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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE LOCI IN *BYRSONIMA CYDONIIFOLIA* (MALPIGHIACEAE) AND CROSS-AMPLIFICATION IN *B. CRASSIFOLIA*[1]

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- *Premise of the study:* Microsatellite markers were developed and characterized in *Byrsonima cydoniifolia* (Malpighiaceae) to allow further investigation of genetic variation in natural populations. Cross-amplification was tested in the related species *B. crassifolia*.
- *Methods and Results:* Seventeen microsatellite markers were isolated by a microsatellite-enriched library protocol. Fourteen polymorphic and three monomorphic loci were identified in *B. cydoniifolia*. The mean number of alleles in the three populations were 6.5, 6.5, and 8.2, ranging from three to 17 for different loci and populations. Mean observed and expected heterozygosities were 0.706 and 0.727, respectively. The fixation index was close to zero for all but two loci. Nine microsatellite loci were successfully cross-amplified in *B. crassifolia*.
- *Conclusions*: This new set of microsatellite markers will be a useful tool for genetic studies of *B. cydoniifolia*, supporting strategies for maintaining the genetic diversity of this species and possibly that of many related species.

Key words: *Byrsonima crassifolia*; *Byrsonima cydoniifolia*; genetic variability; Malpighiaceae; murici; simple sequence repeat (SSR).

Byrsonima cydoniifolia A. Juss. (Malpighiaceae) is a species of fruit tree known as "murici" of the genus *Byrsonima* Rich. ex Kunth, which includes more than 150 species widely distributed in Central and South America (Aguiar et al., 2005). In Brazil, this tree is found in the sandy soils of the cerrado biome, mainly occurring in the floodplains of central Brazil (Pott and Pott, 1994). Species of the genus *Byrsonima* have a mixed mating system that includes pollination by bees and seed dispersal by animals. In various regions of Brazil, several species of this genus are commonly used as a source of raw material for food industries or are consumed directly by humans. The wood is harvested, and the leaves are used in traditional medicine to treat fever, ulcers, and skin infections or as anti-asthmatics (Garritano et al., 2006).

Despite its potential economic, medicinal, and ecological importance, there is a shortage of population genetic studies on *B. cydoniifolia*. Microsatellite markers can provide fine-scale information useful for understanding mating systems, population genetic structure, dispersal, and gene flow. However, such markers are available for only a few species in the genus (Croft and Schaal, 2012). Thus, in this paper, we describe the first development and characterization of microsatellite loci in *B. cydoniifolia*, allowing further studies of genetic diversity and population genetic structure in natural populations of this species. Cross-amplification in a related species (*B. crassifolia* (L.) Kunth) was successfully performed, suggesting that the newly characterized microsatellite markers might also be useful for genetic studies in other related *Byrsonima* species.

METHODS AND RESULTS

Samples from 90 individuals of *B. cydoniifolia* from three localities were analyzed, including 24 individuals from Bom Jardim, Goiás (16°16′41.6″S, 52°02′23.5″W), 30 from Barra do Garças, Mato Grosso (15°30′20.6″S, 52°16′50.3″W), and 36 from Araguaiana, Mato Grosso (14°41′48.1″S, 51°44′19″W).

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Vouchers (CNMT2476, CNMT983, CNMT2465) were deposited at the herbarium of the Universidade Federal de Mato Grosso (CNMT). Cross-amplification was tested for 24 individuals from one population of *B. crassifolia* from Silvânia, Goiás (16°43′6.8″S, 48°13′12.9″W; voucher no. CNMT2479).

A microsatellite-enriched library was constructed for *B. cydoniifolia* using protocols adapted from Billote et al. (1999). Genomic DNA was extracted from leaf tissue of a single adult *B. cydoniifolia* tree using the cetyltrimethylammonium bromide (CTAB) 2% protocol (Doyle and Doyle, 1987). Approximately 250 ng of genomic DNA was completely digested with the restriction enzyme *Afa*I (10 U/μL) (Invitrogen, Carlsbad, California, USA). The fragments were then ligated with the adapters *Rsa*21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and *Rsa*25 (5′-TAGTCCACGCGTAAGCAAGAGCACA-3′) using T4 DNA ligase. To amplify the amount of ligated fragments, 5 μL of the ligation products were amplified with *Rsa*21 (10 μM) in a 50-μL reaction. The PCR conditions consisted of an initial step of 4 min at 95°C; followed by 20 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C; with a final extension at 72°C for 8 min.

The amplified DNA fragments (200–1200 bp in size) containing microsatellites were enriched for repeats by hybridization with a pool of 5′-biotinylated oligonucleotide probes [$(CT)_8$, $(GT)_8$] and captured by streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). Enriched fragments were amplified by PCR using *Rsa*21 (10 μM) as the primer. The PCR products were ligated to a pGEM-T Easy Vector (Promega Corporation), and plasmid DNA was transformed into *Escherichia coli* XL1-Blue Competent Cells (Stratagene, La Jolla, California, USA). A total of 60 positive clones were isolated using the β-galactosidase gene and sequenced in an ABI3500 automated sequencer (Applied Biosystems, Carlsbad, California, USA) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Screening for microsatellites was performed using WebSat software (Martins et al., 2009). Sequences of hybrid clones, duplicates, and those with short flanking sequences were discarded. Twenty-two (37%) of the sequenced clones contained microsatellites with at least seven uninterrupted repeats; 17 of these sequences were suitable for designing locus-specific primers with Primer3 (Rozen and Skaletsky, 2000). The parameters used for microsatellite primer design were (1) a maximum of 3°C difference in melting temperature between the primers; (2) a GC content ranging from 40% to 60%; and (3) a PCR product size ranging from 150 to 200 bp.

The 17 primer pairs were used for the identification of polymorphic loci using a test panel of three *B. cydoniifolia* individuals selected randomly from three populations. Polymorphisms were evaluated in 6% denaturing polyacrylamide gels stained with silver nitrate (Creste et al., 2001) and sized by comparison to a 10-bp DNA ladder standard (Invitrogen). Each of the 17 primer pairs tested successfully amplified a microsatellite region; of these, 14 revealed polymorphic loci, whereas three were monomorphic. To characterize the microsatellite polymorphisms, the forward primers of each pair were labeled with one of three fluorescent dyes (5′ HEX, 5′ NED, or 5′6-FAM) (Table 1). Amplifications were performed in a final volume of 10 μL using 3.75 ng of template DNA and 0.23 mM primers (forward + reverse), 0.23 μM dNTPs, 3.25 mg of bovine serum albumin (25 mg/mL), 1× reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), and 0.75 unit of *Taq* DNA polymerase (5U; Phoneutria, Belo Horizonte, Minas Gerais, Brazil) under the following conditions: 94°C for 5 min (one cycle); 35 cycles of 94°C for 1 min, 46–62°C (depending on primers [Table 1]) for 1 min, and 72°C for 1 min; and 72°C for 45 min (one cycle). The lengths of the amplification products were determined using an

Table 1. Characteristics of 17 microsatellite loci developed in *Byrsonima cydoniifolia.*

Locus	Primer sequence (5′–3′)[a]	Repeat motif	Allele size range (bp)[b]	T_a (°C)	GenBank accession no.
BCY01	F: HEX-AAGTGAGCTAACCTCGGAAGG R: TGCGGAACTTTGACCTATCA	$(TG)_{10}$	160–192	60	KJ001649
BCY02[c]	F: HEX-CATGGAGAAGACATCCACTCTG R: GTGGACGACAGAATGCTTCA	$(AG)_8(GA)_{20}$	149–175	60	KJ001650
BCY03	F: 6-FAM-AAGGGTAGAAGGAGGGCTTG R: ACATCCCATGTTCCCGATT	$(AGGG)_4(TG)_7$	140–160	48	KJ001651
BCY04	F: TGGCTCTGATACCACATGTAAA R: CAGCACAAATTCTGGTTGGA	$(AAG)_4$	176[d]	52	KJ001652
BCY05[c]	F: NED-ATGGATGCTGCCTTACAGGA R: TTATTTTCCAGGCCGTGTTC	$(CA)_9$	177–215	58	KJ001653
BCY06[c]	F: NED-TTGATTGTTGATGCTTAAATGCT R: ACTCCTGTCATGCAAAGACG	$(TG)_8$	169–177	62	KJ001654
BCY07[c]	F: HEX-AGAGGCAAATGGCATTCAAC R: TTTCAATAACCGGACTTTCCTG	$(CA)_7$	180–200	60	KJ001655
BCY08[c]	F: NED-CGTAAACTTGCAAGAAACTGAAA R: TTGACTCGATATACGTCTTCCAC	$(AG)_{14}$	151–167	60	KJ001656
BCY09[c]	F: 6-FAM-TCGCATATCCTTCACAGAGAGA R: CAACGGTCCTTGCAAACTCA	$(AG)_{19}$	175–221	58	KJ001657
BCY10[c]	F: NED-AAAGCACACAGTCCTCACGTT R: TTTGCTCATTGCAAAACACC	$(CA)_{12}$	169–189	59	KJ001658
BCY11	F: 6-FAM-GAATTACAAAATCTAGATCCAAAGTCG R: GCGCAACCCTTAAAAATGTG	$(TG)_7T(AT)_8$	135–189	58	KJ001659
BCY12[c]	F: NED-CGGATATTCTTTTGCTGGGTA R: TGGAAATATTGTCTGCATTTGTG	$(GT)_8$	196–202	62	KJ001660
BCY13	F: 6-FAM-TTTTGCTAGGCCGTCTACCT R: GCCAACGAGTCCACTTTCTC	$(AC)_9$	180–190	46	KJ001661
BCY14[c]	F: HEX-TCGAAGAAAGTCACGGAAGG R: TGCAATATCCCCATGATGTT	$(TC)_{12}$	170–200	62	KJ001662
BCY15	F: CAGCGTGATTGGAAACATTG R: GCAACACACCAGCCACATAC	$(TG)_8$	152[d]	54	KJ001663
BCY16	F: NED-TGGATTTGACATGAAGTGTGC R: TGGGTTTGAGCCTAGAATTGA	$(CT)_{15}$	150–175	64	KJ001664
BCY17	F: GGTGCGTGGATGAATGAGTT R: CCCCATACAAATCCCCTTTC	$(TG)_9$	166[d]	48	KJ001665

Note: T_a = annealing temperature.

[a] For each forward primer of polymorphic loci, the fluorescent label is indicated at the 5′ end.

[b] Fragment size range based on 90 individuals from three populations in Brazil.

[c] Primers that were amplified successfully in cross-amplification with *Byrsonima crassifolia.*

[d] Monomorphic loci not used to analyze genetic variability.

TABLE 2. Results of initial primer screening of 11 polymorphic microsatellite loci in three populations of *B. cydoniifolia* and nine polymorphic microsatellite loci in one population of *B. crassifolia*.[a]

	B. cydoniifolia												*B. crassifolia*			
	Bom Jardim (N = 24)				Barra do Garças (N = 30)				Araguaiana (N = 36)				Silvânia (N = 24)			
Locus	A	H_e	H_o	F_{IS}	A	H_e	H_o	F_{IS}[b]	A	H_e	H_o	F_{IS}[b]	A	H_e	H_o	F_{IS}
BCY01	7	0.638	0.583	0.088	6	0.772	0.600	0.226	13	0.894	0.472	0.475*	NT	NT	NT	NT
BCY02	8	0.830	0.833	−0.004	6	0.545	0.200	0.637*	7	0.612	0.389	0.367*	10	0.786	0.833	−0.061
BCY05	8	0.627	0.542	0.138	7	0.780	0.767	0.018	8	0.815	0.694	0.150	10	0.852	0.792	0.072
BCY06	4	0.630	0.750	−0.195	5	0.693	0.667	0.039	4	0.439	0.333	0.243	5	0.429	0.458	−0.070
BCY07	3	0.635	0.792	−0.254	2	0.440	0.500	−0.139	3	0.562	0.389	0.311	4	0.491	0.417	0.154
BCY08	4	0.578	0.500	0.138	3	0.430	0.433	−0.008	4	0.550	0.500	0.092	9	0.860	0.958	−0.117
BCY09	9	0.717	0.833	−0.166	10	0.828	0.800	0.035	17	0.906	0.917	−0.012	13	0.905	0.917	−0.013
BCY10	6	0.816	0.917	−0.127	8	0.806	0.800	0.007	8	0.750	0.778	−0.038	8	0.636	0.625	0.017
BCY11	12	0.902	0.750	0.172	15	0.903	0.900	0.004	17	0.923	0.861	0.068	NT	NT	NT	NT
BCY12	3	0.568	0.625	−0.102	3	0.555	0.600	−0.082	3	0.549	0.667	−0.218	2	0.223	0.250	−0.122
BCY14	7	0.820	0.875	−0.069	7	0.832	0.800	0.039	6	0.644	0.667	−0.036	7	0.718	0.875	−0.224
Average	6.455	0.706	0.727	−0.031	6.545	0.690	0.642	0.069	8.182	0.695	0.606	0.129	7.556	0.656	0.681	−0.039

Note: A = number of alleles; F_{IS} = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals; NT = microsatellite loci not transferred to *B. crassifolia*.

[a] Loci BCY03, BCY16, and BCY17 showed unclear amplification patterns on the automated sequencer, and thus were excluded from the genetic variability analysis.

[b] Statistically significant deviations from HWE ($P < 0.05$) are indicated with an asterisk (*).

ABI3500 automated sequencer and a GeneScan 500 ROX Size Standard (Applied Biosystems). Allele binning and calling was performed using GeneMapper 5.0 software (Applied Biosystems).

This screening detected that out of the 14 polymorphic loci that were consistently amplified (Table 1), only 11 were adequate and showed good standards on the automated sequencer, which ran three multiplex reactions. However, the genotyping patterns of two of these 11 polymorphic loci (BCY07 and BCY14) were more difficult to interpret, with nonspecific bands in the range of 180–190 bp and 195–205 bp, respectively.

The 11 polymorphic loci were used to estimate genetic variability parameters and the genetic variability among *B. cydoniifolia* individuals (Table 2). The presence of null alleles was analyzed using the program MICRO-CHECKER version 2.2 (van Oosterhout et al., 2004). Analyses of genetic variability, i.e., number of alleles per locus (A), observed (H_o) and expected heterozygosities (H_e), and fixation index (F_{IS}), were performed with Genetic Data Analysis (GDA) software (Lewis and Zaykin, 2000). Tests of Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium using the Bonferroni correction were performed with FSTAT 2.9.3.2 software (Goudet, 2002).

The total number of observed alleles per locus ranged from three to 17 in all loci and populations, with average number of loci equal to 6.5, 6.5, and 8.2 for each of the populations. Null allele analysis indicated that the heterozygote deficiencies are most likely in loci BCY01 and BCY02 for the Barra do Garças and Araguaiana populations, BCY07 for the Araguaiana population, and BCY11 for the Bom Jardim population. Average H_o across loci ranged from 0.606 to 0.727, and H_e ranged from 0.690 to 0.706. Significant deviation from HWE ($P < 0.05$) was observed for locus BCY01 in the Barra do Garças population and for locus BCY02 in the Barra do Garças and Araguaiana populations, with F_{IS} greater than zero (Table 2). No significant departures from linkage disequilibrium ($P > 0.05$) were detected for any pair of loci.

Out of the 17 loci tested, nine amplified successfully and were cross-amplified in *B. crassifolia*, using the same PCR conditions. All were polymorphic loci without null alleles and with no deviation from HWE ($P < 0.05$) (Table 2).

CONCLUSIONS

The new set of microsatellite loci described here will provide a powerful tool for studying genetic diversity, mating system parameters, gene flow, and the spatial genetic structure of *B. cydoniifolia*. This information can then be used to create effective strategies for conservation and the management of future germplasm banks. Furthermore, the success of cross-amplification in *B. crassifolia* suggests that this set of markers will also be useful for future population genetic studies in other species of *Byrsonima*.

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