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A host specialized form of *Ceratocystis fimbriata* causes seed and seedling blight on native *Carapa guianensis* (andiroba) in Amazonian rainforests

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ABSTRACT

Ceratocystis fimbriata Ellis & Halsted recently was recorded causing seed and seedling blight on *Carapa guianensis* Aubl. (andiroba), a tree species native to the Amazon Rainforest and prized for its valuable timber and medicinal seed oil. *C. fimbriata* more commonly causes wilt type diseases in woody hosts, especially on non-native host trees. However, on andiroba the disease occurs on seedlings and seeds, affecting the species regeneration. We studied 73 isolates of *C. fimbriata* on andiroba from three regions of the Amazon Basin to see if they represented natural or introduced populations. Analysis of ITS rDNA sequences and phylogenetic analysis of mating type genes revealed new haplotypes of *C. fimbriata* from the Latin American Clade that were closely related to other Brazilian populations of the fungus. In mating experiments, andiroba isolates were inter-fertile with tester strains of *C. fimbriata* from Brazil and elsewhere, confirming that they belong to a single biological species. Using microsatellite markers, 14 genotypes and populations with intermediate levels of genetic variability were found, suggesting that the fungus is indigenous to the Amazon Basin. Inoculation tests indicated that the andiroba isolates are host-specialized on andiroba, supporting the proposition of the special form *C. fimbriata* f. sp. *carapa*.

1. Introduction

Carapa guianensis (andiroba, Meliaceae) is a woody plant native to the Amazon Rainforest (Tropicos, 2016). It is prized for its highly valued wood and seed oil, which is used for cosmetic and medicinal purposes (Herrero-Jáuregui et al., 2013; Klimas et al., 2012; Souza et al., 2006; Vinhote, 2014). Recently, *Ceratocystis fimbriata sensu lato* (s.l.), was reported on seeds and seedlings underneath naturally established andiroba trees (Halfeld-Vieira et al., 2012). In Brazil, *C. fimbriata* most commonly causes wilt-type diseases in the woody xylem of non-native timber and fruit-producing trees, such as *Mangifera indica* L. (mango) (Carvalho, 1938), *Gmelina arborea* Roxb. Ex Sm. (gmelina) (Muchovej et al., 1978), *Ficus carica* L. (fig tree) (Valarini and Tokeshi, 1980), *Eucalyptus* spp. (Ferreira et al., 1999), *Tectona grandis* L. f. (teak) (Firmino et al., 2012), and *Actinidia deliciosa* (A. Chev) CF Lianget & AR Ferguson (kiwifruit) (Piveta et al., 2013).

Ceratocystis fimbriata is a soilborne pathogen that appears to be native to much of Brazil (Ferreira et al., 2010, 2017; Oliveira et al., 2015a). However, only a few native hosts of *C. fimbriata* have been identified in Brazil. It has been reported on planted *Hevea brasiliensis* M. Arg. (rubbertree) (Albuquerque et al., 1972; Valdetaro et al., 2015), but the *C. fimbriata* strains recovered from this host in Acre (Amazon Region) and Bahia (Northeastern Brazil) may not have been native to those areas (Valdetaro et al., 2015). The species causing wilt on *Theobroma cacao* L. (cacao) was found to be native to the Upper Amazon and was described as a distinct species (*Ceratocystis cacaofunesta* Engelbr. & T.C. Harrin.) that is host-

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specialized to that species and is intersterile with all other tested strains in the *C. fimbriata* complex (Engelbrecht and Harrington, 2005). Recently, *C. fimbriata* was reported on *Caryocar brasiliense*, causing wilting and death of trees in the Cerrado vegetation type in the State of Minas Gerais (Silva et al., 2017). Other important populations of *C. fimbriata* in Brazil have been characterized on non-native hosts, such as mango, eucalyptus, taro, and kiwifruit (Ferreira et al., 2010, 2017; Oliveira et al., 2015a, 2017).

Like *C. cacaofunesta*, the pathogen on andiroba could be a cryptic species native to the Amazon Basin that has specialized to this native host and is no longer sexually compatible with other strains of *C. fimbriata* in Brazil. This work aimed to determine if the population on andiroba is a distinct species by: a) genetically characterizing the pathogen through phylogenetic analyses using ITS rDNA bar-coding and sequences of mating type genes, b) characterizing the population structure of the pathogen using microsatellite markers, c) determining the mating compatibility of andiroba strains with other isolates of *C. fimbriata* from Brazil, and d) evaluating the aggressiveness and host specificity of the pathogen by cross-inoculations on mango, teak, eucalyptus and andiroba.

2. Materials and methods

2.1. Sample collection and fungal isolation

Andiroba seeds and seedlings with stem blight symptoms and gray to black fungal sporulation on their surfaces were collected in native Amazonian rain forests in the states of Acre (AC, Rio Branco), Amazonas (AM, Boa Vista do Ramos/Parintins) and at two sites in Roraima (RR1 and RR2, São João da Baliza) (Fig. 1). At each site, the samples were collected within a 50 m radius around an andiroba tree. Seeds and seedlings were brought to the Laboratory of Forest Pathology, DFP/BIOAGRO at the Universidade Federal de Viçosa (UFV). Diseased tissue was placed on carrot baits (Moller and Devay, 1968) and subsequently incubated at room temperature (approximately 25 °C) to stimulate perithecium production. A single ascospore mass formed at the top of a perithecium was transferred to a plate with MYEA (2 % malt extract, 0.2 % yeast extract, and 2 % agar) (Alfenas and Mafia, 2016; Ferreira et al., 2010). For all isolates, single ascospore strains were obtained from a perithecium formed on MYEA by dispersing an ascopore mass in 10 μL of autoclaved mineral oil and streaking the oil over a fresh plate of MYEA. After 24 h, individual germlings were transferred to fresh MYEA plates (Harrington and McNew, 1997). One self-fertile strain per seed or seedling was stored in 15 % glycerol at −80 °C at Universidade Federal de Viçosa. Some isolates from andiroba were also stored in the collection at Iowa State University, USA.

2.2. DNA extraction

The fungus was grown on MYEA and incubated at 25 °C for about 7 d before extracting DNA using the Wizard® Genomic DNA Purification Kit (Promega Inc. – Madison, USA) with the manufacturer's protocol as modified by Valdetaro et al. (2015). The concentration of purified DNA was quantified with a Nanodrop 2000c® (Thermo Fisher Scientific Inc., Massachusetts, USA) and adjusted to 50–100 ng μL^{-1}.

2.3. ITS and mating type genes sequences

Amplification and sequencing of the ITS rDNA region was performed using the primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). The new ITS sequences were compared with those reported earlier for the LAC (Latin American Clade) (Harrington et al., 2011, 2014) using parsimony analyses.

Partial sequences of mating type genes *MAT1-1-2* (one of the MAT-1 genes) and *MAT1-2-1* (the MAT-2 gene) were amplified and sequenced with primers CFMAT1-F and CFMAT1-R (Harrington et al., 2014) and primers X9978R1R and CFM2-1F, respectively (Harrington et al., 2011). The PCR conditions for amplifying the

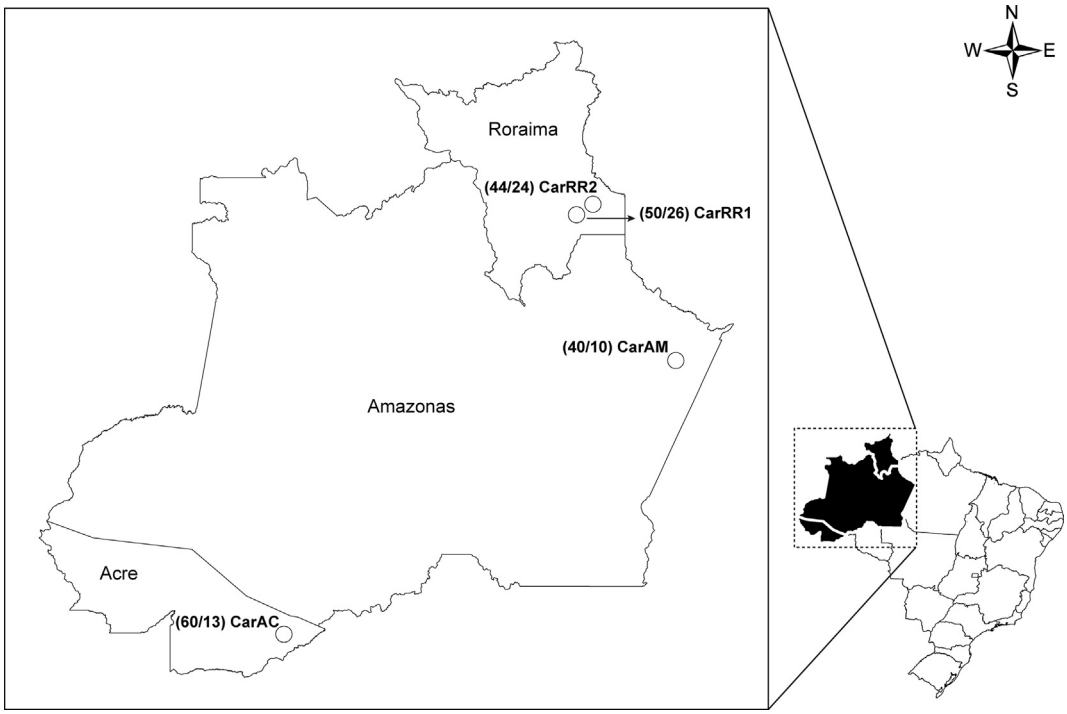

Fig. 1. Geographic distribution of sampling sites of *Ceratocystis fimbriata* populations from *Carapa guianensis* (andiroba) in Brazil. The number of samples and the number of isolates obtained from each population are in parentheses.

three regions were as described previously (Harrington et al., 2014). The PCR products were purified using Ilustra™ GFX™ PCR DNA, and all sequencing was conducted by the Iowa State University DNA Facility. Sequences were analyzed and edited using Sequence Navigator (Applied Biosystems, Foster City, California) software and manually aligned when necessary.

2.4. Phylogenetic analyses based on mating type genes

Sequences of the two mating type genes (*MAT1-1-2* and *MAT1-2-1*) of isolates obtained in this study were compared with representative isolates of the *C. fimbriata* complex, including *C. cacaofunesta*, *C. colombiana* M. van Wyk & M. J. Wingfield and *Ceratocystis platani* (Walter) Engelbrecht & T.C. Harrin. (Table 1). *Ceratocystis variospora* (R.W. Davidson) C. Moreau from the North American Clade (NAC) was used as the outgroup taxon. The partition homogeneity test (PHT) was conducted using PAUP 4.0b1.0 (Swofford, 2003) in order to determine whether the datasets could be combined.

Maximum parsimony (MP) analysis was performed by using PAUP * 4.0b10 (Swofford, 2003) using heuristic searches and the TBR algorithm (Tree Bissection Reconnection), with stepwise addition. Bootstrap values were determined with 1000 random repetitions. Gaps were treated as a fifth base and all characters had equal weight.

Bayesian inference was performed using MrBayes v3.2.1 (Ronquist and Huelsenbeck, 2003). The best model of nucleotide substitution was chosen based on the Akaike information criterion (AIC) of MrModelTest 3.2 (Nylander, 2004). A posterior probability (*PP*) distribution of trees was created using MCMC (Metropolis-coupled Markov chain Monte Carlo), with four chains (one cold and three heated) initiated from a random tree and one million generations executed, discarding the first 25 percent of the trees by means of the burn-in procedure in MrBayers.

2.5. Mating experiments

Self-sterile, single-ascospore strains of isolates from andiroba from each state were selected as MAT-1 (female-only) testers. Self-sterile MAT-2 (male-only) strains were recovered from vegetative sectors that formed spontaneously (Ferreira et al., 2010; Oliveira et al., 2015a). These MAT-2 strains no longer produced perithecia and thus could be used as males only. Other MAT-2 testers included one isolate of *C. fimbriata* from teak, one from *Ipomoea batatas* (sweet potato), one isolate from *Eucalyptus*, three isolates from mango, and one isolate of *C. platani* (Oliveira et al., 2015a). To confirm that the MAT-2 testers had the *MAT1-2-1* gene, DNA was

Table 1
Representatives sequences of isolates of *Ceratocystis fimbriata*, *C. cacaofunesta, C. colombiana, C. platani* and *C. variospora* used for phylogenetic analyses of ITS rDNA and mating type genes.

Species	Hosts	Localization	Representative isolate	ITS sequence acessions	*MAT1-1-2* GenBank accessions	*MAT1-2-1* GenBank accessions
C. fimbriata	*Carapa guianensis*	Acre, Brazil	CarAC-144C3	MH687340	[a]-	–
	Carapa guianensis	Acre, Brazil	CarAC-144B2	–	MH744812	MH744820
	Carapa guianensis	Amazonas, Brazil	CarAM-MA2	MH687341	MH746809	MH746810
	Carapa guianensis	Roraima, Brazil	CarRR1-RR74	–	MH744813	MH744821
	Tectona grandis	Mato Grosso, Brazil	TecMT-QM22	MH687342	MH744814	MH744822
	Tectona grandis	Mato Grosso, Brazil	TecMT-RC353	MH687343	–	–
	Hevea brasiliensis	Acre, Brazil	HevAC-RB08	MH687344	MH744815	MH744823
	Hevea brasiliensis	Bahia, Brazil	HevBA-A50	MH687345	MH744816	MH744824
	Actinidia deliciosa	Rio Grande do Sul, Brazil	PP14	–	MH744817	MH744825
	Actinidia deliciosa	Rio Grande do Sul, Brazil	PM20	–	MF347680	MF347678
	Cajanus cajan	Distrito Federal, Brazil	C2173	MH687346	–	–
	Theobroma cacao	Trinidad and Tobago	C1584	–	MH744818	MH744826
	Ficus carica	São Paulo, Brazil	C1782 (=CBS 115 166)	AY526292	KF482987	HQ157551
	Ficus carica	São Paulo, Brazil	C1857	HQ157542	same as KF482987	same as HQ157551
	Gmelina arborea	Pará, Brazil	C918 (=CBS 115 173)	AY157967	KF482983	HQ157549
	Eucalyptus spp.	Bahia, Brazil	C1442 (=CBS 115 174)	HQ157545	KF482985	HQ157550
	Eucalyptus spp.	Bahia, Brazil	C1440	HQ157544	same as KF482985	same as HQ157550
	Eucalyptus spp.	Bahia, Brazil	C1985	AY157966	same as KF482985	same as HQ157550
	Eucalyptus spp.	Bahia, Brazil	C2123	AF395685	–	–
	Eucalyptus spp.	Paraná, Brazil	C1987	–	KF482990	HQ157552
C. fimbriata	*Mangifera indica*	Pernambuco, Brazil	C1970	[a]-	KF482986	HQ157550
	Mangifera indica	Rio de Janeiro, Brazil	C2055	HQ157548	same as KF482986	same as HQ157550
	Mangifera indica	São Paulo, Brazil	C1657	AY526291	same as KF482986	same as HQ157550
	Mangifera indica	Pernambuco, Brazil	C1968	AY585343	KF482984	HQ157553
	Mangifera indica	Rio de Janeiro, Brazil	C2094	–	KF482987	KF482998
	Mangifera indica	Rio de Janeiro, Brazil	C1558 (=CBS 115 175)	AY157965	KF482988	HQ157552
	Mangifera indica	São Paulo, Brazil	C1655	HQ157546	–	–
	Mangifera indica	São Paulo, Brazil	C1889	HQ157547	–	–
	Mangifera indica	São Paulo, Brazil	C994 (=CBS 600.70)	AY157964	KF482987	HQ157551
	Colocasia esculenta	São Paulo, Brazil	C1905 (=CBS 115 171)	AY526288	KF482989	HQ157552
	Colocasia esculenta	São Paulo, Brazil	C1926	HQ157541	–	–
	Ipomoea batatas	Papua New Guinea	C1476 (=ICMP 8579)	AY157957	KF482992	KF483000
C.cacaofunesta	*Theobroma cacao*	Ecuador	C1004 (=CBS 153.62)	AY157950	KF482993	KF483001
	Theobroma cacoa	Bahia, Brazil	C1587	AY157953	–	–
	Theobroma cacoa	Rondônia, Brazil	C2031	MH687347	MH744819	MH744827
C.colombiana	*Coffea arabica*	Colombia	C1543 (=CBS 135 861)	AY157961	KF482994	KF483002
	Coffea arabica	Colombia	C1024	MH687348	same as KF482994	same as KF483002
C. platani	*Platanus accidentalis*	North Carolina, USA	C1317 (=CBS 115 162)	AY157958	KF482995	KF483003
C. variospora	*Prunus* sp.	Iowa, USA	C1963 (=CBS 135 862)	AY907042	KF482996	KF483004

[a] Not used in analyses.

extracted and PCR was conducted using the primers CFM2-1 and X9978R1R (Oliveira et al., 2015a).

The male (MAT-2) and female (MAT-1) testers were grown on MYEA for 7 d at room temperature. A suspension (1 mL) of conidia (the male tester) was dispersed over the female colony for spermatization (Ferreira et al., 2010; Oliveira et al., 2015a). The cultures were observed during the next 3–4 wk for the presence of perithecia and ascospore masses using a dissecting microscope. Drops of ascospores at the tip of perithecia were characterized as opaque (normal appearance, indicating an intraspecific cross) or watery (indicating a hybrid cross with few or no ascospores). Ascospore masses were spread onto fresh MYEA and colonies were examined after 1 wk to determine if the progeny were viable and a mixture of phenotypes were evident. In successful crosses, the progeny showed the mycelial phenotypes of the two parents. However, in some pairings, the progeny showed a uniform mycelial morphology, indicating an induced selfing had occurred (Ferreira et al., 2010; Oliveira et al., 2015a). The experiment was repeated twice and the two tester strains were considered interfertile if normal acsospore masses (opaque, non-watery) and mixed phenotypes among the progeny were found in at least one of the two crossings.

2.6. Microsatellite analyses

Fourteen microsatellite loci developed by Steimel et al. (2004) and later mapped onto the *C. fimbriata* genome (Simpson et al., 2013) were analyzed. These microsatellite markers have been widely used in studies of population structure involving species within the LAC, especially in other Brazilian populations (Engelbrecht et al., 2004, 2007a; Ferreira et al., 2010, 2011; 2017; Harrington et al., 2015; Li et al., 2016; Ocasio-Morales et al., 2007; Oliveira et al., 2015a; Valdetaro et al., 2015). Each primer pair was specific to the flanking region of a simple sequence repeat, and one of the primers was fluorescently labeled. The PCR conditions were as described (Ferreira et al., 2010), and band sizes of the product were determined using a four-capillary ABI Prism 3100- 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, but most of the microsatellite loci were trinucleotide repeats, and the products differed by increments of 3 bp.

Genetic variation of andiroba populations was compared with representative Brazilian *C. fimbriata* populations on teak, *Colocasia esculenta* (taro) (Oliveira et al., 2017), *Eucalytpus* (Ferreira et al., 2010) and mango (Oliveira et al., 2015a) using the same microsatellite markers. Nei's gene diversity (H) for each population was calculated without and with clone-corrected data using PopGene 1.32 software. Multilocus genotypic diversity was estimated using rarefaction curves based on the smallest sample size (Grünwald et al., 2003) with the Vegan package from CRAN in R v.2.6.1 (R Core Team, 2007). The maximum value of G was 5 for comparisons among individual populations and 13 for comparisons among different host groups and regions.

Relationships among representative genotypes (combinations of the alleles among the 14 microsatellite loci) from andiroba and other hosts of the LAC (Ferreira et al., 2010; Harrington et al., 2015; Oliveira et al., 2015a, 2017; Valdetaro et al., 2015) were examined in PAUP* (Swofford, 2003) using genetic distance (Nei's) matrices and UPGMA (unweigted pair group method with arithmetic mean) trees. Bootstrapping tests utilized 1000 replications.

Nei's genetic distance between populations based on allele frequencies and UPGMA were conducted using POPULATIONS 1.2.30 (Langella, 1999). Bootstrap values were calculated from 100 replications using POPULATIONS 1.2.30.

2.7. Pathogenicity tests

Three representative isolates from andiroba (CarAC-144B2, CarAM-ANDP1 and CarRR1-RR74), one isolate from eucalyptus (EucBA-SBS1), one from mango (ManCE-CEBS13), and one from teak (TecMT-QM29) were used in two inoculation experiments. All of the isolates were inoculated into andiroba seedlings (3-mo-old), into mango seedlings (cultivar Espada, 19-mo-old), into rooted cuttings of hybrid clone 1172 of *E. urophylla* x *E. grandis* (3-mo-old), and into rooted cuttings of teak (6-mo-old). The plants were transplanted into 2 L pots containing the substrate Carolina II (Carolina Soil do Brasil) supplemented with 6 kg m^{-3} of superphosphate and 1.5 kg m^{-3} Basacote® (19-6-10) (ComPo do Brasil). However, the teak plants were grown in potting mix with soil, sand and Carolina II (in proportion 1-1-1). Both experiments were conducted in a completely randomized design, consisting of two factors (host × isolate) with seven replications per treatment. The plants were wounded (approx. 3-mm deep) with a sterile scalpel at 3 cm above the soil level. A volume of 500 μL of the inoculum (3×10^6 spores ml^{-1}) was pipetted into the wound and the inoculation site wrapped with Parafilm to reduce desiccation and contamination. The control plants were wounded and treated with the same volume of sterile distilled water. In the first experiment (inoculated 13 May 2016), the plants were incubated in a greenhouse with an average temperature of 23 °C (range 8–45 °C). In the second experiment (inoculated 24 June 2016), the plants were incubated in a growth chamber maintained at 25 °C (22–30.5 °C) with a 12 h photoperiod and 96 μM photons/s/m^2. After 60 d, the length of xylem discoloration was measured. The carrot baiting method (Moller and Devay, 1968) was used to re-isolate the fungus. The variance analyses (ANOVA) (including isolate, host, and experiment) and Fisher's Protected LSD test ($P < 0.01$) were conducted on the means of the length of xylem discoloration using Statistica® software (StatSoft Inc.).

3. Results

3.1. Sample collection and fungal isolation

The fungus was isolated from 73 of 194 samples of blighted seeds and seedlings at the four locations (Fig. 1). In Acre and the two sites in Roraima, we observed seedlings showing symptoms of *Ceratocystsis* seedling blight (Fig. 2B) as well as fungal sporulation (Fig. 2C and E) and, in some cases, the presence of perithecia on the stem of the seedlings (Fig. 2D). In Amazonas, we collected only seeds, which were covered by fungal sporulation and, in some cases, showed gummosis (Fig. 2F). Particularly high percentages of symptomatic seeds and seedlings were found directly below mature andiroba trees, where seed density was high.

3.2. ITS rDNA haplotypes

Of the 73 isolates, 72 were succeeded to obtain the ITS sequences, only one isolate from Amazonas (CarAM-ANDP2) could not be directly sequenced for the ITS region. This isolate had mixed ITS sequences, that is, the electropherogram generated with the ITS1F primer initially gave clean reads, and then overlapping peaks followed an indel position (Harrington et al., 2014; Oliveira et al., 2015a).

The maximum parsimony analysis found 9 trees of 321 steps with 99 variable characters, 73 of which were parsimony informative in the alignment of 613 bp (TreeBase 23626). In comparisons with the sequences of other LAC sequences, three new ITS haplotypes (ITS17, ITS17a and ITS18) were identified among the sequenced isolates from andiroba (Fig. 3). The ITS haplotype numbers follow the designations by Harrington et al. (2011, 2014) and Oliveira et al. (2015a). The ITS17 and ITS17a sequences were

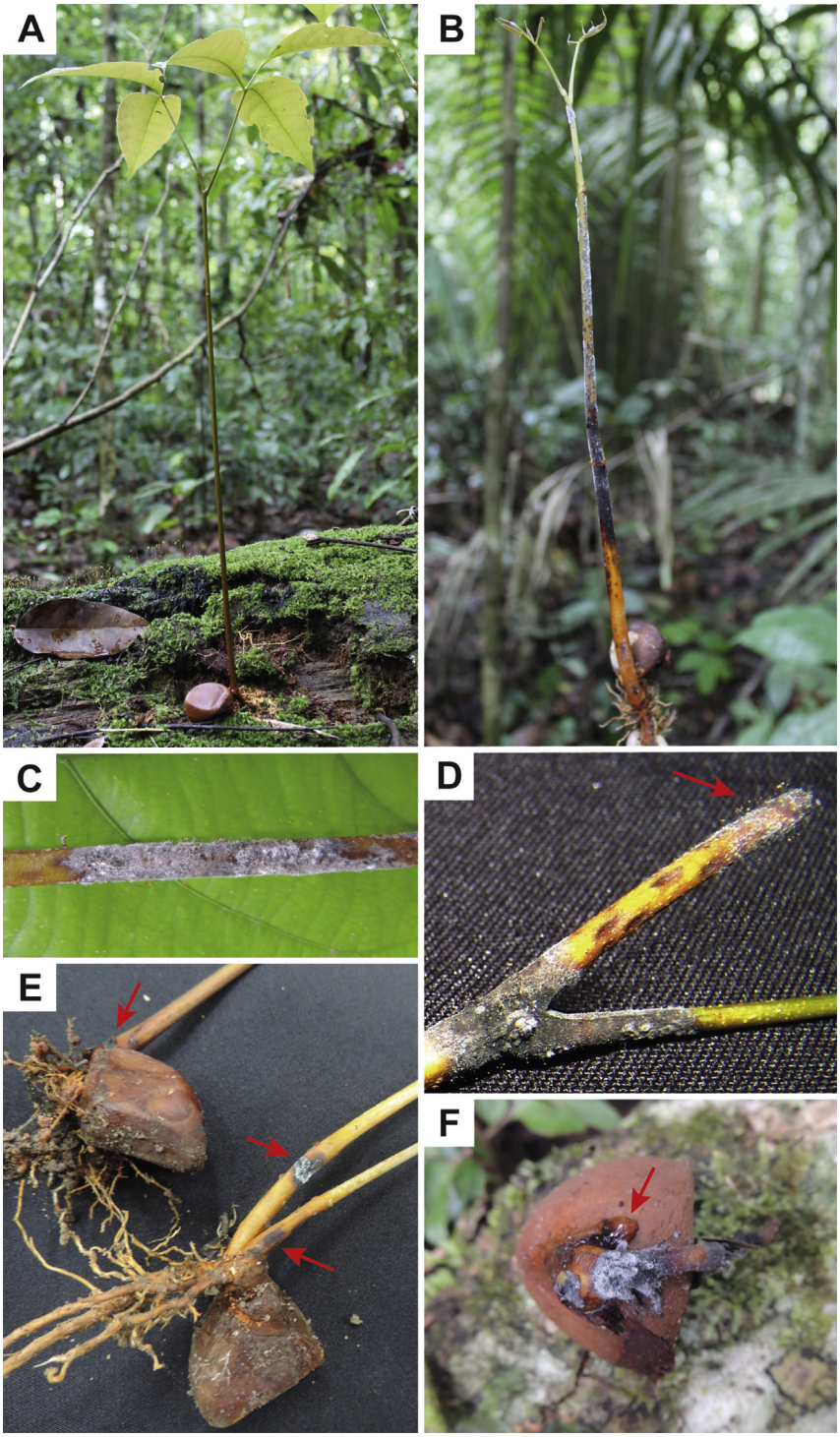

Fig. 2. *Ceratocystis fimbriata* on *Carapa guianensis* (andiroba). (A) Healthy seedling. (B–E) Seedlings with *Ceratocystis* seedling blight and fungus sporulation, perithecia on stem tissues indicated by red arrows. (F) Seed with fungus sporulation and gummosis (red arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

found in two isolates from Acre, and the rest of the isolates had the ITS18 sequence.

3.3. Phylogenetic analyses

Fifteen isolates from andiroba representing the Acre, Amazonas and Roraima populations were sequenced for mating type genes. Each of the 15 isolates had the identical sequence for MAT-1 (*MAT1-1-2*). Minor variation in the MAT-2 (*MAT1-2-1*) sequence also was found among the isolates. The *MAT1-1-2* alignment had 1040 characters, and the *MAT1-2-1* alignment had 1131 characters, with 62 characters varying among the sequences from andiroba and those of other members of the LAC.

Sequences of the two mating type genes were combined for phylogenetic analyses because self-fertile strains have the genes tightly linked in the mating type locus, and the genes are not

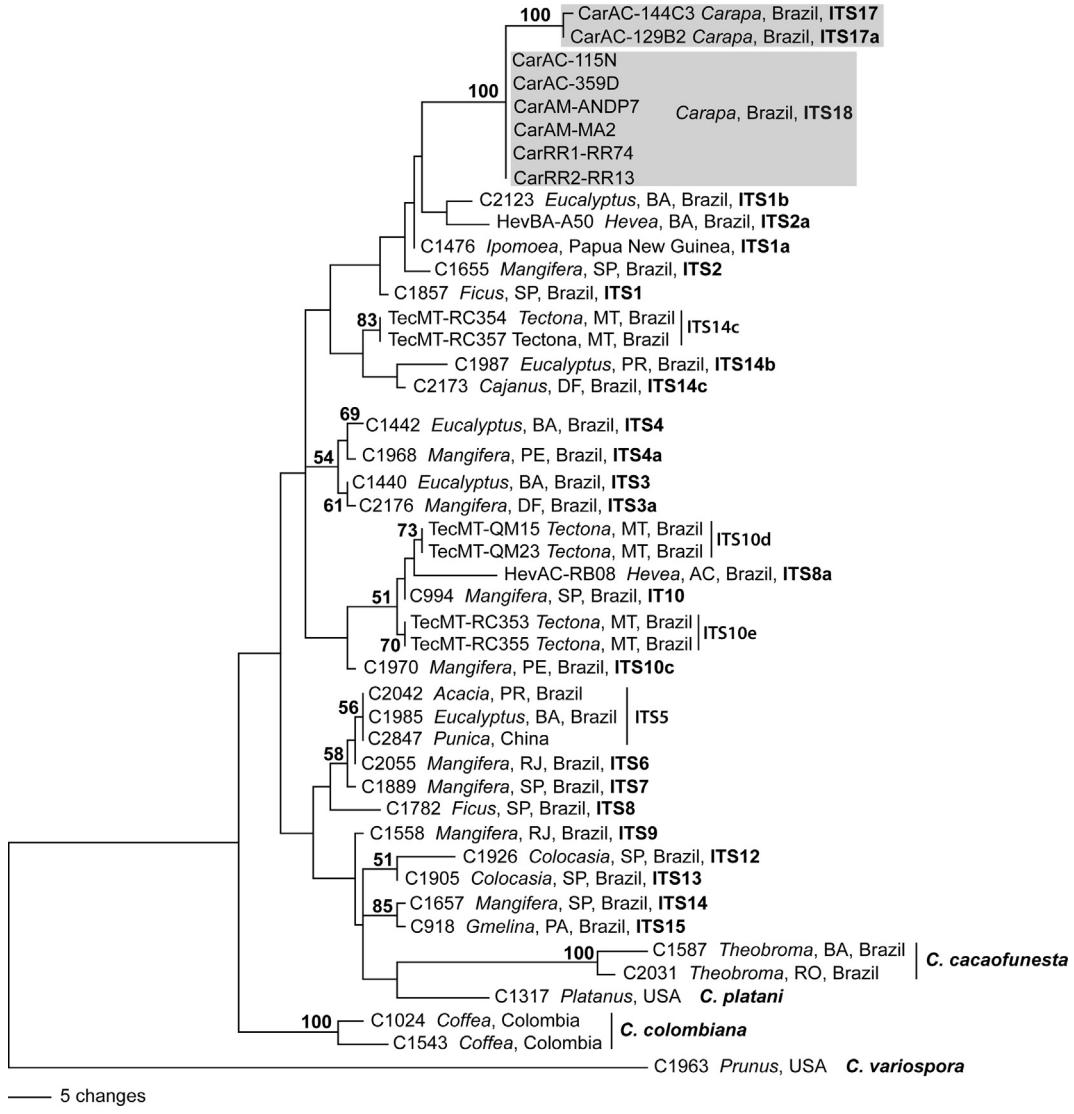

Fig. 3. One of 9 most parsimonious trees based on the ITS rDNA sequences of representative isolates of the Latin American Clade (LAC) of *Ceratocystis*. The tree is rooted to *C. variospora* of the North American Clade (NAC). The host genus, state (AC = Acre; AM = Amazonas; BA = Bahia; DF = Distrito Federal; Mato Grosso = MT; PA = Pará; PE = Pernambuco; PR = Paraná; RJ = Rio de Janeiro; RR = Roraima and SP = São Paulo) or country of origin are given for each isolate. The ITS haplotype designations are indicated to the right. Bootstrap values greater than 50 % are indicated on appropriate branches. Scale bar indicates base pair differences.

thought to recombine in crosses (Harrington et al., 2014). A partition homogeneity test (PHT) performed for the combined dataset of MAT-1 and MAT-2 genes showed that they could be combined ($P = 0.26$) (Cunningham, 1997; Barker and Lutzoni, 2002). The combined dataset of sequences of isolates from andiroba and other representatives of the LAC (Harrington et al., 2014) had an alignment of 2171 characters (TreeBase 23 626). The number of variable characters was 373, and 84 of those characters were parsimony informative. Maximum parsimony (MP) analysis found a single tree of 494 steps, with homoplasy index (HI), consistency index (CI), rescaled consistency index (RC), and retention index (RI) of 0.0486, 0.9514, 0.8816 and 0.9266, respectively.

Evolution model HKY + G was selected and incorporated into the Bayesian analysis, and the level of convergence from two parallel runs after 1 000 000 generations had a mean standard deviation of split frequencies of 0.00665. The likelihood tree from Bayesian analysis had a very similar topology to that of the MP tree. Therefore, the parsimony tree was selected for illustration (Fig. 4). Other species from the LAC (*C. cacofunesta*, *C. colombiana*, and *C. platani*) had bootstrap support of 100 % and posterior probability (PP) of 1.00 (Fig. 4).

The MAT haplotypes from andiroba were placed among the other Brazilian isolates of *C. fimbriata*. Three new mating haplotypes (4e, 4f and 4g, named following the nomenclature proposed by Harrington et al., 2014) were found among the 15 isolates. Haplotype 4e was found in four isolates (two from Roraima and two from Acre), haplotype 4f was found in one isolate from Acre, and the rest of the isolates had haplotype 4g, as did an isolate from cacao in Trinidad and Tobago (C1584). The isolates from andiroba were closely related to each other, with good bootstrap and posterior probability support.

3.4. Mating experiments

The MAT-1 testers from andiroba successfully crossed with the MAT-2 testers of most of the other Brazilian isolates of *C. fimbriata* (Table 2). In successful crosses, there were many fully developed perithecia with thick, creamy ascopore masses within 7 d of

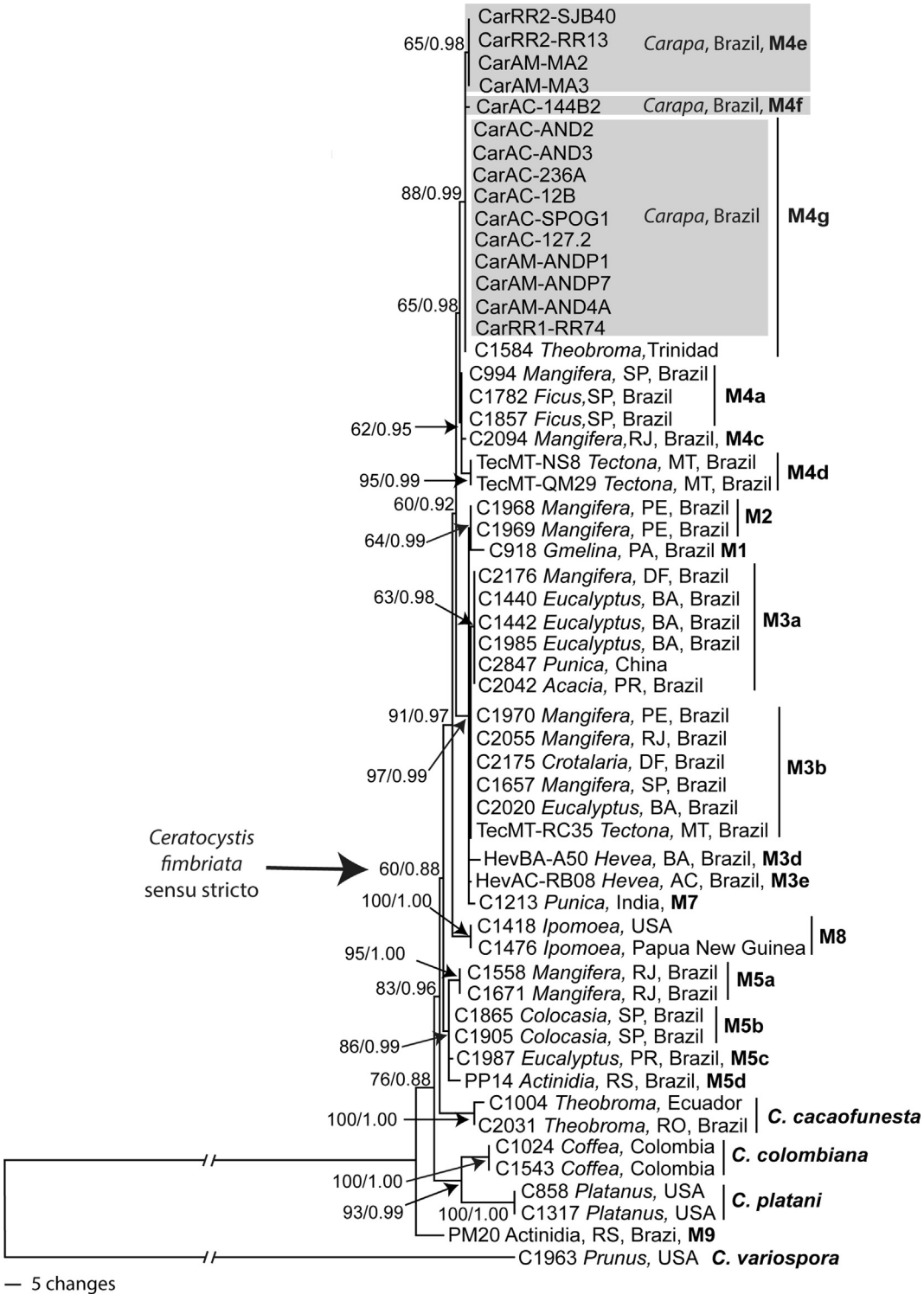

Fig. 4. The single most parsimonious tree of 494 steps based on portions of the *MAT1-1-2* (MAT-1) and *MAT1-2-1* (MAT-2) mating type genes of *Ceratocystis fimbriata* and other members of the Latin American clade (*C. cacaofunesta*, *C. colombiana*, and *C. platani*). The tree was rooted to *C. variopora*, a member of the North American clade of the *C. fimbriata* complex. Bootstrap values greater than 60 %/posterior probability value greater than 0.85 are indicated on appropriate branches. The host genus, and state (Acre = Acre; AM = Amazonas; BA = Bahia; Mato Grosso = MT; PA = Pará; RJ = Rio de Janeiro; RR = Roraima and SP = São Paulo) or country of origin are given for each isolate. The mating type haplotype designations are indicated on the right. Scale bar indicates base pair differences.

spermatization (Fig. 5A), and microscopic examination showed abundant, normal appearing ascopores (Fig. 5C). Ascopores masses from normal crosses streaked onto fresh MYEA plates showed mixed phenotypes among the resulting colonies, demonstrating that the ascopores were not due to a selfing. In unsuccessful crosses, only a few or no perithecia were produced, the ascospores masses were watery (Fig. 5B), and microscopic examination showed mostly misshapen or empty ascospores (Fig. 5D).

Table 2
Normal or watery ascospores masses or lack of perithecia produced from mating experiments of MAT-1/female tester strains of *Ceratocystis fimbriata* from *Carapa guianensis* and MAT-2/male tester strains of *C. platani* and *C. fimbriata* from *Tectona grandis*, *Eucalyptus* spp., *Mangifera indica* and *Ipomoea batatas*.

Species	Host	MAT1, Female[a]	MAT2, Male[a]						
			Tectona grandis	*Eucalyptus* spp.	*Mangifera indica*			*Ipomoea batatas*	*C. platani*
			TecMT-SI69sec	C1347sec	SEMS2-11sec	SESP5-1sec	ERRJ1-10sec	C1418sec	C1343sec
C. fimbriata	*Carapa* guianensis	CarRR1-RR74ss	Normal[b]	Normal	Normal	Normal	Normal	Normal	Watery[c]
		CarAC-127-2ss	Normal	Normal	Normal	Normal	Normal	Normal	Watery
		CarAM-MA2ss	Normal	Normal	Perithecia[d]	Normal	–	–	–
		CarAM-MA3ss	Normal	Normal	Normal	–[e]	–	–	–

[a] sec = strains from MAT2, self-sterile sectors recovered from self-fertile isolates; ss = MAT1, female isolates with protoperithecia.
[b] Normal = masses with abundant, normal appearing ascospores.
[c] Watery = masses with few, mostly misshapen ascospores, or no ascopores.
[d] Perithecia = Perithecia only, no ascospore mass.
[e] – = no perithecia produced.

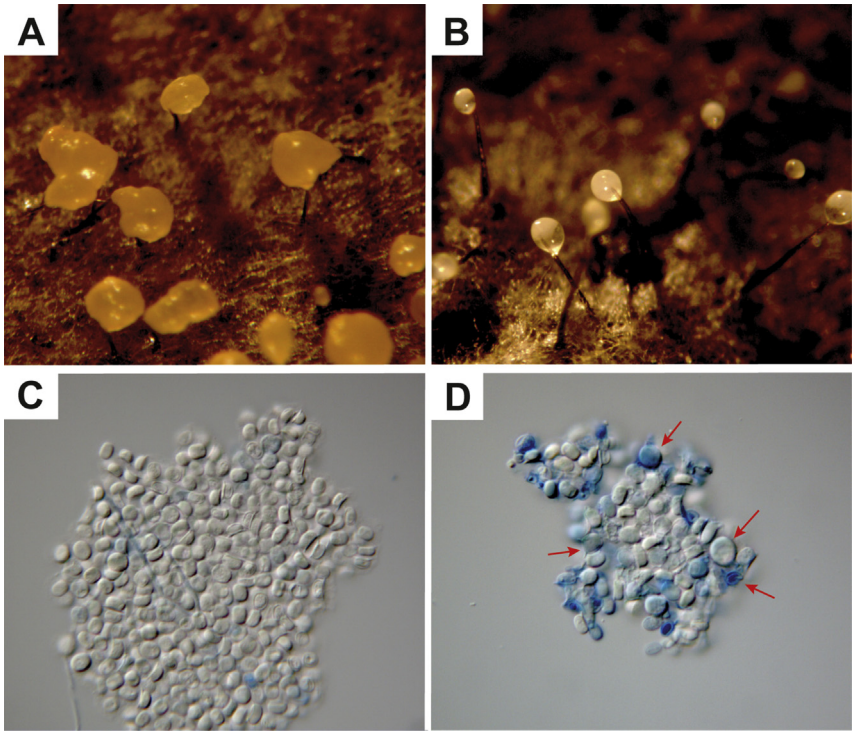

Fig. 5. Normal and abnormal ascospore masses and ascospores from fully inter-fertile and interspecific crosses between isolates of *Ceratocystis*. (A) Perithecia and ascospore mass and (C) normal ascospores from interfetile cross between *C. fimbriata* strains from *Carapa guianensis* (andiroba) (CarA-MA3) and *Eucalyptus* (C1347). Interspecific cross between an andiroba strain of *C. fimbriata* (CarRR1-RR74) and a *Platanus* strain of *C. platani* (C1343) showing small watery ascospore masses (B) and misshapen ascospores (D) (red arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Microsatellite analyses

Of the 14 microsatellite loci tested on the 73 andiroba isolates, eight loci were monomorphic and six were polymorphic (Table 3). The UPGMA tree based on Nei's genetic distance (Fig. 6) found 14 genotypes among the andiroba isolates, and these grouped separately from other *C. fimbriata* isolates with high support (95 %). Three of the 14 andiroba genotypes (AMSAT1, AMSAT2 and AMSAT3) were found in more than one of the four populations (Fig. 6).

The UPGMA tree based on allele frequencies of populations (Fig. 7) showed that andiroba populations grouped separately from the other Brazilian populations of *C. fimbriata* with high bootstrap support (100 %). The UPGMA tree also separated other populations associated with different hosts and geographic locations.

Nei's gene diversity (H) and Stoddart and Taylor's genotypic diversity (G) for populations from andiroba were compared to other Brazilian populations (Table 4). The Amazonas population showed greater gene and genotypic diversity ($H = 0.1486$; $G = 3.3889$) than the other andiroba populations. The Brazilian populations from *Eucalyptus* ($H = 0.3212$; $G = 8.2371$), mango in the Northeast Brazil ($H = 0.3758$; $G = 7.9237$) and taro ($H = 0.1530$; $G = 6.7460$) had greater gene and genotypic diversity than andiroba populations ($H = 0.1271$; $G = 4.8055$) (Table 4).

3.6. Pathogenicity tests

At the end of the inoculation experiments, none of the host plants were killed, but wilt symptoms were observed in some inoculated plants of some host species. Re-isolations of the

Table 3
Estimated sizes (bp) of alleles of 14 microsatellite loci in four *Ceratocystis fimbriata* populations on *Carapa guianensis* (andiroba), with the number of isolates tested in the population shown in parentheses.

Microsatellite Locus	CarAC (13)	CarRR1 (26)	CarRR2 (24)	CarAM (10)
AAG8	183	183	183	183
AAG9	400	400	400	400
CAA9	251 (5)[a], 263 (8)	190	159 (1), 190 (23)	190 (3), 263 (7)
CAA10	127	127	127	127
CAA15	324	324	324	324
CAA38	159 (7), 168 (5), 214 (1)	159 (20), 180 (2), 205 (4)	159 (1), 171 (1), 205 (22)	159 (5), 171 (2), 214 (3)
CAA80	317	296 (1), 299 (25)	299	296 (3), 299 (5), 317 (2)
CAG5	317	317	317	317
CAG15	252	252	252	252
CAG900	194	194	194	194
CAT1	254	248 (1), 254 (25)	248 (8), 254 (16)	248 (3), 254 (7)
CAT12	377	365 (1), 377 (25)	377	377
GACA6K	215	215	215	215
GACA60	187	187 (25), 207 (1)	187	187

[a] The number of isolates with that allele size in parentheses.

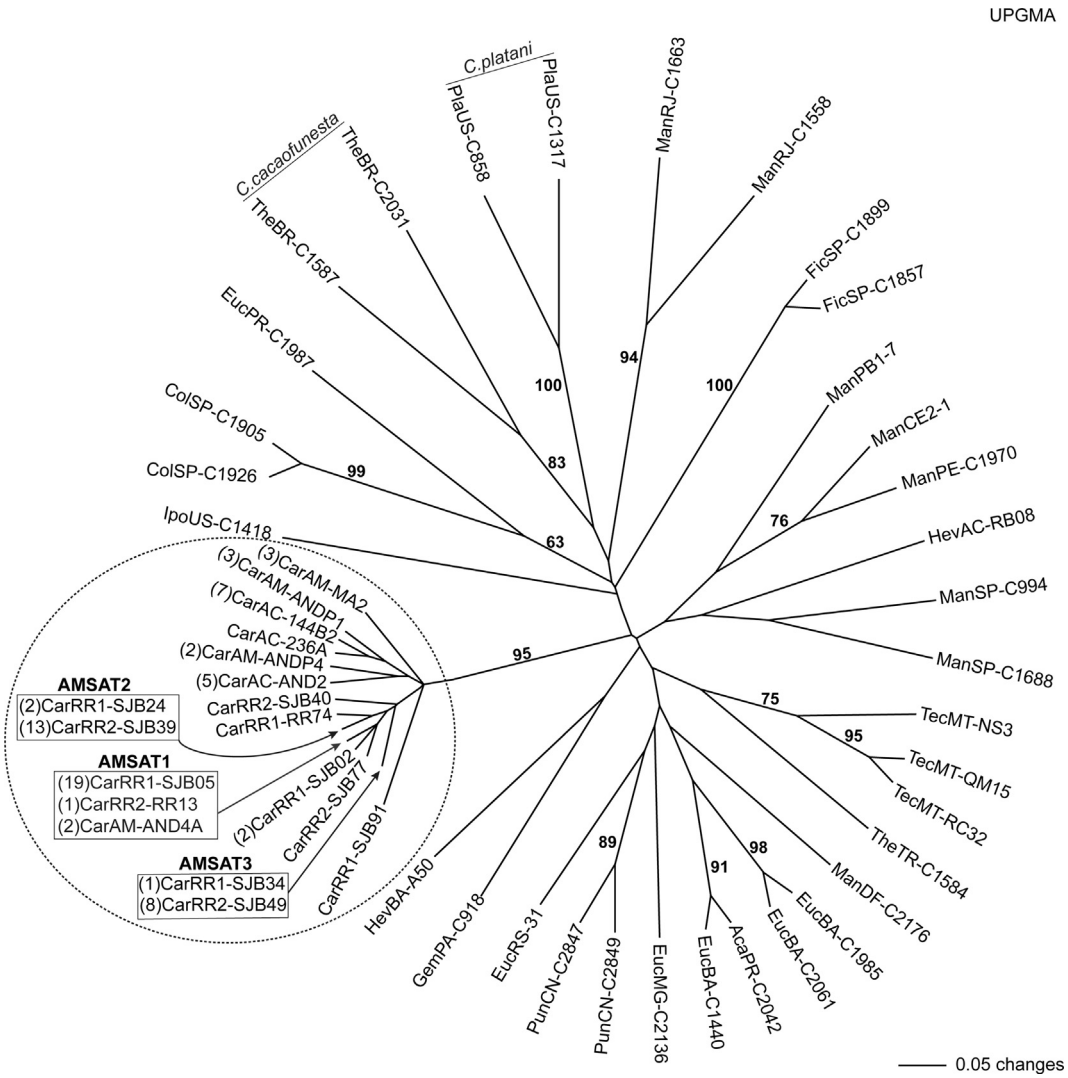

Fig. 6. A UPGMA (unweighted pair group method arithmetic mean) dendrogram of microsatellite genotypes of *Ceratocystis fimbriata*, *C. cacaofunesta* and *C. platani* based on Nei's genetic distance. Bootstrap values are shown along side the branches. The first three letters indicate the host genus (Aca = *Acacia;* Car = *Carapa*; Col = *Colocasia*; Euc = *Eucalyptus*; Fic = *Ficus;* Gme = *Gmelina*; Hev = *Hevea*; Ipo = *Ipomoea*; Man = *Mangifera*; Pla = *Platanus;* Pun = *Punica*; Tec = *Tectona* and The = *Theobroma*), and next two letters indicate the Brazilian state (AC = Acre; AM = Amazonas; AL = Alagoas; BA = Bahia; CE = Ceará; MG = Minas Gerais; MT = Mato Grosso, PA = Pará; PB = Paraíba; RJ = Rio de Janeiro; RS = Rio Grande do Sul; RR = Roraima and SP = São Paulo) or country (BR = Brazil; CN = China; US = United States) of origin. The *Carapa* genotypes are encircled by a dotted line. The number of *Carapa* isolates with that genotype is in parentheses. *Carapa* genotypes found in more than one population are designated by "AMSAT" followed by a number. Scale bar indicates genetic distance.

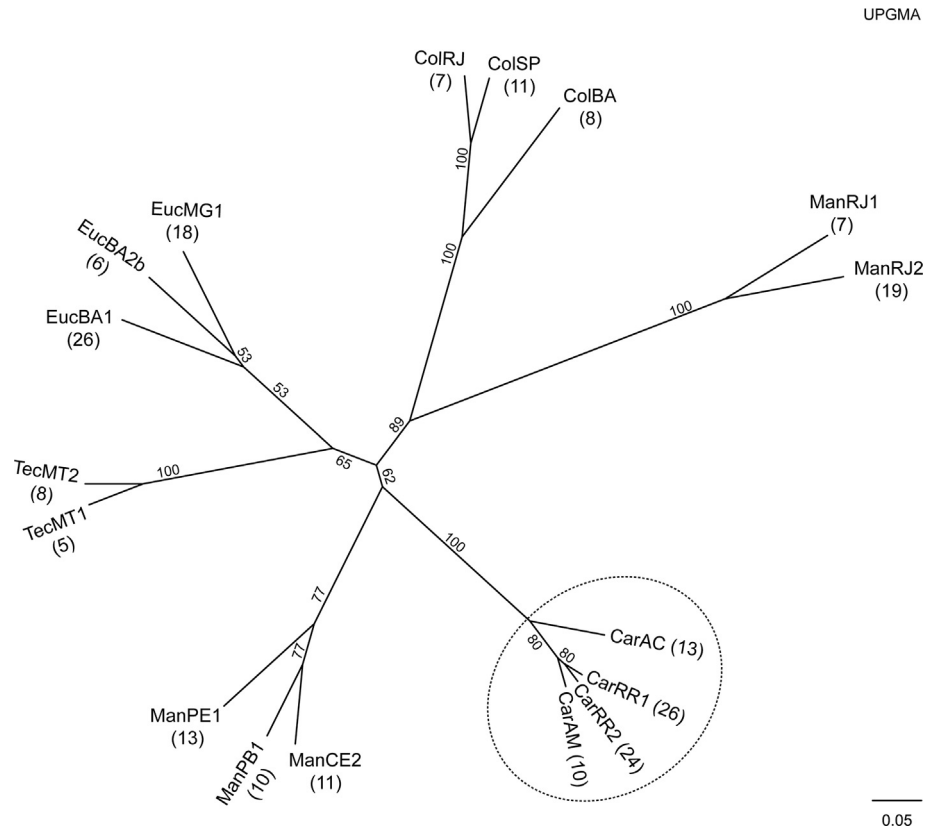

Fig. 7. The UPGMA (unweighted pair group method, arithmetic mean) dendrogram of *Ceratocytis fimbriata* populations from Brazil from different hosts and geographic origins based on allele frequencies of 14 microsatellite loci. Bootstrap values are shown alongside the branches. The first three letters indicate the host genus (Car = *Carapa*; Col = *Colocasia*; Euc = *Eucalyptus*; Man = *Mangifera* and Tec = *Tectona*), and the next two letters indicate the Brazilian states (AC, Acre; AM, Amazonas; BA, Bahia; CE, Ceará; MG, Minas Gerais; MT, Mato Grosso, PB, Paraíba; PE, Pernanbuco; RJ, Rio de Janeiro; RR, Roraima and SP, São Paulo). *Carapa* populations are encircled by a dotted line. The number of isolates sampled from each population is in parentheses.

Table 4
Genetic diversity in populations of *Ceratocystis fimbriata* on *Carapa guianensis* (andiroba) and *Eucalyptus*, *Mangifera indica* (mango), *Colocasia esculenta* (taro) and *Tectona grandis* (teak) based on 14 microsatellite loci.

Host	Population	City/State	No. Isolates	No. geno-types	Genotypic diversity (*G*)[a]	Nei's gene diversity (*H*)	
						All isolates	Clone-corrected
Carapa guianensis	AM	Boa Vista de Ramos/Amazonas	10	4	3.3889	0.1486	0.1429
	RR2	São João da Baliza/Roraima	24	5	2.5114	0.0486	0.0857
	AC	Rio Branco/Acre	13	3	2.3364	0.0735	0.0794
	RR1	São João da Baliza/Roraima	26	6	2.2843	0.0482	0.1230
		All Andiroba Isolates	73	14	4.8055	0.1271	0.1713
Tectona grandis	TecMT1	Nossa Senhora do Livramento/Mato Grosso	5	2	2.0000	0.0686	0.1071
	TecMT2	São José dos Quatro Marcos/Mato Grosso	8	1	1.0000	0.0000	0.0000
		All *Tectona* isolates	13	2	1.7423	0.0913	0.1071
Colocasia esculenta	ColRJ	Rio de Janeiro/Rio de Janeiro	7	5	3.7692	0.1953	0.2343
	ColSP	Sorocaba/São Paulo	11	3	2.4069	0.0472	0.0635
		All *Colocasia* isolates	18	8	6.7460	0.1530	0.2277
Mangifera indica– Rio de Janeiro	ManRJ1	São Fidelis/Rio de Janeiro	7	4	3.3809	0.0816	0.1071
	ManRJ2	São Fidelis/Rio de Janeiro	19	4	1.7895	0.0973	0.1875
		All *Mangifera* isolates from Rio de Janeiro	26	8	2.4717	0.2001	0.2076
Mangifera indica – Northeast	NECE2	Brejo Santo/Ceará	11	7	4.1515	0.2904	0.3469
	NEPB1	Conde/Paraíba	10	6	3.7539	0.3814	0.3889
		All *Mangifera* isolates from the Northeast	21	13	7.9237	0.3758	0.3889
Eucalyptus spp.	EucMG1	Curvelo/Minas Gerais	18	14	4.5899	0.3122	0.3309
	EucBA1	Eunápolis/Bahia	26	13	3.8735	0.2162	0.2832
		All *Eucalyptus* isolates	44	27	8.2371	0.3212	0.3535

[a] Stoddart & Taylor's genotypic diversity (G) 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 5.0 (each isolate in the population of a different genotype). For host population ranged from 1.0 (only one genotype in the population) to a maximum value of 13.0 (each isolate in the population of a different genotype).

pathogen from discoloured tissue of the inoculated plants consistently yielded typical colonies of *C. fimbriata*, even if the lesions were small. Some un-inoculated plants (controls), mostly eucalyptus, showed some wilting of leaves, but these plants had only limited discoloration at the wound site and the pathogen was not re-isolated. In the growth chamber, the temperature was uniform (22 °C - 30.5 °C) during the experiment and the relative humidity was very high (90.76 %), resulting in edema formation on leaves and stems of andiroba. On the other hand, the light intensity was relatively low (95.6 μM photons/s/m^2) and some teak, mango and eucalyptus plants suffered attack from cochineal scales in the growth chamber.

In some cases, the lesions (xylem discoloration) of inoculated plants in the growth chamber was somewhat more extensive than the lesions in the greenhouse experiment (Table 5), and more inoculated plants wilted, but the results of the two experiments were generally similar. In both experiments, wilting occurred in some plants that were inoculated with isolates that originated from that host. Andiroba, mango and eucalyptus isolates inoculated into their respective hosts typically induced dark discoloration that extended continuously from the inoculation point, especially above the inoculation point.

The initial multifactorial ANOVA of length of xylem discoloration showed significant variation between two experiments, so data from the two experiments were analyzed separately. In both the greenhouse and growth chamber analyses, significant variation was found among the isolates (greenhouse, $F = 14.44$, $P < 0.0001$; growth chamber, $F = 47.64$, $P < 0.0001$), the hosts (greenhouse, $F = 25.99$, $P < 0.0001$; growth chamber, $F = 83.36$, $P < 0.0001$), and isolate × host interaction (greenhouse, $F = 15.43$, $P < 0.0001$; growth chamber, $F = 48.58$, $P < 0.0001$).

In both experiments, the three andiroba isolates caused significantly larger lesions in andiroba plants than did the other isolates, whose induced lesion lengths did not differ from those of the controls (Table 5). Variation in the extent of xylem discoloration (pathogen aggressiveness) was found among the andiroba isolates tested. In both experiments, mango and eucalyptus showed longer lesions than the other hosts when inoculated with their respective isolates. The lesions on mango induced by non-host isolates were discontinuous and narrow, unlike the lesions induced by the mango isolate. On mango plants in the growth chamber, all isolates induced xylem discoloration significantly greater than in the controls, but the mango isolate caused significantly greater lesion length. Although the lesions were relatively small in teak compared to the other hosts, isolate TecMT-QM29 from teak caused significantly more xylem discoloration than that seen in the control plants.

4. Discussion

The phylogenetic analysis using sequences of mating type genes (*MAT1-1-1* and *MAT1-2-1*) and ITS-rDNA sequences showed that isolates from andiroba from four sites in three states in the Amazon Basin form a strongly supported lineage closely related to other Brazilian isolates and isolates from sweet potato, on which *C. fimbriata* was originally described (Halsted, 1890). Three similar mating type sequences were found among the isolates from andiroba, and one of the three sequences matched a *C. fimbriata* isolate from cacao in Trinidad (C1584), but that cacao isolate differs from *C. cacaofunesta* and was not pathogenic to cacao plants in earlier studies (Baker et al., 2003; Engelbrecht and Harrington, 2005). That isolate could have been introduced on plant material to Trinidad, but the isolates from andiroba are believed to represent a native Amazonian population of *C. fimbriata*.

Although the population on andiroba was phylogenetically distinct, mating studies showed that they were interfertile with the sweet potato type strain of *C. fimbriata*, on which the species name is based, as well as with Brazilian *Eucalyptus*, mango and teak strains that are considered to be *C. fimbriata*. In contrast, the isolates from andiroba were intersterile with *C. platani*, another species from the LAC (Engelbrecht and Harrington, 2005). Thus, the isolates from andiroba would be considered *C. fimbriata* using the biological species concept (Harrington et al., 2011, 2014; Oliveira et al., 2015a).

Microsatellite analysis identified 14 genotypes among the andiroba isolates, and these isolates grouped separately from other Brazilian genotypes in UPGMA analyses. Three of the 14 genotypes were widely distributed within and among the andiroba populations, and one of the genotypes was found in both Roraima and Amazonas. The genotypic diversity and the gene diversity values of the populations on andiroba were somewhat low, but comparable to some Brazilian populations of *C. fimbriata* on other hosts, strongly suggesting that the andiroba isolates represent natural subpopulations and not introduced genotypes. The analyses of populations based on allele frequencies showed the andiroba subpopulations to be closely related to each other and clearly differentiated from other Brazilian populations on other hosts and other regions in Brazil.

There appears to be a strong regional differentiation of populations of *C. fimbriata* in Brazil, likely due to its soilborne nature, limited dispersal by insects, and a preponderance of selfing (Ferreira et al., 2010, 2017; Harrington, 2013; Oliveira et al., 2015a). The isolates from andiroba represent the first fully studied populations of *C. fimbriata* from the Amazon rain forest. Other differentiated populations of *C. fimbriata* in Brazil have been proposed to be associated with forest types: isolates from *Eucalyptus* and mango

Table 5
Mean xylem discoloration (cm) caused by *Ceratocystis fimbriata* isolates inoculated into four hosts in greenhouse and growth chamber experiments.

Isolates	*Carapa guianensis*		*Tectona grandis*		*Eucalyptus* spp.		*Mangifera indica*	
	GH[b]	GC[c]	GH1	GC	GH1	GC	GH	GC
CarAM-ANDP1[a]	9.13a	8.69ab	2.00ab	1.69b	3.25c	3.38bc	4.25b	6.47b
CarAC-C144B2	7.38ab	10.31a	1.69b	1.75b	2.94c	3.08c	4.38b	7.96b
CarRR-RR174	5.38bc	10.88a	1.88ab	1.75b	3.88bc	3.06c	3.81b	7.03b
TecMT-QM29	3.75cd	5.03bc	2.69a	2.81a	3.44bc	4.50bc	4.34b	7.56b
EucBA-SBS1	3.19cd	3.94bc	2.06ab	1.81b	16.00a	28.38a	6.88b	9.26b
MangCE-CEBS13	2.50cd	3.54bc	2.06ab	1.81b	5.88b	5.81b	15.63a	13.94a
Control	1.81d	1.69c	1.69b	1.69b	2.13c	2.00c	2.69b	2.13c

[a] The first three letters indicate the host genus (Car = *Carapa;* Euc = *Eucalyptus*; Man = *Mangifera* and Tec = *Tectona*) and next two letters indicate the Brazilian state (AC = Acre; AM = Amazonas; BA = Bahia; CE = Ceará; MT − Mato Grosso and RR = Roraima); Means within a column followed by the same lower case letter are not significantly different ($P < 0.01$) based on Fisher's protected least significant difference.
[b] GH = Green House.
[c] GC = Growth Chamber.

native to Cerrado and Caatinga forest types, respectively (Ferreira et al., 2010; Oliveira et al., 2015a); taro isolates along with mango isolates that seem to be native to the coastal Mata Atlântica forest type in Rio de Janeiro (Baker et al., 2003; Harrington et al., 2014; Oliveira et al., 2015a, 2017; Silveira et al., 2006); and kiwifruit isolates in southern Brazil (Ferreira et al., 2017). The Amazonian populations are unique in that andiroba is native to the basin, and it is not a cultivated host. Seeds of the plant are collected from natural forests for extraction of oils, but it is highly unlikely that human activity brought strains from one region of the Amazon to the other. Rather, native strains uniquely pathogenic to andiroba appear to be naturally distributed across the Amazon Basin.

The inoculation tests showed host specialization to andiroba, which is one of the few documented cases of a LAC member of *Ceratocystis* showing specialization to a host native to the Americas. *C. cacaofunesta* is believed native to the Upper Amazon and is specialized to infect cacao and relatives (Baker et al., 2003; Engelbrecht et al., 2007b), *C. platani* is native to eastern USA and is specialized to infect *Platanus* spp., and isolates from *Xanthosoma sagittifolium* and *Syngonium* spp. are phylogenetically related and specialized to infect aroids (Araceae) (Baker et al., 2003; Thorpe et al., 2005). Host specialization has been thought to be a driver of speciation in the group, and it has been considered a phenotypic character warranting species designations (Harrington and Rizzo, 1999; Engelbrecht and Harrington, 2005). However, *Ceratocystis* species have been mostly studied on non-native crop plants, with which they have not evolved (Engelbrecht and Harrington, 2005; Ferreira et al., 2010; Harrington et al., 2002; Johnson et al., 2005; Oliveira et al., 2015a). Isolates from certain non-native hosts have shown higher levels of aggressiveness to those hosts, but not all isolates from those populations have shown the same level of aggressiveness, that is, the populations as a whole are not truly host-specialized (Baker et al., 2003; Harrington et al., 2011; Oliveira et al., 2015b, 2016; Piveta et al., 2016; Zauza et al., 2004).

Host specialization to andiroba could be used as a phenotypic character to delineate this narrowly defined phylogenetic lineage as a new species (Harrington and Rizzo, 1999). However, unlike *C. cacaofunesta* and *C. platani*, the andiroba strains fall within the *C. fimbriata* lineage and were interfertile with a sweet potato isolate, which was phylogenetically related to *C. fimbriata* isolates on other hosts in Ecuador and Colombia (Li et al., 2017). Introduced genotypes of *C. fimbriata* that likely originated from the Northeast of Brazil have been named *C. manginecans* M. van Wyk, A. Adawi & M.J. Wingf., *C. mangivora* M. van Wyk & M.J. Wingf., *C. mangicola* M. van Wyk & M.J. Wingf., *C. acaciovora* Tarigan & M. van Wyk, and *C. eucalypticola* M. van Wyk & M.J. Wingf., but genetic and mating studies clearly indicate that these are genotypes of a single species (Harrington et al., 2014; Li et al., 2017; Oliveira et al., 2015a). These genotypes and andiroba isolates are interfertile with each other and sweet potato isolates of *C. fimbriata* and are morphologically indistinguishable (Li et al., 2017; Oliveira et al., 2015a). In spite of interfertility among the genotypes and with *C. fimbriata* isolates from sweet potato, the species name *C. manginecans* could be used to represent most of the Brazilian populations that tend to be aggressive on mango and eucalyptus, as well as teak and andiroba strains. Other populations on mango and taro from Rio de Janeiro and kiwifruit from Rio Grande do Sul (Ferreira et al., 2017; Oliveira et al., 2017) would also need new species descriptions in this scenario. Regardless, description of the andiroba pathogen as a new species would leave either *C. manginecans* or *C. fimbriata* paraphyletic.

A careful review of South American populations and species in the *C. fimbriata* complex will be necessary to develop a workable species concept that could be consistently applied to this important group of plant pathogens. The andiroba population appears to be native to the Amazon and uniquely pathogenic as a seed and seedling blight pathogen on a native tree species in forests with only limited human disturbance, but recognition of this population as a species does not appear warranted. In lieu of a new species name, this population could be recognized as a special form (forma specialis) of *C. fimbriata*, and we propose *Ceratocystis fimbriata* f. sp. *carapa*. Forma specialis is not a formal taxonomic rank but has been used to recognize physiologically specialized strains of plant pathogens. The other demonstrated host-specialized strain within *C. fimbriata* is the sweet potato pathogen, for which we propose *C. fimbriata* f. sp. *ipomoea*. With these informal designations and in the absence of other distinguishing phenotypic features, we propose to retain the species name *C. fimbriata* for South American and introduced strains that fall within the monophyletic lineage that is interfertile with the sweet potato pathogen.

Acknowledgements

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