

# Species or Genotypes? Reassessment of Four Recently Described Species of the *Ceratocystis* Wilt Pathogen, *Ceratocystis fimbriata*, on *Mangifera indica*

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## ABSTRACT

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*Ceratocystis* wilt is among the most important diseases on mango (*Mangifera indica*) in Brazil, Oman, and Pakistan. The causal agent was originally identified in Brazil as *Ceratocystis fimbriata*, which is considered by some as a complex of many cryptic species, and four new species on mango trees were distinguished from *C. fimbriata* based on variation in internal transcribed spacer sequences. In the present study, phylogenetic analyses using DNA sequences of mating type genes, TEF-1 $\alpha$ , and  $\beta$ -tubulin failed to identify lineages corresponding to the four new species names. Further, mating experiments found that the mango isolates representing the new species were interfertile with each other and

a tester strain from sweet potato (*Ipomoea batatas*), on which the name *C. fimbriata* is based, and there was little morphological variation among the mango isolates. Microsatellite markers found substantial differentiation among mango isolates at the regional and population levels, but certain microsatellite genotypes were commonly found in multiple populations, suggesting that these genotypes had been disseminated in infected nursery stock. The most common microsatellite genotypes corresponded to the four recently named species (*C. manginecans*, *C. acaciivora*, *C. mangicola*, and *C. mangivora*), which are considered synonyms of *C. fimbriata*. This study points to the potential problems of naming new species based on introduced genotypes of a pathogen, the value of an understanding of natural variation within and among populations, and the importance of phenotype in delimiting species.

*Additional keywords:* pathogen diversity, population biology, species concepts.

Sequences of DNA and population-based genetic markers have greatly aided in the delineation of fungal plant pathogens, but they do not always clearly distinguish between cryptic species and genetically isolated populations or introduced genotypes. Distinguishing fungal lineages as new species rather than populations often appears arbitrary without the study of phenotype. The problem of species delineation is particularly evident in the *Ceratocystis fimbriata* Ellis & Halsted complex, especially in the soilborne Latin American Clade (LAC) of the complex, which is native to Eastern United States, Mexico, the Caribbean, Central America, and South America (Harrington et al. 2011). Recently, 14 new species have been described in the LAC as distinct from a widely distributed genotype in storage roots of *Ipomoea batatas* (sweet potato), on which the name *C. fimbriata* is based (Engelbrecht and Harrington 2005; van Wyk et al. 2007, 2009, 2010, 2011a, 2011b, 2012).

The new species in the LAC were mainly distinguished by differences in the internal transcribed spacer (ITS) region of rDNA and mostly using isolates from an introduced population or a limited number of collection sites (Harrington et al. 2014). Two of the new species in the LAC were based on specialization to native hosts as a distinguishing phenotypic character: *C. platani* Engelbr. & T.C. Harrin. on *Platanus occidentalis* in Eastern United States and *C. cacaofunesta* Engelbr. & T.C. Harrin. on *Theobroma cacao* from the Upper Amazon (Engelbrecht and Harrington 2005). Host

specialization to nonnative hosts is suggested by the basionyms of some of the new species, such as *C. manginecans* M. van Wyk, A. Adawi & M.J. Wingf., *C. mangivora* M. van Wyk & M.J. Wingf. and *C. mangicola* M. van Wyk & M.J. Wingf. on *Mangifera indica* (mango), and *C. acaciivora* Tarigan & M. van Wyk on *Acacia* spp. and mango (Tarigan et al. 2011; van Wyk et al. 2007, 2011a). However, host specialization and morphological distinctions have not been documented clearly, and mating studies have found that Brazilian isolates from mango are a single biological species and sexually compatible with a sweet potato strain of *C. fimbriata* (Ferreira et al. 2010).

Mango is native to southern and southeastern Asia (Kostermans and Bompard 1993), but the first report of *Ceratocystis* wilt on mango trees occurred in Brazil in the Northeastern state of Pernambuco (Carvalho 1938). The causal agent was later identified as *C. fimbriata* and confirmed in São Paulo, Brazil (Viégas 1960), where it remains a serious disease in commercial mango plantations. *Ceratocystis* wilt on mango was later confirmed in the states of Bahia (Batista et al. 2008), Rio de Janeiro (Baker et al. 2003; Ferreira et al. 2010; Silveira et al. 2006), and Distrito Federal (Harrington et al. 2014). The disease on mango was observed for the first time outside Brazil in Pakistan (Fateh et al. 2006) and the Sultanate of Oman (Al Adawi et al. 2006), where it causes serious losses to mango and also attacks an important forest species, *Dalbergia sissoo* in Pakistan (Poussio et al. 2010). An isolate from *Punica granatum* (pomegranate) in India (Somasekhara 1999) appears to be the same as the mango pathogen in Pakistan and Oman, which is believed to be an introduced strain from South America (Harrington et al. 2014).

The mango pathogen in Oman and Pakistan was named *C. manginecans* (van Wyk et al. 2007) based on a unique ITS

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sequence, referred to by Harrington et al. (2014) as ITS7b. Later, a second ITS sequence (designated ITS6) was found in Oman, and this was described as a second species, *C. acaciivora*, because the same ITS sequence also was found in isolates from *Acacia* spp. in Indonesia (Tarigan et al. 2011). The ITS sequences of the LAC can vary widely, even within populations (Harrington et al. 2011, 2014), and the presence of both ITS7b and ITS6 was found in single-ascospore isolates of *C. fimbriata* from Oman and Pakistan (Harrington et al. 2014, Naidoo et al. 2013), and ITS6 and other ITS sequences were recovered from a pomegranate isolate in India and a mango isolate in western Rio de Janeiro (Harrington et al. 2014). Two other new species were named from mango isolates in São Paulo as *C. mangicola* and *C. mangivora* based on ITS sequences (van Wyk et al. 2011a), but these species names may merely represent genotypes of *C. fimbriata* that have been distributed in nursery stock (Harrington et al. 2014).

The primary aim of this study was to test the hypothesis that there are cryptic species among isolates of the *C. fimbriata* complex on mango in Brazil, Oman, and Pakistan. Elements of three concepts for species recognition were applied. For genealogical concordance phylogenetic species recognition, species may be delimited by phylogenetic analyses alone, although adequate sampling of populations is necessary (Taylor et al. 2000). The traditional phylogenetic species concept (Harrington and Rizzo 1999) relies on a unique phenotype to designate a phylogenetic lineage as a species, considering phenotype in a broad sense, including morphology, physiology, and ecology. This concept defines a species as "...the smallest aggregation of populations with a common lineage that share unique, diagnosable characters." A third alternative is the traditional biological species concept, in which prezygotic or postzygotic barriers delimit species (Harrington and Rizzo 1999).

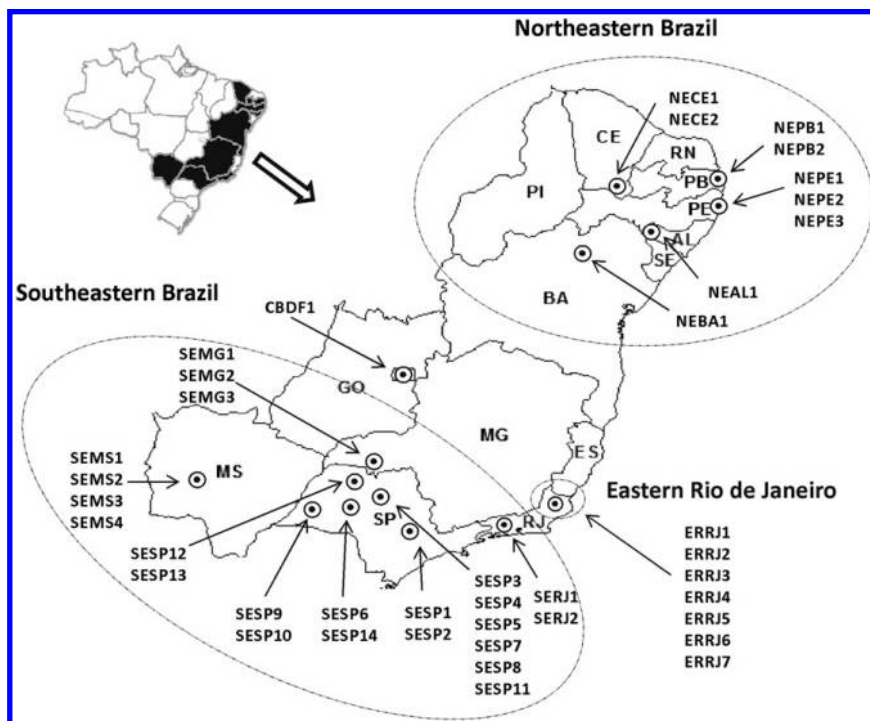
In an attempt to adequately sample both natural and human-mediated populations, isolates were obtained from dead and dying mango trees wherever the disease was known (Oman, Pakistan, and Brazil) and in all other states in Brazil where there had been reports of mango mortality. Sequences of the ITS region were used to place the isolates among the previously described species and identify

new ITS genotypes (Harrington et al. 2014). Phylogenetic analyses were conducted with DNA sequences from four gene-coding regions to identify phylogenetic lineages, and representative isolates from different regions were examined for morphological distinctions. Interfertility tests were conducted to identify possible biological species. Microsatellite markers were utilized to determine the genetic structure of the populations and to see if the recently described species were common genotypes or clones found at multiple locations, implying that they had been moved in infected nursery stock (Harrington et al. 2014).

## MATERIALS AND METHODS

**Collection of isolates.** Isolates were collected from diseased mango trees in plantations, small farms, gardens, and along streets in small cities across Brazil, Oman, and Pakistan. We consulted local agronomists, plant pathologists, and local newspapers for reports of mango mortality and visited all reports of the disease in Brazil. In addition to the five states in Brazil where *Ceratocystis* wilt on mango had been previously reported (Fig. 1), the disease was found in Minas Gerais, Paraíba, Alagoas, and Ceará. Mango trees in Pará, Piauí, Sergipe, and Espírito Santo were also examined, but no mango plant with *Ceratocystis* wilt was found.

Trees showing symptoms of branch death, wilting foliage, bark discoloration, or sap exudation were examined by chopping into the woody xylem at the base of the tree (most infections occur through the root system) or further up the stems for the characteristic xylem discoloration, pieces of which were brought back to the laboratory for isolation attempts (Ferreira et al. 2010). For the new isolates, a single ascospore strain was derived from the original field isolate by dispersing an ascospore mass in a light oil and spreading the spore suspension over the plate; individual germings were subcultured to fresh plates (Harrington and McNew 1997). Aside from 28 mango isolates from Brazil and Pakistan studied previously (Ferreira et al. 2010; Harrington et al. 2011, 2014), 186 new isolates were obtained from mango trees in Brazil and Oman in 2011 to 2013. The fungus can potentially move from a single infection court



**Fig. 1.** Forty sites in Brazil where *Ceratocystis fimbriata* was collected from *Mangifera indica* (mango). The first two letters of each location indicate the geographic region (SE = Southeastern Brazil; NE = Northeastern Brazil; ER = Eastern Rio de Janeiro; and CB = Central Brazil) and the next two letters indicate the state of origin (SP = São Paulo; RJ = Rio de Janeiro; MS = Mato Grosso do Sul; MG = Minas Gerais; DF = Distrito Federal; BA = Bahia; PE = Pernambuco; PB = Paraíba; AL = Alagoas; and CE = Ceará).

throughout a tree, so only one isolate per tree was used in genetic analyses.

Each population in Brazil consisted of at least four isolates from a small city, from a group of small farms in and around a small city, or from a single large plantation. All but a single isolate from Central Brazil (CB) were considered to be from one of three geographic regions: Southeastern Brazil (SE), Northeastern Brazil (NE), and Eastern Rio de Janeiro (ER). *Ceratocystis* wilt on mango is well known and has been well studied in commercial plantations in Southeastern Brazil, primarily in the state of São Paulo, where the pathogen has been associated with nursery stock. Near Limeira, São Paulo, where much of the nursery industry is located, 11 isolates (SESP1) were obtained from a row of diseased mango trees in an abandoned nursery. Other collections in the Southeast were from small cities or farms, but four populations were sampled from four large plantations: SESP7, SEMG1, SESP5, and SESP11. Isolates from the western part of Rio de Janeiro are genetically similar to mango isolates from São Paulo (Ferreira et al. 2010; Harrington et al. 2014), and the earlier studied isolates were included as a Southeastern population (SERJ2), as were additional isolates from the city of Paraíba do Sul (SERJ1). *Ceratocystis* wilt on mango was found in various small cities in the Northeast, but we were unable to locate diseased plantations, even in Pernambuco, the largest mango producing state in Brazil. Earlier studies demonstrated that Eastern Rio de Janeiro isolates were genetically distinct from other mango isolates (Baker et al. 2003; Ferreira et al. 2010; Harrington et al. 2014; Silveira et al. 2006). The earlier isolates were not included, but the newly collected isolates from small cities and one plantation (ERRJ3) in Eastern Rio de Janeiro were confirmed to be genetically similar to the earlier isolates and distinct from all other mango populations. Thus, the Eastern Rio de Janeiro isolates were treated as a third regional group.

In Oman, all the isolates were collected from small farms in the Al-Batinah, Muscat, and Musandam regions in the coastal plains of Northeastern Oman, near the cities of Barka, Almosna'h, Quriyat, Al-Seeb, Swaiq, Sohar, Shinas, Saham, and Madha. The Oman isolates represented a single population, though the collection sites were up to 305 km apart. The Pakistani isolates were collected in a previous study (Harrington et al. 2014) from mango trees in five regions over a distance of about 2,000 km. Isolates from Pakistan were also considered to comprise a single population, and the entire Oman/Pakistan collection was considered to be from a single region.

**Referenced isolates.** Representatives of previously described species and populations on mango used for phylogenetic analyses included isolate CBS 600.70 (=C994), used to describe *C. mangivora*; CBS 114721 (=C1688), the ex-holotype of *C. mangicola*; CBS 115175 (=C1558) as a representative of the Eastern Rio de Janeiro population on mango; CBS 135866 (=C2757) and CBS 135868 (=C2759) from Pakistan; and CBS 135988 (=C2785) and CBS 135867 (=C2787) from Oman. Other reference strains of *C. fimbriata* included CBS 115174 (=C1442) from *Eucalyptus* in Bahia; CBS 115166 (=C1782) from *Ficus carica* in São Paulo; CBS 115171 (=C1905) and CBS 114713 (=C1865) from *Colocasia esculenta* in São Paulo; CBS 115173 (=C918) from *Gmelina arborea* in Pará; and ICMP 8579 (=C1476) from *Ipomoea batatas* in Papua New Guinea. Other isolates included CBS 153.62 (=C1004), representing *C. cacaofunesta*; CBS 135861 (=C1543), representing *C. colombiana*; CBS 115162 (=C1317), representing *C. platani*; and CBS 135862 (=C1963), representing the outgroup taxon *C. variispora*.

**ITS barcoding.** Amplification and direct sequencing of the ITS rDNA region utilized primers ITS1F and ITS4 following conditions described in Harrington et al. (2011). All sequencing was conducted by the Iowa State University DNA Facility. One of the new, single-ascospored isolates from Oman appeared to have a mixed ITS polymerase chain reaction (PCR) product that could not be clearly determined using direct sequencing, so the PCR product was cloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI). Plasmids were extracted using Illustra plasmidPrep Mini Spin Kit (GE Healthcare Life Sciences, Buckinghamshire, UK), and the

cloned fragments were sequenced using vector primers T7-2 and SP6.

The ITS sequences of the 214 mango isolates were compared with those reported earlier for the LAC (Harrington et al. 2011, 2014). Relationships among the mango ITS sequences were examined using genetic distance (Nei's) matrices, unweighted pair group method, arithmetic mean dendrogram (UPGMA) trees, and 1,000 bootstrap replications generated with PAUP 4.0b10 (Swofford 2002).

**$\beta$ -tubulin gene sequences.** Gene sequences of  $\beta$ -tubulin were obtained using PCR (600-bp fragment) followed by direct DNA sequencing with primers BT1- $\alpha$  (5'-TTCCCCCGTCTCCACTTCTT CATG-3') and BT1- $\beta$  (5'-GACGAGATCGTTCATGTTGAACTC-3') (Glass and Donaldson 1995). The thermocycler settings for amplifying the  $\beta$ -tubulin region included initial denaturation at 94°C for 4 min, with 30 cycles of 94°C for 50 s, 55°C for 50 s, 72°C for 50 s, and a final extension at 72°C for 10 min.

**TEF-1 $\alpha$  gene sequences.** The primers EFCF1 (5'-AGTGCG GTGGTATCGACAAG-3'), EFCF2 (5'-TGCTCAACGGGTCTG GCCAT-3'), EFCF3 (5'-ATGGCCAGACCCGTGAGCA-3') and EFCF6 (5'-CATGTACGGACGGCGAAAC-3') were developed for amplification and sequencing in an earlier study (Harrington 2009). The primers EFCF1 and EFCF6 were used for amplification and sequencing, and two additional primers (EFCF2 and EFCF3) were used to sequence about a 1,600-bp region that included portions of three exons and two introns. Thermocycler settings for amplifying the TEF-1 $\alpha$  region included: initial denaturation at 85°C for 2 min followed by 94°C for 1 min and 35 s, with 36 cycles of 60°C for 1 min, 72°C for 1 min and 30 s, and 94°C for 35 s, followed by final extension of 72°C for 15 min. The fragments were purified and sequenced as described above.

**Mating type gene sequences.** Most field isolates of *C. fimbriata* are self-fertile and have genes associated with both the MAT2 and MAT1 mating types in the mating type locus. However, during unidirectional mating-type switching, the gene associated with the MAT2 phenotype (*MATI-2-1*) is deleted, and progeny that have inherited nuclei with the deletion behave as MAT1 and are self-sterile (Harrington and McNew 1997; Wilken et al. 2014; Witthuhn et al. 2000). Portions of the mating type locus associated with the MAT1 phenotype (*MATI-1-2*) and MAT2 phenotype (*MATI-2-1*) of all self-fertile isolates were amplified and sequenced using the primers and protocols of Harrington et al. (2014). Primers CFMAT1-F (5'-CAGCCTCGATTGAKGGTATGA-3') and CFMAT1-R (5'-GGC ATTTTTACGCTGGTTAG-3') were used to amplify and sequence about 1,000 bp of the *MATI-1-2* region. The primers  $\times$ 9978a (5'-GCTAACCTTCACGCCAATTTTGCC-3') and CFM2-1F (5'-GTCA CAAGTGTTCCTCCAAAAG-3') were used to amplify and sequence about 1,150 bp that included most of the *MATI-2-1* gene. The thermocycler settings for amplifying both *MATI-1-2* and *MATI-2-1* regions included: initial denaturation at 94°C for 2 min, with 36 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 10 min. The fragments were purified and sequenced with the PCR primers as described above.

**Phylogenetic analyses.** Datasets for each of the four loci (MAT1, MAT2, TEF-1 $\alpha$ , and  $\beta$ -tubulin) were separately aligned in MAFFT Online version v. 7.0 (Kato and Toh 2010) using the FFT-NS-i (Slow; iterative refinement method) alignment strategy with the 200PAM/K=2 scoring matrix and a gap opening penalty of 1.53 with an offset value of 0.0. Aligned sequences were then manually corrected when necessary using MEGA v. 5 (Tamura et al. 2011). Sequences of the four gene regions were analyzed separately and then a combined dataset was submitted to a partition homogeneity test (PHT) using PAUP 4.0b10 (Swofford 2002) in order to determine whether the datasets could be combined. For maximum parsimony analysis (PAUP 4.0b10), gaps were treated as a fifth base, all characters had equal weight, and the heuristic searches used simple stepwise addition and tree-bisection-reconnection. Bootstrap values were calculated using 1,000 replications. Phylogenetic trees were viewed and edited in Fig. Tree 1.3.1 (<http://tree.bio.ed.ac.uk/software>).

Bayesian analyses were conducted using the Markov chain Monte Carlo algorithm implemented in Mr. Bayes v3.2.1 (Ronquist and Huelsenbeck 2003), and the best model of nucleotide substitution for each gene was selected according to Akaike information criterion using MrModeltest v. 2.3 (Nylander 2004). Bayesian inference was conducted with two sets of four chains (one cold and three heated) from a random tree with 10 million generations, and 25% of the first trees were discarded.

**Mating experiments.** Sexual compatibility (interfertility) was determined following the protocols used in other studies of the *C. fimbriata* complex (Engelbrecht and Harrington 2005; Ferreira et al. 2010; Johnson et al. 2005). Representative mango isolates from each region were selected as male and female testers in mating experiments. Also, isolates from *Eucalyptus* spp., *Ficus carica*, *Colocasia esculenta*, *Gmelina arborea*, and *Ipomoea batatas*, and one representative isolate of *C. cacaofunesta* and one of *C. platani* were selected as female testers in order to compare their interfertility with the mango isolates (Table 1).

*C. fimbriata* and many other species in the genus are homothallic through unidirectional mating type switching (Harrington and McNew 1997; Witthuhn et al. 2000). Most field isolates are self-fertile and have the mating type genes that confer both mating types. In pairings, these field isolates are MAT2 in phenotype and can either cross with MAT1 strains or can self after deletion of the *MAT1-2-1* gene (Wilken et al. 2014; Witthuhn et al. 2000), which changes the phenotype from MAT2 to MAT1. Both MAT2 (self-fertile) and MAT1 (self-sterile due to deletion of the *MAT1-2-1* gene) progeny are recovered from selfings of MAT2 strains (Harrington and McNew 1997; Witthuhn et al. 2000). Because MAT2 strains are generally self-fertile, mating experiments require selection of self-sterile MAT2 (male-only) testers that have lost femaleness (protoperithecia). Otherwise it is difficult to distinguish perithecia due to a cross from perithecia due to a selfing of the MAT2 tester.

The MAT2 testers were obtained by subculturing self-sterile mutants that formed spontaneously and appeared as sectors in colonies of otherwise self-fertile isolates. These MAT2 sectors lacked protoperithecia and thus did not form perithecia through selfing. To confirm that these mutants still were capable of behaving as MAT2 in a cross, genomic DNA of each MAT2, self-sterile sector was extracted, and PCR was performed to amplify a portion of the *MAT1-2-1* gene using the primers CFM2-1F and x9978a. The selected female

MAT1 testers were self-sterile, single ascospore strains recovered from self-fertile isolates and generally formed protoperithecia, thus indicating that they could serve as good female testers.

The MAT1 testers (female recipients) were grown on MYEA for 10 days at room temperature (approximately 23°C) and then spermized by MAT2 testers. The conidial suspensions of MAT2 testers were prepared by flooding a 10-day-old MYEA plate with 10 ml of sterilized deionized water, scraping the mycelium with a sterile spatula. Then, 1 ml of inoculum was dispersed over the MAT1 colony. The colonies were observed for 3 to 4 weeks for the presence of perithecia and ascospore masses. 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. The experiment was repeated twice. The two strains were considered interfertile if they crossed in at least one of the two experiments. In the first experiment, ascospore masses were examined microscopically (×400) to see if there were abundant, normal-appearing ascospores, indicating a good cross, or if there were no ascospores or only a few misshapen ascospores, which is typical of an interspecific cross or hybrid (Ferreira et al. 2010; Harrington and McNew 1998; Johnson et al. 2005).

**Morphology.** Representative mango isolates from the different regions were selected for morphological comparisons. These self-fertile isolates were grown on MYEA for about 10 days at room temperature (approximately 23°C). Measurements of endoconidia and endoconidiophores were made after 4 to 7 days growth, while perithecia and ascospores were measured after 7 to 10 days. Aleurioconidia were measured from cultures that had grown 7 to 20 days. Material from the cultures was mounted in lactophenol cotton blue and observed with Nomarsky interference microscopy. Size ranges were based on at least 10 observations, but some structures were rare or hard to locate in a few isolates, and fewer observations were made. Colony pigmentation was compared with the color chart of Rayner (1970).

**Microsatellite markers.** Variation in simple sequence repeats was used to study population structure and to identify widespread genotypes (genets) of *C. fimbriata* that may have been spread through human activity. We analyzed 14 microsatellite markers developed from an isolate of *C. cacaofunesta* (Steimel et al. 2004) and recently mapped onto the *C. fimbriata* genome (Simpson et al. 2013). The markers had been used in previous population studies on

TABLE 1. Interfertility (I), interspecific hybrid (H) interactions, and no interaction (–) between MAT2, male-only strains of *Ceratocystis fimbriata* from *Mangifera indica* (mango) with MAT1, female strains of *C. fimbriata*, *C. platani*, and *C. cacaofunesta*

Species	Host	MAT1, female <sup>a</sup>	MAT2, male <sup>a</sup>					
			SESP5-1sec <sup>b</sup>	SEMS2-11sec <sup>c</sup>	NECE2-1sec	OMAN1-8sec <sup>d</sup>	ERRJ4-2sec	ERRJ1-10sec
<i>C. fimbriata</i>	<i>Mangifera</i>	SESP5-1ss <sup>b</sup>	I <sup>e</sup>	– <sup>f</sup>	–	I	–	–
		SEMS2-11ss <sup>c</sup>	I	I	I	I	I	I
		NEBA1-10ss	I	–	I	I	I	I
		OMAN5-7ss <sup>d</sup>	I	I	I	I	I	I
		ERRJ4-2ss	–	I	I	I	I	I
		ERRJ2-2ss	I	–	I	I	I	I
	<i>Ficus</i>	C1783ss	–	–	I	I	I	I
	<i>Gmelina</i>	C918ss	–	–	I	I	–	–
	<i>Eucalyptus</i>	C1347ss	I	I	I	I	I	I
	<i>Colocasia</i>	C1926ss	I	–	I	I	I	I
<i>Ipomoea</i>	C1418ss	I	I	I	I	I	I	
<i>C. cacaofunesta</i>	<i>Theobroma</i>	C1587ss	H <sup>g</sup>	H	H	H	H	H
<i>C. platani</i>	<i>Plantanus</i>	C1317ss	H	H	H	H	H	H

<sup>a</sup> sec = strains from MAT2, self-sterile sectors recovered from self-fertile isolates; ss = MAT1, female-competent isolates with protoperithecia.

<sup>b</sup> Isolate representing the previously named species *C. mangicola* (van Wyk et al. 2011a).

<sup>c</sup> Isolate representing the previously named species *C. mangivora* (van Wyk et al. 2011a).

<sup>d</sup> Isolate representing the previously named species *C. manginecans* (van Wyk et al. 2007).

<sup>e</sup> I = interfertile: ascospores abundant, with normal form; exuded ascospore masses creamy. Presence of both parental types among progeny when ascospore mass transferred and spread across a new plate.

<sup>f</sup> – = no perithecia and ascospores produced.

<sup>g</sup> H = hybrid: much cellular debris and few misshapen ascospores inside perithecium; exuded ascospore masses, when present, watery in appearance.

*C. cacaofunesta* (Engelbrecht et al. 2007), *C. platani* (Engelbrecht et al. 2004; Ocasio-Morales et al. 2007), *C. pirilliformis* (Nkuekam et al. 2009), and *C. fimbriata* (Ferreira et al. 2010, 2011; Harrington et al. 2015; van Wyk et al. 2006). Of the 16 markers used in earlier studies, two (CAT3K and CAT9x) were not utilized because their alleles (band sizes) could not be consistently resolved with some mango isolates. One primer of each pair was fluorescently labeled, and PCR amplifications were performed using a 96-well thermal cycler (PTC-100 MJ Research Inc., Watertown, MA) as described in Ferreira et al. (2010). Band sizes of the products were determined using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and ABI Peak Scanner v1.0 Analysis Software (Life Technologies). Each product length (within 1 bp) was considered to be a different allele.

**Microsatellite analyses.** Genetic variation of each mango population was compared with each other and representative *C. fimbriata* populations on *Colocasia* and *Eucalyptus* in Brazil using the microsatellite data (Ferreira et al. 2010). Besides the simple calculation of genotypic diversity ( $D$ , number of genotypes found in the population divided by the number of isolates sampled), multilocus genotypic diversity was estimated with the Stoddart and Taylor's  $G$  index (Stoddart and Taylor 1988). The maximum value of  $G$  is limited by the number of isolates sampled; therefore,  $G$  was scaled by the expected number of genotypes for the smallest sample size being compared (Grünwald et al. 2003). For comparisons among individual populations, 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 2.9.1; R Development Core Team, Vienna). For comparisons among different regions or host groups, the maximum value of  $G$  was 16.

Partition of total variance using analysis of molecular variance (AMOVA) on Euclidean distances was performed using ARLEQUIN 2.0 (Excoffier et al. 2005) in order to determine variation among regions, among populations, and within populations. Nei's gene diversity of microsatellite loci for each population, the matrices of Nei's genetic distance between populations, and a UPGMA dendrogram were constructed using PopGen 1.32 (Yeh and Boyle 1997). 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).

Each unique combination of the alleles among the 14 microsatellite loci was considered a microsatellite genotype. Relationships among the mango genotypes were examined using genetic distance (Nei's) matrices, UPGMA trees, and 1,000 bootstrap replications generated with PAUP 4.0b10 (Swofford 2002).

## RESULTS

An exhaustive search for Ceratocystis wilt on mango in Brazil found that the disease was more widespread than expected, particularly on garden and street trees in the Northeast (Fig. 1). The combined sample of 214 mango isolates from 214 trees included 161 new isolates from Brazil, 25 new isolates from Oman, as well as 15 isolates from Brazil and 13 isolates from Pakistan that had been studied previously (Harrington et al. 2014).

**ITS barcoding.** The PCR product for all but one of the 186 new mango isolates could be directly sequenced for the ITS region. One Omani isolate apparently had a mixture of sequences among the tandem repeats of rDNA, as was earlier found in some mango isolates from Oman and Rio de Janeiro (Harrington et al. 2014). Nine cloned fragments from the ITS PCR product from this new Omani isolate yielded three clones with the ITS7b sequence and six clones with the ITS6 sequence (ITS genotype nomenclature of Harrington et al. 2014).

There was substantial variation among the mango isolates in ITS rDNA sequences, mostly due to variation in insertions/deletions (indels). Twenty-two unique ITS sequences were found among the 214 mango isolates, including 10 previously characterized ITS genotypes (Harrington et al. 2014). New ITS genotypes were designated by

a unique letter suffix and compared with the other mango ITS genotypes using UPGMA analysis (Fig. 2). There was generally little relationship between geographic source of the isolates and relatedness of ITS sequences. However, the ITS7b genotype, described as *C. manginecans* (van Wyk et al. 2007), was only found in Oman and Pakistan (Fig. 2).

The three common ITS genotypes that were found at the most locations in Brazil corresponded with the sequences of three of the four recently named species (Tarigan et al. 2011; van Wyk et al. 2007, 2011a). The ITS6 genotype, described as *C. acaciivora*, was found on mango in 13 locations in Southeastern Brazil, as well as in most of the Oman and Pakistan locations (Fig. 2). The ITS14 genotype, named *C. mangicola*, was found at seven sites in Southeastern Brazil. The ITS10 genotype, named *C. mangivora*, was found at six collection sites in Southeastern Brazil and two collection sites in Northeastern Brazil.

**$\beta$ -tubulin sequences.** Fifty-four geographically representative mango isolates were sequenced for  $\beta$ -tubulin. Among the 564 aligned characters, seven different  $\beta$ -tubulin genotypes, mostly differing from each other at only one or two base positions, were identified. Five sequences were identified in Southeastern Brazil, and three of those sequences were also identified in Northeastern Brazil (GenBank KR002838-43). The isolates from Eastern Rio de Janeiro were uniform and unique for  $\beta$ -tubulin (GenBank KR002844).

Sequences (about 550 bp) of the  $\beta$ -tubulin region (EF433313, EF433308, EF433310, EU588643, EU588636, FJ200272, and FJ200280) from isolates that were used in the description of the four recently named species (Tarigan et al. 2011; van Wyk et al. 2007, 2011a) and those of another 23 isolates from other hosts representing the diversity of the LAC were included in phylogenetic analyses, with *C. variospora* as the outgroup taxon. For maximum parsimony (MP) analysis, 514 characters were constant, 35 were parsimony-uninformative, 15 were parsimony-informative, and there were 204 trees of 55 steps (Fig. 3), with a homoplasy index (HI) = 0.0323, consistency index (CI) = 0.9677, rescaled consistency index (RC) = 0.9558, and retention index (RI) = 0.9877. The evolution model GTR + G for  $\beta$ -tubulin region was selected and incorporated into the Bayesian analysis.

None of the branches with sequences of mango isolates had both high bootstrap and posterior probability support (Fig. 3). Most of the mango isolates had one of two sequences, which differed solely in the number of CT repeats in the intron region. The sequence of the ex-types of *C. mangicola* and *C. mangivora* was identical to that of most of the mango isolates from Southeastern Brazil, and this sequence had one less CT repeat than the sequence from the ex-types of *C. acaciivora* and *C. manginecans* (Fig. 3). The latter sequence was found in isolates from mango, *Eucalyptus*, *Acacia*, and *Gmelina* from Southeastern and Northeastern Brazil, as well as in isolates from Oman, Pakistan, India, Indonesia, and South Africa (Fig. 3).

**TEF-1 $\alpha$  sequences.** The 54 representative mango isolates also were sequenced for TEF-1 $\alpha$  and compared with those of another 19 isolates from other hosts representing the diversity of the LAC, with *C. variospora* as the outgroup taxon. This dataset included 1,377 characters that were constant, 54 that were parsimony-uninformative, and 15 that were parsimony-informative. There were six most parsimonious trees of 74 steps (Fig. 4), with a HI = 0.0412, CI = 0.9588, RC = 0.9090, and RI = 0.9481. Evolution model HKY + I + G was selected and incorporated into the Bayesian analysis. None of the isolates with ITS sequences matching the previously described species formed a monophyletic group in the TEF-1 $\alpha$  tree (Fig. 4). Isolates from Pakistan, Oman and India had the same TEF-1 $\alpha$  sequence as an isolate from *Gmelina arborea* in Pará, Brazil. The sequences of isolates representing *C. mangicola* (including the ex-type isolate C1688) and *C. acaciivora* were found scattered among the different branches. The TEF-1 $\alpha$  sequence of isolates representing *C. mangivora* (including C994 from the original description) was identical to that of most of the isolates of *C. fimbriata* from Brazil and a U.S. isolate from *Ipomoea* (Fig. 4).

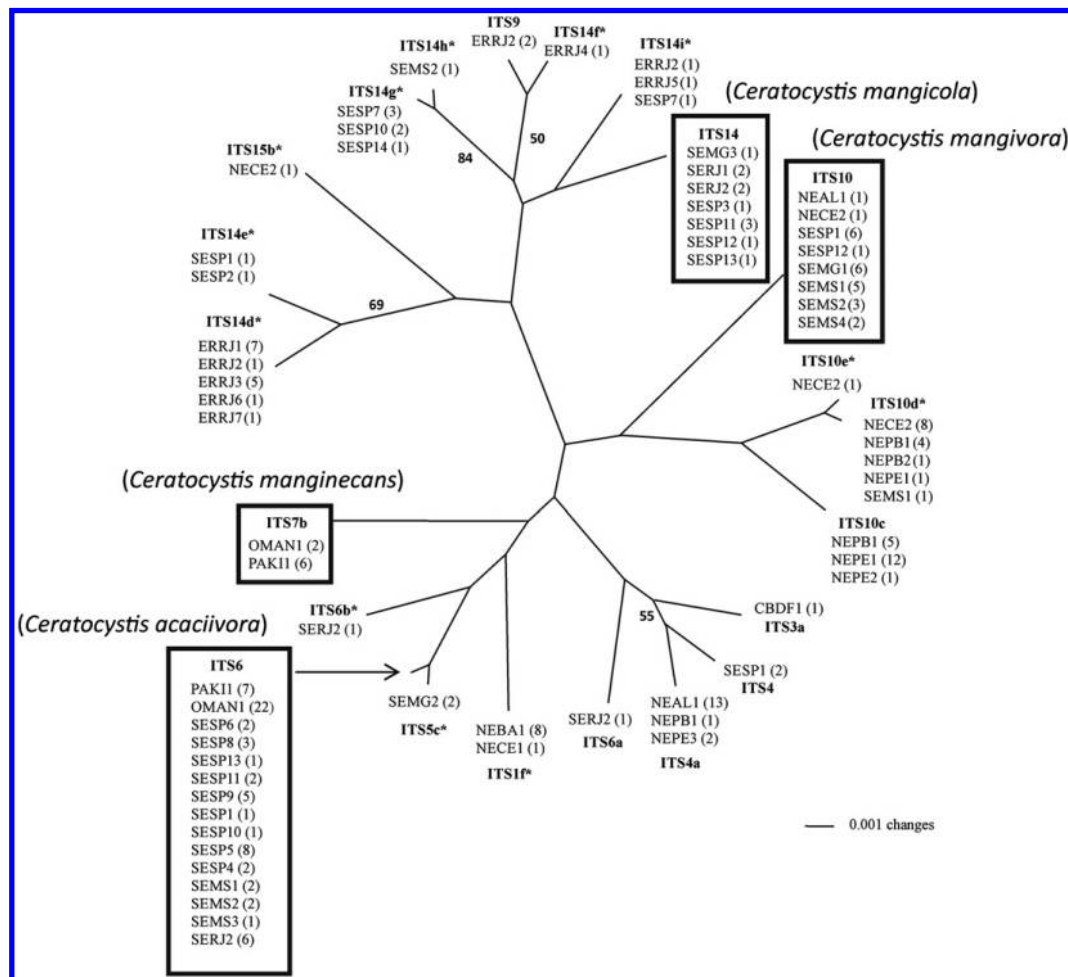
Partial TEF-1 $\alpha$  sequences (664 bp, matching the 5' end of our sequences) for 16 isolates from the original descriptions of the four recently named species (Tarigan et al. 2011; van Wyk et al. 2007, 2011a) were available in GenBank (EF433316-23, EU588646-7, EU588653-4, FJ200285-6, and FJ200293-4). The partial overlap of these sequences with our longer TEF-1 $\alpha$  sequences was used to confirm that our sequences adequately represented the four respective species. Partial sequences (FJ200285, FJ200286, and EF433316, respectively) from isolates CMW28913, CMW28914, and CBS 114721 (=CMW14797 = C1688) representing *C. mangicola* matched the TEF-1 $\alpha$  sequence of some of our mango isolates from São Paulo (e.g., C1688, SESP5-9, SESP11-7, and SESP1-6 in Figure 4). Nine of the partial sequences representing *C. mangivora*, *C. manginecans*, and *C. acaciivora* from Brazil, Pakistan, and Indonesia (Tarigan et al. 2011; van Wyk et al. 2007, 2011a) were identical and matched the common TEF-1 $\alpha$  sequence found in isolates from mango, *Eucalyptus*, and *Acacia* spp. in Northeastern and Southeastern Brazil, the *Ipomoea* isolate, and representatives of *C. cacaofunesta* (Fig. 4).

**Mating type sequences.** An alignment of 1,033 characters had nine characters varying and four *MATI-1-2* genotypes identified among the 214 mango isolates. Each of the four *MATI-1-2* genotypes had been identified previously (Harrington et al. 2014). Of the 214 studied isolates from mango tested using PCR, only 161 were confirmed to have the *MATI-2-1* gene. Many of the new isolates were single-spored, and care was not taken to retain only the self-fertile, MAT2 strains (some recovered progeny switched mating type and lost

the *MATI-2-1* gene). An alignment of 1,139 characters of the *MATI-2-1* gene showed 16 characters varying, and 10 different *MATI-2-1* genotypes were found among the 161 analyzed isolates.

These mating type genes are tightly linked and are not subjected to normal crossover events, and their combined sequences produce robust phylogenetic trees for the *C. fimbriata* complex (Harrington et al. 2014). The combined dataset of *MATI-1-2* and *MATI-2-1* sequences of mango isolates and other representatives of the LAC had an alignment of 2172 characters: 1743 were constant, 352 were parsimony-uninformative, and 76 were parsimony informative. MP analysis found a single tree of 455 steps (Fig. 5), with HI = 0.0384, CI = 0.9616, RC = 0.9232, and RI = 0.9168. Evolution model HKY + G was selected and incorporated into the Bayesian analysis, which produced a tree of identical topology to the MP tree. The other species in the LAC (*C. platani*, *C. colombiana*, and *C. cacaofunesta*) were each well supported and found at the base of the tree, and the branch representing *C. fimbriata* had bootstrap support of 86% and posterior probability of 1.0 (Fig. 5).

Basal within the *C. fimbriata* subclade was a branch (84% bootstrap support and posterior probability of 1.0) comprising mango isolates from Eastern Rio de Janeiro (all 20 isolates had sequence MAT5a), *Colocasia* isolates from São Paulo, and a *Eucalyptus* isolate from Paraná. All 25 isolates from Oman and 13 from Pakistan had the MAT7 genotype, which was placed among other Brazilian isolates of *C. fimbriata* and closest to mango isolate C2092 from Western Rio de Janeiro (Fig. 5). Isolate C1688 (=CBS 11472), the ex-type of



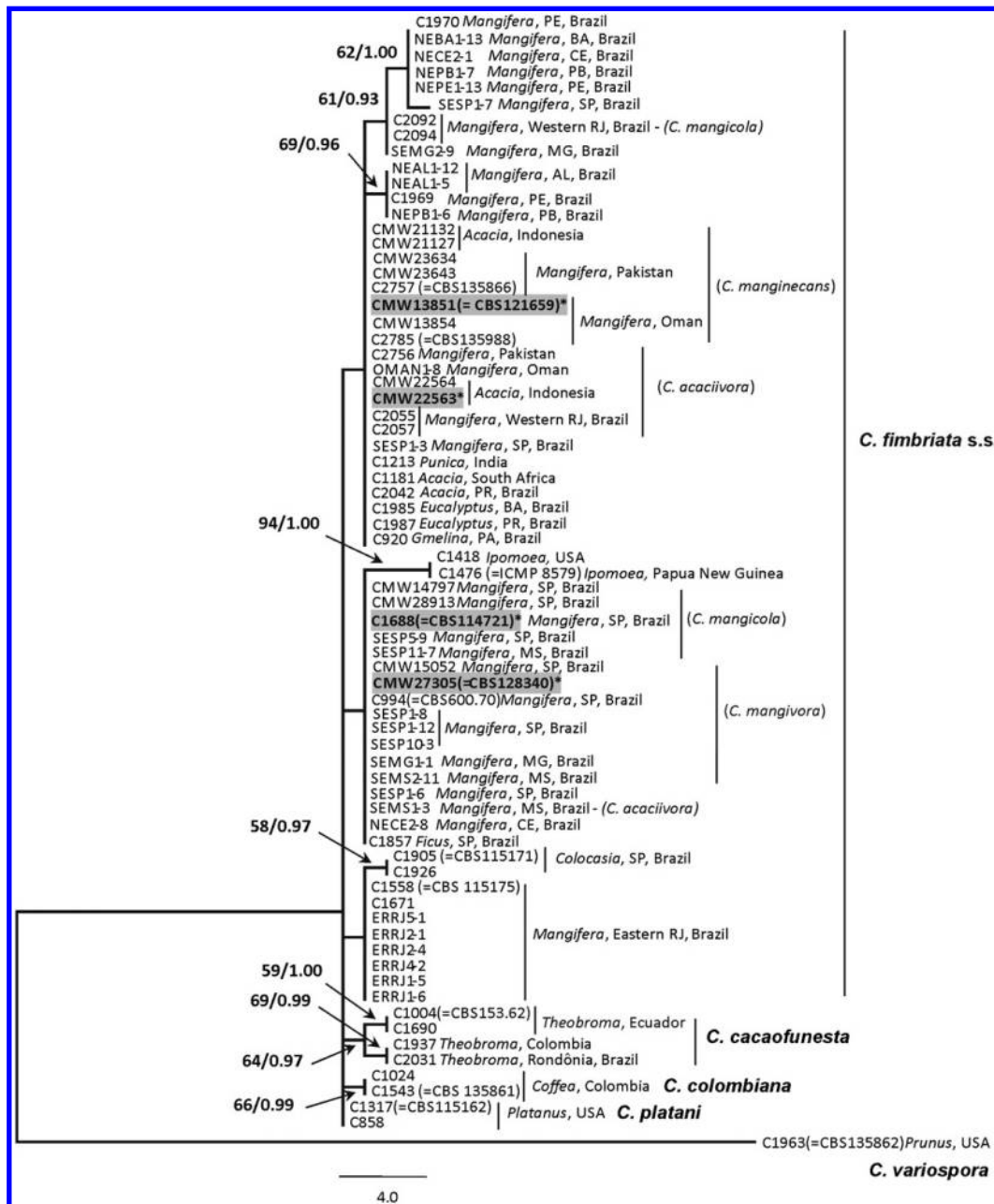
**Fig. 2.** An unweighted pair group method, arithmetic mean dendrogram of the genetic relatedness of internal transcribed spacer (ITS) rDNA genotypes (sequences) of *Ceratocystis fimbriata* from *Mangifera indica* (mango). Ten of the ITS genotypes had been reported by Harrington et al. (2014), with 12 new ITS genotypes designated by unique letters and an asterisk. The names of four previously described species (Tarigan et al. 2011; van Wyk et al. 2007, 2011a) based on distinct ITS sequences are indicated in parentheses above boxes. The locations (Fig. 1) where the ITS genotype was identified are followed by the number of isolates from that location in parentheses. Bootstrap values are shown alongside the branches. Scale bar indicates genetic distance.

*C. mangicola* (van Wyk et al. 2011a), had the MAT3b genotype. Isolate C994 (=CBS 600.70), on which the name *C. mangivora* was based (van Wyk et al. 2011a), had the MAT4a genotype (Fig. 5). However, other mango isolates with ITS rDNA sequences representing *C. mangivora*, *C. mangicola*, and *C. acaciivora* each had two or more mating type sequences that were found scattered throughout the *C. fimbriata* subclade (Fig. 5).

**Multigene phylogeny.** Forty isolates representing the sequence diversity of mango isolates were selected for multigene phylogenetic analyses combining  $\beta$ -tubulin, TEF-1 $\alpha$ , and mating type sequences. A total of 20 different four-gene genotypes were identified among the mango isolates. Another 19 isolates from other hosts representing the

diversity of the LAC and *C. variospora* as the outgroup taxon were included in the combined dataset. The partition homogeneity test conducted for the four gene regions resulted in  $P = 0.01$ , which is considered low (Cunningham 1997). Nonetheless, an analysis of the combined dataset produced 138 trees of 633 steps, and the trees generally were similar in topology to the more robust MP tree from analysis of the mating type genes alone (Fig. 5). Thus, the topology of the combined tree appeared to be governed by the variation in mating type sequences, and the combined tree is not shown.

**Mating experiments.** In attempts to identify biological species among mango isolates, fresh tester strains of opposite mating type derived from isolates from each of three regions of Brazil, an isolate



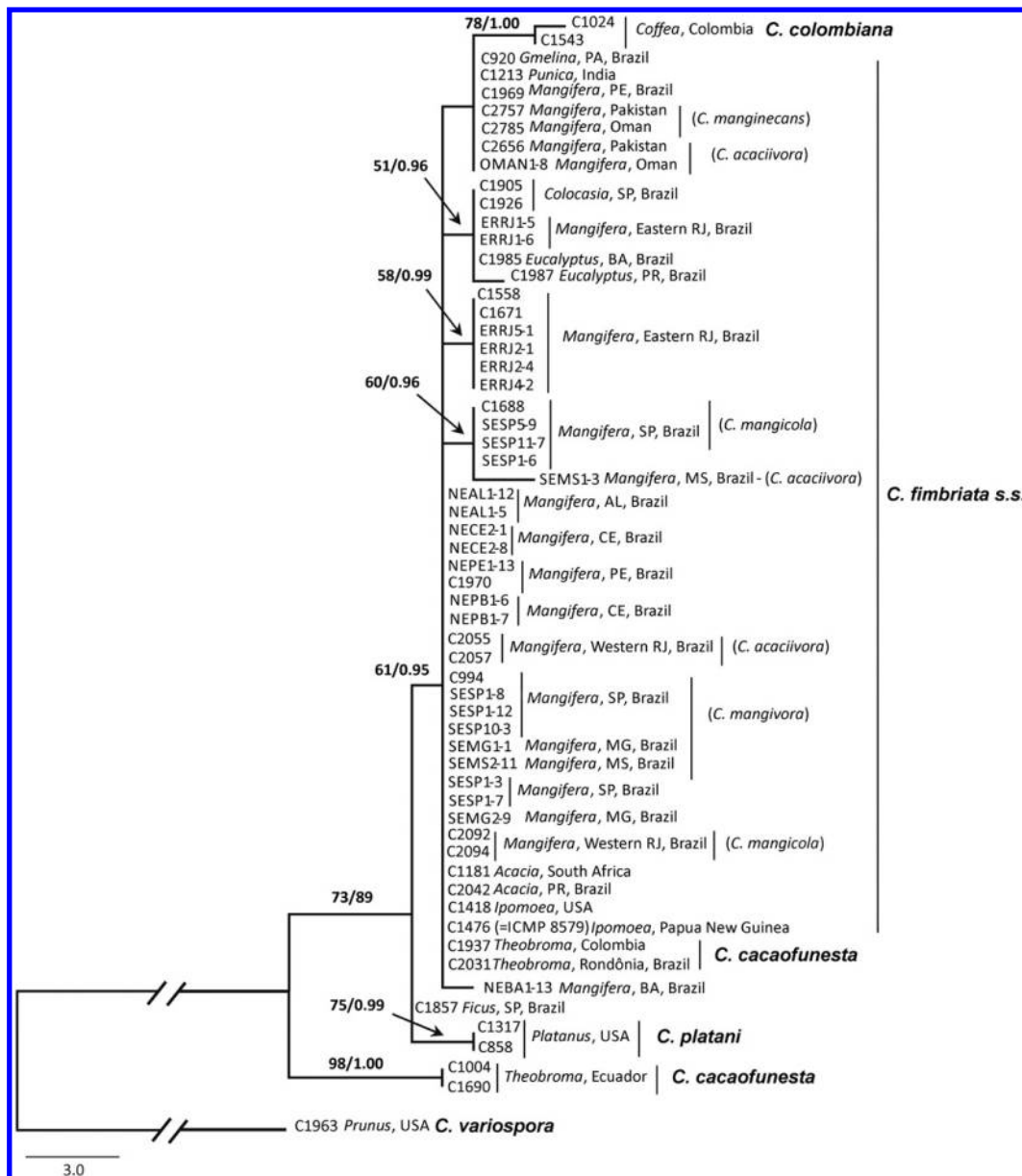
**Fig. 3.** In total, 1 of 204 trees of 55 steps based on maximum parsimony analysis of sequences of a portion of the  $\beta$ -tubulin gene of the Latin American Clade (LAC) of the *Ceratocystis fimbriata* complex. Bootstrap values greater than 50%/posterior probability values greater than 0.9 are indicated on appropriate branches. Isolate numbers are followed by host genus, (abbreviation for Brazilian state, where appropriate), and country. Sequences taken from the holotype of four previously described species on mango (Tarigan et al. 2011; van Wyk et al. 2007, 2011a) are shaded and indicated with an asterisk, and other isolates representing those species based on distinct internal transcribed spacer sequences are indicated in parentheses to the right of the isolate information. Besides *C. fimbriata*, the other accepted species in the LAC are *C. platani*, *C. cacaofunesta* and *C. colombiana* (Harrington et al. 2014). Isolate numbers are based on sample locations (Fig. 1) or are from the collection stored at Iowa State University (C) or the Centraalbureau voor Schimmelcultures (CBS). The tree was rooted to *C. variospora* in the North American Clade (isolate C1963). Scale bar indicates number of base changes.

from Oman, and an isolate from sweet potato successfully crossed with each other in at least one of the two mating experiments (Table 1). In most of the successful, interfertile (I) crosses, there were many fully developed perithecia within a week, with an accumulation of a large, opaque, creamy ascospore masses on the top of each perithecial neck (Fig. 6). However, some of the MAT1 female tester strains performed poorly in crosses, perhaps through loss of femaleness (few protoperithecia produced) (Ferreira et al. 2010). Ascospore masses from crosses were streaked on MYEA, and individual colonies from fully interfertile crosses produced colonies of the mycelial morphology of both the male and female tester strains, demonstrating that the ascospore masses were not due to a selfing.

In contrast, none of the MAT2-male tester strains from mango successfully crossed with MAT1 tester strains of *C. platani* or *C. cacaofunesta*. In some of these interspecific crosses, only a few perithecia were produced and the ascospore masses from these

perithecia were watery, not creamy. Microscopic examination showed aborted asci in the perithecial centrum, and the ascospores were misshapen (Fig. 6), suggesting a postzygotic barrier to meiosis. Consistent with earlier studies (Engelbrecht and Harrington 2005; Ferreira et al. 2010; Harrington and McNew 1997, 1998; Johnson et al. 2005), watery ascospore masses were considered indicative of an interspecific hybrid cross (H).

**Morphology.** All field isolates from mango appeared identical in culture morphology, with similar growth rates and mycelial pigmentation on MYEA. Cultures were initially white or pale brown, turning to dark brown after 5 to 7 days, the agar underneath turned dark brown (Rayner 1970), and the cultures had a banana-like odor. Mango isolates from the three regions of Brazil and Oman were microscopically examined and found to be indistinguishable from each other and sweet potato isolates (Table 2). Bases of perithecia were superficial to partially immersed, black or dark brown, globose, unornamented or



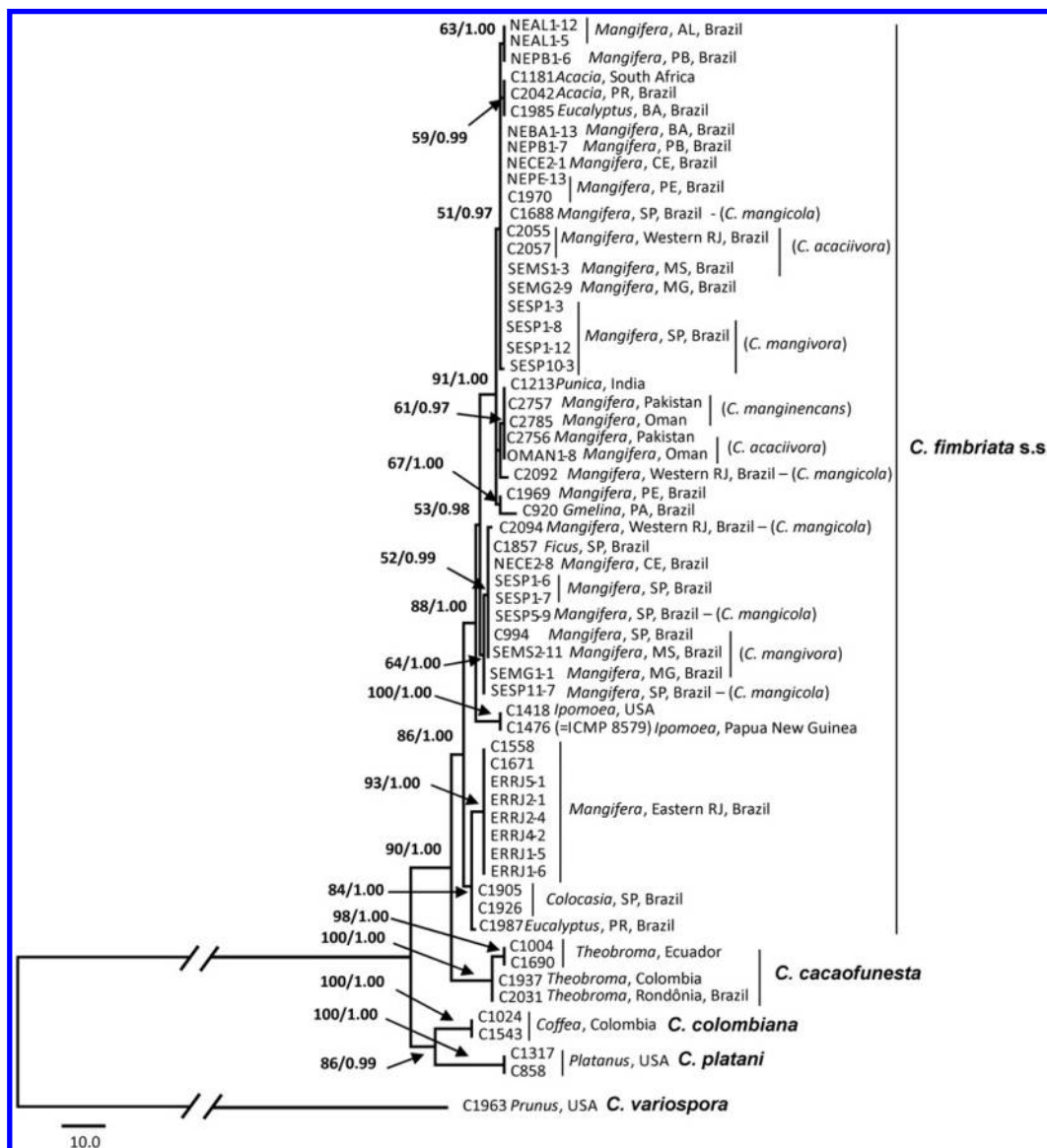
**Fig. 4.** One of six most parsimonious trees of 74 steps of representatives of the Latin American Clade (LAC) of the *Ceratocystis fimbriata* complex based on a portion of the TEF-1 $\alpha$  gene. Bootstrap values greater than 50%/posterior probability values greater than 0.9 are indicated on appropriate branches. Isolate numbers are followed by host genus, abbreviation for Brazilian state (where appropriate), and country. The names of four previously described species on mango (Tarigan et al. 2011; van Wyk et al. 2007, 2011a) based on distinct internal transcribed spacer sequences are indicated in parentheses to the right of the isolate information. Besides *C. fimbriata*, the other accepted species in the LAC are *C. platani*, *C. cacaofunesta*, and *C. colombiana* (Harrington et al. 2014). The tree was rooted to *C. variospora* in the North American Clade (isolate C1963). Scale bar indicates number of base changes.

with undifferentiated hyphae attached. The necks were black or dark brown, slender, with ostiolar hyphae that were hyaline and divergent or convergent. Ascospores had a hat-shaped brim and varied little in size. Endoconidiophores were of two types, one flask-shaped, hyaline to pale brown, septate, producing chains of cylindrical, hyaline endoconidia; and the other endoconidiophore type was less common, usually shorter, not tapering, producing chains of hyaline, doliform (barrel-shaped) endoconidia. Aleurioconidia were produced singly or basipetally in chains, thick-walled, pale brown to dark brown, ovoid or obpyriform, smooth, and similar in size among the studied isolates.

**Microsatellite variation in populations.** Of the 14 microsatellite loci, all but locus CAG900 were polymorphic among the 214 mango isolates (Table 3). At some locations only one, two or three isolates were obtained, and these were not included in the population analyses, leaving 19 mango populations (Table 4), which roughly separated into the four geographic regions in the UPGMA tree (Fig. 7). There were 57 microsatellite genotypes (unique combination of alleles), and 13 of those (MSAT1-MSAT13) were found in more than

one population. The UPGMA tree constructed using the 57 genotypes also tended to separate most of the mango genotypes into the four regional groups (Fig. 8). Further, an AMOVA suggested highly differentiated populations, with more than half of the variation attributed to variation among the four regional groups (Table 5). Within each of the three mango regions in Brazil, more variation was attributable to variation among populations than within populations, with the greatest population differentiation in the Northeast (64%) and the least in the Southeast (56%) (Table 5).

Isolates obtained from Oman and Pakistan showed variation only for locus CAA38, which had seven different alleles, and locus CAA9, which had two different alleles (Table 3). Allele CAA9-211 was found in just one isolate. Microsatellite genotype MSAT7 dominated in both countries (Fig. 8), and this appears to be the same genotype earlier identified in Oman and Pakistan using the same markers (Al Adawi et al. 2014). The genotypic diversity value ( $G$ ) for the 38 isolates from Oman and Pakistan was only 4.40 (maximum possible value of 16 for the regions), and the gene diversity value ( $H$ ) was only 0.04, compared



**Fig. 5.** The single most parsimonious tree of 455 steps based on portions of the *MATI-1-2* and *MATI-2-1* mating type genes for representatives of the Latin American Clade (LAC) of the *Ceratocystis fimbriata* complex. Bootstrap values greater than 50%/posterior probability values greater than 0.9 are indicated on appropriate branches. Isolate numbers are followed by host genus, abbreviation for Brazilian state (where appropriate), and country. The names of four previously described species on mango (Tarigan et al. 2011; van Wyk et al. 2007, 2011a) based on distinct internal transcribed spacer sequences are indicated in parentheses to the right of the isolate information. Besides *C. fimbriata*, the other accepted species in the LAC are *C. platani*, *C. cacaofunesta*, and *C. colombiana* (Harrington et al. 2014). The tree was rooted to *C. variospora* in the North American Clade (isolate C1963). Scale bar indicates number of base changes.

with the respective values of 5.0 and 0.13 for the Eastern Rio de Janeiro populations, 6.0 and 0.29 for the Southeastern populations, and 9.4 and 0.43 for the Northeastern populations (Table 4).

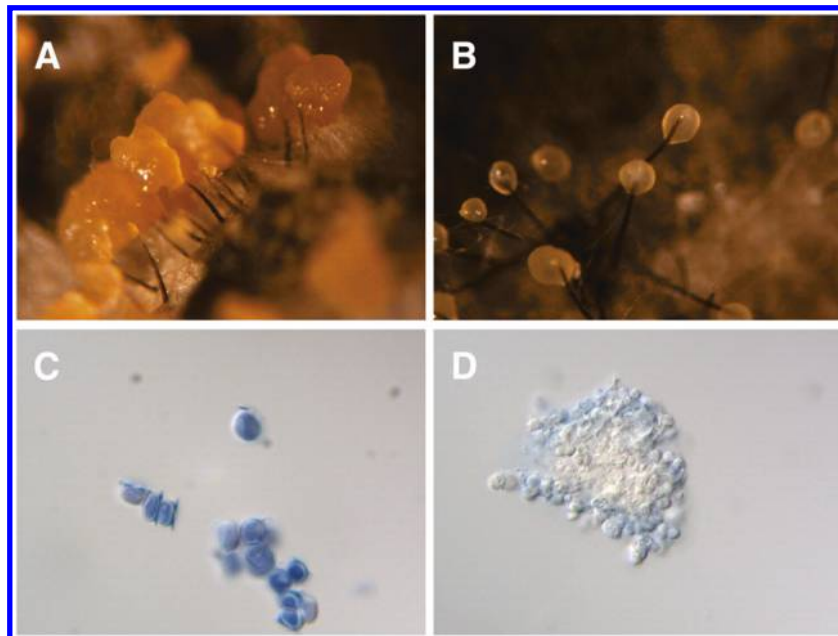
Consistent with the phylogenetic analyses (Figs. 3 to 5), the five Eastern Rio de Janeiro microsatellite genotypes were closely related (Fig. 8) and the populations similar to the *Colocasia* population (Fig. 7). Relatively few microsatellite alleles were found in Eastern Rio de Janeiro (Table 3), and three of the five genotypes were found in more than one population (Fig. 8). The five isolates sampled from a single, large plantation (ERRJ3) were genetically identical (Table 4).

The limited variation in Eastern Rio de Janeiro and the near uniformity of Oman and Pakistan populations contrasted sharply with the *Eucalyptus* populations from three plantations in Bahia and Minas Gerais, which were included as representatives of natural, soilborne populations of *C. fimbriata* (Ferreira et al. 2011, 2013). The three *Eucalyptus* populations were related to each other (Fig. 7), but their combined populations had high values of *G* and *H* (12.5 and 0.34, respectively), comparable only to the combined mango populations in Northeastern Brazil (Table 4).

Although the Northeastern populations had microsatellite alleles that were similar to those in the Southeast (Table 3), the Northeastern

populations showed greater gene and genotypic diversity (Tables 4), and microsatellite genotypes found in the Northeast were rarely found in more than one population (Fig. 8). Only four of the 25 genotypes from Northeastern Brazil were found in more than one Northeastern population (Fig. 8). One isolate from the Southeastern population SEMS1 grouped with Northeastern genotypes, one isolate from NECE1 grouped with Southeastern genotypes, and one isolate from NECE2 had the MSAT12 genotype, which was found in five isolates from two Southeastern plantations (Fig. 8). Aside from the NECE2 isolate, all of the mango isolates from the Northeast had the MAT3b or MAT3c mating type sequence (Fig. 8), showing near uniformity in these genes while the neutral population markers showed a wide variety of alleles (Table 3) in a diversity of combinations (Fig. 8).

The population in and around Seropédica (SERJ2) was the most variable of the Southeastern populations in microsatellite alleles, perhaps because SERJ2 comprises a mixture of introduced genotypes (Ferreira et al. 2010; Harrington et al. 2014). It had relatively high genotypic and gene diversity values (Table 4), as well as four ITS sequences (Fig. 7), including the ITS6 genotype and the mixed (intragenomic) ITS sequences found in some isolates in Oman and Pakistan. The microsatellite genotypes from Western Rio de Janeiro



**Fig. 6.** **A and B,** Perithecia and ascospore masses and **C and D,** ascospores from an interfertile cross (A and C) between two compatible *Mangifera indica* (mango) strains of *C. fimbriata* (isolates NEBA1-10 × OMAN8-1) and a hybrid, interspecific cross between a mango strain of *C. fimbriata* (isolate SESP5-1) and a *Platanus* strain of *C. platani* (isolate C1317) showing small watery ascospore masses (B), aborted asci, and misshapen ascospores (D).

**TABLE 2.** Range in sizes (micrometers) of microscopic features of representative *Ipomoea batatas* and *Mangifera indica* (mango) isolates of *Ceratomyces fimbriata* from each of three regions of Brazil (Northeastern, Southeastern, and Eastern Rio de Janeiro) and Oman, as well as representatives of *C. cacaofunesta* and *C. platani*

Region or previous described species	Isolate	Base of perithecia (height × width)	Ascospores	Length of perithecial neck	Length of ostiolar hyphae	Cylindrical conidia	Doliiform conidia	Aleuroconidia
Southeastern Brazil	SEMS2-11 <sup>a</sup>	115–190 × 105–195	4–6 × 3–5	395–655	37–60	11–29 × 3–5	8–11 × 5–6	10–15 × 8–10
	SESP5-1 <sup>b</sup>	125–195 × 115–185	4–6 × 3–5	370–565	37–55	10–26 × 3–5	7–9 × 5–6	10–14 × 9–12
Northeastern Brazil	NEBA1-10	125–250 × 125–280	4–6 × 3–5	260–680	30–52	14–20 × 3–5	7–9 × 4–6	8–15 × 7–12
Eastern Rio de Janeiro	ERRJ4-2	145–230 × 150–250	5–6 × 4–5	430–620	41–64	12–30 × 3–5	7–11 × 4–6	8–14 × 8–12
Oman	OMAN5-7 <sup>c</sup>	185–260 × 165–245	5–6 × 3–5	367–645	20–38	11–30 × 4–5	7–10 × 4–5	8–15 × 7–12
<i>C. fimbriata</i>	C1418 <sup>d</sup>	120–250 × 110–250	5–7.5 × 3.5–5	440–770	20–120	9–33 × 3–5	Not seen	11–16 × 6.5–12
<i>C. cacaofunesta</i>	C1983 <sup>d</sup>	100–275 × 95–305	4.5–6.5 × 3.5–5.5	310–1,010	30–125	8–40 × 2.5–5	Not determined	10–20 × 3.5–11.5
<i>C. platani</i>	C1317 <sup>d</sup>	175–290 × 175–290	4–6.5 × 3–4.5	535–835	20–90	11–22 × 3–5	6–10 × 3.5–5	10–20 × 6–12

<sup>a</sup> Isolate = *C. mangivora* based on internal transcribed spacer (ITS) sequence (van Wyk et al. 2011a).

<sup>b</sup> Isolate = *C. mangicola* based on ITS sequence (van Wyk et al. 2011a).

<sup>c</sup> Isolate = *C. acaciivora* based on ITS sequence (Tarigan et al. 2011).

<sup>d</sup> Measurements from Engelbrecht and Harrington (2005). Isolate C1418 was from *Ipomoea batatas*.

varied greatly from each other but were generally similar to other genotypes from the Southeast (Fig. 8). Of 13 isolates from Western Rio de Janeiro, 11 had mating type sequences typical of the Northeast (MAT3a and MAT3b), one (C2094) had the MAT4c sequence typical of the Southeastern populations, and one (C2092) had the MAT4b sequence that was similar to the MAT7 sequence found in Oman and Pakistan (Fig. 5).

**Widespread microsatellite genotypes.** Outside of the 13 Western Rio de Janeiro isolates and one isolate from Mato Grosso do Sul, the other 80 isolates from Southeastern Brazil were closely

related to each other, and most had one of two dominant genotypes (Fig. 8). Twenty-four isolates from eight locations had the MSAT1 genotype (Fig. 8), and 37 isolates from 12 locations had the MSAT2 genotype (Fig. 8). The isolate from the holotype of *C. mangicola* (C1688 = CBS 114721) had the MSAT1 microsatellite markers and isolate C994 (=CBS 600.70), which was among the isolates used to initially describe *C. mangivora* (van Wyk et al. 2011a), had the MSAT2 microsatellite markers.

It was hypothesized that the names *C. mangicola* and *C. mangivora* were based on genotypes of *C. fimbriata* that had been distributed in

TABLE 3. Estimated sizes (base pairs) of alleles of 14 microsatellite loci found in *Mangifera indica* (mango) populations of *Ceratocystis fimbriata* in four regions, with the number of isolates tested in each region shown in parentheses

Microsatellite locus	Southeastern Brazil (94)	Northeastern Brazil (61)	Eastern Rio de Janeiro (20)	Oman/Pakistan (38)
AAG8	174, 177, 180, 183	174, 177	186, 198	177
AAG9	391, 397, 400	397, 400, 403, 406	391	403
CAA9	175, 205	175, 178, 205	197, 223	211, 226
CAA10	128, 131, 134	128, 134	128	122
CAA15	321, 324	306, 321, 324	321, 330	324
CAA38	146, 201, 214, 238, 247	146, 153, 162, 174, 183, 202, 211, 214, 223	168, 211	214, 312, 321, 334, 340, 349, 357
CAA80	311, 317, 320, 323, 329, 333	288, 317, 320, 323, 329, 333	311	305
CAG5	298, 317, 320	317, 320	323, 326	317
CAG15	259, 286, 292	268, 286, 292, 295	277, 286	274
CAG900	194	194	194	194
CAT1	257, 261	254, 257, 260, 261	248	254
CAT12	374, 377	371, 374, 377	380	374
GACA6K	215, 219, 221	215, 221	219	215
GACA60	187, 200	187	187, 221	187

TABLE 4. Genetic diversity of *Mangifera indica* (mango) populations of *Ceratocystis fimbriata* from Brazil, Oman, and Pakistan and representative *Eucalyptus* and *Colocasia* populations of *C. fimbriata* from Brazil based on 14 microsatellite loci

Region or host group	Population	State or province	City	Collected from	Number of isolates	Number of genotypes	Genotypic diversity ( $G^a$ )	Nei's gene diversity ( $H$ )
Southeastern Brazil	SERJ2	Rio de Janeiro	Seropédica	Small farms and gardens	10	7	3.14	0.2302
	SESP1	São Paulo	Limeira	One row of nursery trees	11	4	2.58	0.2267
	SEMS2	Mato Grosso do Sul	Aquidauana	Small farms	6	3	2.33	0.1468
	SEMS1	Mato Grosso do Sul	Aquidauana	Small city	8	3	2.29	0.2277
	SESP7	São Paulo	Taquaritinga	Single plantation	4	2	2.00	0.0357
	SEMG1	Minas Gerais	Frutal	Single plantation	7	2	1.93	0.0350
	SESP5	São Paulo	Cândido Rodrigues	Single plantation	11	1	1.00	0.0000
	SESP9	São Paulo	Santo Anastácio	Small city	5	1	1.00	0.0000
	SESP11	São Paulo	Vista Aleg. do Alto	Single plantation	6	1	1.00	0.0000
		All isolates from the Southeastern Region				68	14	6.04
Northeastern Brazil	NECE2	Ceará	Brejo Santo	Small city	11	7	3.48	0.2786
	NEPB1	Paraíba	Conde	Small city	10	6	3.20	0.3814
	NEPE1	Pernambuco	Cabo St. Agostinho	Small farms	13	5	2.67	0.1183
	NEAL1	Alagoas	Água Branca	Small city	14	4	2.65	0.0576
	NEBA1	Bahia	Senhor do Bonfim	Small city	8	2	1.50	0.0156
		All isolates from the Northeastern Region				56	22	9.41
Eastern Rio de Janeiro (Brazil)	ERRJ1	Rio de Janeiro	São Fidélis	Small farms	7	3	2.43	0.0466
	ERRJ2	Rio de Janeiro	Itaocara	Small farms	4	2	2.00	0.1339
	ERRJ3	Rio de Janeiro	Itaocara	Single plantation	5	1	1.00	0.0000
		All isolates from Eastern Rio de Janeiro				16	5	5.00
Oman/Pakistan	OMAN1	Al-Batinah, Muscat and Musandam	Multiple	Small farms	25	5	2.27	0.0361
	PAK11	Multiple	Multiple	Small farms	13	4	1.92	0.0380
		All isolates from Oman and Pakistan				38	7	4.40
<i>Eucalyptus</i>	EucBA1	Bahia	Eunápolis	Single plantation	26	13	3.27	0.2191
	EucBA2b	Bahia	Caravelas	Single plantation	6	4	3.20	0.2262
	EucMG1	Minas Gerais	Curvelo	Single plantation	18	14	3.74	0.3069
		All isolates from <i>Eucalyptus</i>				50	31	12.49
<i>Colocasia</i>	ColSP3	São Paulo	Piedade	Infected corms from local farms	12	4	2.50	0.0784

<sup>a</sup> Stoddart and Taylor's genotypic diversity, with rarefaction. Values of  $G$  with rarefaction for individual populations ranged from 1.0 (only one genotype in the population) to a maximum value of 4.0 (each isolate in the population of a different genotype). Maximum values for region or host groups ranged from 1 to 16.

nursery stock (Harrington et al. 2014), and attempts were made to find the pathogen in commercial nurseries in the largest nursery region in São Paulo. Population SESP1 was a sampling of 11 isolates from a row of mango trees in an abandoned nursery near Limeira (Table 4). Four microsatellite genotypes, including MSAT1/*C. mangivora*, MSAT2/*C. mangicola* and MSAT11 (Fig. 8), as well as four ITS genotypes (Fig. 7) and two mating type sequences (Fig. 8), were recovered from the row of diseased trees. The trees appeared to have been undisturbed since planting some years before, and they were without evidence of stem wounding or attack by bark beetles. The trees were apparently infected from soilborne inoculum because the infections arose from the roots.

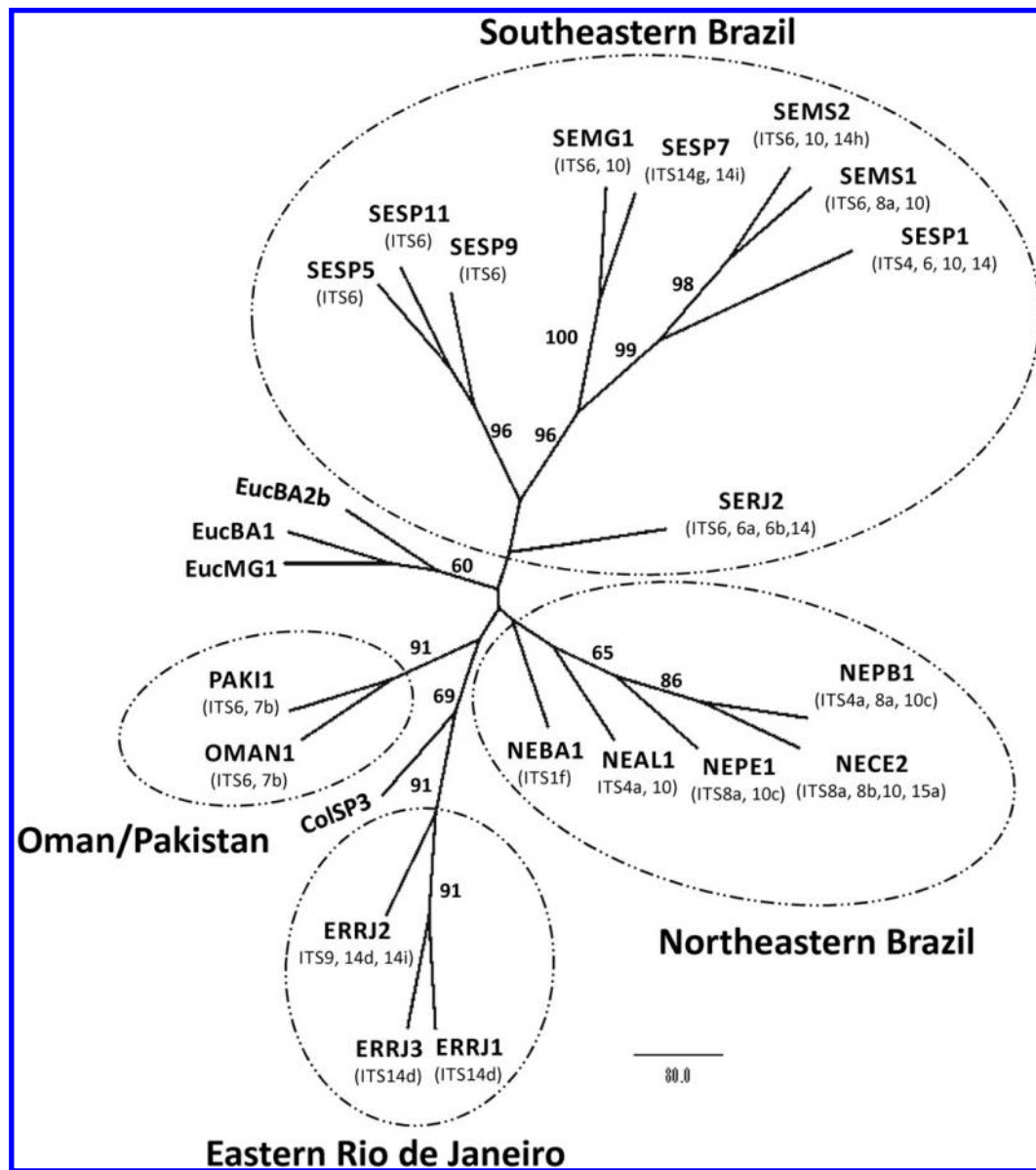
All of the Southeastern populations except SERJ2 from Western Rio de Janeiro had lower genetic diversity values than the nursery population SESP1, and each was dominated by the MSAT1 or MSAT2 genotypes found in the nursery (Table 4, Fig. 8). The four plantation populations in Southeastern Brazil were nearly uniform and had only genotypes MSAT1, MSAT2, and MSAT12, the latter differing from

MSAT1 at only one locus. The isolates recovered from plantations SESP7 and SEMG1 had only the MSAT1 and MSAT12 genotypes, in roughly equal proportions, and only the MSAT2 genotype was recovered from plantations SESP5 and SESP11 (Table 4, Fig. 8). The city population from Santo Anastácio (SESP9) also had only the MSAT2 genotype (Table 4).

Isolates with related microsatellite genotypes generally had identical or similar mating type sequences, except in the Southeastern region (Fig. 8). With the exception of the Mato Grosso do Sul populations, isolates of the MSAT1 genotype had one of the Northeast mating type sequences (MAT3b), while isolates of the MSAT2 genotype had one of the Southeast mating type sequences (MAT4a or MAT4d) (Fig. 8).

## DISCUSSION

There was no evidence of cryptic species among mango isolates of *C. fimbriata*. The new species names were primarily based on

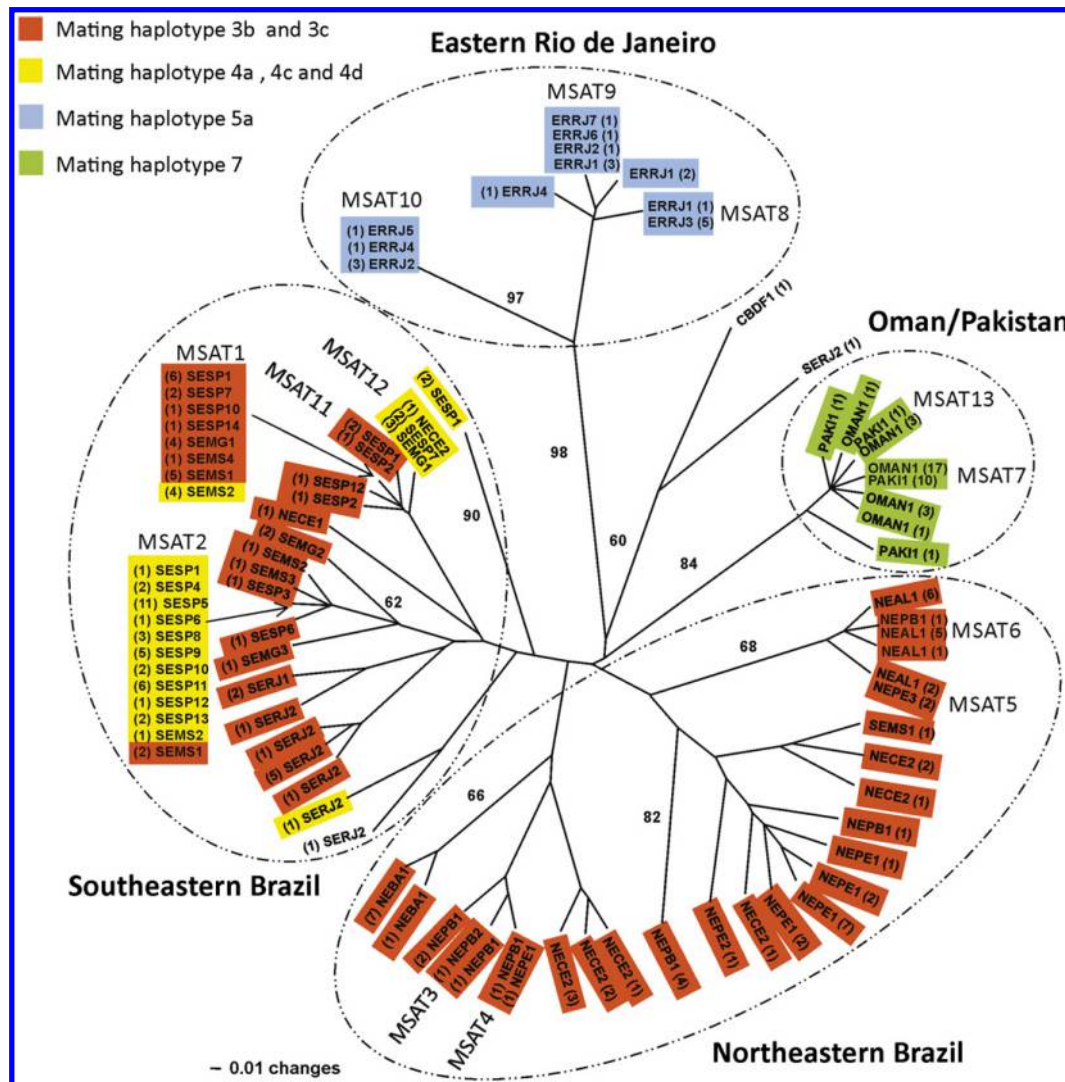


**Fig. 7.** Dendrogram of the relatedness of populations of *Ceratocystis fimbriata* from *Mangifera indica* (mango), *Eucalyptus* (EucBA1, EucBA2b, and EucMG1), and *Colocasia* (ColSP3) generated by unweighted pair group method, arithmetic mean based on allele frequencies (Nei's gene diversity) of 14 microsatellite loci. Bootstrap values greater than 50% are indicated alongside the branches. Dotted and dashed lines encircle *Mangifera* populations from three regions of Brazil (Northeastern, Southeastern, and Eastern Rio de Janeiro), and the Oman/Pakistan region. State designations for the Brazilian populations are AL = Alagoas; BA = Bahia; CE = Ceará; DF = Distrito Federal; MG = Minas Gerais; MS = Mato Grosso do Sul; PA = Pará; PB = Paraíba; PE = Pernambuco; PR = Paraná; RJ = Rio de Janeiro; and SP = São Paulo. The internal transcribed spacer rDNA genotypes found in the population are in parentheses. Scale bar indicates genetic distance.

unique ITS sequences, but phylogenetic analyses of gene coding regions placed the four recently described species among other Brazilian populations of *C. fimbriata* from mango and other hosts, as well as the sweet potato strain, on which the species name *C. fimbriata* was based. No morphological variation was seen among representative mango isolates. Interfertility tests demonstrated that representative mango isolates are within the same biological species as Brazilian isolates from other hosts and the sweet potato strain. Microsatellite markers showed that populations of *C. fimbriata* on mango are highly differentiated within and across regions, and natural populations

rarely shared microsatellite genotypes. On the other hand, representatives of the recently described species had the most common and widespread microsatellite genotypes on mango, supporting the hypothesis that the recently described species were based on introduced strains of *C. fimbriata*.

The strongest case for a genealogical species within *C. fimbriata* was the closely related populations of mango isolates from Eastern Rio de Janeiro, which grouped with *Colocasia* isolates from São Paulo (Ferreira et al. 2010; Harrington et al. 2005) in phylogenetic analyses and in microsatellite makers. These mango and *Colocasia*



**Fig. 8.** An unweighted pair group method, arithmetic mean dendrogram of genotypes of *Ceratocystis fimbriata* from *Mangifera indica* (mango) based on alleles of 14 microsatellite loci (Nei's gene diversity). Bootstrap values are shown alongside the branches. The number of isolates with that genotype from each population is in parentheses. Thirteen genotypes found in more than one population are designated by "MSAT" followed by a number. Microsatellite genotypes encircled by dotted and dashed lines are from one of the three regions of Brazil (Northeastern, Southeastern, and Eastern Rio de Janeiro) or Oman/Pakistan. Related mating type DNA sequences (Fig. 5) of the isolates are color-coded. Scale bar indicates genetic distance.

**TABLE 5.** Analysis of molecular variance of *Mangifera indica* (mango) populations of *Ceratocystis fimbriata* from three regions of Brazil (Northeastern, Southeastern, and Eastern Rio de Janeiro), Oman, and Pakistan based on 14 microsatellite loci

Regions	Among regions			Among populations			Within populations		
	d.f.	%	$P^a$	d.f.	%	$P^a$	d.f.	%	$P^a$
All populations	3	51.40	<0.0001	18	29.88	<0.0001	160	18.68	<0.0001
Northeastern Brazil				4	64.30	<0.0001	51	35.70	<0.0001
Eastern Rio de Janeiro, Brazil				2	60.49	<0.0001	13	39.51	<0.0001
Southeastern Brazil				8	55.71	<0.0001	60	44.29	<0.0001
Oman/Pakistan				1	0.00	0.5318	36	100.00	0.02444

<sup>a</sup> The  $P$  value is for the null hypothesis that there is no significant variation at that level based on 1,023 permutations.

populations appear to be indigenous to a unique ecological zone, a wet, low-elevation coastal region of Brazil that was formerly Mata Atlântica forest type (Harrington et al. 2014). Most of the other studied populations of *C. fimbriata* from Brazil appear to be native to the generally drier Northeast of Brazil, including the seasonally drier Cerrado and Catinga forest types. Although the populations from Eastern Rio de Janeiro may be geographically isolated and ecologically distinct, they were not morphologically distinguishable from other mango isolates of *C. fimbriata*. Further, isolates from this region do not appear to be host specialized, though mango isolates from Eastern Rio de Janeiro may be particularly aggressive to mango and other nonnative hosts (Baker et al. 2003; Harrington et al. 2011; Silveira et al. 2006). Thus, with no diagnosable phenotypic character, this Eastern Rio de Janeiro lineage is not a phylogenetic species in the traditional sense (Harrington and Rizzo 1999). Further, mango tester strains from this region were fully interfertile in crosses with other mango isolates and the sweet potato tester, as was found with similar mango and *Colocasia* isolates in an earlier interfertility study (Ferreira et al. 2010). Therefore, a genealogical species recognition concept may distinguish mango and *Colocasia* isolates from the coastal lowlands as a cryptic species, but the traditional phylogenetic and biological species concepts would recognize it merely as a group of populations.

Species delimitation would ideally be based on understanding of phenotypic variation and population structure (Harrington and Rizzo 1999), but most important fungal pathogens on crop plants are known only by their human-mediated populations. *C. fimbriata* and closely related species in the LAC present interesting case studies because native and introduced populations have been characterized. Patterns of highly variable native populations versus introduced populations that have gone through genetic bottlenecks have been characterized using the same microsatellite and other markers for *C. platani* on *Platanus* spp. (Engelbrecht et al. 2004; Ocasio-Morales et al. 2007), *C. cacaoifunesta* on cacao (Engelbrecht et al. 2007), and *C. fimbriata* populations on various crops in Brazil (Ferreira et al. 2010). Local populations of *C. fimbriata* on *Eucalyptus* that are based on root infections via natural, soilborne inoculum tend to have high gene and genotypic diversity, while local populations based on planting of infected nursery stock are genetically uniform (Ferreira et al. 2011, 2013). Similar evidence suggests that Chinese populations of *C. fimbriata* on pomegranate, *Colocasia* and other hosts have gone through a genetic bottleneck and may have been introduced on *Eucalyptus* cuttings from Brazil (Harrington et al. 2015). This is likely the same pathway for the *C. fimbriata* populations on *Eucalyptus* in South Africa (van Wyk et al. 2006), which are related to Brazilian *Eucalyptus* isolates and Chinese pomegranate isolates (Harrington et al. 2014, 2015).

On mango, the most genetically variable and distinct populations were found in the Northeast, and mostly depauperate populations with widely shared genotypes were found in other regions. Microsatellite markers showed a highly differentiated population structure in the Northeast, suggesting limited gene flow among populations, consistent with expectations for a homothallic, soilborne pathogen with only limited dispersal by insects and no airborne spores (Ferreira et al. 2010, 2011). However, some local populations showed limited genotypic diversity, and localized spread of genotypes is possible through movement of the pathogen on tools (Harrington 2013) or by the mango bark beetle, *Hypocryphalus mangifera* (Coleoptera: Scolytinae), a native of Asia that can serve as a vector of *C. fimbriata* in mango (Al Adawi et al. 2006, 2013a; Masood and Saeed 2012; Rossetto and Medeiros 1967). This mango-specific beetle was common at our collection sites in Oman, Eastern Rio de Janeiro, and Northeastern Brazil, where it may be responsible for local spread of genotypes from mango tree to mango tree. However, humans appear to be the most important long-distance vector, especially in movement of infected grafted or otherwise vegetatively propagated material (Engelbrecht et al. 2007; Ferreira et al. 2011, 2013; Harrington 2013; Harrington et al. 2014, 2015). The genetic data suggest that there is only limited

movement of *C. fimbriata* among populations in the Northeast, but populations in each of the other regions were dominated by a few widespread microsatellite genotypes, consistent with introductions of the pathogen in grafted mango nursery stock (Rossetto and Ribeiro 1990).

Ceratocystis wilt on mango trees was first noted in the Northeast (Carvalho 1938), where we found that the disease remains restricted to scattered locations on garden and street trees, and there are no confirmed reports in large plantations. Decades later, the disease was recognized in São Paulo (Viégas 1960), where it remains a major cause of mango tree mortality in plantations as well as small cities and small farms in São Paulo and neighboring states. The population on the garden and street mango trees in Western Rio de Janeiro was unique because it appeared to be derived from multiple introductions of genetically diverse strains of *C. fimbriata*, some of which genetically resemble the introduced strains found in Oman, Pakistan, India, and Indonesia (Harrington et al. 2014). But elsewhere in the Southeast, either MSAT1 or MSAT2 or both were recovered from each of the sampled populations.

Only one or two of the widespread microsatellite genotypes were recovered from each of the five studied mango plantations. Consistent with the hypothesis that these plantations or neighboring plantings were established with infected nursery stock, the two dominant genotypes of the Southeast were recovered from a row of abandoned and undisturbed mango plants in the sampled nursery in Limeira. Altogether, the MSAT1 and MSAT2 genotypes were isolated from 61 of the 81 sampled trees and from each population sampled in São Paulo, Mato Grosso do Sul and Minas Gerais. The São Paulo isolates used to originally describe *C. mangivora* and *C. mangicola* (C994 = CBS 600.70, and C1688 = CBS 114721, respectively) (van Wyk et al. 2011a) have the respective MSAT1 and MSAT2 microsatellite markers. Representative isolates of these two genotypes were not distinguished from each other or other *C. fimbriata* populations in our phylogenetic analyses, they were fully interfertile in crosses with other mango isolates and with the *C. fimbriata* tester from sweet potato, and they were not distinguished by morphology, host range or other phenotype.

Consistent with our phylogenetic analyses, Fourie et al. (2015) failed to distinguish representatives of *C. mangivora* and *C. mangicola* by DNA sequence analyses. They recommend retention of the species names because they could distinguish the few studied isolates by single nucleotide polymorphisms, which likely correspond to the microsatellite polymorphisms found in MSAT1 and MSAT2. No other Brazilian strains of *C. fimbriata* were studied by Fourie et al. (2015), but with the wider sampling of mango isolates from Brazil, it is clear that it is not practical to name each genotype of *C. fimbriata* as a new species. Similarly, with the widespread introduction of strains of *C. fimbriata* around the world, it is not practical or informative to name each introduced genotype as a new species, especially if the origin of the introduced strains is not known.

The only confirmed reports of Ceratocystis wilt on mango outside of Brazil are in Pakistan and Oman, and our analyses and those of Al Adawi et al. (2014) demonstrate that the pathogen population in those countries is dominated by a single microsatellite genotype. Although the exact combination of microsatellite markers and mating type gene sequences were not identified in Brazil, the Oman, Pakistan, and Indian pomegranate isolates are closest to *Gmelina* isolates from the lower Amazonian state of Pará and mango isolates from Northeastern Brazil and Western Rio de Janeiro. The  $\beta$ -tubulin and ITS rDNA sequences also tie the Oman, Pakistan and Indian strain to the Ceratocystis wilt pathogen on *Acacia* spp. in Indonesia and populations of *C. fimbriata* in the Northeast of Brazil, supporting the hypothesis that the introduced population in Oman and South Asia has a South American origin (Harrington et al. 2014), although the pathway of introduction is not clear.

Some isolates from Pakistan, Oman and India have more than one ITS sequence within a single haploid genome (Al Adawi et al. 2013b; Harrington et al. 2014; Naidoo et al. 2013), as was found for

a mango isolate from Western Rio de Janeiro (Harrington et al. 2014). Such intragenomic variation in ITS sequences may be due to recent crosses between two introduced strains with differing ITS sequences (Harrington et al. 2014). The two most common ITS sequences found among isolates and within genomes in Oman and Pakistan were described as new species, *C. manginecans* (van Wyk et al. 2007) and *C. acaciivora* (Tarigan et al. 2011), but clearly they are a single species based on our results and the more extensive phylogenetic analyses by Fourie et al. (2015). Oman isolates were interfertile with other mango isolates from Brazil and the sweet potato tester strain, showing that they are the same biological species as *C. fimbriata* in the strictest sense. Phylogenetic analyses place the Oman, Pakistan, Indian, and Indonesian strain among Brazilian populations of *C. fimbriata*, and lack of morphological distinction precludes recognition of *C. manginecans* or *C. acaciivora* by the traditional phylogenetic species concept.

Recognition of species by genealogical concordance requires inclusion of representative samples from natural populations of the putative new species and relatives (Taylor et al. 2000). Without representatives of native populations, each independently introduced population could appear as a distinct lineage and could be described as a new species. This is especially likely if the natural populations are highly differentiated, as is the case with *C. fimbriata* in Brazil, and the introductions have different geographic origins.

Many new species of other important fungal genera of plant pathogens have been similarly described based solely on human-mediated populations and only minor DNA sequence variation, mostly in ITS sequences. However, species names without distinguishing phenotype carry little useful information, and recognition of each genotype as a new species is becoming impractical. This work with *C. fimbriata* points to the importance of studying natural populations and the use of phenotype in delineating species of fungi, as is done in the naming of species in other groups of organisms (Harrington and Rizzo 1999).

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