LEONARDO SARNO SOARES OLIVEIRA

CERATOCYSTIS WILT ON *Mangifera indica*, *Colocasia esculenta* AND *Tilia americana*.

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 16 de abril de 2014

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`` A simplicidade é o último grau de sofisticação ``.

Leonardo da Vinci

Aos meus pais,

Antonio de Oliveira Soares e

Maria do Rosário Sarno de Oliveira (*in memorian*)

Dedico.

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BIOGRAFIA

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RESUMO

OLIVEIRA, Leonardo Sarno Soares, D.Sc., Universidade Federal de Viçosa, abril de 2014. **Murcha-de-ceratocystis em *Mangifera indica*, *Colocasia esculenta* e *Tilia americana*.** Orientador: Acelino Couto Alfenas. Coorientador: Dalmo Lopes Siqueira.

Ceratocystis fimbriata sensu lato é considerado atualmente como um complexo de espécies crípticas que afetam principalmente espécies arbóreas no Brasil e no mundo. Nos últimos anos, descrições de algumas espécies desse complexo foram feitas com base apenas na variação de sequencias da região ITS, como por exemplo a descrição de três novas espécies a partir de isolados obtidos de manga. Entretanto, estudos realizados a partir da clonagem de fragmentos da região ITS de isolados monoascospóricos de *C. fimbriata* mostraram haver sobreposição de sequencias, não sendo possível a leitura correta do fragmento amplificado, fazendo com que a descrição de novas espécies seja duvidosa. Com isso, o presente trabalho objetivou estudar a taxonomia do fungo obtido de manga, inhame e de *Tilia americana* por meio da utilização de genes diferentes de ITS e ainda estudar as relações das populações obtidas de manga e inhame por meio de marcadores microssatélite. Análises filogeneticas utilizando genes de *mating type*, TEF-1α e β-tubulina indicaram que isolados de manga e inhame apresentam baixa variabilidade genética e residem em um único grupo juntamente com isolados obtidos de batata-doce, a partir do qual a espécie foi originalmente descrita. Nenhuma variação morfológica significativa foi observada nos isolados de manga e inhame estudados e experimentos de cruzamento mostraram que eles são capazes de cruzar entre si e ainda cruzar com isolados de batata doce, produzindo descendentes férteis, mostrando que todos pertecem a uma única espécie biológica. A partir de valores de diversidade gênica e genotípica, bem como análise de dendrogramas baseados na frequencia alélica, foi possível identificar populações naturais e introduzidas do fungo. Os isolados obtidos de *Tilia americana* foram identificados como uma nova espécie e descrita como *Ceratocystis tiliae*, baseado em análises filogenéticas, diferenças na taxa de crescimento micelial, pigmentação do micélio e especificidade ao hospedeiro.

ABSTRACT

OLIVEIRA, Leonardo Sarno Soares, D.Sc., Universidade Federal de Viçosa, April, 2014. **Ceratocystis wilt on *Mangifera indica, Colocasia esculenta* and *Tilia americana*.** Advisor: Acelino Couto Alfenas. Co-advisor: Dalmo Lopes Siqueira.

Ceratocystis fimbriata is currently recognized as a complex of many cryptic species that affect mainly woody plants in Brazil and worldwide. In recent years, some of the descriptions of new species within this complex were based solely on variation of ITS sequences, such as the description of three new species obtained from mango. However, the cloning of PCR fragments of ITS gene region from single ascospores isolates of *C. fimbriata* showed overlap of sequences, showing that it is not possible to correct the reading of the amplified fragment, making the description of new species doubtful.Thus, the present work aimed to study the taxonomy of the fungus obtained from mango, taro and basswood by using different genes instead ITS and also evaluated the relationships of populations of the fungus obtained from mango and taro using mircrosatellite markers. Phylogenetic analysis of mating type genes , TEF - 1α and β - tubulin indicated that isolates from mango and taro have low genetic variability and reside in a single group that includes isolates from sweet potato, from which the species was originally described. No significant morphological variation was observed in isolates from mango and taro and mating experiments showed that they are able to cross to each other and also cross with isolates from sweet potato, suggesting they all belong to a single biological species. The values of gene and genotypic diversity, and analysis of dendrograms based on allele frequency, it was possible to identify natural and introduced populations of the fungus. The isolates obtained from basswood were identified as a new species and described as *Ceratocystis tiliae*, based on phylogenetic analyzes, differences in mycelial growth, pigmentation of the mycelium and host specificity.

GENERAL INTRODUCTION

Members of the *Ceratocystis fimbriata* complex are wound or soilborne pathogens on root crops or trees and may cause wilting and cankering on some woody hosts (Kile et al, 1996; Johnson et al, 2005). Among the hosts affected by *Ceratocystis* are *Prunus* spp (almonds and other stone fruits), *Populus* spp (aspen and other poplars), *Platanus* spp (sycamore or plane tree), *Theobroma cacao* (cacao), *Coffea arabica* (coffee), *Hevea brasiliensis* (rubber tree), *Eucalyptus* spp (eucalyptus), *Mangifera indica* (mango), *Tectona grandis* (teak), *Acacia* spp (acacia), *Gmelina robusta* (gmelina), *Ficus carica* (edible fig), *Citrus* spp (citrus) and other woody plants (Borja et al, 1995; Engelbrecht & Harrington, 2005; Ferreira et al, 1999; Firmino et al, 2012; Johnson et al, 2005; Marin et al, 2003; Ribeiro, 1982; Ribeiro et al, 1988; Silveira et al, 1994; Valarini & Tokeshi, 1980; Viégas, 1960;). Also, the pathogen can causes the rot of storage roots or corms of *Colocasia esculenta* (taro or inhame) (Harrington et al, 2005) and *Ipomoea patatas* (sweet potato) (Halsted, 1890).

Infection of *Ceratocystis* sp. may occur through the crown and, or the roots. The root penetration is independent on wounds and the plant dies quickly. When occurs through the crown, the disease begins in lateral branches and progress slowly towards the trunk, reach the trunk and cause the death of the plant. In the absence of living plants, the fungus can survive and multiply in the soil and in the dead branches. The dispersion of the pathogen can occur mainly through infested cuttings, agricultural implements and infected tools or through the soil from infected orchard. The fungus can also be disseminated by coleoborer insects of the subfamily *Scolytinae (*Curculionidae) as demonstrated in *Populus* (Hinds, 1972), *Prunus* (Moller et al., 1969) and in *M. indica* (Viégas, 1960; Ribeiro, 1980; Yamashiro and Myazaki, 1985).

The symptoms of Ceratocystis wilt in mango trees resemble those observed in a branch burned by fire. Initially, the apical leaves wilt and become yellowish, following of the complete dry up of the affected branch, but usually the leaves stay in the plant without the occurrence of defoliation. There is sap exudation in some points (gummosis) and, with the evolution of the disease, occurs the death of the infected branch and the adjacent ones, which become progressively infected. The infected wood becomes dark, contrasting with the healthy tissue (Batista et al., 2008).

Currently, *Ceratocystis fimbriata sensu lato* is recognized as a complex of many fungal species. Four phylogenetic clades are present in the complex and they consist of Latin American clade, North American clade, Asian clade and the African species *C. albifundus* M. J. Wingf., De Beer, & M. J. Morris and its relatives (Harrington, 2000; Heath et al., 2009; Johnson et al., 2005; Roux et al., 2001; Thorpe et al., 2005). Problems on distinguishing new species based on ITS rDNA sequences were found in isolates of *C. fimbriata*, and the study of Harrington et al (2014) showed that more than one sequence could be obtained from directly sequencing of PCR fragments. Thereafter, other genes like mating type genes must be applied for distinguishing new *Ceratocystis* species because they give a more robust and reliable information. There are likely other cryptic species within *C. fimbriata* species complex, but these species should be delimited by more than ITS sequences alone (Harrington et al, 2014). Recently, the studies of Ferreira et al (2010) proposed that Brazilian isolates be recognized as a single species, *C. fimbriata*, in part due to the interfertility of Brazilian isolates with isolates from sweet potato, the host from which *C. fimbriata* was originally described.

The present Thesis consists of three chapters. The first chapter aimed to explore the boundaries between species and populations of *Ceratocystis fimbriata* isolates obtained from mango, studing populations from Brazil, Oman and Pakistan. The second chapter aimed to study the genetic variation of populations of *C. fimbriata* obtained from *Colocasia esculenta* in order to establish putatively native and introduced populations of the fungus in Brazil and also evaluate the movement of haplotypes in corms of taro. The third chapter aimed to further determine if *C. fimbriata* strains from basswood are phylogenetically and pathologically distinct from other members of the North American clade.

We expect that the information obtained from this study can significantly contribute to the clarification of the taxonomy of *Ceratocystis* isolates obtained from mango, taro and basswood. Also, with information generated with microsatelite data, we expect to identify natural and introduced populations of the fungus.

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CHAPTER 1

Genetic variation and intersterility among isolates of *Ceratocystis fimbriata* from *Mangifera indica* in Brazil, Oman and Pakistan.

Genetic variation and intersterility among isolates of *Ceratocystis fimbriata* from *Mangifera indica* in Brazil, Oman and Pakistan.

INTRODUCTION

The mango tree (*Mangifera indica* L.), a species from the Anacardiaceae family, is originated in South Asia, more specifically from India and from the Malay Archipelago (Cunha et al., 2002), from where it was taken to other continents, being cultivated in all countries with tropical and subtropical climates. Currently, the mango tree is amongst the most exported tropical fruit trees in the world, being India, China, Thailand, Indonesia, Pakistan, Mexico, and Brazil the main world producers, respectively (FAO, 2011). In Brazil, the main mango producers are the states of Bahia, São Paulo, Pernambuco, and Minas Gerais (IBGE, 2013). Many diseases may affect the mango production and the wilt caused by *Ceratocystis fimbriata* is being pointed as a limiting factor for mango cultivation in areas where the disease occurs.

The first report of *C. fimbriata s.l.* in mango trees occurred in Brazil in the 1930 decade by Carvalho (1938), in the state of Pernambuco being this disease denominated “Mal do Recife” and the causal agent later identified as *C. fimbriata* by Viégas (1960). Later on, it was found and confirmed in the states of São Paulo (Viégas, 1960), Bahia (Batista, 2008), Rio de Janeiro (Baker et al, 2003; Silveira et al, 2006; Ferreira et al, 2010). The disease is known as ``seca da mangueira``, Ceratocystis wilt or mango tree wilt. Recently, the disease was observed for the first time outside Brazil, occurring in Pakistan (Fateh et al, 2006) and in the Sultanate of Oman (Al-Adawi et al., 2006), causing serious damages to this culture and becoming an international problem for the mango cultivation. However, based solely on variation of ITS sequences the strains were described as a new species *Ceratocytis manginecans* M van Wyk, A. Adawi & MJ Wingf. (Van Wyk et al., 2007), although showing similarity with the strains from the Latin American clade (Ferreira et al, 2010; Harrington et al, 2014). Also, two new species infecting mango trees in São Paulo, Brazil, were recently described as *Ceratocystis mangicola* M van Wyk & MJ Wingf. and *Ceratocystis mangivora* M. van Wyk & MJ Wingf. (Van Wyk et al., 2011), but they are apparently ITS genotypes that have been moved in nursery stock to commercial plantations and small farms in the state of São Paulo.

Ceratocystis fimbriata Ellis & Halsted is well known for having a broad host range and are able to infect numerous plant families in over 41 countries spanning in six inhabited continents (CAB International, 2005). The symptoms of the Ceratocystis wilt in mango trees resemble those observed in a branch burned by fire. Initially, the apical leaves wilt and become yellowish, following of the complete dry up of the affected branch, but usually whose leaves stay in the plant without the occurrence of defoliation. There is sap exudation in some points (gummosis) and, with the evolution of the disease, occurs the death of the infected branch and the adjacent ones, which become progressively infected. The infected wood becomes dark, contrasting with the healthy tissue (Batista et al., 2008).

Currently, *C. fimbriata sensu lato* is recognized as a complex of many cryptic species (Harrington, 2000; Engelbrecht & Harrington, 2005; Johnson et al, 2005), and many have been described over the last few years including *C. platani* Engelbr. & TC Harr. (Engelbrecht & Harrington, 2005), *C. cacaofunesta* Engelbr. & T.C. Harr. (Elgenbrecht & Harrington, 2005) and *C. colombiana* M. van Wyk & M. J. Wingfield (Van Wyk et al., 2010). There are four phylogenetic clades within the complex: Latin America clade, the North America clade, the Asian clade and the African clade (Harrington, 2000; Heath et al., 2009; Johnson et al., 2005; Roux et al., 2001; Thorpe et al., 2005). All strains from the complex obtained from Brazil, fall within the Latin America clade and Ferreira et al. (2010) proposed that Brazilian isolates are a single biological species.

In the last years, many strains from the Latin America clade have been described as new species based solely on ITS sequences, though there is very limited morphological variation, physiological (host) specialization, isozyme variation, or DNA sequence variation (Engelbrecht and Harrington, 2005; Johnson et al., 2005; Harrington et al., 2011; Thorpe et al., 2005; Van Wyk et al., 2007, 2009, 2010, 2011a, 2011b, 2012). However, problems with the taxonomy of *C. fimbriata* s.l. using ITS sequences for delimiting species were identified by Harrington et al. (2014) and even being likely the occurrence of new species from the complex, other features must be applied than ITS sequence alone.

Populations of *C. fimbriata* from mango in Brazil were previously studied (Ferreira et al., 2010) and the results indicated that populations from São Paulo and Mato Grosso do Sul are related to each other and also related to the populations of the fungus from eucalyptus. Besides, the populations obtained from mango in the small towns of São Fidélis, located on the eastern part of the state of Rio de Janeiro, were the most distinct population and those isolates were able to infect cultivated annona (sugar-apple, *Annona squamosa*) and the host range of this population appears to be different to the other populations from mango (Baker et al., 2003; Silveira et al., 2006).

Based on previous works, the first objective of this work was to study the phylogeny of *C. fimbriata* isolates from mango obtained from Brazil and Oman, using the phylogenetic species concept supported by Harrington and Rizzo (1999), which consider a solid species only the lineages with unique combination of phenotypic characters. The second objective was to demonstrate the relatedness of *C. fimbriata* isolates obtained from mango in many regions in Brazil, in order to establish the source of introduced populations and also identification of possible native populations. Besides, isolates from Oman and Pakistan were studied in order to show the relatedness to the other Brazilian isolates.

MATERIALS AND METHODS

Collection of Isolates

From information provided by mango experts in Brazil and also information from local newspapers, we were able to identify regions with the possibility to find the disease. Isolates were collected from diseased mango in plantations, small farms or single trees in small cities across Brazil (Fig 1), Oman and Pakistan. Trees showing symptoms of wilting, tissue discoloration alongside the stem and sap exudation (gummosis) were chopped generally on the base of the tree and under the bark in order to find the colonized wood. Thereafter, the fungus was baited from diseased wood tissue by placing the pieces of discoloured tissue between two discs of carrot root. Ascospores masses from perithecia formed on the carrot discs were transferred to agar media for purification and then storage. Only one isolate per tree was stored and used in genetic analysis. Pure cultures were stored at Universidade Federal de Vicosa on glycerol 15% at -80 °C. A total of 214 isolates were obtained along the years of 2011, 2012 and 2013.

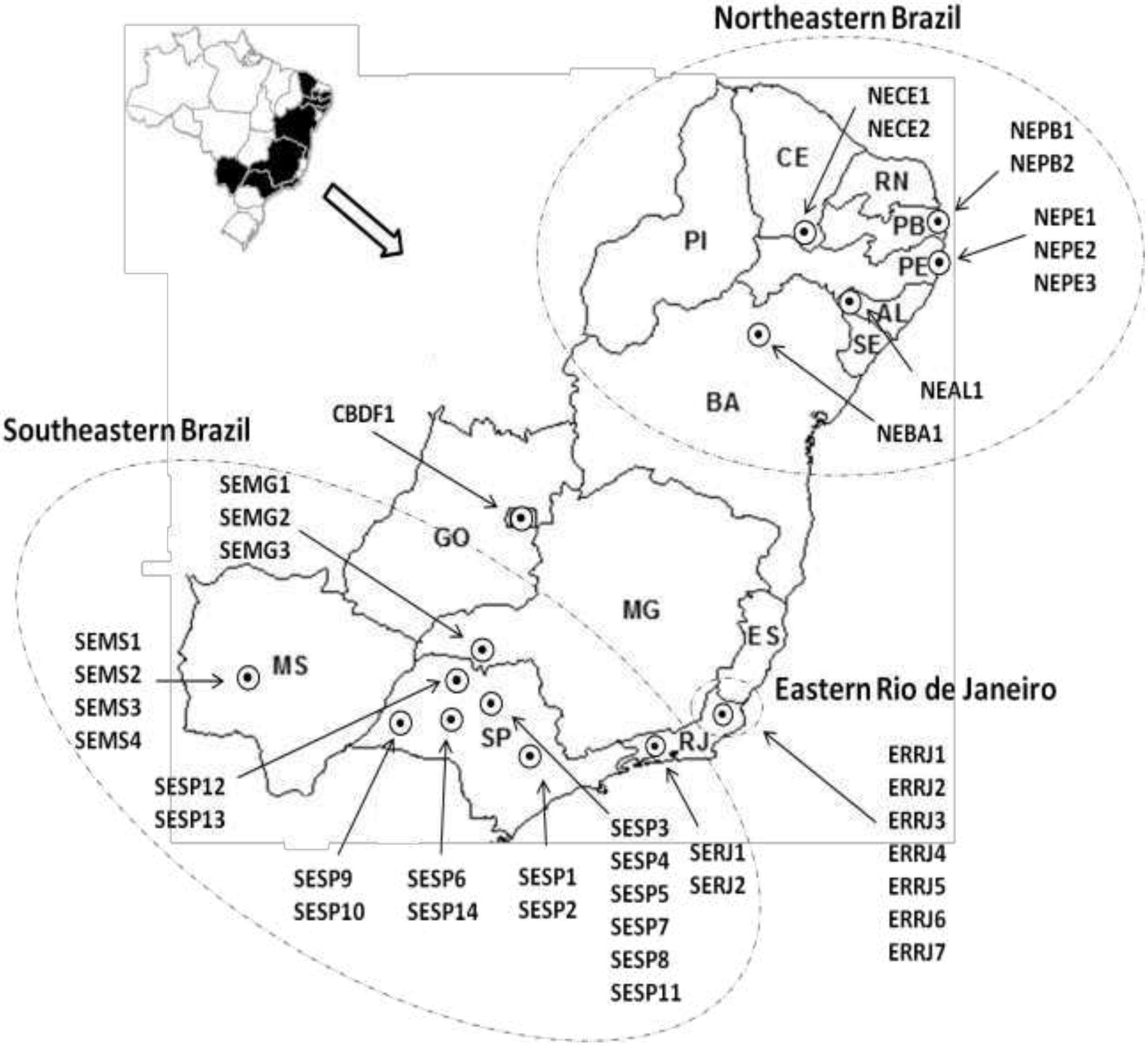

Fig 1. Map of Brazil showing geographic distribution of the 40 sites where *Ceratocystis fimbriata* was collected from mango trees. The first two letters of each population name indicate the geographic region that isolates come from (SE - Southeastern Brazil; NE - Northeastern Brazil; ER - Eastern Rio de Janeiro; CB - Central Brazil). The last 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; CE - Ceará) and the number designates the collection site or population from a given state.

Southeastern Brazil populations

Fungal isolates were obtained from dead or wilting mango trees in the states of São Paulo (SP), Minas Gerais (MG), Rio de Janeiro (RJ) and Mato Grosso do Sul (MS). In SP, the isolates were obtained from comercial plantations in the cities of Monte Alto, Vista Alegre do Alto, Candido Rodrigues and Taquaritinga. Also, isolates were obtained from backyards or single trees along the streets showing disease symptoms in Santo Anastacio, Ribeirao dos Indios, Guaimbe, Lins, Valentim Gentil and Votuporanga. In Limeira, where much of the nursery industry is located, it was possible to identify an abandoned nursery, where mango plants that would be used as rootstocks showed symptoms of wilting and typical tissue discoloration. From a single row of trees we could obtain 11 isolates. In MG, the isolates were obtained from a large plantation with 40,000 trees located in Frutal. In RJ, isolates were collected in the city of Seropedica, nearby the Universidade Federal Rural do Rio de Janeiro, and also in the city of Paraíba do Sul. In MS, we collected isolates from single trees located along the streets of Aquidauana and also in small plantations of a indigenous community located in Limao Verde, nearby Aquidauana. In addition, isolates were also collected in backyards in Campo Grande and Terenos.

Norheastern Brazil populations

The isolates were obtained from dead or wilting mango trees in the states of Bahia (BA), Alagoas (AL), Pernambuco (PE), Paraíba (PB) and Ceará (CE). In BA, we collected all isolates from the small town of Senhor do Bonfim, located about 100 km from the main mango producing area of Brazil. This area has a unique vegetation which is very dry, and is called Caatinga. In AL, all the isolates were obtained from scattered trees along the streets or in backyards of the small town of Agua Branca. This town is also located in a Caatinga region. In PE, we collected the isolates from a small farm with mango trees showing wilting symptoms in Cabo de Santo Agostinho, located in a Atlantic rainforest region. Also, we collected isolates from scattered tree on the city of Recife and Olinda, where the disease was first reported on mango in Brazil. In PB, the isolates were collected from wilting mango trees in the city of Joao Pessoa and suburban neighbor of Conde. They are both located in a Atlantic rainforest region. In CE, the isolates were collected from scattered mango trees along the streets or in backyards of the small town of Brejo Santo, which is located in a Caatinga region.

Eastern Rio de Janeiro (Brazil) populations

Previously studies indicated that isolates obtained from mango in the eastern part of the state of Rio de Janeiro were different from the other mango isolates from Brazil (Baker et al., 2003; Silveira et al., 2006; Ferreira et al., 2010). Based on that information, we collected isolates from wilting mango trees along the roads and streets of the small towns of São Fidélis, Itaocara, Itaperuna, Santo Antonio de Padua, São Francisco do Itabapoana e Campos dos Goytacazes. Besides, we identified a mango plantation in Itaocara with wilting trees and then we could sample one population from that area. Eastern Rio de Janeiro is located near the Atlantic Ocean in a Atlantic rainforest region.

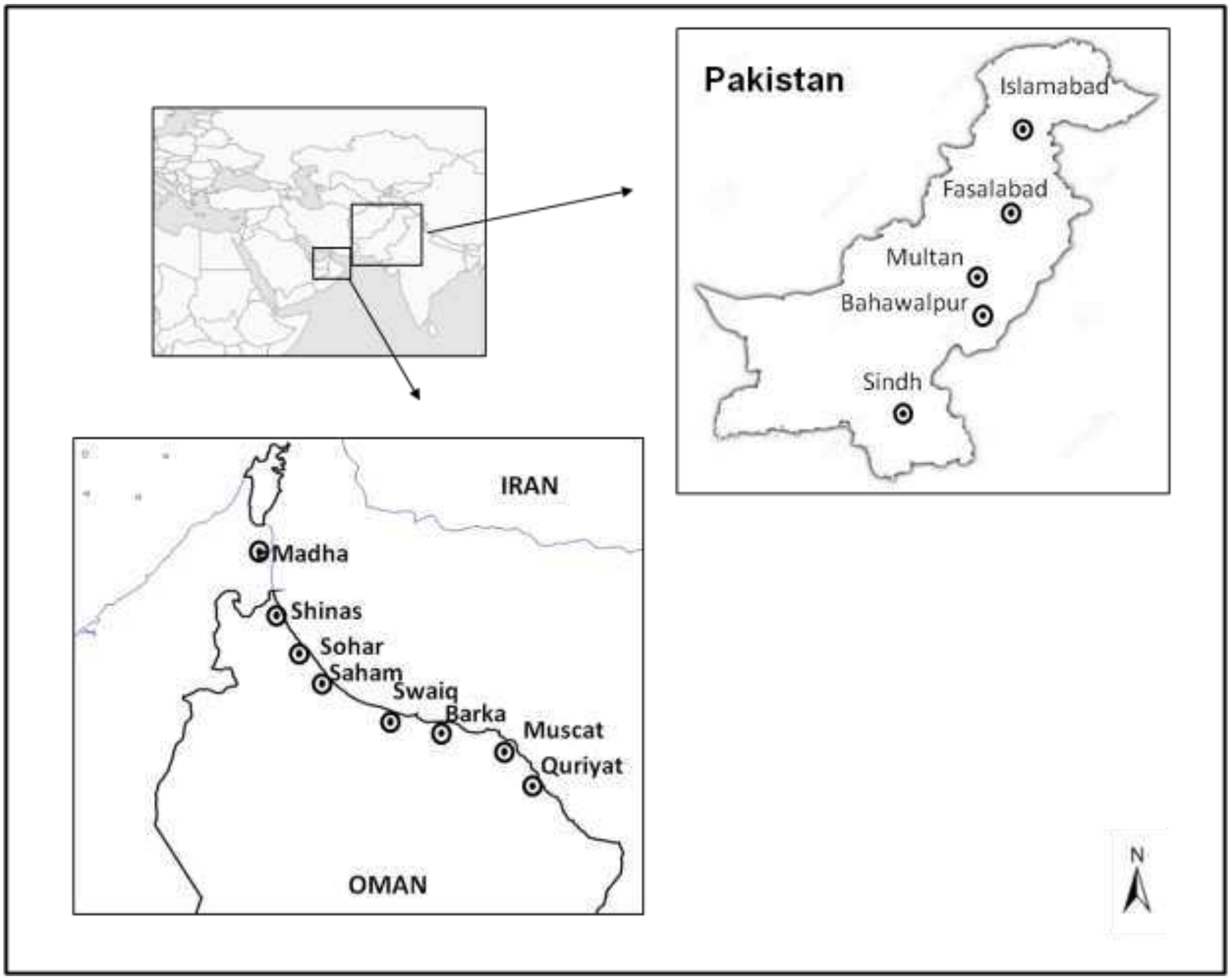

Fig 2. Map of Oman and Pakistan showing geographic distribution of sites where *Ceratocystis fimbriata* was collected from mango trees.

Oman and Pakistan populations

In Oman, all the isolates were collected in the Al-Batinah region (Fig 2). The isolates were obtained from dead or wilting mango trees in small farms from northeastern Oman (coastal plains area) in the cities of Barka, Almosna`h, Quriyat, Al-Seeb, Swaiq, Sohar, Shinas, Saham and Madha. The disease was found moving toward north from the Barka region. In Pakistan, the isolates were collected in an earlier study (Harrington et al. 2014) from mango trees showing wilting symptoms in small farms in Multan, Bahawalpur, Islamabad, Fasalabad and Sindh (Fig 2). The northernmost region (Islamabad) is about 2,000 km far from the southernmost region (Sindh).

Phylogeny

A single ascospore strain were derived from the original field isolate by dispersing an ascospore mass in a light oil and spreading the spore suspension over the plate; individual germlings were subcultured to fresh plates for growth and DNA extraction (Harrington and McNew, 1997). A data set with 48 taxon were used in phylogenetic studies, being 28 of the mango collection of the present work.

Sequences were generated using PCR folowed by direct DNA sequencing of the PCR products with primers primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3) and the following cycling conditions (85 °C for 2 min, 95 °C for 95 sec, and then 36 cycles of 58 °C for 1 min, 72 °C for 80 sec and 95 °C for 70 sec, followed by a 52 °C for 1 min and 72 °C for 15 min (Harrington et al., 2011). The fragments were purified using Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) and sequenced at the Iowa State University DNA Synthesis and Sequence Facility. One of the isolates had ITS PCR product that could not be clearly determined using direct sequencing, so the PCR products were cloned into the pGEM-T Easy vector (Promega Corporation, Madison, Wisconsin). Plasmids were extracted using Ilustra™ plasmidPrep Mini Spin Kit (GE Healthcare Life Sciences, Buckinghamshire, UK), and the cloned fragments were sequenced using primers T7-2 and SP6.

All members of the *C. fimbriata* complex are homothalic through uni-directional mating type switching (Harrington and McNew, 1997; Witthuhn et al, 2000). Homothallic strains have the MAT2 gene (*MAT1-2-1*) in the mating type locus, flanked by two MAT1 genes, *MAT1-1-1* and *MAT1-1-2*. For most isolates, portions of the *MAT1-2-1* and *MAT1-1-2* regions were amplified and sequence in order to compare to earlier-characterized strains of *C. fimbriata* (Harrington et al., 2014). The primers CFMAT1-F (5' –CAGCCTCGATTGAKGGTATGA-3') and CFMAT1-R (5' –GGCATTTTTACGCTGGTTAG-3') were used to amplify and sequence about 1000 bp region of *MAT1-1-2* (Harrington et al., 2013). The primers X9978a (5' -GCTAACCTTCACGCCAATTTTGCC- 3') and CFM2-1F (5' –AGTTACAAGTGTTCCCAAAAG- 3') amplify and sequence about 1150 bp region. The thermocycler settings for amplifying the MAT1 and MAT2 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 at 72 °C for 10 min. The amplified products were sequenced with the PCR primers.

Besides, gene sequences of β-tubulin and TEF-1α were also obtained using PCR folowed by direct DNA sequencing of the PCR products . For β-tubulin, the primers BT1-α (5' -TTCCCCCGTCTCCACTTCTTCATG - 3') and BT1-β (5' -GACGAGATCGTTCATGTTGAACTC - 3') were used to amplify and sequence about 600 bp and thermocycler settings for amplifying the β-tubulin region included: initial denaturation at 94 °C for 4 min, with 30 cycles of 94 °C for 50 sec, 55 °C for 50 sec, 72 °C for 50 sec, and a final extension of at 72 °C for 10 min. For TEF-1α, the primers EFCF1.5 (5' -GCYGAGCTCGGTAAGGGYTC- 3'), EFCF2 (5' -TGCTCAACGGGTCTGGCCAT - 3'), EFCF3 (5' - ATGGCCAGACCCGTGAGCA - 3') and EFCF6 (5' -CATGTCACGGACGGCGAAAC - 3') amplify and sequence about 1600 bp region and thermocycler settings for amplifying the TEF-1α region included: initial denaturation at 85 °C for 2 min folowed by 94 °C for 1 min and 35 sec, with 36 cycles of 60 °C for 1 min, 72 °C for 1 min and 30 sec, and 94 °C for 35 sec, folowed by final extension of 60 °C for 1 min and 72 °C for 15 min.

Sequence datasets for the four genomic loci (MAT1, MAT2, TEF-1α and β-tubulin) were aligned in MAFFT Online version v. 7.0 (Katoh & 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). The likelihood values were calculated and the best model of nucleotide substitution for each gene (TEF-1α, MAT1, MAT2 and β-tubulin) was selected according to Akaike Information Criterion (AIC) using MrModeltest v. 2.3 (Nylander

2004). The multi-gene Bayesian Inference (BI) was conducted for the aligned β-tubulin, MAT1, MAT2 and TEF-1α data set with 48 taxa including outgroup (*Ceratocystis variospora* from *Prunus*) on MrBayes v. 3.1.1 (Ronquist & Heulsenbeck, 2003) using the algorithm of Markov chain Monte Carlo (MCMC) with two sets of four chains (one cold and three heated) and the stoprule option, stopping the analysis at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 1000; the first 25 percent of trees were removed.

Mating experiments

Representative testers isolates of *Ceratocystis fimbriata* from mango, eucalyptus, fig, inhame, gmelina and sweet potato and representative testers of an isolate of *C. cacaofunesta* and *C. platani* (Engelbrecht & Harrington, 2005) were used in mating experiments (Table 1). *Ceratocystis fimbriata* is both a heterothallic and a homothallic fungus, with two mating types; MAT-1 strains are self-sterile, but MAT-2 strains are self-fertile. The MAT-2 strains have both MAT-1 and MAT-2 genes, but during unidirectional mating-type switching, the MAT-2 gene is deleted, and progeny that have inherited nuclei with the deletion behave as MAT-1 and are self-sterile (Harrington and McNew, 1997; Witthuhn et al, 2000). Thus MAT-2 (self-fertile) and MAT-1 (self-sterile) progeny are recovered from selfings of MAT-2 strains. Based on this, the mating experiments were conducted as described by Ferreira et al (2010) (Fig 3).

After recovering the female testers with the male tester, the cultures were observed for 3-4 weeks for the presence of perithecia and ascospore masses. Ascospore masses were examined microscopically (x400) to see if there were abundant, normal-appearing ascospores, indicating a good cross, or if there were few or no ascospores, or if the ascospores were misshapen, which is typical of an interspecific cross or hybrid (Harrington & McNew, 1998; Harrington et al., 2002, Johnson et al, 2005; Ferreira et al, 2010) (Fig 5). Also, ascospore masses from one or more perithecia of a cross were streaked onto fresh MYEA to observe whether the ascospores were viable and if the progeny had a uniform mycelial morphology, indicating an induced selfing had occurred, or if progeny showed the mycelial phenotypes of the two parents, indicating a successful cross (Harrington & McNew, 1998).

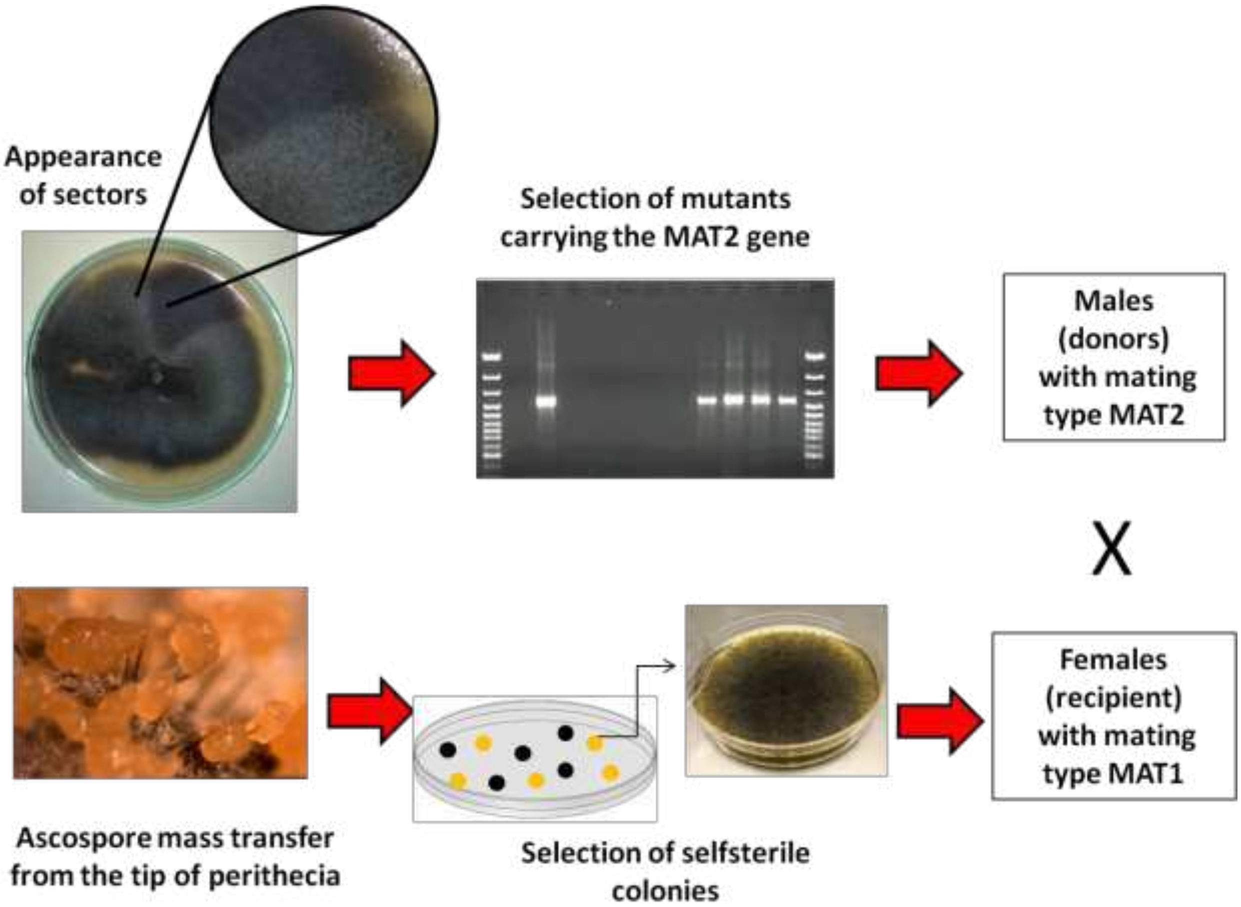

Fig 3. Scheme of mating studies showing the selection of male and female testers.

Morphology

Based on previous results, representative isolates were selected for morphology studies. They were grown on MYEA (2% malt extract, 0.2% yeast extract, and 2% agar) for about 10 days at room temperature (approximately 23 C). Measurements of endoconidia and endoconidiophores were made after 4–7 d growth, while perithecia and ascospores were measured after 7– 10 d. Aleurioconidia were measured from cultures that had grown 7–20 d. Material to be measured was mounted in lactophenol cotton blue and observed with Nomarsky interference microscopy. Perithecia were measured with an eyepiece reticule at 400X magnifications. For most structures 10 observations were recorded per isolate; when measuring endoconidia, however, 20 conidia were measured per isolate. Some structures were rare or hard to locate in a few isolates, and fewer observations were made. Colony pigmentation was compared to the color chart of Rayner (1970).

Microsatellite markers

We analyzed 14 microsatellite loci (CfAAG8, CfAAG9, CfCAA9, CfCAA10, CfCAA15, CfCAA38, CfCAA80, CfCAT1, CfCAT1200, CfCAG5, CfCAG15, CfCAG900, CfGACA60 and CfGACA650) developed from the total genomic DNA of an isolate of *C. cacaofunesta* (Steimel et al, 2004) and used in population studies on *C. cacaofunesta* (Engelbrecht et al., 2007), *C. platani* (Engelbrecht et al., 2004; Ocasio-Morales et al., 2007) and *C. fimbriata* (Ferreira et al., 2010; Ferreira et al., 2011). Of the 16 loci used in earlier studies, two (CfCAT3K and CfCAT9X) were not utilized because their alleles could not be consistently resolved with some isolates. For each primer pair specific to the flanking regions of 14 simple sequence repeat regions, one of the primers was fluorescently labeled. PCR amplifications of all microsatellite loci were performed using a 96-well thermal cycler (PTC-100) MJ research Inc., Watertown, MA) following the earlier described conditions (Ferreira et al., 2010) . The PCR products were electrophoresed using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Band Sizes of the product were determined using marker standards and Peak Scanner 1.0 software (Applied Biosystems Inc.). Each product length (within 1 bp) was considered to be a different allele. The microsatellite regions are trinucleotide repeats, and most alleles of a given locus differed by increments of 3 bp.

Microsatellite analyses

Each population consisted of at least four isolates from one location (one small city, one plantation or a group of small farms in one small city). Nei's gene diversity of microstatellite loci for each population was calculated with and without clone-corrected data using PopGen 1.32 software (Yeh and Boyle, 1997). Clone-corrected datasets were a subset of the population left after removing isolates that were genetically identical, that is, a genotype within a population was counted only once. The clone-corrected value for *H* would be expected to be higher than the uncorrected value if the population was dominated by one or few genotypes, as might occur if a few genotypes were spread within a plantation on tools or equipment or the pathogen was introduced in infected nursery stock.

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, Stoddart and Taylor`s was scaled by the expected number of genotypes for the smallest sample size being compared (Grünwald et al., 2003). For individual plantations, the expected number of genotypes in a sample of four isolates (minimum value = 1.0 and maximum value = 4.0) was estimated based on rarefaction curves using the *R* package (version 2.9.1; R Development Core Team, Vienna). Partition of total variance using analysis of molecular variance (AMOVA) on Euclidean distances was performed using ARLEQUIN 2.0 (Excoffier et al., 2005).

To test for random mating within populations, linkage disequilibrium was analyzed using the index of association (IA) statistic in Multilocus. The populations were clone-corrected before analysis. We used randomization procedures (1000 replications, without replacement) to test the significance of the departure of the observed IA value from zero (randomly mating population).

Nei's genetic distance between populations and UPGMA (unweighted pair group method with arithmetic mean) dendrograms were constructed using PopGen 1.32. Bootstrap values for branches of the population trees were calculated from 100 replicates using SEQBOOT, GENDIST, NEIGHBOR and CONSENSE in PHYLIP version 3.6 (Felsenstein 1989, 1993). Relationships among genotypes were also examined using genetic distance (Nei's) matrices, UPGMA trees, and 1000 bootstrap replications generated with PAUP* (Swofford, 1998).

RESULTS

In addition to the states where the disease was previously reported, the present work identified plants with symptoms of the disease in the states of Minas Gerais, Paraíba, Alagoas and Ceará. Visits were also made in the states of Pará, Piauí, Sergipe and Espírito Santo, however the disease was not found in these states.

Phylogeny

A 4-gene data set including sequences of MAT1, MAT2, TEF-1α and β-tubulin were used to generated a combined phylogenetic tree (Fig 4). The final aligned combined data set contained 47 taxa, with a total of 4182 characters, including gaps. The Bayesian analysis lasted 760.000 generations and the consensus trees and posterior probabilities were calculated from the 1374 trees.

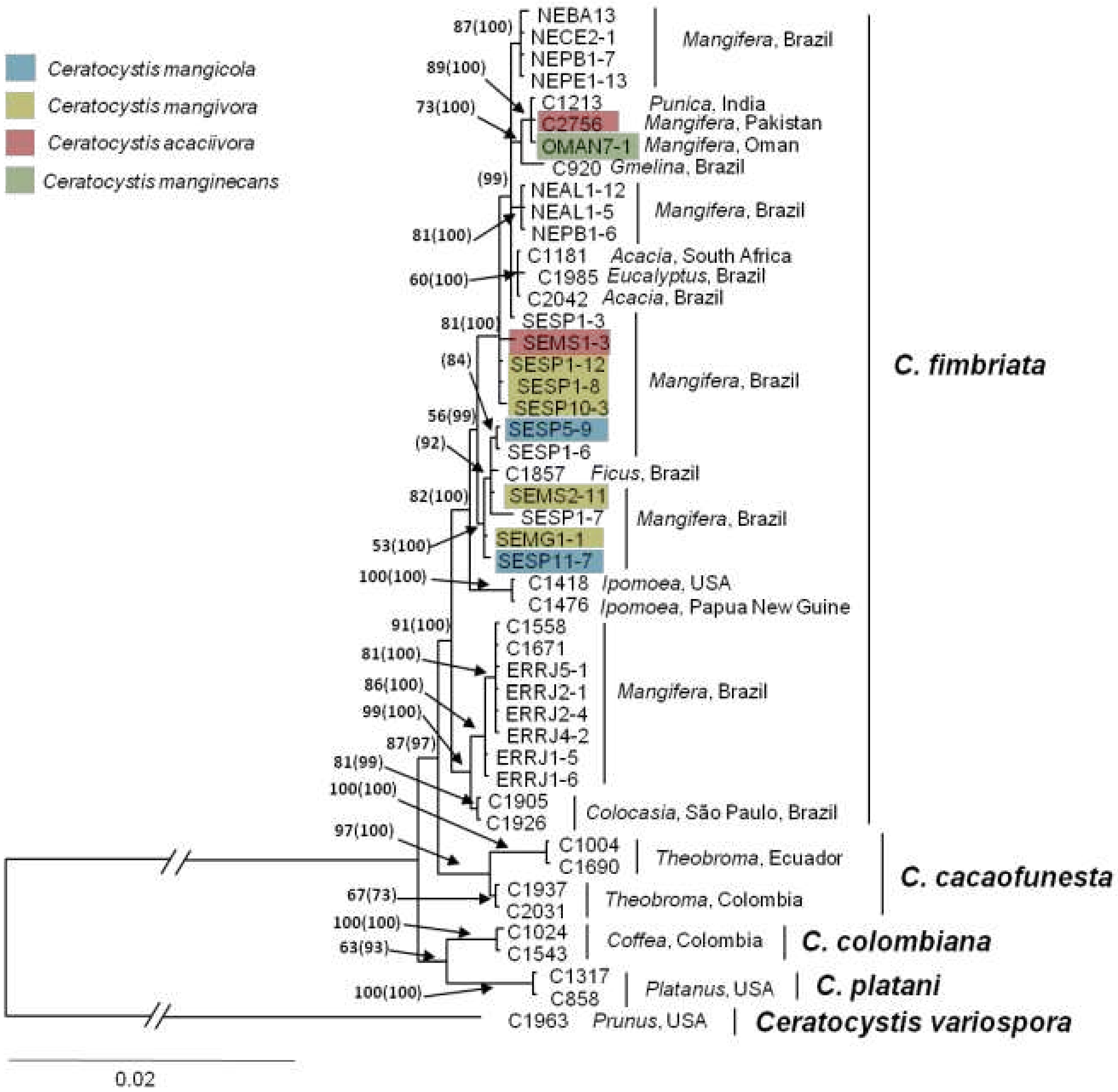

Fig 4. Phylogenetic tree of *Ceratocystis* complex resulting from a Bayesian analysis of the combined 4-gene sequences (MAT1, MAT2, TEF 1α, and β-tubulin). Bootstrap values greater than 50% are indicated on appropriated the branches and Baysian posterior probability values greater than 50% are indicated in brackets. The tree was rooted to *C. variospora* (C1963).

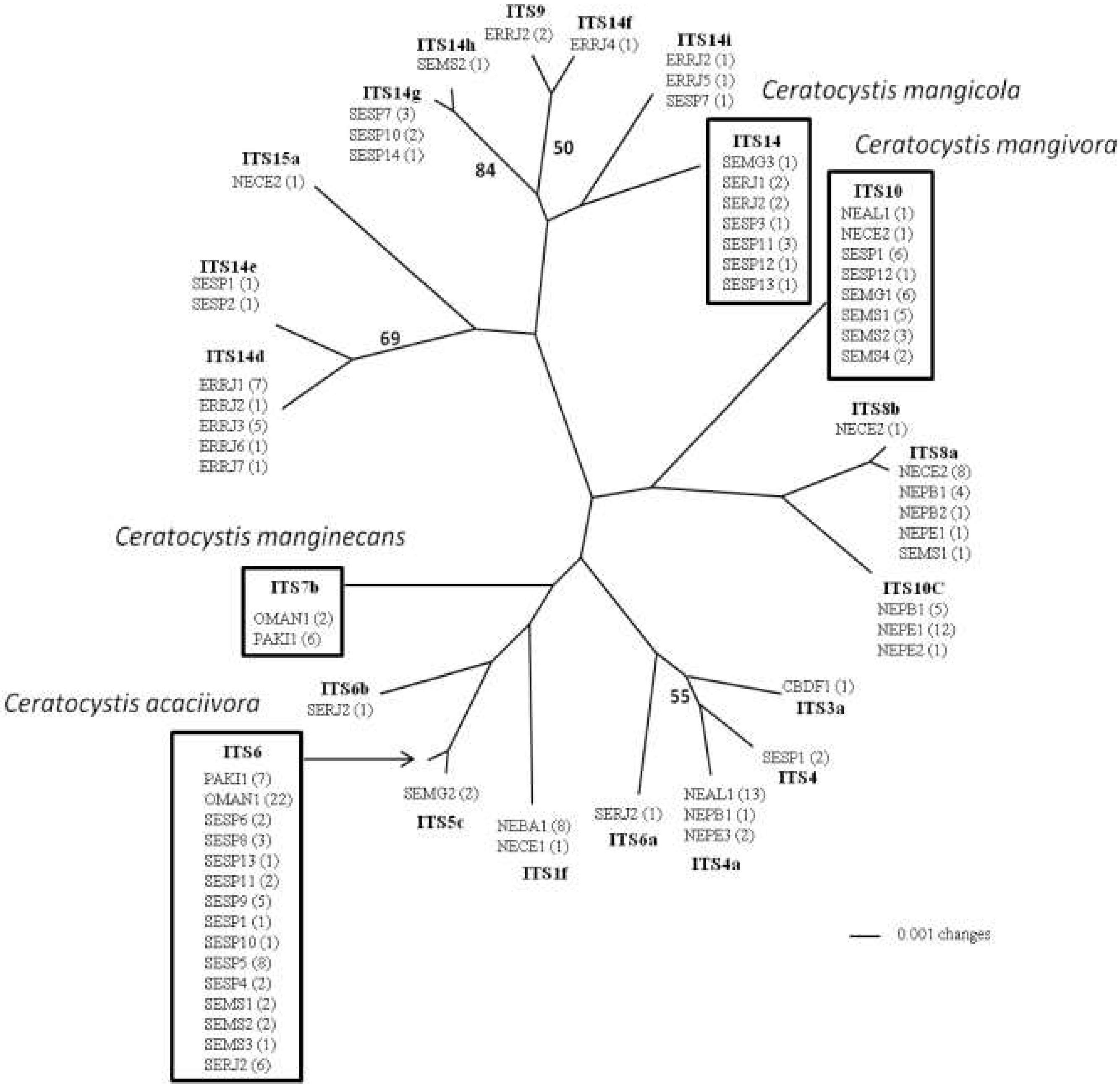

Fig 5. A UPGMA (unweighted pair group method, arithmetic mean) dendrogram of genotypes of *Ceratocystis fimbriata* from mango based on ITS sequences. Bootstrap values are shown alongside the branches. The number of isolates with that genotype from each population is in parentheses. ITS numbers are based on Harrington et al. (2011), with new ITS sequences from Harrington et al. (2014) designated by unique letters.

Among 214 isolates from mango, we identified 22 ITS haplotypes, and 12 of which were not previously reported in the earlier study (Harrington et al. 2014) (Fig 5). The ITS haplotype numbers used by Harrington et al. (2011) were applied, with low case letters to designate new sequences from Harrington et al. (2014) and in the present study. The ITS6 was found in most populations from mango in SE. In ER, the ITS14d was the most common sequence and represents a new ITS haplotype. Four new ITS haplotypes were identified in NE. Among 25 isolates from Oman, we identified 22 as ITS6 and two as ITS7b. One isolate had a mixture of sequences and from the nine cloned fragments obtained we identified three sequences as ITS7b and six as a new ITS haplotype not previously reported. Some of the cloned fragments appeared to have unique single base substitution that were identified as normal PCR error, with random substitution rate of 0.44% (Harrington et al., 2014). In Pakistan, the same ITS haplotypes were identified in mango isolates, being eight as ITS6 and five as ITS7b (Harrington et al. 2014).

The UPGMA tree based on ITS sequences (Fig 5) showed a much different topology than the tree generated by the microsatellite alleles (Fig 8, 9). Some isolates from ER were grouped with isolates from SE and NE, though they have very distinct microsatellite haplotype.

Mating experiments

Testers from mango isolates of each of the three groups from Brazil and one group from Oman successfully crossed to each other (Table 1). Also, there were successful crosses with all mango isolates from Brazil and Oman with the isolate of *Ceratocystis fimbriata s.s.* from sweet potato. In most of these crosses, there were many perithecia observed with normal ascospore masses per plate. In successful crosses, perithecia usually developed within a week and produced thick, creamy ascospore masses at the tips of perithecial necks (Fig 6). However, some of the female testers performed poorly in crosses, perhaps through loss of femaleness (poor protoperithecia or development of perithecia) (Ferreira et al, 2010). From the good crosses, single-ascospore progeny produced colonies of the mycelial morphology of the male and female parents. In the other hand, all the isolates used as MAT2 male tester from mango did not successfully cross with testers of *C. platani* from *Platanus* or *C. cacaofunesta* from *Theobroma*. Only a few perithecia were produced, the ascospore masses from these perithecia were watery, not creamy, and microscopic examination showed there to be misshapen ascospores and aborted asci in the perithecial centrum (Fig 6).

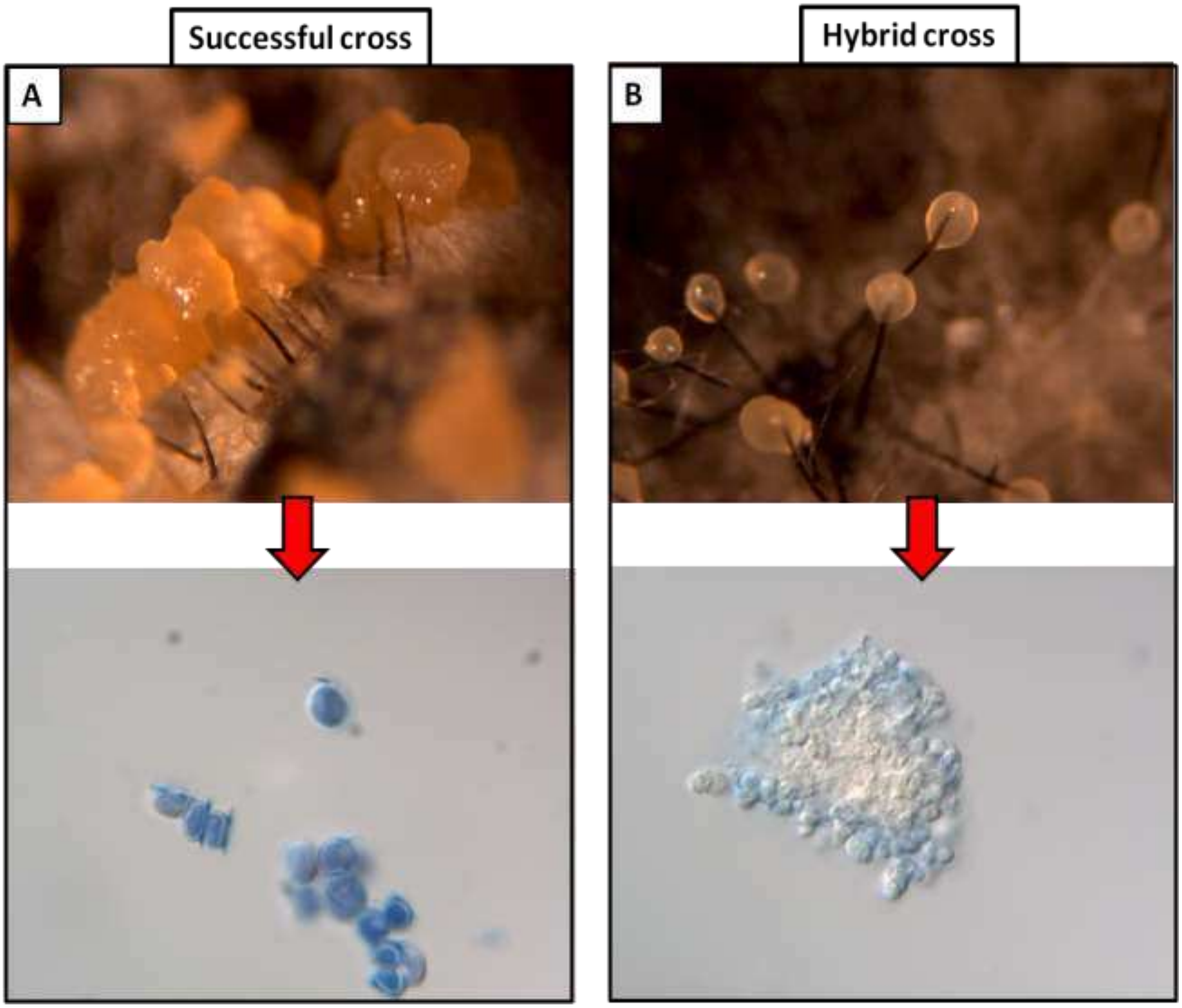

Fig 6. Behavior of perithecia and ascospores in mating experiments. A - Successful cross with many apparently normal perithecia containing normal-appearing ascospores. B - Hybrid cross with watery ascospore masses containing misshapen ascopores.

Table 1. Mating experiments among strains of *Ceratocystis fimbriata*, *Ceratocystis platani* and *Ceratocystis cacaofunesta*. MAT-2 self-sterile sector (sec) strains were used as males and MAT-1 self-sterile (ss) strains as females.

Host	MAT-1, Female[a]	MAT-2, Male[a]					
		SESP5-1sec	SEMS2-11sec	NECE2-1sec	OMAN1-8sec	ERRJ4-2sec	ERRJ1-10sec
Mangifera	SESP5-1ss	I	-	-	I	-	-
	SEMS2-11ss	I	I	I	I	I	I
	NEBA1-10ss	I	-	I	I	I	I
	OMAN5-7ss	I	I	I	I	I	I
	ERRJ4-2ss	-	I	I	I	I	I
	ERRJ2-2ss	I	-	I	I	I	I
Ficus	C1783ss	-	-	I	I	I	I
Gmelina	C918ss	-	-	I	I	-	-
Eucalyptus	C1347ss	I	I	I	I	I	I
Colocasia	C1926ss	I	-	I	I	I	I
Ipomoea	C1418ss	I	I	I	I	I	I
Theobroma	C1587ss	H	H	H	H	H	H
Plantanus	C1317ss	H	H	H	H	H	H

H = Hybrid: much cellular debris and few misshapen ascospores inside perithecium, exuded ascospores masses, when present, watery in appearance; I = Interfertile: ascospores abundant, with normal form; exuded ascospore masses orange or creamy. Presence of both parental types when ascospores mass transferred to a new plate; - = No perithecia and ascospores produced.

[a] sec, strains from MAT-2, self-sterile sectors recovered from self-fertile isolates; ss, MAT-1, female-competent isolates with protoperithecia

Morphology

Isolates from Southeastern Brazil, Northeastern Brazil, Eastern Rio de Janeiro and Oman were examined and all of them showed very similar morphological characteristics (Fig 7) and the range of measurements of all isolates were presented as follow: Cultures on malt yeast-extract agar produced a typical banana odour, starting out whitish or pale brown turning to dark brown after 5-7 days, undersurface of agar turning dark (Rayner, 1970). Where endoconidiophore are being produce stays a pale brown color, areas with perithecia are darker. Perithecia with bases superficial to partially immersed, bases black or dark brown, globose, 105-280 µm diam, 115-260 µm height, unornamented or with undifferentiated hyphae attached; necks black or dark brown, slender, 260-680 µm long, 17-38 µm diam at base and 11-22 µm at the tip; ostiolar hyphae hyaline 20-64 µm long, divergent or convergent; asci not seen; ascospores 4.0-6.0 x 3.0-5.0 µm with outer cell wall forming a hat-shaped brim. Endoconidiophores of two types; one flask-shape, hyaline to pale brown, septate 65-195 µm long, conidiogenous cell 30-115 µm long, width 4.0-7.0 µm at base and 3.0-6.0 µm at the mouth; producing chains of hyaline endoconidia 10-30 x 3.0-5.0 µm; the other endoconidiophore less common, shorter, 55-140 µm long, not tapering, conidiogenous cell 27-80 µm long, width 4.0-6.0 µm at base and 4.0-7.0 µm at the mouth; producing chains of doliform endoconidia, hyaline 7.0-11.0 x 4.0-6.0 µm; aleuroconidia produced blastically, singly or in chains, pale brown to dark brown, ovoid or obpyriform, smooth, 7.0-12.0 x 8.0-15.0 µm.

Fig 7. Morphological characteristics of *Ceratocystis fimbriata* from *Mangifera indica*. A – Perithecium. B – Ostiolar hyphae and emerging ascospores. C – Ascospores. D – Aleuroconidium. E – Flask-shape endoconidiophore producing cylindrical endoconidium. F – Doliform endoconidiophore producing doliform endoconidium. G - Cylindrical endoconidia and doliform endoconidia in a chain. All features from isolate SESP5-1. Bars: A = 100 µm; B = 20 µm; D, E, F, G = 10 µm; C = 5 µm.

Genetic variation of haplotypes based on microsatellite alleles

Of the 14 microsatellite loci tested among the isolates from mango, all were polymorphic with exception of the locus CAG900 (Table 3). At some locations only one, two or three isolates were obtained and these were not included in the population analyses. In total, 214 isolates from mango were studied, and 57 microsatellite genotypes were found. The UPGMA tree constructed using these mango genotypes separated the most of the isolates in four groups found in the population analyses: Southeastern Brazil (SE), Northeastern Brazil (NE), Eastern Rio de Janeiro (ER) and Oman/Pakistan (OMAN/PAKI). Two microsatellite genotypes were commonly found in SE, and we could find this isolates in populations in São Paulo, Minas Gerais and Mato Grosso do Sul (Fig 8). This two isolates were both found in a row of trees in an abandoned nursery of Limeira in the state of São Paulo, where the main nursery industry is located. One of the common genotypes is MSAT1 and it was found in eight different populations in SE and the other common genotype MSAT2 was found in twelve different populations in SE. Few genotypes from NE were found occurring in more than one population. The isolates from ER were the most distinct and they grouped separate to the other mango isolates with well-supported (98%) branch. All the isolates from Oman and Pakistan were very similar, showing variation only for the locus CfCAA38, which had seven different alleles, and the locus CfCAA9, which had two different alleles, but the CAA9-211 allele was found in just one isolate from Pakistan or Oman. The most common microsatellite genotype (MSAT7) dominated in both countries (Fig 8).

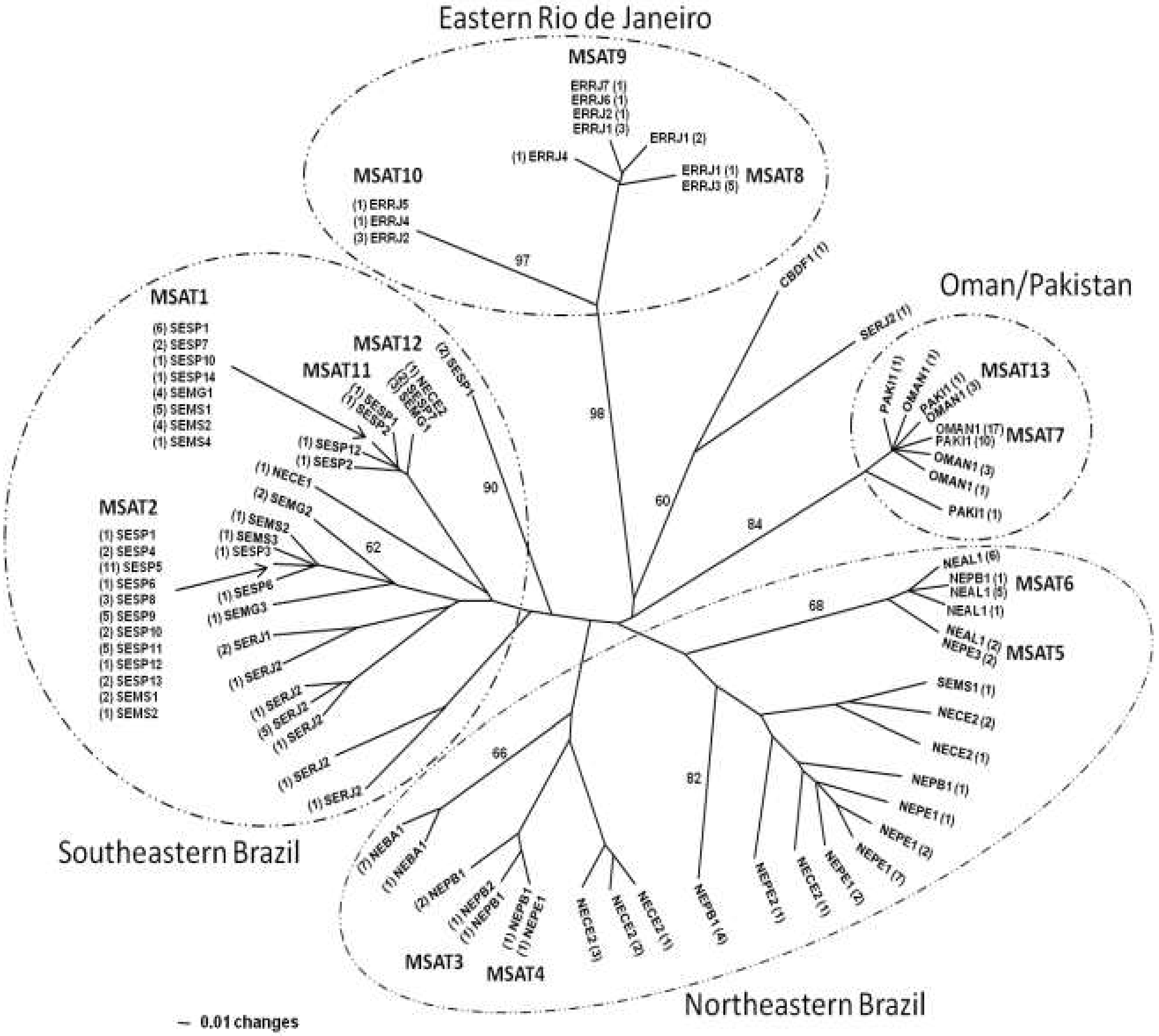

Fig 8. A UPGMA (unweighted pair group method, arithmetic mean) dendrogram of genotypes of *Ceratocystis fimbriata* from mango based on alleles of 14 microsatellite loci. Bootstrap values are shown alongside the branches. The number of isolates with that genotype from each population is in parentheses. Thirteen genotypes found in more than one population are designated by MSAT followed by a number.

Genetic relatedness of populations based on microsatellite alleles

A UPGMA tree based on allele frequencies was constructed in order to compare populations from mango of the present study with populations that appeared to represent natural populations. The tree showed that the 19 populations from mango were separated in four geographic groups: Southeastern Brazil (SE), Northeastern Brazil (NE), Easter Rio de Janeiro (ER), Oman/Pakistan (OMAN/PAKI). Eucalyptus populations from Bahia and Minas Gerais and an inhame population from São Paulo studied earlier (Ferreira et al. 2010) were also included for comparisons. The eucalyptus populations appeared related to each other but were distinct from the mango populations. The inhame population appeared to be somewhat related to mango populations from eastern Rio de Janeiro (Fig 9).

The population SESP1 from the abandoned nursery in Limeira, São Paulo, was from a single, densely-planted row of mango trees, but it had high levels of gene and genotypic diversity (Table 2), including the two most widespread genotypes of *C. fimbriata* on mango (Fig 8). These Limeira trees were planted earlier for rootstock material but were never grafted, and they were apparently infected from soilborne inoculum because the infections rose from the roots.

Populations from São Paulo, Minas Gerais, and Mato Grosso do Sul were each dominated by those genotypes found in the Limeira nursery (Fig 8, 9), and these Southeastern Brazil populations were closely related based on microsatellite analysis (Fig 7). The populations SESP5, SESP9 and SESP11 are connected by a well-supported (96%) branch and they are single genotype populations dominated by one of the common genotypes in SE (MSAT2), leading to no gene and genotypic diversity. Population SEMG1 from Frutal, Minas Gerais, and SESP7 from Taquaritinga, São Paulo, were obtained from commercial plantations and both populations were composed of the same genotypes and were connected by a well-supported (100%) branch. One of the genotypes from the latter populations (MSAT1) is the other common genotype found in the Limeira nursery. The Mato Grosso do Sul populations were very similar each other and were connected by a well-supported (98%) branch. These populations were dominated by the two common genotypes spread in São Paulo, and the MS populations were closely related to the nursery population, connected by a well-supported (99%) branch (Fig 7). Among populations from southeastern Brazil, the western Rio de Janeiro population (SERJ2) were the most distinct (Fig 9).

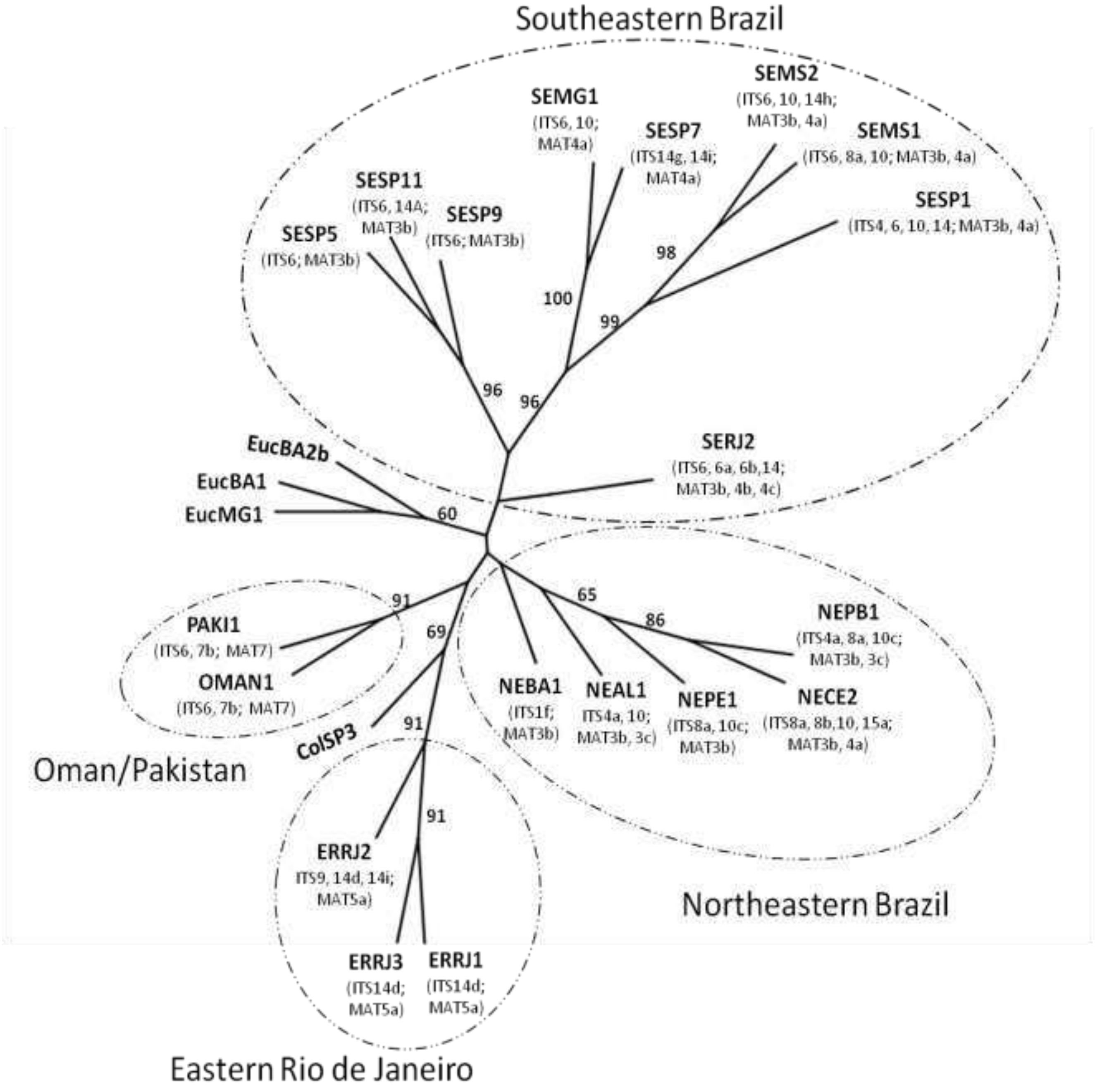

Fig 9. Dendrogram of the relatedness of populations of *Ceratocystis fimbriata* from mango, eucalyptus (EucBA1, EucBA2b, and EucMG1), and inhame (ColSP3) generated by UPGMA (unweighted pair group method, arithmetic mean) based on allele frequencies of 14 microsatellite loci. Bootstrap values are shown alongside the branches. ITS and Mating type gene haplotypes found in a given population are in parentheses.

Northeastern Brazil populations were geographically separated from each other, and they showed the greatest diversity in microsatellite alleles (Table 2). Only a few genotypes were found in more than one population in the Northeast (Fig 8). Populations NEPB1 and NECE2 had the highest levels of gene and genotypic diversity and they were connected by a moderate to high-support (86%) branch (Fig 9). Populations NEAL1, NEBA1 and NEPE1 had relatively low gene diversity and were dominated by one or few genotypes. Short branches suggest that populations from NE appeared to be related to each other (Fig 8, 9).

Populations from Eastern Rio de Janeiro were the most distinct. Populations ERRJ1, ERRJ2 and ERRJ3 were connected by a well-supported (91%) branch. Population ERRJ1 and ERRJ3 had also strong support (91%) for grouping different than ERRJ2. Besides, population ColSP3 that was obtained from inhame in a coastal area in São Paulo, the same Atlantic Rainforest vegetation found in ER, was connected with the ER populations by a moderate-supported (69%) branch.

Populations obtained from Oman and Pakistan were uniform and very similar each other, with gene and genotypic diversity values very low. Both populations were connected by a well-supported (91%) branch, though being close related to the eucalyptus, inhame and mango isolates from Brazil.

Nei's gene diversity (*H*) was calculated for each of the 23 populations of *C. fimbriata* from Brazil, Oman and Pakistan, including isolates from mango, eucalyptus and inhame. A second calculation of gene diversity was made using only unique genotypes from each population (clone-corrected for population) (Table 2). Relatively high levels of gene diversity were found in SESP1, SEMS1, SERJ2, NEPB1 and NECE2 from mango and in EucMG1, EucBA1 and EucBA2b from eucalyptus. A gene diversity value of 0.0 was found in populations SESP5, SESP9, SESP11 and ERRJ3 from mango because they were single-genotypes populations and the data gives a strong support that they are introduced populations. There were relatively low levels of gene diversity found in populations SESP7, SEMG1, SEMS2, NEAL1, NEBA1, NEPE1, ERRJ1, ERRJ2, OMAN1 and PAKI1 from mango, and ColSP3 from inhame. Besides, when we clone-corrected all the populations and compared the groups, the values of gene diversity of the populations from southeastern Brazil were similar to those that apparently are natural populations (Table 2).

The highest genotypic diversity with rarefaction were found in populations SERJ2 (G = 3.14), NEPB1 (G = 3.20) and NECE2 (G = 3.48) from mango and EucMG1(G = 3.74), EucBA1(G

= 3.27) and EucBA2b(G = 3.20) from eucalyptus, with maximum value possible of 4 (Table 2). From single-genotype populations, the expected genotypic diversity value of 1.0 was observed for the populations SESP5, SESP9, SESP11 and ERRJ3 from mango, also supporting that they are introduced populations. The remaining populations had genotypic diversity value G = 1.50 to 2.67.

Based on the relatively high levels of diversity obtained from mango populations, the AMOVA was utilized to determine the amount of variation attributable among groups, among populations within groups and within populations (Table 4). For the three mango groups from Brazil, most of the variation was attributable to variation among populations (SE = 55.71%, ER = 60.49%, NE = 64.30%). In Oman and Pakistan, there was no variation among population (0.0%) and all the variation (100%) was attributable to variation within populations, which is consistent to other results that indicate both as introduced populations (Ferreira et al., 2010). The results considering all populations from mango in Brazil, Oman and Pakistan showed that most of the variation (51.40%) was attributable to variation among groups, 29.88% to the variation among populations within groups, and 18.68% to the variation within populations.

Table 2. Genetic diversity of populations of *Ceratocystis fimbriata* based on fourteen microsatellite loci.

Group	Population	State or Province	City	Collected from	Brazilian vegetation[c]	No. isolates	No. Geno-types	Genotypic diversity (*G*)[a]	Nei`s gene diversity (*H*)	
									All Isolates	Clone corrected[b]
Southeastern Brazil	SERJ2	Rio de Janeiro	Seropedica	small farms and gardens	Mata Atlantica	10	7	3.14	0.2302	0.3003
	SESP1	São Paulo	Limeira	one row of trees	Cerrado/Mata Atlantica	11	4	2.58	0.2267	0.2946
	SEMS2	Mato Grosso do Sul	Aquidauana	small farms	Cerrado/Pantanal	6	3	2.33	0.1468	0.1587
	SEMS1	Mato Grosso do Sul	Aquidauana	small city	Cerrado/Pantanal	8	3	2.29	0.2277	0.3010
	SESP7	São Paulo	Taquaritinga	Single plantation	Cerrado/Mata Atlantica	4	2	2.00	0.0357	0.0357
	SEMG1	Minas Gerais	Frutal	plantation	Cerrado	7	2	1.93	0.0350	0.0357
	SESP5	São Paulo	Cândido Rodrigues	plantation	Cerrado/Mata Atlantica	11	1	1.00	0.0000	0.0000
	SESP9	São Paulo	Santo Anastácio	small city	Cerrado/Mata Atlantica	5	1	1.00	0.0000	0.0000
	SESP11	São Paulo	Vista Aleg. do Alto	plantation	Cerrado/Mata Atlantica	6	1	1.00	0.0000	0.0000
	All					**68**	**14**	**6.04**	**0.2851**	**0.3594**
Northeastern Brazil	NECE2	Ceará	Brejo Santo	small city	Caatinga	11	7	3.48	0.2786	0.3294
	NEPB1	Paraíba	Conde	small city	Mata Atlantica	10	6	3.20	0.3814	0.3889
	NEPE1	Pernambuco	Cabo St. Agostinho	small farms	Mata Atlantica	13	5	2.67	0.1183	0.1943
	NEAL1	Alagoas	Água Branca	small city	Caatinga	14	4	2.65	0.0576	0.0714
	NEBA1	Bahia	Senhor do Bonfim	small city	Caatinga	8	2	1.50	0.0156	0.0357
	All					**56**	**22**	**9.41**	**0.4301**	**0.4328**
Eastern Rio de Janeiro (Brazil)	ERRJ1	Rio de Janeiro	São Fidélis	small farms	Mata Atlantica	7	3	2.43	0.0466	0.0635
	ERRJ2	Rio de Janeiro	Itaocara	small farms	Mata Atlantica	4	2	2.00	0.1339	0.1786
	ERRJ3	Rio de Janeiro	Itaocara	plantation	Mata Atlantica	5	1	1.00	0.0000	0.0000
	All					**16**	**5**	**5.00**	**0.1328**	**0.1508**
Oman/ Pakistan	OMAN1	Al-Batinah	Multiple	small farms	Not known	25	5	2.27	0.0361	0.0571
	PAKI1	Multiple	Multiple	small farms	Not known	13	4	1.92	0.0380	0.0804
	All					**38**	**7**	**4.40**	**0.0376**	**0.0741**
Eucalyptus	EucBA1	Bahia	Eunápolis	plantation	Mata Atlantica	26	13	3.27	0.2191	0.2899
	EucBA2b	Bahia	Caravelas	plantation	Mata Atlantica	6	4	3.20	0.2262	0.2500
	EucMG1	Minas Gerais	Curvelo	plantation	Cerrado	18	14	3.74	0.3069	0.3236
	All					**50**	**31**	**12.49**	**0.3417**	**0.3673**
Colocasia	ColSP3	São Paulo	Piedade	infected corms	Mata Atlantica	12	4	2.50	0.0784	0.1429

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[a] Stoddart and Taylor`s genotypic diversity, with rarefaction. Rarefaction gave estimated values for *G* of 1.0 (only one genotype in the population) to maximum value of 4.0 (all isolates of a different genotype). Maximum value of 16 were applied when comparing all populations for each group.

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

[c] Probable brazilian vegetation types prior to agriculture

Table 3. Estimated sizes (bp) of alleles of 14 microsatellite loci in populations of *Ceratocystis fimbriata* on mango in four regions. Number of isolates tested in each region is shown in parentheses.

Microsatellite Loci	**Southeastern Brazil (94)**	**Northeastern Brazil (61)**	**Eastern Rio de Janeiro (20)**	**Oman/Pakistan (38)**
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
CAG15	259, 286, 292	268, 286, 292, 295	277, 286	274
AAG9	391, 397, 400	397, 400, 403, 406	391	403
AAG8	174, 177, 180, 183	174, 177	186, 198	177
CAA9	175, 205	175, 178, 205	223, 197	211, 226
CAA10	128, 131, 134	128, 134	128	122
CAA15	321, 324	306, 321, 324	321, 330	324
CAT1	257, 261	254, 257, 260, 261	248	254
CAG5	298, 317, 320	317, 320	323, 326	317
GACA6K	215, 219, 221	215, 221	219	215
CAT12	374, 377	371, 374, 377	380	374
GACA60	187, 200	187	187, 221	187
CAG900	194	194	194	194

Table 4. Analysis of molecular variance (AMOVA) of *Ceratocystis fimbriata* populations and regional groups on mango based on fourteen microsatellite loci.

	Among Groups^{b}			**Among Populations**			**Within Populations**		
Group	d.f.	%	*P*^{a}	d.f.	%	*P*^{a}	d.f.	%	*P*^{a}
All populations	3	51.4	<0.0001	15	29.88	<0.0001	160	18.68	<0.0001
Southeastern Brazil				8	55.71	<0.0001	60	44.29	<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
Oman/Pakistan				1	0.00	0.5318	36	100.00	0.02444

[a] The *P* value is for the null hypothesis that there is no significant variation at that level based on 1023 permutations

The index of association (I_A) was used to test for random mating in all populations from SE, NE and ER from mango. The analysis was conducted using clone-corrected data. Because of the very low variability observed in populations from Oman and Pakistan, the analysis was not conducted for this group (Table 5). The value of I_A is expected to be near zero in a randomly mating population, and 1000 randomized replicates were performed to assess whether the value obtained was significantly different from zero. Considering all populations from mango, the I_A obtained showed evidence for non-random mating or asexual reproduction (I_A = 2.0090, $P < 0.001$). The populations in the four groups each showed significant departure from the values expected for random mating, with values of I_A (SE=1.00, $P < 0.001$; ER = 1.26, P = 0.045; NE = 1.39, $P < 0.001$).

Table 5. Index of association of *Ceratocystis fimbriata* populations on mango in Brazil.

Group	**Location**	**No. Genotypes**	**Index of Association (*I*$_A$)**	**Probability**^{a}
SE	Southeastern Brazil	14	1.0037	< 0.001
ER	Eastern Rio de Janeiro	5	1.2619	0.045
NE	Northeastern Brazil	22	1.3932	< 0.001

All populations	2.0090	< 0.001

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

DISCUSSION

Although some lineages were identified in the phylogenetic tree generated with the combined 4-genes dataset, low variation was observed, and the mango lineages, including isolates from Oman and Pakistan, are scattered among the other Brazilian lineages in the *Ceratocystis fimbriata* complex. Mating experiments using representative isolates from the major lineages, showed to be interfertile to each other and also interfertile with isolates from sweet potato *C. fimbriata* s.s, suggesting they are a single biologic species (Ferreira et al, 2010). As the earlier observation of Webster and Butler (1967), studied strains from major lineages or regions do not differ morphologically. The information generated clearly shows that isolates from mango are a single species of *C. fimbriata*, based on phylogenetic species concept supported by Harrington & Rizzo (1999).

Ceratocystis wilt in mango has been recently reported for the first time outside Brazil in Oman (Al-Adawi et al., 2006) and Pakistan (Fateh et al, 2006). Based on microsatellites markers, populations of *C. fimbriata* from mango in Oman and Pakistan appeared to be very uniform, consistent with the results of Al Adawi (2011). The DNA sequences indicate that they were closely related to the South American isolates. Two ITS sequences (ITS6 and ITS7b) were identified among the isolates in both countries, but the first report of the disease just identified one of the ITS haplotype (ITS7b) and named as *C. manginecans* (Van Wyk et al., 2007), currently considered by some to be the name of the causal agent of the disease in Oman and Pakistan. Later on, the same causal agent was reported causing disease in *Dalbergia sissoo* in Pakistan (Poussio et al, 2010; Al-Adawi et al., 2013), *Prosopis cineraria* in Oman (Al-Adawi et al., 2013) and *Acacia mangium* in Indonesia (Tarigan et al., 2011). The second ITS haplotype (ITS6) was recently reported in Oman (Al-Adawi et al., 2013), however earlier study showed that the isolates with that same ITS sequence where observed causing disease on *Acacia mangium* in Indonesia, and named as *C. acaciivora* (Tarigan et al., 2011). Later on, the same ITS haplotype were reported on *Eucalyptus* spp in China (Chen et al., 2013). However, both the ITS6 and ITS7b sequences were found within individual isolates from Pakistan and Oman by repeatedly conducting direct PCR products (Al-Adawi et al, 2013) or cloning PCR fragments (Harrington et al., 2014), suggesting that they are clearly the same species. Problems using the ITS rDNA gene for describing

Ceratocystis spp were pointed by Harrington et al. (2014), and analysis of mating type genes placed the isolates from Oman and Pakistan within the Latin American clade of *C. fimbriata*. Both *C. manginecans* and *C. acaciivora* are considered synonyms of *C. fimbriata*, and strains found in Oman, Pakistan, India, Thailand, Indonesia and China are likely of South American origin (Harrington et al., 2014). The near absence of microsatellite variation among Oman and Pakistan isolates strongly suggests that *C. fimbriata* is not native to these regions. Other description of new species of isolates obtained from mango was observed in Brazil, in the state of São Paulo. Based on the same concept, variation on ITS sequences led to the description *C. mangicola* and *C.mangivora* (Van Wyk et al, 2011). In order to evaluate this information, representative isolates of these described species were analyzed, and based on the robust tree generated with the 4-gene dataset, they all grouped with other mango isolates and also isolates from other hosts, including the sweet potato strain, suggesting they are all a single biologic species and synonyms of *C. fimbriata*.

The data presented indicates that *Ceratocystis fimbriata* populations from mango in Southeastern Brazil (SE) have been moved to other regions in nursery stock, especially from nurseries located in Limeira, where the main nursery industry is located. From one row of trees in an abandoned nursery in Limeira, we identified a relatively high gene and genotypic diversity and the genotypes found were the most common spread genotypes in SE. These trees were apparently infected from soilborne inoculum and very low insect activity was observed. The mango growers from São Paulo and Minas Gerais usually bought their material from Limeira nurseries, and the populations of the fungus obtained from commercial plantations or scattered plants in small towns from those states were all related to each other and dominated by two microsatellite genotypes. Interestingly, these microsatallite genotypes were recently described as *C. mangicola* and *C. mangivora* (Van Wyk et al., 2011), however the present work demonstrate that they are only genotypes of *C. fimbriata* that have been moved in nursery stock. Mango trees planted in Mato Grosso do Sul were also mostly obtained from São Paulo nurseries, and the populations of the fungus were close related to the populations from Limeira nursery. Some of the sampled populations were uniform and dominated by only one or few genotypes, showing little or no genetic diversity, supporting the hypothesis that they are the result of introduced populations supposedly from plant propagative material (Harrington, 2000; Engelbrecht and Harrington, 2005;

Johnson et al., 2005; Thorpe et al., 2005; Engelbrecht et al., 2004, 2007a,b; Ocasio-Morales et al., 2007; Ferreira et al., 2010, 2011, 2013; Harrington et al., 2014).

Besides the dissemination by plant propagative material, high insect activity was observed in mango trees in Brazil and Oman. The mango bark beetle *Hypocryphalus mangifera* Stebbing (Coleoptera: Scolytinae) was abundantly found in NE, ER and Oman, and an intimate association between the mango bark beetle and Ceratocystis wilt was demonstrated (Al-Adawi et al., 2013). Also, in most visited places we frequently identified the presence of ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae) attacking diseased mango trees, and the fungus can be dispersed by insect frass that is pushing out of trees by adult beetles (Iton and Conway, 1961; Baker et al., 2003; Engelbrecht et al, 2007; Ocasio-Morales et al., 2007; Harrington, 2009). As the infections typically occurs to fresh wounds and the fungus is also soilborne (Rosseto & Ribeiro, 1990; Laia et al., 2000; CAB International, 2005) we believe that ambrosia beetle also plays an important role disseminating the disease.

The first report of Ceratocystis wilt was in Brazil in the state of Pernambuco (Carvalho, 1938). Since the disease was found seriously affecting commercial plantations in São Paulo, very little survey was conducted to elucidated the origin of the strains in Northeastern Brazil (NE). Populations sampled in NE were also related to each other but differed to the other Brazilian populations from mango. The higher levels of genetic variation observed for populations in Ceará and Paraíba are similar to those of other putatively natural populations of homothallic *Ceratocystis* spp (Engelbrecht et al., 2007; Engelbrecht et al., 2004; Harrington et al., 1998; Ferreira et al., 2010, 2011) suggesting that these populations appear to be native to the NE region. Populations in Bahia and Alagoas are dominated by one or few genotypes with low genetic diversity and we hypothesized that the most aggressive genotypes have been transmitted to the adjacent plants by insect activity or infected tools.

In accordance with previous studies, the most distinct populations were obtained from Eastern Rio de Janeiro (ER) (Baker et al., 2003; Silveira et al., 2006; Ferreira et al., 2010) and they differed substantially from the other populations in terms of UPGMA trees of microsatellie data and mating type genes sequences. Besides, cultivated annona was also identified as a host (Baker et al., 2003; Silveira et al., 2006), showing that the host range of this population may be different than the other populations on mango. The isolates from ER had exactly the same MAT2 sequence as found in the inhame isolates from São Paulo and a single isolate from *Acacia* from Parana and they also had moderate support for grouping together based on microsatellite loci. The same type of vegetation in ER (Atlantic rainforest) is also found where the inhame is grown in Brazil, suggesting that special investigation should be conducted in this areas for better understanding of this group of isolates.

Analysis of molecular variance showed that most of the genetic variation of populations of the fungus from mango is attributable to variation among regions and variation among populations, suggesting that most of the genotypes have been locally moved, as clearly observed in NE populations. On the other hand, due to very little genetic variation among populations from mango in Oman and Pakistan, all the variation was attributable to diversity within populations.

Ceratocystis fimbriata strains have been moved around the world and reported in many different hosts. With the information about genetic variability obtained so far, we speculate that likely the introduction of *C. fimbriata* strains in Asia occurred through nursery stock that has been moved from South America and spread to many countries in Asia, due to similarity of genotypes.

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CHAPTER 2

Genetic variation and movement of genotypes of *Ceratocystis fimbriata* in corms of *Colocasia esculenta* in Brazil.

Genetic variation and movement of genotypes of *Ceratocystis fimbriata* in corms of *Colocasia esculenta* in Brazil.

INTRODUCTION

Many species of Araceae have a economic or ethnobotanic importance (ornamental, edible or medicinal) and about 10 % of the world use taro corms in feeding. Due to the characteristics of hardiness and nutritional values, taro is a tuberous species suggested by the FAO (Food and Agriculture Organization) as an alternative to increase the food base in developing countries (Pereira et al., 2004). In 2008, the cultivation of taro in the world was about 1.646 million hectares, with production of 11.774 million tonnes and yield of 7.15 tonnes per hectare (FAO, 2010). *Colocasia esculenta* has been pointed as an alternative crop in Brazil with a high potential for production. The state of Espirito Santo stands out as a traditional taro producer in Brazil (Carmo & Puiatti, 2004). However, the crop can be affected by many diseases and the yield and production can then be compromised. *Ceratocystis fimbriata* is an important post harvest pathogen of taro and the spread of the fungus through corms must be contained in order to prevent its introduction into areas free of the disease.

Ceratocystis fimbriata Ellis & Halsted is well known for being an important pathogen that causes wilting and cankering of cultivated woody plants. The fungus has a broad geographic and host range and it is able to infect numerous plant families (CAB International, 2005). Also, *C. fimbriata* can cause black rot of *Ipomoea patatas* (sweet potato) and *Colocasia esculenta* (taro). In Brazil, the disease is commonly found affecting edible corms of taro in supermarkets in the states of São Paulo, Rio de Janeiro, Bahia, Rondonia and Distrito Federal (Harrington et al, 2005). The sporulating areas included numerous conidiophores producing chains of cylindrical conidia, darkwalled aleurioconidia, and black perithecia with sticky drops of ascospores accumulating at the apex of the long necks. Most of the affected corms had a relatively superficial black rot, often only a few millimeter deep, sometimes with pink-toorange discoloration in the starchy interior (Harrington et al, 2005).

Currently, *Ceratocystis fimbriata sensu lato* is recognized as a complex of many fungal species. Four phylogenetic clades are present in the complex and they consist of Latin American clade, North American clade, Asian clade and the African species *C. albifundus* M. J. Wingf., De Beer, & M. J. Morris and its relatives (Harrington, 2000; Heath et al., 2009; Johnson et al., 2005; Roux et al., 2001; Thorpe et al., 2005). Problems on distinguishing new species based on ITS rDNA sequences were found in isolates of *C. fimbriata*, and the study of Harrington et al (2014) showed that more than one sequence could be obtained from directly sequencing of PCR fragments. Thereat, other genes like mating type genes must be applied for distinguishing new *Ceratocystis* species because they give a more robust and reliable information. There are likely other cryptic species within *C. fimbriata* species complex, but these species should not be delimited by ITS sequences alone (Harrington et al, 2014). Recently, the studies of Ferreira et al (2010) proposed that Brazilian isolates be recognized as a single species, *C. fimbriata*, in part due to the interfertility of Brazilian isolates with isolates from sweet potato, the host from which *C. fimbriata* was originally described. So far, the only species besides *C. fimbriata* in Brazil recognized as a solid species is *Ceratocystis cacaofunesta.*

Genetic variation in populations of *C. cacaofunesta* (Engelbrecht et al., 2007), *C. platani* (Engelbrecht et al., 2004; Ocasio-Morales et al., 2007) and *C. fimbriata* (Ferreira et al., 2010; Ferreira et al., 2011) have been studied over the past years using microsatellite markers (Steimel et al, 2004) that were able to distinguish putatively native populations of the pathogen from introduced populations. The present work aimed to study the genetic variation of populations of *C. fimbriata* obtained from *Colocasia esculenta* in order to establish putatively native and introduced populations of the fungus in Brazil and also evaluate the movement of haplotypes of the fungus in corms of taro.

MATERIALS AND METHODS

Fungal isolates and DNA extraction

Isolates were collected from infected edible corms of *Colocasia esculenta* found in many stores in Brazil along the years of 2001, 2002 and 2009. The fungus was baited from infected corms by placing pieces of the infected tissue between two discs of carrot root. Ascospores masses from perithecia formed on the carrot discs were transferred to agar media for purification. After 10 days, pure cultures were stored at Iowa State University on glycerol 15% at -80 °C. Only one isolate per corm was stored and used in genetic analyses. A total of 47 isolates were collected in the states of São Paulo, Rio de Janeiro, Bahia, Rondônia and Distrito Federal (Fig 1).

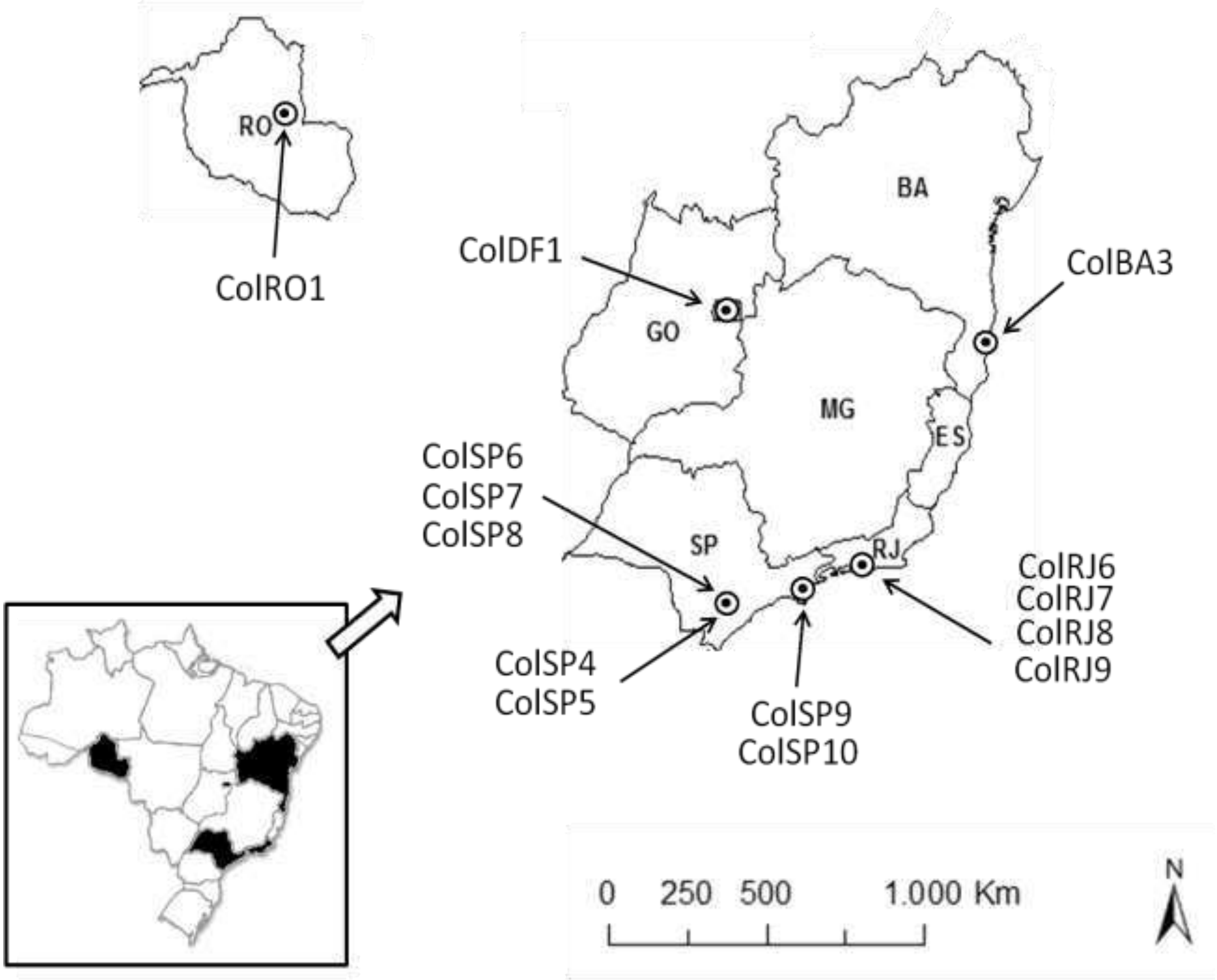

Fig 1. Map of Brazil showing the locations of the 14 *Ceratocystis fimbriata* populations on *Colocasia esculenta*. The first three letters of each population name indicate the host (*C. esculenta*), the last two letters indicate the state of origin (Bahia, Rondonia, São Paulo, Rio de Janeiro, and Distrito Federal) and if more than one population was sampled in that state, they were numbered.

All isolates used in the present study were single ascospore strains obtained from the original field isolate by dispersing an ascospore mass in a light oil and spreading the spore suspension over the plate; individual germlings were then subcultured to fresh plates for growth (Harrington and McNew, 1997). For DNA extraction, the isolates were transferred to MYEA (2% malt extract, 0.2% yeast extract, and 2% agar) for about 10 days at room temperature (approximately 23 °C) and the extraction were conducted using PrepMan™ Ultra (Applied Biosystems, Foster 4 City, CA).

Phylogeny

A data set with 40 taxon were used in phylogenetic studies, being 19 of the taro collection of the present work. Sequences were generated using PCR folowed by direct DNA sequencing of the PCR products with primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3) and the following cycling conditions (85 °C for 2 min, 95 °C for 95 sec, and then 36 cycles of 58 °C for 1 min, 72 °C for 80 sec and 95 °C for 70 sec, followed by a 52 °C for 1 min and 72 °C for 15 min (Harrington et al., 2011). The fragments were purified using Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) and sequenced at the Iowa State University DNA Synthesis and Sequence Facility. The primers CFMAT1-F (5' –CAGCCTCGATTGAKGGTATGA-3') and CFMAT1-R (5' –GGCATTTTTACGCTGGTTAG-3') were used to amplify and sequence about 1000 bp region of *MAT1-1-2* (Harrington et al., 2014). The primers X9978a (5' -GCTAACCTTCACGCCAATTTTGCC- 3') and CFM2-1F (5' –AGTTACAAGTGTTCCCAAAAG- 3') amplify and sequence about 1150 bp region. The thermocycler settings for amplifying the *MAT1-1-2* and *MAT1-2* 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 at 72 °C for 10 min. The amplified products were sequenced with the PCR primers.

Sequence datasets for the two genomic loci (MAT1 and MAT2) were aligned in MAFFT Online version v. 7.0 (Katoh & 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). The likelihood values were calculated and the best model of nucleotide substitution for each gene was selected according to Akaike Information Criterion

(AIC) using MrModeltest v. 2.3 (Nylander 2004). The multi-gene Bayesian Inference (BI) was conducted for the aligned MAT1 and MAT2 data set with 40 taxa including outgroup (*Ceratocystis variospora* from *Prunus*) on MrBayes v. 3.1.1 (Ronquist & Heulsenbeck, 2003) using the algorithm of Markov chain Monte Carlo (MCMC) with two sets of four chains (one cold and three heated) and the stoprule option, stopping the analysis at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 1000; the first 25 percent of trees were removed.

Microsatellite markers

We analyzed 14 loci (CfAAG8, CfAAG9, CfCAA9, CfCAA10, CfCAA15, CfCAA38, CfCAA80, CfCAT1, CfCAT1200, CfCAG5, CfCAG15, CfCAG900, CfGACA60 and CfGACA650) developed from the total genomic DNA of an isolate of *C. cacaofunesta* (Steimel et al, 2004) and used in population studies on *C. cacaofunesta* (Engelbrecht et al., 2007), *C. platani* (Engelbrecht et al., 2004; Ocasio-Morales et al., 2007) and *C. fimbriata* (Ferreira et al., 2010; Ferreira et al., 2011). Of the 16 loci used in earlier studies, two (CfCAT3K and CfCAT9X) were not utilized because their alleles could not be consistently resolved with some isolates. For each primer pair specific to the flanking regions of 14 simple sequence repeat regions, one of the primers was fluorescently labeled. PCR amplifications of all microsatellite loci were performed using a 96-well thermal cycler (PTC-100) MJ research Inc., Watertown, MA) following the earlier described conditions (Ferreira et al., 2010) . The PCR products were electrophoresed using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Band Sizes of the product were determined using marker standards and Peak Scanner 1.0 software (Applied Biosystems Inc.). Each product length (within 1 bp) was considered to be a different allele. The microsatellite regions are trinucleotide repeats, and most alleles of a given locus differed by increments of 3 bp (Table 2).

Microsatellite analyses

For population studies, each population consisted of at least four isolates from one store. Nei's gene diversity of microstatellite loci for each population was calculated with and without clone-corrected data using PopGen 1.32 software (Yeh and Boyle, 1997). Clone-corrected datasets were a subset of the population left after removing isolates that were genetically identical, that is, a genotype within a population was counted only once. The clone-corrected value for *H* would be expected to be higher than the uncorrected value if the population was dominated by one or few genotypes, as might occur if a few genotypes were spread through movement of taro corms.

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, Stoddart and Taylor`s was scaled by the expected number of genotypes for the smallest sample size being compared (Grünwald et al., 2003). For individual plantations, the expected number of genotypes in a sample of four isolates (minimum value = 1.0 and maximum value = 4.0) was estimated based on rarefaction curves using the *R* package (version 2.9.1; R Development Core Team, Vienna).

Nei's genetic distance between populations and UPGMA (unweighted pair group method with arithmetic mean) dendrograms were constructed using PopGen 1.32. Bootstrap values for branches of the population trees were calculated from 100 replicates using SEQBOOT, GENDIST, NEIGHBOR and CONSENSE in PHYLIP version 3.6 (Felsenstein 1989, 1993). Relationships among genotypes were also examined using genetic distance (Nei's) matrices, UPGMA trees, and 1000 bootstrap replications generated with PAUP* (Swofford, 1998).

RESULTS

Phylogeny

A combined data set including sequences MAT1 and MAT2 were used to generated a combined phylogenetic tree (Fig 3). The final aligned combined data set contained 40 taxa, with a total of 2173 characters, including gaps. The MAT-1 haplotype found was 5b and MAT-2 haplotype was 5a. Interestingly, these is the same mating haplotype found in isolates from mango in São Fidélis area in Rio de Janeiro and also in one isolate of *Acacia* from Paraná. Therewith, these results place the isolates from taro with strains of *C. fimbriata* from other hosts (Fig 4).

For ITS analyses, of the 47 taro isolates sequenced, we identified 7 different ITS sequences, being four of them not previously identified (Table 2). The most common haplotype found was ITS13, present in all of the populations studied, with exception of population ColRJ6. In contrast with the ITS variation observed, only one mating haplotype was found in all populations.

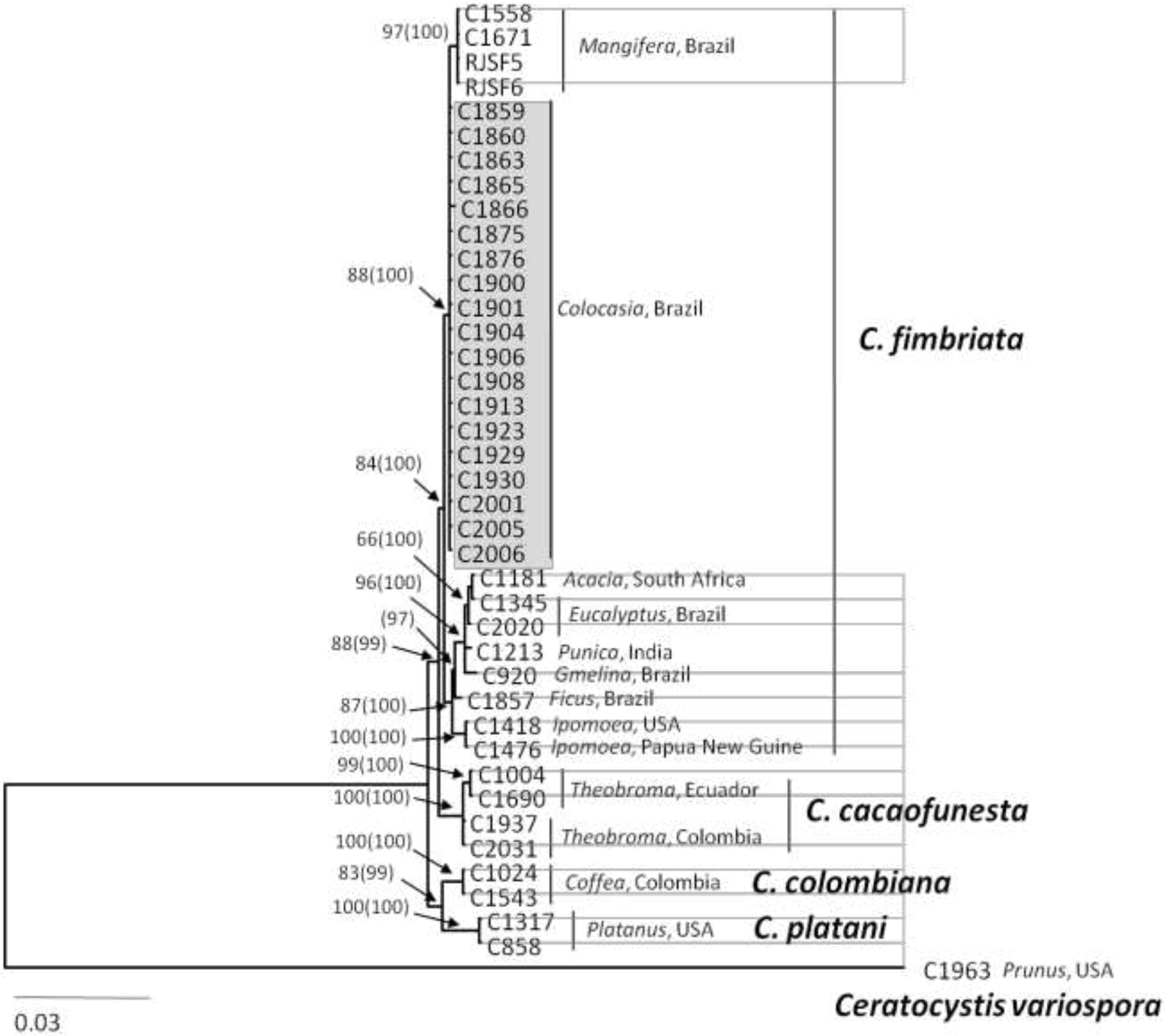

Fig 4. Phylogenetic tree of *Ceratocystis* complex resulting from a Bayesian analysis of the combined 2-genes (MAT1 and MAT2) sequences. Bootstrap values greater than 50% are indicated on appropriated the branches and Baysian support greater than 50% is indicated in brackets. The tree was rooted to *C. variospora* (C1963).

Genetic variation of haplotypes based on microsatellite alleles

Of the 14 microsatellite loci tested among the isolates of taro, all were polymorphic with exception of the locus CAT1200, CAG900 and CAG5 (Table 1). The two most polymorphic loci were CAG15 and CAA38 both with 4 alleles identified. The ranges of allele sizes for the loci were 180 to 208 (AAG8), 400 to 403 (AAG9), 191 to 209 (CAA9), 125 to 128 (CAA10), 321 to 344 (CAA15), 223 to 250 (CAA38), 302 to 314 (CAA80), 249 to 261 (CAT1), 265 to 289 (CAG15), 187 to 214 (GACA60) and 213 to 215 (GACA650). At some locations only one, two or three isolates were obtained and these were not included in the population analyses. In total, 47 isolates from taro were studied, and 13 microsatellite genotypes were found. The UPGMA tree constructed using the taro genotypes (Fig 2) showed that isolates from Bahia are grouped separated from the other isolates and they were the most distinct and grouped separate from the other taro isolates with moderate- supported (72 %) branch. Of the three genotypes found in Bahia, one was dominating. Two microsatellite genotypes (HAPLO 1 and HAPLO2) were commonly found spread in the populations of São Paulo, Rio de Janeiro, Rondonia and Distrito Federal. These two isolates were both found in infected corms of taro from São Paulo, where most of the trade area is located and where the growers sell their material for planting in other states of the country. One of the common genotypes HAPLO1 was found in six populations and the other genotype HAPLO2 in eight populations.

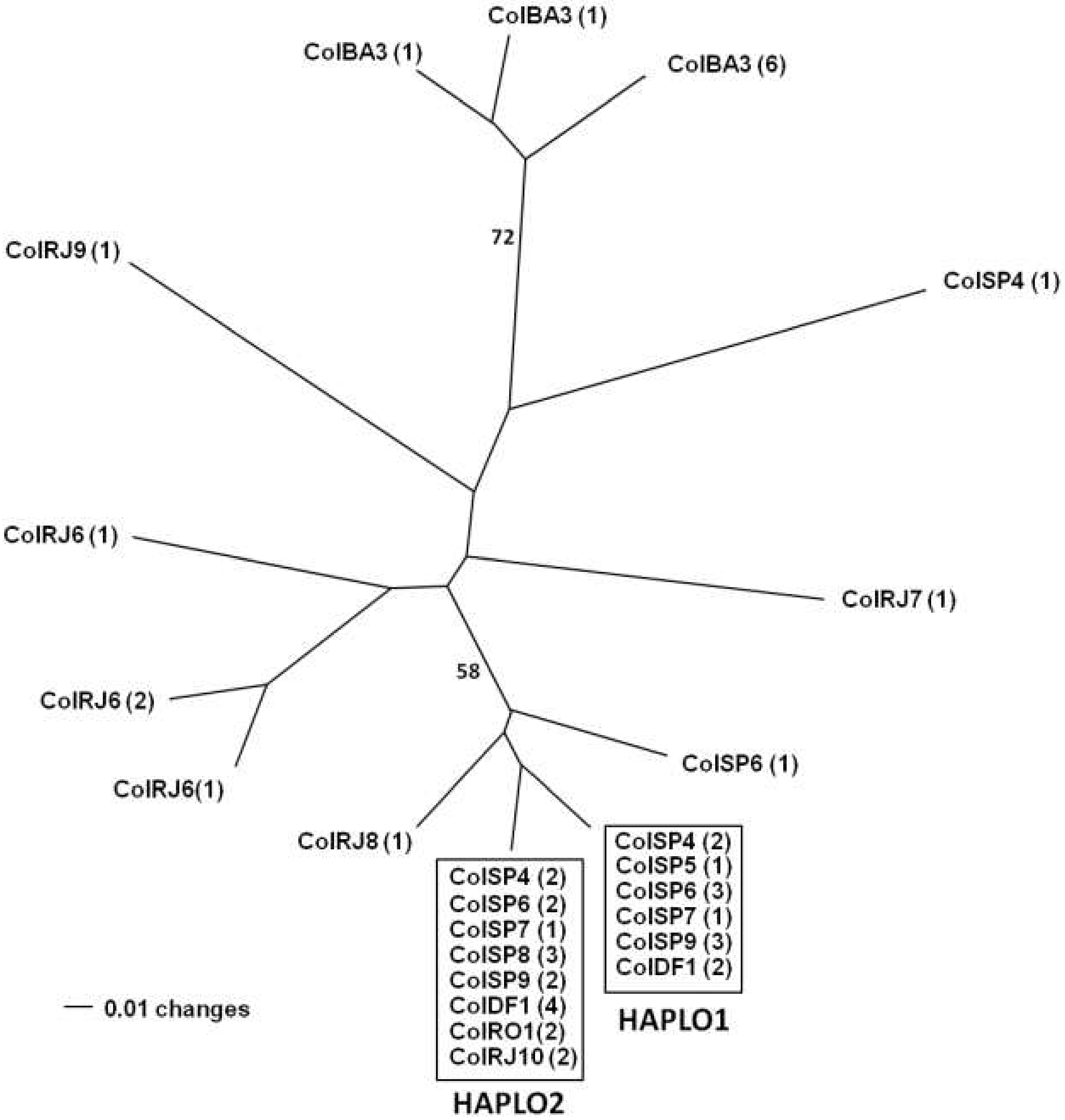

Fig 2. A UPGMA (unweighted pair group method, arithmetic mean) dendrogram of genotypes of *Ceratocystis fimbriata* from *Colocasia esculenta* based on alleles of 14 microsatellite loci. Bootstrap values are shown alongside the branches. The number of isolates with that genotype from each population is in parentheses.

Genetic relatedness of populations based on microsatellite alleles

In order to compare populations from taro of the present study with populations that appeared to represent natural populations from mango and eucalyptus, a UPGMA tree based on allele frequencies was constructed (Fig 3). A total of 17 populations were studied. Six populations from taro were compared to eight populations from mango (Ferreira et al, 2010; Oliveira et al, 2014) and three from eucalyptus (Ferreira et al, 2010). The tree showed three main groups, being one composed by taro populations, the other by mango populations from eastern Rio de Janeiro and the last one by a mixture of populations from eucalyptus and mango. Of the six populations obtained from taro, three were from São Paulo, one from Rio de Janeiro, one from Bahia and one from Distrito Federal and they were all grouped and separated from the other populations with a moderate-supported (79 %) branch. As demonstrated in earlier studies (Ferreira et al, 2010), the populations of eucalyptus and mango were grouped together and had moderate-supported (66 %) branch and the mango population from São Fidélis in Rio de Janeiro consisted of a separated group with a well-supported (90%) branch.

Nei's gene diversity (*H*) was calculated for each of the 17 populations of *C. fimbriata*, including populations from taro, mango and eucalyptus. A second calculation of gene diversity was made using only unique genotypes from each population (clone-corrected for population) (Table 2). Low levels of gene diversity were found in all taro populations suggesting that the populations sampled are apparently introduced populations. Also low levels of genotypic diversity were identified in all taro populations, with exception of the population ColRJ6 from Rio de Janeiro with value of 3.0. Even when the taro populations were clone corrected, no substantial increase were notice in gene divestity.

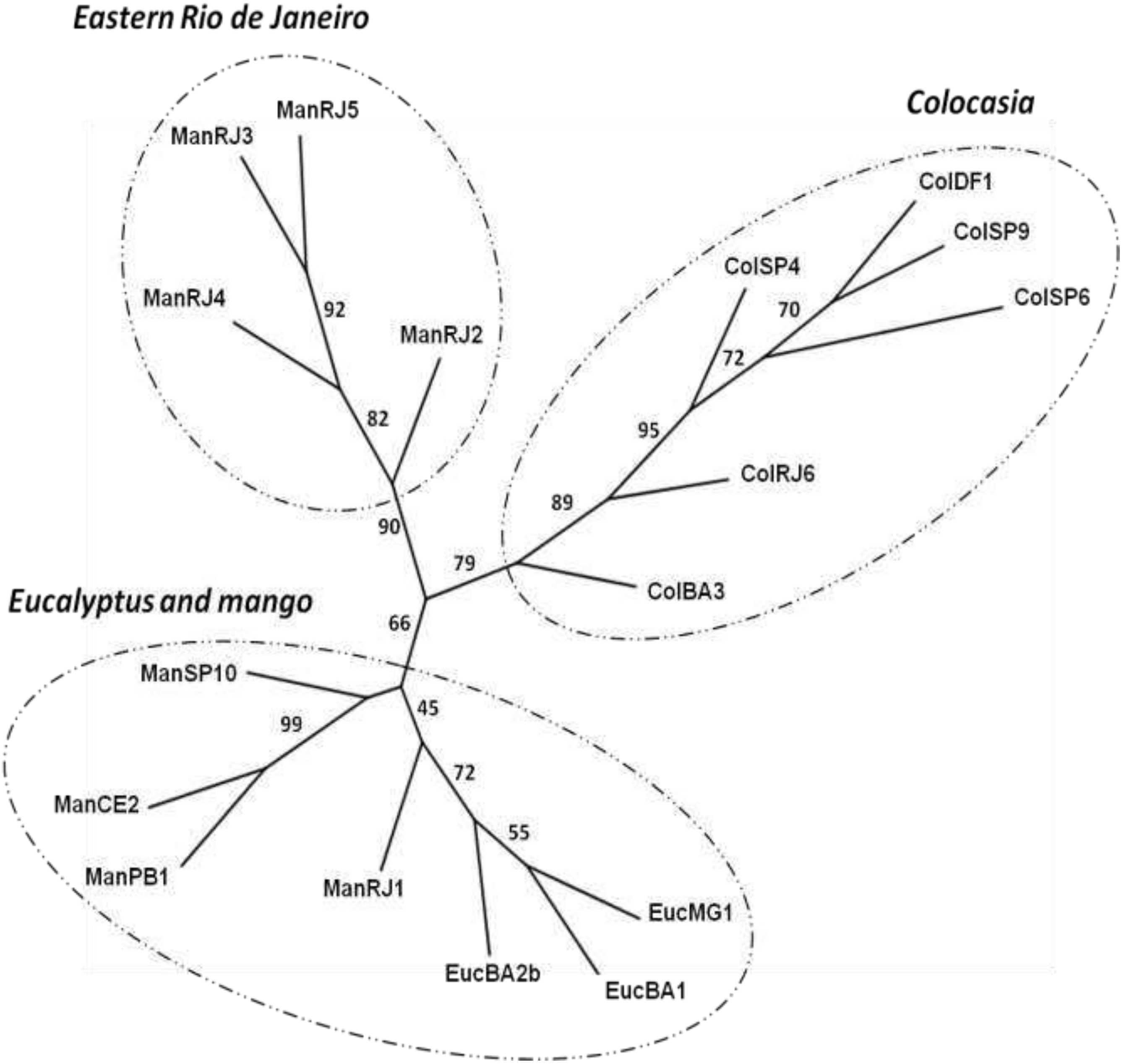

Fig 3. Dendrogram of populations of *Ceratocystis fimbriata* generated by UPGMA (unweighted pair group method, arithmetic mean) based on allele frequencies of 14 microsatellite loci. Bootstrap values are shown alongside the branches. The first three letters of each population name indicate the host (*C. esculenta*), the last two letters indicate the state of origin and if more than one population was sampled in that state, they were numbered.

Table 1. Microsatellite alleles, based on approximate band sizes as determined by PeakScanner analysis of *Ceratocystis fimbriata* populations on *Colocasia esculenta*, with umber of isolates tested in the population shown in parentheses.

Loci	**ColSP4(5)**	**ColSP5 (1)**	**ColSP6 (6)**	**ColSP7 (2)**	**ColSP8 (3)**	**ColSP9 (5)**	**ColSP10 (2)**	**ColRJ6 (4)**	**ColRJ 7(1)**	**ColRJ8 (1)**	**ColRJ9 (1)**	**ColDF1 (6)**	**ColRO1 (2)**	**ColBA3 (8)**
CAG15	265, 274,	274	274	274	274	274	274	265	289	274	265	274	274	265, 280
CAA38	223	223	223	223	223	223	223	241, 250	223	223	232	223	223	223
CAA80	314	314	314, 302	314	314	314	314	314	314	314	314	314	314	314
GACA60	187, 214	214	187, 214	187, 214	187	187, 214	187	187	187	187	187	187, 214	187	187
CAA15	321, 324	324	324	324	324	324	324	321, 324	324	324	344	324	324	324
CAT1	249, 261	261	261	261	261	261	261	261	249	261	261	261	261	261
AAG9	400, 403	400	400	400	400	400	400	400	400	400	400	400	400	403
AAG8	180	180	180	180	180	180	180	180	180	208	208	180	180	180
CAA9	209	209	209	209	209	209	209	209	209	209	209	209	209	191
CAA10	128	128	128	128	128	128	128	128	125	128	128	128	128	128
GACA650	213	213	213	213	213	213	213	213	213	213	213	213	213	215
CAT1200	373	373	373	373	373	373	373	373	373	373	373	373	373	373
CAG5	326	326	326	326	326	326	326	326	326	326	326	326	326	326
CAG900	194	194	194	194	194	194	194	194	194	194	194	194	194	194

Table 2. Genetic diversity of populations of *Ceratocystis fimbriata* on *Colocasia esculenta*, *Mangifera indica* and *Eucalyptus* spp. based on fourteen microsatellite loci and sequences of three different gene regions.

					Microsatellites alleles				
							Nei`s gene diversity (*H*)		
Group	**Population**	**State**	**City**	**No. isolates**	**No. genotypes**	**Genotypic diversity *(G)*[a]**	**All isolates**	**Clone Corrected[b]**	**ITS[d] haplotype**
Colocasia	ColRJ6	Rio de Janeiro	Rio de Janeiro	4	3	3.00	0.0893	0.0952	11b
	ColSP4	São Paulo	Tapirai	5	3	2.80	0.1257	0.1587	9, 12c, 13
	ColSP6	São Paulo	Sorocaba	6	3	2.60	0.0516	0.0635	12, 13
	ColSP9	São Paulo	Ubatuba	5	2	2.00	0.0343	0.0357	13
	ColBA3	Bahia	Porto Seguro	8	3	2.00	0.0424	0.0635	13c
	ColDF1	Distrito Federal	Brasilia	6	2	1.93	0.0317	0.0357	13,13b
Eastern Rio de Janeiro	ManRJ1	Rio de Janeiro	São Fidélis	7	4	2.97	0.0641	0.0804	14d
	ManRJ2	Rio de Janeiro	Itaocara	4	2	2.00	0.1339	0.1786	9, 14d, 14i
	ManRJ4	Rio de Janeiro	São Fidélis	19	4	1.63	0.0973	0.1875	ND[c]
	ManRJ3	Rio de Janeiro	Itaocara	5	1	1.00	0.0000	0.0000	14d
Eucalyptus and mango	EucMG1	Minas Gerais	Curvelo	18	14	3.74	0.3086	0.3258	ND
	ManCE1	Ceará	Brejo Santo	11	7	3.48	0.2680	0.3149	8a, 8b, 10, 15a
	EucBA1	Bahia	Eunapolis	26	13	3.27	0.2079	0.2697	ND
	EucBA1	Bahia	Caravelas	6	4	3.20	0.2262	0.2500	ND
	ManPB1	Paraíba	Conde	10	6	3.20	0.3814	0.3889	4a, 8a, 10c
	ManRJ5	Rio de Janeiro	Seropedica	11	7	3.14	0.2420	0.3178	3, 6, 6a, 14, 14a
	ManSP1	São Paulo	Limeira	11	4	2.58	0.2267	0.2946	4, 6, 10, 14a

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

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

[c] ND = not determined.

[d] Haplotype numbers follow the designations of Harrington et al. (2014).

DISCUSSION

Over the past few years, a large number of species of the *Ceratocystis fimbriata* complex have been described based on variation of ITS rDNA gene sequence, not considering studies on the biology of the fungus has been neglected. Variation in ITS sequences were found among the isolates of *C. fimbriata* from taro in Brazil, but those sequences are hypervariable and insufficient to phylogenetically distinguish species in the *C. fimbriata* complex (Harrington et al, 2014). In the other hand, sequences of mating type gene *MAT1-1-2* (MAT1) and *MAT1-2* (MAT2) showed no variation among all isolates obtained in the present study. Interestingly, these sequence is the same as found in isolates of *C. fimbriata* on mango from eastern Rio de Janeiro, in the São Fidélis region and in one single isolate obtained from *Acacia* in the state of Paraná (Harrington et al, 2011; Harrington et al, 2014). Besides, cultivated annona was identified as a host (Baker et al., 2003; Silveira et al., 2006) of *C. fimbriata* in the São Fidélis region, showing that the host range of this population may be different than the other populations in Brazil. Also, studies using microsatellite markers demonstrated the relatedness among populations of *C. fimbriata* obtained from mango in São Fidélis with population obtained from taro (Chapter 1). Curiously, these related strains come from the coastal Atlantic area in Brazil, which is covered by Atlantic rain forest vegetation. Those populations are genetically the most distinct populations of *C. fimbriata* s.l. found so far, but represent lineages of geographically separated populations of one single biological species (Ferreira et al, 2010).

Populations of *Ceratocystis fimbriata* from *Colocasia esculenta* had very low gene and genotypic diversity values, similar to those found in introduced populations (Engelbrecht et al., 2004; Engelbrecht et al., 2007; Harrington et al., 1998; Ferreira et al., 2010, 2011; Ocasio-Morales et al., 2007). However, those populations grouped together and formed a separate group from the other populations from other hosts, as seen on the UPGMA tree generated. The similarity of genotypes found spread in Brazil is explained by the commercialization of taro rhizomes in São Paulo, where most of the agriculture trade is located. As speculated before, populations on taro from São Paulo with limited diversity may have been introduced from some other region on corms (Ferreira et al, 2010). Two haplotypes were found spread in many populations of the fungus, being one of them (HAPLO2) found in all states where the disease is reported (Harrington et al, 2005), with exception of Bahia. The most distinct population found was from Bahia, but low levels of gene and genotypic diversity indicate that these population is introduced.

The collections of isolates were made by isolating the fungus from infected corms, selecting aggressive strains to taro corms. It is likely that a genetic bottleneck may have been created. These artificial populations are difficult for interpretation, because even finding the right location of native populations of the fungus, we are not counting the genotypes present in the soil or seedlings of alternative hosts.

Dissemination of isolates of *C. fimbriata* complex in plant propagative material have been demonstrated before (Harrington, 2000; Engelbrecht & Harrington, 2005; Johnson et al., 2005; Thorpe et al., 2005; Engelbrecht et al., 2004, 2007; Ocasio-Morales et al., 2007; Ferreira et al, 2011) and introductions of the pathogen into areas free of the disease may cause a important economic and ecological issue. The movement of haplotypes of *C. fimbriata* in corms of taro and sweet potato in Brazil and around the world is the proof that a better sanitization process is needed.

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CHAPTER 3

A new wilt disease of *Tilia americana* caused by *Ceratocystis tiliae* sp. nov.

A new wilt disease of *Tilia americana* caused by *Ceratocystis tiliae* sp. nov.

INTRODUCTION

Tilia americana is a large, rapid-growing tree of eastern and central hardwood woodlands. It is commonly called american basswood and it is an important timber tree, especially in the Great Lakes States. The soft, light wood works exceptionally well and is valued for hand carving, and the inner bark can be used as a source of fiber. The tree is also well known as a honey-tree, and the seeds and twigs are eaten by wildlife. In the eastern states it is called American linden, where is commonly planted as a shade tree in urban areas. Only three species of *Tilia* are now recognized in the United States, *T. americana* L., *T caroliniana* Mill., and *T. heterophylla* Vent. (Jones, 1968).

Members of the *Ceratocystis fimbriata* complex are wound or soilborne pathogens on root crops or trees and may cause wilting and cankering on some woody hosts (Kile et al, 1996; Johnson et al, 2005). Among the hosts affected by *Ceratocystis* are *Prunus* spp (almonds and other stone fruits), *Populus* spp (aspen and other poplars), *Platanus* spp (sycamore or plane tree), *Theobroma cacao* (cacao), *Coffea arabica* (coffee), *Hevea brasiliensis* (rubber tree), *Eucalyptus* spp (eucalyptus), *Mangifera indica* (mango), *Tectona grandis* (teak), *Acacia* spp (acacia), *Gmelina robusta* (gmelina), *Ficus carica* (edible fig), *Citrus* spp (citrus) and other woody plants (Borja et al, 1995; Engelbrecht & Harrington, 2005; Ferreira et al, 1999; Firmino et al, 2012; Johnson et al, 2005; Marin et al, 2003; Ribeiro, 1982; Ribeiro et al, 1988; Silveira et al, 1994; Valarini & Tokeshi, 1980; Viégas, 1960;). Also, the pathogen can causes the rot of storage roots or corms of *Colocasia esculenta* (taro or inhame) (Harrington et al, 2005) and *Ipomoea patatas* (sweet potato) (Halsted, 1890).

Johnson et al (2005) studied members of the complex on wounds of trees in Iowa and elsewhere and revised the taxonomy of the North Amercan Clade (NAC) of the *C. fimbriata* complex, resurrecting the name *C. variospora* and describing three new: *C. populicola, C. caryae*, and *C. smalleyi*. There were several lineages within *C. varispora* that were considered to be potential new species, including one isolated from wounds on Amercian basswood. Isolates from wounds on basswood in Iowa were not interfertile with other strains of *C. variospora* and had ITS rDNA sequences that differed slightly from other species in the NAC.

Isolations from other wounded basswood trees in Iowa and Nebraska recovered isolates of similar morphology and ITS rDNA sequences, indicating that the basswood fungus was a unique lineage within the NAC that may be specialized to colonize wounds of this host. The aim of this study was to further determine if basswood strains are phylogenetically and pathologically distinct from other members of the NAC.

MATERIALS AND METHODS

Collection of isolates

Isolates were collected from four *Tilia americana* trees at three locations. The tree at the Ogden, Iowa site was artificially wounded, and isolates of the fungus (C1954 and C1959) were recovered from the edge of the wounded tissue (Johnson et al., 2005). A small diameter tree in Ames, Iowa was damaged from a larger fallen tree, and the fungus was recovred (C2131) from the wounded area. A row of large trees showing substantial branch dieback was examined in Omaha, Nebraska. The trees had suffered storm damage several years earlier, and many of the major branches had sapwood discoloration coming down from the dead branches. Isolate attempts from two of the trees yielded six isolates (C2525 and C2623 from tree 1 and C2620, C2621, C2622, and C2624 from tree 2). In most cases, the fungus was baited from the wounded area of discolore sapwood tissue using the carrot sandwich technique (Johnson et al., 2005). Ascospores masses from perithecia formed on the carrot discs were transferred to agar media for purification and then storage. Only one isolate was used in genetic analysis.

DNA extraction and gene sequencing

Isolates were grown on MYEA (2% malt extract, 0.2% yeast extract, and 2% agar) for 10 days at room temperature (approximately 23 °C), and DNA was extracted using PrepMan™ Ultra (Applied Biosystems, Foster 4 City, CA).

The LSU rDNA gene region was amplified and sequenced with the primers LROR (5' - ACCCGCTGAACTTAAGC - 3'), LR5 (5' - TCCTGAGGGAAACTTCG - 3') and LR3 (5' - CCGTGTTTCAAGACGGG - 3') and thermocycler settings included: 85 °C for 120 sec, 95 °C for

95 sec, 36 cycles of 58 °C for 60 sec, 72 °C for 80 sec, and 95 °C for 70 sec, followed by 52 °C for 60 sec and 72 °C for 15 min. The TEF-1α were generated using PCR folowed by direct DNA sequencing of the PCR products with primers EFCF1.5 (5' - GCYGAGCTCGGTAAGGGYTC-3'), EFCF2 (5' -TGCTCAACGGGTCTGGCCAT - 3'), EFCF3 (5' - ATGGCCAGACCCGTGAGCA - 3') and EFCF6 (5' -CATGTCACGGACGGCGAAAC - 3'). Thermocycler settings for amplifying the TEF-1α region included an initial denaturation at 85 °C for 120 sec folowed by 94 °C for 95 sec, with 36 cycles of 60 °C for 60 sec, 72 °C for 90 sec, and 94 °C for 35 sec, folowed by final extension of 60 °C for 60 sec and 72 °C for 15 min. The Cerato-platanin gene region was sequenced and amplified with the primers CP-2F (5' - TCCTACCCATGATTGCCAGC - 3') and CP-1R (5' - ACAACAGCGTACTGCCTTCAT - 3') and thermocycler settings adjusted as in the LSU.

Phylogenetic analyses

Isolate C1476 (ICMP 8579) of *C. fimbriata* from sweet potato in Papua New Guinea was used as the outgroup taxon for TEF-1α and Cerato-platanin analyses. Isolate C2239 (CBS 118128) of *C. piriliforms* from eucalyptus in Australia was used as outgroup taxon for LSU rDNA analysis. The sequences were manually aligned with some ambiguity between the ingroup taxa and the outgroup in TEF-1α introns, so the intron regions were eliminated from the analysis. The aligned sequences of the three gene regions were analyzed for maximum parsimony (MP) separately using PAUP 4.0b10 (Swofford, 1998). 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 analyses also were conducted in PAUP with 1000 replications. Bayesian posterior probability was determined with Mr. Bayes 3.2.1 (Ronquist and Huelsenbeck 2003), in which gaps are treated as missing data. Estimates were calculated by majority rule consensus of the trees after burn-in.

Pathogenecity tests

Host specialization was tested in two cross inoculation experiments with two hosts, *T. americana* and *Quercus macrocarpa* (bur oak) and three isolates (C1954, C2131, C2622) from *T. americana*) and three isolates of *C. variospora* (C1837, C1846, C1964) from *Q. rubra*, *Q. robor* *and Q. macrocarpa*, respectively in Iowa. Six inoculation treatments, consisting of three isolates from each of the two hosts and a control, were applied to each host and the experiment was repeated twice in a completely random design with three replicates (seedlings) per treatment. For both experiments, single-ascospore strain of each isolate was transferred onto MYEA and grown at room temperature (≈ 23 °C) for 10 days. Twelve-month-old bareroot seedlings of basswood and bur oak were grown in 6-inch pots in greenhouse soil amended with Osmocote slow-release fertilizer in a greenhouse and inoculated 6 wk after bud break. The plants were wounded by making an 3-mm-deep, downward-slanting cut from the outer bark into the wood with a sterile scalpel at 3 cm above the groundline. Discs of mycelium were then placed into the wound and the inoculation site was wrapped with Parafilm. After 60 days or at the time of death, each stem was sectioned vertically and the length of xylem discoloration above and below the point of inoculation was measured.

Morphological characterization

Representative isolates were grown on MYEA (2% malt extract, 0.2% yeast extract, and 2% agar) for 10 days at room temperature (approximately 23 C). Measurements of endoconidia and endoconidiophores were made after 4–7 d growth, while perithecia and ascospores were measured after 7– 10 d. Aleurioconidia were measured from cultures that had grown 7–20 d. Material was mounted in lactophenol cotton blue and observed with Nomarsky interference microscopy. Perithecia were measured with an eyepiece reticule at 400X magnification. For most structures 10 observations were recorded per isolate; when measuring endoconidia, however, 20 conidia were measured per isolate. Some structures were rare or hard to locate in a few isolates, and fewer observations were made. Colony pigmentation was compared to the color chart of Rayner (1970).

The growth rate of the isolates from *T. americana* (C1954, C1954, C2131 and C2622) were compared to isolates of *C. variospora* from *Quercus* spp. (C1837, C1846 and C1964). Three replicate plates of each isolate were grown on MYEA at 25 °C. The diameters of the colonies were assessed after 7 days.

RESULTS

Phylogenetic analyses

An alignment of 601 characters of LSU rDNA showed little variation among the 19 isolates analyzed, with 560 characters constant and only 9 parsimony informative characters. Parsimony analysis resulted in one most parsimonious trees of 29 steps (Fig. 1) with homoplasy index (HI) = 0.1379, consistency index (CI) = 0.8621, rescaled consistency (RC) = 0.7871, retention index (RI) = 0.9130. The three isolates from *T. americana* were not distinguished from other isolates of *C. variospora*.

The aligned sequences of 36 isolates TEF-1α consisted of 1410 characters, 1304 of which were constant and 67 parsimony informative. Parsimony analysis resulted in 60 most parsimonious (MP) trees of 146 steps with homoplasy index (HI) = 0.1918, consistency index (CI) = 0.8082, rescaled consistency (RC) = 0.7726 and retention index (RI) = 0.9560. Three well-supported lineages within the *C. variospora* group were evident, including a grouping of three isolates from *T. americana* (Fig. 2).

The dataset with sequences of the Cerato-platanin gene for 46 isolates resulted in an alignment of 502 characters, 324 of which were constant and 94 were parsimony informative. Parsimony analysis resulted in three most parsimonious trees of 222 steps with homoplasy index (HI) = 0.1081, consistency index (CI) = 0.8919, rescaled consistency (RC) = 0.8726 and retention index (RI) = 0.9784. The *C. variospora* group showed substantial sequence variation, and there was support for the grouping of four isolates from *T. americana* (Fig 3).

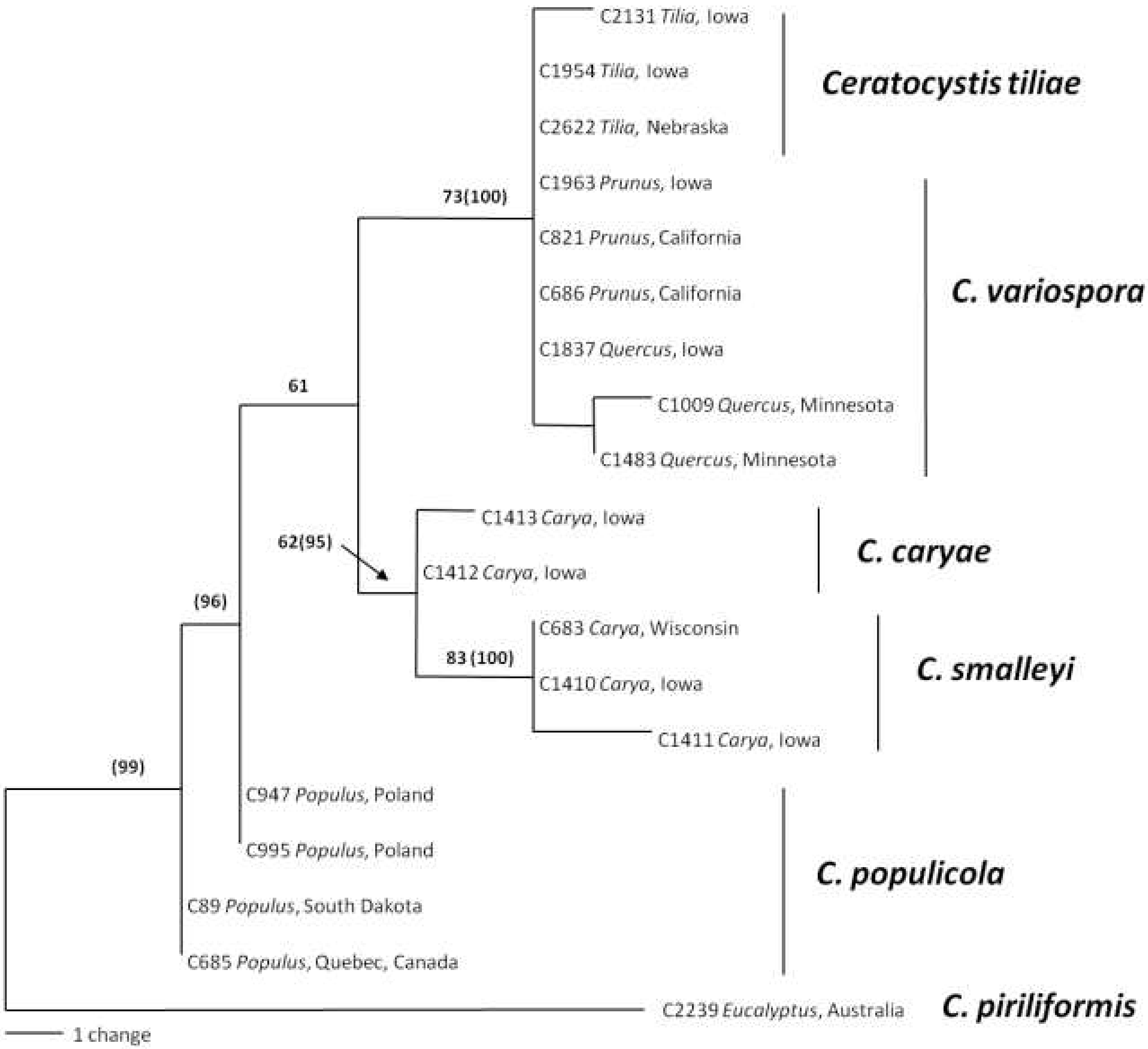

Fig 1. A single most parsimonious tree based on LSU gene sequences of *Ceratocystis fimbriata* from the North American clade. Bootstrap values greater than 50% are indicated above the branches while Baysian support greater than 50% is indicated in brackets. *Ceratocystis piriliforms* from the Asian clade was used as an outgroup taxon. Isolate numbers, host genus and state (USA) or country of origin of the isolates are given on the tree.

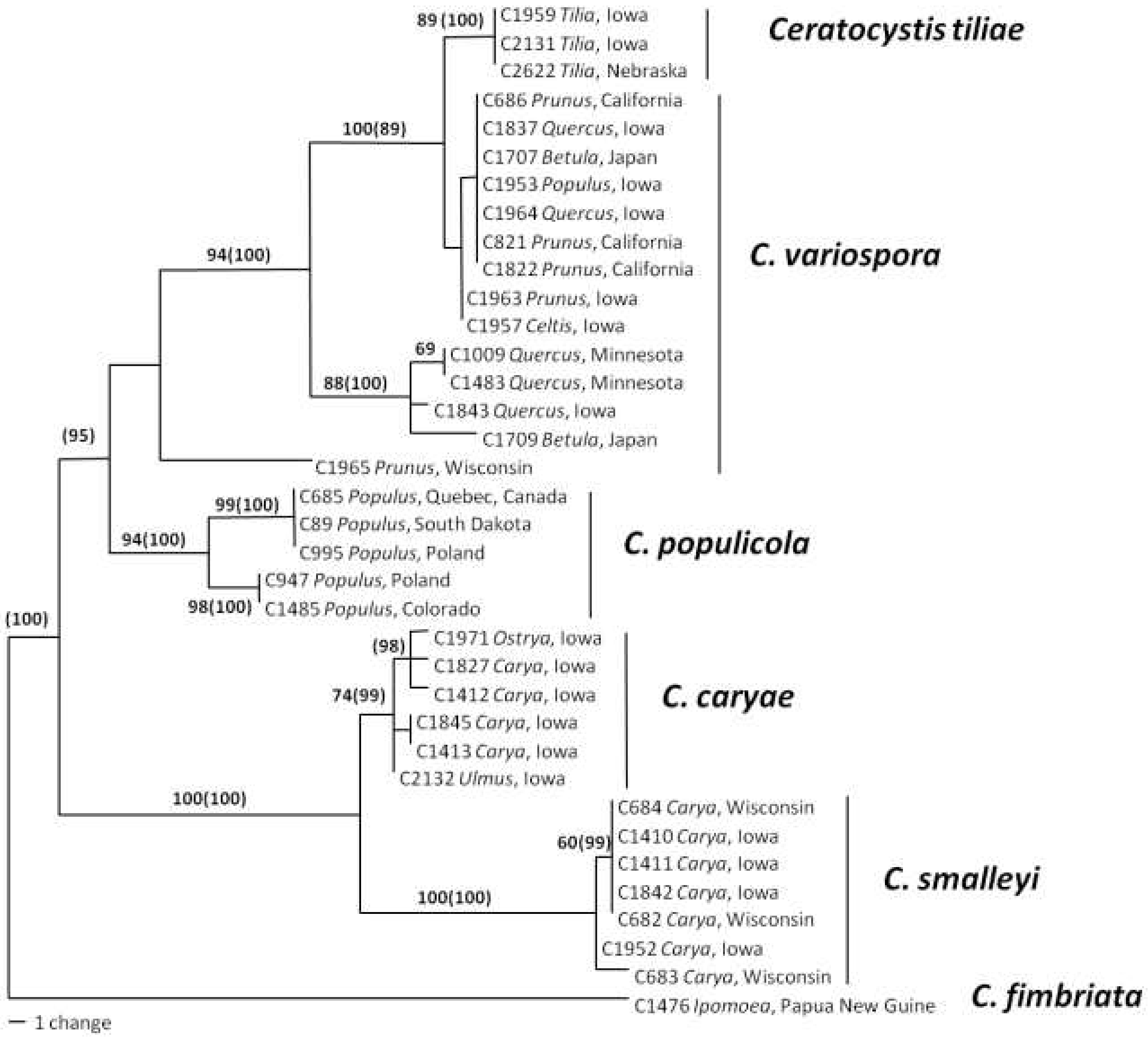

Fig 2. One of 60 most parsimonious trees based on TEF-1α gene sequences of *Ceratocystis fimbriata* from the North American clade. Bootstrap values greater than 50% are indicated above the branches while Baysian support greater than 50% is indicated in brackets. *Ceratocystis fimbriata* from the Latin American clade was used as an outgroup taxon. Isolate numbers, host genus and state (USA) or country of origin of the isolates are given on the tree.

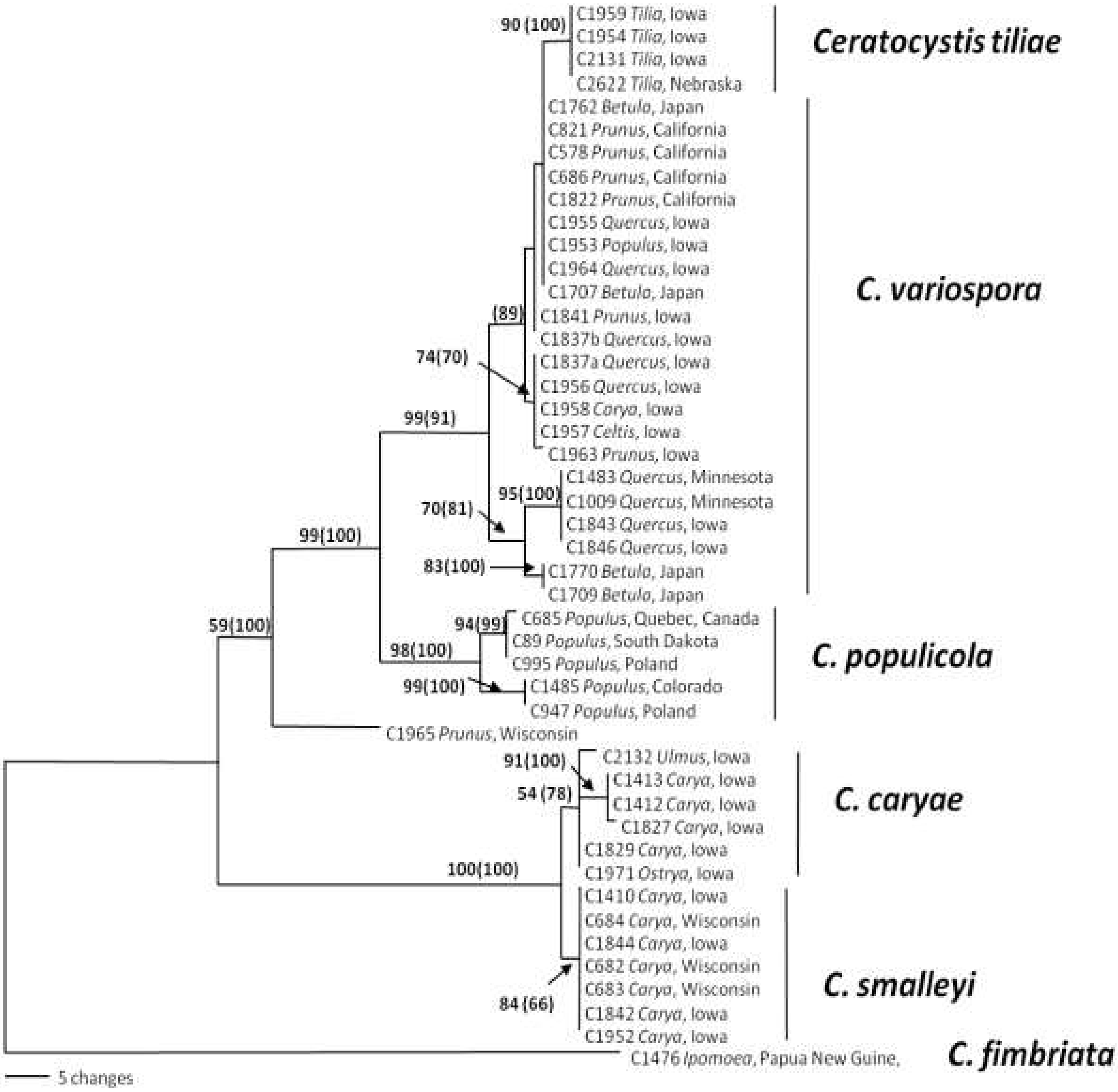

Fig 3. One of 3 most parsimonious trees based on Cerato-platanin gene sequences of *Ceratocystis fimbriata* from the North American clade. Bootstrap values greater than 50% are indicated above the branches while Baysian support greater than 50% is indicated in brackets. *Ceratocystis fimbriata* from the Latin American clade was used as an outgroup taxon. Isolate numbers, host genus and state (USA) or country of origin of the isolates are given on the tree.

Pathogenecity tests

All plants showed xylem discoloration by the end of the two experiments. In both experiments, the isolates from *T. americana* and *Quercus* spp. were able to cause greater xylem discoloration in their respective hosts than the other host (*Q. macrocarpa* and *T. americana*) (Fig 4, 5). The three *T. americana* seedlings inoculated with isolate C1954 died in the first experiment, and two of the three *T. americana* seedlings inoculated with isolate C2131 died in the second experiment.

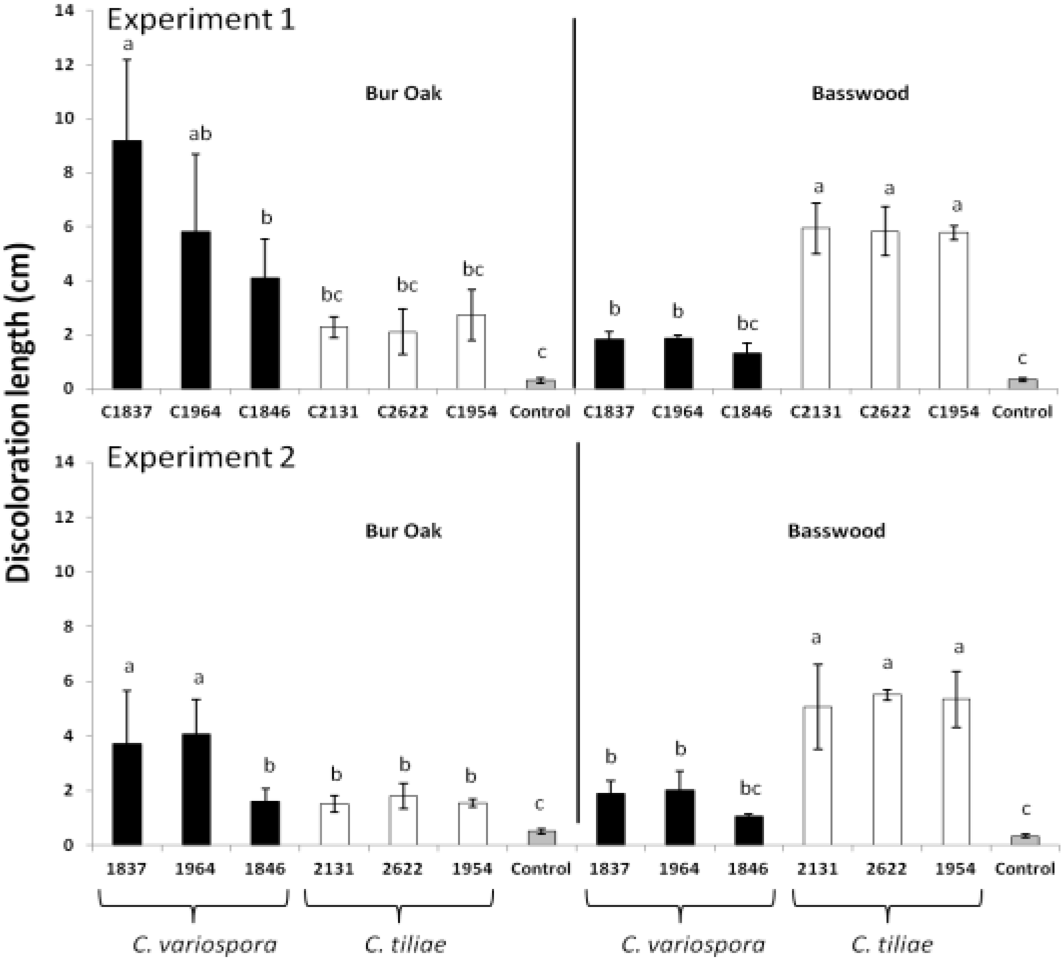

Fig 4. Average length of xylem discoloration (cm) in *Quercus macrocarpa* (bur oak) and *Tilia americana* (basswood) cross inoculated at a greenhouse with isolates of *Ceratocystis variospora* and *C. tiliae* obtained from *Quercus* spp. and *T. americana*. Bars on the graph indicated with the same letter are not significantly different from each other using Tukey`s test ($P < 0.05$).

The ANOVA showed significant variation in the length of xylem discoloration among the six isolates and between the two inoculated host species, and there was significant isolate x host interaction in both experiments ($F = 13.38$, $P < 0.0001$ and $F = 14.59$, $P < 0.0001$, respectively), indicating host specialization. There was significant difference between the two experiments, so they could not be combined because variances of the two experiments were not homogeneous (Fig. 4). The controls remained asymptomatic and had only a trace of xylem discoloration at the inoculation point, After evaluation of xylem discoloration, the inoculated pathogen was recovered from each of the seedlings, but no fungus was recovered from the controls.

Morphological characterization

The isolates obtained from *T. americana* can be distinguished from *C. variospora* isolates from Quercus spp. in the pigmentation of their mycelia and growth rate (Fig 5). *Ceratocystis variopora* isolates from *Quercus* spp. are more darkly pigmented, tending to dark brown, while isolates from *T. americana* start out whitish and then turning to pale brown grey. The growth rate of the isolates from *T. americana* (C1954, C1954, C2131 and C2622) was slower than isolates of *C. variospora* (C1837, C1846 and C1964), exhibiting a smaller diameter after seven days (Fig. 5).

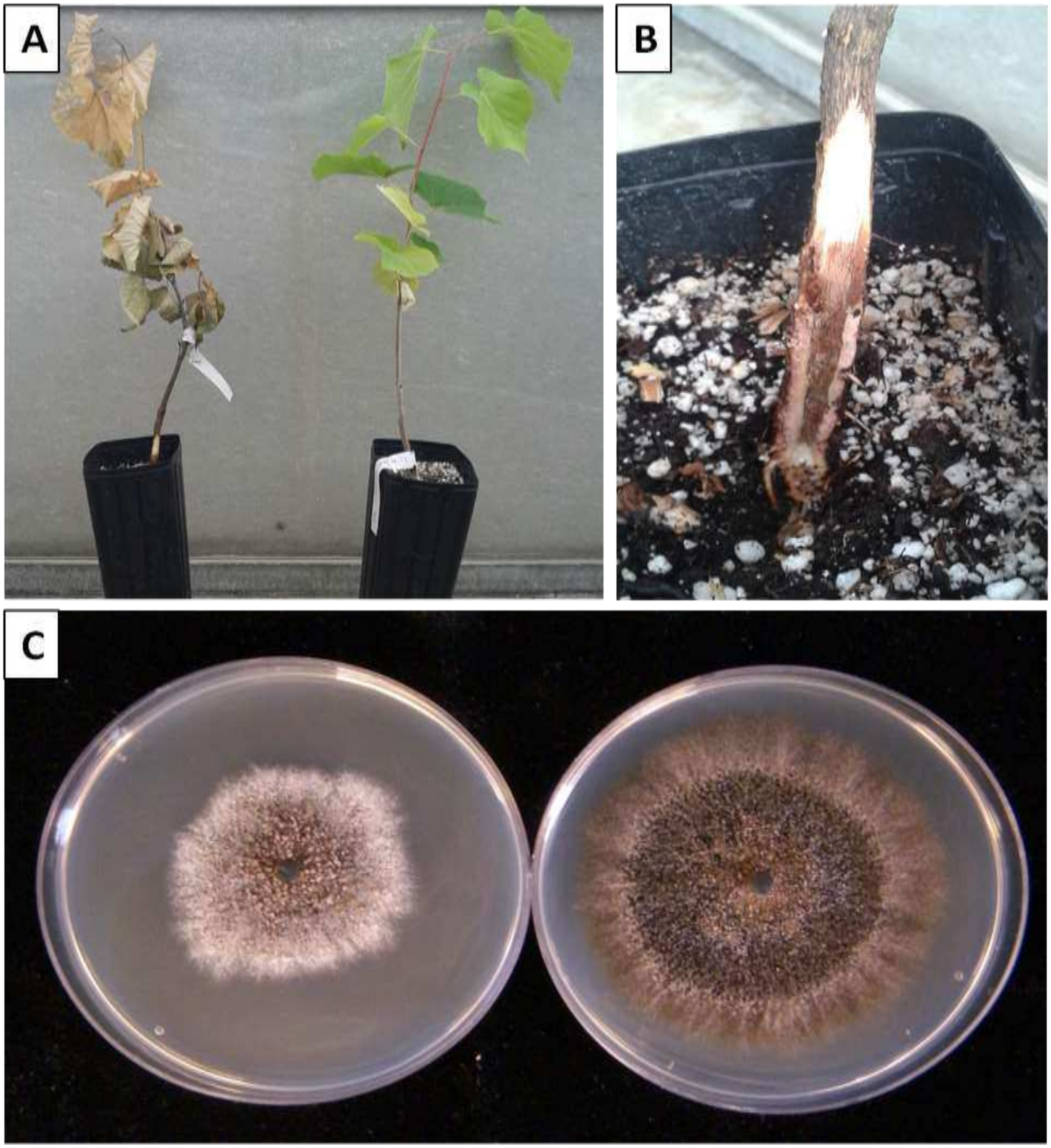

Fig 5. Pathogenicity and colony characteristics of isolates of *Ceratocystis tiliae*. A – Wilting of a *Tilia americana* seedling (left) inoculated with a *C. tiliae* isolate (C1954 = CBS 137354) and a control seedling (right). B – Xylem discoloration caused by *C. tiliae* in *T. americana*. C – Difference in the extent of radial growth and pigmentation between *C. tiliae* isolate C1954 (left) and *C. variospora* isolate C1837 (right) after 10 days on malt yeast extract agar.

Taxonomy

Ceratocystis tiliae L.S.S. Oliveira, D. McNew & T.C. Harrington. **sp. nov. (**Fig. 6a-f)

MycoBank:

Cultures on malt yeast-extract agar white, turning to pale brown gray after 7-10 days at 25 C, undersurface of agar turning dark, especially under areas where perithecia are produced; diameter 35 mm at 7 days; odor sweet, with banana scent. Perithecia with bases superficial to partially immersed, bases black or rarely dark brown, globose, 175-350 µm diam, unornamented or with undifferentiated hyphae; possessing a collar at the base of the neck 50-100 µm wide; necks black or rarely dark brown, slender, 425-915 µm long, 25-45 µm diam at base and 15-35 µm at the tip; ostiolar hyphae hyaline, 40-90 µm long, tapering to a blunt tip; asci not seen; ascospores 5.0-6.0 x 4.0-4.5 µm with outer cell wall forming a hat-shaped brim. Endoconidiophores of two types; one flask-shape, hyaline to pale brown, septate 80-160 (330) µm long, conidiogenous cell 25-50 (120) µm long, width 4.0-5.5 µm at base and 3.5-4.5 µm at the mouth; producing chains of hyaline endoconidia 15-40 x 3.0-5.5 µm; other endoconidiophores rarely found, shorter, 50-90 µm long, not tapering, often flared at mouth, conidiogenous cell 25-50 µm long, width 4.5-5.5 µm at base and 4.0-6.0 µm at the mouth; producing chains of doliform endoconidia, hyaline 6.5-9.0 x 4.5-6.0 µm; aleuroconidia produced singly or in chains, pale brown to dark brown, ovoid or obpyriform, smooth, 7.5-12.5 x 8.0-11.5 µm.

HOLOTYPE: USA. IOWA. Ogden, from *Tilia americana*, June 2002, J. A. Johnson, BPI: XXXXX, dried culture from isolate C1954 (=CBS 137354).

Etimology: *tiliae*, Latin = on *Tilia*

Cultures examined: USA. IOWA. Ogden, from *Tilia americana*, June 2002, J. A. Johnson, BPI: XXXXX, dried culture from isolate C1954 (=CBS 137354). Ogden, from *Tilia americana*, June 2002, J. A. Johnson, isolate C1959. Ames, from *Tilia americana*, August 2004, T. C. Harrington, isolate C2131 (=CBS 137355). Omaha, from *Tilia americana*, September 2009, T. C. Harrington, isolate C2622 (=CBS 137356).

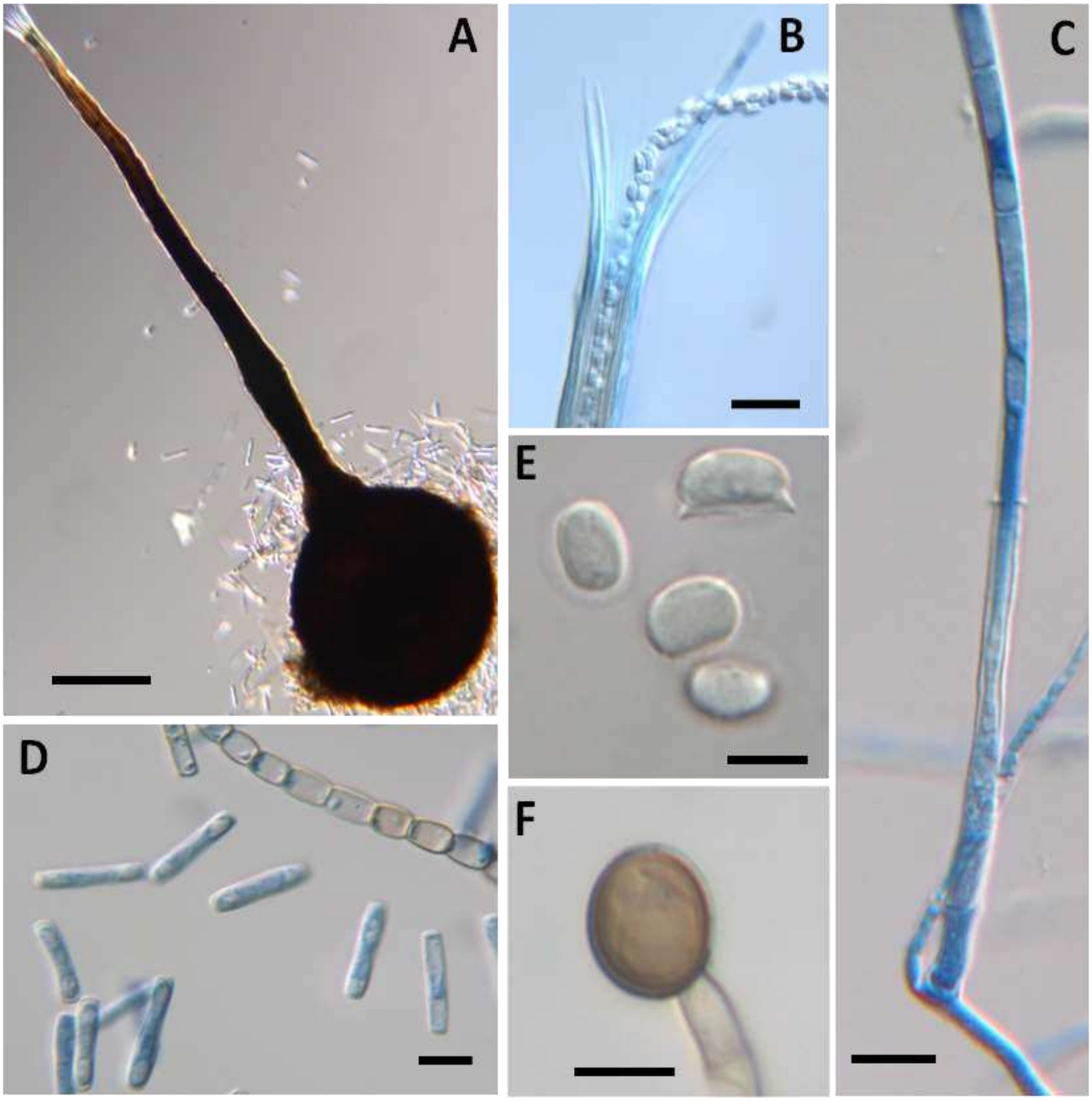

Fig 6. Morphological characteristics of *Ceratocystis tiliae*. A – Perithecium. B – Ostiolar hyphae and emerging ascospores. C – Flask-shape endoconidiophore producing cylindrical endoconidium. D – Cylindrical endoconidia and doliform endoconidia in a chain. E – Ascospores. F – Aleuroconidium. All features from the isolate (CBS 137354) from the hologype , except Fig. A, which was from isolate CBS 137355. Bars: A = 100 µm; B = 20 µm; C, D, F = 10 µm; E = 5 µm.

DISCUSSION

Analyses of TEF-1α and Cerato-platanin showed that isolates of *C. tiliae* are lineages distinct from the others present in the North American clade and they are connected by a moderate to well-supported branches for both genes. Analyses of LSU rDNA gene region placed some of the isolates of *C. tiliae* with isolates of *C. variopora*, its closest relative species. Despite having not been possible to combine the three gene sequence dataset, the grouping of the lineages were clear for the three phylogenetic trees, showing that isolates from *T. americana* can be easily distinguish from the others. Some of the isolates used in the present work were studied before (Johnson et al, 2005), and they behave typically as the cherry lineage in ITS sequence, allozymes and morphology. However, the intesterility studies using pairings between mutant MAT-2 (male tester) strains that had lost the ability to self and MAT-1 (female testers) strains that are self-sterile, showed that isolates from *T. americana* were able to mate only with themselves (Johnson et al, 2005), supporting *C. tiliae* as a distinct biological species.

Ceratocystis species are well known for their association with insects and they require the host plant to be wounded to cause infections (Kile, 1993). *C. tiliae* isolates were obtained from wounded trees of *T. americana* in the states of Iowa and Nebraska, and pathogenicity experiments were performed to test whether isolates obtained from *T. americana* were host specific and could be distinguish from the other lineages based on host range. It is interesting that isolates of *C. tiliae* behaved specific to *T. americana* in cross-inoculation experiments with its closest relative species *C. variopora* from oak. The tested isolates showed to be aggressive strains that were isolated from wounded trees, demonstrating that one of the strategies for controlling the disease could be done by reducing the pruning activity in the periods that the disease is more severe.

The ITS rDNA analysis presented by Johnson et al. (2005) suggested that there were likely cryptic species within *C. variospora*, but morphological comparisons and inoculation studies were unable to characterize species based on a phylogenetic species concept (Harrington and Rizzo 1999). However, the Tilia pathogen is characterized by intersterility with *C. variospora* isolates from other hosts (Johnson et al., 2005), pathogenicity to *T. americana*, growth rate, and pigmentation of mycelia. Both the TEF-1α and Ceratoplatanin sequence analyses show that isolates from *T. americana* are a separate lineage. Recognition of *T. americana* pathogen as *C.*

tiliae leaves *C. variospora* as a paraphyletic taxon, and other cryptic species are likely to be found in this group.

Although isolates of *C. tiliae* were aggressive in inoculation tests, and the fungus has been recovered from the xylem below branches of *T. americana* with dieback, it is not clear *C. tiliae* kills *T. americana* in nature. It is clear, however, that *C. tiliae* is able to aggressively colonize the xylem of wounded *T. americana*.

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