Grosso do Sul; PA, Pará; BR, Brazil; EC, Eucador; WW, worldwide.

Analyses of single-ascospore progeny from thick, creamy ascospore masses demonstrated that the ascospore masses were not from an induced selfing. Single-ascospore progeny produced colonies of the mycelial morphology of the male and female parents in a roughly 1:1 ratio. Ten sets of progeny from 10 crosses were analysed for three microsatellite markers (Table 7). Eight to 24 progeny were tested for each set of progeny. All sets showed normal segregation (not deviating from 1:1:6 by the chi-squared test) for male-parental:female-parental:non-parental types (Table 7), confirming that there had been a successful cross and meiotic segregation of three unlinked loci.

Some of the female testers performed poorly in crosses, perhaps through loss of femaleness (poor protoperithecia or development of perithecia). Four female/MAT-1 testers (C925, C1590, C1783 and C1858) usually produced only a few perithecia per plate when paired with what appeared to be compatible MAT-2 testers (Table 6). Three of the four female/MAT-1 testers (C918, C920 and C925) from *G. arborea* formed few or no perithecia with ascospores when spermatized with MAT-2 testers from eucalyptus, mango, or inhame. However, when MAT-2 testers from *G. arborea* were used as males, they successfully crossed with all of the female/MAT-1 testers from Brazil.

The Brazilian isolates did not successfully cross with testers of *C. platani* or *C. cacaofunesta*, except when fewer than 25 perithecia with ascospores formed between the female/MAT-1 tester C1657 from mango and the male/MAT-2 *C. platani* tester (Table 6). By contrast, when the MAT-2 tester of *C. fimbriata* from sweet potato was used as a male, abundant perithecia with numerous ascospores were produced by many of the Brazilian female/MAT-1 testers (Table 6). The crossings between the male tester from sweet potato and the female/MAT-1 tester from eucalyptus (C1347) and female/MAT-1 tester from *G. arborea* (C924) were confirmed by the presence of recombinant progeny as determined by microsatellite markers (Table 7). By contrast, when the female/MAT-1 tester from sweet potato was spermatized with male/-MAT-2 testers of Brazilian isolates, few perithecia were produced, the ascospore masses from these perithecia were watery, not creamy, and microscopic examination showed there to be misshapen ascospores and aborted asci in the perithecial centrum.

Discussion

Ceratocystis fimbriata populations on mango in São Paulo and Rio de Janeiro and on eucalyptus in Minas Gerais and Bahia have gene diversity (*H*) values ranging from 0·1889 to 0·3813, comparable to the values (using the same microsatellite markers) of what have been considered native populations of other homothallic species in the *C. fimbriata* complex (Engelbrecht *et al.*, 2004, 2007b). Using other neutral markers, similar values were found for putatively native populations of *Ceratocystis albifundus* in South Africa (Roux *et al.*, 2001; Barnes

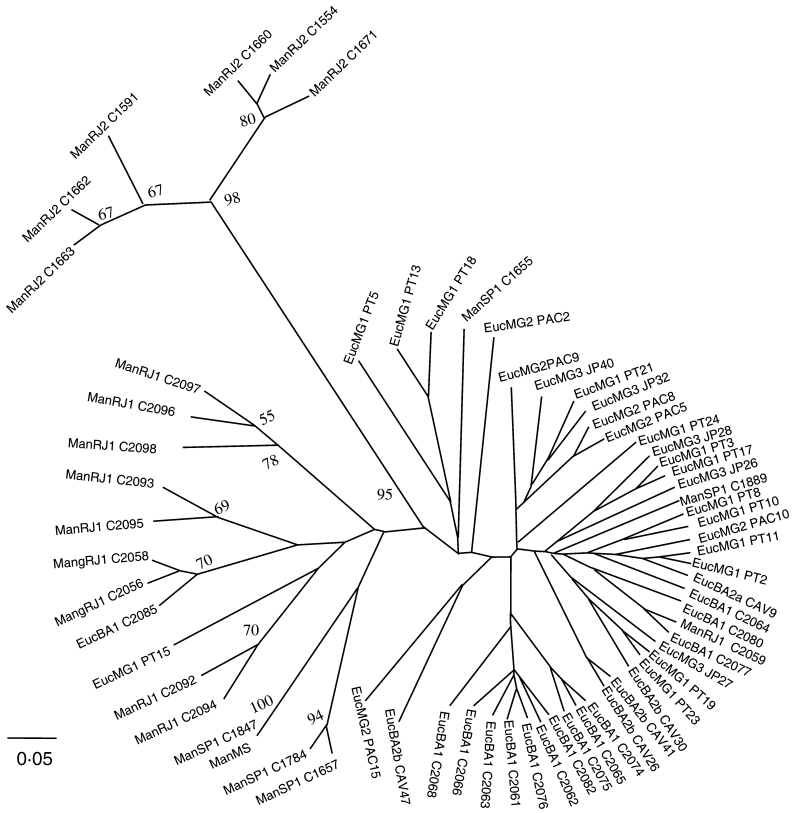

Figure 4 Dendrogram generated by UPGMA (unweighted pair group method, arithmetic mean) based on Nei's genetic distance among *Ceratocystis fimbriata* populations on *Eucalyptus* spp. and *Mangifera indica* in Brazil. Bootstrap values greater than 50 are shown alongside the branches. Isolate numbers following a 'C' are stored at Iowa State University; all other isolates are stored at the Universidade Federal de Viçosa. SP, São Paulo; BA, Bahia; RJ, Rio de Janeiro; MG, Minas Gerais; MS, Mato Grosso do Sul.

Table 4 Analysis of molecular variance (AMOVA) of *Ceratocystis fimbriata* populations on eucalyptus and mango in five regions in Minas Gerais, Bahia, Rio de Janeiro and São Paulo in Brazil based on 15 microsatellite loci

Source of variation	d.f.	Sum of squared deviations	Variance components	Proportion of variance components (%)	P^a
Among regions[b]	4	216·940	2·270	48·14	<0·001
Among populations within regions	3	19·778	0·528	11·20	<0·001
Within populations	95	182·204	1·917	40·66	0·003
Total	102	418·922	4·716		

[a]The *P* value is for the null hypothesis that there is no significant variation at that level based on 1023 permutations.
[b]The populations were distributed in five regions: (1) ManRJ1; (2) ManRJ2; (3) ManSP1; (4) EucBA1 and EucBA2b; (5) EucMG1, EucMG2 and EucMG3.

et al., 2005). Gene diversity values are relatively low, but expected, for homothallic *Ceratocystis* species (Harrington *et al.*, 1998). High index of association values found in populations from eucalyptus and mango suggest that much of the reproduction is asexual or via selfing, as found in *C. cacaofunesta* (Engelbrecht *et al.*, 2007b), *C. platani* (Engelbrecht *et al.*, 2004) and *C. albifundus* (Barnes *et al.*, 2005). Selfing and asexual reproduction, as well as limited dispersal by insects or in insect frass, should lead to isolated populations that are highly differentiated from each other and with relatively little gene flow between them (Harrington *et al.*, 1998; Harrington, 2000; Baker *et al.*, 2003).

Although some of the Brazilian populations were highly differentiated from each other, mating studies indicated that they constitute a single biological species that is interfertile with *C. fimbriata* from sweet potato on which the species is based (Engelbrecht & Harrington, 2005). By contrast, crosses between different species of *Ceratocystis* produce no interaction when paired, or they form only a few perithecia with few ascospores that are misshapen, germinate poorly and produce colonies of

Table 5 Index of association of *Ceratocystis fimbriata* populations on eucalyptus and mango in five regions in Bahia, Minas Gerais, Rio de Janeiro and São Paulo, Brazil

Population	Host	Location(s)	Index of association (I_A)	Probability[a]
ManRJ1	Mango	Southwestern Rio de Janeiro	1·037	<0·001
ManRJ2	Mango	Northeastern Rio de Janeiro	0·745	0·044
ManSP1	Mango	São Paulo	1·913	<0·001
EucBA1, EucBA2b	Eucalyptus	Eunápolis and Caravelas, Bahia	0·658	0·010
EucMG1, EucMG2, EucMG3	Eucalyptus	Curvelo, João Pinheiro, and Paracatu, Minas Gerais	0·583	0·002
All populations			2·680	<0·001

[a]Probability that the index of association does not differ from a purely sexually outcrossing population.

Table 6 Mating experiments between strains representing different populations of *Ceratocystis fimbriata, C. platani* (from *Platanus* spp.), *C. cacaofunesta* (from *Theobroma cacao*) using MAT-2, self-sterile sector (sec) strains as males and MAT-1 self-sterile (ss) strains as females

		MAT-2, Male[a]								
		Gmelina arborea		*Eucalyptus*		Mango	Inhame	Sweet potato	*Platanus*	*Cacao*
Host	MAT-1, Female[a]	920sec (GmePA)	925sec (GmePA)	1347sec (EucBA2)	1440sec (EucBA2)	1657sec (ManSP1)	1907sec (ColSP2)	1418sec (IpoWW)	1317sec (PlaUS)	1587sec (CacBR)
Gmelina arborea	918ss (GmePA)	C	C	–	C (1)	–	–	–	H (2)	–
	920ss (GmePA)	C	C	–	–	–	–	–	–	–
	924ss (GmePA)	C	C	C	C	C[b]	C	C[b]	H	–
	925ss (GmePA)	C	C	C (Few)	C (2)[b]	C (2)	–	C (1)	H (Few)	–
Eucalyptus	1347ss (EucBA2)	C[b]	C	C	C	C (Few)	–	C[b]	H (Few)	–
	1440ss (EucBA2)	C	C[b]	C	C	C	C (Few)	C (1)	H (Few)	–
Mango	1590ss (ManRJ2)	C (Few)	C	C	C (Few)	C (Few)	C (Few)	C	H	–
	C1657ss (ManSP1)	C	C[b]	C[b]	C	C	C	C	C (Few)	–
Fig	1783ss (FicSP3)	C (Few)	C	C	C (Few)	C (1)	C (Few)	C (1)	–	–
	1858ss (FicSP3)	C (Few)[b]	C	C	C	–	C (Few)	–	H (Few)	–
Inhame	1907ss (ColSP2)	C	C	C	C[b]	C	C	C	H	–
	1926ss (ColSP2)	C	C	C	C	C (Few)	C	C (Few)	H	–
Sweet potato	1418ss (IpoWW)	H	H	H	H	H	H	C	H	–
Platanus	1317ss (PlaUS)	H	–	H	H	H	H	H	C	–
Cacao	1587ss (CacBR)	H	H	–	H (Few)	H	H	H	H	–

C: successful cross with many perithecia per plate (greater than 25) and apparently normal ascospore masses and the mycelial morphology of each parent found among the progeny; H: hybrid cross with either watery ascospore masses of few spores and no germination of ascospores when plated on MYEA, typical of a cross between two different species. –, no perithecia produced.
In parentheses: number indicating the actual number of perithecia with ascospore masses; Few, less than 25 perithecia produced.
[a]sec, strains from MAT-2, self-sterile sectors recovered from self-fertile isolates; ss, MAT-1, female-competent isolates with protoperithecia.
[b]Progeny compared to parents for segregation of microsatellite markers (see Table 7).

aberrant morphology and aberrant segregation of markers (Harrington & McNew, 1997; Harrington *et al.*, 2002; Engelbrecht & Harrington, 2005; Johnson *et al.*, 2005). The genetic analyses of progeny from crosses between the different Brazilian populations and with the male tester of *C. fimbriata* from sweet potato demonstrated that the ascospore masses were the result of a crossing of two sexually compatible parents. With one exception, the Brazilian testers were not interfertile with *C. platani* or *C. cacaofunesta*. Thus, no biological species could be distinguished among the Brazilian isolates and *C. fimbriata sensu stricto*. Phylogenetic analyses of rDNA and DNA sequences of a mating type gene also failed to distinguish Brazilian host-based lineages and *C. fimbriata* isolates from sweet potato, although isolates of *C. platani* and *C. cacaofunesta* were phylogenetically distinct (Baker *et al.*, 2003; Thorpe, 2004; Thorpe *et al.*, 2005). More extensive phylogenetic analysis is needed to more clearly delineate clades and potential species within the Latin American clade of the *C. fimbriata* complex.

Based on genetic distance analyses of populations and genotypes, as well as values of theta, the eucalyptus and mango populations from Minas Gerais, Bahia, São Paulo and southwestern Rio de Janeiro were closely related. The analysis of molecular variance showed that most of the genetic variation among these populations was attributable to variation among regions and variation within populations, and little to variation among populations within regions. Thus, even in this limited part of Brazil, there are regional differences among populations.

Table 7 Segregation of alleles of three microsatellite loci among single ascospore progeny from crosses between strains of *Ceratocystis fimbriata* from Brazil or a sweet potato strain (C1418) from North Carolina, USA

Parents[a]						
Male parent	Female parent	Total progeny	Male parental type	Female parental type	Non-parental type	Deviation from 1:1:6 (*P*)[b]
C1347sec	C1657ss	12	0	0	12	0·1801
C920sec	C1347ss	20	3	5	12	0·5455
C1440sec	C925ss	8	3	3	2	0·1353
C925sec	C1657ss	10	1	2	7	0·8967
C920sec	C1858ss	8	1	2	5	0·8089
C1418sec	C1347ss	10	0	4	6	0·2397
C1440sec	C1907ss	20	5	4	11	0·4076
C1657sec	C924ss	19	2	2	15	0·9591
C925sec	C1440ss	17	6	4	7	0·1290
C1418sec	C924ss	24	1	5	18	0·4724
Total		148	22	31	95	0·0953

[a]sec: self-sterile MAT-2 strain; ss: MAT-1 strain.
[b]Probability of lack of deviation from 1:1:6 segregation of male parental:female parental: non-parental genotypes based on chi-squared tests.

The eucalyptus plantations that had the *C. fimbriata* populations with the highest genetic diversity were on sites that were formerly Cerrado forest or were previously farmland. It is presumed that the eucalyptus trees were infected from soilborne inoculum (Laia *et al.*, 2000), probably in the form of aleurioconidia that were originally formed in the wood of infected wild or cultivated trees, perhaps expelled from the trees by the tunnelling of ambrosia beetles or other wood-boring insects (Viégas, 1960; Iton & Conway, 1961; Goitia & Rosales, 2001; Ocasio-Morales *et al.*, 2007).

Surprisingly, few native hosts of *C. fimbriata* have been identified in Brazil, and then only on cultivated native hosts, such as *Hevea brasiliensis* (CAB International, 2005). However, the high incidence of the disease and relatively high genetic diversity of the pathogen in eucalyptus plantations of Minas Gerais on sites that were recently Cerrado forest suggest that *C. fimbriata* is native to this relatively dry forest type. At least one of the diverse Bahia populations (EucBA1) was on a site that was previously a farm, but the natural vegetation history of the Bahia populations and the sites of the mango isolates is not known. However, these regions of Bahia, Rio de Janeiro and São Paulo would have been Mata Atlântica rainforest prior to agriculture or urban development. Thus, the region of the greatest population diversity of *C. fimbriata* in Brazil spans the very different Cerrado and Mata Atlântica forest types.

One of the populations from mango, near the town of São Fidélis in northeastern Rio de Janeiro, differed substantially from the other populations in terms of UPGMA trees and values of theta. The level of gene diversity for the São Fidélis population was somewhat low, and it is possible that this population is not native. The isolates were from scattered, small farms, so greater diversity was expected. However, much of this region was in pasture before cattle grazing and there may not have been much woody vegetation prior to establishment of these small farms. Besides mango, cultivated annona (sugar-apple, *Annona squamosa*) is also a host in this area (Baker *et al.*, 2003; Silveira *et al.*, 2006), so the host range of this population appears to differ from the other populations on mango.

Some of the sampled populations in Brazil had little or no genetic diversity and were presumably the result of introductions of the fungus on plant propagative material, as observed in other populations of the *C. fimbriata* complex (Harrington, 2000; Engelbrecht & Harrington, 2005; Johnson *et al.*, 2005; Thorpe *et al.*, 2005; Engelbrecht *et al.*, 2004, 2007a,b; Ocasio-Morales *et al.*, 2007). Only five closely related genotypes were found among the 12 inhame isolates from a small commercial growing area in São Paulo, and the gene diversity value for this population was somewhat less than expected. Isolates of *C. fimbriata* and other *Ceratocystis* species were readily dispersed on corms of inhame and related Araceae (Thorpe *et al.*, 2005), and it was earlier speculated that much of the *C. fimbriata* on inhame in Brazil originated from São Paulo (Harrington *et al.*, 2005). Limited diversity in the population on inhame from São Paulo suggests that *C. fimbriata* may have been introduced from some other region on corms. Alternatively, selection for aggressiveness to inhame may have created a genetic bottleneck in an otherwise diverse local population of *C. fimbriata*.

Eucalyptus is usually planted in Brazil as rooted plants from field-collected cuttings emanating from fresh stumps or from special mini-hedges in nurseries (Alfenas *et al.*, 2004). The fungus can be introduced to new sites in symptomless, rooted cuttings (Baker *et al.*, 2003), and this is believed to be the case with plantation EucBA2a, where all six of the sampled isolates had exactly the same genotype, which was similar to the genotypes found at the site where the cuttings were collected (EucBA2b). Recent reports of *C. fimbriata* on eucalyptus in Africa and Uruguay (Barnes *et al.*, 2003; Roux *et al.*, 2004; van Wyk *et al.*, 2006) suggest that the fungus has been introduced to other countries on infected cuttings of eucalyptus. Likewise, *C. fimbriata* was probably introduced on mango nursery stock to Mato Grosso do Sul, where ceratocystis wilt was recently recognized. The mango

population on street trees in Mato Grosso do Sul was of a single genotype, and this genotype was also isolated from a mango tree in São Paulo, where there are many nurseries producing mango seedlings and ceratocystis wilt has been recognized for more than 70 years (Costa & Krug, 1935; Viégas, 1960).

Mango has been reported as a host of *C. fimbriata* only in Brazil (CAB International, 2005) until reports of serious mortality of mango in Oman (Al Adawi *et al.*, 2006) and Pakistan (Fateh *et al.*, 2006), where anecdotal evidence suggested that the pathogen was introduced from Brazil in mango seedlings or grafted material. The DNA sequences of mango isolates from Oman and Pakistan indicated that they were closely related to South American isolates of *C. fimbriata* and it was proposed that the mango pathogen in Oman and Pakistan be distinguished from *C. fimbriata* as a new species, *C. manginecans*, on the basis of the presence of barrel-shaped conidia (van Wyk *et al.*, 2007). Isolates of the sweet potato form of *C. fimbriata* do not produce barrel-shaped conidia (Engelbrecht & Harrington, 2005), but this character has been found to be variable among other isolates of the Latin American clade of *C. fimbriata* (T. C. Harrington, unpublished data). The ITS rDNA, β-tubulin and elongation factor 1-α sequences of the mango isolates from Oman and Pakistan (van Wyk *et al.*, 2007) are similar to those of other South American isolates of *C. fimbriata*, but DNA sequences of these genetic regions do not resolve Brazilian populations of *C. fimbriata* (T. C. Harrington, unpublished data). However, the history of ceratocystis wilt on mango and DNA sequence analyses suggest that *C. manginecans* is probably based on one or a few isolates of *C. fimbriata* from Brazil, perhaps from São Paulo. More detailed comparisons of mango isolates from Brazil, Oman and Pakistan are needed.

Gmelina arborea is known as a host of *C. fimbriata* only in Pará, near the mouth of the Amazon. By contrast, isolates from the other Brazilian hosts were from regions previously in the Mata Atlântica or Cerrado forest types. It was assumed that the fungus was native to the lower Amazon because *G. arborea* plants were observed within 2 years of planting of seedlings in recently cleared, natural rainforest (Muchovej *et al.*, 1978; Fearnside, 1988). Only five isolates from five *G. arborea* trees in a single plantation were sampled, and all were of a single, unique genotype, suggesting that they were not from natural, soilborne inoculum, but more isolates need to be sampled from more plantations.

The fig isolates were collected from various plantations in a small area where most of the commercial figs in Brazil are grown. Only two genotypes were identified among the 20 isolates studied. Two of the plantations were on sites previously in pasture for cattle, so it is likely that the fungus was introduced on vegetatively propagated figs. The disease in these and the other fig plantations appeared in discrete, circular foci of dead and dying trees, suggesting that one plant had been infected initially and the fungus spread to adjacent plants through root systems.

Isolates of *C. fimbriata* from Brazil vary in aggressiveness to various hosts in inoculation studies, but they do not appear to be highly host specialized, except that *G. arborea* isolates from Pará (population GemPA) are particularly aggressive on *G. arborea* seedlings, and two isolates from eucalyptus (population EucBA2) were especially aggressive on eucalyptus (Baker *et al.*, 2003; Thorpe, 2004; Thorpe *et al.*, 2005). Zauza *et al.* (2004) tested 18 commercial eucalyptus clones using the same two isolates from eucalyptus, and there was significant clone × isolate interaction in the amount of discoloration found in the inoculated hosts.

These inoculation studies and the population genetic analyses presented here suggest that geographically isolated populations of *C. fimbriata* tend to be particularly aggressive on different exotic hosts, or at least there appears to have been selection for genotypes with particular aggressiveness (Pariaud *et al.*, 2009). Most of the isolates studied have been from dead and dying cultivated plants, and thus the samples are biased towards the portion of the population aggressive to these exotic hosts. The most aggressive phenotypes may be further selected as they are spread and maintained on vegetatively propagated material. In the most extreme example of such a genetic bottleneck, it is likely that isolates from sweet potato around the world originated from a single, aberrant form (Steimel *et al.*, 2004) selected by humans on propagative material (storage roots), perhaps taken from northern South America, where cultivated sweet potato may have originated (Engelbrecht & Harrington, 2005). Thus, the name *C. fimbriata* is based on a single, highly selected form of the fungus on sweet potato, taken from an unstudied population, probably a population of wound-colonizers that originally varied in aggressiveness to a wide variety of hosts (Engelbrecht & Harrington, 2005; Johnson *et al.*, 2005).

Recognizing that populations of *C. fimbriata* tend to be geographically isolated and highly differentiated, care must be taken to distinguish between populations and species. Mechanisms of speciation and the species concept will continue to be controversial issues (Taylor *et al.*, 2000; Kohn, 2005), but a functional species definition is needed for important plant pathogens like *C. fimbriata*. Harrington & Rizzo (1999) proposed that a fungus species is 'the smallest aggregation of populations with a common lineage that share unique, diagnostic phenotypic characters.' Using phylogenetics, matings, inoculations and morphology, this definition was applied to the North American clade of the *C. fimbriata* complex, resulting in four newly recognized species (Johnson *et al.*, 2005). Within the Latin American clade, *C. cacaofunesta* and *C. platani* were distinguished by an ecologically significant phenotype (host specialization to native hosts), and phylogenetic analyses and intersterility tests indicated that *C. cacaofunesta* and *C. platani* are each monophyletic (Baker *et al.*, 2003; Engelbrecht &

Harrington, 2005). However, two geographically isolated populations (Ecuador vs. other populations) of the phenotypically uniform *C. cacaofunesta* (Baker *et al.*, 2003; Engelbrecht & Harrington, 2005; Engelbrecht *et al.*, 2007b) are highly differentiated based on microsatellite alleles. The microsatellite markers used in the present study are too sensitive to mutation and homoplasy to be useful for phylogenetic analyses (Dettman & Taylor, 2004). New microsatellite alleles have arisen in just decades after strains of *C. platani* were introduced to new regions (Engelbrecht *et al.*, 2004; Ocasio-Morales *et al.*, 2007), so *Ceratocystis* populations isolated by geography or propagated hosts can quickly accumulate unique mutations.

Although phylogenetic analyses of the Latin American clade have been limited, sequence analyses of multiple genes have failed to distinguish the Brazilian populations from each other or from the sweet potato form of *C. fimbriata* (Baker *et al.*, 2003; Thorpe, 2004; Thorpe *et al.*, 2005; T.C. Harrington, unpublished data). Furthermore, Brazilian isolates were interfertile with themselves and the male tester strain from sweet potato. More cryptic species will probably be delimited in the *C. fimbriata* complex, and perhaps more species will be recognized in Brazil. However, the evidence to date suggests that much of the variation in Brazil is found among geographically isolated populations of a single species, and these populations vary in aggressiveness to certain exotic hosts. The level of aggressiveness to some of these cultivated hosts and the ease of spread of these aggressive forms to new regions in propagative or woody material is becoming an increasing concern.

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