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Development and characterization of microsatellite loci for *Tabebuia cassinoides* (Bignoniaceae)

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ABSTRACT. *Tabebuia cassinoides* (Lam.) DC., popularly known as caxeta, is a tree species that belongs to the plant family Bignoniaceae. This species is endemic to the Brazilian Atlantic Forest and is widely exploited commercially. To date, little is known about its genetic structure, preventing the establishment of adequate management plans for this taxon. The objective of this study was to construct a microsatellite-enriched genomic library for *T. cassinoides* to

select polymorphic loci, and standardize polymerase chain reaction amplification conditions. Of the 15 loci examined, 5 were polymorphic. The number of alleles per locus ranged from 2 to 8, with a mean of 4.4. The microsatellite loci described here represent the basis for detailed population genetic studies of this species, which will greatly contribute for the development of better conservation strategies for this taxon.

Key words: Brazilian Atlantic Forest; Microsatellite primers; Bignoniaceae; *Tabebuia cassinoides*

INTRODUCTION

Tabebuia cassinoides (Lam.) DC. is a tree from the swampy forests of the Atlantic Rain Forest of eastern Brazil, which occurs from the State of Paraná to the northern portions of the State of Espírito Santo (Gentry, 1992). This species is popularly known as caxeta because of its durable wood that is often used to produce boxes ("caixas" in Portuguese). Despite its economic importance, little is known about the genetic structure of *T. cassinoides* along the Atlantic Rainforest of Brazil, preventing the establishment of adequate management plans for this taxon.

Plant population genetic studies have increased substantially in the past years (Collevatti et al., 2012; Diniz-Filho et al., 2012; Pinheiro et al., 2010, 2011, 2013). Yet, the development of molecular markers with adequate levels of variation to investigate the genetic structure within and among populations remains one of the greatest limitations of these types of studies. More recently, special attention has been devoted to microsatellite loci (Sunnucks, 2000), which are thought to be selectively neutral (Schlötterer, 1998) and to present high levels of polymorphism and variation. The objective of this study was to develop a set of polymorphic microsatellite markers for *T. cassinoides* to be used as the basis to describe the genetic structure of the various populations of this threatened species.

MATERIAL AND METHODS

Total DNA was extracted from silica-dried leaves of *T. cassinoides* using the DNA extraction kit Invisorb spin plant mini kit (Invitek-Berlin, Berlin, Germany) and following the manufacturer protocol with minor modifications. Marker isolation involved the construction of a genomic library that was partially enriched for $(CT)_n$ and $(GT)_n$ repeats through the use of biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles (Kijas et al., 1994) and the modifications suggested by Billotte et al. (1999). DNA fragments enriched with microsatellites were combined with the vector pGEM-T Easy (Promega) as described by the supplier, and the product was used to transform competent cells of *Escherichia coli* XL1-Blue. Ninety-six recombinant colonies were obtained and sequenced by Macrogen (South Korea).

Geneious Pro 5.3.5 (Drummond et al., 2011) was used to edit forward and reverse sequences and to locate the regions containing microsatellites with the Phobos plugin (Mayer, 2010). Primers were designed using the Primer3 software (Rozen and Skaletsky, 2000). Among the clones containing microsatellite regions, 19 allowed primer design. Forward primers were synthesized with a 19-bp long, 5' M13 tail (5'-CACGACGTTGTAAAACGAC-3') by the

method of Schuelke (2000).

To determine polymerase chain reaction (PCR) amplification conditions and general genetic parameters, we analyzed 67 individuals from two Brazilian populations: Linhares (36 samples) and Iguape (31 samples). Total DNA was extracted following the procedures described above. PCRs were performed following the protocols described by Francisco et al. (2011) and using the following amplification conditions: 94°C for 3 min; 35 cycles of 94°C for 30 s, 60°C for 1 min or 54°C for 1 min (Table 1), and 72°C for 1 min; and 72°C for 10 min. PCR products were separated by electrophoresis in an Applied Biosystems 3730 Genetic Analyzer. GeneScan 500 LIZ (Applied Biosystems) was used as a size standard.

Table 1. Polymorphic microsatellite loci characterized for *Tabebuia cassionoides* and descriptive genetic levels of diversity for two populations (Iguape and Linhares).

Locus	Primer sequences	T(A)	Repeat	N_A	Size (bp)	Iguape		Linhares		GenBank
						H_O	H_E	H_O	H_E	
Tcass B5	(F) ACAAGTCTTAACCTTTTCAGATGC (R) ACAGGCACCTCAGATTTAGAAC	54	$(CT)_{15}CCCT(CT)_4$	8	238-254	0.0869	0.4917*	0.6666	0.6685	JQ905595
Tcass B11	(F) GCCAGCAGGACGGTTGTGAT (R)TGGCGTCATCCCGCTCTATG	54	$(AG)_2AC(AG)_8$	5	212-234	0.3333	0.4028	0.7272	0.4806*	JQ905596
Tcass E2	(F) TGGAGTTGGGATTTCCCCGTTGA (R) ACAACGACACTCTGGTGAATCTTGC	60	$TTTC(T)_6(TC)_2$ $(TTC)_4(TC)_8(T)_4TC$	3	265-278	0.57143	0.5785	0.1176	0.1123	JQ905597
Tcass F9	(F) ACCCAAAGCTCCCAAAGCAGCA (R) CAGGTTCGTCGCTTGGCTATGGA	54	$(GA)_{11}$	2	222-226	0.45833	0.4388	Mono	Mono	JQ905598
Tcass H3	(F) GAGAATCCTGAGGCGATAACATGAGGT (R) ACATGCAAATGGTGGCCTTAATCAGA	54	$(CATA)_2CAT(GA)_{12}$	4	193-207	0.0434	0.2637*	0.5128	0.4315	JQ905599

T(A) = optimal annealing temperature in ^{0}C; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; Mono = monomorphic locus. *P < 0.01.

GeneMarker 1.85 (SoftGenetics) was used to automatically genotype individuals. The genotypes were verified by visual inspection. Genetic diversity indices were calculated by GenAlEx 6.4 (Peakall and Smouse, 2006), and tests to determine the deviation from Hardy-Weinberg equilibrium were implemented in ARLEQUIN 3.5 (Excoffier and Lischer, 2010). Linkage disequilibrium was calculated in GENEPOP 4.1 (Rousset, 2008).

The cross-amplification of 21 pairs of primers that were developed for *Tabebuia aurea* (Silva Manso) Benth. & Hook.f. ex S. Moore (Braga et al., 2007) was tested in *T. cassinoides* using the same amplification protocol described above.

RESULTS AND DISCUSSION

Fifteen of 19 primer pairs amplified their respective loci successfully. Out of these 15 loci, five were polymorphic (Table 1). The number of alleles per locus ranged from two to eight. Expected and observed heterozygosities ranged from 0.1123 to 0.6685 (mean = 0.4298) and 0.0434 to 0.7272 (mean = 0.3908), respectively. No polymorphisms were encountered at locus Tcass F9 in the population from Linhares (Espírito Santo). Three loci, namely, Tcass B5 and Tcass H3 from Iguape (São Paulo) and Tcass B11 from Linhares (Espírito Santo), showed significant departure from the Hardy-Weinberg equilibrium (P < 0.001), because of the heterozygote deficiency in the population from Iguape and heterozygote excess in the population from Linhares (Table 1). The natural subdivision of populations of *T. cassinoides* may be the

cause of the disequilibrium that was observed in these loci. No linkage disequilibrium between any pair of loci was detected. The cross-amplification in *T. cassinoides* using primers that were designed for *T. aurea* resulted in seven amplifications for the 21 primer pairs, of which three were polymorphic (14.29%) (Table 2).

Table 2. Cross amplification test in *Tabebuia cassinoides* with primers of *Tabebuia aurea*.

Locus	Reference	Status amplification	Result genotyping
Tau 07	Braga et al., 2007	--	--
Tau 08	Braga et al., 2007	--	--
Tau 09	Braga et al., 2007	--	--
Tau 10	Braga et al., 2007	--	--
Tau 13	Braga et al., 2007	+	--
Tau 14	Braga et al., 2007	+	Polymorphic
Tau 15	Braga et al., 2007	+	Polymorphic
Tau 16	Braga et al., 2007	--	--
Tau 17	Braga et al., 2007	+	Monomorphic
Tau 18	Braga et al., 2007	--	--
Tau 19	Braga et al., 2007	--	--
Tau 20	Braga et al., 2007	--	--
Tau 21	Braga et al., 2007	+	Polymorphic
Tau 22	Braga et al., 2007	+	--
Tau 23	Braga et al., 2007	--	--
Tau 24	Braga et al., 2007	--	--
Tau 27	Braga et al., 2007	+	--
Tau 28	Braga et al., 2007	--	--
Tau 30	Braga et al., 2007	--	--
Tau 31	Braga et al., 2007	--	--

Successful amplifications (+) and failed amplifications or genotypings (--) are indicated.

The microsatellite loci of *T. cassinoides* that were described here represent the basis of further studies on the population genetics and conservation of this taxon (Pretti VQ, Pinheiro F, Meyer D and Lohmann LG, unpublished results). Given the economic importance of *T. cassinoides*, genetic studies of this taxon are especially needed so that adequate management plans can be established.

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