Movement of Genotypes of *Ceratocystis fimbriata* Within and Among *Eucalyptus* Plantations in Brazil

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ABSTRACT

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Ceratocystis wilt on eucalyptus, caused by *Ceratocystis fimbriata*, was first recognized in 1997 in the state of Bahia, Brazil, but is now known in five other states and in four other countries. *C. fimbriata* is a native, soilborne pathogen in some parts of Brazil but we hypothesized that genotypes of the pathogen have been moved among plantations in rooted cuttings collected from diseased trees and within plantations on cutting tools. We used six microsatellite markers to identify 78 genotypes of *C. fimbriata* among 177 isolates from individual trees in 20 eucalyptus plantations. The highest gene and genotypic diversity values were found in plantations on formerly wild Cerrado forest in Minas Gerais, suggesting that the fungus was in the soil prior to planting eucalyptus. In contrast, one or only a few genotypes were found in plantations on previous pastureland (with no woody hosts) in Bahia and São Paulo, and most of these genotypes were found in a Bahian nursery or in one of two Bahian plantations that were sources for rooted cuttings. Sources of cuttings tended to be dominated by one or a few genotypes that may have been spread within the plantation on cutting tools.

Additional keywords: Atlantic rainforest.

Eucalyptus spp. and their hybrids are the most planted forest species worldwide, and eucalyptus is among the most important crops in Brazil, covering over 4 million ha and typically producing wood at >40 m^3/ha/year (1,2). One of the most important diseases on eucalyptus in Brazil is Ceratocystis wilt, caused by the fungus *Ceratocystis fimbriata* Ellis & Halst., which is soilborne and appears to be native to some parts of Brazil but may be increasing in incidence and distribution (14). Most eucalyptus in Brazil is planted with rooted cuttings of fast-growing, hybrid clones of these Australasian species, and *C. fimbriata* can be disseminated in symptomless propagation material taken from diseased hedge plants in nurseries or infected stump sprouts from harvested plantations (4,18).

Once inside the host, *C. fimbriata* generally colonizes the xylem, including radial parenchyma cells, eventually darkening infected tissues and causing wilting and death of the whole plant or individual branches (3,16). Eucalyptus plants may be infected through their roots by soilborne inoculum in the form of aleurioconidia (21), or they may be infected through fresh wounds by inoculum in the form of conidia or ascospores on tools or on insects, or the pathogen may be dispersed in airborne or rain-splashed insect frass (7,10). For all but human-facilitated spread, dispersal is for relatively short distances.

In addition to eucalyptus, *C. fimbriata* causes lethal, wilt-type diseases or cankers in other important plantation or orchard crops in Brazil, such as mango (*Mangifera indica*), *Gmelina arborea*, rubber tree (*Hevea brasiliensis*), inhame (*Colocasia esculenta*), and fig (*Ficus carica*) (6,14,20). Brazilian isolates of *Ceratocystis fimbriata* vary greatly in aggressiveness to different hosts but there does not appear to be strong host specialization (20). Although *C. fimbriata* has been recognized as a pathogen on other hosts in Brazil for more than 75 years (14), *C. fimbriata* was first observed on eucalyptus in the south of Bahia in 1997 (15). Isolates from eucalyptus in Bahia and Minas Gerais, where the disease was recognized in 2003, are genetically related to some *C. fimbriata* isolates from mango (14), though mango isolates are not particularly aggressive on eucalyptus clones, and even eucalyptus isolates vary substantially in their aggressiveness to various eucalyptus clones (4,20,36). The disease on eucalyptus is now common in Bahia and was discovered from 2001 to 2006 in six other states (Fig. 1) (3,16; A. C. Alfenas, *unpublished*). Ceratocystis wilt on eucalyptus also has been recognized in Uruguay (5) and Africa (31,34), where *C. fimbriata* may have been introduced from Brazil (14,20).

Populations of *Ceratocystis* spp. introduced on contaminated tools or propagative material typically show little or no genetic variation because of genetic bottlenecks and lack of sexual outcrossing (4,6,10–12,14,18,28,34). *Ceratocystis* spp. readily produce perithecia and sticky ascospore masses, which are suitable for insect dispersal, but *C. fimbriata* is homothallic, and most reproduction is via selfing or is asexual (14). An introduced population of a homothallic *Ceratocystis* sp. may remain genetically uniform for decades (11,12,28). However, some populations of *C. fimbriata* on eucalyptus from Bahia and Minas Gerais have substantial diversity (14). Other populations on eucalyptus show limited genetic diversity and may be derived from introductions in infected cuttings (14). Some plantations may have mixtures of introduced strains and natural, soilborne inoculum. The predominance of selfing and asexual reproduction (14) allows for tracing the movement of genotypes of *C. fimbriata* from sources of eucalyptus cuttings to plantations.

This study explored the possibility that genotypes of *C. fimbriata* have been moved to new areas in rooted eucalyptus cuttings collected from diseased trees and that some genotypes have been spread within plantations on tools used for collecting cuttings. Six polymorphic microsatellite loci were used to determine the relatedness of populations of the pathogen in Minas

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Gerais, Bahia, and São Paulo and to determine whether some populations may have been derived from a common source. Gene diversity and genotypic diversity of populations were determined to distinguish natural populations from putatively introduced populations. The diversity of *C. fimbriata* populations from eucalyptus planted on former pastureland was compared with the diversity from plantations on former forested sites. The geographic distribution of common genotypes was determined to see whether they may have been moved within plantations or from sources of rooted cuttings to plantations of the same eucalyptus clone.

MATERIALS AND METHODS

Collection of isolates. We sampled 20 Brazilian populations of *C. fimbriata* from *Eucalyptus* spp. and hybrids in three states: Bahia, São Paulo, and Minas Gerais (Table 1; Fig. 1). Each population was from a single plantation of a single clone of *Eucalyptus*, and only one isolate was used per tree. Ceratocystis wilt in eucalyptus plantations typically occurs in scattered pockets of a few affected trees. An attempt was made to collect samples from trees scattered throughout the plantation but, in some cases, only one small part of the plantation was affected, and all suitable trees were sampled. In most cases, living trees with crown dieback or recent branch wilting were examined by chopping into the lower stem, and samples of discolored xylem were collected if it appeared to be freshly colonized.

Isolates were baited from dead or dying trees by placing pieces of discolored xylem tissue between two discs of carrot root (26). Ascospore masses from perithecia forming on the carrot discs were transferred to agar media for purification and then storage. Only one isolate per tree was stored and used in genetic analyses.

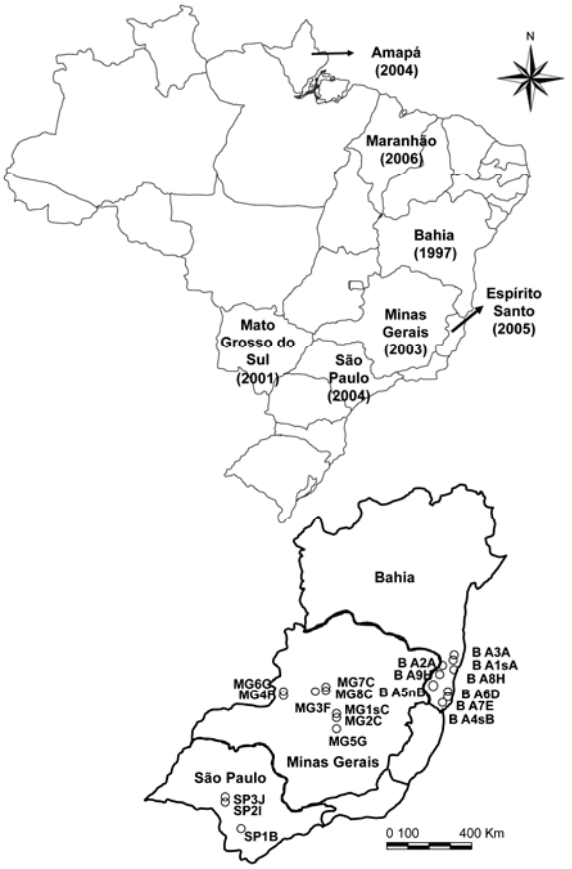

Fig. 1. States in Brazil where Ceratocystis wilt on eucalyptus has been reported and year when first recognized (left), and geographic distribution of sampling sites of *Ceratocystis fimbriata* populations from eucalyptus (right).

Pure cultures were stored on malt agar at −80°C or at room temperature (7).

Bahia plantations. Nine plantations of five eucalyptus clones were sampled near the cities of Eunápolis, Itabela, Teixeira de Freitas, and Caravelas in southern Bahia, just north of the state of Espírito Santo, in a well-established region of the eucalyptus industry (Fig. 1). This region is in a tropical, high-rainfall area that would naturally be in the Atlantic Rainforest vegetation type; however, most of the eucalyptus plantings in the region are on former pastureland.

The BA1sA plantation, north of Eunápolis, was a small farm of unknown crops immediately prior to clearing and planting of eucalyptus (Table 1). Plantation BA1sA (the letter "s" designates this plantation as a source of cuttings for rooting in the nursery, and "A" designates the eucalyptus clone) was heavily damaged in a windstorm in 2000 and, in 2001, sprouts from wind-damaged stumps were a source material for the production of rooted cuttings that were planted elsewhere in south Bahia, including the BA2A and BA3A plantations. Plantation BA1sA was heavily diseased when sampled in 2003 but only a small percentage of eucalyptus trees showed symptoms in plantations BA2A and BA3A (Table 1).

Plantations BA8H and BA9H were planted in 2003 with a different hybrid of *Eucalyptus grandis* × *E. urophylla* but the rooted cuttings originated from the same nursery where the BA1sA cuttings were rooted, and the plantations were located in the same region of Bahia as plantations BA1A, BA2A, and BA3A (Fig. 1). Plantations BA8H and BA9H, sampled in 2006, were in their first rotation, and the vegetation previous to eucalyptus was pastureland that had substantial woody regeneration prior to clearing and planting (Table 1).

South of Eunápolis, Ceratocystis wilt of eucalyptus is more common, and source plantation BA4sB was among the first locations where the disease was recognized. Eucalyptus clone B later became a widely planted clone, and stump sprouts from plantation BA4sB were used as source material for many cuttings that were rooted in a nearby nursery, BA5nB ("n" signifies that the population was in a nursery). At the time of sampling, site BA4sB was in its second rotation of eucalyptus, and the site was in pasture before eucalyptus (Table 1). However, the plantation was adjacent to natural forest vegetation.

The cuttings for plantations BA6D and BA7E came from a nursery in Espírito Santo. Plantation BA6D, sampled in 2007, was in a second rotation of a *E. grandis* × *E. urophylla* clone. Previous to eucalyptus, the BA6D site was in pasture (Table 1). Plantation BA7E, also sampled in 2007, was planted with a different *E. grandis* × *E. urophylla* hybrid in its third rotation, started in 1989, but the vegetation previous to eucalyptus is not known.

São Paulo plantations. Three eucalyptus plantations were sampled near the towns of Lençóis Paulista and Angatuba (Fig. 1). Plantation SP1B was in a region that would have once been Atlantic Rainforest but plantations SP2I and SP3J were in a region that would have been in a transitional zone between Cerrado and Atlantic Rainforest types. The SP1B plantation was in its fourth eucalyptus rotation, most recently planted with cuttings of clone B from BA4sB that were rooted at nursery BA5nB (Table 1). The vegetation at site SP1B before eucalyptus planting was pasture with some citrus trees. The SP2I and SP3J populations were from two different *E. grandis* × *E. urophylla* hybrids but the vegetation before these plantings is not known (Table 1), and the source of the cuttings is also unknown.

Minas Gerais plantations. Plantations of three different *Eucalyptus* spp. or hybrids were sampled near the towns of Curvelo, Paraopeba, João Pinheiro, Paracatu, and Buritizeiro (Fig. 1). These Minas Gerais plantations were in natural Cerrado forest vegetation type before eucalyptus cultivation, though some of the plantations had a mixture of agroforestry or *Pinus* spp. cultivation along with natural Cerrado forest immediately before planting

eucalyptus (Table 1). Sites MG1sC and MG2C were near each other and planted with the same clone but MG1sG was used as a source of cuttings. However, it is not clear whether any of these cuttings were planted in MG7C or MG8C, which were also in close proximity and where there had been some *Pinus* spp. cultivation after removal of Cerrado forest 30 years earlier (Table 1). Plantations MG3F and MG4F were in an agroforestry system, where agronomic plants were interplanted with eucalyptus. The MG4F plantation had been first planted with eucalyptus seedlings 21 years before, and a neighboring plantation was planted to mango 33 years before. Sites MG5G and MG6G were planted with an *E. grandis* × *E. urophylla* hybrid after clearing Cerrado forest.

DNA extraction. Two methods were used to obtain DNA from cultures for use as template in polymerase chain reaction (PCR). Isolates were grown in 25 ml of liquid medium (2% malt extract and 0.2% yeast extract) at room temperature for 2 weeks, and DNA extraction followed the method of DeScenzo and Harrington (9) or a cetyltrimethylammonium bromide-based protocol (27).

Microsatellite markers. We analyzed six highly polymorphic, PCR-based microsatellite loci (CfCAA9, CfCAA10, CfCAA15, CfCAA38, CfCAA80, and CfCAG15) developed from the genomic DNA of an isolate of *C. cacaofunesta* (32). These primer pairs were selected because they were the most polymorphic of 15 markers used in an earlier study of Brazilian isolates of *C. fimbriata* (14) and, thus, they were most likely to distinguish genotypes. For each primer pair, one of the primers was fluorescently labeled. PCR amplifications of all microsatellite loci were performed using a 96-well thermal cycler (PTC-100; MJ Research Inc., Watertown, MA) following the earlier described conditions (14). The PCR products were electrophoresed using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). 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 to be a different allele. The microsatellite regions are trinucleotide repeats, and most alleles of a given locus differed by increments of 3 bp.

Analyses. Based on the potentially large size ranges of microsatellite alleles within and among populations and species of *Ceratocystis* (11,12,14,28), an infinite isoalleles model of mutation was assumed. Alleles of a locus that were similar in length, such as a 3-bp difference, were not considered more closely related to each other than alleles differing by 9 or 12 bp, and each

TABLE 1. Description of plantations, disease incidence, and genetic diversity of populations of *Ceratocystis fimbriata* based on six microsatellite loci

State, Population	City	Vegetation before eucalyptus	Eucalyptus clone	Rotation of eucalyptus	Age (mo)	Disease incidence (%)	Number of		Genotypic diversity (G)[a]	Nei's gene diversity (H)	
							Isolates	Genotypes		All isolates	Clone corrected[b]
Bahia											
BA1s[c]A[d]	Eunápolis	Small farm	Source for 43	First	84	14.8	31	14	3.33	0.3836	0.4932
BA2A	Eunápolis	Pasture	43, from BA1sA	First	17	2.3	7	2	1.57	0.0180	0.1667
BA3A	Eunápolis	Pasture	43, from BA1sA	First	19	3.8	7	4	2.71	0.2857	0.3333
BA4sB	Teixeiras de Freitas	Pasture	Source for 1172	Second	ND[e]	ND	27	13	3.18	0.3356	0.4280
BA5n[f]B	Teixeiras de Freitas	Nursery	1172, from BA4sB	Nursery		ND	4	3	3.00	0.2292	0.2593
BA6D	Caravelas	Pasture	1501	Second	12	13.3	6	1	1.00	0.0000	0.0000
BA7E	Caravelas	ND	6011	Third	10	1.3	6	4	3.20	0.4352	0.4583
BA8H	Eunápolis	Pasture, woody regeneration	1028	First	40	39.8	8	6	3.42	0.3958	0.4074
BA9H	Itabela	Pasture, woody regeneration	1028	First	40	48.9	7	4	2.97	0.3265	0.3333
Mean ± SE							11.44	5.67	2.71 ± 0.28	0.2677 ± 0.0529	0.3199 ± 0.0525
São Paulo											
SP1B	Angatuba	Pasture, citrus	1172, from BA5nB	Fourth	48	ND	6	3	2.33	0.1389	0.2222
SP2I	Lençóis Paulista	ND	H13	ND	ND	ND	6	4	3.20	0.3958	0.4167
SP3J	Lençóis Paulista	ND	TC30	ND	ND	ND	6	4	3.00	0.2407	0.2500
Mean ± SE							6.0	3.67	2.84 ± 0.26	0.2585 ± 0.0747	0.2963 ± 0.0607
Minas Gerais											
MG1sC	Curvelo	Cerrado	Source for 1288	ND	ND	50.1	6	4	3.00	0.4537	0.5208
MG2C	Curvelo	Cerrado	1288	ND	ND	15.6	12	12	4.00	0.6145	0.6145
MG3F	Paracatu	Cerrado, Agroforestry	8B	Fourth	120	ND	6	5	3.60	0.5741	0.6000
MG4F	João Pinheiro	Cerrado, Agroforestry	8B	First	75	ND	7	5	3.60	0.4352	0.4267
MG5G	Paraopeba	Cerrado	VM3	First	24	8	7	5	3.25	0.4762	0.5067
MG6G	João Pinheiro	Cerrado	VM3	First	29	ND	6	5	3.60	0.4537	0.4933
MG7C	Buritizeiro	Cerrado, pine	1288	First	37	ND	6	6	4.00	0.5093	0.5093
MG8C	Buritizeiro	Cerrado, pine	1288	First	35	ND	7	3	2.68	0.4762	0.4815
Mean ± SE							7.13	5.63	3.47 ± 0.16	0.4991 ± 0.0225	0.5191 ± 0.0218
Mean ± SE, all							8.9	5.35	3.03 ± 0.06	0.3588 ± 0.0372	0.3960 ± 0.0344

[a] Stoddart and Taylor's genotypic diversity, with rarefaction. Rarefaction gave estimated values for G of 1.0 (only one genotype in the population) to maximum value of 4.0 (all isolates of a different genotype).

[b] Clone correction removed isolates that had genotypes identical to other isolates from the same site.

[c] s = source of cuttings for clonal propagation of *Eucalyptus* spp.

[d] Letter designates clone of *Eucalyptus* sp. or hybrid.

[e] ND = not determined.

[f] n = nursery where cuttings from BA4sB were rooted.

allele of a locus was considered independent from the others in all analyses.

Multilocus genotypic diversity was estimated with the Stoddart and Taylor's *G* index (33). However, the maximum value of *G* is limited by the number of isolates sampled; therefore, Stoddart and Taylor's *G* was scaled by the expected number of genotypes for the smallest sample size being compared (17). For individual plantations, the expected number of genotypes in a sample of four isolates (minimum value = 1.0 and maximum value = 4.0) was estimated based on rarefaction curves using the R package (version 2.6.1; R Development Core Team, Vienna). For state-wide populations, the maximum value for *G* was 18.0, the number of isolates sampled from São Paulo.

Nei's gene diversity (*H*) for each population was calculated with and without clone-corrected data using PopGen 1.32 software (35). 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. The clone-corrected value for *H* would be expected to be higher than the uncorrected value if the population was dominated by one or a few genotypes, as might occur if a few genotypes were spread within a plantation on tools or equipment.

A dendrogram comparing the relatedness of populations was generated based on a matrix of Nei's genetic distances (based on allele frequencies) between populations clustered with unweighted pair-group method with arithmetic mean (UPGMA) using PopGen 1.32 (35). Bootstrap values for the population tree were calculated from 100 bootstrap replicates of 100 UPGMA trees using SEQBOOT, GENDIST, NEIGHBOR, and CONSENSE in PHYLIP version 3.6 (13).

The relationship between genetic distance and geographic distance of the *C. fimbriata* populations was estimated by Mantel's test (22), implemented in Arlequin 3.11. The Mantel test was carried out using 1,000 random permutation tests.

RESULTS

All six microsatellite loci were polymorphic among eucalyptus isolates from the 20 Brazilian plantations. In all, 11, 3, 3, 14, 9, and 10 alleles were identified for loci CfCAA9, CfCAA10, CfCAA15, CfCAA38, CfCAA80, and CfCAG15, respectively. The ranges of allele sizes for the respective loci were 171 to 247, 127 to 133, 319 to 325, 145 to 333, 302 to 329, and 256 to 287 bp. The length of the alleles generally differed by increments of 3 bp, which is expected because the six loci are each trinucleotide repeats.

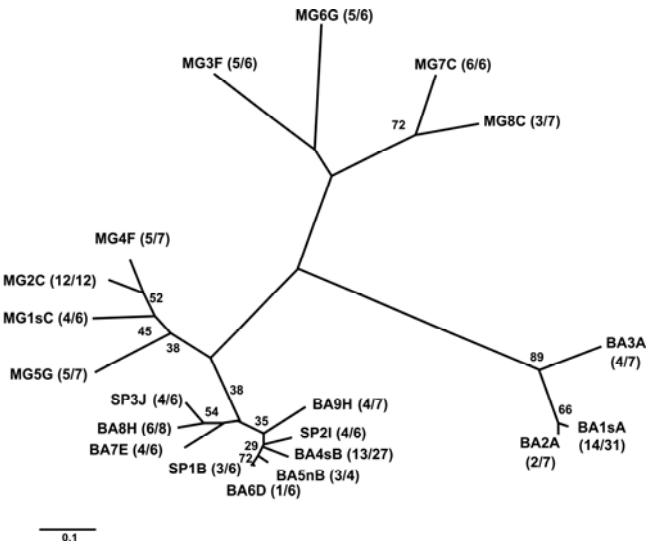

Fig. 2. Dendrogram of 20 *Ceratocystis fimbriata* eucalyptus populations generated by unweighted pair-group method with arithmetic mean based on allele frequencies of six microsatellite loci. Bootstrap values are shown alongside the branches. Populations are designated by state (two-letter abbreviation), plantation number, and clone of eucalyptus (capital letter). A nursery population is designated with a "n" and three populations that were used as a source for rooted cuttings are designated with an "s". The number of genotypes/number of isolates from each population are in parentheses. Bar indicates genetic distance.

In total, 78 multilocus genotypes were identified among 177 isolates tested using the six loci. In comparison with data from 62 isolates from five plantations that were also included in the earlier study with 15 microsatellite loci (14), the 6 polymorphic loci distinguished 40 genotypes, while 43 genotypes were distinguished using all 15 loci. Because the 6 loci were among the most polymorphic of the original 15 loci, the gene diversity values were approximately twice as high when the 6 loci were used than when all 15 loci were used (data not shown). Thus, values of *H* based on the six loci cannot be directly compared with the values of *H* in the earlier study (14).

Genetic relatedness of populations. The UPGMA tree constructed using allele frequencies of populations (plantations) showed that some of the populations were closely related to each other (Fig. 2). Most notably, populations BA1sA, BA2A, and BA3A from the Eunápolis region of Bahia were unique and connected by a well-supported (89%) branch. Plantations BA2A and BA3A were planted with cuttings collected from stump sprouts at plantation BA1sA (Table 1), which was unique in that it was planted on a former farm site. Other populations (i.e., BA8H and BA9H) from the same region (Fig. 1) were unrelated to the BA1sA/BA2A/BA3A populations (Fig. 2), though the rooted cuttings planted on BA8H and BA9H originated from the same nursery where the BA1sA cuttings were rooted.

Aside from the BA1sA/BA2A/BA3A cluster, the Bahia and São Paulo populations were related because they were connected by short branch lengths and 38% bootstrap support (Fig. 2). This latter group of populations included the clone (B) and area (Fig. 1) where Ceratocystis wilt on eucalyptus was first discovered in Brazil, in the area of source plantation BA4sB, which is adjacent to natural Atlantic Rainforest vegetation. Cuttings of stump sprouts from plantation BA4sB were rooted in nursery BA5nB, a major source for rooted cuttings. The populations from plantations SP1B (planted with rooted cuttings from nursery BA5nB) and BA6D were connected with moderate bootstrap support (72%), and populations from BA8H and SP3J were connected with low bootstrap support (54%).

The Minas Gerais populations also were distinct from the BA1sA/BA2A/BA3A cluster (Fig. 2). Most of the populations from Minas Gerais had long branch lengths and were distinct from each other in the UPGMA tree (Fig. 2), suggesting that they have a more natural genetic structure (14). However, the branch connecting populations MG7C and MG8C had moderate bootstrap support (Fig. 2), and these nearby sites were planted at approximately the same time with the same eucalyptus clone (Table 1; Fig. 1). In contrast, the source plantation MG1sC and plantation MG2C were of the same clone as the MG7C and MG8C plantations but the populations of *C. fimbriata* from MG1sC and MG2C were not closely related to the MG7C and MG8C populations. There was some support for grouping the MG1sC, MG2C, MG4F, and M5G populations but populations MG3F and MG6G were distinct from the other populations on those respective clones (Fig. 2).

Genetic distance versus geographic distance. Mantel tests comparing the matrix of genetic distances (ΦST) with the matrix of geographical distances (using the mean coordinate of each sampling site) resulted in a statistically significant correlation (P = 0.02; 10,000 randomizations) with a low correlation coefficient (r = 0.32). This may have been due to the close genetic and geographic relatedness of some of the Bahia populations (especially BA1sA, BA2A, and BA3A), and the three São Paulo

populations also were genetically related to each other (Fig. 2). When only populations from Minas Gerais were analyzed, there was no correlation between genetic distance and geographic distance ($r = 0.31$; $P = 0.07$ with 10,000 randomizations).

Diversity of populations in Bahia, São Paulo, and Minas Gerais. There were 38 genotypes among the 103 isolates from the nine Bahia populations ($G = 11.47$ with rarefaction, maximum possible = 18), and the overall gene diversity value of the combined Bahia populations was $H = 0.5526$ without clone correction. The nine Bahia populations had lower gene diversity (H of 0.0 to 0.4352, mean $H = 0.2677$) and genotypic diversity (G of 1.00 to 3.42, mean $G = 2.71$, maximum possible = 4.0) than the Minas Gerais populations (Table 1).

Only seven unique genotypes and a relatively low value of $G = 4.15$ were found among the 18 São Paulo isolates from the three plantations, suggesting that the São Paulo populations may have been derived from a common source. Also, the overall gene diversity value was lower for the 18 São Paulo isolates ($H = 0.3323$) than for the 103 isolates from Bahia ($H = 0.5526$) and the 56 isolates from Minas Gerais ($H = 0.6732$). However, the mean values of gene diversity (mean $H = 0.2585$) and genotypic diversity (mean $G = 2.84$) for the three São Paulo populations did not differ from the means of the nine Bahia populations (Table 1).

The Minas Gerais populations were much more diverse than the Bahia and São Paulo populations. Combining the eight Minas Gerais populations together, there were 40 genotypes out of the 56 isolates sampled, with an overall gene diversity value of $H = 0.6732$ and a genotypic diversity value of $G = 16.01$ (with rarefaction, maximum possible value of 18). Compared with populations from Bahia and São Paulo, high levels of gene diversity (H of 0.4352 to 0.6145, mean $H = 0.4991$) and genotypic diversity (G of 2.68 to 4.00, mean $G = 3.47$) were found in populations from Minas Gerais (Table 1).

Prior vegetation. We were able to identify the previous vegetation type of 16 of the 20 eucalyptus plantations (Table 1). The populations with the highest gene diversity values (mean $H = 0.4991$) and genotypic diversity values (mean $G = 3.46$) tended to be from the eight plantations that followed natural Cerrado forest vegetation in Minas Gerais (Tables 1 and 2). Five plantations (four in Bahia and one in São Paulo) were on former pastureland, presumably without hosts for *C. fimbriata* and without soilborne inoculum, and these five populations had much lower gene diversity (mean $H = 0.1556$) and genotypic diversity (mean $G = 2.16$) than the plantations on former Cerrado forest (Table 2). The source plantation BA4sB was in a second rotation on former pastureland but it was adjacent to natural Atlantic Rainforest vegetation; it had the highest values of H and G of any of the five former pastureland sites (Table 1).

Isolates from eucalyptus clone A were genetically distinct from isolates from all other clones (Fig. 2), and the source of clone A cuttings (BA1sA) was planted on a former farm site. The BA1sA population had an average gene diversity of $H = 0.3836$ and genotypic diversity of $G = 3.33$, higher than that found on the former pastureland sites (Table 2), suggesting that there was soilborne inoculum from previously cultivated plants.

Two Bahia plantations of H (BA8H and BA9H) were on former pasture sites that had substantial woody regeneration before clearing and planting (Table 1); therefore, there may have been soilborne inoculum on the site prior to planting. The populations sampled from these sites had a mean gene diversity value of $H = 0.3611$ and a mean genotypic diversity value of $G = 3.19$, higher than that found on former pastureland sites without woody regeneration (Table 2).

Sources of common genotypes. Of the 78 genotypes identified in this study, 17 were found in more than one population. More than half of the isolates sampled (95 of 177) fell into one of these 17 multipopulation genotypes (Table 3). In all, 64 of 103 isolates

TABLE 2. Average genotypic diversity and gene diversity based on six microsatellite loci of *Ceratocystis fimbriata* populations found in eucalyptus plantations planted on four different previous vegetation types

Vegetation before planting eucalyptus	Plantations	Mean G[a]	Mean population gene diversity (H)	
			All isolates	Clone corrected[b]
Pasture	BA2A, BA3A, BA4sB, BA6D, SP1B	2.16 ± 0.39	0.1556 ± 0.0681	0.2300 ± 0.0731
Pasture with woody regeneration	BA8H, BA9H	3.19 ± 0.22	0.3611 ± 0.0346	0.3703 ± 0.0370
Small Farm	BA1sA	3.33	0.3836	0.4932
Cerrado Forest	MG1sC, MG2C, MG3F, MG4F, MG5G, MG6G, MG7C, MG8C	3.46 ± 0.164	0.4991 ± 0.0225	0.5191 ± 0.0218

[a] Mean Stoddart & Taylor's genotypic diversity (G) with rarefaction. Mean and standard error of the populations of that prior vegetation type. Maximum value of G for each plantation is 4.0.
[b] Clone correction removed isolates that had genotypes identical to other isolates from the same site.

TABLE 3. Genotypes of *Ceratocystis fimbriata* from eucalyptus found in more than one population based on six microsatellite loci

Genotype[a]	Number of isolates	Populations (number of isolates)
CBCICA	21	BA1sA (10), BA2A (6), BA3A (5)
BBCIDA	2	BA1sA (1), BA2A (1)
BCCFDA	4	BA1sA (1), BA9H (3)
BCBJEA	2	BA1sA (1), SP1B (1)
BBBJDA	20	BA4sB (2), BA5nB (2), BA6D (6), BA8H (1), BA9H (2), SP1B (4), SP2I (2), SP3J (1)
BCBJDA	12	BA4sB (11), BA1sA (1)
BABKEA	3	BA4sB (1), BA5nB (1), MG2C (1)
BBBGDA	3	BA5nB (1), SP1B (1), SP2I (1)
ABCKEA	2	BA4sB (1), MG6G (1)
BABJDA	2	BA8H (1), BA9H (1)
ABBGDA	9	BA7E (2), BA8H (3), SP2I (1), SP3J (3)
BCCJEF	3	SP2I (2), MG2C (1)
ABBJEE	2	MG1sC (1), MG2C (1)
BCBKGF	4	MG1sC (3), MG2C (1)
BCBKAI	2	MG4F (1), MG6G (1)
ACCLEI	2	MG3F (1), MG7C (1)
ECCEIJ	2	MG7C (1), MG8C (1)

[a] Different alleles are designated by different letters for six respective loci: CfCAA9, CfCAA10, CfCAA15, CfCAA38, CfCAA80, and CfCAG15.

(62%) from Bahia and 16 of 18 isolates (89%) from São Paulo had multipopulation genotypes (Table 3), suggesting that there had been considerable spread of genotypes among plantations. In contrast, only 27% (15 of 56) of the isolates from Minas Gerais had multipopulation genotypes, and no genotype in Minas Gerais was found in more than three plantations (Table 3). The Bahia and São Paulo populations were dominated by four genotypes (CBCICA and three closely related genotypes: BBBJDA, BCBJDA, and ABBGDA), which were represented by 21, 20, 12 and 9 isolates, respectively (Table 3).

The most common genotypes could be traced to plantations where cuttings were collected. Of the 17 multipopulation genotypes, 11 were found in populations BA1sA, BA4sB, BA5nB, or MG1sC (Table 3). Sixty-nine isolates had one of the nine multipopulation genotypes found in Bahia source populations BA1sA or BA4sB or in nursery BA5nB. Four of the seven genotypes identified in São Paulo were also found in Bahia, and half of the 18 isolates from São Paulo had genotypes that also were identified in nursery BA5nB (Table 3).

The population from the source plantation BA1sA was closely related to populations from BA2A and BA3A (Fig. 2), which were planted with cuttings from BA1sA. The BA2A and BA3A populations had lower gene diversity (H = 0.0180 and 0.2857, respectively) and lower genotypic diversity (G = 1.57 and 2.71, respectively) than the source population for this clone (H = 0.3836 and G = 3.33) (Table 1). Source plantation BA1sA was dominated by one genotype (10 of 31 isolates were genotype CBCICA), and this genotype dominated (11 of 14 isolates) the BA2A and BA3A plantations (Table 3). In contrast, genotype CBCICA was not found in the other 16 plantations studied (Table 3), including the nearby Eunápolis plantations BA8H and BA9H (Fig. 1), which had populations more closely related to population BA4sB (Fig. 2).

Plantation BA4sB had a population of diverse genotypes (Table 1) but this source population also had a predominant genotype: 11 of the 27 isolates were genotype BCBJDA (Table 3). Cuttings collected from BA4sB were rooted in nursery BA5nB, which showed a lower gene diversity (H = 0.2292) and lower genotypic diversity (G = 3.00) than the source population BA4sB (H = 0.3356 and G = 3.18) (Table 1), and genotype BCBJDA was only found in one other population, BA5nB (Table 3). A second genotype found in BA5nB (BBBJDA) also was found in the source plantation BA4sB (Table 3), and the third genotype found in BA5nB (BBBGDA) was closely related to BBBJDA. The BBBJDA genotype also was found in three other Bahia plantations and in each of the three São Paulo plantations (Table 3). Plantation SP1B was planted with cuttings rooted in nursery BA5nB, and five of the six isolates from SP1B had genotypes identical to those found in BA5nB, including four isolates of BBBJDA (Table 3). Genotype BBBJDA also was found in four eucalyptus clones besides clone B (Table 3), suggesting that spread of genotypes may not be restricted to movement in cuttings. Bahia plantation BA6D was in the second rotation of eucalyptus following pasture, and all six isolates were genotype BBBJDA (Table 3), but the rooted cuttings planted in BA6D did not come from nursery BA5nB (Table 1).

Half of the isolates from source plantation MG1sC were genotype BCBKGF (Table 3), and this genotype was found in MG2C. A second isolate from MG2C had a genotype that also was found in source plantation MG1sC (Table 3). However, most isolates from Minas Gerais were unique and could not be traced back to source population MG1sC (Table 3).

Clone-corrected gene diversity and disease incidence in source plantations. Each of the three plantations that were a source of cuttings (BA1sA, BA4sB, and MG1sC) had moderate levels of genotypic diversity but a third to a half of the isolates sampled were of a single genotype (Tables 1 and 3). These source plantations were entered frequently to collect cuttings, and spread of a few genotypes on cuttings tools was likely. The clone-corrected value of H (0.4932) was much higher than the uncorrected value of H (0.3836) for population BA1sA (Table 1). The clone-corrected gene diversity values of populations BA4sB and MG1sC also were substantially higher than their uncorrected gene diversity values (Table 1), further indicating that the dominance of one or a few genotypes masked some of the allele variation found in source plantations. For most of the other 16 plantation populations, there was little difference between the clone-corrected and uncorrected values of H (Table 1).

The three plantations that were entered frequently to collect cuttings had relatively high levels of disease incidence. Of all the plantations with quantified disease levels, MG1sC had the highest incidence of disease (50%), much higher than that in MG2C (16%), which was near MG1sC and planted with the same clone (Table 1). After cuttings were collected from source plantation BA1sA, the stump sprouts were managed for another rotation and, only 2 years later, nearly 15% of the sprout trees were dead or symptomatic (Table 1). Stump sprouts from source plantation BA4sB were thinned and used to develop a second rotation, and disease incidence in this second rotation was well over 50% at the time of sampling; however, the disease incidence level was not quantified.

DISCUSSION

The *C. fimbriata* populations in Minas Gerais had high levels of gene and genotypic diversity, supporting the hypothesis that *C. fimbriata* is native to the Cerrado (14) and that eucalyptus trees can be infected by soilborne inoculum that has carried over from native vegetation. Populations of *C. fimbriata* on eucalyptus planted after clearing natural forests or other woody vegetation are among the most genetically diverse in Brazil (14). In contrast, the least diverse populations in Bahia and São Paulo tended to be from eucalyptus plantations on former pastureland, and most of the genotypes found in those plantations were also found in one of the two Bahian plantations that were used as a source of cuttings or in a nursery where the cuttings were rooted. Source plantations in Bahia and Minas Gerais where the plantations were entered frequently to collect cuttings had high disease incidence and tended to be dominated by one of a few genotypes. These genetic data suggest a complex epidemiology where both natural, soilborne inoculum and infected cuttings are important sources of inoculum, and the pathogen also may be spread tree to tree on contaminated tools or other equipment.

The unique disease cycle and reproductive biology of *Ceratocystis* spp. make comparisons with other pathogens difficult (24,25), even when similar markers are used (8), but the levels of gene diversity and genotypic diversity found in Minas Gerais are similar to those of other putatively natural populations of homothallic *Ceratocystis* spp. (11,12,19). Earlier work (14) also indicated that Minas Gerais populations of *C. fimbriata* have a high degree of selfing or asexual reproduction but appear to be natural. Although some genotypes in Minas Gerais plantations may have been introduced in infected cuttings, such inoculum would appear to be unimportant where there is abundant soilborne inoculum.

In contrast, populations in Bahia and São Paulo are generally less diverse than in Minas Gerais, and they have a more complex genetic structure and history. At least five of the sites were planted on former pastureland, which would not have woody hosts for *C. fimbriata* and, thus, little soilborne inoculum. Genetic diversity was limited in populations on former pastureland sites, and most of the genotypes could be traced back to source plantations. If only disease-free cuttings were planted on former pastureland sites in these states, then it may be possible to avoid Ceratocystis wilt for many rotations.

One of the sources of cuttings, BA1sA, was formerly a small farm, and the unique population found there suggests that the

BA1sA genotypes were introduced from some other region of Brazil to the farm in propagative material of fruit trees or other hosts for *C. fimbriata* (14). Rooted cuttings collected from BA1sA were planted on two former pastureland sites, and 11 of the 14 isolates from these two plantations were of a single unique genotype, CBCICA, the most common genotype found at the source plantation. Nearby plantations of cuttings of another clone (clone H) rooted as the same nursery where the clone A cuttings were rooted had genetically distinct populations. The uniqueness of the genotypes from clone A and the decrease in genetic diversity from the source population BA1sA to the BA2A and BA3A populations planted on former pastureland provide strong evidence for movement of *C. fimbriata* in rooted eucalyptus cuttings.

Another source of cuttings in Bahia, plantation BA4sB, was planted on former pastureland adjacent to native Atlantic Rainforest vegetation, which may have been a source of inoculum. The genetic diversity of the *C. fimbriata* population from this plantation was relatively high, and it is possible that inoculum in the form of insect frass had blown into the site from the nearby wild trees (14). Although it is well known that *C. fimbriata* is soilborne (21,23,29), the source of soilborne propagules needs further investigation.

The population of source plantation BA4sB was genetically diverse and had higher gene diversity than the nursery where the cuttings were rooted, and even lower gene diversity was seen in the SP1B planting of clone B from the nursery. Thus, the SP1B population may have gone through several genetic bottlenecks, from native Atlantic Rainforest forest to nearby source plantation BA4sB to nursery BA5nB to plantation SP1B. Genotype BBBJDA from BA4sB was found in nursery BA5nB, plantation SP1B, and in four other eucalyptus clones, perhaps spread to these other clones in nurseries or plantations on contaminated tools or equipment.

Eucalyptus nurseries in Brazil typically produce millions of rooted cuttings each year, and plantations used as sources of cuttings may be entered frequently to collect stump sprouts. High incidence of disease in the three sampled source plantations and the dominance of one or a few genotypes of the pathogen in those plantations suggest that there was substantial tree-to-tree spread of certain genotypes of *C. fimbriata*. Sanitation of tools may reduce within-plantation incidence of the disease as well as minimize spread of the pathogen among clones in the nursery (6).

We had speculated that *C. fimbriata* populations from Bahia and Minas Gerais would differ genetically because of the different native forest types but the results presented here and in the earlier study (14) do not support that contention. The close relatedness of some Bahia populations and the São Paulo populations also was not expected. Half of the isolates on eucalyptus in São Paulo could be traced back to genotypes from source plantation BA4sB and nursery BA5nB in Bahia. *C fimbriata* has long been known on other crops in São Paulo, especially on mango, but genotypes of the fungus on inhame (taro) and fig in São Paulo are genetically distinct from those on mango and eucalyptus (14), and mango isolates are generally not very aggressive on eucalyptus (4,20). The history of the disease in Brazil and the data presented here suggest that at least some of the genotypes of *C. fimbriata* on eucalyptus in São Paulo were introduced from Bahia.

The potential for moving *C. fimbriata* in symptomless eucalyptus cuttings was recognized early on based on isolations of *C. fimbriata* from rooted cuttings at nursery BA5nB (15,18). *C. fimbriata* was first observed on eucalyptus in south Bahia (15), initially on only a few clones, especially on clone B in the vicinity of plantation BA4sB, and then several years later on clone B and other clones in other states (3,16; A. C. Alfenas, *unpublished data*). *C. fimbriata* also has been found in eucalyptus plantations in Uruguay and Africa (5,31,34), and South African populations on eucalyptus have limited genetic diversity (34). The internal transcribed spacer ribosomal DNA sequences of the Uruguayan and African isolates from eucalyptus are similar to those of eucalyptus isolates from Bahia (20,34; T. C. Harrington, *unpublished data*), and it is likely that the pathogen was moved from Brazil to new countries on infected eucalyptus cuttings, on another host, or perhaps in wood (14,34).

The disease on eucalyptus may be harder to manage in Minas Gerais than elsewhere because there appears to be abundant inoculum in the Cerrado forest type. Resistance to particular genotypes or populations of *C. fimbriata* (2,36) may be possible where the fungus is already established in the soil, as has been found for Ceratocystis wilt on mango (29,30). However, isolates of *C. fimbriata* vary in aggressiveness to eucalyptus and other hosts (4,20,36), and the greater genetic diversity found in Minas Gerais may make selection for resistance difficult (36). In all locations where the disease is present, it is important that eucalyptus cuttings are collected from mother plants free of *C. fimbriata*. Carefully monitored minihedges in nurseries would appear to be the best source of disease-free cuttings. In addition, cleaning of tools should be practiced after working with potentially infected plants in the field and in the nursery.

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LITERATURE CITED

1. ABRAF, Associação Brasileira de Produtores de Florestas Plantadas. 2008. Anuário Estatístico da ABRAF, Brasília.
2. Alfenas, A. C., and Ferreira, E. M. 2008. Emerging diseases in eucalyptus plantations. Trop. Plant Pathol. 33:25-28.
3. Alfenas, A. C., Zauza, E. A. V., Mafia, R. G., and Assis, T. F. 2009. Clonagem e doenças do eucalipto, 2nd ed. Editora UFV, Universidade Federal de Viçosa, Viçosa, Brasilia.
4. Baker, C. J., Harrington, T. C., Krauss, U., and Alfenas, A. C. 2003. Genetic variability and host specialization in the Latin American clade of *Ceratocystis fimbriata*. Phytopathology 93:1274-1284.
5. Barnes, I., Roux, J., Wingfield, B. D., O'Neill, M., and Wingfield, M. J. 2003. *Ceratocystis fimbriata* infecting *Eucalyptus grandis* in Uruguay. Australas. Plant Pathol. 32:361-366.
6. CAB International. 2005. *Ceratocystis fimbriata* (original text prepared by T. C. Harrington). In: Crop Protection Compendium. CAB International, Wallingford, UK.
7. Castellani, A. 1939. Viability of some pathogenic fungi in distilled water. J. Trop. Med. Hyg. 24:270-276.
8. Chen, W., and Harrington, T. C. 2006. Genetic diversity of poplar leaf rust populations in the north-central United States. Can. J. For. Res. 36:2047-2057.
9. DeScenzo, R. A., and Harrington, T. C. 1994. Use of $(CAT)_5$ as a DNA fingerprinting probe for fungi. Phytopathology 84:534-540.
10. Engelbrecht, C. J., Harrington, T. C., and Alfenas, A. C. 2007. Ceratocystis wilt of cacao—a disease of increasing importance. Phytopathology 97:1648-1649.
11. Engelbrecht, C. J., Harrington, T. C., Alfenas, A. C., and Suarez, C. 2007. Genetic variation of populations of the cacao wilt pathogen, *Ceratocystis cacaofunesta*. Plant Pathol. 56:923-933.
12. Engelbrecht, C. J. B., Harrington, T. C., Steimel, J., and Capretti, P. 2004. Genetic variation in eastern North American and putatively introduced populations of *Ceratocystis fimbriata* f. *platani*. Mol. Ecol. 13:2995-3005.
13. Felsenstein, J. 1993. PHYLIP (phylogeny inference package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.
14. Ferreira, E. M., Harrington, T. C., Thorpe, D. J., and Alfenas, A. C. 2010.

Genetic diversity and interfertility among highly differentiated populations of *Ceratocystis fimbriata* in Brazil. Plant Pathol. 59:721-735.
15. Ferreira, F. A., Demuner, A. M. M., Demuner, N. L., and Pigato, S. 1999. Murcha de Ceratocystis em eucalipto no Brasil. (Abstr.) Fitopatol. Bras. 24:284.
16. Ferreira, F. A., Maffia, L. A., Barreto, R. W., Demuner, N. L., and Pigatto, S. 2006. Sintomatologia da murcha de *Ceratocystis fimbriata* em eucalipto. Rev. Árvore 30:155-152.
17. Grünwald, N. J., Goodwin, S. B., Milgroom, M. G., and Fry, W. E. 2003. Analysis of genotypic diversity data for populations of microorganisms. Phytopathology 93:738-746.
18. Harrington, T. C. 2000. Host specialization and speciation in the American wilt pathogen *Ceratocystis fimbriata.* Fitopatol. Bras. 25:262-263.
19. Harrington, T. C., Steimel, J., and Kile, G. 1998. Genetic variation in three *Ceratocystis* species with outcrossing, selfing and asexual reproductive strategies. Eur. J. For. Pathol. 28:217-226.
20. Harrington, T. C., Thorpe, D. J., and Alfenas, A. C. 2011. Genetic variation and variation in aggressiveness to native and exotic hosts among Brazilian populations of *Ceratocystis fimbriata*. Phytopathology 101:555-566.
21. Laia, M. L., Alfenas, A. C., and Harrington, T. C. 2000. Isolation, detection in soil, and inoculation of *Ceratocystis fimbriata*, causal agent of wilting, die-back and canker in *Eucalyptus*. (Abstr.) Fitopatol. Bras. 25:384.
22. Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27:209-220.
23. Marin, M., Castro, B., Gaitan, A., Preisig, O., Wingfield, B. D., and Wingfield, M. J. 2003. Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. J. Phytopathol. 151:395-405.
24. McDonald, B. A. 1998. The population genetics of fungi: Tools and techniques. Phytopathology 87:448-453.
25. Milgroom, M. G. 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34:457-477.
26. Moller, W. J., and DeVay, J. E. 1968. Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. Phytopathology 58:123-124.
27. Murray, M. G., and Thompson, W. F. 1980. Rapid isolation of high molecular weight DNA. Nucleic Acids Res. 8:4321-4325.
28. Ocasio-Morales, R. G., Tsopelas, P., and Harrington, T. C. 2007. Origin of *Ceratocystis platani* on native *Platanus orientalis* in Greece and its impact on natural forests. Plant Dis. 91:901-904.
29. Rossetto, C. J., and Ribeiro, I. J. A. 1990. Mango wilt. XII. Recommendations for control. Rev. Agric. 65:173-180.
30. Rossetto, C. J., Ribeiro, I. J. A., Gallo, P. B., Soares, N. B., Sabino, J. C., Martins, A. L. M., Bortoletto, N., and Paulo, E. M. 1997. Mango breeding for resistance to diseases and pests. Acta Hortic. 455:299-304.
31. Roux, J., Wingfield, M. J., Bouillet, J. P., Wingfield, B. D., and Alfenas, A. C. 2000. A serious new wilt disease of Eucalyptus caused by *Ceratocystis fimbriata* in Central Africa. For. Pathol. 30:175-184.
32. Steimel, J., Engelbrecht, C. J. B., and Harrington, T. C. 2004. Development and characterization of microsatellite markers for the fungus *Ceratocystis fimbriata*. Mol. Ecol. Notes 4:215-218.
33. Stoddart, J. A., and Taylor, J. F. 1988. Genotypic diversity: estimation and prediction in samples. Genetics 118:705-711.
34. van Wyk, M., van der Merwe, N. A., Roux, J., Wingfield, B. D., Kamgan, G. N., and Wingfield, M. J. 2006. Population genetic analyses suggest that the *Eucalyptus* pathogen *Ceratocystis fimbriata* has been introduced into South Africa. S. Afr. J. Sci. 102:259-263.
35. Yeh, F. C., and Boyle, T. J. 1997. Population genetic analysis of co-dominant and dominant markers and quantitative traits. Belg. J. Bot. 129:157.
36. Zauza, E. A. V., Alfenas, A. C., Harrington, T. C., Mizubuti, E. S., and Silva, J. F. 2004. Resistance of *Eucalyptus* clones to *Ceratocystis fimbriata*. Plant Dis. 88:758-760.