Genetic variability in populations of *Chrysoporthe cubensis* and *Chr. puriensis* in Brazil

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Abstract

Chrysoporthe puriensis, a sibling species of the well-known *Eucalyptus* canker pathogen *Chr. cubensis*, has recently been described from Brazil. Both species are thought to be native to South America, but previous population genetic analyses were conducted prior to the ready availability of robust markers such as microsatellites to test this hypothesis. The objective of this investigation was to analyse the structure and genetic variability of *Chr. cubensis* and *Chr. puriensis* populations from non-native Myrtaceae and native Melastomataceae hosts in Brazil, using microsatellite markers developed for this purpose. The fungal isolates were obtained from *Eucalyptus* species, *Corymbia citriodora* and *Tibouchina* species in different regions of the country. Isolates were separated into sub-populations based on host families (Melastomataceae and Myrtaceae) and on region of origin. There was high genetic variability in all sub-populations with the highest levels detected within, rather than among sub-populations. Gene and genotypic diversities were higher for the isolates from the Melastomataceae than the Myrtaceae isolates. High levels of gene flow were found between sub-populations based on host and geographic distribution of the sub-populations. The presence of genetically diverse *Chr. cubensis* and *Chr. puriensis* populations on native hosts in Brazil supports a Latin American centre of origin for the two pathogens. Both undergo sexual and asexual reproduction and have a high potential for gene flow.

Key-words: Canker disease; *Tibouchina* species; *Eucalyptus* species; Microsatellites; Phylogeny; Primers

Introduction

Species of *Chrysoporthe* are important canker pathogens of trees in natural and planted forests (Hodges et al. 1976, 1979; Sharma et al. 1985; Wingfield et al. 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 et al. 1986; Barreto et al. 2006). *Chrysoporthe cubensis* (as *Cryphonectria cubensis)* was first described on *Eucalyptus* in Cuba (Bruner 1917; Hodges 1980) and it has subsequently been reported from many other countries of the world where these trees are grown, mainly as non-natives propagated for commercial forestry purposes (Alfenas et al. 1982; Gryzenhout et al. 2004; Seixas et al. 2004; Roux et al. 2005; Barreto et al. 2006; Nakabonge et al. 2006). Studies

applying phylogenetic inference based on DNA sequence data have shown that *Chr. cubensis* is distinct from species in *Cryphonectria* and that *Chrysoporthe* accommodates a number of cryptic species (Myburg et al. 2003, 2004; Gryzenhout et al. 2004; van der Merwe et al. 2010).

Phylogenetic analyses of sequence data for multiple gene regions have led to the description of nine species of *Chrysoporthe* including *Chr. cubensis, Chr. austroafricana*, *Chr. deuterocubensis*, *Chr. doradensis*, *Chr. hodgesiana*, *Chr. inopina*, *Chr. syzygiicola*, *Chr. zambiensis* and *Chr. puriensis* (Gryzenhout et al. 2004; Gryzenhout et al. 2006; Van der Merwe et al. 2010; Chungu et al. 2009; Oliveira et al. 2021). These fungi are all pathogens that are typically found in tropical and sub-tropical regions of the world, including Southeast Asia, Africa, South and Central America and Australia (Hodges et al. 1986; Sharma et al. 1985; Wingfield et al. 1989; Nakabonge et al. 2006; Pegg et al. 2010; Oliveira et al. 2021). For many years, *Chr. cubensis* was believed to be restricted to *Eucalyptus* hosts. The pathogen was originally thought to be native to Indonesia and accidentally introduced into South America on clove trees (Myrtaceae) as part of the spice trade (Hodges et al. 1986). An intriguing discovery was made by Wingfield et al. (2001) who showed that *Chr. cubensis* occurs on *Tibouchina lepidota* and *Tibouchina urvilleana,* native trees in the Melastomataceae*,* occurring in natural forests of Colombia. The fungus has subsequently been found on many different species of Melastomataceae and Myrtaceae in South and Central America (Myburg et al. 2002; Seixas et al. 2004; Soares et al. 2018) where it appears to have undergone a host-shift to infect non-native *Eucalyptus*. *Chrysoporthe cubensis* is thought to be native to south and central America whereas its sibling species *Chr. austroafricana* and *Chr. deuterocubensis* are likely native species in southern Africa and Asia respectively (Gryzenhout et al. 2004; van der Merwe et al. 2010).

In Brazil, three species of *Chrysoporthe* have been reported including *Chr. cubensis*, *Chr. doradensis* and *Chr. puriensis* (Hodges et al. 1976; Soares et al. 2018; Oliveira et al. 2021). All three cause similar stem canker diseases on *Eucalyptus* and, particularly in the case of *Chr. cubensis,* are important constraints to plantation forestry (Wingfield 1999, 2003; Soares et al. 2018). The disease can cause quantitative and qualitative damage, including value depreciation for timber processing, lower cellulose yields and decreased calorific value (Souza 2008). On susceptible *Eucalyptus* species and clones, cankers can lead to stem breakage and occasionally tree death (Hodges et al. 1976; Sharma et al. 1985). In this regard, canker caused by *Chrysoporthe* species is considered one of the most important diseases of *Eucalyptus* in South America (Ferreira and Milani 2004).

Both *Chr. cubensis* and *Chr. puriensis* are known to undergo sexual reproduction in Brazil (van Zyl et al. 1998; Oliveira et al. 2021). The study by Van Zyl et al. (1998) revealed a high level of phenotypic variability in Brazilian populations of the *Chr. cubensis*. But these phenotypic data failed to provide a comprehensive description of diversity. Very little is known regarding genetic diversity of this or other *Chrysoporthe* species anywhere in the world. There is consequently a need to identify and develop informative genetic markers for these important pathogens. In this regard, microsatellite markers have previously been developed for *Chr*. *cubensis*, but they were not polymorphic across all of the *Chrysoporthe* species (van der Merwe et al. 2013).

The aim of this study was to develop informative microsatellite markers that could be used to assess the genetic diversity of *Chr. cubensis* and *Chr. puriensis* populations in Brazil. These populations were collected in order to compare the genetic diversity of isolates from native *Tibouchina* and non-native *Eucalyptus* species grown in plantations. Isolates were also collected from trees growing in different geographic areas of Brazil and having distinct climatic conditions.

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 stem cankers caused by *Chrysoporthe* species. Fungal isolates were collected from native *Tibouchina* species (Melastomataceae), and non-native *Eucalyptu*s species and *Corymbia citriodora* (Myrtaceae), which all reside in the Myrtales. Trees with sexual or asexual fruiting structures of *Chrysoporthe* species on the surface of cankers were sampled.

A single spore isolate was made from a fruiting body (pycnidium or perithecium) of *Chrysoporthe* species for each tree sampled. For this purpose, a spore mass 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 24 h. Single germinating spores were transferred to fresh PDA plates and incubated at 28 °C for 7 days. For genomic DNA isolation, cultures were grown in liquid malt extract broth (20% w/v) at 28 °C for 7–10 days in the dark. The mycelium was harvested by filtration through sterilized filter paper. Total genomic DNA was extracted using a Wizard Genome DNA Purification Kit (Promega, USA) according to the manufacturer's instructions.

Phylogenetic analyses

The identities of collected isolates were verified by PCR amplification and sequencing of the internal transcribed spacer (ITS) regions of the ribosomal DNA operon using primers ITS1 and ITS4 (White et al. 1990) and the β-tubulin gene region using primers Bt1a/Bt1b and Bt2a/Bt2b (Glass and Donaldson 1995). Sequencing was performed using Big Dye terminator sequencing kits (Life Technologies) on an ABI 3100 sequencer (Applied Biosystems) following the instructions of the manufacturer and as described by van der Merwe et al. (2010). Sequences were manually edited where appropriate using CLC Genomics Workbench v 9.1 (CLC Bio, Arhus, Denmark).

Additional reference sequences for comparison were obtained from the NCBI GenBank database (Table 1). Sequence alignments were performed using the online interface of MAFFT v 7.182 (Katoh et al. 2017). For phylogenetic analyses, *Amphilogia gyrosa* was used as the outgroup taxon (Table 1). A partition homogeneity test (PHT; Farris et al. 1994) of the combined ITS and β-tubulin sequences was performed using PAUP* 4.0 (Swofford 2002) with 1000 replicates. A combined alignment was subjected to Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses.

Table 1. Reference sequences of *Chrysoporthe* species 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 puriensis**	CT13	MN590029	MN590041	MN590041
	TCL01	MN590030	MN590042	MN590042
	TGCD01	MN590037	MN590049	MN590049
	TGPNI01	MN590032	MN590044	MN590044
	THS01	MN590039	MN590051	MN590051
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

*Isolates from collection of the Forest Pathology Laboratory (LPF) of Federal University of Lavras, Brazil

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 branch points were determined using 1000 bootstrap replicates. Maximum likelihood (ML) analysis was performed 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 calculated using 1000 bootstrap replicates.

Microsatellite marker development

MSATCOMMANDER (Faircloth 2008) was used to identify microsatellite sequences with a motif length of three to six nucleotides from whole genome sequences of *Chr. austroafricana* (CMW2113, GenBank accession: JYIP00000000.2), *Chr. deuterocubensis* (CMW8650, GenBank accession: LJDD00000000.2) and *Chr. cubensis* (CMW10028, GenBank accession: LJCY00000000.2) (Wingfield et al. 2015a, b). Custom python scripts were used to extract and group the microsatellite sequences based on motif and motif length adding 200 bp on either flank. Primers were designed from the conserved flanking regions of sequences containing similar motifs in each of the three *Chrysoporthe* species using Primer 3 v0.40 (Untergasser et al. 2012). Primers were tested for cross-species amplification on sample isolates of eight *Chrysoporthe* species including *Chr. austroafricana*, *Chr. cubensis*, *Chr. deuterocubensis*, *Chr. doradensis*, *Chr. hodgesiana*, *Chr. inopina*, *Chr. syzigiicola* and *Chr. zambiensis* (Supplementary Information 1) using standard PCR conditions. All isolates utilized in this study have been preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Forward primers from primer sets that successfully amplified microsatellite regions in all eight *Chrysoporthe* species were fluorescently labelled and synthesized by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. Based on estimated allelic ranges, these primers were grouped into panels using Multiplex manager v1.0 (Holleley and Geerts 2009) and tested by PCR on eight *Chrysoporthe* species. Amplicons were analysed for polymorphism using GeneScan® analyses (Life Technologies) with a LIZ500 size standard (Life Technologies) on an ABI 3500 machine (Applied Biosystems). Genemapper v4.1 was used to analyse the data (Applied Biosystems), and heterozygosity of each locus was calculated with GenAlEx (Peakall and Smouse 2006).

Microsatellite marker genotyping

Microsatellite marker genotyping was performed for each confirmed Brazilian *Chrysoporthe* isolate using labelled polymorphic microsatellite primers. Single-plexes of the ten markers were prepared for each isolate, each 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). The PCR conditions were similar to those used during primer testing described above.

Population genetic analyses

Population genetic analyses were conducted to determine whether populations of *Chr. cubensis* and *Chr. puriensis* found in Brazil were significantly different from each other. These analyses were also carried out on each of the *Chrysoporthe* species separately. Population genetic analyses for each species were calculated for sub-populations based on two host families (Myrtaceae and Melastomataceae) and three regions of Brazil (North/Northeast, Central-West and Southeast).

To infer the evolutionary history of a population, the unweighted pair-group method with an arithmetic mean (UPGMA) and a minimum expansion network (MEN) was performed. The UPGMA analyses were conducted in PAUP* 4.0 (Swofford 2002). The percentage of trees in which the associated taxa clustered together was calculated using 1000 bootstrap replicates. The evolutionary distances were computed using the number of differences method (Nei and Kumar 2000). A MEN was built using the Network software (http://www.fluxus-engineering.com). For this analysis, we used the total number of genotypes observed. The coalescence and the connections between the genotypes, given by the minimum number of mutations among them, were the basis of the analysis to generate a tree showing the frequency and the relation with the distances in number of mutations that separate each haplotype from others.

In order to determine the genetic structure of the population and the likely number of clusters (*K*), the allelic data were analysed without assigning population priors 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 and 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 species, hosts and regions, and the same parameters were used to run the Structure simulations in both cases.

Molecular variance analysis (AMOVA) was performed using GenAlEx v. 6.501 (Peakall and Smouse 2006) to estimate genetic (gene) variation within and between populations and sub-populations. Additionally, gene flow was calculated using two measures. The theoretical number of migrants per generation ($\widehat{M}$) between populations and sub-populations was estimated (Slatkin 1993, 1995), and population genetic differentiation (Θ) (Weir and Cockerham 1984) was calculated among pairs of populations and sub-populations using the program Multilocus v. 1.3 (Agapow and Burt 2001). Furthermore, the compositional allelic uniqueness (φ) of populations and a sub-population (the probability of sampling a unique/private allele in each) was calculated following the procedure of van der Merwe et al. (2013).

Nei's gene diversity ($\overline{H}$) (Nei 1973) was calculated using GenAlEx v. 6.501 (Peakall and Smouse 2006). Genotypic diversity ($\hat{G}$) was calculated according to Stoddart and Taylor (1988). Data generated from 1000 randomizations of the genotypic diversity per locus for each population and each sub-population based on host and region using Multilocus v. 1.3

(Agapow and Burt 2001) were plotted to determine whether the sample size was appropriate and whether the number of loci was 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 differences between genotypic diversities across populations and sub-populations (Stoddart and Taylor 1988).

The gametic (linkage) disequilibrium statistic (normalised $\overline{r}_d$) was calculated using Multilocus v. 1.3 (Agapow and Burt 2001) to infer panmixia (random mating) within the different populations and sub-populations. The significance levels of the $\overline{r}_d$ values were determined by calculating a *p*-value at a 95% confidence interval by generating 10,000 randomised $\overline{r}_d$values of data sets per population and sub-populations.

Results

Sampling and phylogenetic analyses

A total of 96 isolates were collected from different regions in Brazil (Table 2). The partition homogeneity tests showed that the ITS-rDNA and β-tubulin sequence data sets had no significant conflict (*P* = 0.013) and could thus be combined (Gryzenhout et al. 2006). The combined sequence dataset, comprising the ITS-rDNA fragment and β-tubulin gene sequences, produced 1131 aligned sequence characters of which 1012 were constant, 14 were parsimony uninformative, and 105 were parsimony informative. Phylograms obtained by maximum parsimony and maximum likelihood analyses had similar topologies.

A total of 53 isolates were confirmed as *Chr. cubensis* and 43 isolates were confirmed as *Chr. puriensis* (Table 2) with bootstrap support values of > 81% (Fig. 1). The *Chr. cubensis* isolates were predominantly from the North/Northeast regions, with 34 obtained in these regions and another 8 from West-central and 11 from the Southeast. In the case of *Chr. puriensis*, 42 isolates came from the Southeast and only 1 isolate was identified from the Northeast collections. All of the isolates of this species were isolated from native *Tibouchina* trees. The *Chr. cubensis* isolates were found on *Corymbia* species (30), *Tibouchina* (20) and *Eucalyptus* (3) trees.

Microsatellite marker

Genome mining for microsatellites in *Chr. austroafricana*, *Chr. cubensis* and *Chr. deuterocubensis* genomes yielded 30 microsatellite loci with conserved flanking sequences. Primers designed to amplify these microsatellite loci successfully amplified across the three species. The amplicons showed inter-species polymorphism evident from the differing amplicon sizes and number of motifs (Table 3). Most (23) of the microsatellite markers identified were trinucleotide repeats, three were tetranucleotide repeats, one was a pentanucleotide repeat, and two primer sets amplified compound microsatellites. The motif AGC was the most abundant observed for trinucleotides, while the most common tetranucleotide motif was TGGC (Table 3).

Table 2. *Chrysoporthe s*pp isolates used in this study

Species	Isolates	Number of isolates	Host		Location	
			Family	Species	City-State	Region
Chr. cubensis	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, CT110	2			São João del Rei-MG	
	THS01,	1		*Tibouchina heteromalla*	Silveirânia-MG	
	THSC05, THSC06	2			São Roque de Minas-MG	
	TIPASC01	1		*Tibouchina* sp.	São Roque de Minas-MG	
	TGII11	1		*Tibouchina* sp.	Itumirim-MG	
	TUC01, TUC02, TUC03	3		*Tibouchina urvilleana*	Carrancas-MG	
Chr. puriensis	TGCD01	1	Mela	*T. granulosa*	Lençóis-BA	Northeast
	TCL01	1	Mela	*T. candolleana*	Lavras-MG	Southeast
	CT05, TGL02, TGL03 TGL05	4		*T. granulosa*	Lavras-MG	
	TIL01	1		*T. heteromalla*	Lavras-MG	

	TGDR01, CT07, CT11	3		*T. granulosa*	São João del Rei-MG
	TGSC01, TGSC03, TGSC04, TGSC07, TGSC09, TGSC11, TGSC13, TGSC14	8		*T. granulosa*	São Roque de Minas-MG
	THSC01, THSC04	2		*T. heteromalla*	São Roque de Minas-MG
	TISC02	1		*Tibouchina* sp.	São Roque de Minas-MG
	TIST01	1		*Tibouchina* sp.	São Roque de Minas-MG
	TGS01, TGS02, TGS03, TGS04, TGS06, TGS07, TGS08	7		*T. granulosa*	Silveirânia-MG
	TGT02, TGT03	2		*T. granulosa*	Tiradentes-MG
	TGPNI01, TGPNI03, TGPNI04, TGPNI08, TGPNI09, TGPNI10, TGPNI11, TGPNI12, TGPNI13, TGPNI14, TGPNI16, TGPNI19	12		*T. granulosa*	Itatiaia-RJ

Myrt Myrtaceae, *Mela* Melastomataceae

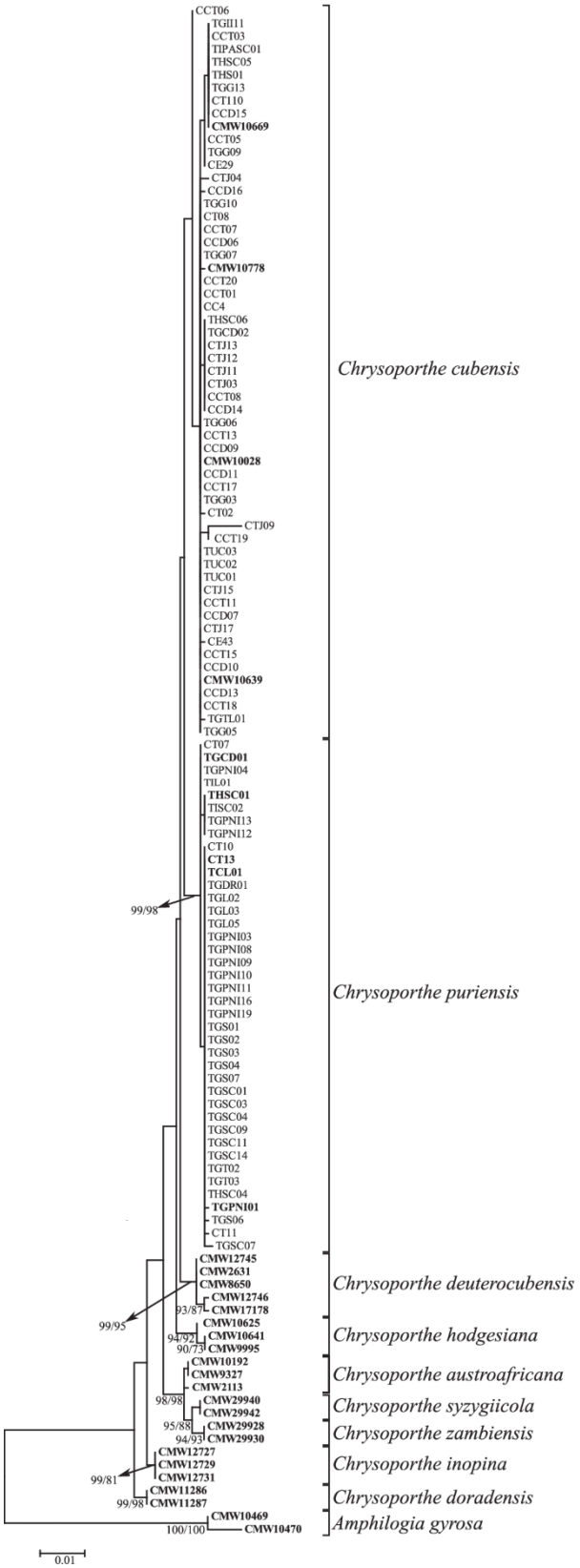

Fig. 1. Maximum Likelihood (ML) tree generated using combined ITS, BT1 and BT2 regions gene regions. Bootstrap values above 80% are indicated above each branc (ML/MP). Reference sequences are in bold

Table 3. Characteristics of homologous microsatellite markers identified in silico from genomes of three *Chrysoporthe* species

Motif	#repeats	Forward primer		Reverse primer		Predicted PCR product length (bp)			Scaffold		
		ID	Sequence (5' to 3')	ID	Sequence (5' to 3')	*cub*	*deu*	*aus*	*cub*	*deu*	*aus*
GCCTGG	5,2,4	Chry1F	TCTTGTTGCCAGCAGAGGAC	Chry1R	CGCCGCGCGAGAAGAC	168	150	162	72	563	759
TGGC	7,10, 6	Chry2F	CAGCCCTTCCCCTACTTTGG	Chry2R	ATCCCATGCGGCACAGAC	204	216	200	107	69	302
AGCC	7,8,12	Chry3F	TTCGTACAGTGATCTGCCCG	Chry3R	GCCACTGTGGAGATATTCGC	165	169	185	179	379	396
AGC	8,14,11	Chry4F	CAGCCAAAGGACACGAAAAT	Chry4R	ACCCATTTGATTTCCAAGCA	199	217	208	110	105	26
TGC	7,9,10	Chry5F	AGGAACGAAGAGGAGGAAGC	Chry5R	CTCTCGCTGCACAAATCCTC	224	230	233	162	88	31
AGC	**7,8,9**	**Chry6F**	**CCCCAAGCTCGCCTACTTC**	**Chry6R**	**GACGCTCTCGGGTATTGTCA**	**214**	**217**	**220**	**59**	**9**	**9**
AGT	**10,13,11**	**Chry7F**	**TTGGTCGGTTCGGTAGAAAG**	**Chry7R**	**CATGACGGGAGAGCGATAAG**	**223**	**232**	**226**	**44**	**127**	**41**
ACT	6,10,8	Chry8F	CCAATCTCGGTCCGGGTATC	Chry8R	CGCGACAGTGAACAAGAGAC	269	281	275	74	16	50
TGC	**7,20,10**	**Chry9F**	**CCTTCTCTTTCCCAATTTTGC**	**Chry9R**	**CACCTCGTACAAGGCCTCTC**	**208**	**247**	**217**	**1**	**5**	**62**
TGGC	**3,4,2,**	**Chry10F**	**TGCGAGTGGGTGAGTGAGTA**	**Chry10R**	**ACACGGACGGTCATAGCAC**	**157**	**161**	**153**	**66**	**67**	**49**
AGC	**9,12,8**	**Chry11F**	**GCTGAAGGCACAGAAAGAGC**	**Chry11R**	**AGTCTGCGTTGATCCTTGCT**	**156**	**165**	**159**	**105**	**21**	**73**
TGC	12,9,8	Chry12F	AACAACCACAACCCACCCC	Chry12R	CTGCCGTTCTCACTGTTGG	161	152	149	50	34	59
TGGTC	**8,7,9**	**Chry13F**	**GTGTCGTCGACTCAAGCTCA**	**Chry13R**	**GACGGAGAGGACACTGAAGC**	**213**	**208**	**218**	**5**	**202**	**120**
AAG	8,17,13	Chry14F	AACCTTCTATCACCGCCAGA	Chry14R	CTTGCAGGCTTTGTCGAGAT	214	241	229	166	172	121
TGA	**12,9,7**	**Chry15F**	**AAGGCCAGATTGTCCAAATG**	**Chry15R**	**CAGCAACGAACCAAAATCAA**	**253**	**244**	**238**	**157**	**91**	**140**
ACG	8,7,6	Chry16F	GTCTCGTCTCTGGGCTCATC	Chry16R	CTCCTCTCGTCACGGTTCAC	155	152	149	75	28	255
ACC	**11,9,6**	**Chry17F**	**AGATCCAAGTGGGCTTTCCT**	**Chry17R**	**CTTAGCTTGACCCAGGGATG**	**162**	**156**	**147**	**53**	**409**	**256**
ACG	9,8,12	Chry18F	CTGCATCAAGAAGCAGATCG	Chry18R	TGAGACCAGAGTTGAGATCCG	266	263	275	63	308	324
AGC_AAC	9,10,8	Chry19F	GCACTGCACATGGATCATTC	Chry19R	CTCGTGGCCCTAATGTTGTT	225	228	222	195	272	63
GCC	5,11,8	Chry20F	CTCCCGGGTAGGTCTGTCA	Chry20R	TTCTCCCTCCTGATGCTCAC	166	184	175	48	510	65
ATC	11,8,10	Chry21F	CATGCAGCTCTTCCCTCTTC	Chry21R	CAGCTGGCTGTGACAAAGAA	188	179	185	452	582	468
TGC	13,19,9	Chry22F	CGATTACCCACCAATTGTCC	Chry22R	GGAAAAAGCAAGCGGTGAC	203	221	191	49	15	100

AGC	**7,6,10**	**Chry23F**	**TGTCCTGCATCTAGGTCGTG**	**Chry23R**	**GTCCCAGTCCTCTTGGATCA**	**148**	**145**	**157**	**43**	**122**	**117**
ATC	**8,12,7**	**Chry24F**	**GTGCGGGCATCTGTTCTACT**	**Chry24R**	**GCGCATTGTGAGGCGTTAAG**	**183**	**195**	**180**	**132**	**124**	**558**
ACC	**10,7,8**	**Chry25F**	**TATCTGCGCAACTACCACCA**	**Chry25R**	**GGGAAGTGTGTCTCCTCCTG**	**173**	**164**	**161**	**44**	**252**	**911**
ACC	7,8,9	Chry26F	AGAGTGGTCCGAGCTTCCTT	Chry26R	GCCTTCGATTGATGTCGATT	170	173	179	336	479	1002
AGC	**7,6,14**	**Chry27F**	**GCCGCCTACAGAGACTATAACG**	**Chry27R**	**CGACGAAGACGTAGTTGCAC**	**232**	**229**	**253**	**57**	**132**	**139**
TGG	**6,7,9**	**Chry28F**	**GATATGCCGGTCATGGTGAT**	**Chry28R**	**TACTTGAGGGCGAAGGAAAG**	**226**	**229**	**235**	**93**	**751**	**1058**
TGC_TGG	8,11,9	Chry29F	CCGTAAGCTGGATCCTGAAC	Chry29R	AAGCCCCGAGATTAGGACAT	235	244	238	14	131	322
ACC	6,9,8	Chry30F	GCGCGGATACTTCATCGTTC	Chry30R	TGAAGCCTGGGAGAGGAGAG	178	187	184	918	467	1200

Data highlighted in bold refer to microsatellite markers with cross-species amplification in *Chrysoporthe*

[#]repeats: Number of repeats of the microsatellite motif in *C. cubensis*, *C. deuterocubensis and C. austroafricana* respectively

ID: Primer identity

bp: Base pairs

cub.: Chr. cubensis

deu.: Chr. deuterocubensis

aus.: Chr. Austroafricana

Table 4. Characteristics of polymorphic microsatellite markers used for genotyping *Chrysoporthe* species isolates from different regions and hosts in Brazil

Microsatellite markers	Motif	Primer		Ta[a] (°C)	*Chrysoporthe* species		*Chrysoporthe cubensis*		*Chrysoporthe puriensis*	
					AR[b] (bp[c])	N. of alleles[c]	AR[b] (bp[c])	N. of alleles[c]	AR[b] (bp[c])	N. of alleles[c]
CHRY06	AGC	F[d]:	CCCCAAGCTCGCCTACTTC	63	210–216	3	210–216	3	210–216	2
		R[e]:	GACGCTCTCGGGTATTGTCA							
CHRY07	AGT	F[d]:	TTGGTCGGTTCGGTAGAAAG	60	216–255	8	255	1	216–255	8
		R[e]:	CATGACGGGAGAGCGATAAG							
CHRY09	TGC	F[d]:	CCTTCTCTTTCCCAATTTTGC	64	210–219	3	210	1	216–219	2
		R[e]:	CACCTCGTACAAGGCCTCTC							
CHRY10	TGGC	F[d]:	TGCGAGTGGGTGAGTGAGTA	60	164–172	3	164	1	168–172	2
		R[e]:	ACACGGACGGTCATAGCAC							
CHRY13	TGGTC	F[d]:	GTGTCGTCGACTCAAGCTCA	63	195–265	10	215–265	8	195–205	2
		R[e]:	GACGGAGAGGACACTGAAGC							
CHRY15	TGA	F[d]:	AAGGCCAGATTGTCCAAATG	58	246–348	13	246–348	11	249–276	6
		R[e]:	CAGCAACGAACCAAAATCAA							
CHRY17	ACC	F[d]:	AGATCCAAGTGGGTTTCCT	60	147–186	10	147–171	8	147–186	8
		R[e]:	CTTAGCTTGACCCAGGGATG							
CHRY24	ATC	F[d]:	GTGCGGGCATCTGTTCTACT	61	180–195	5	186–189	2	180–195	5
		R[e]:	GCGCATTGTGAGGCGTTAAG							
CHRY25	ACC	F[d]:	TATCTGCGCAACTACCACCA	61	162–189	9	162–189	7	162–186	8
		R[e]:	GGGAAGTGTGTCTCCTCCTG							
CHRY27	AGC	F[d]:	GCCGCCTACAGAGACTATAACG	63	228–234	3	231–234	2	228–231	2
		R[e]:	CGACGAAGACGTAGTTGCAC							
CHRY28	TGGTC	F[d]:	GATATGCCGGTCATGGTGAT	60	225–250	5	230	1	225–250	4
		R[e]:	TACTTGAGGGCGAAGGAAAG							

[a]Annealing temperature

[b]Allelic range

[c]Base pairs

[d]Forward primer

[f]Reverse primer

Of the 30 microsatellite loci identified, 13 primer sets were selected for genotyping based on their ability to amplify across the *Chrysoporthe* species (Supplementary Information 2). Genotyping of *Chr. austroafricana*, *C. cubensis* and *Chr. deuterocubensis* isolates showed that polymorphisms were present for these 13 markers (Table 3). No null alleles were observed in any of the 13 microsatellite markers. Heterozygosity of each marker ranged from 0.568 to 0.833 (Supplementary Information 2).

The microsatellite markers were developed before the *Chr. puriensis* genome became available and were thus tested empirically on isolates of this species. Eleven polymorphic microsatellite markers were used for microsatellite marker genotyping of the *Chrysoporthe* isolates from Brazil (Table 4). The markers Chry07, Chry13, Chry15, Chry17 and Chry25 had eight or more alleles present in the meta-population, while Chry06, Chry09, Chry10, Chry24, Chry27 and Chry28 had five or fewer alleles (Table 4). The marker with the largest number of alleles as well as the largest allelic range was Chry15 with 13 alleles ranging from 246 to 348 bp (Table 4).

Combined population genetic analyses of Chr. cubensis and Chr. puriensis

The UPGMA-based dendrogram emerging from the microsatellite analyses exhibited spatial clustering and divided all 96 isolates in two distinct groups, separating the isolates from Brazil as either *Chr. cubensis* or *Chr. puriensis* (Fig. 2a). The *Chr. cubensis* cluster consisted of 53 isolates, collected in different regions of Brazil. Among these, 33 were collected from Myrtaceae (*Eucalyptus* or *Corymbia*) and 20 from Melastomataceae (*Tibouchina*). The *Chr. puriensis* cluster consisted of 43 isolates, collected mostly from the Southeast region of Brazil and all were collected from *Tibouchina* (Melastomataceae). Several sub-groups were observed in both clusters, indicating genetic variability within and between species.

The MEN diagram illustrated the phylogenetic structure of the 81 genotypes found to represent the two *Chrysoporthe* species from Brazil (Fig. 2b,c). The genotypes do not have a structure defined by region or host but were separated based on their respective identities as *Chr. cubensis* or *Chr. puriensis*. All *Chr. puriensis* genotypes were interconnected to only two *Chr. cubensis* genotypes. There were nine genogroups, with each genogroup accommodating between 2–4 isolates. The remaining genotypes found in the study were unique. For *Chr. puriensis* there were 41 unique genotypes and one genogroup that accommodated two isolates. For *Chr. cubensis* there were 31 unique genotypes and eight genogroups.

Differentiation between *Chr. cubensis* and *Chr. puriensis* populations was significant (Θ = 0.508). Similarly, in the structure analysis, the number of *Chrysoporthe* species populations in Brazil, as inferred by Structure and analysed by Structure Harvester, was K = 2 (Fig. 3a), suggesting that there were two genetically distinct clusters. Thus, *Chr. cubensis* and *Chr. puriensis* could be readily separated from each other based on this result. These data also highlighted the fact that the two species are each characterized by markedly different allelic compositions, although a low level of admixture was detected.

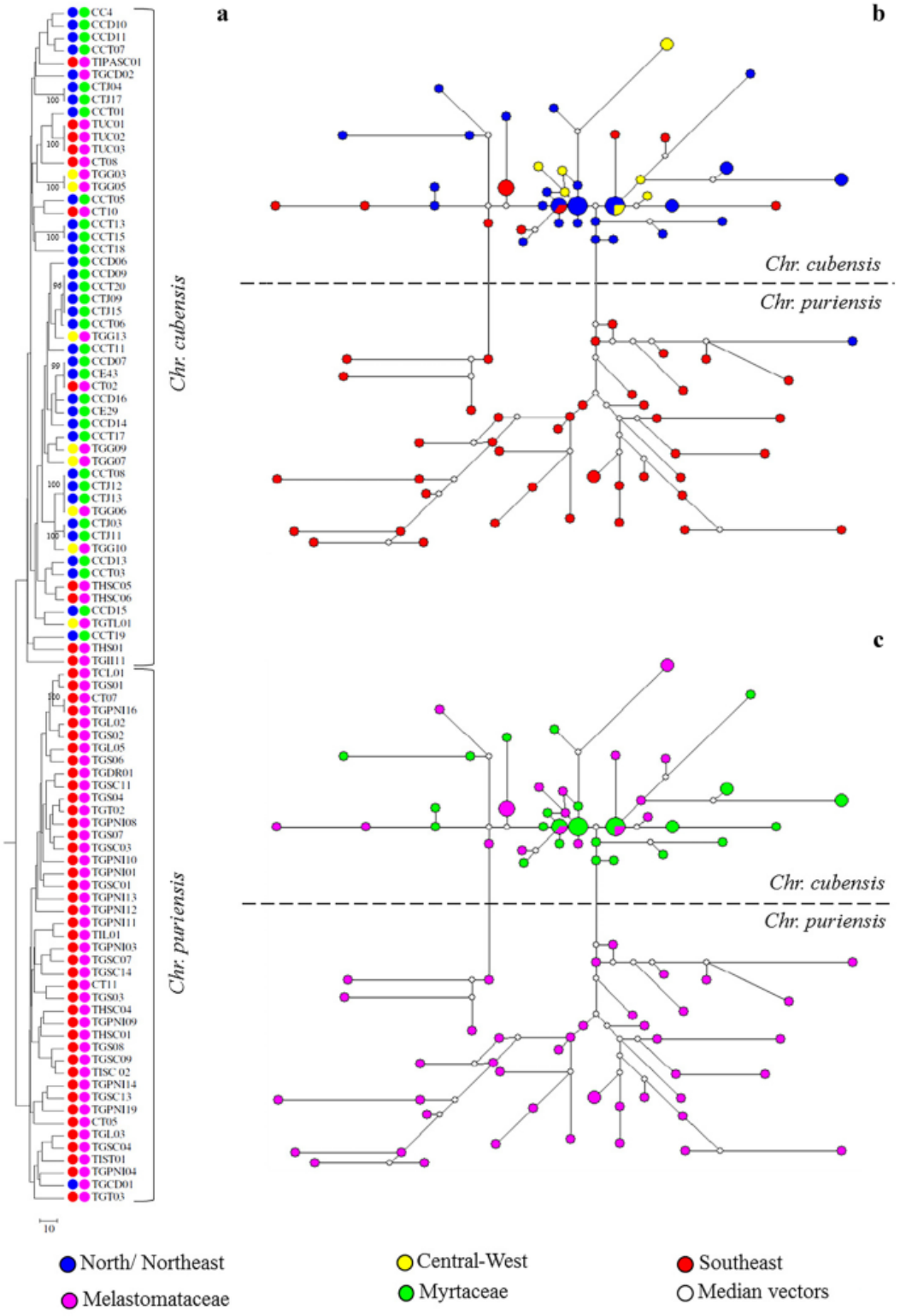

Fig. 2. Evolutionary history of population of the 96 isolates of *Chrysoporthe* species from Brazil. (**a**) UPGMA dendrogram based on Nei's genetic distance. Bootstrap values above 95% are indicated above each branch. Host and region of each isolate are indicated with a coloured label. (**b,c**) Minimum expansion network (MEN). It shows the frequency and relationship among genotypes and genogroups. Sub-populations and median vectors are indicated with a coloured label. (**a**) Genotypes based on region and (**b**) Genotypes based on host

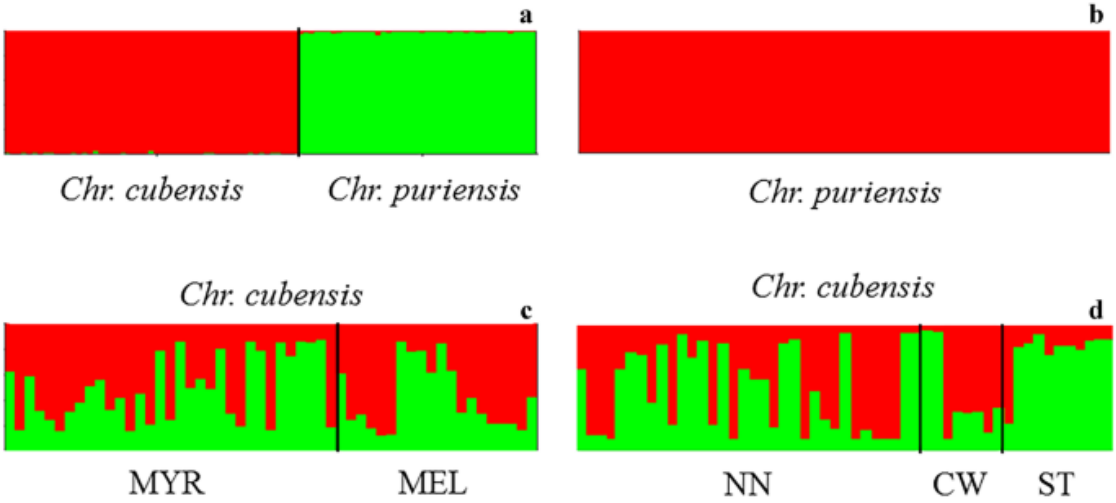

Fig. 3. Genetic structure of the population of *Chrysoporthe* species populations in Brazil. (**a**) Structure plot of *Chr. cubensis* and of *Chr. puriensis* populations. (**b**) Structure plot of *Chr. puriensis* populations. (**c,d**) Structure plot of *Chr. cubensis* populations. (**c**) Isolates based on host, MYR = Myrtaceae, MEL = Melastomataceae. (**d**) Isolates based on region, NN = North/Northeast, CW = Central-West, ST = Southeast

Analysis of molecular variance revealed that the genetic variation was highest within than among the populations analysed ($P < 0.01$). Genetic variation within populations was 49% and 51% among populations. Low number of migrants value between *Chr. cubensis* and *Chr. puriensis* populations was observed ($\widehat{M} = 0.485$).

Seventy-one alleles were found, distributed among the eleven loci analysed. Of these, 31% of the alleles were shared between the two species. The *Chr. puriensis* population had 37% ($\varphi = 0{,}818$) of private alleles and the remaining 32% ($\varphi = 0.587$) are private alleles of the *Chr. cubensis* population (Table 5).

The Stoddart and Taylor (1988) and the Agapow and Burt (2001) methodologies were used to determine how many microsatellite loci should be analysed to reach the maximum genotypic diversity and to determine whether the number of isolates sampled was sufficient to analyse the maximum genotypic diversity in both the *Chr. cubensis* and *Chr. puriensis* populations (Fig. 4a,b). This was also tested in the case of the *Chr. cubensis* populations from different regions (Fig. 4c,d). In all cases, the number of loci used in this study was sufficient to determine genotypic diversity appropriately. On average, the genotypic diversity of the populations and sub-populations remained constant from four loci (Fig. 4a,b,c,d). Seven loci were sufficient to reach the maximum genotypic diversity (Fig. 4b,d). *Chr. puriensis* population reached the maximum genotypic diversity with the sampled isolates (Fig. 4a). The number of isolates sampled in *Chr. cubensis* meta-population and sub-populations was not sufficient to analyse the maximum genotypic diversity, with the exception of the Central-West sub-population (Fig. 4c).

Table 5. Gene diversity, genotypic diversity, private alleles and gametic (linkage) disequilibrium in the meta-population and sub-populations of *Chrysoporthe* species from different regions and hosts in Brazil and other populations from other parts of the world

Countries	Species	Population		Number of Isolates (N)	Gene diversity $(\overline{H})$	Genotypic diversity $(\widehat{G})$	Private allele (φ)	Gametic (linkage) disequilibrium $(\bar{r}_d)$
Brazil	*Chr. puriensis*	Meta-population		43	0.456	1.000	0.818	0.012
	Chr. cubensis	Meta-population		53	0.304	0.535	0.587	0.048[a]
		Host	Myrtaceae	33	0.273	0.673	0.250	0.142[a]
			Melastomataceae	20	0.321	0.769	0.766	0.030
		Region	North/Northeast	34	0.278	0.680	0.520	0.140[a]
			Central-West	8	0.287	1.000	0.147	0.263[a]
			Southeast	11	0.275	0.733	0.428	0.091[a]
Colombia[b]	*Chr. cubensis*	Host	Myrtaceae	59	0.445	1.000	0.530	-
			Melastomataceae	32	0.398	0.314	0.249	-
South Africa[c]	*Chr. cubensis*	Region	Kenya	10	0.100	0.172	-	-
			Malawi	51	0.290	0.053	-	-

[a]The populations are in gametic disequilibrium ($P < 0.05$)

[b]vander Merwe et al. (2013)

[c]Nakabonge et al. (2007)

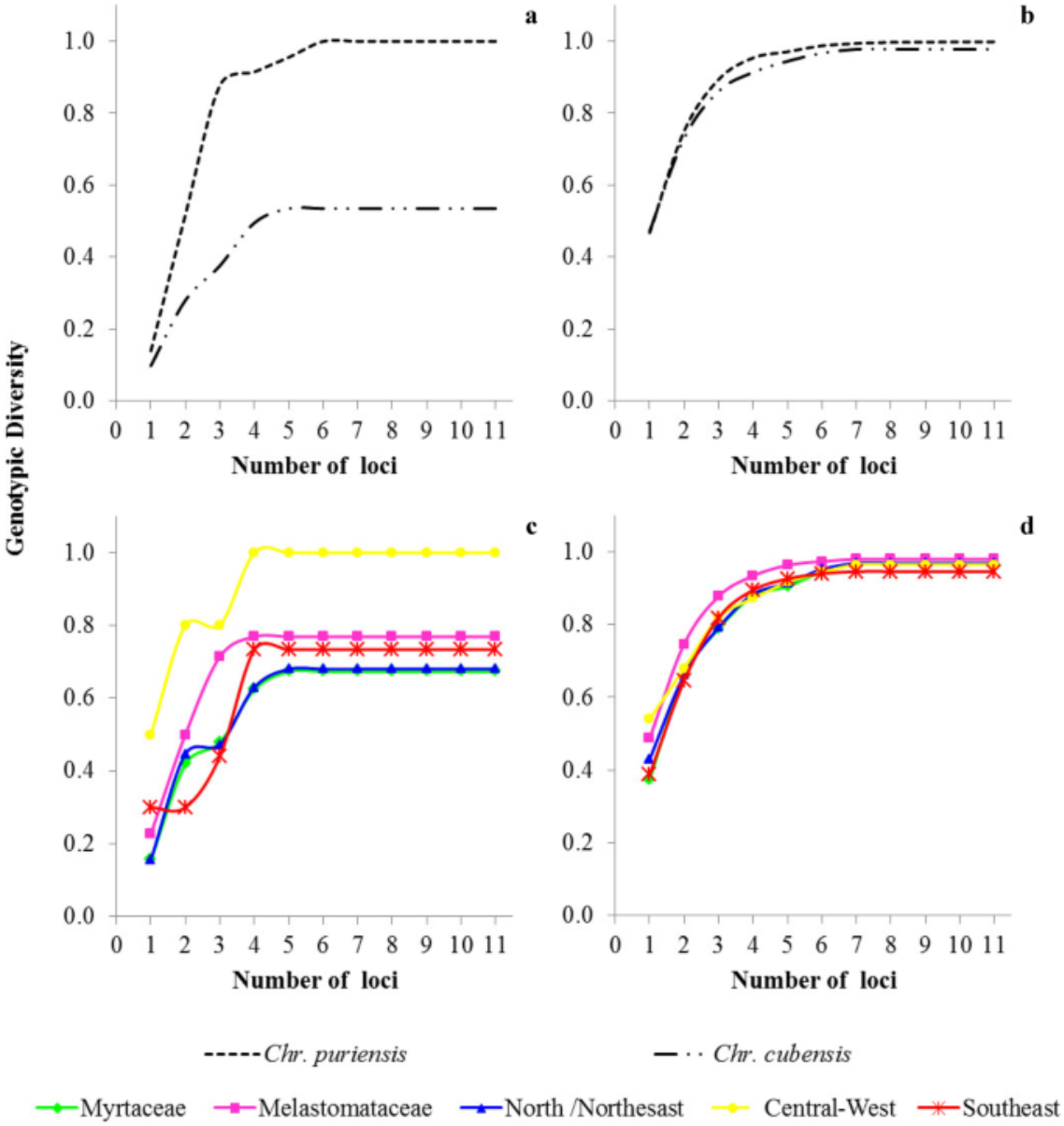

Fig. **4.** Genotypic diversity of the populations of *Chrysoporthe* species from Brazil per number of loci. (**a,c**) Calculated according to Stoddart and Taylor (1988); (**b,d**) Calculated according to Agapow and Burt (2001), data generated from 1000 randomizations using Multilocus v. 1.3. (**a,b**) Genotypic diversity of *Chrysoporthe cubensis* and *Chrysoporthe puriensis* per number of loci. (**c,d**) Genotypic diversity of the sub-populations of *Chrysoporthe cubensis* from Brazil per number of loci

Population genetic analyses of Chr. cubensis isolates

The probable number of *Chr. cubensis* sub-populations in Brazil, as inferred by Structure and analysed by Structure Harvester, was $K = 2$, which suggests that there were two genetically distinct clusters. Admixture was observed in the bar plot both with data grouping according to the host (Fig. 3c) and according to the region (Fig. 3d). Considering the sub-populations based on the host, gene and genotypic diversities were slightly higher in the sub-population

from Melastomataceae ($\overline{H}$ = 0.508 and $\hat{G}$= 0.769) than in the sub-population from Myrtaceae ($\overline{H}$ = 0.427 and $\hat{G}$= 0.673) (Table 5).

For *Chr. cubensis* sub-populations based on regions, the highest gene diversity and the highest genotypic diversity was found in the Central-West sub-population ($\overline{H}$ = 0.287 and $\hat{G}$= 1), while the North/Northeast and Southeast sub-populations had values of gene and genotypic diversity of $\overline{H}$ = 0.278, $\hat{G}$ = 0.680 and $\overline{H}$ = 0.275, $\hat{G}$== 0.733 respectively (Table 5). 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 other than the Central-West sub-population, had different genotypic diversities ($P < 0.05$) 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 (> 88%). Genetic variation among sub-populations based on hosts, *i.e.* Melastomataceae and Myrtaceae, was 4%. Among the North/Northeast and Central-West sub-populations, the genetic variation was lowest and there was no significant difference between these sub-populations. The highest genetic variation among the sub-populations was 12%, which was detected between the Southeast sub-population and North/Northeast and Central-West sub-populations. Among the sub-populations based on the host, the genetic differentiation was significant (Θ = 0.043), with a considerable number of migrants ($\widehat{M}$ = 11.03) (Fig. 5a).

For the different regions, the highest number of migrants was observed between the North/Northeast and Central-West sub-populations ($\widehat{M}$ = 45.28), coinciding with the lowest value of genetic differentiation (Θ = 0.011), which was not significant. The number of migrants was low both between Southeast and North/Northeast sub-populations ($\widehat{M}$ = 3.68), and between Southeast and Central-West sub-populations ($\widehat{M}$=3.58). The differentiation was significant both between Southeast and North/Northeast sub-populations (Θ = 0.120), and between Southeast and Central-West sub-populations (Θ = 0.123) (Fig. 5b).

The number of private or shared alleles among sub-populations is presented in a Venn diagram (Fig. 5c,d). The composition of private alleles (φ) for each sub-population (Table 5) supports the data in the Venn diagram. The meta-population had 45 alleles distributed among all eleven loci analysed. Considering the two host-based sub-populations, 55.6% of the alleles were shared. The Myrtaceae sub-population had 28.9% (φ = 0.464) of private alleles and the remaining 15.6% (φ = 0.412) private alleles were in the Melastomataceae sub-population (Fig. 5c, Table 5). In the sub-populations based on regions, 31.1% of the alleles were shared among all sub-populations, while 11.1% were shared between the North/Northeast and between Central-West and North/Northeast and Southeast sub-populations. Only 2.2% were shared between the Southeast and Central-West sub-populations (Fig. 5d). The North/Northeast sub-population had the highest proportion of private alleles (33.3%; φ = 0.520), while the Central-West sub-population presented the lowest proportion (2.2%; φ = 0.147) (Table 5).

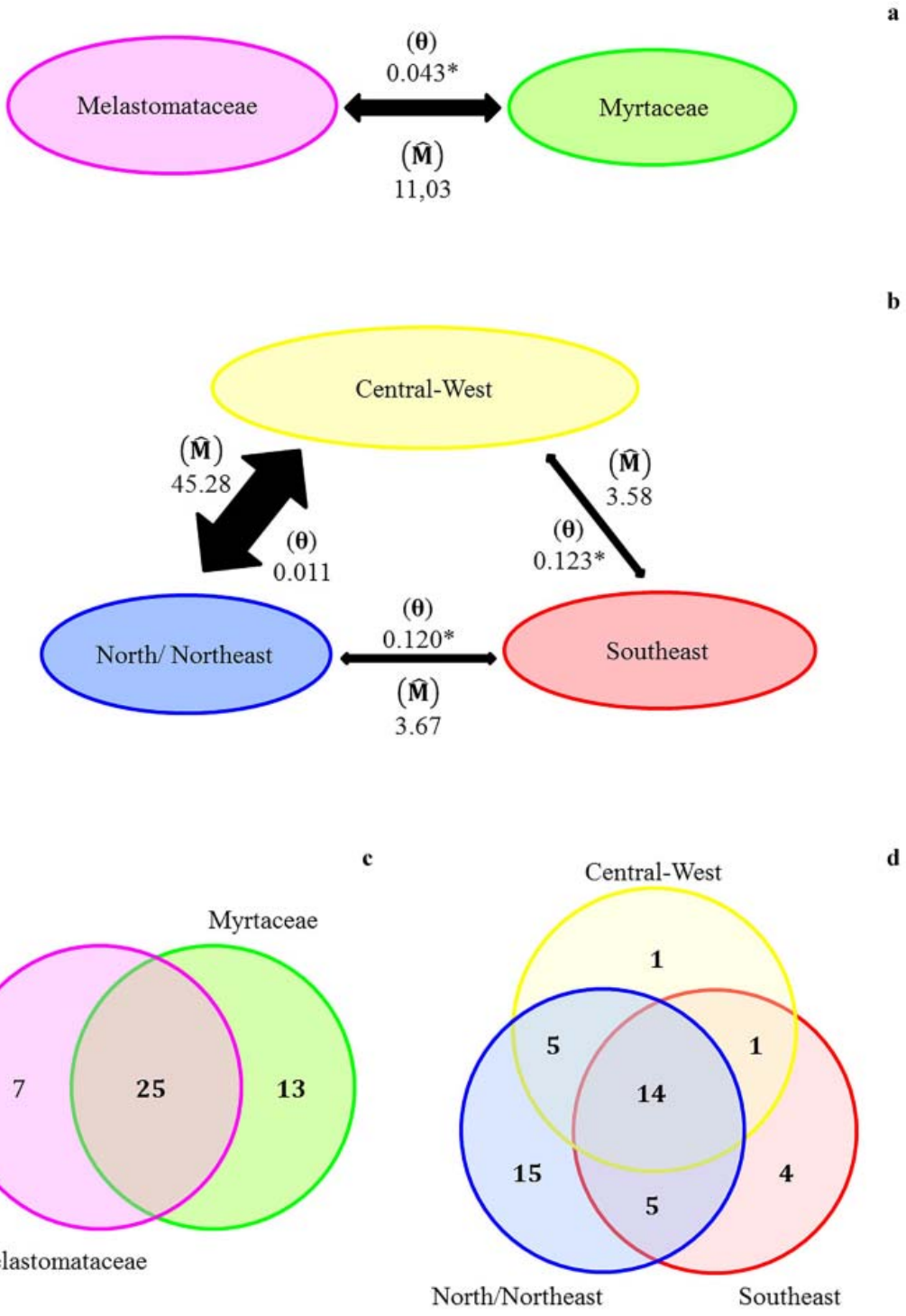

Fig. 5. (**a,b**) Plot of gene diversity ($\overline{H}$), genotypic diversity ($\hat{G}$), gene flow ($\widehat{M}$) and genetic differentiation (Θ) of *Chrysoporthe cubensis* isolates from sub-populations based on different hosts (**a**) and regions (**b**). Thickness and shade of lines with arrows show amount of gene flow ($\widehat{M}$) between subpopulation. Shape of circles show diversity in each subpopulation; horizontal width representing genotypic diversity ($\hat{G}$) and vertical width representing gene diversity ($\overline{H}$). *Significance ($P \leq 0.05$). (**c,d**) Venn diagram representing the distribution of the private and shared alleles among the sub-populations of *Chr. cubensis* isolates, based in hosts (**c**) and region (**d**)

The results of $\bar{r}_d$ for the meta-population and other sub-populations, with the exception of the Melastomataceae sub-population, were significant ($P < 0.05$) (Table 5), implying that these sub-populations were in gametic disequilibrium. In contrast, the null hypothesis of random association of alleles in loci for the Melastomataceae sub-population was accepted.

Population genetic analyses of Chr. puriensis isolates

The result of the population structure test for *Chr. puriensis*, highlighted that this represented a single population (Fig. 3b), and that it did not include sub-populations. The *Chr. puriensis* population also showed slightly higher gene and genotypic diversities ($\overline{H}$= 0.456 and $\hat{G}$= 1.0) than that of *Chr. cubensis* (Table 5). The results of $\bar{r}_d$ for *Chr. puriensis* population was not significant (P < 0.05) (Table 5), which implies that the alleles in the population is in linkage equilibrium.

Discussion

This is the first study to consider genetic diversity of populations of *Chr. cubensis* isolates collected from different regions of Brazil and from different hosts in the non-native Myrtaceae and native Melastomataceae. Furthermore, we were able to consider the genetic diversity of the newly discovered *Chr. puriensis* and how this relates to its sibling species *Chr. cubensis*.

Microsatellite markers that can now be used on all known *Chrysoporthe* species were designed. The availability of whole genome sequences for three species of *Chrysoporthe* made it possible to develop these microsatellite markers. They will clearly also be useful in the future for population genetic and evolutionary biology studies on this group of pathogens from different parts of the world.

Population genetic analyses for a relatively large collection of isolates from different species of trees and applying microsatellite markers revealed a high level of diversity for both *Chr. puriensis* and *Chr. cubensis* in Brazil. The fact that these pathogens occur commonly on native *Tibouchina* species and that isolates from these trees were associated with a high level of genetic diversity provides strong evidence that they are native to Brazil. This confirms the results of earlier studies on *Chr. cubensis*, which were conducted using vegetative compatibility tests (van Zyl et al. 1998). The existence of some shared alleles between *Chr. cubensis* and *Chr. puriensis* as well as evidence of some gene-flow between the species, suggests that these are cryptic species with a common ancestry (Taylor et al. 2000; van der Merwe et al. 2010).

An important result of this study was that the structure and genetic variability of *Chr. cubensis* in Brazil appears not to be strongly influenced by its geographical distribution or the hosts on which it occurs. Isolates collected from the same host did not group in a single cluster implying that there is movement of the pathogen between hosts. This result is in contrast to those of van der Merwe (2013) who studied a South African population of the sibling species *Chr. austroafricana* and showed that partition of the population is linked to host.

Significant differences were observed in gene and genotypic diversity between the sub-populations of *Chr. cubensis* from Myrtaceae and Melastomataceae that represent non-native and native hosts respectively. There were high levels of gene flow between sub-populations from Myrtaceae and Melastomataceae. This explains the highly admixed allelic composition of these sub-populations, where more than 50% of the alleles were shared. This is consistent with the fact that high levels of genetic variability would be expected in native or better-established populations (Tsutsui et al. 2000). The results also show that *Chr. cubensis* is well established in Brazil, both on native and non-native hosts.

A high level of genetic variability was expected in the sub-population of *Chr. cubensis* collected from Melastomataceae. This is because trees in this Family are generally native hosts of the pathogen (Nakabonge et al. 2006; Gryzenhout et al. 2009). However, the gene and genotypic diversities were only slightly higher for Melastomataceae (native hosts) than for Myrtaceae (non-native hosts). This high level of variability in the sub-population from Myrtaceae can be explained by two factors that may be acting individually or in combination. Firstly, the sexual state of the pathogen is present on trees in the Myrtaceae, and populations of pathogens undergoing sexual reproduction would be expected to exhibit high levels of genotypic diversity (McDonald and Linde 2002). Secondly, although *Eucalyptus* species and *Corymbia citriodora* are not native in Brazil, these trees were introduced into the country more than 100 years ago (Fishwick 1975). The first reports of *Chr. cubensis* on *Eucalyptus* in Brazil was in 1973 (Hodges et al. 1973), and there has been at least 40 years for the pathogen to generate high levels of genetic variability on non-native hosts.

Asexual reproduction combined with non-random mating could have resulted in gametic disequilibrium (Brewer et al. 2012) in the sub-populations of *Chr. cubensis* in Brazil. Non-random mating might occur because *Chr. cubensis* is a homothallic fungus with frequent self-fertilization. However, we believe that outcrossing, which is possible but not common in homothallic fungi (Billiard et al. 2012), has been the driver of the observed high genotypic diversity. The presence of gametic disequilibrium indicates that the outcrossing frequency has not been sufficient to randomize alleles across the sub-populations.

The high genetic variability of *Chr. cubensis* in Brazil could explain why clones of *Eucalyptus* selected in the Southeast region lose their resistance to the pathogen when they are introduced in the North and Northeast. This would be consistent with the fact that the durability of disease resistance is affected by the evolutionary potential of pathogens. Thus, those strains most likely to overcome host resistance would undergo both sexual and asexual reproduction and have a high potential for gene flow (Mc Donald and Linde 2002). All these characteristics were found in the population of *Chr. cubensis* in Brazil. It is consequently important that the breeding and selection programs for *Eucalyptus* clones based on artificial inoculation (van Heerden et al. 2005) utilize several isolates having different genotypes from different regions and hosts.

This study has provided strong support for the view that *Chr. cubensis* and *Chr. puriensis* are native to Brazil. There are substantial implications of this finding, not only for *Eucalyptus* forestry in Brazil but also for global tree health in general. Due to a host shift from native trees (Slippers et al. 2005), *Chr. cubensis* has become a new pathogen of *Eucalyptus* in Brazil. The same can happen with *Chr. puriensis*, which has the potential to become a new

threat to the *Eucalyptus* forestry in Brazil as was discussed by Oliveira et al. (2021). In this respect, it is similar to the globally invasive myrtle rust pathogen *Austropuccinia psidii* (Coutinho et al. 1998; Glen et al. 2007) native to South America and that now threatens many species of Myrtaceae where these trees are native.

Global trade in wood and wood products raises the risk that *Chr. cubensis* and *Chr. puriensis* could be introduced into new areas of the world where it might devastate native Myrtaceae and Melastomataceae (Wingfield et al. 2015c; Burgess and Wingfield 2017). In this regard it is relevant to recognise that *Chr. cubensis* and *Chr. puriensis* result in a disease very similar to chestnut blight, which is caused by the closely related *Cryphonectria parasitica*. Every effort should thus be made to avoid the trade in *Eucalyptus* timber that could be infected with *Chr. cubensis* or *Chr. puriensis*.

Data availability statement

Data openly available in a public repository that does not issue DOIs.

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The data that support the findings will be available in NCBI Nucleotide at https://www.re3data.org/search?query=ncbi. The Genbank numbers are given in the text of the manuscript. The data is under embargo until a manuscript is accepted for publication. It will then be openly available. Should reviewers wish to see the data they will be made available.

Conflict of interest

The authors declare that they have no conflict of interest.

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