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Development and characterization of microsatellite loci for *Campomanesia xanthocarpa* (Myrtaceae) and cross amplification in related species

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ABSTRACT. *Campomanesia xanthocarpa* is a native tree, of common occurrence in almost all Brazilian Forest formations, which has its fruits and timber with high commercial value. Using an enriched genomic library we isolated and characterized microsatellite loci for *C. xanthocarpa* (Myrtaceae), in order to estimate genetic diversity parameters for this and related species. Twenty-eight microsatellite loci were identified and ten of them successfully amplified and showed polymorphism in a sample of 96 individuals, from four natural populations. The number of alleles per locus ranged from two to eight, and the observed and expected heterozygosities varied from 0.042 to 1.000 and from 0.294 to 0.855, respectively. These markers were tested and validated in two related species (*C. eugenioides* and *C. guazumifolia*). The microsatellite markers will be used in further studies of population genetics of *C. xanthocarpa*, in order to understand the genetic variability and to define the strategies needed for the conservation of the species.

Keywords: gabiroba, genetic diversity, molecular marker, native tree, simple sequence repeat (SSR).

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Introduction

Campomanesia xanthocarpa O. Berg (Myrtaceae) is a tree species commonly known as Gabirobeira or just Gabiroba, which can reach from 10 to 20 m high with an elongated and dense top (Lorenzi, 2002). This species occurs naturally in Brazil in almost all forest formation and it can also be found in Uruguay, Argentina and Paraguay (Biavatti et al., 2004).

Campomanesia xanthocarpa produces a large quantity of seeds that are widely disseminated by the avifauna. The species has an important role in environmental preservation, mainly for the restoration of riparian vegetation and for the recovery of degraded areas. The fruits of *C. xanthocarpa* have great economic value, either as consumed 'fresh' or in the preparation of candies, sorbets and homemade liqueurs (Lorenzi, 2002). Its wood is used in the production of musical instruments, agriculture, firewood, coal, fence and plank. The species presents medicinal value in the fight against dysentery, fever, scurvy, and urinary tract diseases (Cravo, 1994; Alice, Siqueira, Mentz, Silva, & José, 1995).

Due to the selective logging, *C. xanthocarpa* is undergoing severe loss of genetic variability that can lead to the extinction of many natural populations, thus, it is necessary to know the genetic structure of the species in order to maintain their genetic variability (Chaves et al., 2015).

The simple sequence repeat (SSR) marker is a powerful technique for characterizing allelic diversity and it is frequently used in population studies due to their high level of polymorphism and codominant inheritance (Ellegren, 2004; Deng, Wang, Zhu, Wen, & Yang, 2015). The purpose of our study was to develop and characterize microsatellite loci for *C. xanthocarpa* for further application in population genetic studies and to boost strategies for conserving the genetic resources of this species and to assess cross-amplification in two related species.

Material and methods

Genomic DNA from *C. xanthocarpa* and related species was extracted using the Doyle and Doyle (1987) protocol, with some modification in which CTAB (Cationic hexadecyl trimethyl ammonium bromide) was replaced by MATAB (Mixed Alkyltrimethylammonium Bromide). A microsatellite-enriched library was developed for *C. xanthocarpa* using the hybridization capture method (Billotte, Lagoda, Risterucci, & Baurens, 1999), with biotin-labeled $(CT)_8$, $(GT)_8$ and $(AGA)_5$ probes in the enrichment step. Genomic DNA (5 *µ*g) from *C. xanthocarpa* was digested with *Rsa*I (Promega, Madison, USA) and blunt-ended fragments were linked to adapters *Rsa*-21 and *Rsa*-25 (Edwards, Barker, Daly, Jones, & Karp, 1996). Fragments containing repeats were selected by hybridization with the biotinylated oligonucleotides and recovered by streptavidin-coated magnetic beads (Invitrogen-Dynal, Lillestrøm, Norway). Microsatellite-rich fragments were amplified by PCR with the *Rsa* 21 adapter, cloned into the pGEM-T Easy vector II (Promega, Madison, WI, USA) and transformed into *Escherichia coli* XL1Blue MRF supercompetent cells (Agilent Technologies, Stratagene Products Division, La Jolla, CA, USA). Plasmids from 192 individual colonies were isolated, and the inserts were sequenced using the sequencing kit BigDye terminator version 3.1 (Applied Biosystems, Foster City, CA, USA) and the automated sequencer 3500 x L Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence fragments were screened for microsatellites using the Gramene program markers database (Ware et al., 2002). Fragments containing microsatellite with dinucleotides or trinucleotides repeated more than four times and surrounded by a flanking region suitable for primer design were chosen for further study. Although the $(CT)_8$, $(GT)_8$ and $(AGA)_5$ probes were used for pre-cloning enrichment, other repeat motifs were also found in the cloned products (Table 1). Primer pairs complementary to sequences flanking the repeat elements were designed using the PRIMER3 version 0.4.0 (Rozen & Skaletsky, 2000). A total of 28 primer pairs were synthesized and tested for amplification, 10 primers showed clear and consistent amplification patterns.

Table 1. Characteristics of 10 microsatellite loci developed in *Campomanesia xanthocarpa*.

Locus	Primer sequences (5'– 3')	Repeat motif	Size range (bp)	*T*a (ºC)	PIC	GenBank accession no.
CXAN1	F: TCATCACAATCGGTTCATCG R:GGTTCAGTGTCACACCAAGTG	$(GC)_3(GA)_2(GT)_2(CT)_2$	121-330	52ºC	0.851	MH230138
CXAN2	F: CTCGGTTAGATGCGATGGAT R: GCTTCTTCATGGGACAATGG	$(CAT)_5$	114-130	63ºC	0.679	MH230139
CXAN5	F:GGGAGGAGAACTGGAGAAGG R: CAAGCTTCGATCAATTC	$(AAG)_5$	257-279	61.3ºC	0.492	MH230140
CXAN6	F: AAAGCATTGGAGGATGATGC R: TGTTGCAGTTTCCAGTTTGC	$(CA)_3TGTTGC(CA)_3$	127-147	63ºC	0.697	MH230141
CXAN10	F: GTTGGGTTTGCTAGGGACAA R: TGAAAACAATTCCTCGCACA	$(GA)_4A(GA)$	124-139	53.8ºC	0.537	MH230142
CXAN13	F: TTCACGCGTCCTTCACATAG R:GAAAGCCATATCCTCCTCCAC	$(AAAT)_4$	121-139	63ºC	0.716	MH230143
CXAN16	F: CCAATGACGTCCAACTCAGA R: CGATTGGGACAAGAAAGGTG	$(CAC)_3n(TC)_3$	154-173	58.6ºC	0.493	MH230144
CXAN18	F: TTGGCAGTGAGTTGCATAGG R: ATCCACATCTTCCGATGACC	$TG)_3GAT(TA)_3$	121-160	52ºC	0.693	MH230145
CXAN27	F: GAAAGGGCACTTGCTTATGG R: GTCAGATTTGGCCGATGGT	$(CCA)_5$	124-133	53.8ºC	0.531	MH230146
CXAN28	F: TGCGATGTTGTCCTTTAGGG R: GGTTTGCTAGGGACAACCAA	$(TC)_4$	129-156	53.8ºC	0.695	MH230147

Note: *T*a = annealing temperature (ºC); PIC = polymorphic information content;

PCR amplification and the efficiency of each primer pair were tested in a sample of 96 individuals from four populations of *C. xanthocarpa* (24 individual per population). Three collection sites were from Paraná state, including Fazenda Doralice (23º16'00.0"S and 51º03'00.0"W; Voucher FUEL 17164), Parque Estadual Mata São Francisco (23º27'00.0"S and 51º15'00.0"W; Voucher FUEL 55710) and RPPN Fazenda Duas Barras (23°0'2"S and 52°54′50"W; Voucher UPCB 18338); and the last one was from São Paulo state, Horto Florestal de Palmital (22º48´00.0"S and 50º16´00.0" W; Voucher FUEL 55282). Cross-amplification was tested in other two species of *Campomanesia*; *C. eugenioides* (24° 1'12.23"S and 50°53'48.77"W; Voucher

FUEL 42813) and *C. guazumifolia* (23°19'43.37"S and 51°12'10.86"W; Voucher FUEL 55400) using 10 plants from each species.

PCR reactions were performed using a 10 µL volumes containing 4.5 µL GoTaq Green Master Mix (2 × buffer, pH 8,5, 1600 µM dNTP e MgCl 3mM; Promega, Madison, WI, USA), 0.08 µL of M13-tailed (5'-TGTAAAACGACGGCCAGT-3') forward primer (5pM), 0.32 µL of reverse primers (5pM) and 0.32 µL of 5µM solution of M13-labelled (6-FAM, HEX, NED or PET) primers, 2 µL (10 ng) of genomic DNA and 2.78 µL of nuclease free water. Primer amplification was performed on a PTC200 (MJ Research, California, USA) under the cycling conditions: denaturation at 95°C for 3 min.; 35 cycles of 94°C for 40 s, specific annealing temperature (Table 1) for 40 s, 72°C for 40 s, 7 cycles at 94°C for 30 s, 53°C 45 s, 72°C for 45 s and final extension at 60°C for 40 min. PCR products were purified using ExoProStar 1 step purification kit (GE Healthcare Life Science, NY. USA) and run in the ABI 3500-xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Genetic analysis of the fragments was carried out using the GeneMarker® v.2.3 software (Soft Genetics, State College, PA, USA) with manual correction to reduce the chance of increasing the occurrence of null alleles. For characterization of the polymorphic loci, it was applied the standard population genetic statistics, such as number of alleles (K), observed (*Ho*) and expected (*He*) heterozygosities, polymorphic information content (PIC), calculated by Cervus v. 2.0 software (Marshall, Slate, Kruuk, & Pemberton, 1998). The exact test for linkage disequilibrium (Lewontin, 1964) ($p < 0.05$) was assessed using the software Arlequim version 3.5 (Excoffier & Lischer, 2010) and the presence of null alleles was tested using the software Micro-Checker (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004).

Results and discussion

The sequences of the 10 microsatellite loci were submitted to Genbank and registered under the accession numbers cited in Table 1. The genotyping of 96 individuals of *C. xanthocarpa* identified a total of 61 alleles, with the sizes of the fragments varying from 114 (Cxan2) to 330 bp (Cxan1) (Table 2). The polymorphic information content varied from 0.492 (Cxan5) to 0.851 (Cxan1). According to Botstein, White, Skolnick, and Davis (1980), the level of polymorphism at a specific loci can be considered high when the PIC > 0.5 (Table 1), therefore, most of the analyzed loci had high polymorphic information content. The number of alleles per locus ranged from two to eight (Table 2), and the mean of alleles per locus across all populations was 3.95. Means of observed (Ho) and expected (He) heterozygosities were, respectively, 0.274 and 0.528 (Fazenda Doralice), 0.262 and 0.528 (Parque Estadual Mata São Francisco), 0.236 and 0.613 (Horto Florestal de Palmital), 0.329 and 0.590 (RPPN Fazenda Duas Barras) (Table 2).

Table 2. Genetic parameters of the 10 microsatellites loci developed *Campomanesia xanthocarpa.*

Locus	Fazenda Doralice (n = 22)			Parque Estadual Mata São Francisco (n = 24)			Horto Florestal de Palmital (n = 26)			RPPN Fazenda Duas Barras (n = 24)		
	A	*Ho*	*He*	*A*	*Ho*	*He*	*A*	*Ho*	*He*	*A*	*Ho*	*He*
Cxan1	5	0.909	0.796	6	0.958	0.796	5	0.923	0.756	5	1.000	0.708
Cxan2	3	0.000	0.385	3	0.000	0.465	6	0.000	0.627	5	0.000	0.582
Cxan5	3	0.333	0.446	3	0.304	0.517	2	0.250	0.481	3	0.773	0.591
Cxan6	3	0.227	0.475	3	0.250	0.503	7	0.346	0.855	7	0.708	0.790
Cxan10	2	0.000	0.474	3	0.000	0.543	5	0.000	0.670	4	0.000	0.684
Cxan13	3	0.045	0.551	4	0.042	0.653	6	0.000	0.673	4	0.000	0.635
Cxan16	2	0.333	0.303	3	0.238	0.556	2	0.150	0.450	4	0.063	0.542
Cxan18	5	0.762	0.639	4	0.792	0.733	4	0.577	0.577	3	0.750	0.550
Cxan27	2	0.000	0.512	2	0.000	0.454	3	0.000	0.486	3	0.000	0.294
Cxan28	8	0.136	0.701	5	0.042	0.648	5	0.115	0.556	3	0.000	0.528

Note: *A* = number of alleles sampled; *Ho* = observed heterozygosity; *He* = expected heterozygosity; *n* = number of individuals sampled.

Pairwise comparisons for multiple tests among the polymorphic loci showed significant linkage disequilibrium between loci (Table 3), while 8 loci showed significant evidence for the presence of null alleles (Cxan2, Cxan5, Cxan6, Cxan10,Cxan13, Cxan16, Cxan27, Cxan28) according to Bonferroni correction ($p \leqslant 0.05$), which can indicate homozygosis excess.

The 10 loci were tested for cross-amplification using annealing temperature gradients in two species of the genus *Campomanesia*: *C. guazumifolia* and *C. eugenioides* (Table 4). The primers Cxan 2, Cxan13 and Cxan27 showed inconsistent amplification for all species tested, while primers Cxan1, Cxan5, Cxan6 and

Cxan18 amplified for all species. Loci Cxan10 and Cxan16 showed amplification only for *C. guazumifolia* and Cxan28 only for *C. eugenioides*.

Table 3. Significant linkage disequilibrium between 39 pairs of combinations pairs for ten microsatellite loci of *Campomanesia xanthocarpa*.

Locus	Cxan 1	Cxan 2	Cxan 5	Cxan 6	Cxan 10	Cxan 13	Cxan 16	Cxan 18	Cxa n27	Cxan 28
Cxan1										
Cxan2	+									
Cxan5	+	+								
Cxan6	+	+	+							
Cxan10	+	+	-	+						
Cxan13	+	+	-	+	+					
Cxan16	+	+	+	-	+	+				
Cxan18	+	+	-	+	-	+	+			
Cxan27	+	+	-	+	+	+	+	+		
Cxan28	+	+	+	+	+	+	+	+	+	

$p \leqslant 0.05$

The patterns of genetic polymorphism observed in *C. xanthocarpa* for 10 microsatellite loci suggest that these markers can be used as valuable sources of information for the actual level of genetic conservation of *C. xanthocarpa* in their distribution area. A similar study was carried out by Ruas et al. (2009) and the microsatellite loci developed by those authors were employed by Conson et al. (2013), to verify if the forest fragmentation that began last century, in South and Southeast Brazil affected the genetic variability of the tree species *Luehea divaricata*. Chaves et al. (2016) used microsatellite loci, developed by Ramos et al. (2011), to demonstrate the apomictic and sexual reproduction, polen and seed dispersion of the tree species *Aspidosperma polyneuron*.

We also tested the developed markers for cross-species amplification, with a success in the transferability rate of five and six loci for *C. eugenioides* and *C. guazumifolia*, respectively (Table 4).

Table 4. Cross-amplification of 10 microsatellite loci tested in two species of *Campomanesia*.

Locus	*C. eugenioides* (Cambess.) D. Legrand	*C. guazumifolia* (Cambess) O.Berg
Cxan1	+	+
Cxan2	-	-
Cxan5	+	+
Cxan6	+	+
Cxan10	-	+
Cxan13	-	-
Cxan16	-	+
Cxan18	+	+
Cxan27	-	-
Cxan28	+	-

The transferability of polymorphic microsatellite markers in plants is likely to be successful, mainly within genera (success rate close to 60% in eudicots and close to 40% in the monocots). Between genera, transfer rates are approximately 10% for eudicots, and researchers of monocots, such as orchids or grasses, are very unlikely to get away without isolating novel markers from the genomes of new target genera. An exception is the large adaptive radiations with low levels of DNA sequence divergence such as Bromeliaceae, where polymorphic markers are readily transferred between species of the same subfamily and beyond (Barbará et al., 2007).

Comparing our results employing the 10 microsatellite loci developed and validated for *C. xanthocarpa* with similar work cited in literature we observe the high potential of these markers, to estimate parameters of genetic diversity and population structure in further studies for this species, as well, for other related species tested for cross-amplification. Also, the produced data will be valuable in conservation and management programs aiming the recovering of degraded areas.

Conclusion

In this study, we developed 28 microsatellite markers for the tree species *C. xanthocarpa*, 10 of which were polymorphic. Tested for cross-amplification, five and six loci successfully amplified in the related

species *C. eugenioides* and *C. guazumifolia*, respectively. The development of novel microsatellite markers will be an important tool to understand the consequences of selective logging that have affected the genetic diversity of *C. xanthocarpa* and other related species present in the same area.

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