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LAVRAS-MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Patologia Florestal, para a obtenção do título de Doutora.

Profa. Dra. Maria Alves Ferreira
Orientadora

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Aprovada em 08 de março de 2018.

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Orientadora

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Aos meus pais, Silmara e Elysio, os alicerces da
minha vida, que sempre me deram suporte para
conquistar mais essa etapa da minha vida.
A um anjo que está no céu, mas também ao meu lado
o tempo todo, José Bissolatti Neto, *in memoriam*.
Dedico

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RESUMO

Entre as oito espécies de *Chrysoporthe* descritas no mundo, somente *Chrysoporthe cubensis* e *Chrysoporthe doradensis* foram reportadas no Brasil. O patógeno tem como hospedeiros várias plantas da família Myrtaceae e Melastomataceae, sendo os gêneros *Eucalyptus* e *Tibouchina*, os mais atacados. O fungo causa lesões no câmbio das plantas, levando a ruptura dos tecidos (tronco e galhos), reduzindo seu crescimento, podendo resultar na morte da planta. Existem indícios que no Brasil haja grande diversidade genética de *Chrysoporthe* spp. bem como, possíveis espécies ainda desconhecidas. Desta forma, os objetivos deste trabalho foram: identificar isolados *Chrysoporthe* oriundos de hospedeiros nativos e não nativos de diferentes regiões do Brasil e analisar a estrutura e a diversidade genética de populações de *Chrysoporthe* spp. no Brasil, utilizando marcadores microssatélites. Para isso o presente estudo foi dividido em dois artigos, o primeiro teve como objetivo estudar a estrutura e diversidade genética em população de *C. cubensis* no Brasil. Enquanto o segundo artigo, objetivou-se estudar a filogenia, a morfologia, a patogenicidade e a estrutura da população de uma nova espécie descoberta neste trabalho. Os isolados de *Chrysoporthe* foram obtidos em árvores de eucalipto em plantios comerciais e em árvores de espécies nativas em parques florestais e áreas urbanas. Os isolados foram submetidos às análises filogenéticas utilizando duas regiões gênicas, β-tubulina e espaço interno transcrito ITS-rDNA. Os resultados das análises filogenéticas e morfológicas confirmaram a existência de uma nova espécie de *Chrysoporthe*, que foi descrita neste trabalho como *Chrysoporthe puriensis*. De acordo com as análises da diversidade genética de populações em *C. cubensis*, constatou-se alta diversidade genética no Brasil. Foi possível verificar que os isolados de *C. cubensis* agrupam-se em subpopulações de acordo com sua a distribuição geográfica e que a maior variabilidade genética ocorre dentro das subpopulações do que entre as subpopulações. Em relação a análise populacional dos isolados de *C. puriensis*, verificou-se a presença de uma única população com altas diversidades gênica e genotípica. A alta variabilidade genética em *C. cubensis* e *C. puriensis* no Brasil destaca a importância da continuidade dos estudos genético-molecular de *Chrysoporthe*. Possivelmente com estudos mais abrangentes, com novos isolados coletados de áreas ainda não estudadas, novas espécies serão encontradas. Isso reflete nos programas de melhoramento e na seleção de clones de eucalipto deverão ficar atentos e considerar as diferentes espécies e genótipos do fungo durante a fenotipagem quanto a resistência dos clones a espécies de *Chrysoporthe*. Os resultados também apontam para a importância ambiental de *Chrysoporthe* spp. quando associados a espécies de *Tibouchina*. Durante o estudo, observou-se muitas árvores de *Tibouchina* spp. morrendo devido ao ataque destes patógenos. É importante que sejam realizados estudos futuros para verificar a abrangência dos patossistemas, tanto em florestas nativas quanto em arborização urbana e reflorestamento, e iniciar estudos de seleção de progênies de *Tibouchina* spp. resistentes a espécies de *Chrysoporthe* para utilização na arborização urbana.

Palavras-chave: Cryphonectriaceae, diferenciação populacional, filogenia.

ABSTRACT

Among the eight *Chrysoporthe* species described in the world, only *Chrysoporthe cubensis* and *Chrysoporthe doradensis* were reported in Brazil. The pathogen has as hosts several plants of the family Myrtaceae and Melastomataceae, being the genera *Eucalyptus* and *Tibouchina*, the most attacked. The fungus causes lesions in the exchange of the plants, causing the rupture of the tissues (stem and branches), reducing their growth, which can result in the death of the plant. It is believed that in Brazil there is high genetic diversity of *Chrysoporthe* spp. as well as possible unknown species. The objective of this work was to verify the occurrence of new *Chrysoporthe* species from native and non-native hosts from different regions of Brazil and to analyse the structure and genetic diversity of populations of *Chrysoporthe* spp. in Brazil, using microsatellite markers. For this the present study was divided into two papers, the first one was designed to study the structure and genetic diversity in the population of *C. cubensis* in Brazil. While the second, the objective was to study the, phylogeny, morphology, pathogenicity and population structure of a new species reported in this work. *Chrysoporthe* isolates were obtained from eucalyptus trees in commercial plantations and *Tibouchina* native tree species in forest parks and urban areas. The isolates were submitted to phylogenetic analyses using two gene regions, β-tubulin and transcribed internal space. The results of the phylogenetic and morphological analyses confirmed the existence of a new species of *Chrysoporthe*, which was described in this work as *Chrysoporthe puriensis*. Genetic diversity analyses of *C. cubensis* populations showed high genetic diversity in Brazil. And it was possible to verify that the isolates of *C. cubensis* are grouped in sub-populations according to their geographic distribution and that the highest genetic variability occurs within the sub-populations than among the sub-populations. In relation to the population analyses with the isolates of *C. puriensis*, the presence of a single population with very high gene and genotypic diversities were observed. The high genetic variability in *C. cubensis* and *C. puriensis* in Brazil highlights the importance of the continuity of the genetic-molecular studies of *Chrysoporthe* in Brazil. Possibly with more ample studies new species will be found. This reflects in breeding programs and selection of eucalyptus clones that should be careful and consider the different species and genotypes during the screening of the resistance of the clones to *Chrysoporthe* species. The results also point to the environmental importance of *Chrysoporthe* spp. when associated with *Tibouchina* species. During the study, many trees of *Tibouchina* spp. were found dying due to the attack of these pathogens. It is very important be performed future studies to verify how ample these pathosystems is in both native forests and in urban afforestation and reforestation. And initiate selection studies of progenies of *Tibouchina* spp. resistant to *Chrysoporthe* species.

Keywords: Cryphonectriaceae, population differentiation, phylogeny.

SUMÁRIO

FIRST PART

1 INTRODUCTION

The genus *Chrysoporthe* represents a group of phytopathogenic ascomycetes belonging to the family Cryphonectriaceae (GRYZENHOUT et al., 2004). The species of this genus are responsible for causing cankers and lesions in the exchange of the plants, causing the rupture of their stems, reducing their growth, being able to result in the death of the plant (HODGES et al., 1976; HODGES; GEARY; CORDELL, 1979; SHARMA; MOHANAN; FLORENCE, 1985; WINGFIELD; SWART; BEAR, 1989). The host range of these fungi includes eucalyptus and other members of the families Melastomataceae (WINGFIELD et al., 2001; RODAS et al., 2005) and Myrtaceae (HODGES; ALFENAS; FERREIRA, 1986; BARRETO; ROCHA; FERREIRA, 2006). The morphological and physiological differences, as well as the phylogenetic analysis of multiple gene regions, elucidated eight species, being: *C. austroafricana*, *C. cubensis*, *C. deuterocubensis*, *C. doradensis*, *C. hodgesiana*, *C. inopina*, *C. syzygiicola* e *C. zambiensis* (GRYZENHOUT et al., 2004; GRYZENHOUT et al., 2005; VAN DER MERWE et al., 2010; CHUNGU et al., 2009). These pathogens are typically found in tropical and subtropical regions of the world, including Southeast Asia, Africa, South and Central America and Australia (HODGES; ALFENAS; FERREIRA, 1986; SHARMA; MOHANAN; FLORENCE, 1985; WINGFIELD; SWART; BEAR, 1989; NAKABONGE et al., 2006; PEGG et al., 2010).

In Brazil have been reported the presence of two species, *C. cubensis* e *C. doradensis* (SANTOS, 2015). The most important in the country is *C. cubensis*, because it causes the disease known as eucalyptus canker, an economically important disease in eucalyptus plantations (WINGFIELD, 1999, 2003). *Chrysoporthe cubensis* is also attacking native species of *Tibouchina*, both in its natural habitat and in urban afforestation. It has been common to find *Tibouchina* spp. with canker symptoms and dying. There is no way to control the effects and decrease the incidence of *C. cubensis* in species of *Tibouchina*. For eucalyptus, control of this disease occurs through the use of resistant clones that are selected and propagated (ALFENAS; JENG; HUBBES, 1983; CONRADIE; SWART; WINGFIELD, 1990).

Despite the importance of the fungus, Brazil lacks research that conducts a good sampling throughout the national territory and that considers the various hosts that occur naturally in the country. Due to the great territorial extension and to the several biomes that occur in the country, together with its condition of tropical climate, it is consistent to hypothesize that there is great genetic diversity of *Chrysoporthe* spp. as well as possible unknown species.

The information on genetic diversity of a fungi population can support the understanding of the evolutionary relationship of the pathogen and its hosts. In addition, provide valuable information regarding the contribution of key evolutionary factors, such as gene flow, selection pressure, mating and recombination, and genetic drift (MCDONALD, 1997).

Genetic variation of a population of phytopathogenic fungi can be evaluated by molecular markers (MCDERMOTT; MCDONALD, 1993; MILGROOM; FRY, 1997). Molecular markers are any molecular patterns from any expressed or non-expressed segments of DNA that are genetically inherited and can be used to differentiate from one or more individuals (FERREIRA; GRATTAPAGLIA, 1998; MILACH, 1998). They behave as characters of simple and predictable inheritance and are unaffected by variations in the environment. Thus, molecular markers allow the quantification of genetic diversity, follow the movement of individuals, estimate inbreeding, characterize new species and evaluate the historical patterns of dispersion of the species or populations in question (FREELAND, 2005).

In order to verify the genetic variability of *Chrysoporthe* spp., in Brazil, through the identification of new species and analysing the population diversity, this study had as main objectives: Characterization of *Chrysoporthe* isolates from eucalyptus plantations, urban afforestation areas and forest parks in Brazil and to study the genetic structure and diversity of populations of *Chrysoporthe* spp. in Brazil, using microsatellite markers. Thus, it was divided into two papers, the first one had as objective to study the structure and genetic diversity in population of *Chrysoporthe cubensis* in Brazil. In the second, the objective was to study the morphology, phylogeny, pathogenicity and structure and diversity of the population of *Chrysoporthe puriensis*, a new species discovered in this work.

REFERENCES

ALFENAS, A.; JENG, R.; HUBBES, M. Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. **Forest Pathology**, v. 13, n. 4, p. 197-205, 1983

BARRETO, R.W.; ROCHA, F.B.; FERREIRA, F.A. First record of natural infection of *Marlierea edulis* by the eucalyptus canker fungus *Chrysoporthe cubensis*. **Plant pathology**, v. 55, n. 4, p. 577-577, 2006.

CHUNGU, D. et al. Taxonomy and pathogenicity of two novel *Chrysoporthe* species from *Eucalyptus grandis* and *Syzygium guineense* in Zambia. **Mycological Progress**, v. 9, n. 3, p. 379-393, 2009.

CONRADIE, E.; SWART, W. J.; WINGFIELD, M. J. *Cryphonectria* Canker of Eucalyptus, an Important Disease in Plantation Forestry in South Africa. **South African Forestry Journal**, v. 152, n. 1, p. 43-49, 1990.

FERREIRA, M. E.;GRATTAPAGLIA, D. **Introdução ao uso de marcadores moleculares em análise genética**. 3. ed. Brasília: EMBRAPA-CENARGEN, 1998.

FREELAND, J. The evolution of population biology: past, present and future. **Journal of Biogeography**, v. 32, n. 11, p. 2039-2040, 2005.

GRYZENHOUT, M. et al. *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. **Studies in Mycology**, v. 50, p. 119-142, 2004.

GRYZENHOUT, M. et al. *Chrysoporthe doradensis* sp. nov. pathogenic to *Eucalyptus* in Ecuador. **Fungal Diversity**, v. 20, p. 39-57, 2005.

HODGES, C.S.; ALFENAS, A.C.; FERREIRA, F.A. The conspecificity of *Cryphonectria cubensis* and *Endothia eugeniae*. **Mycologia**, p. 343-350, 1986.

HODGES, C.S.; GEARY, T.F; CORDELL, C.E. The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii and Puerto Rico. **Plant Disease Reporter**, v. 63, n. 3, p. 216-220, 1979.

HODGES, C.S. et al. O cancro do eucalipto causado por *Diaporthe cubensis*. **Fitopatologia Brasileira**, v. 1, p. 129-170, 1976.

MCDERMOTT, J. M.; MCDONALD, B. A. Gene flow in plant pathosystems. **Annual Review of Phytopathology**, v. 31, n. 1, p. 353-373, 1993.

MCDONALD, B. A. The population genetics of fungi: tools and techniques. **Phytopathology**, v. 87, n. 4, p. 448-453, 1997.

MILACH, S.C.K. **Marcadores moleculares em plantas**. Porto Alegre: UFRGS, 1998. 140 p.

MILGROOM, M. G.; FRY, W. E. Contributions of population genetics to plant disease epidemiology and management. In: **Advances in Botanical Research**. Academic Press, 1997. 1-30 p.

NAKABONGE, G.; ROUX, J.; GRYZENHOUT, M.; WINGFIELD, M. J. Distribution of *Chrysoporthe* canker pathogens on *Eucalyptus* and *Syzygium* spp. in eastern and southern Africa. **Plant Disease**, v. 90, n. 6, p. 734-740, 2006.

PEGG, G. S. et al. The Eucalyptus canker pathogen *Chrysoporthe cubensis* discovered in eastern Australia. **Australasian Plant Pathology**, v. 39, n. 4, p. 343-349, 2010.

RODAS, C.A. et al. Discovery of the *Eucalyptus* canker pathogen *Chrysoporthe cubensis* on native *Miconia (*Melastomataceae*)* in Colombia. **Plant Pathology**, v. 54, n. 4, p. 460-470, 2005.

SANTOS, T.P.F. **Caracterização morfológica, filogenética e patogênica de *Chrysoporthe* spp. associado ao cancro em *Eucalyptus* spp. e *Tibouchina* spp**. Lavras, Brasil, Universidade Federal de Lavras. PhD Thesis, 2015.

SHARMA, J.K.; MOHANAN, C.; FLORENCE, E.J.M. **Disease survey in nurseries and plantations of forest tree species grown in Kerala:** research report. Kerala: Kerala Forest Research Institute, 1985, 268 p.

VAN DER MERWE, N.A. et al. Multigene phylogenetic and population differentiation data confirm the existence of a cryptic species within *Chrysoporthe cubensis*. **Fungal Biology**, v. 114, n. 11-12, p. 966-979, 2010.

VAN ZYL, L. et al. Population diversity among Brazilian isolates of *Cryphonectria cubensis*. **Forest Ecology and Management**, v. 112, n. 1-2, p. 41-47, 1998.

WINGFIELD, M. J. Daniel McAlpine memorial lecture: increasing threat of diseases to exotic plantation foreste in the Sourthen Hemisphere: lessons from *Chryphonectria* canker on *Tibouchina* in Colombia. **Forest Pathlogy**, New York, v. 32, n. 2, p. 133-139, 2003.

WINGFIELD, M. et al. *Cryphonectria* canker on *Tibouchin*a in Colombia. **Forest Pathology**, v. 31, n. 5, p. 297-306, 2001.

WINGFIELD, M.; SWART, W.; ABEAR, B. First record of *Cryphonectria* canker of *Eucalyptus* in South Africa. **Phytophylactica**, v. 21, n. 3, p. 311-313, 1989.

WINGFIELD, M.J. Pathogens in exotic plantation forestry. **The International Forestry Review**, p. 163-168, 1999.

SECOND PART – ARTICLES

Norms according to the journal that the articles will be submitted: Plant Pathology

ARTICLE 1: Genetic variability in populations of *Chrysoporthe cubensis* in Brazil.

ABSTRACT

The objective of this work was to analyse the structure and genetic variability of *Chrysoporthe cubensis* populations of native and non-native host, using microsatellite markers. The fungal isolates were obtained from *Eucalyptus* spp., *Corymbia citriodora* and *Tibouchina* spp. in different regions of Brazil. Isolates were separated into two sub-populations based on the host (Melastomataceae and Myrtaceae) and three sub-populations based on the region (North/Northeast, Central-West and Southeast). There was high genetic variability in all sub-populations. The highest genetic variability was found more within than among sub-populations. Gene and genotypic diversities were higher in the Melastomataceae than in the Myrtaceae. For the fungal sub-populations (based on regions), the highest gene diversity was found in the North/Northeast and the highest genotypic diversity was found in the Central-West. Southeast sub-population presented the lowest values of gene and genotypic diversities. High gene flow was observed between sub-populations based on host and North/Northeast and Central-West sub-populations. The presence of *C. cubensis* in native hosts of Brazil allied with the high genetic variability found are in support of the hypothesis that the center of origin of *C. cubensis* is Latin America. It was observed that the population of *C. cubensis* in Brazil has a mixed reproduction system, with sexual and asexual reproduction and the high potential of gene flow. Thus, it is important that the selection programs of eucalyptus clones use isolates with different genotypes from different regions and hosts during the screening of the resistance of the clones to *C. cubensis*. During the study, it was observed that many

trees of *Tibouchina* spp. decline and die due to the pathogen's attack. It is very important that future studies be done to verify the current situation and possible damages caused by *C. cubensis* in *Tibouchina* spp. both in native forests as well as in urban afforestation and reforestation. Also, studies aiming the selection of progenies of *Tibouchina* spp. resistant to *C. cubensis* should be prioritized.

INTRODUCTION

The fungus *Chrysoporthe cubensis*, pathogen of *Eucalyptus* spp. and other species of the order Myrtales, is predominantly found in Central and South America, Central Africa, Southeast Africa and Australia (Alfenas *et al.*, 1982; Gryzenhout *et al.*, 2004; Seixas *et al.*, 2004; Roux *et al.*, 2005, Baretto *et al.*, 2006; Nakabonge *et al.*, 2006). It causes lesions in the cambium of the plants, leading to the rupture of their stems, reducing their growth, which can result in the death of the plant (Hodges *et al.*, 1976; Sharma *et al.*, 1985). The center of origin of the *C. cubensis* has already been widely discussed, but after the description of *Chrysoporthe deuterocubensis* in 2010 (Van der Mewer *et al.*, 2010) the most accepted hypothesis is that *C. cubensis* is native to Latin America.

Canker, caused by *C. cubensis*, is an economically important disease in plantations of *Eucalyptus* species (Wingfield, 1999, 2003). It can cause quantitative and qualitative damages, as it decreases the growth of the plant and the affected wood besides having its value harmed for use in sawmill, also reduced the yield of cellulose and calorific value (Souza, 2008). In the 1970s, Brazil recorded severe economic losses in eucalyptus culture due to the high incidence of this disease, characterizing eucalyptus canker as the most important disease of the country's plantations (Ferreira *et al.*, 2004).

Since then, the only option to control the disease is the use of pathogen-resistant eucalyptus species or clones (Hodges *et al.*, 1976, Alfenas *et al.*, 1983; Sharma *et al.*, 1985). Brazil was the first country to use vegetative propagation of *Chrysoporthe*-resistant trees (Wingfield, 2003;

Alfenas *et al.*, 2009). Because it is a method of effective and economical control to this pathogen in *Eucalyptus* spp., programs for selection of resistant progenies and clones have been carried out by forest companies in several countries (Alfenas *et al.*, 1983; Sharma *et al.*, 1985; Heerden & Wingfield, 2002; Wingfield, 2003).

In Brazil it has been observed that resistant clones that were selected in the Southeast region are losing resistance when introduced in other regions of the country, such as North and Northeast. In these regions the occurrence of canker caused by *C. cubensis* in clonal plantations is high (authors' personal observations). Studies by Van Zyl *et al.* (1998), evaluating vegetative compatibility groups in *C. cubensis* isolates from eucalyptus plantations suggested high genetic variability of *C. cubensis* in Brazil.

Chrysoporthe cubensis has been attacking native species of *Tibouchina*, both in its natural habitat and in urban afforestation. *Tibouchina* spp. have two centers of origin in Latin America, one in the Central-West and Southeast regions of Brazil, and the other, less expressive, in northwestern South America (Guimarães, 1997; Peralta, 2002). *Tibouchina* spp. are widely used in urban afforestation, with landscaping purposes, due to the beauty of its flowers, and are also used in the recovery of degraded areas and reforestation (Lorenzi, 1992; Backes & Irgang, 2004). In the cities it has been common to find trees of *Tibouchina* spp. with symptoms caused by *C. cubensis* and dying. The situation in the forests has unfortunately not been different. The high mortality rate of *Tibouchina* spp. by *C. cubensis* in Brazil is causing a great concern.

Despite the importance of *C. cubensis* for trees in Brazil, little attention has been directed to genetic-molecular studies of the fungus. In order to understand the evolutionary relationship of the pathogen with its hosts, as well as to determine the genotypic and genetic composition of this fungus, this study aimed to analyse the structure and genetic variability of *C. cubensis* populations from native and non-native hosts of Brazil, using microsatellite markers.

MATERIAL AND METHODS

Sampling and DNA extraction

Eucalyptus plantations, urban afforestation areas and forest parks in six states of Brazil (Bahia, Goiás, Maranhão, Mato Grosso do Sul, Minas Gerais e Tocantins) were surveyed for the presence of *C. cubensis*, mainly on trees of *Tibouchina* spp. belonging to the family Melastomataceae represented the native hosts of Brazil and on trees of *Eucalyptu*s spp. and *Corymbia citriodora* belonging to the Myrtaceae family represented the non-native hosts of Brazil. Trees displaying sexual or asexual fruiting structures of *C. cubensis* were sampled.

From each tree sampled, an isolate was obtained from a single spore (Table 1). The cultures of a single spore were obtained from the exudate of the fruiting body of the fungus, which with a sterile needle was suspended in sterile water and plated out on 20% w/v potato dextrose agar (PDA). The plates were incubated in the dark at 28 °C for 24h. From each plate, single spores germinated were transferred to fresh PDA plates and incubated at 28 °C for 7 days.

The isolates were grown at 28 °C for 7-10 days in the dark in liquid malt extract (20% w/v). The mycelium was harvested by filtration. Total genomic DNA was extracted using a Wizard Genome DNA Purification Kit (Promega, USA) according to the manufacturer's instructions.

Phylogenetic analyses

The identity of the species of the isolates obtained was verified by amplification with the polymerase chain reaction (PCR) and sequencing two nuclear regions, namely Internal transcribed spacer (ITS) in the conserved 5.8 S gene of the ribosomal DNA (primers ITS1 and ITS4, White *et al.*, 1990) and the β-tubulin gene region with two pair primers (primers Bt1a/Bt1b and Bt2a/Bt2b, Glass & Donaldson, 1995). Sequencing was performed using Big Dye terminator sequencing kits (Life Technologies) on an ABI 13100 machine (Applied Biosystems) as per the instructions of the

manufacturers and as published previously by Van der Merwe *et al.*, (2010). Sequences were manually edited when needed.

Additional reference sequences for comparison were obtained from Genbank. For the purpose of this study, sequence alignments were done using the online interface of MAFFT (Katoh *et al.*, 2017). For phylogenetic analyses, *Amphilogia gyrosa* was used as the outgroup taxa. Partition homogeneity test (PHT) (Farris *et al.*, 1994) was applied using PAUP* 4.0 (Swofford, 2002) to the combined data of rDNA ITS and β-tubulin sequences, using 1000 replicates, to ascertain whether they could be collectively analysed. Combined gene alignment was subjected to Maximum likelihood (ML) and Maximum parsimony (MP) analyses.

Maximum parsimony (MP) analysis was performed using PAUP* 4.0 (Swofford, 2002). Only parsimony informative characters were used. The Heuristic search option with random stepwise addition and tree bisection reconnection (TBR) was used as the swapping algorithm. Confidence levels of the branching points were determined using 1000 bootstrap replicates.

Maximum likelihood (ML) analysis was conducted using MEGA 6 (Tamura *et al.*, 2013), incorporating the Tamura 3-parameter model of evolution as determined by MEGA 6 (Tamura, 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites. All positions containing gaps and missing data were eliminated. The confidence in branches was tested using 1000 bootstrap replicates.

Microsatellite marker genotyping

Microsatellite marker genotyping was done on each confirmed *C. cubensis* isolate using ten labelled polymorphic microsatellite primers (Table 2) developed by Mkabili *et al.*, (unpublished). Single-plexes of the ten markers were prepared for each isolate, comprising of reaction buffer A, 1.5 mM $MgCl_2$, 0.5 U Kapa Taq polymerase (Kapa Biosystems), 0.2 µM of each primer, 200 µM dNTPs, and 50 ng genomic DNA. Reactions were adjusted to a final volume of 10 µl with nuclease

free water (Adcock Ingram) and subjected to denaturation at 94 °C (5 min). This was followed by 35 cycles of 94 °C (35 s), annealing temperatures specific to each primer pair ranging from 58–64 °C (35 s), and 72 °C (1 min). A final extension step was performed at 72 ºC (7 min).

Amplicons were grouped into panels based on estimated allelic ranges and analysed using GeneScan® analyses (Life Technologies) with a LIZ500 size standard (Life Technologies) on an ABI 3500 machine (Applied Biosystems). Genemapper 4.1 (Applied Biosystems, Life Technologies) was used to analyse the data.

Population analyses

In order to determine the genetic structure of the population and the likely number of clusters (*K*), the allelic data was analysed without assigning populations using Structure v. 2.3.4 (Pritchard *et al*., 2000). The parameters included a burn-in of 100,000 steps, followed by 100,000 MCMC steps under an admixture model with correlated allele frequencies, and 10 replicates from *K*=1 to *K*=10. The results of the posterior probability values for each set of runs for each *K* were analysed using Structure Harvester (Earl & Von Holdt, 2012) and the ΔK value was determined using Evanno's method to estimate the most likely number of clusters (*K*) that best fit the data (Evanno *et al*., 2005). The data were grouped based on hosts and on regions, and the same parameters were used to run the Structure simulations.

Independently of the number of clusters (*K*) inferred by structure, population statistics were calculated for sub-populations based on two host families (Myrtaceae and Melastomataceae) and three regions of Brazilian (North/Northeast, Central-West and Southeast),aiming to verify the influence of different hosts and different regions of Brazil on the genetic variability of *C. cubensis* isolates.

Nei's gene diversity ($\overline{H}$) (Nei, 1973) was calculated using GenAlEx v. 6.501 (Peakall & Smouse, 2006). Genotypic diversity ($\hat{G}$) was calculated according Stoddart & Taylor (1988).

Data generated from 1000 randomizations of the genotypic diversity per locus for each sub-population based on host and region using Multilocus v. 1.3 (Agapow & Burt, 2001) were plotted to determine whether a sufficient amount of diversity was sampled and the number of loci sufficient to reach the maximum of genotypic diversity.

A two-tailed *t*-test at a 95% confidence interval with $(N_1 + N_2) - 2$ degrees of freedom was used to calculate the significance of the difference between genotypic diversities across sub-populations (Stoddart & Taylor, 1988).

In order to estimate genetic (gene) variation within and between populations, molecular variance analysis (AMOVA) was performed using GenAlEx v. 6.501 (Peakall & Smouse, 2006).

Gene flow was calculated using two measures. The theoretical number of migrants per generation ($\widehat{M}$) between sub-populations was calculated (Slatkin, 1993; Slatkin, 1995), and population genetic differentiation (θ) (Weir & Cockerham, 1984) was calculated among pairs of populations using the program Multilocus v. 1.3 (Agapow & Burt, 2001).

Furthermore, the uniqueness alleles (φ) of a sub-population (the probability of sampling a unique/private allele in each sub-population) was calculated according Van der Merwe *et al*,. (2013).

The gametic (linkage) disequilibrium statistic (normalised $\bar{r}_d$) was calculated using Multilocus v. 1.3 (Agapow & Burt, 2001) to infer panmixia (random mating) within the different sub-populations. This statistic is derived from a traditional measure of linkage disequilibrium, the Index of Association (I_A), which measures the probability that two alleles in a population are randomly associated, but which is dependent on sample size (Agapow & Burt, 2001). The significance levels of the $\bar{r}_d$ values were determined by calculating a *p*-value at a 95% confidence interval by generating 10000 randomised $\bar{r}_d$ values of data sets per sub-population. The null hypothesis for this analysis is that there is random association of alleles, *i.e.* the population is in linkage equilibrium.

Determination of mating type

The mating type of each *C. cubensis* isolate was determined by amplifying a *MAT-1* (*MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3*) and *MAT-2* (*MAT 1-2-1*) gene regions (Kanzi *et al*., unpublished). For each primer pair, each reaction comprised of reaction buffer A, 1.5 mM $MgCl_2$, 0.5 U Kapa Taq polymerase (Kapa Biosystems), 0.2 µM of each primer, 200 µM dNTPs, 50 ng genomic DNA, and adjusted to a final volume of 25 µl with nuclease free water (Adcock Ingram). DMSO (2.5 µl) was include in the PCR reaction to amplify portions of *MAT1-1-1* and *MAT1-1-2* gene. The reaction mixes were subjected to denaturation, followed by 35 cycles of 94 °C (35 s), 35 s at a gene region specific annealing temperature (60°C for *MAT1-1*-1, 60.4 °C for *MAT1-1-2*, 58.4 °C for *MAT1-1-3* and 59 °C for *MAT1-2-1*), and 72 °C (1 min). A final extension step was performed at 72 °C (7 min) (Kanzi *et al*., unpublished).

RESULTS

Phylogenetic analyses

The partition homogeneity test PHT analysis showed that the ITS-rDNA and β-tubulin sequence data sets did not have any significant conflict (P=0.013) and could thus be combined (Gryzenhout *et al.*, 2006). The combined sequence dataset, comprising ITS-rDNA fragment and β-tubulin gene sequences, produced 1191 aligned sequence characters of which 1073 were constant, 14 parsimony uninformative, and 104 parsimony informative.

Phylograms obtained by maximum parsimony and maximum likelihood analyses were similar topologies. A total of 53 isolates were confirmed as *C. cubensis* (Table 1), strongly supported by bootstrap values of >97%. Maximum likelihood analysis was chosen for presentation,

where of the 53 isolates analysed, 10 isolates from different hosts and regions were selected to construct an illustrative phylogenetic tree (Figure 1).

Microsatellite marker genotyping

Ten microsatellite markers were divided into two panels based on allelic frequencies and dye colours of fluorescent markers (Table 2). The markers Chry06, Chry13, Chry15, Chry17 and Chry25 had five alleles or more present in the meta-population, while Chry07, Chry24, Chry27, Chry23 and Chry28 had three alleles or less present. The marker with the largest number of alleles as well as the largest allelic range was Chry15 with 13 alleles ranging from 245 bp to 346 bp. The markers with the least number of alleles was Chry07, Chry23 and Chry28 which had two alleles ranging between 225 bp to 226 bp, 149 bp to 150 bp and 229 bp to 230 bp respectively (Table 2).

Population analyses

The probable number of *C. cubensis* sub-populations in Brazil, as inferred by Structure and analysed by Structure Harvester, was K=2 (Figure 2a), suggesting that there were two genetically distinct clusters. Admixture was observed in the bar plot with data grouped according to host (Figure 2b) than data grouped according to region (Figure 2c).

Considering the sub-populations based on the host gene and genotypic diversity was higher in the sub-population from Melastomataceae ($\overline{H}$= 0.462 and $\hat{G}$= 0.909) than in the sub-population from Myrtaceae ($\overline{H}$= 0.455 and $\hat{G}$= 0.891) (Table 3).

For sub-populations based on regions, highest gene diversity was found in the North/Northeast sub-population ($\overline{H}$= 0.462) and the highest genotypic diversity was found in the Central-West sub-population ($\hat{G}$ = 1). While the Southeast sub-population presented the lowest values of gene and genotypic diversity ($\overline{H}$= 0.358 and $\hat{G}$= 0.815) (Table 3).

The number of samples in all as sub-populations was sufficient to analyse the genotypic diversity successfully (Figure 3). On average, seven loci are already sufficient to reach the maximum of the genotypic diversity of the sub-populations (Figure 3).

Two-tailed t-tests showed that there was a significant difference between genotypic diversity of sub-populations based on host and region. Also, all sub-populations, except the Central-West sub-population, had different genotypic diversity ($P<0.05$) from the meta-population.

Analysis of molecular variance revealed that the genetic variation was higher within than among the sub-populations analysed. Genetic variation within all sub-populations, based on both host and region was high, greater than 90% (Table 4).

Genetic variation among sub-populations based on the host Melastomataceae and Myrtaceae was 5% (Table 4). Among the North/Northeast and Central-West sub-populations, the genetic variation was lowest, 1% and there was no significant difference. The highest genetic variation among the sub-populations was 13% and occurred between the Southeast sub-population and the two other North/Northeast and Central-West sub-populations.

Among the sub-populations based on the host Myrtaceae and Melastomataceae the genetic differentiation was significant (θ = 0.045), presenting a considerable number of migrants ($\widehat{M}$ = 10.55) (Figure 4a).

For region, highest number of migrants value between the regions was observed between the North/Northeast and Central-West sub-populations ($\widehat{M}$ = 49.34), coinciding with lowest value of differentiation genetic (θ = 0.01), which was not significant. The number of migrants was low both between Southeast and North/Northeast sub-populations ($\widehat{M}$ = 3.43), and between Southeast and Central-West ($\widehat{M}$ = 3.49). The differentiation was significant both between Southeast and North/Northeast sub-population (θ = 0.127), and between Southeast and Central-West (θ = 0.125) (Figure 4b).

The number of private or shared alleles among subpopulations was shown in the Venn diagram (Figure 4c,d). The proportion of private alleles (φ) of each subpopulation (Table 3)

strengthens data from the Venn diagram. The meta-population has 58 alleles distributed among all ten loci analysed. Considering the sub-populations separated by hosts, 44.82% of the alleles are shared between the two sub-populations. Myrtaceae sub-population has 36.20% (φ = 0.582) of private alleles and the remaining 18.96% (φ = 0.503) are private alleles of the sub-population Melastomataceae (Figure 4c and Table 3). In the sub-populations based on the regions, 27.6% of the alleles were shared among all sub-populations, while 10.34% were shared between the North/Northeast and Central-West, 6.9% were shared between North/Northeast and Southeast, only 1.72% were shared between the Southeast and Central-West sub-populations (Figure 4d). The North/Northeast sub-population presents the highest proportion of private alleles 38.65% (φ = 0.618), while the Central-West sub-population presents the lowest proportion 5.17% (φ = 0.343) (Table 3).

The results of $\bar{r}_d$ for all sub-populations and also for the meta-population were significant ($P<0.05$) (Table 3), this means that the sub-populations are in gametic disequilibrium. The hypothesis of random association of alleles at loci (i.e., random mating) was rejected.

Determination of mating type

The 53 isolates of *C. cubensis* amplified all mating type tested (MAT1-1-1, MAT1-1-2, MAT1-1-3 and MAT1-2-1). This result confirms that all isolates were homothallic.

DISCUSSION

This is the first study on genetic diversity of populations of *C. cubensis* performed with isolates from different regions of Brazil and from native and non-native hosts, using molecular tools. The structure and genetic variability of *C. cubensis* in Brazil were influenced by geographic distribution and hosts.

In this study, analyses of population structure suggest two genetically distinct clusters. The isolates collected from same host did not form into one cluster (Figure 2a). For region, the clusters were also mixed. However, better the separation of the two clusters observed. Southeast and Central-West sub-populations in one cluster for each while the North/Northeast region was shown to be composed of the mixture of the two clusters (Figure 2b). The results indicate that the geographic distribution has more influence on the structure of the population than the host. This result contrasts with the results obtained by Van der Merwe (2012), which for populations of *C. austroafricana* showed a population structure associated to the host. However, other studies indicate that pathogen sub-populations can be influenced by the geographic distribution, as for example in the work performed by Tschurtschenthaler *et al.*, (2012), it was observed that *Phakopsora pachyrhizi* sub-populations were grouped separately into groups representing the Central and Southern regions of Brazil.

There is significant gene and genotypic difference between the sub-populations Myrtaceae and Melastomataceae. However, the high gene flow between Myrtaceae and Melastomataceae justifies the highly admixed allelic composition in which more than 40% of the alleles are shared. The two host-based sub-populations presented high genetic and genotypic diversities. According to Tsutsui *et al.*, (2000), high genetic variability is expected in native or better-established populations. Thus, indicating that *C. cubensis* is well established in Brazil, both on native and on non-native hosts.

High genetic variability was expected in Melastomataceae because this family represents native hosts, which are probably hosts of the pathogen (Nakabonge *et al.*, 2006; Gryzenhout *et al.*, 2009). However, the gene and genotype diversities values were a little higher in Melastomataceae (native hosts) than in Myrtaceae (non-native hosts). This high variability in Myrtaceae can be explained by two factors that may be acting individually or in combination. The first factor is the presence of the sexual phase of the pathogen, which is very common in Myrtaceae family hosts and according to McDonald & Linde (2002), the populations of sexual pathogens usually exhibit a high

genotype diversity degree. The second factor is that although *Eucalyptus* spp. and *Corymbia citriodora* were not native from Brazil, but these species were introduced in the country more than 100 years (Sampaio, 1975; Fishwick, 1975). The first reports of *C. cubensis* in eucalyptus in Brazil were in 1973 (Hodges *et al.*, 1973), thus the pathogen can be evolved along with these non-native hosts for at least 40 years. This coevolution may be contributing to the establishment of the pathogen in non-native hosts and consequently generating high genetic variability.

In sub-populations based on the regions, genotypic diversity was different among all sub-populations. However, the gene diversity was not different to the North /Northeast and Central-West sub-populations. The North/Northeast and Central-West sub-populations have many common alleles and the combination of these alleles generate different genotypes. The very high gene flow between these two subpopulations explains this allele sharing, contributing to the high values of genetic and genotypic diversity in both sub-populations.

With lower gene and genotypic diversities, the Southeast sub-population showed a better-defined grouping of alleles, differing from other sub-populations based on the regions. This higher differentiation can be supported by the low gene flow observed between the Southeast region and the other regions. The lower genetic variability in the sub-population of the Southeast region may be due to the rare occurrence of the sexual phase of the pathogen in this region. Only in one isolate the sexual phase was found. However, in the Central-West region, no isolates were found presenting the sexual phase, and even so, it was the region with the highest genotypic diversity. Thus, in addition to the lack of sexual reproduction of the pathogen, the Southeast sub-population is likely to suffer a genetic drift, which occurs when a small, randomly chosen subset of a population survives an event that causes a severe reduction in population size (McDonald & Linde, 2002). The geographic distance between the North/Northeast, Central-West and Southeast regions may be narrowing the gene flow and caused isolation (genetic drift) of the Southeast region, reducing its genetic variability.

The presence of both asexual reproduction and sexual reproduction in the *C. cubensis* isolates was observed in this study. In the hosts of the Myrtaceae family were found perithecia and pycnidia. While in most of the hosts family Melastomataceae were found only pycnidia. The highest genetic variability found in the Melastomataceae sub-populations indicates that the sexual phase of the pathogen could be present although it was not found in most samples of this family. Possibly the sexual phase of the pathogen was underestimated because the sexual phase is not as common as asexual allied with relative limited sampling of this study.

All isolates of *C. cubensis* analysed in this work are homothallic. This means that they are capable of self-fertilization, but can also perform outcrossing (Burnett, 2003). Van Zyl *et al.*, (1998) studying vegetative compatibility groups (VCG) in *C. cubensis* population in Brazil, found that *C. cubensis* is represented by a large number of VCGs, resenting a very diverse population and that this would be an indicator that the fungus performs a high level of outcrossing. In the present study, the VCGs were not evaluated, so it cannot be affirmed that the sexual phases found in this study were from self-fertilization or outcrossing. However, the high number of genotypes found in sub-populations suggests that outcrossing is present, since outcrossing is responsible for generation of a large number of genotypes (Van der Mewer *et al.*, 2012).

Probably asexual reproduction combined with non-random mating were the possible causes of gametic disequilibrium (Brewer *et al.*, 2012) in the sub-populations of *C. cubensis* in Brazil. Non-random mating occurs because *C. cubensis* is a homothallic fungus with frequent self-fertilization. However as previously reported it is believed that outcrossing is occurring being responsible for the high genotypic diversity found, but the gametic disequilibrium indicates that the outcrossing frequency is not being sufficient to balance the population.

High genetic variability found in the *C. cubensis* population in Brazil in this study. Van der Mewer *et al*. (2012) also found the high diversity values in Colombia (Table 3) which results contrasted with the low values of diversity genetics found by Nakabongea *et al*. (2007), in *C. cubensis* populations in South Africa. The presence of *C. cubensis* in native hosts of Brazil allied

with the high genetic variability found support the hypothesis that the center of origin of *C. cubensis* is Latin America. However, in order to clarify which Latin American country is the center of origin of *C. cubensis*, more studies are needed with more isolates from different countries.

The high genetic variability of *C. cubensis* in Brazil is probably contributing to the fact that the clones selected in the Southeast region lose their resistance when introduced in the North and Northeast. According to McDonald & Linde (2002), the durability of disease resistance is affected by the evolutionary potential of the pathogen population. Pathogens with greatest risk of breaking down resistance genes possess a mixed reproduction system, with sexual and asexual reproduction, and a high potential for gene flow. All these characteristics were found in the population of *C. cubensis* in Brazil. Therefore, it is very important that the breeding and selection programs of eucalyptus clones use several isolates with different genotypes from different regions and hosts during the screening of the resistance of the clones to *C. cubensis*.

The results of this study also point to the environmental importance of *C. cubensis* when associated with *Tibouchina* species. During the study, it was observed that many trees of *Tibouchina* spp. declined and died due to the pathogen's attack. It is very important that future studies be done to verify the current situation and possible damages caused by *C. cubensis* in *Tibouchina* spp. both in native forests as in urban afforestation and reforestation, as well as initiating studies of selection of progenies of *Tibouchina* spp. resistant to *C. cubensis*.

REFERENCES

Agapow PM, Burt A. 2001. Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes,* **1**, 101-102.

Alfenas A, Hubbes M, Couto L. 1982. Effect of phenolic compounds from *Eucalyptus* on the mycelial growth and conidial germination of *Cryphonectria cubensis*. *Canadian Journal of Botany,* **60**, 2535–2541.

Alfenas A, Jeng R, Hubbes M. 1983. Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. *European Journal of Forest Pathology* **13**, 197-205.

Backes P, Irgang B. 2004. *Mata Atlântica. As árvores e a paisagem*. Editora Paisagem do Sul, Porto Alegre.

Barreto RW, Rocha FB, Ferreira FA. 2006. First record of natural infection of Marlierea edulis by the eucalyptus canker fungus *Chrysoporthe cubensis*. *Plant Pathology*, **55**, 577-577.

Brewer MT, Frenkel O, Milgroom MG. 2012. Linkage Disequilibrium and Spatial Aggregation of Genotypes in Sexually Reproducing Populations of *Erysiphe necator*. *Phytopathology*, **102**, 997-1005.

Burnett JH. 2003. *Fungal populations and species. Oxford University Press, Oxford*, 346 p.

Earl DA, Vonholdt BM. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, **4**, 359-361.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, **14**, 2611-2620.

Farris JS, Källersjö M, Kluge AG, Bult C. 1994. Testing significance of incongruence. *Cladistics*, **10**, 315-319.

Ferreira AF, Milani D. 2004. Avaliação de resistência de clones de Eucalipto às infecções naturais de *Cryphonectria cubensis*, com nova metodologia. *Revista Árvore*, **28**, 313-316.

Fishwick RW. 1975. Forestry in Brazil. *The Commonwealth Forestry Review*, 53-63.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology,* **61**, 1323-1330.

Gryzenhout M, Myburg H, Van der Merwe NA, Wingfield BD, Wingfield MJ. 2004. *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. *Studies in Mycology,* **50**, 119-142.

Gryzenhout M, Rodas CA, Portales JM, Clegg P, Wingfield BD, Wingfield MJ. 2006. Novel hosts of the Eucalyptus canker pathogen *Chrysoporthe cubensis* and a new *Chrysoporthe* species from Colombia. *Mycological research*, **110**, 833-845.

Gryzenhout M, Wingfield BD, Wingfield MJ. 2009. *Taxonomy, phylogeny, and ecology of bark-inhabiting and tree-pathogenic fungi in the Cryphonectriaceae*. Prestoria, South Africa: American Phytopathological Society (APS Press), 119 p.

Guimarães LMDS, Resende MDVD, Lau D, Rosse LN, Alves AA, Alfenas AC. 2010. Genetic control of *Eucalyptus urophylla* and *E. grandis* resistance to canker caused by *Chrysoporthe cubensis*. *Genetics and Molecular Biology,* **33**, 525-531

Heerden SW, Wingfield MJ. 2002. Effect of environment on the response of Eucalyptus clones to inoculation with *Cryphonectria cubensis*. *Forest Pathology*, **32**, 395-402.

Hodges CS, Reis MS, Ferreira FA, Henfling JDM. 1976. O cancro do eucalipto causado por *Diaporthe cubensis*. *Fitopatalogia Brasileira*, Brasília, **1**, 129-170.

Hodges CS, Reis MS, May L. 1973. Duas enfermidades em plantações de essências florestais exóticas no Brasil. *Brasil Florestal*, **4**, 5-12.

Katoh K, Rozewicki J, Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization, *Briefings in Bioinformatics*, https://doi.org/10.1093/bib/bbx108.

Lorenzi, H. (1992). *Árvores brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil*. Nova Odessa: Editora Plantarum 352 p.

McDonald BA, Linde C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annual review of phytopathology*, **40**, 349-379.

Nakabonge G, Gryzenhout M, Wingfield BD, Wingfield MJ, Roux J. 2007. Genetic diversity of *Chrysoporthe cubensis* in eastern and southern Africa. *South African Journal of Science*, **103**, 261-264.

Nakabonge G, Roux J, Gryzenhout M, Wingfield M J. 2006. Distribution of *Chrysoporthe* canker pathogens on Eucalyptus and Syzygium spp. in eastern and southern Africa. *Plant Disease*, **90**, 734-740.

Nei M. 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences*, **70**, 3321-3323.

Peakall ROD, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Resources*, ***6***, 288-295.

Peralta, P. 2002. Las especies del género Tibouchina (Melastomataceae) en Argentina. *Darwiniana*, 107-120.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics*, ***155***, 945-959.

Roux J, Meke G, Kanyi B, Mwangi L, Mbaga A, Hunter GC, Nakabongea G, Heatha RN, Wingfield MJ. 2005. Diseases of plantation forestry trees in eastern and southern Africa. *South African Journal of Science*, **101**, 409-413.

Seixas CD, Barreto RW, Alfenas AC, Ferreira FA. 2004. *Cryphonectria cubensis* on an indigenous host in Brazil: a possible origin for eucalyptus canker disease?. *Mycologist*, **18**, 39-45.

Sharma JK, Mohanan C, Florence EJM.1985. *Disease survey in nurseries and plantations of forest tree species grown in Kerala***:** research report. Kerala: Kerala Forest Research Institute, 268 p.

Slatkin M. 1993. Isolation by Distance in Equilibrium and Non-Equilibrium Populations. *Evolution*, **47**, 264-279.

Slatkin. 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**, 457-462.

Souza HG. 2008. Resistencia do eucalipto ao cancro de *Chrysoporthe cubensis* e *Botryosphaeria* sp.. Piracicaba, Brasil: Universidade Estadual Paulista "Julio de Mesquita Filho", PhD thesis.

Stoddart JA, Taylor JF. 1988. Genotypic diversity: estimation and prediction in samples. *Genetics*, **118**, 705-711.

Swofford DL. 2002. PAUP: Phylogenetic analysis using parsimony Version 4.0 beta. Sunderland, MA: Sinauer Associates Inc, software.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, **30**, 2725-2729.

Tamura K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+ C-content biases. *Molecular biology and evolution*, **9**, 678-687.

Tschurtschenthaler NN, Vieira ESN, Dalla Nora T, Schuster I. 2012. Variabilidade genética de Phakopsora pachyrhizi avaliada por meio de marcadores microssatélites. *Pesquisa Agropecuária Brasileira*, **47**, 181-186.

Tsutsui ND, Suarez AV, Holway DA, CASE TJ. 2000. Reduced genetic variation and the success of an invasive species. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 5948-5953.

Van der Merwe NA, Gryzenhout M, Steenkamp ET, Wingfield BD, Wingfield MJ. 2010. Multigene phylogenetic and population differentiation data confirm the existence of a cryptic species within *Chrysoporthe cubensis*. *Fungal biology*, **114**, 966-979.

Van der Merwe NA, Steenkamp ET, Rodas C, Wingfield BD, Wingfield MJ. 2013. Host switching between native and non-native trees in a population of the canker pathogen *Chrysoporthe cubensis* from Colombia. *Plant Pathology*, **62**, 642-648.

Van der Merwe NA. 2012. *Phylogeography and population biology of Chrysoporthe austroafricana and allied species.*Pretoria, South Africa: University of Pretoria, PhD thesis.

Van Zyl L, Wingfield MJ, Alfenas AC, Crous P. 1998. Population diversity among Brazilian isolates of *Cryphonectria cubensis*. *Forest Ecology and Management*, **112**, 41-47.

Weir BS, Cockerham CC. 1984. Estimating F-Statistics for the Analysis of Population Structure. *Evolution*, **38**, 1358-1370.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, **18**, 315-322.

Wingfield MJ, Roux J, Slippers B, Hurley BP, Garnas J, Myburg AA, Wingfield BD. 2013. Established and new technologies reduce increasing pest and pathogen threats to Eucalypt plantations. *Forest Ecology and Management*, **301**, 35-42.

Wingfield MJ. 2003. McAlpine Memorial Lecture Increasing threat of diseases to exotic plantation forests in the Southern Hemisphere: lessons from Cryphonectria canker. *Australasian Plant Pathology*, **32**, 133-139.

Wingfield MJ.1999. Pathogens in exotic plantation forestry. *International Forestry Review*, **1**, 163-8.

Table 1. Information for *Chrysoporthe cubensis* isolates used in the population study.

Isolates	Number of isolates	Host		Location	
		Family	Species	City-State	Region
CCD06*, CCD07, CCD09, CCD10, CCD11, CCD13, CCD14, CCD15, CCD16	9	Myrt.	*Corymbia citriodora*	Dueré-TO	North
CCT01*, CCT03, CCT05, CCT06, CCT07, CCT08, CCT11, CCT13, CCT15, CCT17, CCT18, CCT19, CCT20,	13			Figueirópolis-TO	
CTJ03*, CTJ04, CTJ09, CTJ11, CTJ12, CTJ13, CTJ15, CTJ17	8			Alvorada-TO	
CC4*, CE29 , CE43	3	Myrt.	*Eucalyptus* spp.	Açailândia-MA	Northeast
TGCD02*	1	Mela.	*Tibouchina granulosa*	Lençóis-BA	
TGG03*, TGG05, TGG06, TGG07, TGG09, TGG10, TGG13	7	Mela.	*Tibouchina granulosa*	Goiânia-GO	Central-West
TGTL01*	1			Três Lagoas-MS	
CT02	1	Mela.	*Tibouchina granulosa*	Lavras-MG	Southeast
CT08, CT10	2			São João del Rei-MG	
THS01*,	1		*Tibouchina heteromalla*	Silveirânia-MG	
THSC05, THSC06*	2			São Roque de Minas-MG	
TIPASC01	1		*Tibouchina* spp.	São Roque de Minas-MG	
TGII11	1			Itumirim-MG	
TUC01*, TUC02, TUC03	3		*Tibouchina urvilleana*	Carrancas-MG	

*Selected isolates for the construction of the illustrative phylogenetic tree.
Myrt.= Myrtaceae; Mela.=Melastomataceae

Table 2. Characteristics of polymorphic microsatellite markers used for genotyping *Chrysoporthe cubensis* isolates from different regions and hosts in Brazil.

Microsatellite markers	**Motif**	**Forward primer Sequence (5' to 3')**	**Reverse primer Sequence (3' to 5')**	**Ta* (^{o}C)**	**Allelic Range (bp**)**	**Number of alleles**	**Panel**
CHRY06	AGC	CCCCAAGCTCGCCTACTTC	GACGCTCTCGGGTATTGTCA	63	211-217	5	A
CHRY07	AGT	TTGGTCGGTTCGGTAGAAAG	CATGACGGGAGAGCGATAAG	60	225-226	2	A
CHRY13	TGGTC	GTGTCGTCGACTCAAGCTCA	GACGGAGAGGACACTGAAGC	63	216-267	11	B
CHRY15	TGA	AAGGCCAGATTGTCCAAATG	CAGCAACGAACCAAAATCAA	58	245-346	13	B
CHRY17	ACC	AGATCCAAGTGGGTTTCCT	CTTAGCTTGACCCAGGGATG	60	148-172	8	A
CHRY23	AGC	TGTCCTGCATCTAGGTCGTG	GTCCCAGTCCTCTTGGATCA	61	149-150	2	B
CHRY24	ATC	GTGCGGGCATCTGTTCTACT	GCGCATTGTGAGGCGTTAAG	61	185-188	3	B
CHRY25	ACC	TATCTGCGCAACTACCACCA	GGGAAGTGTGTCTCCTCCTG	61	162-188	9	A
CHRY27	AGC	GCCGCCTACAGAGACTATAACG	CGACGAAGACGTAGTTGCAC	63	231-235	3	A
CHRY28	TGGTC	GATATGCCGGTCATGGTGAT	TACTTGAGGGCGAAGGAAAG	60	229-230	2	B

*****Annealing temperature
******base pairs

Table 3. Gene diversity, genotypic diversity, private allele and gametic (linkage) disequilibrium in meta-population and sub-populations of *Chrysoporthe cubensis* from different regions and hosts in Brazil.

Countries	**Sub-population**		**Number of Isolates (N)**	**Gene diversity** $(\bar{H})$	**Genotypic diversity** $(\widehat{G})$	**Private allele** (φ)	**Gametic (linkage) disequilibrium** $(\bar{r}_d)$
Brazil	Host	Myrtaceae	33	0.455	0.891	0.582	0.059*
		Melastomataceae	20	0.462	0.909	0.503	0.053*
	Region	North/Northeast	34	0.456	0.894	0.618	0.056*
		Central-West	8	0.444	1.000	0.343	0.125*
		Southeast	11	0.358	0.846	0.415	0.070*
	Meta-population		53	0.478	0.815	-	0.048*
Colombia**	Host	Myrtaceae	59	0.445	1.000	0.530	-
		Melastomataceae	32	0.398	0.314	0.249	-
South Africa***	Region	Kenya	10	0.100	0.172	-	-
		Malawi	51	0.290	0.053	-	-

*The populations are in gametic disequilibrium (P<0.05)

**Van der Merwe *et al.*, 2013

***Nakabongea *et al.*, 2007

Table 4. Genetic variation among and within sub-populations of *Chrysoporthe cubensis*, from different regions and hosts in Brazil. Estimated by analysis of molecular variance (AMOVA).

	Source	df	SS	MS	Variance Component	Percentage of total Variance (%)
Melastomataceae	**Among Pops**	1	5.350	5.350	0.116	5%
vs	**Within Pops**	51	125.159	2.454	2.454	95%
Myrtaceae	**Total**	52	130.509		2.570	100%
North/Northeast	**Among Pops**	1	2.755	2.755	0.025^{ns}	$1\%^{ns}$
vs	**Within Pops**	40	97.412	2.435	2.435	99%
Central-West	**Total**	41	100.167		2.460	100%
Central-West	**Among Pops**	1	5.488	5.488	0.338	13%
vs	**Within Pops**	17	40.091	2.358	2.358	87%
Southeast	**Total**	18	45.579		2.696	100%
Southeast	**Among Pops**	1	8.075	8.075	0.344	13%
vs	**Within Pops**	43	101.503	2.361	2.361	87%
North/Northeast	**Total**	44	109.578		2.704	100%

ns= non-significant (P>0,05)

df=degrees of freedom

SS=sum of squares

MS=mean square

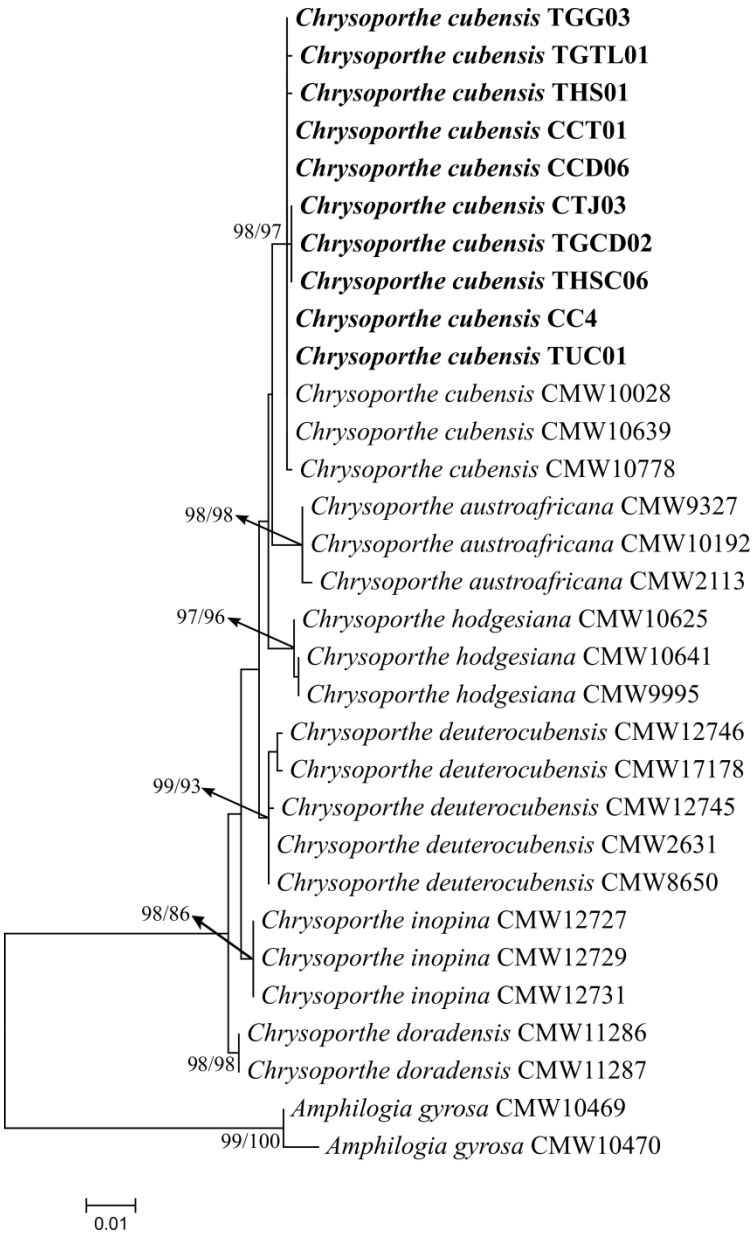

Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood (ML) combined DNA sequence data set of regions of the Internal Transcribed Spacer of rRNA gene (ITS), and Beta-tubulin (BT1 and BT2 regions). Bootstrap values above 85% are indicated above each branch (ML/MP). The isolates of *Chrysoporthe cubensis* isolated from this study are highlighted.

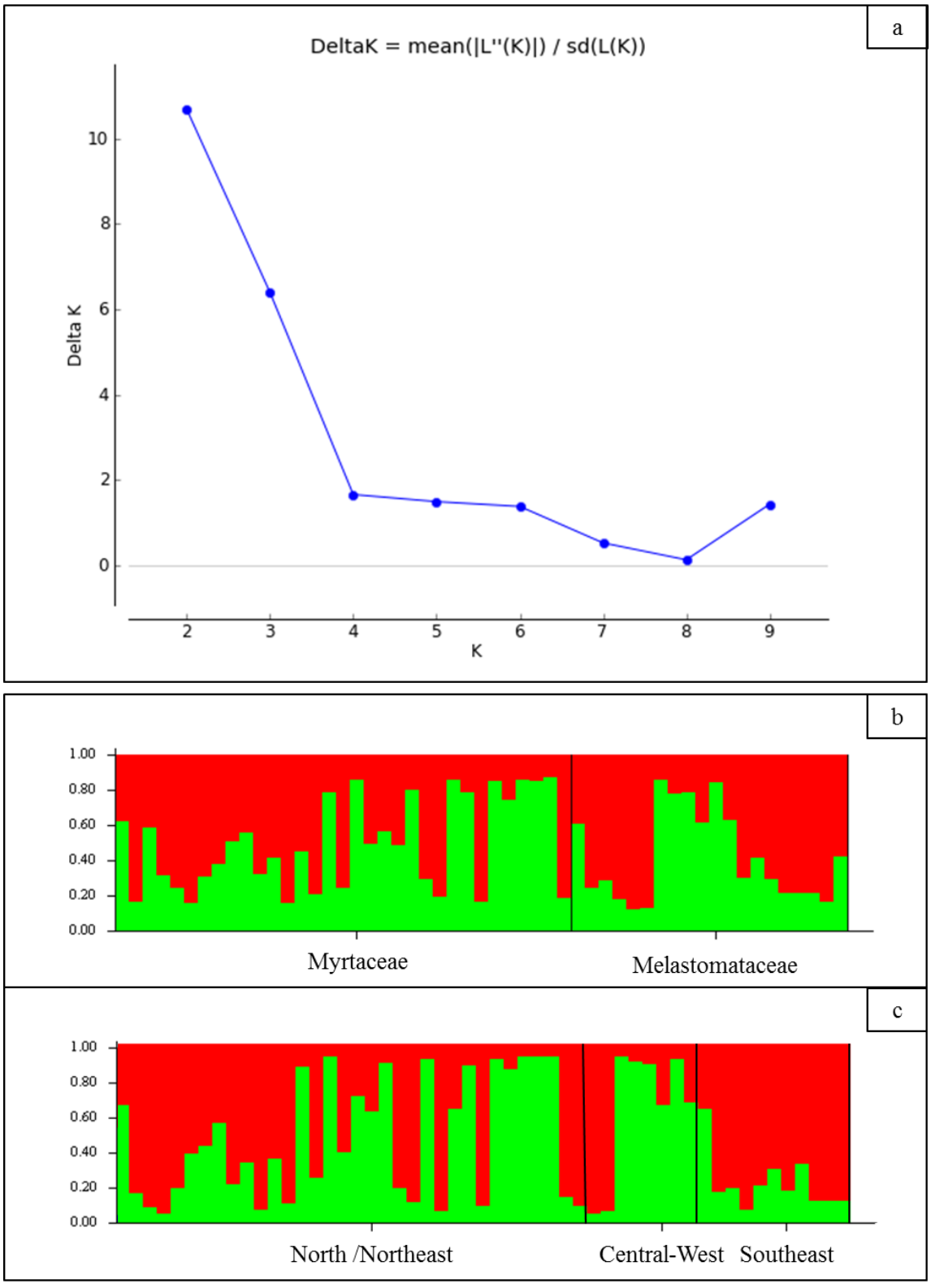

Figure 2. (a) Delta K plot generated from posterior probability values obtained from structure analysis of microsatellite data. (b,c) Structure plot of *Chrysoporthe cubensis* populations in Brazil. (b) Isolates based on host. (c) Isolates based in region.

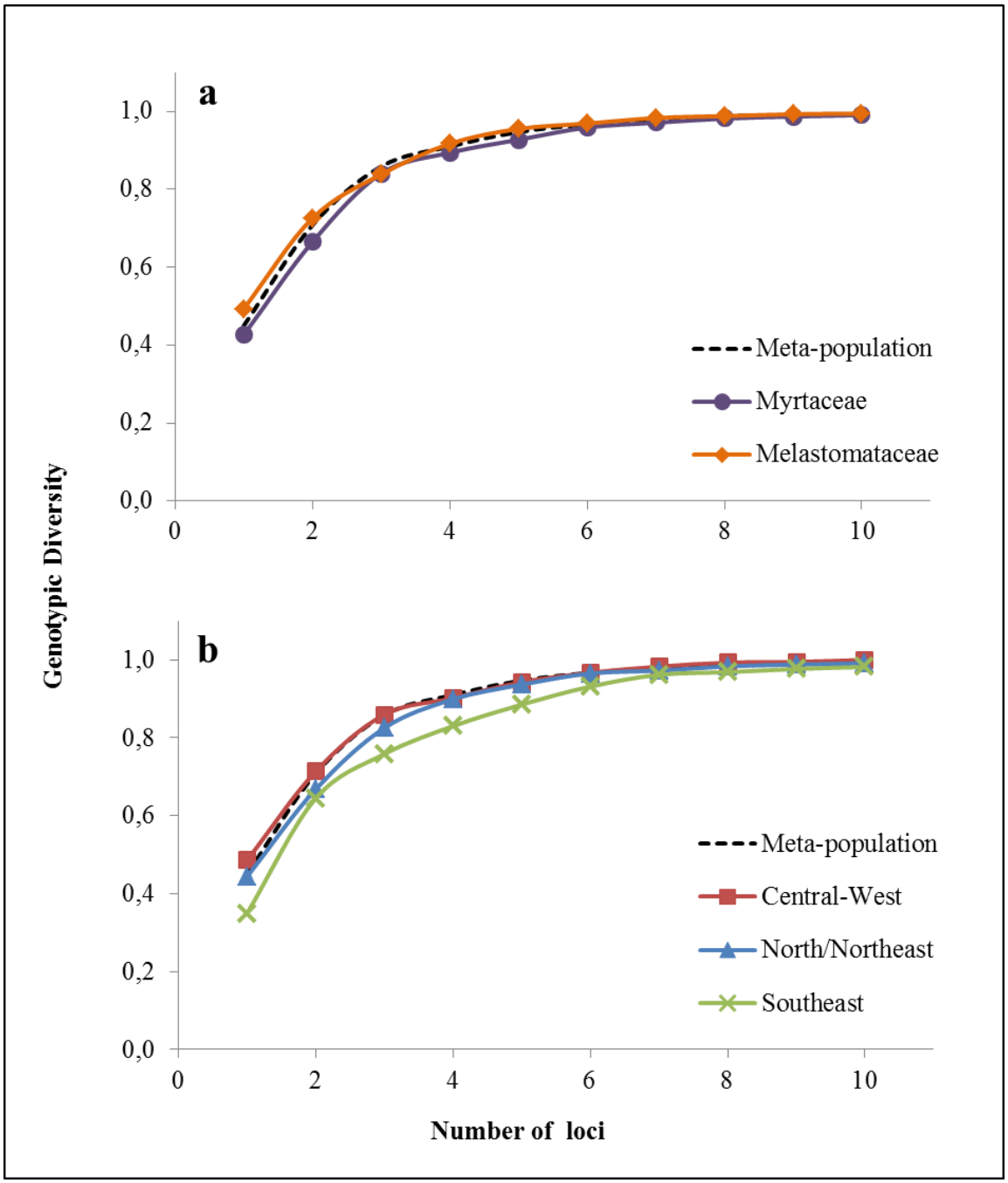

Figure 3. Genotypic diversity per number of loci for *Chrysoporthe cubensis*. (a) isolates from different hosts; (b) isolates from different regions.

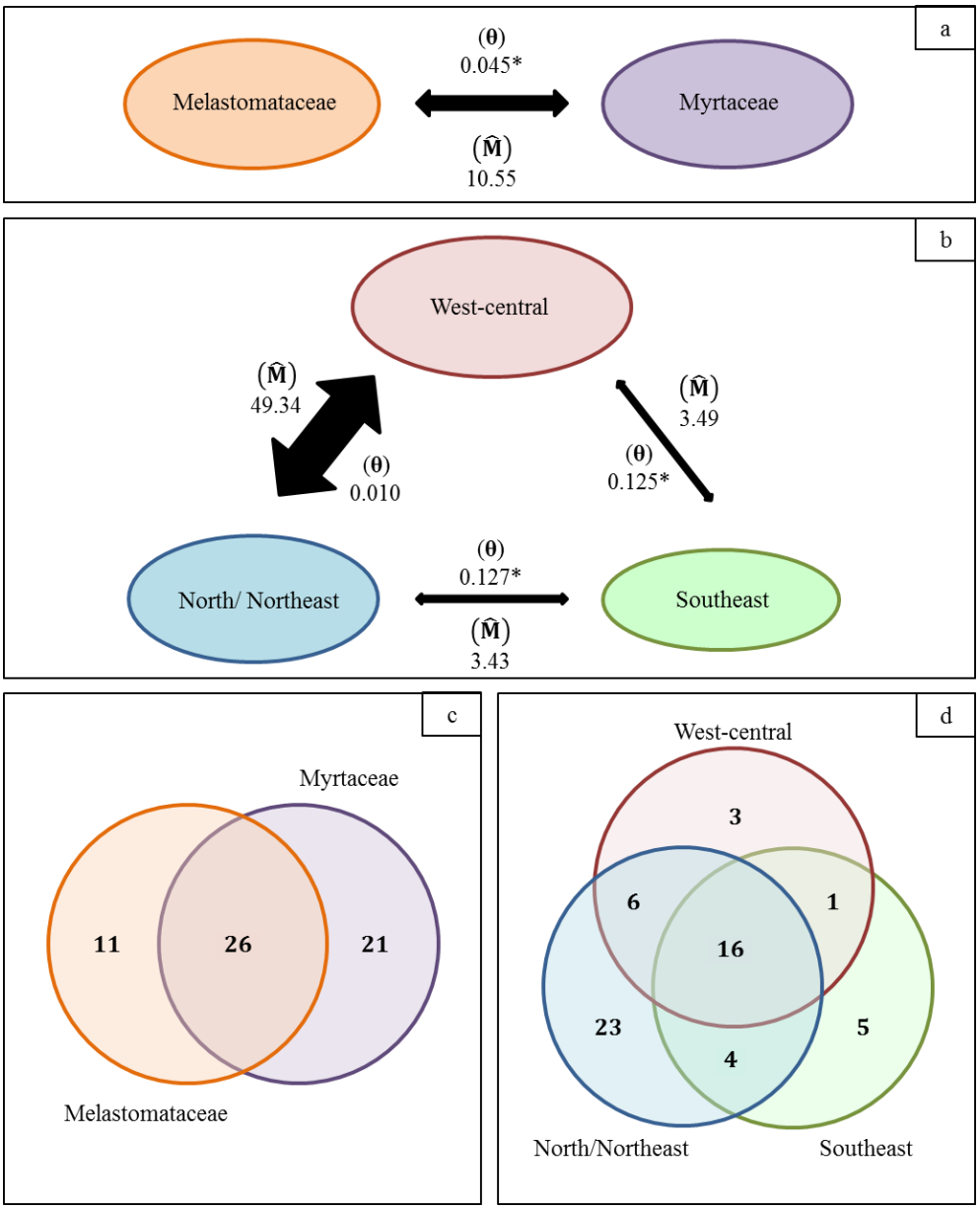

Figure 4. (a,b) Plot of gene diversity $(\bar{H})$., genotypic diversity $(\hat{G})$, gene flow $(\hat{\mathrm{M}})$ and genetic differentiation (θ) of *Chrysoporthe cubensis* isolates from sub-populations based on different hosts (a) and regions (b). Thickness and shade of lines with arrows show amount of gene flow$(\hat{\mathrm{M}})$ between subpopulation. Shape of circles show diversity in each subpopulation; horizontal width representing genotypic diversity $(\hat{G})$ and vertical width representing gene diversity $(\bar{H})$. *Significance (P≤0.05). (c,d) Venn diagram representing the distribution of the private and shared alleles among the sub-populations of *C. cubensis* isolates, based in hosts (c) and region (d).

ARTICLE 2: *Chrysoporthe puriensis* sp. nov. from *Tibouchina* spp. in Brazil: phylogeny, morphology, pathogenicity and population structure.

ABSTRACT

Unknown species of *Chrysoporthe* may be occurring in the trees of *Tibouchina* spp. in Brazil, causing canker and death in both urban afforestation and native forest. In order to verify the occurrence of unknown species, the present study performed phylogenetic, morphological, and pathogenic and population characterization of *Chrysoporthe* sp. isolated from *Tibouchina* spp. in Brazil. In this work, 45 isolates, from different states of Brazil, formed a distinct clade from other *Chrysoporthe* species using the combined data of rDNA ITS and β-tubulin sequences and morphological characters, mainly the stromatic tissue *textura*, differentiate one new species, named as *Chrysoporthe puriensis.* This novel species on *Tibouchina* spp. tree causes branch die-back, stem cankers, and death of plants. Fruiting sexual or asexual bodies of a fungus were found between the cankers and on dead areas of the stem. The microsatellites analyses showed differentiation between *C. cubensis* and *C. puriensis* populations. Low number of migrants value between *C. cubensis* and *C. puriensis* populations was observed. *Chrysoporthe puriensis* population showed higher gene and genotypic diversities reaching maximum value of genotypic diversity (G=100%). Pathogenicity tests on *T. granulosa, T. heteromalla* and hybrids of *Eucalyptus grandis* x *E. urophylla* showed that *C. puriensis* is not host-specific. The results have important implications for trees of *Tibouchina* spp. and *Eucalyptus* spp.. For *Tibouchina* spp., *C. puriensis* has affected the ecosystem caused tree deaths in both natural habitats, in urban environments and reforestation areas. For *Eucalyptus* spp., *C. puriensis* presents a potential

risk to the plantations, since it does not know if the species and clones currently used present resistance to its attack.

INTRODUCTION

Chrysoporthe species are known as causal agent of canker in several hosts on Myrtaceae, Melastomataceae and Lyrtaceae families (Hodges *et al*., 1979, Gryzenhout *et al*., 2005, Rodas *et al*., 2005, Gryzenhout *et al*., 2006).

Chrysoporthe cubensis was the first described species, initially identified as *Diaporthe cubensis*, causing canker and threatening eucalyptus plantations in Cuba (Bruner, 1917). After this description, new reports have been recorded worldwide, including the occurrence of new species described in several countries of South and Central America, South Africa, Southeast Asia, and Australia (Gryzenhout *et al*., 2004; Nakabonge *et al*., 2006, Roux *et al*., 2005), which can be distinguished through morphological characteristics and molecular studies.

Through comparative studies of DNA sequences of the β-tubulin and histone H3 genes, it was initially suggested that *Chrysoporthe cubensis* isolates represented three different lineages (Myburg *et al*., 2002). One of them comprised isolates, mainly from Southeast Asia and Australia, while the others represented isolates from South Africa and South America (Myburg *et al*., 2002; Myburg *et al*., 2003; Myburg *et al*., 2004, Gryzenhout *et al*., 2004). Subsequently the specimens representing the lineage of South Africa were differentiated by morphological characters and were described as *Chrysoporthe austroafricana* (Gryzenhout *et al*., 2004). Isolates representing the South American and Southeast Asian lineage are indistinguishable morphologically, but phylogenetic analyses of sequences for four variable gene regions, rDNA ITS, actin, β-tubulin, and eukaryotic translation elongation factor 1-a, separated representative isolates in two species: *C. cubensis*,

the lineage South American and *C. deuterocubensis*, the lineage Southeast Asian (Van der Merwe *et al*., 2010).

Currently, eight species are described into the genus (Bruner, 1917; Gryzenhout *et al*., 2004; Gryzenhout *et al*., 2005; Gryzenhout *et al*., 2006; Van der Merwe *et al*., 2010; Chungu *et al*., 2009) and its wide distribution is due to the favourable climate in the countries of occurrence, mainly in tropical and subtropical areas, predominated by high temperatures and humidity (Hodges *et al*., 1976; Sharma *et al*. 1985).

In Brazil, *C. cubensis* and *C. doradensis* have been reported (Santos, 2015). Since *C. cubensis* was found to occur in *Syzygium aromaticum*, *Plinia edulis*, *Corymbia citriodora*, *Eucalyptus* spp. and species of *Tibouchina*. While *C. doradensis* was found occurring in *Eucalyptus* spp. and *Tibouchina* spp. (Hodges *et al*., 1973; Seixas *et al*., 2004; Baretto *et al*., 2006, Santos, 2015). All *Chrysoporthe* species reported in Brazil cause damage to trees of *Tibouchina* spp.. *Tibouchina* species are widely used in urban afforestation and in the recovery of degraded areas. Many trees are being found dying or dead in both urban and native forest, with canker symptoms similar to those caused by *Chrysoporthe* species.

Considering that Latin America a potential center of origin of the *Chrysoporthe* species, we hypothesized that there is unknown species causing canker disease on trees of *Tibouchina* spp. in Brazil. The present study aimed to characterize the phylogenetic, morphological, pathogenic and population of *Chrysoporthe* sp. isolated from *Tibouchina* spp. in Brazil.

MATERIAL AND METHODS

Symptoms and Isolates

Trees of *Tibouchina* spp. from native forests and urban afforestation in three Brazilian states (Bahia, Minas Gerais and Rio de Janeiro), were examined to verify the presence of cankers that justified the death of trees. Bark of the plants covered by structures similar to those from *Chrysoporthe* species were sampled.

From each tree sampled, an isolate was obtained from a single spore. The cultures of a single spore were obtained from the exudate of the fruiting body of the fungus, which with a sterile needle was suspended in sterile water and plated out on 20% w/v potato dextrose agar (PDA). The plates were incubated in the dark at 28 °C for 24h. From each plate, germinated single spores were transferred to fresh PDA plates and incubated at 28 °C for 7 days. The isolates were stored in microtubes containing 0.85% NaCl and kept at room temperature (16.5–23 °C) (Castellani, 1939) and in microtubes with 15% glycerol and stored at -80 °C. The Castellani's method was used to maintain the isolates during the experiments.

The resultant cultures were maintained in the culture collection of the Forest Pathology Laboratory (LPF) of Federal University of Lavras, Brazil, and representative isolates have been deposited in culture collection Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. The original bark specimens collected to the isolations have been deposited in the herbarium of the Forest Pathology Laboratory (LPF) of Federal University of Lavras, Brazil.

The isolates were grown at 28 °C for 7-10 days in the dark in liquid malt extract (20% w/v). The mycelium was filtered. Total genomic DNA was extracted using a Wizard Genome DNA Purification Kit (Promega of USA) according to the manufacturer's instructions.

DNA sequence comparisons

Polymerase chain reaction (PCR) was used to amplify two nuclear regions, namely internal transcribed spacer (ITS) the conserved 5.8 S gene of the ribosomal DNA (primers ITS1 and ITS4, White *et al.*, 1990) and the β-tubulin gene region with two pair primers (primers Bt1a/Bt1b and Bt2a/Bt2b, Glass & Donaldson, 1995). Sequencing was performed using Big Dye terminator sequencing kits (Life Technologies) on an ABI 13100 machine (Applied Biosystems) as per the instructions of the manufacturers and as published previously by Van der Merwe *et al.*, (2010). Sequences were manually edited when needed.

Sequences of forty five isolates obtained in this study (Table 1), as well as of 25 representatives for the other known species of *Chrysoporthe* and *Amphilogia gyrosa*, as the outgroup taxa, were used in phylogenetic analyses. Sequences of the reference species of *Chrysoporthe* and *Amphilogia gyrosa* were obtained from Genbank (Table 2). For the purpose of this study, sequence alignments were done using the online interface of MAFFT (Katoh *et al.*, 2017). Partition homogeneity test (PHT) (Farris *et al.*, 1994) was applied using PAUP* 4.0 (Swofford, 2002) to the combined data of rDNA ITS and β-tubulin sequences, using 1000 replicates, to ascertain whether they could be collectively analysed. Combined gene alignment was subjected to Maximum likelihood (ML) and Maximum parsimony (MP) analyses.

Maximum parsimony (MP) analysis was realized using PAUP* 4.0 (Swofford, 2002). Only parsimony informative characters were used. The Heuristic search option with random stepwise addition and tree bisection reconnection (TBR) was used as the swapping algorithm. Confidence levels of the branching points were determined using 1000 bootstrap replicates.

Maximum likelihood (ML) analysis was conducted using MEGA 6 (Tamura *et al.*, 2013), incorporating the Tamura 3-parameter model of evolution as determined by MEGA 6 (Tamura, 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites. The confidence in branches was tested using 1000 bootstrap replicates.

Population genetic analyses

In this study all isolates confirmed as "*Chrysoporthe puriensis*" and 53 isolates of *C. cubensis* (Oliveira, unpublished, article 1) were used for population genetic analyses. The inclusion of *C. cubensis* isolates was necessary to determine if there was significant differences between populations of isolates representing "*C. puriensis*" and *C. cubensis*. Population structure, genetic and genotypic differentiation, and gene flow between these species were estimated. For "*C. puriensis*", gene and genotypic diversities and gametic disequilibrium were also estimated. For this purpose, 12 labelled polymorphic microsatellite markers microsatellite markers (Table 3) developed by Mkabili *et al.*, (unpublished) were used following previously described methods (Mkabili *et al.*, unpublished; Mara E. S. Oliveira, unpublished data, article 1 of this thesis).

A test for population division was performed using STRUCTURE v. 2.3.1 (Pritchard *et al*., 2000). The allelic data was analysed assigning two populations, *i.e*., *C. cubensis* and "*C. puriensis*", were used as priors. The parameters included a burn-in of 100 000 steps, followed by 100 000 MCMC steps under an admixture model with correlated allele frequencies, and 10 replicates from K=1 to K=5. The results of the posterior probability values for each set of runs for each K were analysed using Structure Harvester (Earl & Von Holdt, 2012) and the ΔK value was determined using Evanno's method to estimate the most likely number of clusters (K) that best fit the data (Evanno *et al*., 2005).

To estimate genetic (gene) variation within and between populations, molecular variance analysis (AMOVA) was performed using GenAlEx v. 6.501 (Peakall & Smouse, 2006).

Gene flow was calculated using two measures. The theoretical number of migrants per generation ($\widehat{M}$) between populations was calculated (Slatkin, 1993; Slatkin, 1995), and population genetic differentiation (θ) (Weir & Cockerham, 1984) was calculated among populations using the program Multilocus v. 1.3 (Agapow & Burt, 2001). Additionally, the uniqueness alleles (φ) of a population (the probability of sampling a unique/private allele in each population) was calculated according Van der Merwe *et al.,* (2013).

For "*C. puriensis*" population gene diversity ($\overline{H}$) (Nei, 1973) was calculated using GenAlEx v. 6.501 (Peakall & Smouse, 2006) and genotypic diversity ($\hat{G}$) was calculated according Stoddart & Taylor (1988). The gametic (linkage) disequilibrium statistic (normalised $\bar{r}_d$) was calculated using Multilocus v. 1.3 (Agapow & Burt, 2001) to infer panmixia (random mating). The significance levels of the $\bar{r}_d$ values were determined by calculating a *p*-value at a 95% confidence interval by generating 10000 randomised $\bar{r}_d$ values of data sets per sub-population. The null hypothesis for this analysis is that there is random association of alleles, *i.e.* the population is in linkage equilibrium. Data generated from 1000 randomizations of the genotypic diversity per locus using Multilocus v. 1.3 (Agapow & Burt, 2001) were plotted to determine whether a sufficient amount of diversity was sampled and how much locus is sufficient to reach the maximum of genotypic diversity.

Morphological analyses

For morphological characterization, fruiting structures from bark specimens were observed and sectioned. Sections were mounted in lactophenol and examined using light microscopy (LABOMED Lx400, Labo America, Fremont, Canada) equipped with an iVu 500 camera and Software Capture Pro 2.8.8.5. Fifty asci, ascospores, conidiophores and conidia were measured, and a range was obtained for ascostromata and conidiomata. Measurements

were represented as (min –)(average – SD) – (average + SD)(– max) μm, where SD is the standard deviation. The pictures were taken under Zeiss observer Z.1 motorized inverter microscope using differential interference contrast (DIC).

Pathogenicity

The pathogenicity of "*C. puriensis*" was tested on *T. granulosa*, *T. heteromalla* seedlings (18 months old) and on hybrids of *Eucalyptus grandis* x *E. urophylla* seedlings (4 months old) in a greenhouse, room temperature (15-27 °C). The isolate CT13 randomly selected was used.

The isolate was grown on 2% MEA and maintained in the dark at 28 °C for 7 days. Ten seedlings each host were inoculated with *C. puriensis* and ten others with a sterile water agar plug to serve as control. A 5-mm-diameter cork borer was used to remove a disc of bark from seedlings to expose the cambium, and a mycelial plug of equal size was taken from actively growing cultures and placed into the wounds with the mycelia facing the cambium. Wounds were sealed with parafilm 'M' (American National Can™ Chicago, USA) to avoid desiccation (Chungu *et al*., 2009).

Lesion lengths were recorded 8 weeks after inoculation (w.a.i.) to determine the pathogenicity of the *C. puriensis*. Re-isolations were made from lesions and fruiting structures. The identification was performed observing the morphological characteristics of fruiting structures under a light-microscope.

Lesion length averages were analysed using the Scott-Knott test (Scott & Knott, 1974). To verify the significance among the averages, the t-test was used and the values $P \leq 0.05$ were considered as significant.

RESULTS

Symptoms and Isolates

A total of 45 isolates of "*C. puriensis*" were collected from *T. granulosa*, *T. candolleana*, *T. heteromalla* and *Tibouchina* spp. trees in seven cities in Brazil (Table 1). Symptoms on *Tibouchina* spp. tree cracked bark, branch die-back (Figure 1a), canker on stem (Figure 1b) and tree death. Fruiting sexual or asexual bodies of a fungus of "*C. puriensis*" were found between the cankers and on dead areas of the branches and stems (Figure 1c).

DNA sequence comparisons

PCR products were approximately 438 bp (ITS) and 696 bp (β-tubulin) in size. The combined sequence dataset, which includes ITS-rDNA fragments and β-tubulin gene, produced 1134 sequences aligned characters, which 1020 were constant, 12 non-informative parsimony and 102 informative parsimony. The partition homogeneity test PHT analysis showed that the ITS-rDNA and β-tubulin sequence data sets did not have any significant conflict (P=0.01) and could thus be combined (Gryzenhout *et al.*, 2006; Chungu *et al.*, 2009).

Phylograms obtained by maximum parsimony and maximum likelihood analyses were similar and consistent topologies with well supported branches. Tree statistics by maximum parsimony were: alignment length in base pairs (length) 124, consistency index (CI) 0.880, retention index (RI) 0.962 and homoplasy index (HI) 0.120. Maximum likelihood analysis was chosen for presentation (Figure 2). The *Chrysoporthe* isolates in the phylogram generated from the combined sequence data set resided in nine sub-clades (1–9), clustering separately from the outgroup taxa represented by *A. gyrosa*. Clades 2–9 represented known isolates of

Chrysoporthe that included *C. cubensis*, *C. deuterocubensis*, *C. hodgesiana*, *C. austroafricana*, *C. syzygiicola*, *C. zambiensis*, *C. inopina* and *C. doradensis*. Each clade was strongly supported by bootstrap values of >70%. Clade 1 represented "*C. puriensis*' from *Tibouchina* spp. in Brazil, distinct from those representing known species (>90% bootstrap support), residing in clade most closely related to *C. cubensis* (Figure 2). The *Chrysoporthe* isolates comprising the nine clades in the phylogenetic analyses could be distinguished by 44 nucleotides in the ITS-rDNA and β-tubulin gene regions (Table 3). Six nucleotides were different between "*C. puriensis*" and other species of *Chrysoporthe* (Table 4).

Population analyses

Differentiation between *C. cubensis* and "*C. puriensis*" populations was significant (θ = 0.414). Similarly, the results of population structure tests suggested that populations of *C. cubensis* and "*C. puriensis*" can be readily separated (Figure 3a). These data highlighted the fact that the two populations were each characterized by markedly different allelic compositions, although a low level of admixture was detected.

Analysis of molecular variance revealed that the genetic variation was highest within than among the populations analysed. Genetic variation within populations was 62% and among populations was 38% (Figure 3b). Low number of migrants value between *C. cubensis* and "*C. puriensis*" populations was observed ($\widehat{M} = 0.804$b) (Figure 3).

Ninety-nine alleles were found, distributed among the 12 locus analysed. Of which 27% of the alleles are shared between the two populations. "*C. puriensis*" population has 39,4% (φ = 0,879) of private alleles and the remaining 33,6% (φ = 0.604) are private alleles of *the C. cubensis* population (Table 5).

"*Chrysoporthe puriensis*" population presented higher gene and genotypic diversities ($\bar{H}$= 0.524 and $\hat{G}$= 1.0) (Table 5). The results of $\bar{r}_d$ by "*C. puriensis*" population was significant (P<0.05) (Table 5), this means that the populations are in gametic disequilibrium. The number of samples of "*C. puriensis*" population was sufficient to analyse the genotypic diversity successfully. Seven loci are already sufficient to reach the maximum of the genotypic diversity of the population (Figure 4).

Morphological analyses

The sexual and asexual phases were found in samples collected from *Tibouchina* spp. in different states of Brazil. The morphological characteristics resembled those observed in the isolates from the *Chrysoporthe* species already reported (Table 6). Ascostromata was semi-immersed in bark, fuscous-black to cinnamon, with cylindrical perithecial necks. Ascospores were hyaline, 1-septate, oval to ellipsoid, with septum central. Conidiomata was occurring on the surface of ascostroma or as separate structures, superficial to lightly immersed, matte black, pyriform to pulvinate. Conidiophores were hyaline, conidiogenous cells were phialidic. Conidia was hyaline, aseptate, oblong, fusoid to oval, masses exuded as bright luteous droplets, eventually with excessive exudation (Figure 5).

However, some morphological characteristics stood out because they were different from the *Chrysoporthe* species already described. The most relevant characteristics that differentiate "*C. puriensis*" were: the size of the asci, which were smaller than in the other *Chrysoporthe* spp.; perithecia with tissue of *textura epidermoidea*; conidiomata with stromatic base tissue of *textura globulosa* and *textura epidermoidea* and neck tissue of *textura porrecta* and *textura epidermoidea* and occasionally long cylindrical paraphyses, occurring between conidiophores (Figure 5).

Taxonomy

Morphological, phylogenetic and population analyses performed in this study differentiated the isolates of this study from the other *Chrysoporthe* species reported. These isolates represent a new species of *Chrysoporthe* associated with canker in *Tibouchina* spp. in Brazil, described as follows:

Chrysoporthe puriensis, M.E.S. Oliveira, T.P.F. Soares & M.A. Ferreira, sp. nov

Mycobank: CML3738, the same CT13.

Etymology: Tribute to an extinct indigenous tribe called "puris" in the collection region.

Ascostromata semi-immersed in bark, fuscous-black to cinnamon, cylindrical perithecial necks, and in some cases, erumpent, orange ascostromatic tissue, 130-230 µm high, above level of bark, 140-440 µm diam. **Perithecia** valsoid, bases immersed in bark, fuscous-black, top of perithecial bases covered with cinnamon to orange, limited stromatic tissue present around upper above the bark surface, extending necks up to 370 µm long emerging through bark covered in umber stromatic tissue of *textura epidermoidea*, appearing fuscous-black (Figure 5a,b). **Asci** (14-)17.5-24(-28) x (3.5-)4-6 (-7) µm, fusoid to ellipsoidal, 8-spored (Figure 5c). **Ascospores** (3-)3.5-6(-9) x (1-)1.5-3(-4) µm, hyaline, 1-septate, oval to ellipsoid, ends tapered, with septum central (Figure 5d).

Conidiomata occurring on the surface of ascostroma or as separate structures, superficial to lightly immersed, matte black, pyriform to pulvinate, with one to four necks, but usually one, masses exuded as bright luteous droplets, eventually with excessive exudation.

(Figure 5e). Conidiomatal bases above the bark surface 70-1350 μm height, 95-470 μm length. Conidiomatal locules with plane to oval inner surfaces, occasionally multilocular (Figure 5f). Stromatic base tissue of *textura globulosa* and *epidermoidea* and neck tissue of *textura porrecta* and *epidermoidea (*Figure 5g). **Conidiophores** hyaline, consisting of a basal cell branched irregular at the base or above into cylindrical cells, with or without separating septa, 2.3-4.0 x 1.2-2.1 and 9.2-17.0 μm (Figure 5h). Occasionally long cylindrical paraphyses, occurring between conidiophores (Figure 5i). Conidiogenous cells phialidic, apical or lateral on branches bellow a septum, cylindrical to flask-shaped with attenuated apices, 1.5 - 2.5 μm length, collarette and periclinal thickening inconspicuous. **Conidium** hyaline, aseptate, oblong, fusoid to oval, (3-)3.5-5(-6.5) × 1.5-2(-2.5) μm (Figure 5j).

Cultural characteristics Colonies in MEA showed white and fluffy mycelial growth when younger and turned to orange when older, smooth margin, showing orange discoloration in the growth medium. Rapid growth at the optimum temperature of 28 °C, covering the plate 90 mm of diameter in seven days.

Substrate Bark of *Tibouchina* spp. trees, such as *T. granulosa*, *T. heteromalla* and *T. candolleana*.

Distribution Three states of Brazil: Bahia, Minas Gerais and Rio de Janeiro

Material examined BRAZIL, Minas Gerais, *Tibouchina* spp., October 2016, M.A. Ferreira. Holotype LPFCT13, ex type culture CT13.

Pathogenicity

Lesions were found in all seedlings inoculated with *C. puriensis*, 8 w.a.i.. No lesion was developed in any of the control inoculations. Analysis of variance revealed significant differences among hosts ($P \leq 0.05$). *Tibouchina* spp., presented lengths of lesions larger than

hybrids of *Eucalyptus grandis* x *E. urophylla*. The mean lengths of lesions that were observed: Eucalyptus, 3.36 cm, *T. granulosa,* 5.1 cm and *T. heteromalla,* 7.7 cm (Figure 6). In *Tibouchina* spp., fruiting bodies of pathogens were observed 8 w.a.i at the inoculated site.

DISCUSSION

The combination of morphological, phylogenetic and population characterization confirmed the occurrence of a new species of *Chrysoporthe* in Brazil in a native host, which was named as *C. puriensis*. The symptoms observed in *Tibouchina* spp. caused by *C. puriensis* were similar to those caused by *Chrysoporthe* spp. in *Tibouchina* spp, such as branch die-back and stem cankers (Wingfield *et al*., 2001; Myburg *et al*., 2002; Seixas *et al*., 2004).

Chrysoporthe puriensis shares morphological characteristics with other species of *Chrysoporthe*, however, the characters that differentiate this species from the others were smaller asci, perithecia with tissue of *textura epidermoidea* and conidiomata with tissue of *textura globulosa, epidermoidea* and *porrecta.* Tissue of *textura epidermoidea* doesn't occur in others species of *Chrysoporthe*. For phylogenetic analyses with combined data of ITS-rDNA and β-tubulin sequences, the *C. puriensis* isolates formed a strongly supported branch, clearly showing the distinction from the *Chrysoporthe* species already described. Population analyses showed *C. cubensis* isolates were grouped in different population of *C. puriensis*, confirming the existence of two distinct species. Cryptic species can be separated based on low levels of interspecific gene flow (Taylor *et al*., 2000; Van der Merwe *et al*., 2010). Low genic flow was present between the two populations, making evident the existence vectors of ancestry, supported by the presence of shared alleles between the two populations (Van der Merwe *et al*., 2010).

With the description of *C. puriensis* increases the number of species known in the genus *Chrysoporthe* to nine, including *C. deuterocubensis* (Van der Merwe *et a*l., 2013), *C. zambiensis, C. syzygiicola* (Chungu *et al*., 2009), *C. austroafricana*, *C. cubensis*, *C. doradensis*, *C. inopina* and *C. hodgesiana* (Gryzenhout *et al*., 2005; Gryzenhout *et al*., 2009). Besides *C. puriensis*, four of these species have already been described occurring in *Tibouchina* species in South America. *C. cubensis* and *C. doradensis* were found occurring in *Tibouchina* sp. in Brazil (Seixas *et al*., 2006; Santos, 2015; Oliveira *et al*., 2018). *C. hodgesiana* and *C. inopina* were found occurring in *Tibouchina* sp. in Colombia (Gryzenhout *et al*., 2006). This suggests, the existence of a species complex, occurring in native species in South America (Gryzenhout *et al*., 2006). Species as *C. hodgesiana* and *C. puriensis* have been described causing canker exclusively in species of *Tibouchina,* belonging to the family Melastomataceae, a host widely distributed in Latin America. The association of these species with hosts native to South America suggests that these species occur naturally in South America, and thus, strengthening the hypothesis that the center of origin of most *Chrysoporthe* species is Latin America (Van der Merwe *et al*., 2010).

High gene and genotypic diversities were found in the *C. puriensis* population. This result was expected, since on native populations are expected to have high genetic variation (Tsutsui *et al*., 2000). High genetic variability has also been found in populations of *C. cubensis* in Colombia and Brazil, South America (Van der Mewer *et al*., 2013, Oliveira *et al*., 2018) and in population of *C. austroafricana* in Mozambique, Africa (Table 4), reaffirming the association of high genetic variability with the center of origin of the pathogen. The gametic disequilibrium analysis produced unexpected results. Since *C. puriensis* being a native species with sexual and asexual phases present, it was expected since this population was in gametic equilibrium. This result indicates that the population was non-random mating, probably caused by asexual reproduction.

The pathogenicity test showed *C. puriensis* is highly virulent for species of *Tibouchina*. The results also show that although *C. puriensis* has been found to cause canker in *Tibouchina* spp., it is also a potentially important pathogen of *Eucalyptus* species. *C. puriensis* is not host-specific. This result is supported by other studies showing that *Chrysoporthe* spp. has no specificity for host, provided that these hosts belong to Myrtales (Hodges *et al*., 1986, Gryzenhout *et al*. 2006, Seixas *et al*., 2004, Van der Merwe *et al*., 2010). In addition, the relative susceptibility of *Eucalyptus* spp. to stem canker disease induced by different *Chrysoporthe* spp. has been shown in a number of studies in which *C. austroafricana*, *C. cubensis*, *C. doradensis* and *C. hodgesiana* were used to inoculate trees (Roux *et al*., 2003; Gryzenhout *et al*., 2004; Gryzenhout *et al*., 2005; Gryzenhout *et al*., 2006; Van der Merwe *et al*., 2010; Chungu *et al*., 2009). Van der Merwe *et al*., (2013), in a genetic study on vegetative compatibility groups (VCG) with *Chrysoporthe* populations of native and non-native species in Colombia, observed that crossbreed events between two perithecia from different progenies may lead to the generation of a large number of different genotypes. These crossbreeds can be efficient in order to guarantee fungus pathogenicity in other hosts, thus threatening commercial crops, such as eucalyptus.

This study has important implications for *Tibouchina* trees, since *C. puriensis* has affected the ecosystem causing tree deaths in both natural habitats and in urban environments and reforestation areas. In addition, research involving the extinction of tree species or shrubs is not directed at the losses caused by these fungi. In most cases, the studies are specifically aimed at the tree species themselves or the animals of these ecosystems, and the fungal portion is extremely important for the conservation of the natural forests.

The results also contribute to the eucalyptus plantations in Brazil. With high genetic diversity and ability to cause disease in eucalyptus, *C. puriensis* may cause problems if not included in the genetic improvement programs of eucalyptus plantations. Breeding programs

that aim to control eucalyptus canker should include isolates of *C. puriensis* during the screening of the resistance of the clones.

Chrysoporthe puriensis have a high potential to cause damage in native and non-native hosts in Brazil. Future researches with more isolates are necessary to know better geographical distribution of *C. puriensis*, such as to describe species still unknown in Brazil from native hosts.

REFERENCES

Agapow PM, Burt A. 2001. Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes,* **1**, 101-102.

Barreto RW, Rocha FB, Ferreira FA. 2006. First record of natural infection of *Marlierea edulis* by the eucalyptus canker fungus *Chrysoporthe cubensis*. *Plant Pathology*, **55**, 577-577.

Bruner SC. 1917. Una enfermedad gangrenosa de lós eucaliptos. *Estaciòn Experimental Agronòmica,* Bulletin, **37**, 1-33.

Castellani A. 1939. Viability of some pathogenic fungi in distilled water. *Journal of Tropical Medicine & Hygiene,* **24**, 270-276.

Chungu D, Gryzenhout M, Muimba-Kankolongo A, Wingfield MJ, Roux J. 2009. Taxonomy and pathogenicity of two novel *Chrysoporthe* species from *Eucalyptus grandis* and *Syzygium guineense* in Zambia. *Mycological Progress,* **9**, 379-393.

Earl DA, Vonholdt BM. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources,* **4**, 359-361.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, **14**, 2611-2620.

Farris JS, Källersjö M, Kluge AG, Bult C. 1994. Testing significance of incongruence. *Cladistics*, **10**, 315-319.

Guimarães, P. J. F., & Martins, A. B. 1997. *Tibouchina* sect. Pleroma (D. Don) Cogn.(Melastomataceae) no estado de São Paulo. *Brazilian Journal of Botany*, **20**, 11-33.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology*, **61**, 1323-1330.

Gryzenhout M, Myburg H, Van der Merwe NA, Wingfield BD, Wingfield MJ. 2004. *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. *Studies in Mycology*, **50**, 119-142.

Gryzenhout M, Myburg H, Wingfield BD, Montenegro F, Wingfield MJ. 2005. *Chrysoporthe doradensis* sp. nov. pathogenic to *Eucalyptus* in Ecuador. *Fungal Diversity*, **20**, 39-57.

Gryzenhout M, Rodas CA, Portales JM, Clegg P, Wingfield BD, Wingfield MJ. 2006. Novel hosts of the Eucalyptus canker pathogen *Chrysoporthe cubensis* and a new *Chrysoporthe* species from Colombia. *Mycological research*, **110**, 833-845.

Gryzenhout M, Wingfield BD, Wingfield MJ. 2009. *Taxonomy, phylogeny, and ecology of bark-inhabiting and tree-pathogenic fungi in the Cryphonectriaceae*. Prestoria, South Africa: American Phytopathological Society (APS Press), 119 p.

Hodges CS, Reis MS, May L. 1973. Duas enfermidades em plantações de essências florestais exóticas no Brasil. *Brasil Florestal*, **4**, 5-12.

Hodges CS, Reis MS, Ferreira FA, Henfling JDM. 1976. O cancro do eucalipto causado por *Diaporthe cubensis*. *Fitopatalogia Brasileira*, Brasília, **1**, 129-170.

Hodges CS, Geary TF, Cordell CE. 1979. The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii and Puerto Rico. *Plant Disease Reporter*, **63**, 216-220.

Hodges CS, Alfenas AC, Ferreira FA. 1986. The conspecificity of Cryphonectria cubensis and Endothia eugeniae. *Mycologia*, **78**, 343-350.

Katoh K, Rozewicki J, Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization, *Briefings in Bioinformatics*, https://doi.org/10.1093/bib/bbx108.

Myburg H, Gryzenhout M, Heath RN, Roux J, Wingfield BD, Wingfield MJ. 2002. *Cryphonectria* canker on *Tibouchina* in South Africa. *Mycological Research*, **106**, 1299-1306.

Myburg H, Gryzenhout M, Wingfield BD, Wingfield MJ. 2003. Conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*: A re-evaluation based on morphology and DNA sequence data. *Mycoscience*, **104**, 187-196.

Myburg H, Gryzenhout M, Wingfield BD, Wingfield MJ, Stipes RJ. 2004. A reassessment of the fungal genera *Cryphonectria* and *Endothia* based on DNA sequence data. *Mycologia*, **96**, 990-1001.

Nakabonge G, Roux J, Gryzenhout M, Wingfield M J. 2006. Distribution of *Chrysoporthe* canker pathogens on *Eucalyptus* and *Syzygium* spp. in eastern and southern Africa. *Plant Disease*, **90**, 734-740.

Nei M. 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences*, **70**, 3321-3323.

Peakall ROD, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Resources*, ***6***, 288-295.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics*, ***155***, 945-959.

Rodas CA, Gryzenhout M, Myburg H, Wingfield BD, Wingfield MJ. 2005. Discovery of the *Eucalyptus* canker pathogen *Chrysoporthe cubensis* on native *Miconia (*Melastomataceae*)* in Colombia. *Plant Pathology*, **54**, 460-470.

Roux J, Myburg H, Wingfield BD, Wingfield MJ. 2003. Biological and phylogenetic analyses suggest that two Cryphonectria species cause cankers of *Eucalyptus* in Africa. *Plant Disease*, **87**, 1329-1332.

Roux J, Meke G, Kanyi B, Mwangi L, Mbaga A, Hunter GC, Nakabongea G, Heatha RN, Wingfield MJ. 2005. Diseases of plantation forestry trees in eastern and southern Africa. *South African Journal of Science*, **101**, 409-413.

Santos TPF. 2015. *Caracterização morfológica, filogenética e patogênica de Chrysoporthe spp. associado ao cancro em Eucalyptus spp. e Tibouchina spp*. Lavras, Brasil, Universidade Federal de Lavras. PhD Thesis.

Scott AJ, Knott M. 1974. A cluster analysis method for grouping means in the analysis of variance. *Biometrics*, 507-512.

Seixas CD, Barreto RW, Alfenas AC, Ferreira FA. 2004. *Cryphonectria cubensis* on an indigenous host in Brazil: a possible origin for eucalyptus canker disease? *Mycologist*, **18**, 39-45.

Sharma JK, Mohanan C, Florence EJM. 1985. *Disease survey in nurseries and plantations of forest tree species grown in Kerala***:** research report. Kerala: Kerala Forest Research Institute, 268 p.

Slatkin M. 1993. Isolation by Distance in Equilibrium and Non-Equilibrium Populations. *Evolution,* **47**, 264-279.

Slatkin. 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics,* **139**, 457-462.

Stoddart JA, Taylor JF. 1988. Genotypic diversity: estimation and prediction in samples. *Genetics,* **118**, 705-711.

Swofford DL. 2002. PAUP: Phylogenetic analysis using parsimony Version 4.0 beta. Sunderland, MA: Sinauer Associates Inc, software.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, **30**, 2725-2729.

Tamura, K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+ C-content biases. *Molecular biology and evolution*, **9**, 678-687.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC, 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology, **31**, 21-32.

Tsutsui ND, Suarez AV, Holway DA, CASE TJ. 2000. Reduced genetic variation and the success of an invasive species. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 5948-5953.

Van der Merwe NA, Gryzenhout M, Steenkamp ET, Wingfield BD, Wingfield MJ. 2010. Multigene phylogenetic and population differentiation data confirm the existence of a cryptic species within *Chrysoporthe cubensis*. *Fungal biology*, **114**, 966-979.

Van der Merwe NA, Steenkamp ET, Rodas C, Wingfield BD, Wingfield MJ. 2013. Host switching between native and non-native trees in a population of the canker pathogen *Chrysoporthe cubensis* from Colombia. *Plant Pathology*, **62**, 642-648.

Weir BS, Cockerham CC. 1984. Estimating F-Statistics for the Analysis of Population Structure. *Evolution,* **38**, 1358-1370.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications,* **18**, 315-322.

Wingfield MJ, Rodas C, Wright J, Myburg H, Venter M, Wingfield BD. 2001. First report of Cryphonectria canker on *Tibouchina* in Colombia. *Forest Pathology*, **31**, 1-10.

Table 1. Detailed information for *Chrysoporthe puriensis* isolates used in this study.

Isolates	**Number of isolates**	**Host**	**Location**	
			State	**City**
TGCD01	1	*T. granulosa*	Bahia	Lençóis
TCL01	1	*T. candolleana*	Minas Gerais	Lavras
CT05, TGL02, TGL03, TGL05	4	*T. granulosa*	Minas Gerais	Lavras
TIL01	1	*T. heteromalla*	Minas Gerais	Lavras
CT07, CT10, CT11, CT13	4	*T. granulosa*	Minas Gerais	São João del Rei
TGDR01	1	*T. granulosa*	Minas Gerais	São João del Rei
TGSC01, TGSC03, TGSC04, TGSC07, TGSC09, TGSC11, TGSC13, TGSC14,	8	*T. granulosa*	Minas Gerais	São Roque de Minas
THSC01, THSC04	2	*T. heteromalla*	Minas Gerais	São Roque de Minas
TISC02	1	*Tibouchina* sp.	Minas Gerais	São Roque de Minas
TIST01	1	*Tibouchina* sp.	Minas Gerais	São Tomé das Letras
TGS01, TGS02, TGS03, TGS04, TGS06, TGS07, TGS08	7	*T. granulosa*	Minas Gerais	Silveirânia
TGT02, TGT03	2	*T. granulosa*	Minas Gerais	Tiradentes
TGPNI01, TGPNI03, TGPNI04, TGPNI08, TGPNI09, TGPNI10, TGPNI11, TGPNI12, TGPNI13, TGPNI14, TGPNI16, TGPNI19	12	*T. granulosa*	Rio de Janeiro	Itatiaia

Table 2. Reference sequences of *Chrysoporthe* ssp. and *Amphilogia gyrosa* used in this study.

Species	Isolate number	GenBank accession numbers		
		ITS	BT1	BT2
Chrysoporthe cubensis	CMW10669	GQ290154	GQ290177	AF535126
	CMW10778	GQ290155	GQ290178	GQ290189
	CMW10639	AY263421	AY263419	AY263420
	CMW10028	GQ290153	GQ290175	GQ290186
Chrysoporthe deuterocubensis	CMW12745	DQ368764	GQ290183	DQ368781
	CMW12746	HM142105	HM142121	HM142137
	CMW17178	DQ368766	AH015649	AH015649
	CMW2631	GQ290157	GQ290184	AF543825
	CMW8650	AY084001	AY084024	GQ290193
Chrysoporthe hodgesiana	CMW10641	AY692322	AY692326	AY692325
	CMW9995	AY956969	AH014904	AH014904
Chrysoporthe austroafricana	CMW10192	AY214299	GQ290176	GQ290187
	CMW9327	GQ290158	GQ290185	AF273455
	CMW2113	AF046892	AF273067	AF273462
Chrysoporthe syzygiicola	CMW29940	FJ655005	FJ805230	FJ805236
	CMW29942	FJ655007	FJ805232	FJ805238
Chrysoporthe zambiensis	CMW29928	FJ655002	FJ858709	FJ805233
	CMW29930	FJ655004	FJ858711	FJ805235
Chrysoporthe inopina	CMW12729	DQ368778	AH015656	AH015656
	CMW12727	DQ368777	AH015657	AH015657
	CMW12731	DQ368779	AH015655	AH015655
Chrysoporthe doradensis	CMW11286	AY214290	AY214218	AY214254
	CMW11287	GQ290156	GQ290179	GQ290190
Amphilogia gyrosa	CMW10469	AF452111	AF525797	AF525714
	CMW10470	AF452112	AF535708	AF525715

CMW: Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria

Table 3. Characteristics of polymorphic microsatellite markers used for genotyping *Chrysoporthe puriensis* isolates from different regions and hosts in Brazil.

Microsatellite markers	**Motif**	**Forward primer Sequence (5' to 3')**	**Reverse primer Sequence (3' to 5')**	**Ta (^{o}C)**	**Allelic Range (bp)**	**Number of alleles**
CHRY06	AGC	CCCCAAGCTCGCCTACTTC	GACGCTCTCGGGTATTGTCA	63	211-218	4
CHRY07	AGT	TTGGTCGGTTCGGTAGAAAG	CATGACGGGAGAGCGATAAG	60	216-254	11
CHRY09	TGC	CCTTCTCTTTCCCAATTTTGC	CACCTCGTACAAGGCCTCTC	64	216-219	2
CHRY10	TGGC	TGCGAGTGGGTGAGTGAGTA	ACACGGACGGTCATAGCAC	60	167-170	3
CHRY13	TGGTC	GTGTCGTCGACTCAAGCTCA	GACGGAGAGGACACTGAAGC	63	196-206	2
CHRY15	TGA	AAGGCCAGATTGTCCAAATG	CAGCAACGAACCAAAATCAA	58	248-275	8
CHRY17	ACC	AGATCCAAGTGGGTTTCCT	CTTAGCTTGACCCAGGGATG	60	149-186	8
CHRY23	AGC	TGTCCTGCATCTAGGTCGTG	GTCCCAGTCCTCTTGGATCA	61	149-150	2
CHRY24	ATC	GTGCGGGCATCTGTTCTACT	GCGCATTGTGAGGCGTTAAG	61	179-194	6
CHRY25	ACC	TATCTGCGCAACTACCACCA	GGGAAGTGTGTCTCCTCCTG	61	162-185	11
CHRY27	AGC	GCCGCCTACAGAGACTATAACG	CGACGAAGACGTAGTTGCAC	63	229-232	2
CHRY28	TGGTC	GATATGCCGGTCATGGTGAT	TACTTGAGGGCGAAGGAAAG	60	226-248	7

Ta: Annealing temperature
bp: base pairs

Table 4. Summary of polymorphic nucleotides found within sequences of the ribosomal ITS region and two regions in the β-tubulin genes for all known *Chrysoporthe* species, including "*C. puriensis*" described in this study. Polymorphic nucleotides unique to *C. puriensis* are highlighted.

Species	Beta-tubulin 2 (Bt2a/Bt2b)														Beta-tubulin 1 (Bt1a/Bt1b)																												ITS1/5.8S/ITS4									
	10	49	58	79	146	196	202	250	251	252	273	281	290	320	404	431	455	458	509	527	561	568	571	582	586	595	598	600	601	605	608	619	620	621	696	716	750	754	779	780	787	840	859	1066							
"*C. puriensis*"	G	C	T	C	A	C	C	G	T	**T**	C	G	C	**T**	T	C	G	C	C	T	**T**	T	T	T	A	A	A	G	G	C	C	**T**	T	T	-	**T**	C	G	T	**T**	A	-	C	A							
C. cubensis	G	C	T	C	A	C	C	G	T	C	C	G	C	C	T	C	G	C	T	T	C	T	T	T	A	A	A	A	G	C	C	C	T	C	-	-	C	A	-	C	A	-	C	A							
C. doradensis	G	C	T	T	G	T	C	G	C	C	T	G	C	C	T	A	G	C	C	T	C	C	C	T	A	A	A	G	A	C	T	C	C	C	-	-	C	A	-	C	A	-	C	A							
C. hodgesiana	A	C	C	C	A	C	C	G	T	C	C	A	C	C	T	C	G	C	T	C	C	T	T	T	G	A	A	G	G	C	C	C	C	C	-	-	C	G	-	C	A	-	C	G							
C. inopina	G	C	T	T	A	T	C	A	C	C	T	G	C	C	T	C	G	C	C	C	C	C	C	T	A	A	A	G	G	C	C	C	C	C	-	-	C	G	-	C	A	-	C	G							
C. austroafricana	G	C	T	C	A	C	C	G	T	C	C	G	T	C	T	C	G	C	C	C	C	C	C	C	A	C	C	G	G	C	C	C	C	C	-	-	C	A	-	C	A	-	T	A							
C. syzygiicola	G	C	T	C	A	A	C	G	T	C	C	G	T	C	T	C	T	A	C	C	C	C	C	C	A	C	C	G	G	T	C	C	C	C	G	-	C	A	-	C	A	-	C	A							
C. zambiensis	G	C	T	C	A	A	A	G	T	C	C	G	T	C	A	C	G	C	C	C	C	C	C	C	A	C	C	G	G	T	C	C	C	C	C	-	A	A	-	C	A	-	C	A							
C. deuterocubensis	G	T	T	T	A	C	C	G	C	C	C	G	C	C	T	C	G	C	C	T	C	T	T	T	A	A	A	G	G	C	C	C	C	C	-	-	C	G	T	T	G	C	C	A							

Table 5. Gene diversity, genotypic diversity, private allele and gametic (linkage) disequilibrium in *Chrysoporthe puriensis* and *C. cubensis* populations in Brazil and other species of *Chrysoporthe* populations from other parts of the world.

Country	**Population**	**Number of Isolates (N)**	**Gene diversity** $(\bar{H})$	**Genotypic diversity** $(\hat{G})$	**Private allele** (φ)	**Gametic (linkage) disequilibrium** $(\bar{r}_d)$
Brazil	*C. puriensis*	43	0.524	1.000	0.879	0.0288*
Brazil[1]	*C. cubensis*	53	0.478	0.815	0.604	0.0483*
Colombia[2]	*C. cubensis*	91	0.410	0.840	-	-
South Africa[3]	*C. cubensis*	61	0.190	0.110	-	-
South Africa[4]	*C. austroafricana*	70	0.420	0.240	-	-
Mozambique[4]	*C. austroafricana*	49	0.610	0.900	-	-
Zambia[4]	*C. austroafricana*	13	0.240	0.250	-	-

*The populations are in gametic disequilibrium ($P<0.05$).

References: [1]Oliveira, unpublished, article 1; [2] Van der Merwe *et al.*, 2012; [3]Nakabongea *et al*., 2007; [4]Mkabili *et al*., unpublished.

Table 6. Comparison of *Chrysoporthe puriensis* with other *Chrysoporthe* species.

Species	**Optimal temp. for growth**	**Conidiomata base width (µm)**	**Conidiomata shape**	**Conidiomata textura**	**Presence of Paraphyses**	**Conidium shape**	**Spore mass**	**Teleomorph**
C. puriensis[1]	28°C	95-470	Pyriform to pulvinate	Globulosa, epidermoidea, porrecta	Sometimes	Oblong	Bright luteous	Know
C. cubensis[2]	30°C	100-950	Pyriform to pulvinate	Globulosa, porrecta	No found	Oblong	Bright luteous	Know
C. deuterocubensis[3]	30°C	100-950	Pyriform to clavate, pulvinate	Globulosa, porrecta	No found	Oblong	Bright luteous	Know
C. doradensis[4]	30°C	100–290	Pyriform to pulvinate	Globulosa, porrecta	No found	Oblong to ovoid to cylindrical	Pale luteous	Know
C. hodgesiana[5]	25°C	145–635	Pulvinate, occasionally pyriform	Globulosa, porrecta	No found	Oblong	Bright luteous	Unknown
C. inopina[5]	25°C	70–710	Subulate to pyriform to pulvinate	Globulosa, porrecta	No found	Oblong	Orange to luteous	Know
C. austroafricana[6]	25–30°C	80–120	Pyriform to pulvinate	Globulosa, porrecta	No found	Oblong to ovoid	Bright luteous	Know
C. syzygiicola[7]	30°C	250–500	Ovoid with tapering neck	Globulosa, porrecta	No found	Oblong	Bright yellow luteous	Unknown
C. zambiensis[7]	30°C	208–310	Pyriform to rostrate	Globulosa, porrecta	No found	Oblong	Pale luteous	Unknown

Continuous in next page

Table 6 (continuation). Comparison of *Chrysoporthe puriensis* with other *Chrysoporthe* species.

Species	Conidium size (µm)		Ascus size (µm)		Ascospore size (µm)	
	Length	Width	Length	Width	Length	Width
C. puriensis[1]	(3-)3.5-5(-6,5)	1.5-2(-2.5)	(14-)17.5-24(-28)	(3.5-)4-6 (-7)	(3-)3.5-6(-9)	(1-)1.5-3(-4)
C. cubensis[2]	4.5(–5)	(3–)3.5	(19-)22-26.5(-28)	(4.5-)5-6.5(-7)	(5.5-)6.5-7.5(-8)	2-2.5(-3)
C. deuterocubensis[3]	(3-)3.5-4.5(-5)	(1.5-)2(-2.5)	(19-)22-26.5(-28)	(4.5-)5-6.5(-7)	(5.5-)6.5-7.5	(-8)2-2.5(-3)
C. doradensis[4]	(3-)3-5(-6.5)	1.5-2(-2.5)	(19.5-)21.5-24(-25)	(4-)4.5-6(-7)	(4.5-)5.5-7.5(-8.5)	2-2.5
C. hodgesiana[5]	(3-)3.5-5(-5.5)	1.5-2(-2.5)	-	-	-	-
C. inopina[5]	(3-)3.5-4	(1.5-)2-2.5	(27.5-)29.5-34(-35)	(4.5-)5.5-6.5(-7)	(4.5-)6-7.5(-8)	2.5-3.5
C. austroafricana[6]	3-4(-4.5)	1.5-2	(25-)27-32(-34)	(4-)5.5-7(-7.5)	(5.5-)6-7	(2-)2.5
C. syzygiicola[7]	(2.1-)2.5-3.5(-4.0)	(1.2-)1.5-2.0	-	-	-	-
C. zambiensis[7]	(2.5-)3.0-3.5(-4.0)	(1.0-)1.5-2.0	-	-	-	-

Reference: [1]This work; [2] Roux *et al*., 2003, Gryzenhout *et al*., 2004; [3]Van der Merwe *et al*., 2010; [4]Gryzenhout *et al*., 2005; [5]Gryzenhout *et al*., 2006; [6]Gryzenhout *et al*., 2004, Chungu *et al*., 2009; [7]Chungu *et al*., 2009.

Figure 1. Symptomatology. (a) *Tibouchina* tree showing branch die-back caused by *Chrysoporthe puriensis*. (b) Cracked bark and canker on the stem of a *Tibouchina* tree infected by *C. puriensis*. (c) Signs of *C. puriensis* in bark *Tibouchina* tree.

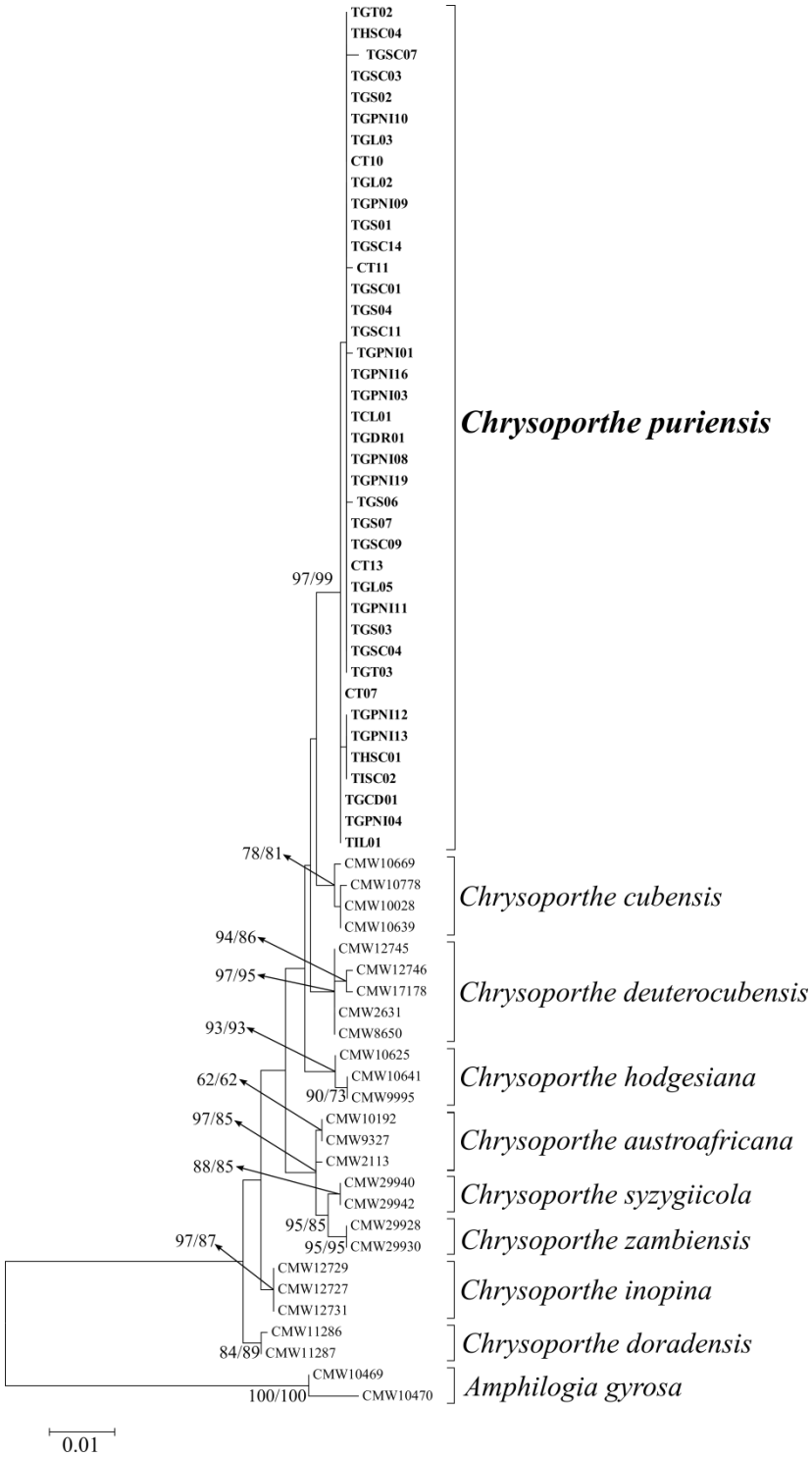

Figure 2. Molecular Phylogenetic analysis by Maximum Likelihood (ML) combined DNA sequence data set of regions of the Internal Transcribed Spacer of rRNA gene (ITS), and Beta-tubulin (BT1 and BT2 regions). Bootstrap values above 60% are indicated above each branch (ML/MP). The isolates of *Chrysoporthe puriensis* isolated from this study are highlighted.

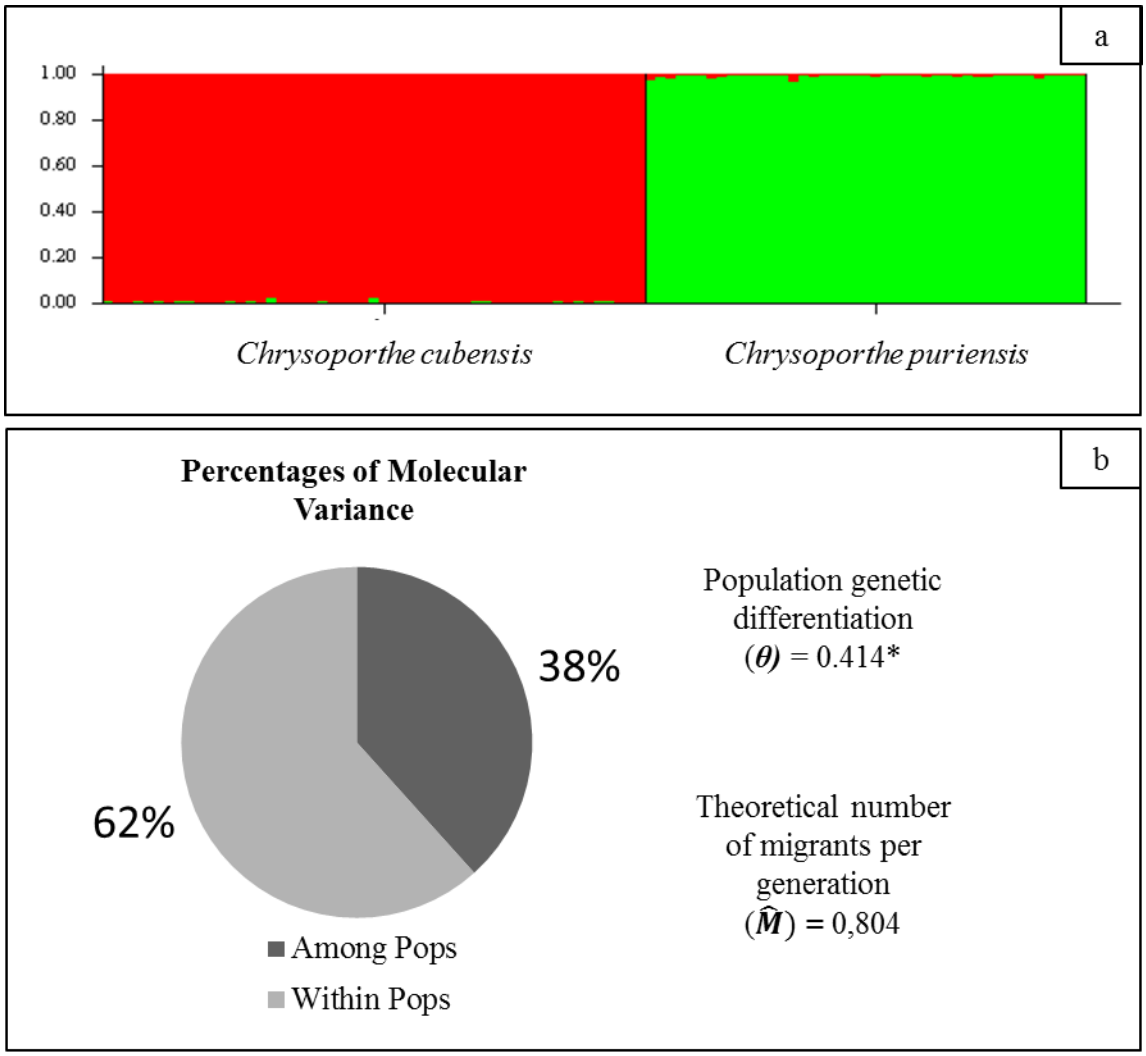

Figure 3. (a) Structure plot of *Chrysoporthe cubensis* and of *C. puriensis* populations in Brazil (b) Genetic variation among and within populations of *C. cubensis* and of *C. puriensis*. Estimated by analysis of molecular variance (AMOVA), theoretical number of migrants per generation and genetic differentiation between populations of *C. cubensis* and of *C. puriensis*. *Significance (P≤0.05).

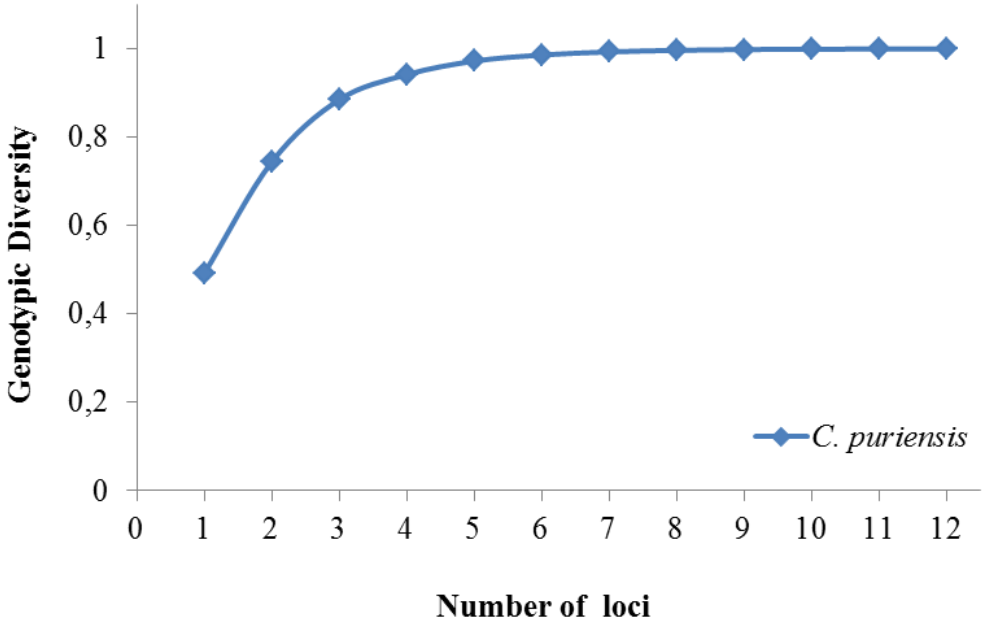

Figure 4. Genotypic diversity per number of loci for *Chrysoporthe puriensis*.

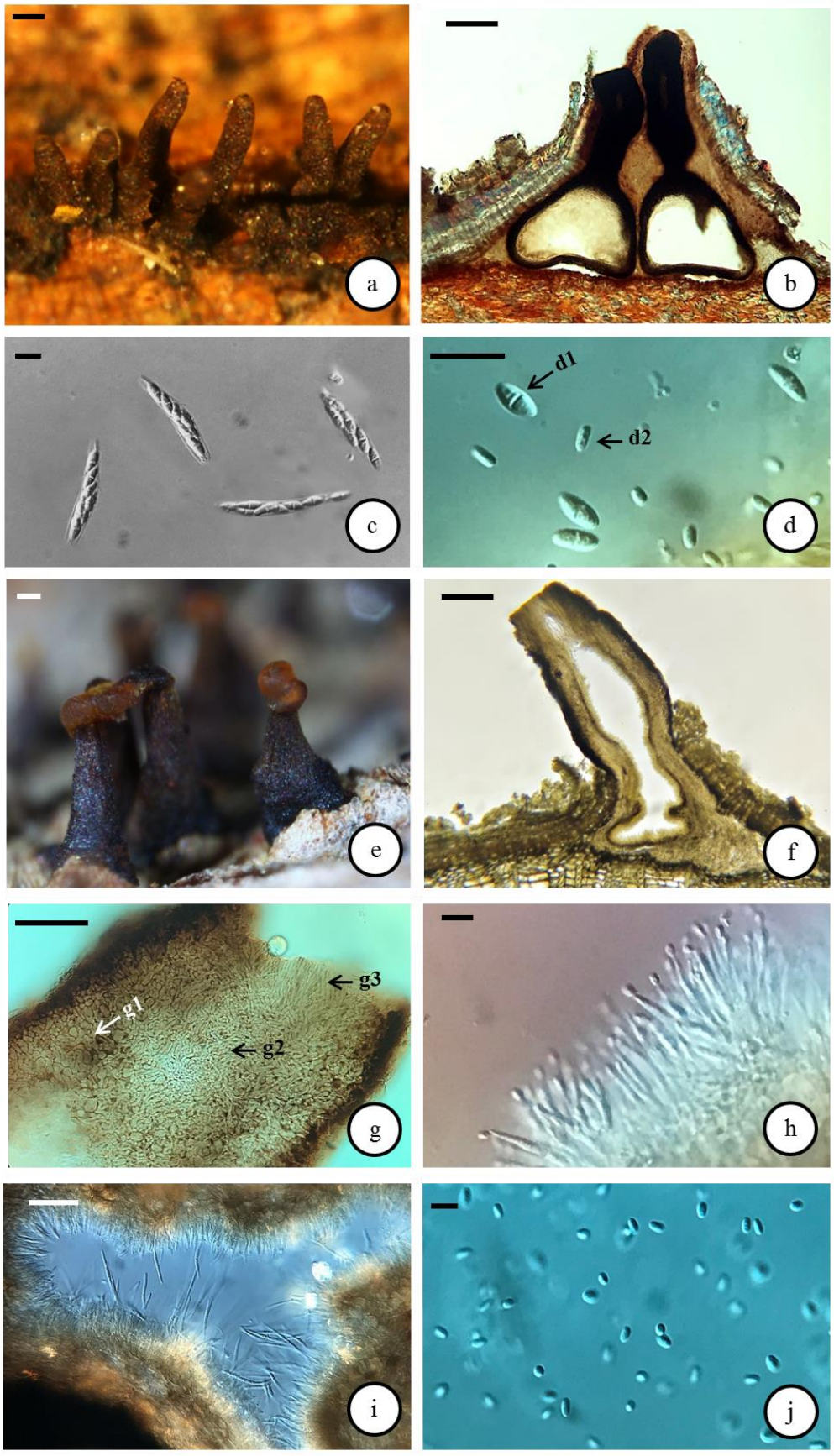
a
b
c
d1
d2
d
e
f
g3
g1
g2
g
h
i
j

Figure 5. Fruiting structures of *Chrysoporthe puriensis*. (a) Ascostromata on bark. (b) Longitudinal section through ascostroma. (c) Asci. (d) d1= Ascospores, d2=Conidium. (e) Conidioma on bark. (f) Longitudinal section through conidiomata. (g) Tissue of textura globulosa (g1), textura epidermoidea (g2) and textura porrecta (g3) for the neck. (h) Conidiophores. (i) Paraphyses. (j) Conidium. Scale bars: a,b,e,f (100 µm); g,i (20 µm); c,d,h,j (10 µm).

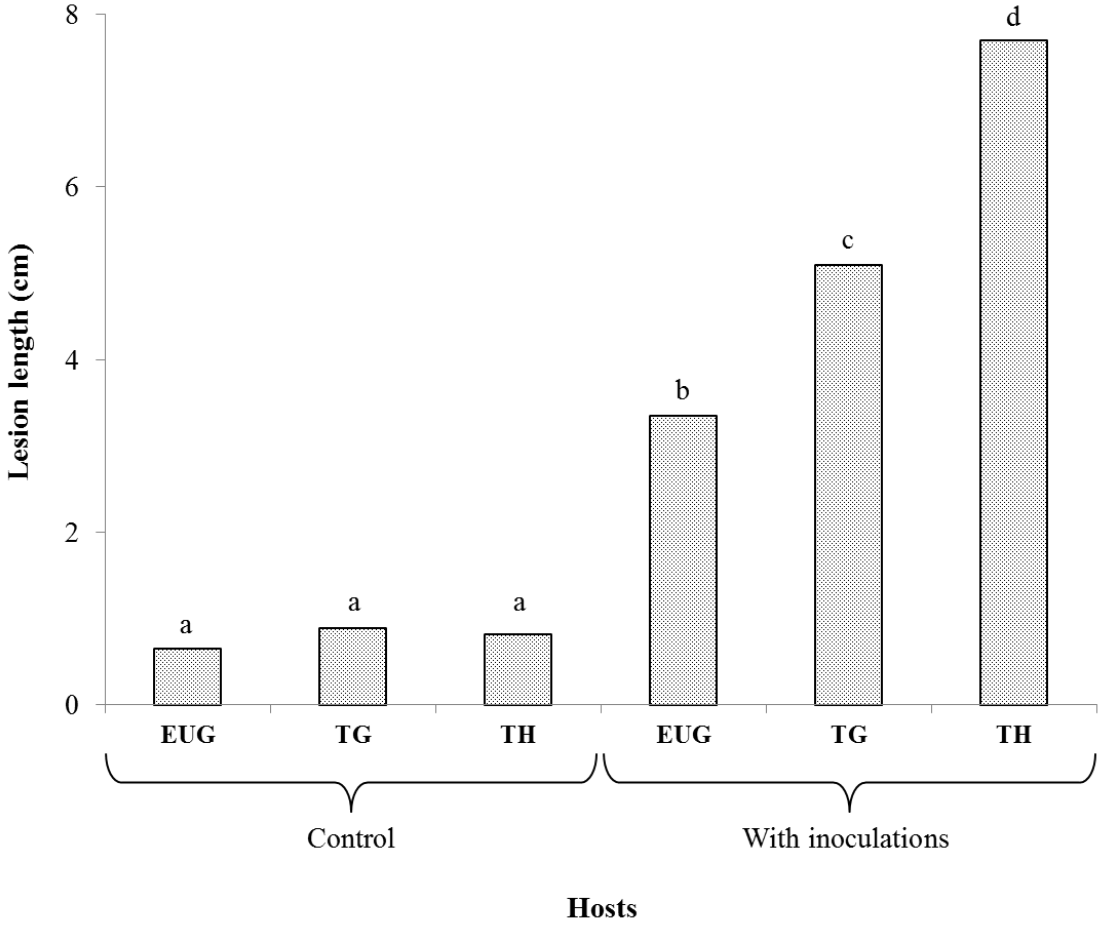

Figure 6. Lesion length (cm) of *Chrysoporthe puriensis* in different hosts, 8 weeks after the inoculation. Mean followed by the different letter were not grouped by the Scott-Knott test (P≤0.05). EUG = Hybrids of *Eucalyptus grandis* x *E. urophylla*, TG = *Tibouchina granulosa*, TH = *Tibouchina heteromalla*.