DENISE CRISTINA DE OLIVEIRA FRANCO VALDETARO

GENETIC VARIABILITY OF *Ceratocystis fimbriata* ON *Tectona grandis* AND *Carapa guianensis* IN BRAZIL

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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"Tudo vale a pena quando a alma não é pequena."

Fernando Pessoa.

"Não se deixe levar pelo desânimo. Siga sempre por caminhos com otimismo e esperança de que tudo vale a pena. Tudo podemos e tudo será sempre uma flor em nossa vida através da nossa existência."

Gotas de otimismo – Chiquinho da Floresta

Aos meus pais Ciloni e Maria

Ao meu marido Erlon e minha filha Rafaela

Ao meu sogro Rafael Smiderle Valdetaro (*In memorian*)

DEDICO

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RESUMO

VALDETARO, Denise Cristina de Oliveira Franco, D.Sc., Universidade Federal de Viçosa, outubro de 2016**. Variabilidade genética de *Ceratocystis fimbriata* em *Tectona grandis* e *Carapa guianensis* no Brazil.** Orientador: Acelino Couto Alfenas. Coorientadores: Thomas Charles Harrington e Leonardo Sarno Soares Oliveira.

Recentemente, *Ceratocystis fimbriata* Ellis & Halsted foi relatado em *Tectona grandis* L. f (teca) e *Carapa guianensis* Aubl. **(**andiroba) no Brasil. Em teca, o fungo provoca descoloração do lenho e morte das plantas afetadas, o que compromete a produção de madeira nobre para serraria. Em andiroba, infecta e mata sementes e mudas, o que pode afetar negativamente sua regeneração natural e consequentemente sua biodiversidade. A identificação de *C. fimbriata* nessas hospedeiras foi baseada apenas em características morfológicas e em sequências gênicas da região ITS. Além disso, não foram analisadas suas relações filogenéticas com outros isolados e espécies de *Ceratocystis* e nem a variabilidade genética e fisiológica de suas populações. Tais estudos são fundamentais para embasar as estratégias de controle da doença. Deste modo, o presente estudo teve como objetivos: 1) Avaliar as relações entre os isolados do fungo de ambas as espécies hospedeiras com isolados de outros hospedeiros do Clado da América Latina por meio dos genes "mating type" e da região ITS rDNA. 2) Avaliar a interesterilidade por meio de cruzamentos entre isolados obtidos de diferentes espécies hospedeiras. 3). Determinar a variabilidade genética nas populações de *C. fimbriata*, por meio de marcadores microssatélites e sua especialização fisiológica por meio de inoculações cruzadas. Dentre 24 isolados do fungo, obtidos de teca de quatro localidades do Mato Grosso e 73 isolados de *C. fimbriata* de andiroba do Acre, Roraima e Amazonas, foram identificados novos haplótipos de ITS e mating type, distribuídos entre outros isolados dentro do Clado da América Latina (CAL), que residem em um único grupo juntamente com isolados de

batata doce, a partir do qual a espécie foi originalmente descrita. Os isolados oriundos de teca e andiroba foram inter férteis com outras linhagens de *C. fimbriata*¸ mostrando que pertencem a uma única espécie biológica de *C. fimbriata*. As análises de microssatélites da população do fungo em teca mostraram uma baixa variabilidade genética. Nas inoculações, apesar das mudas de teca apresentarem lesões relativamente pequenas, dois isolados de teca diferiram da testemunha. Os isolados de eucalipto não foram patogênicos em teca. As populações de *C. fimbriata* de andiroba dos três estados (AC, RR e AM) apresentaram 14 genótipos e variabilidade genética intermediária em relação a outras populações nativas do fungo. Inoculações cruzadas do fungo em andiroba, teca, eucalipto e mangueira, sugerem que os isolados de *C. fimbriata* destas espécies são hospedeiro-específicos e que existe variabilidade em agressividade entre os isolados do fungo de andiroba.

ABSTRACT

VALDETARO, Denise Cristina de Oliveira Franco, D.Sc., Universidade Federal de Viçosa, October, 2016**. Genetic variability of *Ceratocystis fimbriata* on *Tectona grandis* and *Carapa guianensis* in Brazil.** Adviser: Acelino Couto Alfenas. Co-advisers: Thomas Charles Harrington and Leonardo Sarno Soares Oliveira.

Recently, *Ceratocystis fimbriata* Ellis & Halsted was reported in *Tectona grandis* L. f (teak) and *Carapa guianensis* Aubl. (andiroba) in Brazil. In teak, the fungus causes death and wood discoloration, which affects the production of high valuable timber. In andiroba, the fungus infects and kills seeds and seedlings, which may affect negatively its natural regeneration and, probably, its biodiversity. The identification of *C. fimbriata* on these hosts was based only on morphological characteristics and analysis of ITS rDNA sequences. Furthermore, the genetic and physiological variability of *C. fimbriata* populations as well as their phylogenetic relationships with other isolates and species of *Ceratocystis* were not analyzed . Such studies are essential to support disease management strategies. Therefore, this study aimed to: 1) Assess the relationship among isolates of the fungus from both host species with isolates from other hosts of the Latin American Clade (LAC) by using mating type genes and ITS rDNA region. 2) Assess the interesterility through crossings between isolates from different host species. 3) Determine the genetic variability in populations of *C. fimbriata* through microsatellite markers and their physiological especialization by f cross-inoculation experiments. Out of 24 isolates of the fungus obtained from teak from of locations in the state of Mato Grosso, and of 73 isolates of *C. fimbriata* from andiroba in the states of Acre, Roraima and Amazonas, new ITS haplotypes and mating types identified were distributed among other isolates within the LAC and reside in a single group that includes isolates from sweet potato, from which the species was originally described. Teak and andiroba isolates were inter fertile with

other strains of *C. fimbriata,* showing that this is a single biological species of *C. fimbriata*. Microsatellite analysis of the fungal population on teak showed low genetic variability. In cross-inoculations, although teak plants presented relatively small lesions, two isolates differed from the control. The eucalyptus isolates were not pathogenic on teak. The populations of *C. fimbriata* from andiroba of the three states (AC, AM, and RR) showed 14 genotypes and intermediary levels of genetic variability compared to other native populations of the fungus in Brazil. Cross-inoculations of andiroba, teak, eucalyptus and mango isolates suggest that the isolates of *C. fimbriata* from these species are host specific and that there is variability in aggressiveness among isolates of the fungus from andiroba.

GENERAL INTRODUCTION

Ceratocystis fimbriata Ellis & Halsted is an important pathogen for a large number of trees and herbaceous plants. Initially, it was reported as the causal agent of the black rot of *Ipomoea batatas* (L.) Lam. (sweet potato), in New Jersey, USA (Halsted, 1890). Since then, the fungus has been reported in many countries, infecting plants of different botanical families (CAB International, 2005). In Brazil, the first report of *C. fimbriata* was on *Crotalaria juncea* L. (crotalaria), described by Costa and Krug (1935). From the 1930's to the 1960's, the fungus was reported in legumes such as *Cajanus indicus* (L.) Millsp. (pigeon pea) (Viégas, 1944) and tree species as *Mangifera indica* L. (mango) (Carvalho, 1938) and *Cassia fistula* L. (golden shower tree) (Galli, 1958). Between 1970-80, *Hevea brasiliensis* M. Arg. (rubber tree) (Albuquerque et al., 1972), *Gmelina arborea* Roxb. Ex Sm. (gmelina) (Muchovej et al., 1978) and *Cassia renigera* Wall. (Ribeiro et al., 1987) also became part of the host range of this pathogen. In 1997, the fungus was identified infecting *Eucalyptus* spp. in the southeast of the state of Bahia (Ferreira et al., 1999) and has recently been identified in *Tectona grandis* L. f. plantations (teak) in midwest Brazil (Firmino et al., 2012), *Carapa guianensis* Aubl. (andiroba) seedlings in native forest in state of Roraima (Halfeld-Vieira et al., 2012) and *Actinidia deliciosa* (A. Chev) CF Lianget & AR Ferguson (kiwifruit) orchards in southern Brazil (Piveta et al., 2013).

Ceratocytsis fimbriata belongs to the class Pyrenomycetes, order Microascales, family Ceratocystidaceae (Webster & Weber, 2007). *Ceratocystis fimbriata* is the type species of the genus, and its taxonomy remained confusing for more than 50 years. Initially, Saccardo (1892) transferred the species to the genus *Sphaeronaema*. Then, Elliott (1923) designated it as *Ceratostomella*, but later, Melin & Nannfeldt (1934) reclassified it in *Ophiostoma* and, finally, Davidson (1935) transferred the species to

the genus *Endoconidiophora*. In 1950, the genus *Ceratocystis* was recognized again in the work of gender review by Bakshi (1950). The anamorph of *C. fimbriata* was, for many years, accommodated in the genus *Chalara*, but Montoya & Wingfield (2006), based on phylogenetic analyzes of DNA sequence data, placed it in the gender *Thielaviopsis*.

With the development of modern molecular techniques, some fungi species have become considered a species complex for comprising cryptic species, i.e., species which are not morphologically or reproductively distinct (Kohn, 2005; Milgroom, 2015). Phylogenetic analyzes indicate that there are at least five species complexes within the genus *Ceratocystis*: i) *C. coerulenscens*; ii) *C. paradoxa*; iii) *C. moniliformis*; iv) *Thielaviopsis* anamorph; and v) *Ceratocystis fimbriata* (Harrington, 2009; Harrington, 2013). The complex *C. fimbriata* can be further divided into four phylogenetic clades including the Latin American Clade, North American Clade, the Asian Clade and the African species *C. albofundus* MJ Wingf., De Beer, & MJ Morris (Harrington, 2000; Johnson et al., 2002; Harrington et al., 2011). Extensive studies have been conducted with the aim of better characterize the species according with the host studied within each clade, mainly from Latin America Clade (Baker et al., 2003; Thorpe et al., 2005; Harrington et al., 2011; Oliveira et al., 2015; Valdetaro et al., 2015).

Baker et al. (2003) studied differents isolates of *C. fimbriata*, from the Latin America clade, infecting *Theobroma cacao* L. (cacao), *Herrania* sp., *I. batatas* (sweet potato), *Platanus* sp. (sycamore), *Coffea* sp. (coffee), *Xanthosoma* sp. (dasheen, malanga, cocoyam, yautia), *M. indica* (mango), *Annona* sp , *Eucalyptus* sp. and *G. arborea* (gmelina) and found, based on the analysis of sequences of the genomic region ITS (*Internal Transcribed Spacer*) including the 5.8s region of the rDNA gene and

cross inoculations, the existence of three monophyletic lineages with specialization for cocoa, sweet potato and *Platanus* spp. Subsequently, Engelbrecht & Harrington (2005) using interesterility tests and morphological characteristics described the cocoa's pathogen as a new species called *C. cacaofunesta* and upgraded the identification of the pathogen from *Platanus* sp., previously *C. fimbriata* f. sp. *platani*, to *C. platani*. The sweet potato pathogen was kept as *C. fimbriata*, as originally described and named *C. fimbriata sensu stricto*. In addition, the evolution and divergence of species of the *C. fimbriata* complex and other complexes as *C. coerulescens*, may have been conducted by host specialization given that only minor morphological differences have been observed (Webster & Buttle, 1967; Harrington et al., 2002; Johnson et al., 2002; Baker et al., 2003; Engelbrecht et al., 2004; Johnson et al., 2005; Thorpe et al., 2005).

The description of new species within the complex *C. fimbriata*, however, is not always performed considering the phylogenetic concept of species, being, in many cases, only based on analyzes of the sequence of gene regions such as ITS, jeopardizing this description. According to Harrington et al. (2014), more than one ITS sequence may be present in a single monoascosporic isolate of the fungus, causing this gene region not to be suitable for evolutionary inferences and neither for the description of new species. For example, two new species of the complex *C. fimbriata* been described, one in Oman and Pakistan infecting mango tree, named *C. manginecans* M. van Wyk, A. Adawi & M.J.Wingf. (haplotype ITS7b) (van Wyk et al., 2007), and one in Indonesia infecting acacia (*Acacia mangium* Willd), named *C. acaciivora* Tarigan & M.van Wyk (haplotype ITS6) (Tarigan et al., 2011). However, ITS sequences that characterized these two new species (haplotypes ITS7b and ITS6) were found in a single monoascosporic isolate of mango tree (Harrington et al., 2014; Oliveira et al., 2015). Therefore, these are not new species, but different genotypes

within the population of *C. fimbriata*. In addition, according to studies by Harrington et al. (2011), the gene "mating type" MAT1-2 showed less variation in relation to the ITS region and this variation corresponded more closely with the host of origin, therefore, being more suitable for separating species of the complex *C. fimbriata*.

Besides the correct choice of gene region, the inclusion of natural populations of the pathogen is extremely important for the recognition of new species. Without this, each independently introduced population could appear as a distinct lineage and could be described as a new species (Harrington et al., 2014; Oliveira et al., 2015). However, the movement of infected plant material at various regions of the world makes it difficult to analyze whether the *C. fimbriata* populations are native or introduced in a given region (CAB International, 2005). Studies of *C. fimbriata* populations using microsatellite markers have pointed to an intense movement of contaminated materials from different hosts (Roux et al., 2000; Santini & Capretti, 2000; Engelbrech et al., 2004; Ocasio-Morales et al., 2007 ; Ferreira et al., 2010; Ferreira et al., 2011; Harrington et al., 2011;. Harrington et al., 2014; Harrington et al., 2015; Li et al., 2016). In Brazil, analysis of isolates from eucalyptus, mango and kiwi, using microsatellite markers showed that the fungus seems to be native from regions with vegetation type of Cerrado (Brazilian savannah), Caatinga (Brazilian semi-arid biome) and from in the state of Rio Grande do Sul, respectively, due to the high variability found in these localities (Ferreira et al., 2010; Ferreira et al., 2013b; Oliveira et al., 2015).

Recently, two new hosts of the *C. fimbriata* complex were described: teak (Firmino et al., 2012) and andiroba (Halfeld-Vieira et al., 2012). Teak is an Asian tree introduced in Brazil 80 years ago (Sampaio, 1930). Its large-scale cultivation started in 1971 in the state of Mato Grosso (Cáceres Florestal, 2006) and its wood is valuable

due to its light weight, durability, strength, good dimensional stability, no corrosion in contact with metals, and resistance to chemical and to the weather (Keogh, 2013). As in most tree species, the disease symptoms in teak can vary from wilting with discoloration of the sapwood, leaf yellowing, leaf fall to death (Ferreira et al., 2013a). The fungus can cause direct damages with the death of the plant and indirect with the loss of timber quality due to discoloration of the sapwood, besides the risk of spreading the disease with the exportation of wood products. *Carapa guinensis* (andiroba) is a species native from the Amazon rainforest, which is prized for its wood and especially for the oil extracted from its seeds, widely used in the field of cosmetic and medicines (Souza et al., 2006). Unlike the other hosts, in Andiroba, *C. fimbriata* affects seeds and seedlings, which may affect negatively the species regeneration due the death of the infected plants and seeds (Halfeld-Vieira 2012).

The correct identification of the causal agent of a given disease is essential in the search for efficient control methods. Moreover, understanding the spread of the disease and knowing the origin of the pathogen, it may give subsidy in decision-making to prevent the spread of the pathogen. Hence, this Thesis was divided into two chapters. In the first, we studied the taxonomy of the fungus through phylogenetic analyses of mating type genes and interesterelity tests. Analyses of ITS rDNA region were used as fingerprinting as well as to study the genetic variation of teak isolates in this region. By using microsatellite markers, we estimated their genetic variability and by cross inoculations, we evaluated the pathogenicity and aggressiveness of the fungal isolates. In the second chapter, populations of *C. fimbriata* on andiroba were studied. Andiroba is the only host of the fungus in Brazil whose disease occurrence happened in natural conditions (Halfeld-vieira et al., 2012). Therefore, studying this fungus's population in this host is extremely important due to the assumption that it is a

pathosystem without human interference and with great chances of being a native population from the Amazon region. Hence, by using phylogenetic analyses of the mating type genes and intersterility tests, we studied the taxonomy of the fungus. Additionally, we used the ITS rDNA region as fingerprinting and to determine the genetic variation of teak isolates in this region. By using microsatellite markers, we evaluated whether the fungus is native to Amazon, and if there is host specialization through cross inoculations.

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CHAPTER 1

ARTICLE 1

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Genetic variability of *Ceratocystis fimbriata* on *Tectona grandis* in Brazil.

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ABSTRACT

Ceratocystis wilt is currently one of the most worldwide important diseases in forest and agriculture crops. The disease, that was recently reported on teak, in Brazil, can kill the infected trees and reduce the wood quality for timber. Teak is one of the most important and valuable hardwoods in the world. Because the fungus was very recently reported on teak, there are few studies on this pathosystem. Therefore, this work aimed to study the taxonomy, as well as the genetic and physiological variability of the fungus through phylogenetic analyses by using the mating type gene sequences and intersterility tests, ITS rDNA fingerprinting, microsatellite analyses, and aggressiveness of selected isolates by cross inoculations. Analyses of ITS rDNA sequences of 24 isolates showed three new ITS haplotypes among the teak isolates scattered among other isolates of the Latin American Clade (LAC). Phylogenetic analysis, using mating type genes (MAT-1 and MAT-2) from representatives of the *C.*

fimbriata complex from around the world clearly place the teak isolates in the LAC and formed two distinct groups, one of them is unique. Furthermore, isolates from teak were inter-fertile with tester strains of *C. fimbriata*. Thus, isolates from teak belong to the same biological species of *C. fimbriata* ss from sweet potato. The analyses of 14 microsatellite loci showed three genotypes among teak isolates that formed a distinct group with low genetic diversity. Results from cross inoculations of the fungus from teak and eucalyptus infected only their respective host species.

Keywords: Ceratocystis wilt, teak tree, microsatellite, mating type, ITS

1 Introduction

Ceratocystis fimbriata Ellis & Halsted is considered as a complex of many cryptic species that are morphologically similar to each other and are able to cause disease on a wide range of economically important hosts with worldwide distribution (CAB International 2005; Ferreira et al. 2013a; Harrington 2013). In Brazil, where the pathogen is native (Ferreira et al. 2010; Harrington et al. 2011, 2014; Ferreira et al. 2013b; Oliveira et al. 2015a), Ceratocystis wilt is one of the most important diseases of woody plants under field conditions. Recently, in Brazil, teak tree (*Tectona grandis* L. f.) was reported as a host of *C. fimbriata* in the region of Cáceres, MT, Brazil (Firmino et al. 2012). Teak is one of the most important and valuable hardwoods in the world (Kollert and Cherubini 2012). It is a highly durable Asian tree, water- and pest-resistant tropical hardwood, used in the manufacturing of boats, residential and commercial architecture and veneer furniture making (Arias 2013; Midgley et al. 2015). Worldwide area of teak plantation is estimate in 6.8 Mha, concentrated mainly in Asia, Africa, Latin America and Caribbean (Midgley et al. 2015). In Brazil, teak

plantations are estimated currently in about 67,000 ha, distribuited in the states of Mato Grosso, Pará and Roraima (ABRAF 2013). However, up to now, the disease on teak has been identified only in Mato Grosso.

Ceratocystis wilt symptoms on teak are similar to those observed in other woody plants like *Eucalyptus* sp., *Acacia* spp., *Gmelina arborea* Roxb. Ex Sm. (Ferreira et al. 2013a). The symptons range from wilt, yellowing and leaves to dry resulting in the death of the plant. The fungus infects through the wounds and move through sapwood tissue, mostly in ray parenchyma, causing sapwood discoloration (Ferreira et al. 2013a).

According to phylogenetic studies using DNA sequences, *C. fimbriata* complex was outlined in four geographic groups: the Latin American Clade (LAC), North American Clade (NAC), Asian Clade and African species (Wingfield et al. 1996; Harrington 2000; Johnson et al. 2005; Harrington et al. 2011). The study of Ferreira et al. (2010) using microsatellite markers and intersterility tests with *C. fimbriata* isolates from different hosts, suggested that Brazilian isolates constitute a single biological species within the LAC, regardless the host studied [*Eucalyptus* spp., *Mangifera indica* L., *Ficus carica* L., *Gmelina arborea* Roxb. Ex Sm. and *Ipomoea batatas* (L.) Lam.]. In inoculations studies, Brazilian isolates did not show host-based lineage, instead they vary greatly in aggressiveness (Baker et al. 2003; Thorpe et al. 2005; Harrington et al. 2011; Oliveira et al. 2015b; Oliveira et al. 2016).

Additionally, studies using microsatellite markers were able to distinguish possible native populations of *C. fimbriata* from introduced populations (Engelbrecht et al. 2004, 2007a; van Wyk et al. 2006; Ocasio-Morales et al. 2007; Ferreira et al. 2010; Harrington et al. 2015; Oliveira et al. 2015a; Li et al. 2016). Normally, introduced populations show lower genetic variability than native populations.

Furthermore, populations that were recently introduced by human activity can be highly differentiated from other populations due limited dispersal distance and a high degree of selfing or asexual reproduction (Engelbrecht et al. 2004; Ferreira et al. 2010).

Thus, this work aimed to: a) study the relationship of the isolates of *C. fimbriata* from teak with isolates from other hosts in LAC, by using mating type gene sequences (*MAT1-1-2* and *MAT1-2-1*); b) analyze the ITS rDNA region as fingerprinting; c) determine the interesterelity among *C. fimbriata* isolates from other host species; d) estimate the genetic variability and spread of genotypes of *C. fimbriata* on teak, by using microsatellite markers; e) evaluate the aggressiveness of the fungal isolates by cross-inoculations on teak and eucalyptus.

2 Materials and Methods

2.1 Sample collection and fungal isolation

Isolates from wilted teak trees were collected four farms in Mato Grosso, Brazil: Nossa Senhora do Livramento (NS), São José dos Quatro Marcos (QM), São José do Rio Claro (RC) and Sinop (SI) (Fig. 1). Plantations were established in 2000, and the farm RC was located in a region of secondary forest. In this farm, there was *Eucalyptus* plantation near the teak plantation where the isolates of C. *fimbriata* were also collected. Prior to teak plantations, NS and SI farms were usually cultivated with rice, while in QM farm was under pasture land. The samples collected in the farms NS and SI were from an area of the 200 ha, approximately, while the samples of QM and RC farms were collected from an area of 150 ha. The samples of the discoloured tissue were conducted to the Laboratory of Forest Pathology, DFP/BIOAGRO at the Universidade Federal de Viçosa (UFV), and the fungus was isolated from diseased tissue by the carrot bait method (Moller and DeVay 1968). Subsequently, single

ascospore strains were obtained by dispersing ascospore mass in about 10 µL of mineral oil autoclaved and the spore suspension was spread over the plate with MYEA (2% malt extract, 0.2% yeast extract, and 2% agar) media (Ferreira et al. 2010; Alfenas and Mafia 2016). Then, 12 h later individual germlings were transferred to fresh plates (Harrington and McNew 1997). One isolate per tree (self-fertile strain) was stored in 15% glycerol at -80 °C at the Universidade Federal de Viçosa. Some teak isolates were also stored in the Plant Pathology Laboratory, at Iowa State University, Iowa, USA.

2.2 DNA extraction

The fungus was grown on MYEA (Malt Yeast Extract Agar) and incubated at 28 °C for about 15 days before DNA extracting using the Wizard® Genomic DNA Purification Kit (Promega Inc. – Madison, USA) with the manufacturer's protocol modified by Valdetaro et al. (2015). The concentration of purified DNA was quantified with a Nanodrop 2000c® (Thermo Fisher Scientific Inc., Massachusetts, USA) and adjusted to 50 - 100 ng μL^{-1}.

2.3 ITS and mating type genes sequences

Sequences of ITS rDNA region were generated using PCR folowed by direct DNA sequencing of the PCR products with primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardens and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3) (White et al. 1990). The mating type genes (*MAT1-1-2* and *MAT1-2-1*) was amplified with the set of primers CFMAT1-F (5' – CAGCCTCGATTGAKGGTATGA-3') and CFMAT1-R (5' – GGCATTTTTACGCTGGTTAG-3') for *MAT1-1-2* (Harrington et al. 2014), and X9978R1R (5'-GCTAACCTTCACGCCAATTTT-3') and CFM2-1F (5'–

AGTTACAAGTGTTCCCAAAAG- 3') for *MAT1-2-1* (Harrington et al. 2011) were used to amplify and sequence about 1040 bp and 1131 bp regions, respectively.

The thermocycler settings for amplifying the three regions were used as described previously by Harrington et al. (2014). The PCR products amplified were sequenced with the PCR primers. The fragments were purified using Ilustra™ GFX™ PCR DNA and all sequencing was conducted by Iowa State University DNA Synthesis and Sequencing Facility. Sequences were analyzed and edited using Sequence Navigator (Applied Biosystems, Foster City, California) software and subsequently manually aligned when necessary.

The ITS sequences obtained of the teak isolates were compared with those reported earlier for the LAC (Table 1) (Harrington et al. 2011, 2014; Oliveira et al. 2015a) using parsimony analyses.

2.4 Phylogenetic analyses based on mating type genes

Datasets of mating type gene sequences were manually aligned and submitted to a partition homogeneity test (PHT) using PAUP 4.0b1.0 (Swofford 2003) in order to determine whether the datasets could be combined.

Maximum Parsimony (MP) and Bayesian Inference (BI) were used to construct phylogenetic trees. The MP analysis was performed with PAUP * 4.0b10 (Swofford 2003) using heuristic searches with the TBR algorithm (Tree Reconnection Bissection) and stepwise addition with 1000 random repetitions. Gaps were treated as a fifth base and all characters had equal weight.

Bayesian inference was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The substitution model was chosen based on the Akaike information criterion (AIC) of MrModelTest 3.2 (Nylander 2004). A posteriori

probability (PP) distribution of trees was created using MCMC (Metropolis-coupled Markov chain Monte Carlo) and two chains initiated from a random tree with 25 million generations and discarding the first 25 percent of the trees.

Sequences of isolates obtained in this study were compared with sequences of representative of isolates of the *C. fimbriata* complex, including *C. cacaofunesta* Engelbr. & T.C. Harrin., *C. colombiana* M. van Wyk & M. J. Wingfield and *C. platani* Engelbr. & T.C. Harrin. One isolate of *Ceratocystis variospora* (R.W.Davidson) C. Moreau (C1963) from the North American Clade (NAC) was used as the outgroup taxon (Table 1).

2.5 Mating experiments

Representative teak isolates from each of the four sites were selected as MAT-1 (female-only) testers. MAT-1 isolates from *Eucalyptus* spp., *F. carica, Colocasia esculenta* (L.) Schott, *G. arborea, M. indica* and *I. batatas*, and one representative isolate from *C. cacaofunesta*, one of *C. platani* and one of *C. colombiana* were also included. The MAT-1 testers self-sterile (females) were obtained from single ascospore isolation from field isolates that were self-fertile.

Self-sterile isolates, MAT-2 (male-only), were selected for mating experiments: one isolate from teak (Sinop), one from *I. batatas*, one from *C. platani*, one from *Eucalyptus* and three isolates from *M. indica* (Oliveira et al. 2015a). The MAT-2 testers were recovered from sectors, formed, spontaneously by subculturing, from otherwise self-fertile isolates. For theses mutants, DNA was extracted and PCR was performed to confirm the presence of MAT-2 region (*MAT1-2-1*). The primers CFM2-1 and X9978R1R (Harrington et al. 2011) were used to amplify the MAT-2 gene.

The MAT-1 and MAT-2 testers were grown on MYEA for 7 days at room temperature (25 ^{o}C +/- 2 ^{o}C). The conidial suspension of the MAT-2 tester was prepared as described previously (Ferreira et al. 2010). Then, 1 mL was dispersed over the MAT-1 colony to spermatization.

During 3 to 4 weeks, the cultures were checked for the presence of perithecia and ascospore masses. Ascospore masses were examined microscopically (400 × magnification) to see the spores quality (normal and mixed indicating intraspecific pairing or watery indicating hybrid). Ascospore masses from progeny was spreaded onto fresh MYEA to observe the mycelial morphology. In successful crosses, the progeny showed both mycelial phenotypes of the parents (Ferreira et al. 2010; Oliveira et al. 2015a). The experiment was performed twice and the strains were considered interfetile if they crossed in at least one of the two experiments.

2.6 Microsatellite analyses

We analyzed 14 microsatellite loci (AAG8, AAG9, CAA9, CAA10, CAA15, CAA38, CAA80, CAT1, CAT1200, CAG5, CAG15, CAG900, GACA60 and GACA6K) (Steimel et al. 2004). For each primer pair specific to the flanking regions of fourteen simple sequence repeat regions, one of the primers was fluorescently labeled. The microsatellite analyses were done in the Laboratory of Forest Pathology at the Universidade Federal de Viçosa (UFV), Brazil and at Iowa State University (ISU), USA. The microsatellite data, which were obtained in the Forest Pathology Laboratory/Bioagro/UFV were also calibrated in the ISU. PCR amplifications of all microsatellite loci were performed using a Veriti® 96-well thermos cycler (Thermo Fisher Scientific Inc., Pittsburgh, USA) (UFV) and PCT 100 (MJ Research Inc) (ISU) following the earlier described conditions (Ferreira et al. 2010). Band sizes of the

product were determined using a four-capillary ABI Prism 3500 – Genetic Analyser (Applied Biosystems Inc., Foster City, CA) and GeneMapper v4.1 Software (Applied Biosystems) (UFV) and ABI Prism 3100- Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and ABI Peak Scanner v1.0 Analysis Software (Life Technologies) (ISU). Each product length (within 1 bp) was considered to be a different allele. Most of the microsatellite loci contained trinucleotide repeats, and most alleles of a given locus differed by increments of 3 bp.

Nei's gene diversity (*H*) for teak populations using the microsatellite data were calculated without and with clone-corrected data using PopGene 1.32 software. Multilocus genotypic diversity was estimated with the Stoddart and Taylor's *G* index (Stoddart and Taylor 1988). For comparisons among individual populations, *G* index was scaled by the expected number of the genotypes for the smallest sample size (Grünwald et al. 2003) and the smallest population had five isolates, so the expected number of the genotypes in a sample of five isolates (minimum value = 1.0 and maximum value = 5) was estimated based on rarefaction curves using the Vegan package from CRAN in R v.2.6.1 (R Core Team 2007).

Relationships among the teak genotypes (combinations of the alleles among the 14 microsatellite loci) of *C. fimbriata* and representative genotypes from other hosts of the LAC (Ferreira et al. 2010; Harrington et al. 2015; Oliveira et al. 2015a) were examined in PAUP* (Swofford 2003) using genetic distance (Nei's) matrices and UPGMA trees. Bootstrapping tests utilized 1000 replications.

2.7 Pathogenicity tests

In Mato Grosso, plantations of eucalyptus and teak are commonly found next to each other. Therefore, cross inoculations of selected teak and eucalyptus isolates

were performed on teak and eucalyptus clones. The isolates of both host species were selected according to the genetic variability and geographic origin. The isolates were inoculated on 6-month old teak plants (clone A3) and on the hybrid clone 1172 of *E. urophyla* x *E. grandis* (three-month old). The eucalyptus plants were transplanted into 2 L pots containing the substrate Carolina II (Carolina Soil do Brasil) supplemented with 6 kg m^{-3} of superphosphate and 1,5 kg m^{-3} Basacote® (19-6-10) (ComPo do Brasil). The teak plants were grown into 2 L pots mix with soil, sand and Caroline II substrate (1:1:1) supplemented with 6 kg m^{-3} of superphosphate and 1,5 kg m^{-3} Basacote® (19-6-10) (ComPo do Brasil).

The fungus was grown MYEA at 28 ºC for 10 days. Subsequently, 10 mL of sterile water were added to each plate, and the surface of the colony was scraped and filtered through a double layer of gauzes, and the inoculum concentration was adjusted to 3×10^6 spores mL^{-1}. The plants were wounded (around 3-mm deep) with a sterile scalpel at 3 cm, approximately, above the soil line, and a volume of 500 µL of the inoculum was applied. The inoculation site was wrapped with parafilm®. The control plants were wounded and treated with the same volume of sterile distilled water.

The experiment was repeated twice. The first experiment was conducted from May 6th to July 6th of 2016 and the second experiment from May 16th to July 16th of 2016, when greenhouse temperatures ranged from 7.9 - 45.2 ºC. Each experiment used a completely randomized design, consisting of two factors (host × isolate), with seven replicates, considering each plant as a sampling unit. The plants were incubated in the greenhouse for 60 days, when the xylem discoloration was measured. To re-isolate the fungus, the carrot bait method (Moller and DeVay 1968) was used. A multifactorial analyses of variance (ANOVA) (including isolate, host, and experiment) and Fisher's

Protected LSD test ($P < 0.01$) (data from two experiments combined) was conducted using Statistica® software (StatSoft Inc.).

3 Results

3.1 Sample collection and fungal isolation

Twenty-four *Ceratocystis* sp. isolates were obtained from discolored wood tissue collected from 66 trees (Fig. 1). The isolates had uniform mycelial morphology and presented similar growth rate. All isolates recovered from diseased trees were self-fertile.

Trees, varying from 7 – 14 years old, with typical wilt symptoms caused by *C. fimbriata* (Fig. 2B) as well as infected asymptomatic trees were found scattered in the collection sites. From the discolored tissue in symptomatic trees, we observed that infections started from the roots (Fig. 2E) and moved upwards in the stem (Fig. 2F), culminating with the death of the tree (Fig. 2B). In this case, the root infections can start from soil-borne inoculum. In most cases, however, we observed that infections started from the pruning points of the trees, moving both ways, upwards and downwards (Fig. 2G). Stem infection may arise from trees previously infected by soil inoculum starting from the roots and, subsequently, the fungus can be disseminated by pruning tools to healthy plants. Pruning is a common practice in teak plantation to produce clear wood, carried out annually until the seventh year and then sporadically for removal of side branches. In transverse sections of the stem and roots, grayish discoloration was observed in the xylem (Fig. 2D) compared to cross sections of healthy trees (Fig. 2C). In addition, on teak, bark beetle and ambrosia beetle activities were not common.

3.2 ITS

ITS sequences of 16 isolates were compared with a dataset of ITS sequences of *C. fimbriata* isolates from the Latin American Clade (LAC) (Harrington et al. 2011). Eight isolates (TecMT-NS3, TecMT-NS7, TecMT-NS8, TecMT-QM16, TecMT-QM24, TecMT-QM28, TecMT-QM29 and TecMT-RC32) were not considered in ITS analyses, because they had a mixed ITS PCR product that could not be clearly determined using direct sequencing. The ITS sequence generated with the ITS1F primer initially gave a clean read in electropherogram, and then overlapped peaks followed (Harrington et al. 2014).

In an aligment of 613bp, the number of variable characters was 99, and 63 of those characters were parsimony informative. Maximum parsimony analysis found 45 trees of 303 steps, with the teak isolates grouping with other Brazilian isolates (Fig. 3). Three ITS sequences (haplotypes) were identified among the 16 teak isolates (Fig. 3). All ITS haplotypes were unique to teak isolates and did not match to any other sequence previously reported. The ITS haplotypes numbers follow the designations by by Harrington et al. (2011). The letters were named following the sequence previously described (Harrington et al. 2014; Oliveira et al. 2015a).

The five isolates from RC town had the ITS10g haplotype. The ITS14j haplotype was found in six isolates spread in NS, RC and SI towns, and the ITS10f was found in five isolates from NS and QM.

3.3 Phylogenetic analyses based on mating type genes

The mating-type genes are more suitable than ITS for separating species of the *C. fimbriata* complex (Harrington et al. 2011). Then, representative isolates from each of the four locations were selected for sequencing of mating type genes. For MAT-1

(*MAT1-1-2*), an alignment of 1040 characters showed two haplotypes among the 10 teak isolates tested. For MAT-2 (*MAT1-2-1*), an alignment of 1131 characters showed 24 characters varying and two different MAT-2 haplotypes were found among 10 analyzed isolates.

The mating type genes are tightly linked, they are not subjected to normal crossover events, therefore, when combined, they produce robust phylogenetic tree of the *C. fimbriata* complex (Harrington et al. 2014). The PHT test resulted in a high P-value (P=0.36) suggesting that the dataset of MAT-1 and MAT-2 genes could be combined (Cunningham 1997; Barker and Lutzoni 2002).

The combined dataset of MAT-1 and MAT-2 sequences of teak and different mating haplotypes found in the LAC (Harrington et al. 2014) had alignment of 2171 characters. The number of variable characters was 368, and 77 of those characters were parsimony informative. Maximum parsimony (MP) analysis found a single tree of 475 steps, with homoplasy index (HI) = 0.0379, consistency index (CI) = 0.9621, rescaled consistency index (RC) = 0.9121, and retention index (RI) = 0.9480. Evolution model HKY = I was selected and incorporated the Bayesian analysis. The MP tree and Baeysian-inferred tree had very similar topology. Then, the single parsimony tree generated was selected for illustration (Fig. 4). Two mating haplotypes were found among 10 teak isolates. The mating haplotypes were named following the sequences previously described (Harrington et al. 2014). The number of the haplotype was defined according to the number of the isolate more closely related and the letters follow the sequence used previously by Harrington et al. (2014).

The 4d haplotype was unique and separated by highly supported (95% boostrap and 0.99 posterior probability), and these teak haplotype was closely related to isolates on *Ficus carica* from São Paulo and *Mangifera indica* from Brasília and Rio de

Janeiro. While, the two other teak isolates (TecMT-RC35 and TecMT-RC356) had the mating haplotype 3b (Fig 3) that was identified previously in isolates collected from *Eucalyptus* (BA), *Mangifera* (BA, CE, PE, PB, SP and RJ), *Crotalaria* (DF), *Cajanus* (DF), *Acacia* (PR) and *Punica* (India) (Harrington et al. 2014; Oliveira et al. 2015a).

3.4 Mating experiments

MAT-1 and MAT-2 testers from teak isolates successfully crossed with the majority of the other Brazilian testers of opposite mating type (Table 2). In most of the successful crosses, normal and mixed crosses, there were many fully developed perithecia within a week and produced thick, creamy ascopore masses at the tips of perithecial necks (Fig. 5A). Microscopic examination showed abundant, normal appearing ascopores (Fig. 5C), but crosses between MAT-2 tester from teak isolate and MAT-1 tester from sweet potato showed junk, empty and normal ascopores. Because they had more normal ascopores, they was considered successful crosses. To demonstrate that ascopore masses is not due to a selfing, ascopores masses from normal and mixed crosses were streaked on MYEA. The colonies produced mycelia morphologically similar to both male and female testers strains. In unsuccessful crosses, only a few perithecia were produced and ascospores masses were watery (Fig. 5B) and microscopic examination showed few misshapen ascospores (Fig. 5D). Unsuccessful crosses occurred when testers from teak isolates crossed with opposite mating testers from *C. platani*, *C. cacaofunesta* and *C. colombiana*.

3.5 Microsatellite analyses

Of the 14 microsatellite loci tested on the 24 teak isolates, ten loci were monomorphic (AAG8 = 174; AAG9 = 397; CAA9 = 175; CAA10 = 134; CAA38 =

156; CAT1 = 253; CAT12X = 371; CAG5 = 317; CAG900 = 194; and GACA60 = 187) and four were polymorphic, and the respective allele size – base pairs (number of isolates) were: CAA15 = 318 (10), 324 (14); CAA80 = 311 (5), 317 (19); CAG15 = 289 (19), 297 (5); and GACA6K = 213 (18), 215 (6).

Among the 24 teak tree isolates studied, three genotypes were found. The UPGMA tree constructed (Fig. 6) showed that three very closely related microsatellite genotypes from teak grouped separately from the other isolates. Microsatellite analyses identified genotypes from teak that appeared to be very widespread. Two genotypes were found infecting trees in more than one locality. The third genotype was found only in NS town (Fig. 1 and Fig. 6).

Nei's gene diversity (*H*) and Stoddart and Taylor's genotypic diversity (*G*) for all teak populations was $H = 0.0967$, or $H = 0.1270$ when clone corrected, and was $G = 2.5410$, respectively (Table 3).

3.6 Pathogenicity tests

There were no wilting symptoms in eucalyptus or teak within 60-day period. The control plants remained asymptomatic and limited discoloration (wound reaction) was observed, but only near the wound. At the end of the experiments, the fungus was re-isolated from infected tissue of each host and the cultures were typical of *C. fimbriata*. The ANOVA showed that size of xylem discoloration varied significantly by isolate ($F = 41.82$; $p < 0.0001$), host ($F = 225.99$; $p < 0.0001$) and isolate × host interaction ($F = 41.77$; $p < 0.0001$). There were no significant differences between the two experiments ($F = 0.0063$; $p = 0.9366$), therefore, the means showed in the Table 4 are for the combined data of the two experiments.

Teak plants inoculated with teak isolates did not differ from each other, but two teak isolates (TecMT-NS5 and TecMT-QM29) differed significantly from the control, despite the little difference in length of xylem discoloration between isolates (Table 4). Teak isolates did not infected eucalyptus plants and vice-versa. The fungus was re-isolated from the inoculated plants even in those plants with small xylem discoloration, which indicates that the fungus was alive but could not colonize extensively the host tissue. Differences in aggressiveness were also found among eucalyptus isolates on *Eucalyptus* plants.

4 Discussion

The sequence comparisons of mating type genes (*MAT1-1-2* and *MAT1-2-1*) from representatives of the *C. fimbriata* complex from around the world clearly place the teak isolates in the Latin American Clade, among other Brazilians isolates, as well as the *Ipomoea* strain of *C. fimbriata*, from which the fungus was originally described (Halsted 1890). Furthermore, the teak isolates belong to two lineages within LAC, but one of them was unique. Additionally, the teak isolates were interfertil with sweet potato strain and they were intersterile with other species from LAC (*C. cacaofunesta*, *C. platani* and *C. colombiana*) (Engelbrecht and Harrington 2005; van Wyk et al. 2010). Thus, based on the information generated in the present study, isolates from teak are a single biological species, *C. fimbriata*, as Brazilian isolates from another hosts and the sweet potato strain (Harrington et al. 2011; Harrington et al. 2014).

The ITS rDNA region is hypervariable so that it can be used only as fingerprint and not for evolutionary inference neither for description of new species (Harrington et al. 2014). Fifty-seven ITS haplotypes had been reported within LAC (Harrington et al. 2014; Oliveira et al. 2015a; Piveta et al. 2016). The higher variability was found

within *Eucalyptus* and *Mangifera* populations (Harrington et al. 2014; Oliveira et al. 2015a). In the presente study, the analysis of rDNA-ITS sequences showed three new haplotypes of the fungus in teak population belonging the *C. fimbriata* complex in LAC.

The microsatellite data showed that the *C. fimbriata* isolates from teak formed a distinct group, but closely related to isolates from *Gmelina arborea* and *Hevea brasiliensis*. The teak population had gene and genotypic diversity values similarly to those of introduced populations of *C. fimbriata* and other relative species in the LAC (Engelbrecht et al. 2004; Ferreira et al., 2010; Harrington et al. 2015; Oliveira et al. 2015a; Li et al. 2016), suggesting that the *C. fimbriata* isolates from teak in Mato Grosso is not natural. It occurs, especially in populations of recent introduction, as previously found for *C. platani* on *Platanus acerifolia* (Ait.) Willd. (Santini and Caprini 2000). However, in our studies we were not able to explain through microsatellite analysis from where the genotypes found on teak came from. Then, to confirm whether the teak population was introduced or, in fact, it is native will be necessary to collect more isolates from teak and others hosts in Mato Grosso.

Members of the LAC are mostly soil-borne pathogens (Rossetto and Ribeiro 1990; Laia et al. 2000; Marin et al. 2003; Engelbrecht et al. 2007a; Ferreira et al. 2010), and they can be dispersed by ambrosia beetle frass, soil, water infestation, pruning activities and for long-distance by vegetatively propagated material and package materials, among other ways (Panconesi 1999; Harrington 2000; Englebrecht et al. 2007b; CAB International 2005; Ferreira et al. 2011; Masood and Saeed 2012; Ferreira et al. 2013a; Harrington 2013). In the present study, we observed infections starting from the roots, which may indicate that the soil is the main source of primary inoculum. It is possible that only a few pathogenic genotypes from soil infected teak trees, and

than they were spreaded by pruning tools. This may have influenced the low genetic diversity found in this work along with the relatively small number of isolates (24) studied and the sampling method used.

Additionally, we found that the fungus was scattered between farms. This happened because of the teak wood trading, mainly, between RC and SI farms. In addition, we observed log and sawn wood and decks, made of teakwood, for exportation with discoloration caused likly by the fungus. Pathogens introduced into new geographic areas have the potential for rapid emergence and devastating consequences if they encounter native host species or host population that have not previously been exposed to the pathogen (Milgroom 2015). Knowledge of the disease spread is important to avoid the introduction of the pathogen into new areas. Thus, certification for sanitary quality of the products made from teakwood for exportation may avoid or retard the spread of the pathogen into new disease free areas.

The results showed that the fungal isolates tested were only pathogenic on their respective host species. There was also variation in aggressiveness of the isolates on their respective hosts and the teak isolates were relatively much less aggressive than the eucalyptus ones. Previous studies showed that the isolates of *C. fimbriata* varied in aggressiveness on different hosts (Zauza et al. 2004; Baker et al. 2003; Marin et al. 2003; Harrington et al. 2011; Oliveira et al. 2015b, 2016; Valdetaro et al. 2015; Piveta et al. 2016). Addtionally, studies on the aggressiveness of isolates from eucalyptus and mango showed that there was no correlation between aggressiveness and genetic variability, or between aggressiveness and geohraphic origin (Oliveira et al. 2015b; Olivera et al. 2016). However, Nunes (2015) using many more isolates from mango showed that there was correlation between aggressiveness and geographic origin.

Probably, the low aggressiveness of teak isolates may be due the variability in aggressiveness as it has been found in other isolates of *C. fimbriata*.

Based on phylogenetic analyses and on intersterility tests, the teak isolates belong to the American Latin Clade of *C. fimbriata* ss. Furthermore, if the low genetic variability of *C. fimbriata* indicates that selection and breeding for durable resistance have more chances of success.

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Tables and figures

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

Species	Hosts	Localization	Representative isolate	ITS sequence acessions	[a]*MAT1-1-2* GenBank accessions	[a]*MAT1-2-1* GenBank accessions
C. fimbriata	*Tectona grandis*	Mato Grosso, Brazil	TecMT-RC353	[A]xxxx	[B]-	-
	Tectona grandis	Mato Grosso, Brazil	TecMT-RC354	xxxx	-	-
	Tectona grandis	Mato Grosso, Brazil	TecMT-QM15	xxxx	-	-
	Tectona grandis	Mato Grosso, Brazil	TecMT-NS8	-	xxxx	xxxx
	Tectona grandis	Mato Grosso, Brazil	TecMT-RC35	-	KF482986	HQ157550
	Hevea brasiliensis	Acre, Brazil	HevAC-RB08	xxxx	xxxx	xxxx
	Hevea brasiliensis	Bahia, Brazil	HevBA-A50	xxxx	xxxx	xxxx
	Cajanus cajan	Distrito Federal, Brazil	C2173	xxxx	-	-
	Eucalyptus spp.	Bahia, Brazil	C2123	AF395685	-	-
	Eucalyptus spp.	Bahia, Brazil	C1442 (=CBS 115174)	HQ157545	KF482985	HQ157550
	Eucalyptus spp.	Bahia, Brazil	C1440	HQ157544	KF482985	HQ157550
	Eucalyptus spp.	Bahia, Brazil	C1985	AY157966	KF482985	HQ157550
	Eucalyptus spp.	Paraná, Brazil	C1987	-	KF482990	HQ157552
	Mangifera indica	Distrito Federal, Brazil	C2176	-	KF482985	HQ157550
	Mangifera indica	Pernambuco, Brazil	C1970	-	KF482986	HQ157550
	Mangifera indica	Pernambuco, Brazil	C1968	AY585343	KF482984	HQ157553
	Mangifera indica	Rio de Janeiro, Brazil	C1558 (=CBS 115175)	AY157965	KF482988	HQ157552
	Mangifera indica	Rio de Janeiro, Brazil	C2094	-	KF482987	KF482998
	Mangifera indica	Rio de Janeiro, Brazil	C2055	HQ157548	KF482986	HQ157550
	Mangifera indica	São Paulo, Brazil	C1655	HQ157546	-	-
	Mangifera indica	São Paulo, Brazil	C1889	HQ157547	-	-
	Mangifera indica	São Paulo, Brazil	C994 (=CBS 600.70)	AY157964	KF482987	HQ157551
	Mangifera indica	São Paulo, Brazil	C1657	AY526291	KF482986	HQ157550

[A] The sequence will be deposite in GenBank;
[B] Not used in analyses.

Table 1. Continued

Species	Hosts	Localization	Representative isolate	ITS sequence acessions	*MAT1-1-2* GenBank accessions	*MAT1-2-1* GenBank accessions
C. fimbriata	*Ficus carica*	São Paulo, Brazil	C1782 (=CBS 115166)	AY526292	KF482987	HQ157551
	Ficus carica	São Paulo, Brazil	C1857	HQ157542	KF482987	HQ157551
	Colocasia esculenta	São Paulo, Brazil	C1905 (=CBS 115171)	AY526288	KF482989	HQ157552
	Colocasia esculenta	São Paulo, Brazil	C1926	HQ157541	-	-
	Gmelina arborea	Pará, Brazil	C918 (=CBS 115173)	AY157967	KF482983	HQ157549
	Ipomoea batatas	Papua New Guinea	C1476 (=ICMP 8579)	AY157957	KF482992	KF483000
C.cacaofunesta	*Theobroma cacoa*	Ecuador	C1004 (=CBS 153.62)	[B]-	KF482993	KF483001
	Theobroma cacoa	Bahia, Brazil	C1587	AY157953	-	-
	Theobroma cacoa	Rondônia, Brazil	C2031	[A]xxxx	xxxx	Xxxx
C.colombiana	*Coffea arabica*	Colombia	C1543 (=CBS 135861)	AY157961	KF482994	KF483002
	Coffea arabica	Colombia	C1024	xxxx	KF482994	KF483002
C. platani	*Platanus accidentalis*	North Carolina, USA	C1317 (=CBS 115162)	AY157958	KF482995	KF483003
C. variospora	*Prunus* sp.	Iowa, USA	*C*1963 (=CBS 135862)	xxxx	KF482996	KF483004

[A] The sequence will be deposite in GenBank;
[B] Not used in analyses.

Table 2. Normal or watery ascospores masses or lack of perithecia produced from mating experiments between MAT1/female and MAT2/male strains of *Ceratocytsis fimbriata* from *Tectona grandis*, *Eucalyptus* spp., *Mangifera indica, Ficus carica, Colocasia esculenta, Gmelina arborea* and *Ipomoea batatas,* and *C. cacaofunesta, C. platani,* and *C. colombiana.*

Species	Host	MAT1, Female[a]	MAT2, Male[a]						
			Tectona TecMT-SI69sec	*Eucalyptus* C1347sec	*Mangifera* ERRJ1-10sec	*Mangifera* SEMS2-11sec	*Mangifera* SESP5-1sec	*Ipomoea* C1418sec	*Platanus* C1343sec
C. fimbriata	*Tectona*	TecMT-QM29ss	Normal[b]	Normal	Normal	Normal	Normal	Normal	Watery
		TecMT-RC35ss	Normal	Normal	Normal	Normal	Normal	Normal	Watery
		TecMT-SI69ss	Normal	Normal	Normal	—	—	—	—
		TecMT-NS5ss	Normal	Normal	—[f]	—	—	—	—
		TecMT-NS8ss	Normal	Normal	—	—	—	—	—
	Mangifera	SEMS2-11ss	Normal	Normal	Normal	Normal	Normal	Mixed	Watery
		NEBA1-10ss	Normal	Normal	Normal	Normal	Normal	Normal	—
		ERRJ4-2ss	Normal	Normal	Normal	—	—	—	—
	Eucalyptus	C1347ss	Normal	Normal	—	—	—	—	—
	Gmelina	C918ss	Normal	Normal	Normal	Normal	—	—	—
	Ficus	C1783ss	Normal	Normal	—	Normal	—	Normal	—
	Colocasia	C1926ss	Normal	Normal	—	Normal	—	—	Watery
	Ipomoea	C1418ss	Mixed[d]	Mixed	Normal	Mixed	Mixed	Normal	Perithecia[e]
C. platani	*Platanus*	C1317ss	Watery	Watery	Watery	Watery	Watery	Watery	Normal
C. cacaofunesta	*Theobroma*	C1587ss	Watery[c]	—	Watery	Watery	Watery	Watery	—
C. colombiana	*Coffea*	C1564ss	Watery	Watery	Watery	Watery	Watery	—	Watery

[a] sec = strains from MAT2, self-sterile sectors recovered from self-fertile field isolates; ss = MAT1, female-component isolates with protoperithecia;
[b]Normal = abundant, creamy appearing ascospore masses and normal ascospores;
[c]Watery = watery ascospore masses and misshapen ascospores or no ascopore;
[d]Mixed = Some perithecia with normal ascospore masses and other perithecia with watery ascospore masses and mishapened ascospores.
[e]Perithecia = Perithecia only, no ascospore mass;
[f]— = no perithecia produced;

Table 3. Genetic diversity of *Tectona grandis* (teak) populations of *Ceratocystis fimbriata* from Mato Grosso based on 14 microsatellite loci.

Host	Towns	Nº isolates	ITS rDNA haplotype	Nº mixed ITS sequences	Nº Genotypes	Stoddart & Taylor's Genotypic diversity (*G*)[a]	Nei's gene diversity (*H*)	
							All isolates	Clone-corrected
Tectona grandis	Nossa Senhora do Livramento (NS)	5	10d (1)[y], 14d (1)	3	2	2.0000	0.0686	0.1071
	São José dos Quatros Marcos (QM)	8	10d (4)	4	1	1.0000	0.0000	0.0000
	São José do Rio Claro (RC)	9	10e (5), 14d (3)	1	1	1.0000	0.0000	0.0000
	Sinop (SI)	2	14d (2)	-	2	NA	NA	NA
	All isolates from teak	24			3	**2.5410**	**0.1054**	**0.1270**

[a]Stoddart & Taylor's genotypic diversity (G) with rarefaction, maximum value = 5
[y]number of isolates

Table 4. Mean xylem discoloration (cm) caused by six *Ceratocystis fimbriata* isolates inoculated in clones of *Eucalyptus grandis* x *Eucalyptus urophylla* and *Tectona grandis*.

Isolates	Host	
	Eucalyptus clone	*Tectona grandis*
EucSP1	19.71a	2.43ab
EucMS3	16.36ab	2.18ab
EucBASBS1	15.00b	2.04ab
TecMT-QM29	3.61c	2.50a
TecMT-RC35	3.32c	2.18ab
TecMT-NS5	2.75c	2.61a
Control	2.14c	1.79b

The first three letters indicate the host genus (Euc = *Eucalyptus* and Tec = *Tectona*) and next two letters indicate the Barzilian state (BA = Bahia; MT – Mato Grosso; MS = Mato Grosso do Sul);
Means within a column followed by the same lower case letter are not significantly different from each other (P<0.01) based on Fisher's protected least significant difference.

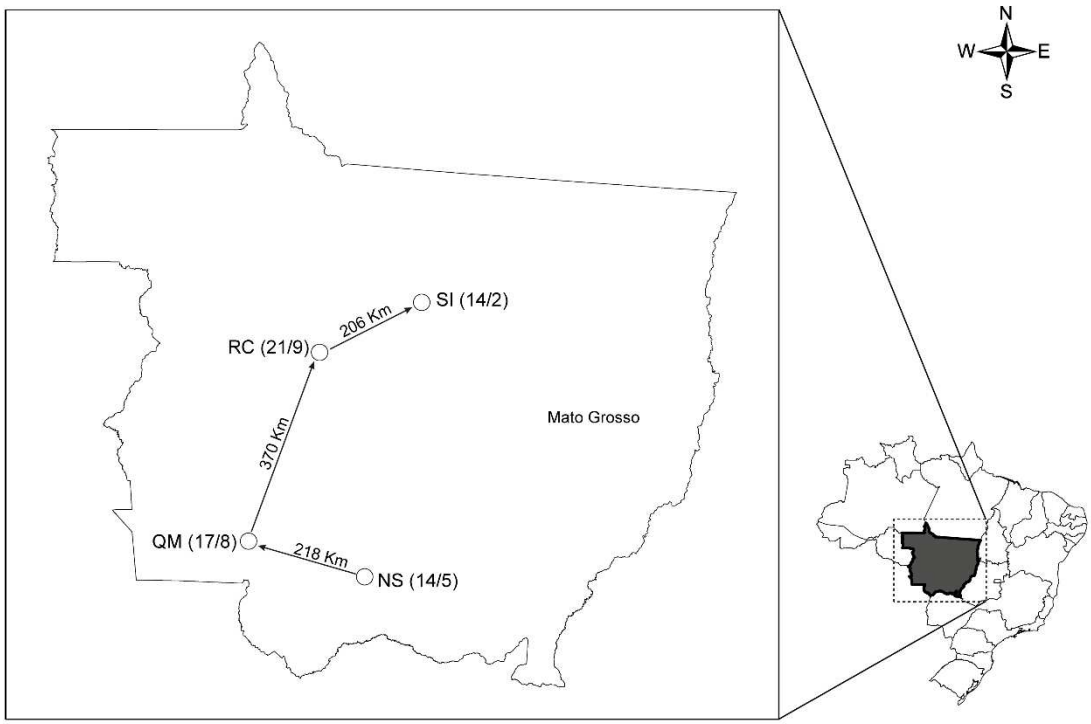

Fig. 1. Sampling sites of *Ceratocystis fimbriata* from teak (*Tectona grandis*) in Mato Grosso. The first two letters indicate the town (NS = Nossa Senhora do Livramento; QM = São José dos Quatro Marcos; RC = São José do Rio Claro; SI = Sinop). In parentheses, the number of samples and the number of isolates that were obtained from each locality.

Fig. 2. Ceratocystis wilt on *Tectona grandis* caused by *Ceratocystis fimbriata* (teak). (a) teak plantation at 4 years old in Mato Grosso; (b) teak tree with wilt symptoms; (c) sections of the health tree; (d) xylem discoloration caused by *C. fimbriata*; (e) root infection reaching the trunk; (f - g) infection through wound (red arrow) in the trunk.

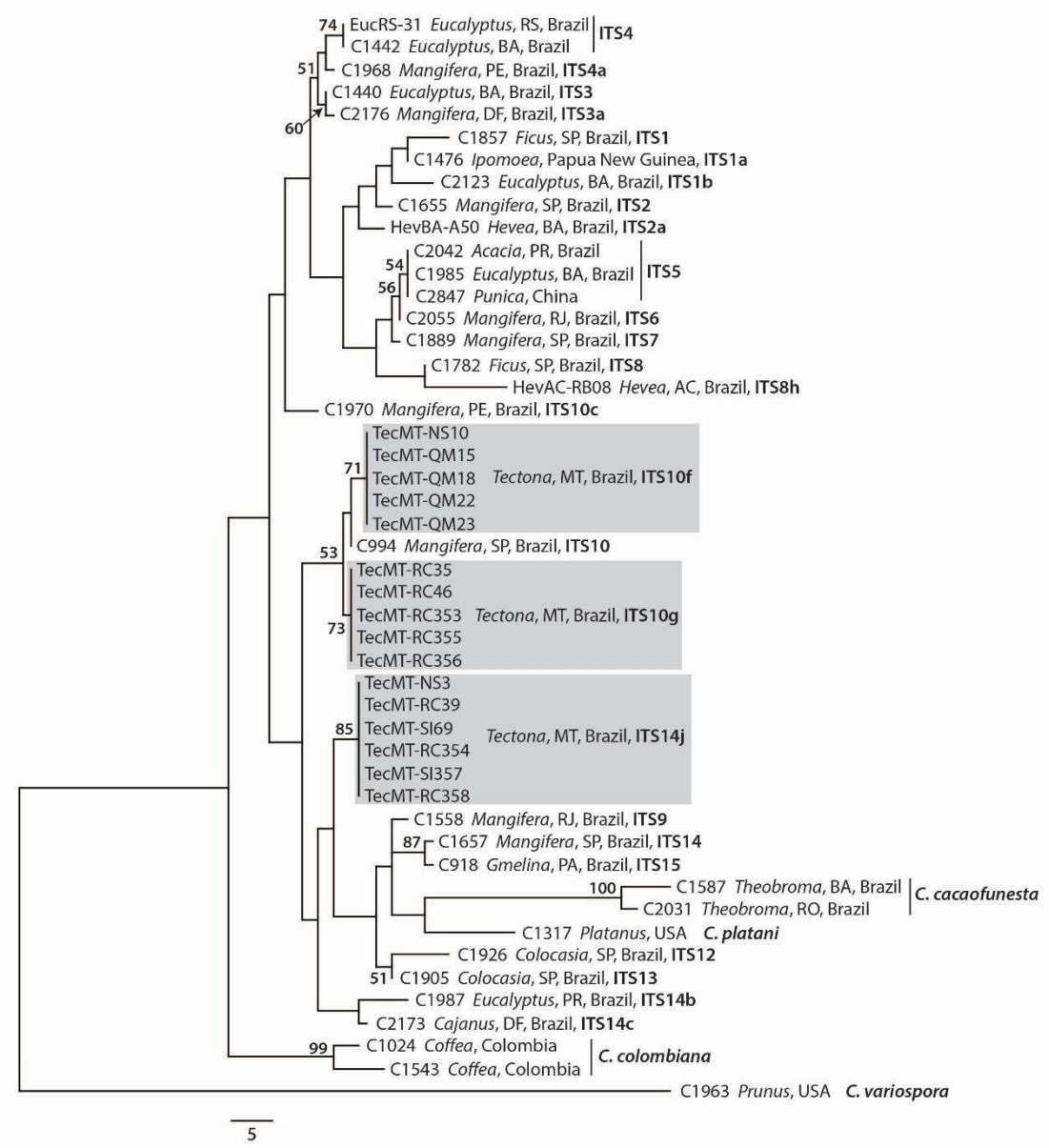

Fig. 3. One of 45 most parsimonious trees based on the ITS rDNA sequences of representative isolates of the Latin American Clade (LAC) of the *Ceratocytsis fimbriata* complex. The tree is rooted to *C. variospora* of the North American Clade (NAC), and all other isolates are considered to be in the LAC. The host genus, state (BA = Bahia; DF = Distrito Federal; Mato Grosso = MT; PA = Pará; PE = Pernambuco; PR = Paraná; RJ = Rio de Janeiro and SP = São Paulo) or country of origin are given for each isolate. The ITS haplotype designations are indicated in the right. Bootstrap values greater than 50% are indicated on appropriate branches. Scale bar indicates base pair differences.

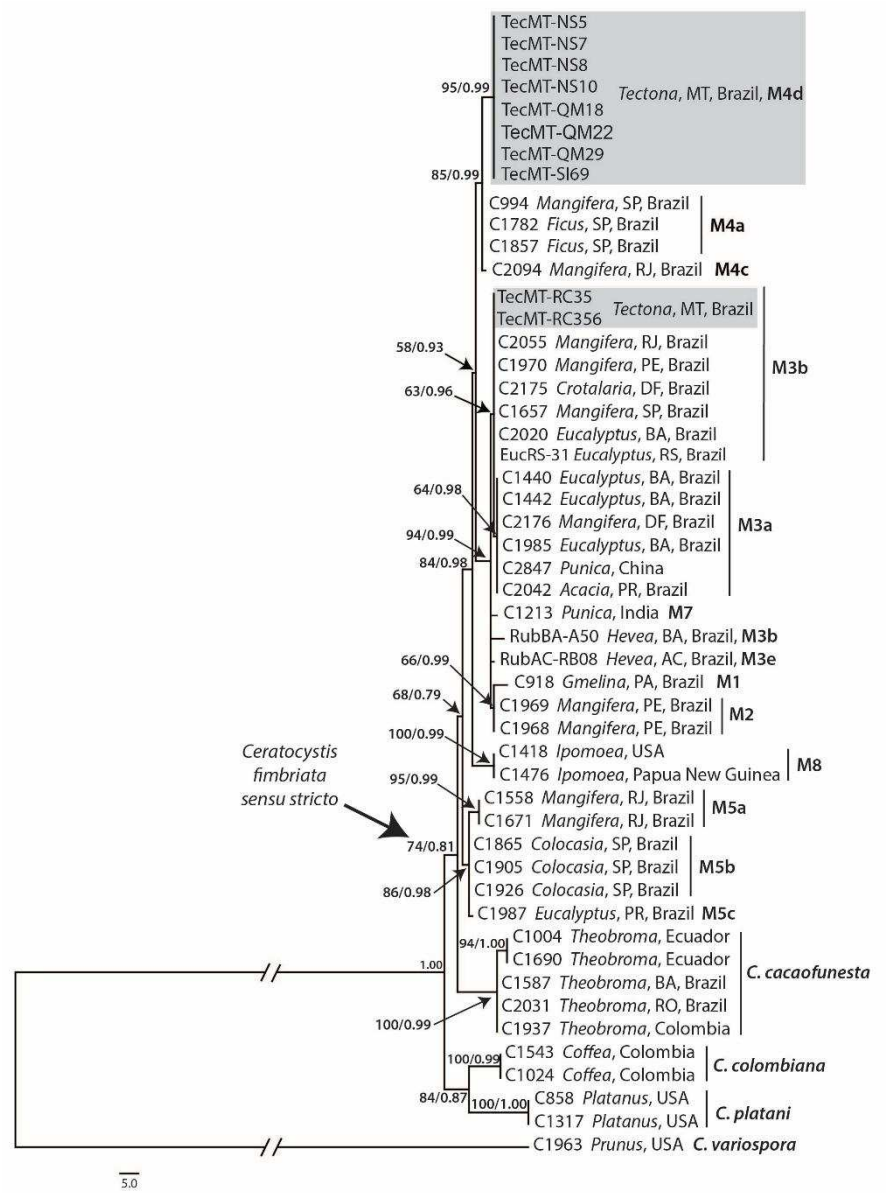

Fig. 4. The single most parsimonious tree of the 475 steps based on portions of the MAT1-1-2 (MAT1) and MAT1-2-1 (MAT2) mating type genes *Ceratocystis fimbriata* and other members of the LAC (*C. cacaofunesta*, *C. colombiana*, and *C. platani*). The tree was rooted to *C. variospora*, a member of the North American Clade of the *C. fimbriata* complex. Bootstrap values greater than 50% /posterior probability value greater than 0.85 are indicated on appropriate branches. The host genus, state (BA = Bahia; Mato Grosso = MT; PA = Pará; RJ = Rio de Janeiro and SP = São Paulo) or country of origin are given for each isolate. The mating type haplotype designations are indicated in the right. Scale bar indicates base pair differences.

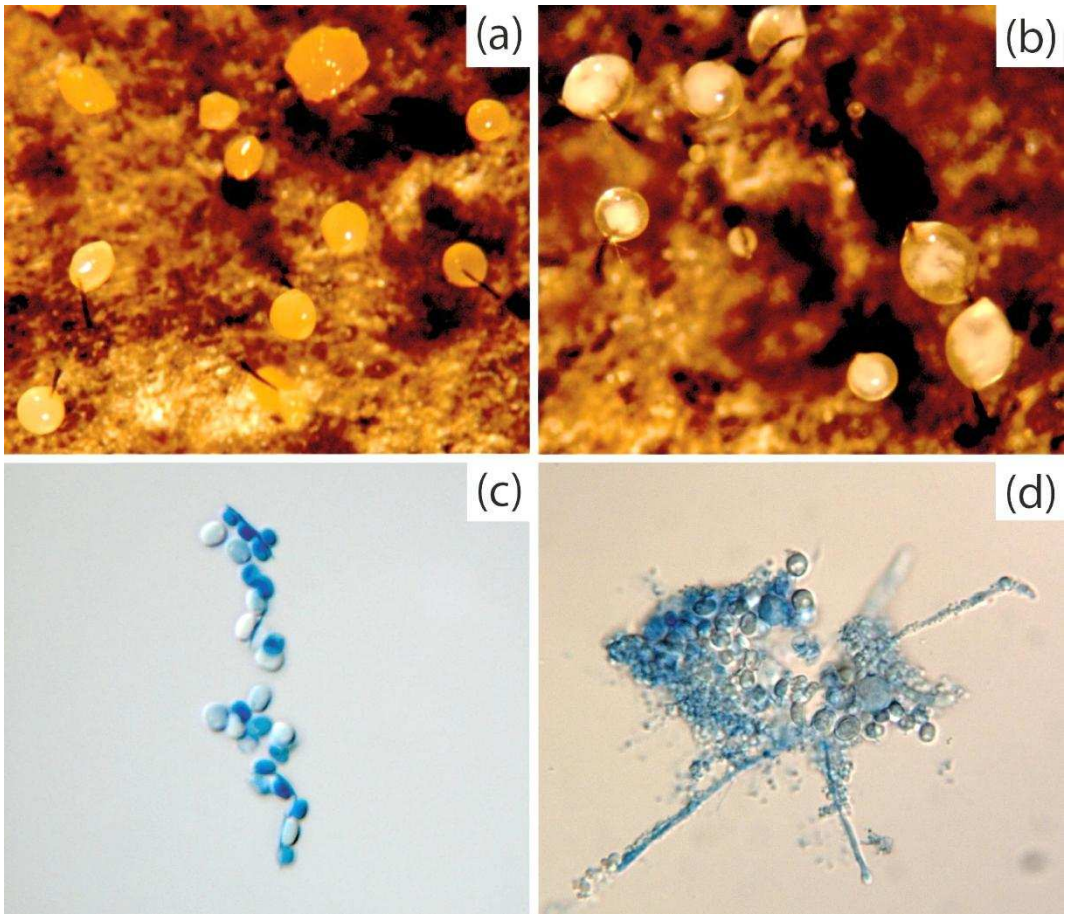

Fig. 5. Example of inter-fertile and inter-sterile crosses between isolates of *Ceratocystis* spp. (a) Perithecia and ascospore mass and (c) ascospore from interfetile cross between *C. fimbriata* strains from *Tectona grandis* (teak) (TecMT-RC35) and from *Eucalyptus* (C1347); (b) and (d) interspecific cross between an teak strain of *C. fimbriata* (TecMT-RC35) and a *Platanus* strain of *C. platani* (C1343) showing small watery ascospore masses (b) and misshapen ascopore (d).

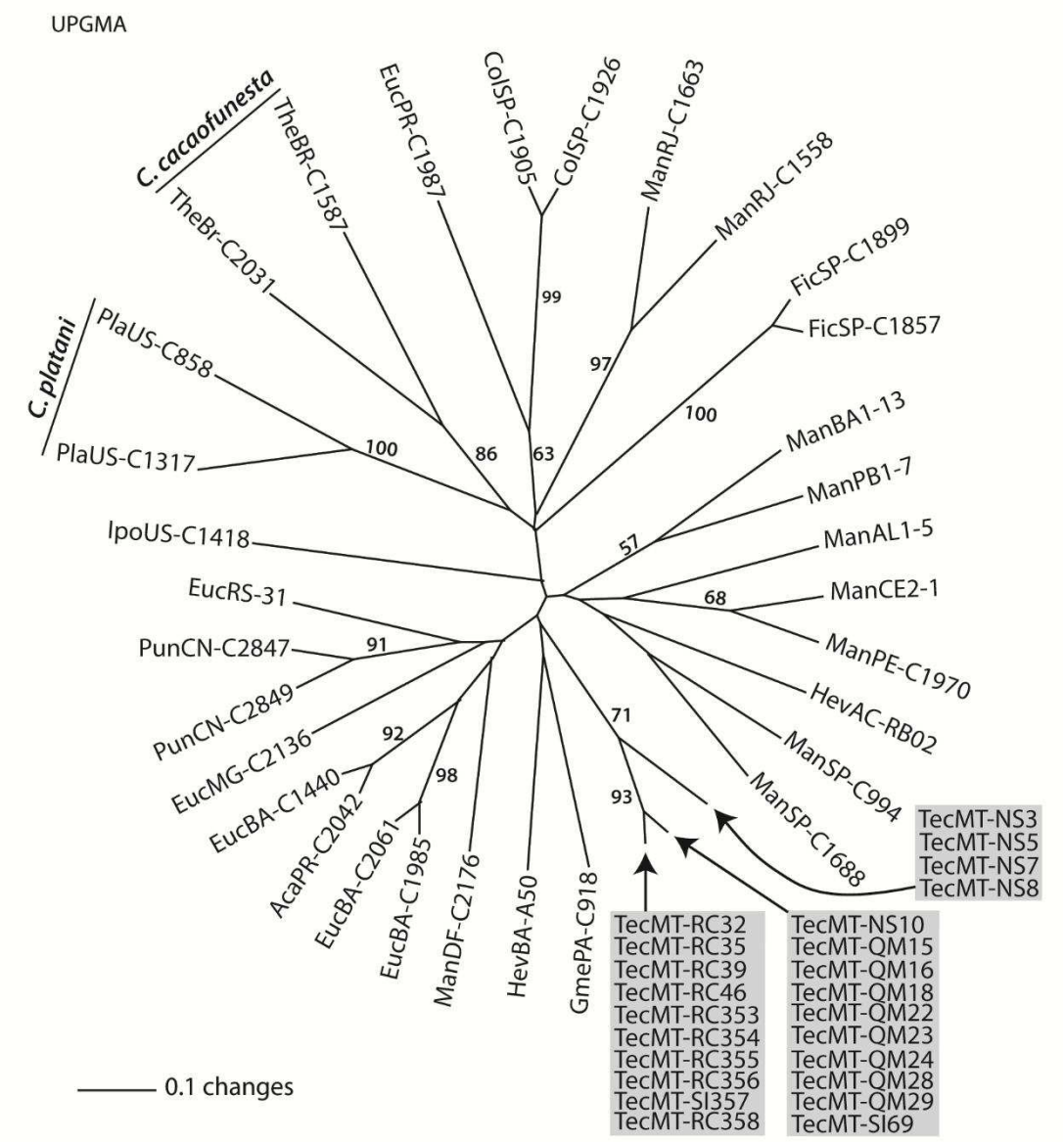

Fig. 6. A UPGMA (unweighted pair group method arithmetic mean) dendrogram of genotypes of *Ceratocystis fimbriata*, *C. cacaofunesta* and *C. platani* based on alleles of 14 microsatellite loci. The first three letters indicate the host genus (Aca = *Acacia;* Col = *Colocasia*; Euc = *Eucalyptus*; Fic = *Ficus*; Gme = *Gmelina*; Hev = *Hevea*; Ipo = *Ipomoea*; Man = *Mangifera*; Pla = *Platanus*; Pun = *Punica*; Tec = *Tectona* and The = *Theobroma*), and next two letters indicate the Brazilian state (AC = Acre; AL = Alagoas; BA = Bahia; CE = Ceará; MG = Minas Gerais; MT = Mato Grosso; PA = Pará; PB = Paraíba; RJ = Rio de Janeiro and SP = São Paulo) or country (BR = Brazil; CN = China and US = United States) of origin. Scale bar indicates genetic distance.

CHAPTER 2

ARTICLE 2

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

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ABSTRACT

Recently, *Ceratocystis fimbriata* Ellis & Halsted was recorded causing seedling blight on *Carapa guianensis* (andiroba), a native tree species to the Amazon Rainforest. This species is prized for its high valuable timber and medicinal seed oil production. Generally, wilt-type disease caused by *C. fimbriata* is more common in woody hosts; however, on andiroba the disease occurs in seedlings and seeds, affecting the species regeneration. In Brazil, few native host species have been identified and the host-specialized pathogen may co-evolve with the plants they affect. Then, this work aimed to study the taxonomy of the fungus obtained from andiroba through phylogenetic analyses using the mating type genes and intersterility tests, ITS rDNA

fingerprinting, estimate the genetic variability using microsatellite markers and evaluate the aggressiveness and host specificity by cross inoculations. Analysis of ITS rDNA sequences and phylogenetic analysis using the mating type genes (MAT-1 and MAT-2) of 73 isolates showed new haplotypes of *C. fimbriata* from LAC. In the mating experiments, andiroba isolates were inter-fertile with tester strains of *C. fimbriata*, confirming that they belong to a single biological species. Fourteen genotypes and intermediary levels of genetic variability were found in andiroba populations. In addition, the results of inoculation tests showed evidence of host specialization.

Keywords: American Latin Clade, microsatellite, genetic diversity, mating type genes, ITS barcoding.

Introduction

Carapa guianensis Aubl. (andiroba), a woody plant that belongs to the Meliaceae , is native to the Amazon Rainforest region (Tropicos 2016). It is prized for its high valuable timber and seed oil production, used for cosmetic and medicinal purposes (Souza et al. 2006). Furthermore, this species has great social and economic values for Amazon extractive populations because its products increase income as well as oil from seed, used for medicinal purposes. (Klimas et al. 2012; Herreno-Jáuregui et al. 2013; Vinhote 2014). Recently, seedling blight, caused by *Ceratocytsis fimbriata sensu lato* (s.l.) was recorded on andiroba (Halfeld-Vieira et al. 2012). *Ceratocystis fimbriata* s.l. involves a complex of many cryptic species with a large number of economically important hosts (CAB International 2005; Harrington 2009; Harrington et al. 2011; Harrington 2013). Generally, wilt-type disease caused by *C. fimbriata* is

more common in woody hosts as *Mangifera indica* L. (Carvalho 1938), *Gmelina arborea* Roxb. Ex Sm. (Muschovej et al. 1978); *Ficus carica* L. (Valarini & Tokeshi 1980); *Eucalyptus* spp. (Ferreira et al. 1999); *Tectona grandis* L. f (Firmino et al. 2012), among others. In andiroba, the disease occurs in seedlings, and was characterized by the presence of fungal sporulation on stems and petioles, affecting the species regeneration (Halfeld-Vieira et al. 2012).

Despite *C. fimbriata* affects mainly woody plants, its first report was as the causal agent of black rot on sweet potato (*Ipomoea batatas* (L.) Lam.) (Halsted 1890), considered the type species of the genus. Currently, the fungus from sweet potato is treated as *C. fimbriata sensu stricto* (*s.s.*) while isolates of cryptic species of the complex are treated as *C. fimbriata sensu lato* (*s.l.*) (van Wyk et al. 2010; Harrington et al. 2014). Although there is limited morphological variation within the complex (Webster & Butler 1967), in Latin American Clade (LAC) (Harrington 2000; Harrington 2009; Harrington et al. 2011) new species have been recognized based on DNA sequence variation (Rodas et al. 2008; van Wyk et al. 2009; 2010, 2011a, 2011b). However, according to Harrington & Rizzo (1999), to describe new species is necessary that lineages show unique and diagnosable phenotypic characters. Host specialization may have been a driving force in relatively recent speciation events (Harrington et al. 2002; Harrington et al. 2014). In the LAC, three host specialized lineages were identified on *Theobroma cacao* (cacao), *Ipomoea batatas* (sweet potato) and *Platanus* spp. (sycamore) (Baker et al. 2003). Later, studies of intersterility and morphological characterization allowed to describe the cacao pathogen as a new species, *C. cacaofunesta* Engelbr. & T.C. Harrin., and elevate the sycamore pathogen to a species level named as *C. platani* Engelbr. & T.C. Harrin., and sweet potato pathogen remaining as *C. fimbriata s.s.* (Englebrecht & Harrington 2005). In North

America Clade (NAC), two lineages host specialized to *Populus tremuloides* Michx. and *Carya illinoensis* (Wangenh.) K. Koch. have been defined to new species, *C. populicola* J.A. Johnson & Harrington and *C. caryae* J.A. Johnson & Harrington, respectively (Jonhson et al. 2005). Besides the *C. fimbriata* complex, in *Ceratocystsis coerulescens* complex, lineage host specialized have also been identified (Harrington et al. 2002).

Ceratocystis fimbriata is a soilborne pathogen that seems to be native to Brazil (Ferreira et al. 2010; 2103; Oliveira et al. 2015). Nevertheless, few native hosts of *C. fimbriata* has been identified in Brazil, which includes *Hevea brasiliensis* L., (Alburqueque et al. 1972; CAB International 2005; Valdetaro et al. 2015) and *Theobroma cacao* L. (Englebrecht & Harrington 2005). However, *C. fimbriata* populations from *H. brasiliensis* were introduced in a cultivated area (Valdetaro et al. 2015) and on *T. cacao* the fungus seems to be native from upper Amazon region, but was considered as a new species within the *C. fimbriata* complex (Englebrecht & Harrington 2005).

Then, this work aimed to: a) study the taxonomy of the fungus from andiroba through phylogenetic analyses using the mating type genes and intersterility test; b) analyze the ITS rDNA as fingerprimting; c) estimate the genetic variability using microsatellite markers to determine whether *Ceratocystis* populations on andiroba are native to Amazon region; d) evaluate the aggressiveness and host specificity of the fungal by cross-inoculations on mango, teak, eucalyptus and andiroba.

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Materials and Methods

Sample collection and fungal isolation

Andiroba seedlings with stem blight symptoms and fungal sporulation on stem and seeds were collected in native Amazonian Rain Forests in the states of Acre (AC), Amazonas (AM) and Roraima (RR) (Fig. 1). Four populations were analyzed: two from Roraima (São João da Baliza), one fromin Acre (Rio Branco) and one from Amazonas (Boa Vista do Ramos/Parintins) (Fig 1). The samples were collected in an area of 50 m radius around andiroba trees. Seeds as well as seedlings infected with the fungus were brought to the Laboratory of Forest Pathology, DFP/BIOAGRO at the Universidade Federal de Viçosa (UFV). The infected tissue was transferred to carrot baits (Moller & DeVay 1968) and subsequently incubated at room temperature (approximately 25 °C) to stimulate ascomatal production. Then, the ascospore mass formed in the apice of perithecia on the material incubated was transferred to plate with MYEA (2% malt extract, 0.2% yeast extract, and 2% agar) (Ferreira et al. 2010; Alfenas & Mafia 2016). For all isolates, the single ascospore strains were obtained by collecting the ascopore masses and dispersing in about 10 µL of oil autoclaved. Then, the spore suspension was streaked over the fresh plate with MYEA. After 12h, approximately, individual germlings were transferred to fresh plates (Harrington & McNew 1997). One self-fertile isolate per tree was stored in 15% glycerol at -80 °C at the Forest Pathology Laboratory at the Universidade Federal de Viçosa, Brazil. Some andiroba isolates were also stored in the Forest Pathology Laboratory at Iowa State University, USA.

DNA extraction

The fungus was grown on MYEA (Malt Yeast Extract Agar) and incubated at 28 °C for about 15 days before DNA extracting using the Wizard® Genomic DNA Purification Kit (Promega Inc. – Madison, USA) with the manufacturer's protocol modified by Valdetaro et al. (2015). The concentration of purified DNA was quantified with a Nanodrop 2000c® (Thermo Fisher Scientific Inc., Massachusetts, USA) and adjusted to 50 - 100 ng μL^{-1}.

ITS and mating type genes sequences

For all andiroba isolates, amplification and sequencing of the ITS rDNA region, including 5.8s gene, was performed using the primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). Portion of the MAT-1 (*MAT1-1-2*) and MAT-2 (*MAT1-2-1*) genes were amplified and sequenced. The primers CFMAT1-F and CFMAT1-R (Harrington et al. 2014) were used to amplify and sequence about 1040 bp region of MAT-1. The primers X9978R1R and CFM2-1F (Harrington et al. 2011) amplify and sequence about 1131 bp region of MAT-2 .

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

The ITS sequences obtained of andiroba isolates were compared with those reported earlier for the LAC (Harrington et al. 2011, 2014) using parsimony analyses.

Phylogenetic analyses based on mating type genes

Mating type genes (*MAT1-1-2* and *MAT1-2-1*) sequences were analyzed separately. The partition homogeneity test (PHT) was conducted using PAUP 4.0b1.0 (Swofford 2003) in order to determine whether the datasets could be combined. Maximum parsimony (MP) and Bayesian inference (BI) were used to construct phylogenetic trees. The MP analysis was performed by using PAUP * 4.0b10 (Swofford 2003). The heuristic search based on parsimony with the TBR algorithm (Tree Bissection Reconnection) and stepwise addition with 1000 random repetitions were used. Gaps were treated as a fifth base and all characters had egual weight.

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

Sequences of isolates obtained in this study were compared with representative isolates of the *C. fimbriata* complex, including *C. cacaofunesta*, *C. colombiana* M. van Wyk & M. J. Wingfield and *C. platani* (Table 1). *Ceratocystis variospora* (R.W. Davidson) C. Moreau from the North American Clade (NAC) was used as the outgroup taxon.

Mating experiments

Carapa guianensis (andiroba) isolates from each state were selected as MAT-1 (female-only) testers. The self-sterile MAT-1 testers (females) were obtained from single ascospore isolation from self-fertile field isolates.

Self-sterile isolates, MAT-2 (male-only), were selected for mating experiments: one isolate of *C. fimbriata* from *T. grandis* (teak) (Sinop), one from *I. batatas* (sweet potato), one from *Eucalyptus* spp. and three isolates from *M. indica* (mango), and one isolate of *C. platani* (Oliveira et al. 2015). The MAT-2 testers were recovered from sectors, formed spontaneously by subculturing the colonies systematically. The DNA was extracted and PCR was conducted using the primers CFM2-1 and X9978R1R in order to confirm the presence of MAT-2 region. The male (MAT-2) and female (MAT-1) testers were grown on MYEA for 7 days at room temperature. The conidial suspension of the male tester was prepared as described previously (Ferreira et al. 2010; Oliveira et al. 2015). Then, 1 mL was dispersed over the female colony for spermatization.

During 3 to 4 weeks, the cultures were observed for the presence of perithecia and ascospore masses. Ascospore masses were examined microscopically (400 × magnification) to see the spores quality (normal appearance indicating intraspecific pairing or watery indicating hybrid). To observe the mycelial morphology, ascospore masses from the progeny were spread onto fresh MYEA. In successful crosses, the progeny showed the both mycelial phenotype of the parents. However, unsuccessfully pairing, the progeny showed the uniform mycelial morphology due the selfing (Ferreira et al. 2010; Oliveira et al. 2015).

The experiment was repeated twice and the strains were considered interfetile if they crossed in at least one of the two experiments.

Microsatellite analyses

Fourteen microsatellite loci developed by Steimel et al. (2004), and recently mapped onto the *C. fimbriata s.s.* genome sequence (Simpson et al., 2013), were analyzed. This microsatellite markers are widely used in studies of population structure involving species within the Latin American Clade (Engelbrecht et al. 2004; Engelbrecht et al. 2007b; Ocasio-Morales et al. 2007; Ferreira et al. 2010, 2011; Oliveira et al. 2015; Valdetaro et al. 2015; Harrington et al., 2015; Li et al., 2016). For each primer pair specific to the flanking regions of fourteen simple sequence repeat regions, one of the primers was fluorescently labeled. The microsatellite analyses were conducted in Iowa State University (ISU), USA. PCR amplifications of all microsatellite loci were performed using a PCT 100 (MJ Research Inc) following the earlier described conditions (Ferreira et al. 2010). Band sizes of the product were determined using a four-capillary ABI Prism 3100- Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and ABI Peak Scanner v1.0 Analysis Software (Life Technologies). Each product length (within 1 bp) was considered to be a different allele. Most of the microsatellite loci contained trinucleotide repeats, and most alleles of a given locus differed by increments of 3 bp.

Genetic variation of andiroba populations was compared with representative *C. fimbriata* populations on *T. grandis*, *Colocasia esculenta* (L.) Schott (taro), *Eucalytpus* spp. (Ferreira et al. 2010) and *M. indica* (Oliveira et al. 2015) in Brazil using the microsatellite data. Nei's gene diversity (*H*) for each population was calculated without and with clone-corrected data using PopGene 1.32 software. Multilocus genotypic diversity was estimated with the Stoddart and Taylor's *G* index (Stoddart & Taylor 1988). *G* values were estimated based on rarefaction curve according the number of genotypes in the smallest size sampled (Grünwald et al. 2003)

using the Vegan package from CRAN in R v.2.6.1 (R Core Team 2007). For comparisons among individual populations, the maximum value of *G* was 5, and for comparisons among different host groups and regions was 13.

Nei's genetic distance between populations and UPGMA (unweigted pair group method with arithmetic mean) dendrogram were constructed using POPULATIONS 1.2.30 (Langella 1999). Bootstrap values were calculated from 100 replications using POPULATIONS 1.2.30.

Partition of total variance using analysis of molecular variance (AMOVA) on Euclidean distance was performed using ARLEQUIN v3.11 (Excoffier et al. 2005) to determine variation associated with different levels of genetic structure (host-associated groups, among populations within groups, and within populations).

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

Pathogenicity tests

Three representative isolates from andiroba (CarAC-144B2, CarAM-ANDP1 and CarRR1-RR74) and one isolate from eucalyptus (EucBA-SBS1), one from mango (ManCE-CEBS13) and one from teak (TecMT-QM29) were used in two inoculation experiments. All of the isolates were inoculated on andiroba seedlings (three-months old), on mango seedlings (cultivar Espada, 19-months old), on the hybrid clone 1172 of *E. urophylla* x *E. grandis* (three-month old) and on teak seedlings (six-months old). The plants were transplanted into 2 L pots containing the substrate Carolina II

(Carolina Soil do Brasil) supplemented with 6 kg m^{-3} of superphosphate and 1,5 kg m^{-3} Basacote® (19-6-10) (ComPo do Brasil). However, the teak plants were grown in potting mix with soil, sand and substrate Carolina II (in proportion 1-1-1). Both experiments were conducted in a completely randomized design, consisting of two factors (host × isolate) with seven replications per treatment. The plants were wounded (around 3-mm deep) with a sterile scalpel at 3 cm above the ground. A volume of 500 µL of the inoculum (3×10^6 spores ml^{-1}) was applied into the wound and the inoculations site wrapped with Parafilm to reduce desiccation and contamination. The control plants were wounded and treated with the same volume of sterile distilled water. The first experiment (inoculation day – May 13th 2016), the plants were incubated in the greenhouse at 23.08 ºC (7.9 ºC – 44.6 ºC). The second experiment (inoculation day – June 24th 2016) was performed during the winter, and because of weather conditions, the plants were incubated in growth chamber at 25.45 ºC (22 ºC - 30.5 ºC), 12 h photoperiod and 95.66 µM photons/s/m^2. After 60 days, the length of xylem discoloration was measured. To re-isolate the fungus, the carrot bait method (Moller and DeVay 1968) was used. The variance analyses (ANOVA) of the data was conducted and for each experiment, means of xylem discoloration length were compared by Fisher's Protected LSD test ($P< 0.01$) using Statistica® software (StatSoft Inc.).

Results

Sample collection and fungal isolation

A total of 194 samples, including infected seeds and seedlings, was collected and the fungus was successfully isolated from 73 samples (Fig. 1). The isolates showed uniform mycelial morphology and were self-fertile. In Acre and Roraima, we observed

seedlings showing symptoms of *Ceratocystsis* seedling blight (Fig 2B) as well as fungal sporulation (Fig 2C and 2E) and, in some cases, the presence of perithecia on the stem (Fig 2D). In Amazonas population, only seeds were collected. The infected seeds were covered by fungal sporulation and, sometimes, the presence of gummosis was observed (Fig 2F), likely in response to the fungus infection. A high number of seedlings affected by *C. fimbriata* was observed bellow the andiroba trees in the field. In some cases, one or a very few healthy seedlings were observed coming from the same fruit (Fig 3). These plants could be resistant or just escaped from the disease.

ITS

For 73 andiroba isolates, only one from Amazonas (CarAM-ANDP2) could not be sequenced for the ITS region. This isolate had mixed ITS sequences, that is, the sequence generated with the ITS1F primer initially gave clean reads in electropherogram and then overlapping peaks followed (Harrington et al. 2014; Oliveira et al. 2015).

The Maximum parsimony analysis found 9 trees of 321 steps with 99 number of variable characters. From those characters, 73 were parsimony informative in aligment of the 613 bp. The ITS haplotype numbers follow the designations by Harrington et al. (2011). The letters were named following the sequences previously described (Harrington et al. 2014; Oliveira et al. 2015).

Three new ITS haplotypes (ITS17, ITS17a and ITS18) were identified among the sequenced andiroba isolates (Fig. 4). The ITS17 and ITS17a were found in two isolates from Acre. All of the other isolates sequenced had ITS18 haplotype, but only two isolates per each state was showed in the ITS tree.

Phylogenetic analyses based on mating type gene

For phylogenetic analyses, the mating-type genes are more suitable than ITS. Then, fifteen andiroba isolates out of 73 from Acre, Amazonas and Roraima states were selected for sequencing of mating type genes. For MAT-1 (*MAT1-1-2*), an alignment of 1040 characters one haplotype were identified among the isolates tested. For MAT-2 (*MAT1-2-1*), an alignment of 1131 characters showed 62 characters varying and three different haplotypes were found among the isolates analyzed.

Mating type genes when combined produced a robust phylogenetic tree from the *C. fimbriata* complex because they are tightly linked and, different of the other genes, cannot be recombined during the crossover events (Harrington et al. 2014). Even so, the partition homogeneity teste (PHT) was performed for the combined dataset of MAT-1 and MAT-2 genes and showed that they could be combined (P = 0.26) (Cunningham 1997; Barker & Lutzoni 2002).

The combined dataset of MAT-1/MAT-2 sequences of andiroba (fifteen isolates) and from other hosts found in the LAC (Harrington et al. 2014) had an alignment of 2171 characters. The number of variable characters was 373, and 84 of those characters were parsimony informative. Maximum parsimony (MP) analysis found a single tree of 494 steps, with homoplasy index (HI), consistency index (CI), rescaled consistency index (RC), and retention index (RI) were 0.0486, 0.9514, 0.8816 and 0.9266, respectively. Evolution model HKY+G was selected and incorporated into the Bayesian analysis, and the level of convergence from two parallel runs after 1000 000 generations had a mean standard deviation of split frequencies of 0.00665.

The likelihood tree from Bayesian analysis had very similar topology of the MP tree. Therefore, the parsimony tree was selected for illustration (Fig. 5). Other

species from the LAC (*C. cacofunesta*, *C. colombiana*, and *C. platani*) had bootstrap support of 100% and posterior probability (PP) of 1.00 (Fig 5).

All andiroba isolates haplotypes were placed along with the other Brazilian isolates. Three new mating haplotypes (4e, 4f and 4g) were found closely related to each other among the 15 isolates analyzed. The mating haplotypes were named following the sequence previously described by Harrington et al. (2014). The number of the haplotype was defined according with the haplotype number of the isolate more closely related and the letters followed the sequence used previously by Harrington et al. (2014) for this number.

The 4e and 4f haplotypes did not match to any other sequence previously described. The haplotype 4e was found in four isolates (two from Roraima and two from Acre) and was separated by moderately-supported (65%) boostrap and a high posterior probability value (= 0.98). The haplotype 4f was found in one isolate from Acre. However, the haplotype 4g was found among the isolates from andiroba and cacao (C1584).

Mating experiments

MAT-1 testers from andiroba isolates successfully crossed with the majority of the other Brazilians testers of opposite mating type (Table 2). In most of the successful crosses, (normal and mixed), there were many fully developed perithecia within a week and they produced thick, creamy ascopores masses at the tips of perithecia necks (Fig. 6A). Microscopic examination showed abundant, normal appearing ascopores (Fig. 6C). Ascopores masses from normal cross were streaked on fresh plates to demonstrated that ascopore masses is not due to a selfing. The colonies produced mycelial morphology of the both male and female testers strains. In unsuccessfully

crosses, only a few perithecia were produced and ascospores masses were watery (Fig. 6B), and microscopic examination showed few misshapen ascospores (Fig. 6D).

Microsatellite analyses

Of the 14 microsatellite loci tested on the 73 andiroba isolates, eight loci were monomorphic and six were polymorphic (Table 3). The UPGMA tree constructed based on Nei's genetic distance (Fig. 7) showed that andiroba genotypes grouped separately from the other isolates with high support (95%). Fourteen genotypes were found among the andiroba isolates and three microsatellite genotypes (AMSAT1, AMSAT2 and AMSAT3) were found in more than one population (Fig. 7).

The UPGMA tree constructed using allele frequencies of populations (Fig. 8) showed that andiroba populations grouped separately from the other Brazilian populations and had high bootstrap support (100%). The UPGMA tree showed clear separation according to the host and geographic distribution. That was confirmed by analysis of molecular variance (AMOVA), which shows that more than a half (62%) of the genetic variation was attributed to variation among host-associated groups (Table 4). The AMOVA also showed that 21% was attributable to diversity within populations, and 17% to variation among populations within groups.

Nei's gene diversity (*H*) and Stoddart and Taylor's genotypic diversity (*G*) (Table 5) were calculated for populations of *C. fimbriata* from andiroba and these values were compared to other Brazilian isolates from different hosts and geographic distribution. The Amazonas population showed greater gene and genotypic diversity (H = 0.1486; G = 3.3889) (Table 5) among andiroba populations. Furthermore, Brazilian populations from *Eucalyptus (*H = 0.3212; G = 8.2371), from *Mangifera* in the Northeast Brazil (H = 0.3758; G = 7.9237) and *Colocasia* (H = 0.1530; G = 6.7460)

had greater gene and genotypic diversity than andiroba population (H = 0.1271; G = 4.8055) (Table 5).

Pathogenicity tests

At the end of the experiments, none of the plants were killed, however wilting was observed in some inoculated plants. Re-isolations of the pathogen from discoloured tissue of the inoculated plants yielded typical colonies of *C. fimbriata* even in those with small lesions, which shows that the fungus was alive but the fungus was confined in a small portion of the host tissue. The controls remained asymptomatic and we observed limited discoloration (wound reaction) near the wound.

The multifactorial ANOVA found significant variation in the length of xylem discoloration between two experiments as well as among the isolates inoculated (greenhouse - F=14.44, P < 0.0001 and growth chamber – F = 47.64, P < 0.0001), the hosts (greenhouse – F = 25.99, P < 0.0001 and growth chamber – F = 83.36, P < 0.0001) and isolate × host interaction (greenhouse – F = 15.43, P < 0.0001 and growth chamber - F=48.58, P < 0.0001).

In the growth chamber, despite the temperature had been uniform (22 ^{o}C - 30.5 ^{o}C) during the experiment, the relative humidity was very high (90.76 %) resulting in edema formation on leaves and stem of andiroba. On the other hand, the light intensity was relatively low (95.6 μM photons/s/m^2) and some hosts as teak, mango and eucalyptus suffered from cochineal attack probably because of unsubtle conditions for plant growth for such a long period of incubation (60 days). In both experiments, wilting occurred in hosts, from which the isolates were originally obtained. Although, some un-inoculated plants (controls), mostly eucalyptus, also wilted and displayed only wound reaction. In the growth chamber, the lesions were generally bigger (Table

6) and more plants wilted, probably because of the high favorable conditions for the fungal infection and especially because of less favorable conditions for growth and reaction of the plants. The lesions on mango induced by non-host isolates were discontinuous and narrow, differently from the lesions induced by the mango isolate.

Andiroba, mango and eucalyptus isolates inoculated in theirs respective hosts caused typical discoloration, extended continuouslly from the inoculation point, especially upwards.

In both experiments (greenhouse and growth chamber), all three andiroba isolates caused significantly bigger lesions in andiroba plants than did the other isolates, which did not differ from the controls. However, on mango under growth chamber conditions, all isolates induced xylem discoloration significantly different from the controls, but the mango isolate caused significantly greater lesion. In both experiments, mango and eucalyptus showed bigger lesions than the other hosts, inoculated with their respective isolates. Although the lesion was relatively small, compared to other hosts and isolates, TecMT-QM29 caused xylem discoloration significantly different from the control. Variation in aggressiveness was found among the andiroba isolates tested.

Discussion

Analysis of ITS rDNA sequences found two new haplotypes that belong to *C. fimbriata* from LAC. The phylogenetic analysis, using sequences of mating type genes (*MAT1-1-1* and *MAT1-2-1*), showed that andiroba isolates form a single group strongly supported and closely related to other Brazilian isolates, including the isolate from sweet potato, on which the fungus was originally described (Halsted 1890). The andiroba group, formed on the MP tree of the mating type genes, showed three mating

type haplotypes, which are not correlated to the states they were isolated from. However, the andiroba isolates from the three states formed a group, sharing the same recent ancestor. Interestingly, one of three haplotypes matched with one cacao from Trinidad (C1584) (Engelbrecht & Harrington, 2005). However, the isolate C1584 differed genetically from isolates of *C. cacaofunesta* and was not pathogenic to cacao plants, and it was intersterile with members of *C. cacaofunesta* (Engelbrecht & Harrington 2005; Baker et al. 2003). Therefore, we hypothesized that this isolate could have been introduced on plant material from Upper Amazon or Northern South American into Trinidad and it may be in fact *C. fimbriata*.

Although the andiroba populations showed to be a distinct group, the phylogenetic analysis and mating studies showed that they are not reproductively isolated because they were interfertile with the sweet potato type strain of *C. fimbriata*, as well as with *Eucalyptus*, *Mangifera* and *Tectona* strains. In contrast, the andiroba isolates were intesterile with *C. platani*, another species from LAC (Engelbrecht & Harrington 2005). Thus, the andiroba isolates can be consider *C. fimbriata* using the biological species concept (Harrington et al. 2011; Harrington et al. 2014).

Microsatellite analysis showed that fourteen andiroba genotypes grouped separately from the other Brazilian genotypes. Three genotypes were widely distributed within and among the three andiroba populations. However, one of the genotypes was found in Roraima and Amazonas. At present, it is not known how this genotype was moved between these places. Future work will be necessary to understand how this happened.

The populations from andiroba were differentiated with strong bootstrap support from populations of other hosts. Furthermore, analysis of molecular variance showed that the differentiation among populations was due the variation among

populations located in different regions of Brazil. This is expected because of the high genetic variability found in some populations (Engelbrecht et al. 2007a; Ferreira et al. 2010; Oliveira et al. 2015).

Furthermore, andiroba populations of *C. fimbriata* had intermediary levels of genetic diversity, compared to other populations considered native to Brazil, such as those of *Eucalyptus* and *Mangifera* from Cerrado and Caatinga forest type, respectively (Ferreira et al. 2010; Oliveira et al. 2015), and *Colocasia* population along with *Mangifera* population from eastern Rio de Janeiro, which seems to be native to coastal Mata Atlântica forest type (Baker et al. 2003; Silveira et al. 2006; Harrington et al. 2014; Oliveira et al. 2015). However, differently from the values found in *Mangifera*, *Eucalyptus* and *Colocasia* populations, the gene diversity within the andiroba populations was limited. *Ceratocystis fimbriata* is homothallic through uni-directional mating type switching (Harrington & McNew, 1997; Witthuhn et al. 2000) its reproduction is mostly asexual or through selfings, wich could, probably, result in the low gene diversity (*H*). Among the andiroba populations, the population from Amazonas state had highest genetic diversity. Isolates in this region were obtained mostly from contaminated seeds. In the Amazon forest, rubber tree and andiroba are the only native hosts of *C. fimbriata* identified so far. Considering the results of microsatellite analysis, *C. fimbriata* on andiroba seems to be native to the Amazon rain forest.

Additionally, the results of inoculation tests suggest host specialization. The andiroba isolates infected only andiroba plants, except mango kept in growth chamber, in which all isolates caused xylem discoloration. Infection on mango by all isolates tested can be attributed to the plant´s predisposition for the fungus colonization and highly conducive condition for disease development. Host-specialized pathogens

sometimes evolve in the same area as the plants they affect (Engelbrecht et al. 2007b), like *C. cacaofunesta* with *Theobroma*, showing that a co-evolution process is present. Additionally, in Brazil the strains from non-native or cultivated host did not showed evidence of host specialization, instead they varied widely in aggressiveness to various host species (Ito et al. 1988; Baker et al. 2003; Harrington et al. 2011; Valdetaro et al. 2015). Host specialization is helping in delimitation of species within *Ceratocytsis* complex, but the intersterility test have been very important to complete the identification of cryptic species (Harrington et al. 2002; Johnson et al. 2005; Engelbrecht & Harrington 2005; Ferreira et al. 2010; Oliveira et al. 2015). Despite to the interfertility between andiroba isolates and sweet potato isolate (*C. fimbriata s. s.*), the fungus from andiroba may be under speciation process. In the process to splitting of the one species into two (speciation process), the intersterility can occur at early or late stages of speciation (Giruad et al. 2008). According to the latest referred authors, the intersterility is the stage at which the process has become irreversible, but this stage may take very long to be achieved. Additionally, according Baker et al. (2003), lineages of *C. fimbriata* that have adapted to American hosts may represent distinct species or populations in the process of speciation.

The infected andiroba samples were collected in a forest site with some human activity by researchers or simply transit of people. Deforestation and human activities in native forests may affect the equilibrium between both native pathogen and host. Thus, the more aggressive strains may dominate and impact negatively andiroba regeneration in this area, located near to private properties.

Therefore, phylogenetic analysis and intersterility tests confirmed that the andiroba isolates belong to *C. fimbriata* ss of the American Latin Clade. Furthermore, recognition of andiroba populations as native to Amazon Rain Forests and host

specialization may contribute to the disease management and avoid introduction of isolates into new areas.

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Tables and figures

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

Species	Hosts	Localization	Representative isolate	ITS sequence acessions	*MAT1-1-2* GenBank accessions	*MAT1-2-1* GenBank accessions
C. fimbriata	*Carapa guianensis*	Acre, Brazil	CarAC-144C3	[A]xxxx	[B]-	-
	Carapa guianensis	Acre, Brazil	CarAC-129B2	xxxx	-	-
	Carapa guianensis	Acre, Brazil	CarAC-144B2	-	xxxx	xxxx
	Carapa guianensis	Amazonas, Brazil	CarAM-MA2	xxxx	xxxx	xxxx
	Carapa guianensis	Roraima, Brazil	CarRR1-RR74	xxxx	xxxx	xxxx
	Tectona grandis	Mato Grosso, Brazil	TecMT-RC353	xxxx	-	-
	Tectona grandis	Mato Grosso, Brazil	TecMT-RC354	xxxx	-	-
	Tectona grandis	Mato Grosso, Brazil	TecMT-QM15	xxxx	-	-
	Tectona grandis	Mato Grosso, Brazil	TecMT-NS8	-	xxxx	xxxx
	Tectona grandis	Mato Grosso, Brazil	TecMT-RC35	-	KF482986	HQ157550
	Hevea brasiliensis	Acre, Brazil	HevAC-RB08	xxxx	xxxx	xxxx
	Hevea brasiliensis	Bahia, Brazil	HevBA-A50	xxxx	xxxx	xxxx
	Actinidia deliciosa	Rio Grande do Sul, Brazil	PP14	-	xxxx	xxxx
	Actinidia deliciosa	Rio Grande do Sul, Brazil	PM20	-	xxxx	xxxx
	Cajanus cajan	Distrito Federal, Brazil	C2173	xxxx	-	-
	Ficus carica	São Paulo, Brazil	C1782 (=CBS 115166)	AY526292	KF482987	HQ157551
	Ficus carica	São Paulo, Brazil	C1857	HQ157542	KF482987	HQ157551
	Gmelina arborea	Pará, Brazil	C918 (=CBS 115173)	AY157967	KF482983	HQ157549
	Eucalyptus spp.	Bahia, Brazil	C1442 (=CBS 115174)	HQ157545	KF482985	HQ157550
	Eucalyptus spp.	Bahia, Brazil	C1440	HQ157544	KF482985	HQ157550
	Eucalyptus spp.	Bahia, Brazil	C1985	AY157966	KF482985	HQ157550
	Eucalyptus spp.	Bahia, Brazil	C2123	AF395685	-	-
	Eucalyptus spp.	Paraná, Brazil	C1987	-	KF482990	HQ157552

[A] The sequence will be deposite in GenBank;

[B] Not used in analyses.

Table 1. Continued

Species	Hosts	Localization	Representative isolate	ITS sequence acessions	*MAT1-1-2* GenBank accessions	*MAT1-2-1* GenBank accessions
C. fimbriata	*Mangifera indica*	Distrito Federal, Brazil	C2176	[B]-	KF482985	HQ157550
	Mangifera indica	Pernambuco, Brazil	C1970	-	KF482986	HQ157550
	Mangifera indica	Pernambuco, Brazil	C1968	AY585343	KF482984	HQ157553
	Mangifera indica	Rio de Janeiro, Brazil	C2055	HQ157548	KF482986	HQ157550
	Mangifera indica	Rio de Janeiro, Brazil	C2094	-	KF482987	KF482998
	Mangifera indica	Rio de Janeiro, Brazil	C1558 (=CBS 115175)	AY157965	KF482988	HQ157552
	Mangifera indica	São Paulo, Brazil	C1657	AY526291	KF482986	HQ157550
	Mangifera indica	São Paulo, Brazil	C1655	HQ157546	-	-
	Mangifera indica	São Paulo, Brazil	C1889	HQ157547	-	-
	Mangifera indica	São Paulo, Brazil	C994 (=CBS 600.70)	AY157964	KF482987	HQ157551
	Colocasia esculenta	São Paulo, Brazil	C1905 (=CBS 115171)	AY526288	KF482989	HQ157552
	Colocasia esculenta	São Paulo, Brazil	C1926	HQ157541	-	-
	Ipomoea batatas	Papua New Guinea	C1476 (=ICMP 8579)	AY157957	KF482992	KF483000
C.cacaofunesta	*Theobroma cacao*	Ecuador	C1004 (=CBS 153.62)	AY157950 -	KF482993	KF483001
	Theobroma cacoa	Bahia, Brazil	C1587	AY157953	-	-
	Theobroma cacoa	Rondônia, Brazil	C2031	xxxx	Xxxx	Xxxx
C.colombiana	*Coffea arabica*	Colombia	C1543 (=CBS 135861)	AY157961	KF482994	KF483002
	Coffea arabica	Colombia	C1024	xxxx	KF482994	KF483002
C. platani	*Platanus accidentalis*	North Carolina, USA	C1317 (=CBS 115162)	AY157958	KF482995	KF483003
C. variospora	*Prunus* sp.	Iowa, USA	*C*1963 (=CBS 135862)	xxxx	KF482996	KF483004

[A] The sequence will be deposite in GenBank;
[B] Not used in analyses.

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

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

[a] sec = strains from MAT2, self-sterile sectors recovered from self-fertileisolates; ss = MAT1, female-component isolates with protoperithecia;
[b]Normal = abundant, normal appearing ascospores;
[c]Watery = few misshapen ascospores or there are no ascopore;
[d]Perithecia = Perithecia only, no ascospore mass;
[e]---- = no perithecia produced;

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

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

*The number of isolates in the allele size in parentheses.

Table 4 - Analysis of molecular variance (AMOVA) of *Ceratocystis fimbriata* populations on *Carapa* guianensis (andiroba), *Mangifera indica* (mango), *Tectona grandis* (teak), *Colocasia esculenta* (taro) and *Eucalyptus* spp. in Brazil based on 14 microsatellite loci.

Source of variation	d.f	Sum of squared desviations	Variance components	Percentage of variation (%)	*P*[a]
Among host-associated groups[b]	5	564.814	3.06639	61.96	<0.001
Among populations within groups	10	117.559	0.84786	17.13	<0.001
Within populations	195	201.779	1.03476	20.91	<0.001
Total	210	884.152	4.94902		

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

[b] The populations were distributed in 6 groups: (1) CarAC, CarRR1, CarRR2 and CarAM; (2) TecMT1 and TecMT3; (3) ManERRJ1 and ManRJ2; (4) EucBA1, EucBa2b and EucMG1; (5) ManNEPB1, ManNEPE1 and ManNECE2; (6) ColSP and ColRJ.

Table 5 - Genetic diversity of *Carapa guianensis* (andiroba) populations of *Ceratocystis fimbriata* and representative *Eucalyptus*, *Mangifera indica* (mango), *Colocasia esculenta* (taro) and *Tectona grandis* (teak) populations from Brazil based in 14 microsatellite loci.

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

[a] Stoddart & Taylor's genotypic diversity (G) with rarefaction. Values of G with rarefaction for individual populations ranged from 1 (only one genotype in the population) to a maximum value of 5 (each isolate in the population of a different genotype). For host population ranged from 1 (only one genotype in the population) to a maximum value of 13 (each isolate in the population of a different genotype).

Table 6 - Mean xylem discoloration (cm) caused by *Ceratocytsis fimbriata* isolates inoculated in four host plants in greenhouse and growth chamber.

Isolates	*Carapa guianensis*		*Tectona grandis*		*Eucalyptus* spp.		*Mangifera indica*	
	GH[y]	GC[z]	GH1	GC	GH 1	GC	GH	GC
CarAM-ANDP1	9.13a	8.69ab	2.00ab	1.69b	3.25c	3.38bc	4.25b	6.47b
CarAC-C144B2	7.38ab	10.31a	1.69b	1.75b	2.94c	3.08c	4.38b	7.96b
CarRR-RR174	5.38bc	10.88a	1.88ab	1.75b	3.88bc	3.06c	3.81b	7.03b
TecMT-QM29	3.75cd	5.03bc	2.69a	2.81a	3.44bc	4.50bc	4.34b	7.56b
EucBA-SBS1	3.19cd	3.94bc	2.06ab	1.81b	16.00a	28.38a	6.88b	9.26b
MangCE-CEBS13	2.50cd	3.54bc	2.06ab	1.81b	5.88b	5.81b	15.63a	13.94a
Control	1.81d	1.69c	1.69b	1.69b	2.13c	2.00c	2.69b	2.13c

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

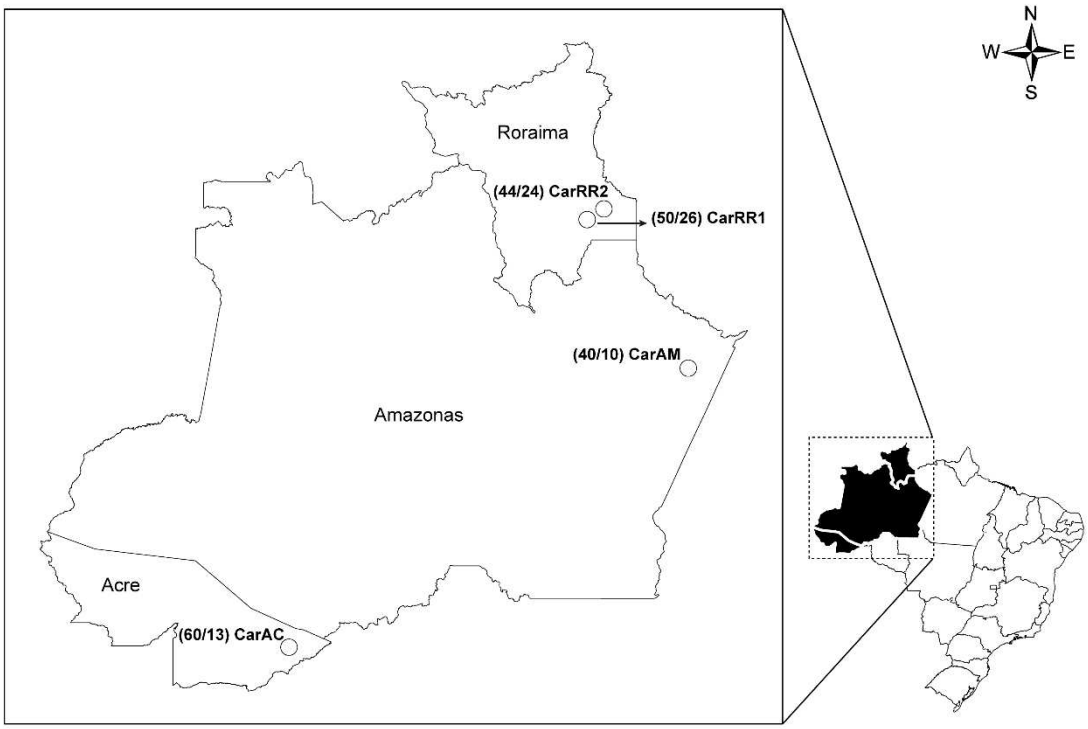

Fig 1 - Geographic distribuition of sampling sites of *Ceratocystis fimbriata* populations from *Carapa guianensis* (andiroba). In parentheses, the number of samples and the number of isolates that were obtained from each population.

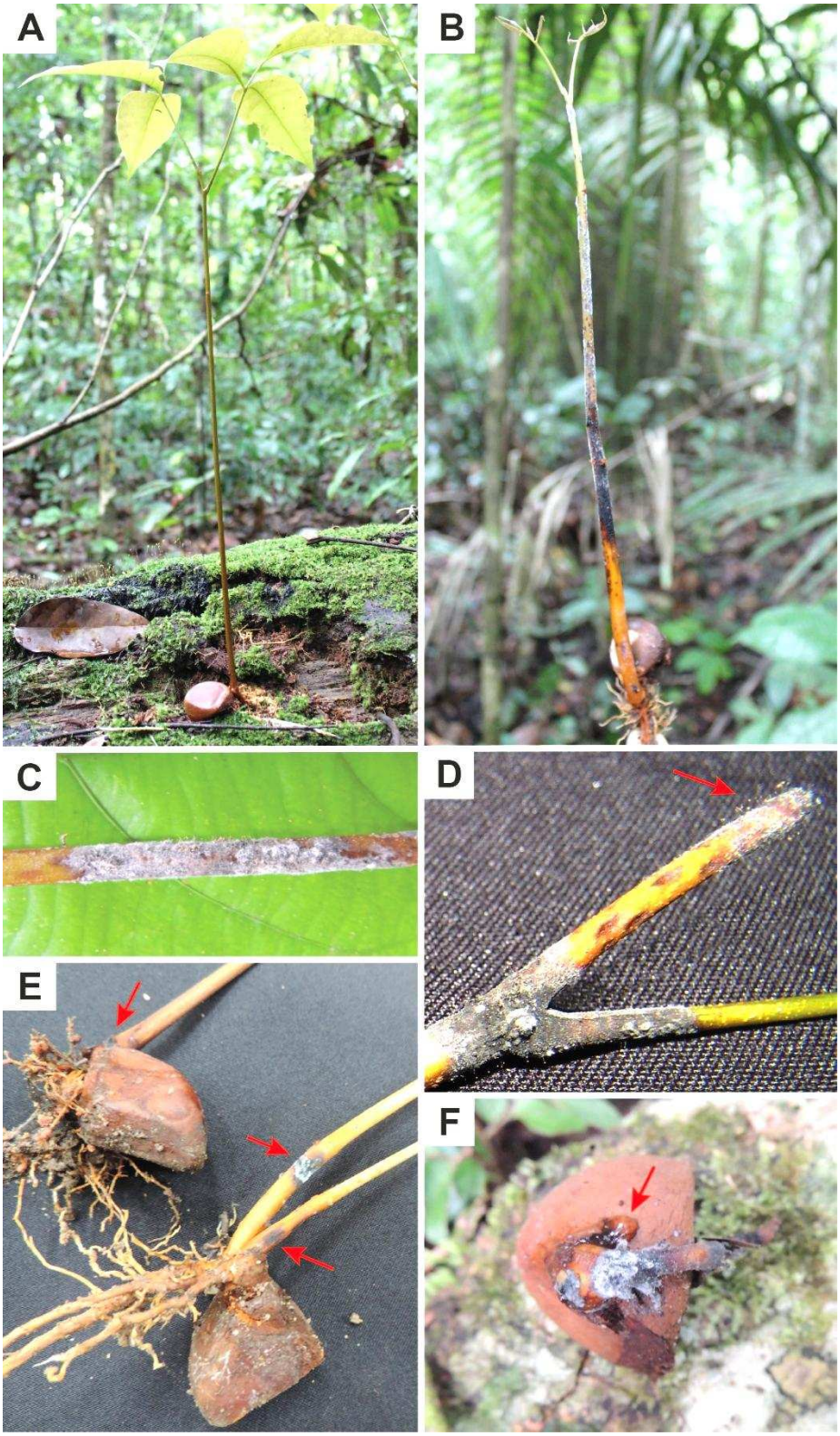

Fig 2 - *Ceratocystis fimbriata* on *Carapa guianensis* (andiroba). (A) Healthy seedling. (B) Seedling with *Ceratocystis* seedling blight. (C, E) Stem with fungus sporulation. (D) Stem with perithecia indicated by the red arrow. (F) Seed with fungus sporulation and gummosis (red arrow).

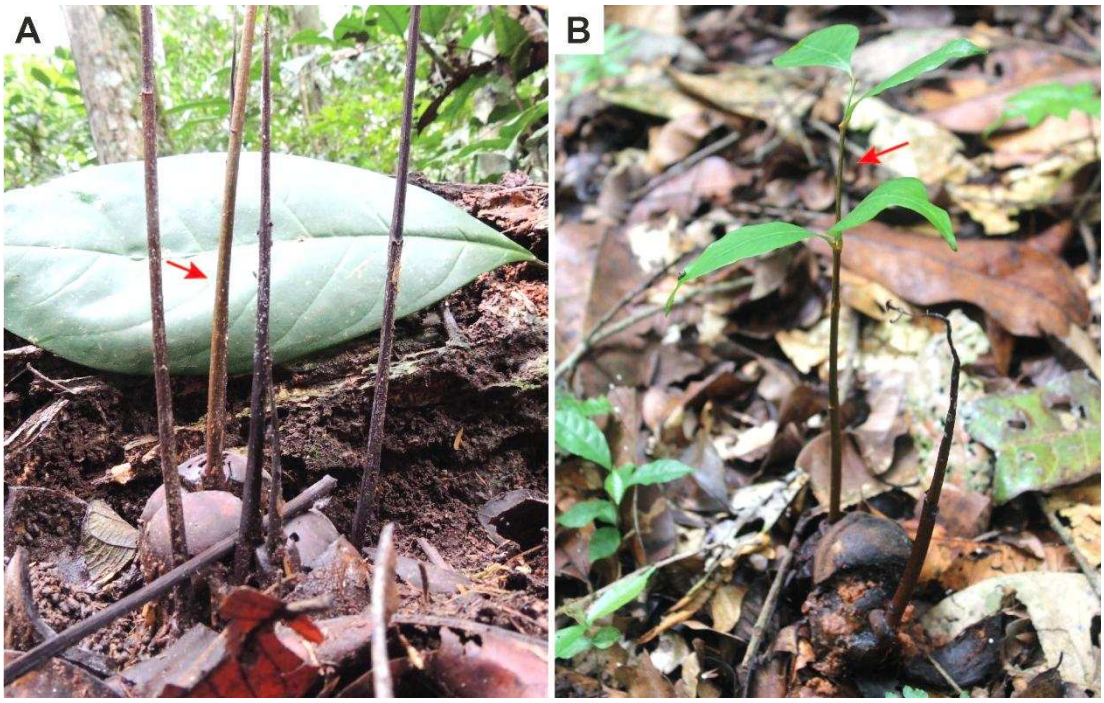

Fig 3 - *Ceratocystis fimbriata* on *Carapa guianensis* (andiroba). (A) and (B) Healthy seedling (red narrow) and with *Ceratocystis* seedling blight coming from the same fruit.

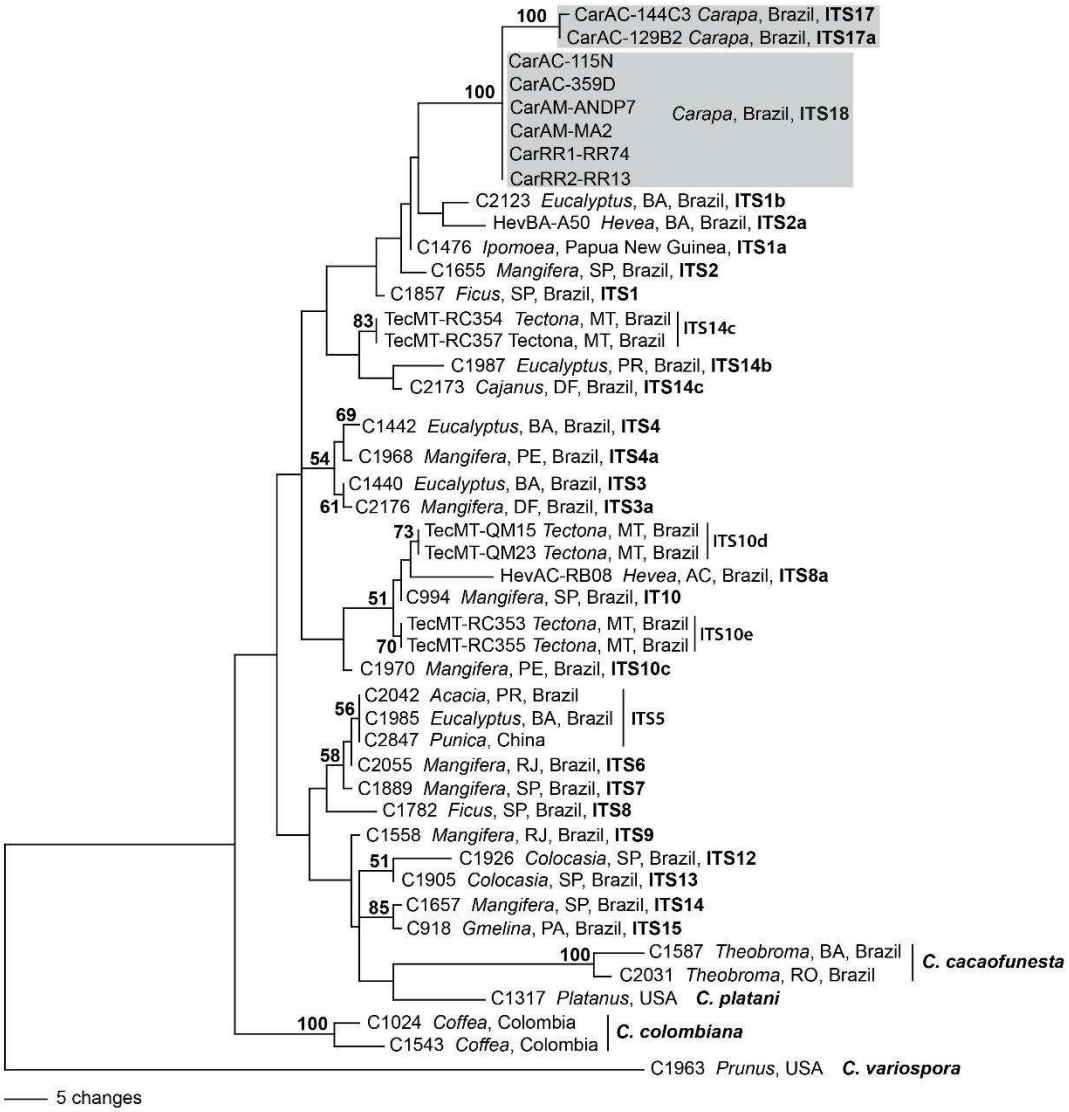

Fig 4 - One of 9 most parsimonious trees based on the ITS rDNA sequences of representative isolates of the Latin American Clade (LAC) of the *Ceratocytsis fimbriata* complex. The tree is rooted to *C. variospora* of the North American Clade (NAC), and all other isolates are considered to be in the LAC. The host genus, state (AC = Acre; AM = Amazonas; BA = Bahia; DF = Distrito Federal; Mato Grosso = MT; PA = Pará; PE = Pernambuco; PR = Paraná; RJ = Rio de Janeiro; RR = Roraima and SP = São Paulo) or country of origin are given for each isolate. The ITS haplotype designations are indicated in the right. Bootstrap values greater than 50% are indicated on appropriate branches. Scale bar indicates base pair differences.

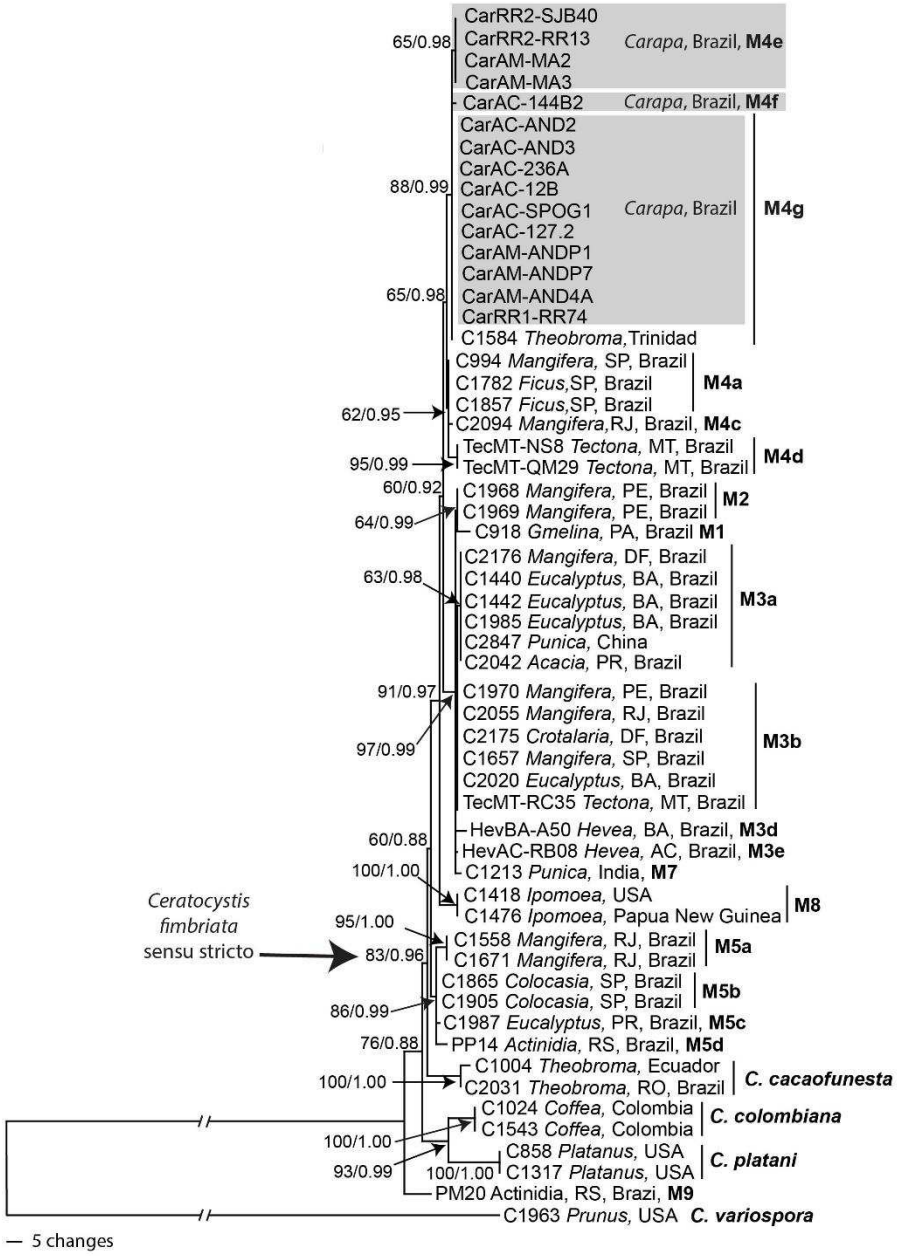

Fig 5 - The single most parsimonious tree of the 494 steps based on portions of the MAT1-1-2 (MAT-1) and MAT1-2-1 (MAT-2) mating type genes *Ceratocystis fimbriata* and other members of the LAC (*C. cacaofunesta*, *C. colombiana*, and *C. platani*). The tree was rooted to *C. variopora*, a member of the North American Clade of the *C. fimbriata* complex. Bootstrap values greater than 60%/posterior probability value greater than 0.85 are indicated on appropriate branches. The host genus, state (Acre = Acre; AM = Amazonas; BA = Bahia; Mato Grosso = MT; PA = Pará; RJ = Rio de Janeiro; RR = Roraima and SP = São Paulo) or country of origin are given for each isolate. The mating type haplotype designations are indicated in the right. Scale bar indicates base pair differences.

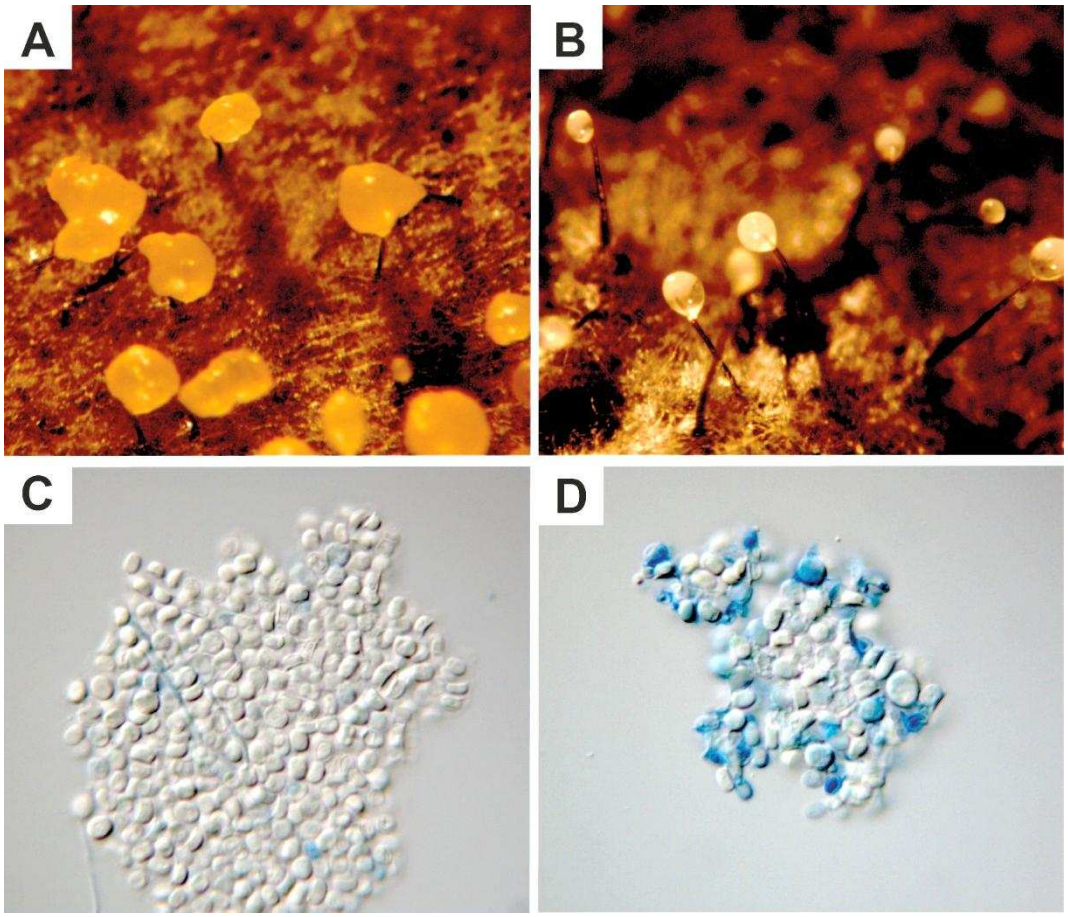

Fig 6 – Inter-fertile and inter-sterile crosses between isolates of *Ceratocytsis* spp. (A) Perithecia and ascospore mass and (C) ascospore from interfetile cross between *C. fimbriata* strains from *Carapa guianensis* (andiroba) (CarA-MA3) and from *Eucalyptus* (C1347). Interspecific cross between an andiroba strain of *C. fimbriata* (CarRR1-RR74) and a *Platanus* strain of *C. platani* (C1343) showing small watery ascospore masses and (B) misshapen ascopore (D).

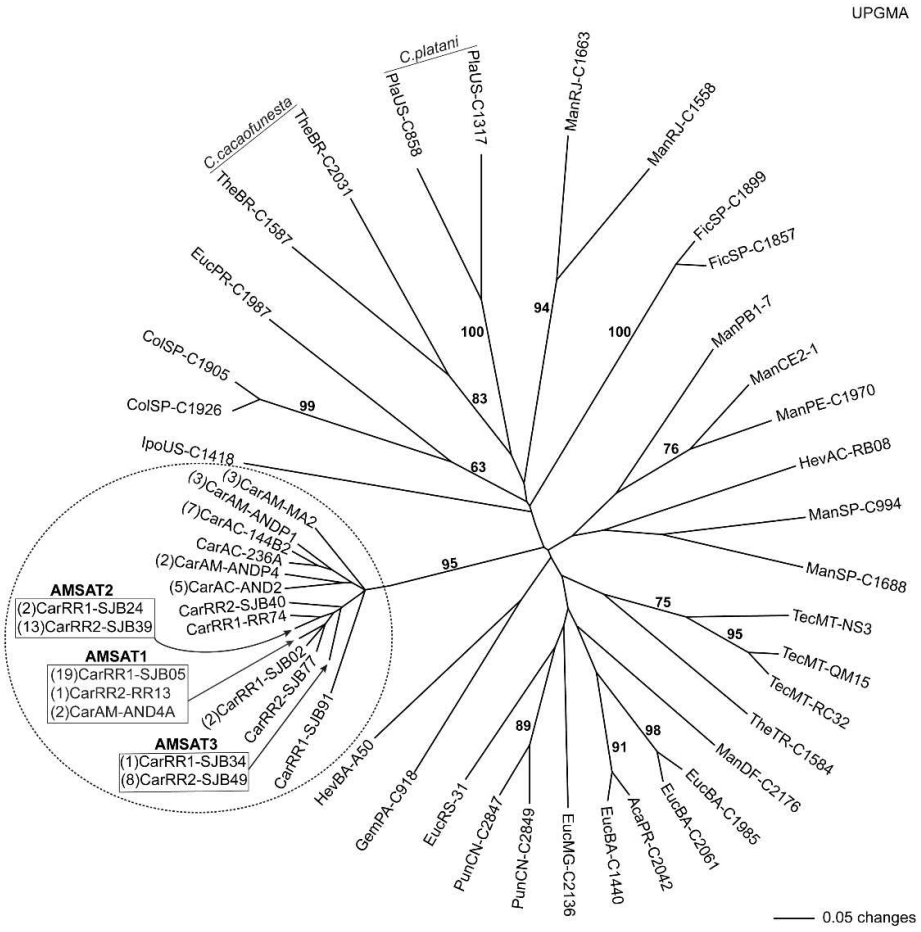

Fig 7 - A UPGMA (unweighted pair group method arithmetic mean) dendrogram of genotypes of *Ceratocystis fimbriata*, *C. cacaofunesta* and *C. platani* based on Nei's genetic distance. Bootstrap values are shown along side the branches.The first three letters indicate the host genus (Aca = *Acacia;* Car = *Carapa*; Col = *Colocasia*; Euc = *Eucalyptus*; Fic = *Ficus;* Gme = *Gmelina*; Hev = *Hevea*; Ipo = *Ipomoea*; Man = *Mangifera*; Pla = *Platanus;* Pun = *Punica*; Tec = *Tectona* and The = *Theobroma*), and next two letters indicate the Brazilian state (AC = Acre; AM = Amazonas; AL = Alagoas; BA = Bahia; CE = Ceará; MG = Minas Gerais; MT = Mato Grosso, PA = Pará; PB = Paraíba; RJ = Rio de Janeiro; RS = Rio Grande do Sul; RR = Roraima and SP = São Paulo) or country (BR = Brazil; CN = China; US = United States) of origin. *Carapa* genotypes encircled by dashed line. The number of isolates with that *Carapa* genotypes is in parentheses. In addition, *Carapa* genotypes found in more than one population are designated by "**AMSAT**" followed by a number. Scale bar indicates genetic distance.

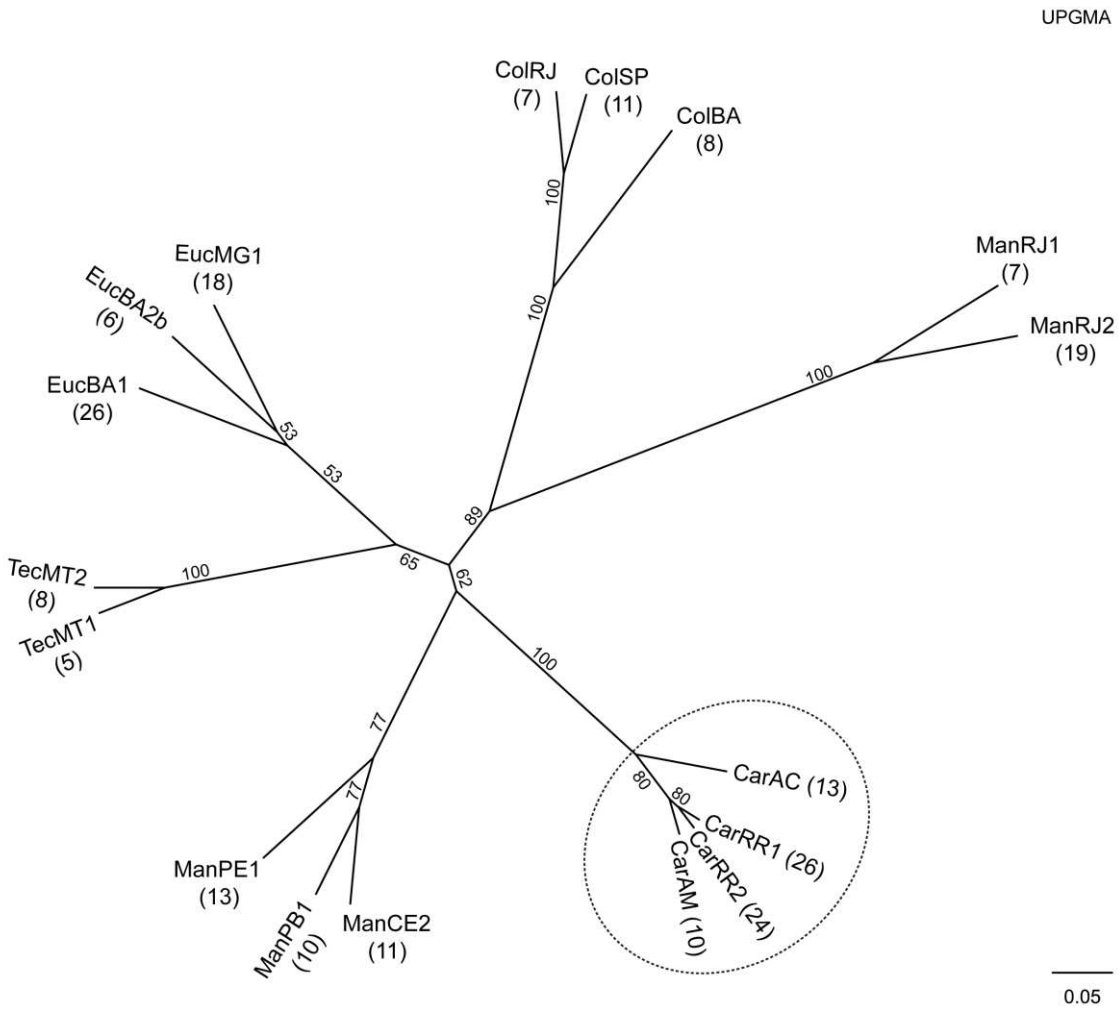

Fig 8- Dendrogram of *Ceratocytis fimbriata* populations from Brazil according the host and geographic distribution generated by UPGMA (unweighted pair group method, arithmetic mean) based on allele frequencies of the 14 microsatellite loci. Bootstrap value are shown alongside the branches. The first three letters indicate the host genus (Car = *Carapa*; Col = *Colocasia*; Euc = *Eucalyptus*; Man = *Mangifera* and Tec = *Tectona*), and the next two letters indicate the Brazilian states (AC, Acre; AM, Amazonas; BA, Bahia; CE, Ceará; MG, Minas Gerais; MT, Mato Grosso, PB, Paraíba; PE, Pernanbuco; RJ, Rio de Janeiro; RR, Roraima and SP, São Paulo). *Carapa* populations encircled by dashed line. The number of isolates sampled from each population is in parentheses.

GENERAL CONCLUSIONS

The results of this study allow concluding that:

1. The isolates from teak and andiroba belong to *C. fimbriata*;
2. The genetic variability of *Ceratocystis fimbriata* populations from teak was relatively low;
3. *Ceratocystis fimbriata* populations from andiroba are native the Amazonian Rain Forest.
4. There is variability in aggressiveness among isolates of the fungus from andiroba and teak.

SUPPLEMENT

Table. Collection sites of isolates of *Ceratocystis fimbriata* from *Carapa guianensis* (andiroba) and *Tectona grandis* (teak) in Brazil.

Isolate	Host	City	State	Collector	Geographic Coordenate
CarAC-AND2	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	S10º 01' 57.3" W67º 41' 13.75"
CarAC-AND3	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-236A	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-144B2	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-144B3	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-129B2	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-SPO1G	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-SPO3D	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-359D	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-12B	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-127/2	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-115N	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarAC-127/1	Andiroba	Rio Branco	Acre	Denise/Acelino/Leonardo	
CarRR1-RR74	Andiroba	São João da Baliza	Roraima	Denise	N0º 52' 19.01" W59º 59' 2.10"
CarRR1-SJB02	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB05	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB06	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB07	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB08	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB13	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB14	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB15	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB18	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB19	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB22	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB24	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB26	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB28	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB29	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB33	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB84	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB85	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	

Table. Continued

Isolate	Host	City	State	Collector	Geographic Coordenate
CarRR1-SJB86	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB87	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB89	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB90	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB91	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR1-SJB94	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-RR13	Andiroba	São João da Baliza	Roraima	Denise	N0º 57' 2" W59º 54' 41"
CarRR2-SJB39	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB40	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB41	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB42	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB44	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB49	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB51	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB52	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB56	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB57	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB58	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB59	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB62	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB63	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB64	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB68	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB69	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB70	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB73	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB74	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB77	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB81	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarRR2-SJB82	Andiroba	São João da Baliza	Roraima	Denise/Leonardo	
CarAM-MA2	Andiroba	Boa Vista do Ramos	Amazonas	Walter	S2º 58' 28.27" W57º 35' 16.30"
CarAM-MA3	Andiroba	Boa Vista do Ramos	Amazonas	Walter	
CarAM-AND4A	Andiroba	Boa Vista do Ramos	Amazonas	Walter	

Table. Continued

Isolate	Host	City	State	Collector	Geographic Coordenate
CarAM-AND5A	Andiroba	Boa Vista do Ramos	Amazonas	Walter	
CarAM-AND10A	Andiroba	Boa Vista do Ramos	Amazonas	Walter	
CarAM-ANDP1	Andiroba	Parintins	Amazonas	Walter	S2º 38' 15.31" W56º 44' 4.42"
CarAM-ANDP2	Andiroba	Parintins	Amazonas	Walter	
CarAM-ANDP4	Andiroba	Parintins	Amazonas	Walter	
CarAM-ANDP5	Andiroba	Parintins	Amazonas	Walter	
CarAM-ANDP7	Andiroba	Parintins	Amazonas	Walter	
TecMT-NS3	Teak	Nossa Senhora do Livramento	Mato Grosso	Denise/Lucas	S16°2'46.2" W056°23'08.4"
TecMT-NS5	Teak	Nossa Senhora do Livramento	Mato Grosso	Denise/Lucas	
TecMT-NS7	Teak	Nossa Senhora do Livramento	Mato Grosso	Denise/Lucas	
TecMT-NS8	Teak	Nossa Senhora do Livramento	Mato Grosso	Denise/Lucas	
TecMT-NS10	Teak	Nossa Senhora do Livramento	Mato Grosso	Denise/Lucas	
TecMT-QM15	Teak	São José dos Quatro Marcos	Mato Grosso	Denise/Lucas	S15°37'16.9"W 58°18'34.2"
TecMT-QM16	Teak	São José dos Quatro Marcos	Mato Grosso	Denise/Lucas	
TecMT-QM18	Teak	São José dos Quatro Marcos	Mato Grosso	Denise/Lucas	
TecMT-QM22	Teak	São José dos Quatro Marcos	Mato Grosso	Denise/Lucas	
TecMT-QM23	Teak	São José dos Quatro Marcos	Mato Grosso	Denise/Lucas	
TecMT-QM24	Teak	São José dos Quatro Marcos	Mato Grosso	Denise/Lucas	
TecMT-QM28	Teak	São José dos Quatro Marcos	Mato Grosso	Denise/Lucas	
TecMT-QM29	Teak	São José dos Quatro Marcos	Mato Grosso	Denise/Lucas	
TecMT-RC32	Teak	São José do Rio Claro	Mato Grosso	Denise/Lucas	S12°29'24.4" W057°08'5.9"
TecMT-RC35	Teak	São José do Rio Claro	Mato Grosso	Denise/Lucas	
TecMT-RC39	Teak	São José do Rio Claro	Mato Grosso	Denise/Lucas	
TecMT-RC46	Teak	São José do Rio Claro	Mato Grosso	Denise/Lucas	
TecMT-RC353	Teak	São José do Rio Claro	Mato Grosso	Acelino	
TecMT-RC354	Teak	São José do Rio Claro	Mato Grosso	Acelino	
TecMT-RC355	Teak	São José do Rio Claro	Mato Grosso	Acelino	
TecMT-RC356	Teak	São José do Rio Claro	Mato Grosso	Acelino	
TecMT-RC358	Teak	São José do Rio Claro	Mato Grosso	Acelino	
TecMT-SI357	Teak	Sinop	Mato Grosso	Acelino	S11°39'42.1" W055°26'32.9"
TecMT-SI69	Teak	Sinop	Mato Grosso	Denise/Lucas	