Population characterization of Brazilian isolates of *Ceratocystis* spp using microsatellites

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Introduction

The genus *Ceratocystis* includes several species widely distributed all over the world. In Brazil, there are reports of five species: *Ceratocystis cacaofunesta*, *C. paradoxa*, *C. mangicola e C. mangivola* and *C. fimbriata*, the latter being most relevant and causer of diseases in a large number of woody plants and in some herbaceous plants of great economic importance. During the past decade, numerous new and cryptic species in the *C. fimbriata* complex have been described. Many different DNA fingerprinting techniques have been used to define fungal populations. There is, however, an increasing interest in using co-dominant markers in population studies due to their ability to detect and characterize multiple aleles at a given locus. Microsateilite regions provide an attractive source of polymorphisms between isolates, and many properties favour their use as genetic markers.

Objectives

This study aimed to characterize the population structure and diversity of isolates of *Ceratocystis fimbriata* sensu lato collected from diseased *Eucalyptus* spp. and to compare with isolates from cacao (*Theobroma cacao*), , mango (*Mangifera indica*) , teak (*Tectona grandis*), fig *(Ficus carica*) , rubber (*Hevea brasiliensis*) and atemoia (hibrid de *Annona cherimola* com *A. squamosa*) with using microsatellites.

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Material and Methods

➢**Sample collection and isolation**: The samples were obtained from forest pathology mycology collection of Faculdade de Ciências Agronômicas of the UNESP, Brazil, Sao Paulo state, Botucatu City.

➢ **DNA Extraction, PCR amplification and separation of SSR loci**: Cultures were grown on MEA in Petri dishes for two weeks and mycelium scraped from the surface of the plates for DNA extraction as described by de Murray and Thompson (1980). Ten fluorescently labelled SSR-PCR products were multiplexed and analyzed according to the methodology described by Fourie (2015).

➢ **Gene and genotypic diversity**: analyzed according to the methodology described by Bihon et al (2014).

➢ **Population differentiation and clustering**: Clone corrected populations were analyzed using the program Multilocus (Agapow and Burt, 2000), with an estimate of Wright's, FST as Θ = Q-q/1-q to calculate population differentiation theta (Θ) where Q is the probability that two alleles from the same population are the same and q is the probability that two alleles from different populations are the same (Weir, 1997). Population structure was inferred and assigned in STRUCTURE 2.2 that clusters individuals into K distinct populations (clusters) and permits mixed ancestry (Pritchard et al., 2000).

➢ **Index of Association:** Association of alleles for a clone-corrected D. pinea population was inferred by calculating the Index of Association (IA) and rBarD (r-D) using the program Multilocus (Agapow and Burt 2000).

➢ **Analysis of molecular variance (AMOVA):** was conducted to differentiate the sources of variation between and within populations (9999 permutations), using the software GeneAlEx version 6.2 (Peakall and Smose 2006).

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Results

Table 1: Grouping of isolates according to population *

Population	Isolate	City/State	Host
Population 1	ACF25	Montes Claros/MG	*Eucalyptus*
Population 1	ACF26	Montes Claros/MG	*Eucalyptus*
Population 1	ACF27	Araraquara/SP	*Eucalyptus*
Population 1	ACF28	Itararé/SP	*Eucalyptus*
Population 1	ACF32	Avaré/SP	*Eucalyptus*
Population 1	ACF33	Avaré/SP	*Eucalyptus*
Population 1	ACF35	Avaré/SP	*Eucalyptus*
Population 1	ACF36	Agudos/SP	*Eucalyptus*
Population 1	ACF38	João Pinheiro/MG	*Eucalyptus*
Population 1	ACF39	Manduri/SP	*Eucalyptus*
Population 1	ACF40	Olhos D'agua/MG	*Eucalyptus*
Population 1	ACF41	Paraopeba/MG	*Eucalyptus*
Population 1	ACF42	Paraopeba/MG	*Eucalyptus*
Population 1	ACF43	Paraopeba/MG	*Eucalyptus*
Population 1	ACF52	Bocaiuva/MG	*Eucalyptus*
Population 1	AFC53	Bocaiuva/MG	*Eucalyptus*
Population 1	ACF29	Itararé/SP	*Eucalyptus*
Population 2	ACF54	Bocaiuva/MG	*Eucalyptus*
Population 1	ACF60	Agudos/SP	*Eucalyptus*
Population 2	ACF70	Nova Adamantina/MS	*Eucalyptus*
Population 1	ACF61	Agudos/SP	*Eucalyptus*
Population 2	ACF71	Cuz das Almas/BA	*Eucalyptus*
Population 1	ACF24	Botucatu/SP	Anona
Population 2	ACF1	Votuporanga/SP	Mango
Population 1	ACF64	Ilha Solteira	*Hevea*
Population 1	ACF72	Valinhos/SP	*Ficus carica*
Population 1	ACF73	Valinhos/SP	*Ficus carica*
Population 2	ACF37	João Pinheiro/MG	*Eucalyptus*
Population 2	ACF44	Itatinga/SP	*Eucalyptus*
Population 2	ACF46	Itatinga/SP	*Eucalyptus*
Population 2	ACF47	Itatinga/SP	*Eucalyptus*
Population 2	ACF45	Itatinga/SP	*Eucalyptus*
Population 2	ACF48	Itatinga/SP	*Eucalyptus*
Population 2	ACF49	Itatinga/SP	*Eucalyptus*
Population 2	ACF75	Itatinga/SP	*Eucalyptus*
Population 2	ACF74	Atibaia/SP	*Ficus carica*
Population 2	ACF3	Santa Bárbara d'Oeste/SP	Mango
Population 2	ACF65	Tanhaçu/BA	Passion fruit
Population 2	ACF51	Cáceres/MT	Teak
Population 3	ACF5	Camacan/BA	Cocoa
Population 3	ACF6	Canavieiras/BA	Cocoa
Population 3	ACF9	Ilhéus/BA	Cocoa
Population 3	ACF10	Camacan/BA	Cocoa
Population 3	ACF11	Ilhéus/BA	Cocoa
Population 3	ACF14	Ilhéus/BA	Cocoa
Population 3	ACF16	Belém/PA	Cocoa
Population 3	ACF17	CEPEC/CEPLAC/BA	Cocoa
Population 3	ACF18	Itamari/BA	Cocoa
Population 3	ACF20	Nazaré/BA	Cocoa

* Isolates with lines of the same colors were identical alleles are therefore treated as one individual in the analysis

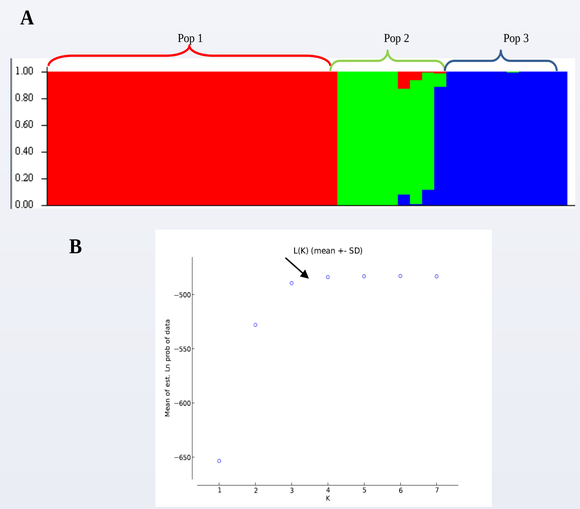

Figura 1: Affiliation of individual genotypes assessed using Structure 2.2 and separated into discrete vertical bars that are organized by sampling groups (**A**) and different K vs LnK values (**B**). Differences in colour within a vertical bar (A) indicate a multi-population affiliation of an individual genotype.

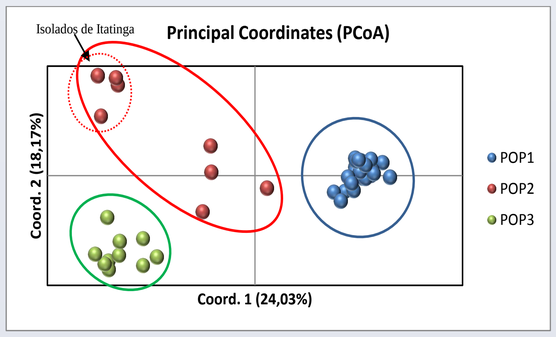

Figure 2: Principal analysis of molecular variance performed on GENALEX 6.1 components using the matrix of genetic divergence PhiPT. The first two axes explain 42.13% of the total variance.

Table 2: Gene diversities (H) and contingency chi-square tests for differences in allele frequencies for 10 SSR loci across clone corrected population.

Population	Gene diversities **(H)**				
	"locus"	GL	ChiSq	Prob	Signif
POP1	AF2	15	120,000	0,000	***
POP1	AF3	1	24,000	0,000	***
POP1	AF4	Monomorphic			
POP1	AF5	3	48,000	0,000	***
POP1	AF6	1	24,000	0,000	***
POP1	AF7	3	48,000	0,000	***
POP1	AF8	10	96,000	0,000	***
POP1	AF9	10	96,000	0,000	***
POP1	AF11	3	48,000	0,000	***
POP1	AF12	28	168,000	0,000	***
POP2	AF2	10	36,000	0,000	***
POP2	AF3	3	18,000	0,000	***
POP2	AF4	10	36,000	0,000	***
POP2	AF5	Monomorphic			
POP2	AF6	15	45,000	0,000	***
POP2	AF7	3	18,000	0,000	***
POP2	AF8	6	27,000	0,000	***
POP2	AF9	3	18,000	0,000	***
POP2	AF11	10	36,000	0,000	***
POP2	AF12	6	27,000	0,000	***
POP3	AF2	15	50,000	0,000	***
POP3	AF3	1	10,000	0,002	**
POP3	AF4	15	50,000	0,000	***
POP3	AF5	Monomorphic			
POP3	AF6	3	20,000	0,000	***
POP3	AF7	3	20,000	0,000	***
POP3	AF8	Monomorphic			
POP3	AF9	6	30,000	0,000	***
POP3	AF11	Monomorphic			
POP3	AF12	1	10,000	0,002	**

*Significant difference at alpha (P) ≤ 0.05, ** Significant difference at P ≤ 0.01, *** highly significant difference at P ≤ 0.001, NS = non-significant difference at P ≤ 0.05, df = degree of freedom = (Number of alleles – 1) * (Number of populations – 1).

Table 3: Genotypic diversity (G) and percentage of genotypic diversity (G *)

Population	G	G* (%)
All population	29,34	68,25
Pop. 1	16,33	77,77
Pop. 2	4,1	52,08
Pop. 3	10	100

Table 4: Observed IA and r-d values of each populations

Population	Ia	rd	p
All population	1,91	0,22	0,16
1	0,52	0,64	0,02
2	2,21	0,27	0,02
3	0,39	0,68	0,05

Table 5: Analysis of molecular variance of Brazilian Ceratocystis populations hierarchically partitioned.

Source of Variation	gl	SQ	MQ	Est. Var.	%
Among Pops	2	44,528	22,264	1,446	41%
Within Pops	44	91,727	2,085	2,085	59%
Total	46	136,255		3,530	100%

Conclusions

➢Ten SSR markers developed previously were used and were polymorphics. These markers produced a total of 76 alleles for the 49 *Ceratocystis* isolates used in this study.

➢Three distinct populations were identified.

➢Analysis of population differentiation show a Θ = 0.27 and p ≤ 0.04.

➢High values of genotypic diversity were obtained for the combined (68.25%) and separate populations (77.77%, 52.08% and 100%).

➢The combined population, the Association Index (Ia) and baD values (Rd) were 1.91 and 0.22, respectively, indicating a high rate of recombination among the isolates.

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