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Source: Applications in Plant Sciences, 1(11)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1300055

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Applications in Plant Sciences 2013 1(11): 1300055

PRIMER NOTE

CHARACTERIZATION OF 10 MICROSATELLITE LOCI FOR *BATHYSA AUSTRALIS* (RUBIACEAE)[1]

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- *Premise of the study: Bathysa australis* is a common subcanopy tree from the Atlantic Forest that is pollinated by bees and wasps and produces autochoric seeds. This species exhibits great phenotypic plasticity along the elevational gradient of Serra do Mar in southeastern Brazil. We expect to assess the genetic diversity and gene flow between populations of this species along the elevational gradient.
- *Methods and Results:* We developed a microsatellite-enriched genomic library for *B. australis*, and 10 microsatellite loci were successfully amplified, ranging from one to 13 alleles per locus. The observed and expected heterozygosities ranged from 0.333 to 0.900 (average: 0.629) and 0.564 to 0.900 (average: 0.742), respectively.
- *Conclusions:* These are the first microsatellite markers developed for the genus *Bathysa* and may be useful in other species of the Condamineeae tribe. These primers will be an important tool for studies of population ecology and conservation genetics.

Key words: Atlantic Forest; *Bathysa australis*; conservation genetics; medicinal plant; polymorphism; population ecology.

Bathysa australis (A. St.-Hil.) Hook. f. ex K. Schum. (Rubiaceae) is a subcanopy tree that is widespread along the elevational gradient (100–1000 m a.s.l.) of the Atlantic Forest of Serra do Mar in São Paulo State, Brazil. This species is a common plant in parts of the Atlantic Forest (e.g., Ramos et al., 2011) and has an important role in ecosystem functioning, e.g., providing a nectar source to a variety of insects (Andrich, 2008). Furthermore, its bark is used in folk medicine (Germano-Filho, 1999), which indicates its social value in addition to its ecological value. *Bathysa australis* displays great phenotypic plasticity in leaf size and color along its elevational gradient, is pollinated mainly by bees and wasps (Andrich, 2008), and presents autochoric seed dispersal (Pedroni, 2001). Because we believe the elevational gradient might function as a barrier for *B. australis* gene flow, the investigation of the spatial distribution of the dispersal, pollination, and genetic diversity of this plant could generate important information regarding its population biology. Above all, the Brazilian Atlantic Forest is significantly threatened (Myers et al., 2000), and the microsatellites developed in this study should serve as tools to evaluate the impacts and define conservation strategies.

[1]Manuscript received 20 June 2013; revision accepted 22 July 2013.

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) through fellowships (CNPq 141590/2010-6, 140813/2008-0, and 308748/2010-7) and financial support (CNPq 474530/2010-8). The authors also thank Projeto Temático Gradiente Funcional (BIOTA/FAPESP 03/12595-7) for logistical support.

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doi:10.3732/apps.1300055

METHODS AND RESULTS

We extracted genomic DNA from leaf tissue samples using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). A microsatellite-enriched library was then developed following Billotte et al. (1999). The DNA samples were digested using the *Rsa*I restriction enzyme (Invitrogen, Carlsbad, California, USA) for 3 h at 37°C, and the resulting fragments were ligated to *Rsa*I adapters for 2 h at 20°C. The fragments containing microsatellites were selected by hybridization with $(CT)_8$- and $(GT)_8$-biotinylated oligonucleotides, followed by capture with Streptavidin MagneSphere Paramagnetic Particles (Promega Corporation, Fitchburg, Wisconsin, USA). The selected DNA fragments were PCR-amplified in a final volume of 100 µL containing 20 µL of selected fragments, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 4 pmol of primer Rsa21, and 2.5 U of *Taq* DNA polymerase. A PTC-100 thermal cycler (MJ Research, Waltham, Massachusetts, USA) was used with the following program: 95°C for 1 min, followed by 25 cycles of denaturation at 94°C for 40 s, 60°C for 1 min, extension of 72°C for 2 min, and a final extension of 72°C for 5 min. The amplification products were cloned into the pGEM-T Easy Vector (Promega Corporation). Plasmids were transformed into *Escherichia coli* XL1-Blue competent cells, and positive clones were selected using the β-galactosidase gene and grown overnight in an HM/FM medium with ampicillin. A total of 96 positive clones were bidirectionally sequenced using an automated ABI PRISM 377 sequencer (Applied Biosystems, Foster City, California, USA) with T7 and SP6 primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were assembled and edited using SeqMan Pro (DNAStar Inc., Madison, Wisconsin, USA). The repetitive regions were identified using the Simple Sequence Repeat Identification Tool (Temnykh et al., 2001), and 30 primer pairs were designed using WebSat (Martins et al., 2009). Ten primer pairs amplified microsatellite regions and were selected for screening (Table 1). The remaining loci were discarded due to amplification failures or nonspecific amplification patterns. The forward primer for each pair was labeled with fluorochromes (HEX and TET).

PCR amplifications were performed in a 15-µL volume containing 15 ng DNA, 1× PCR buffer, 0.15 mM each dNTP, 0.8 mM each primer, 0.04% bovine serum albumin (BSA), 1.5 mM $MgCl_2$, and 1 U *Taq* DNA polymerase. A PTC-100 thermal cycler (MJ Research) was used with the following program: 96°C for

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TABLE 1. Characteristics of 10 microsatellite markers developed for *Bathysa australis*.

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Allele size range (bp)	GenBank accession no.
BA02	$(CT)_8$	F: CTTGCCAAACTGAGCTTCTG R: GGTGATGGTGCTCCTCTTTC	62	150–180	KF267877
BA14	$(TC)_7$	F: CAGCAAAGTCCACAGCACA R: TGCGTGCACGTGTGAGT	62	140–200	KF267878
BA15	$(CA)_9$	F: TCCCATTTTCCTGGTCGT R: TGGCATCCAAGACTCTGCTA	55	270–300	KF267879
BA16	$(CA)_{11}$	F: TCACAGATCCTACAACAGCACC R: AGAAGGAGAACGCAAATACCC	55	190	KF267880
BA22	$(AG)_6$	F: CCACAGGTTTGTGTTTGTTCTC R: GTCCCATTCCTTTCATATTCCA	55	330–360	KF267881
BA24	$(GA)_{30}$	F: ACAGCGAAGCTCACACACAT R: TCTGTGGAAGAAGAGTGGGAAT	55	170–230	KF267882
BA25	$(AC)_{40}$	F: TGCCCAGTAAATAGGAGAGATTG R: TTATGCTGCTGGAATGGTATTG	55	150–180	KF267883
BA26	$(CT)_{25}$	F: AGGTGCATTGGAAAGGTATTGA R: GTTTGAGGCTTTGGACATACATC	65	360–400	KF267884
BA28	$(TG)_7$	F: AGGACTTCCATTTTGTTGGGTA R: GGGTTTTAATTTCGTGACTTGC	55	340–400	KF267885
BA30	$(CT)_{33}$	F: CTTGAATGCTGCTGGTAAAGC R: GCATCCTTTTGGACTCAATTTC	65	290–370	KF267886

Note: T_a = annealing temperature.

1 min, followed by 30 cycles of denaturation at 94°C for 1 min, 1 min at a specific annealing temperature (T_a), and a final extension of 72°C for 5 min. The obtained products were verified by electrophoresis on 3% agarose gels containing 0.1 mg ethidium bromide per milliliter in 1× TBE buffer and genotyped using 6% denaturing polyacrylamide gels dyed with silver nitrate (Creste et al., 2001). We estimated the allele sizes by comparison to a 10-bp DNA ladder (Invitrogen).

The amplicons were electrophoretically separated using an ABI 377 automated sequencer (Applied Biosystems) with GeneScan 500 TAMRA marker as the size standard (Applied Biosystems). The fragment size and allele identification were determined using GeneMarker version 2.2 software (SoftGenetics, State College, Pennsylvania, USA). Cross-species amplifications were evaluated using five other species from the Rubiaceae family with varying phylogenetic proximity to *B. australis*: *B. mendoncaei* K. Schum., *B. stipulata* (Vell.) C. Presl, and *Rustia formosa* (Cham. & Schltdl.) Klotzsch (Condamineeae tribe, Ixoroideae subfamily); *Rudgea jasminoides* (Cham.) Müll. Arg. (Psychotrieae tribe); and *Coussarea accedens* Müll. Arg. (Coussareeae tribe, Rubioideae subfamily) (Bremer and Eriksson, 2009).

We characterized the preliminary genetic diversity of *B. australis* populations from lowland (20 individuals; 23.3762°S, 45.0806°W, Ubatuba, São Paulo, Brazil) and upland (20 individuals; 23.3259°S, 45.0710°W, São Luis do Paraitinga, São Paulo, Brazil) Serra do Mar. Descriptive statistics and Hardy–Weinberg equilibrium tests were performed using GenAlEx version 6.5 (Peakall and Smouse, 2006). The same nine loci were polymorphic in both populations (Table 2) and for these the average number of alleles was 8.6, ranging from five to 13 alleles per locus. The observed and expected heterozygosities ranged from 0.333 to 0.900 (average: 0.629) and 0.564 to 0.900 (average: 0.742), respectively. The fixation index ranged from –0.089 to 0.611, with an average of 0.147. Four loci in the lower population and three loci in the upper population showed significant deviations from Hardy–Weinberg equilibrium ($P < 0.05$). These results indicated some slight excess of homozygotes, which might have resulted from mating between relatives and/or the inbreeding generated by selfing; *B. australis* is a self-compatible species (Andrich, 2008). All 10 loci amplified successfully in the other *Bathysa* C. Presl species, but only four primers performed well for *Rustia formosa* (Table 3); all primers failed for *Rudgea jasminoides* and *Coussarea accedens*.

TABLE 2. Results of initial primer screening of lower (23.3762°S, 45.0806°W) and upper (23.3259°S, 45.0710°W) *Bathysa australis* populations. Only polymorphic loci are shown.

Population	Locus	A	A_e	H_o	H_e	F	HWE[a]
Lower	BA02	5	2.417	0.450	0.601	0.232	ns
(N = 20)	BA14	12	5.270	0.842	0.832	–0.039	ns
	BA15	8	5.369	0.850	0.835	–0.045	ns
	BA22	5	2.222	0.500	0.564	0.091	ns
	BA24	12	2.462	0.500	0.609	0.158	*
	BA25	7	5.026	0.353	0.825	0.559	**
	BA26	11	8.163	0.600	0.900	0.316	ns
	BA28	8	5.755	0.900	0.847	–0.089	*
	BA30	12	6.968	0.333	0.881	0.611	**
Upper	BA02	5	2.606	0.600	0.632	0.026	ns
(N = 20)	BA14	11	6.422	0.647	0.870	0.234	**
	BA15	8	3.404	0.750	0.724	–0.062	ns
	BA22	6	2.548	0.550	0.623	0.095	ns
	BA24	11	4.938	0.800	0.818	–0.003	ns
	BA25	7	4.040	0.750	0.772	0.003	ns
	BA26	13	7.407	0.650	0.887	0.249	**
	BA28	5	3.162	0.450	0.701	0.342	***
	BA30	10	4.396	0.800	0.792	–0.036	ns

Note: A = number of alleles; A_e = effective number of alleles; F = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium tests; N = number of individuals sampled.

[a] Significant departures from HWE are indicated at the following levels: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; ns = nonsignificant.

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TABLE 3. Results from the cross-amplification tests using primers designed for *Bathysa australis*.

Locus	*Bathysa stipulata*	*Bathysa mendoncaei*	*Rustia formosa*	*Coussarea accedens*	*Rudgea jasminoides*
BA02	+	+	+	–	–
BA14	+	+	–	–	–
BA15	+	+	–	–	–
BA16	+	+	–	–	–
BA22	+	+	+	–	–
BA24	+	+	–	–	–
BA25	+	+	–	–	–
BA26	+	+	+	–	–
BA28	+	+	–	–	–
BA30	+	+	+	–	–

Note: + = successful amplification; – = failed amplification.

CONCLUSIONS

The microsatellite markers described here are the first developed for the genus *Bathysa* and will be useful for genetic, ecological, and conservation management evaluations. Cross-species amplifications suggest that some of these loci may be useful in other species from the tribe Condamineeae (subfamily Ixoroideae).

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APPENDIX 1. Voucher information for species used in this study.

Species	Voucher specimen accession no.	Collection locality
Bathysa australis (A. St.-Hil.) Hook. f. ex K. Schum.	HRCB 60163	Rio Claro, SP
Bathysa stipulata (Vell.) C. Presl	HRCB 60107	Rio Claro, SP
Bathysa mendoncaei K. Schum.	HRCB 59786	Rio Claro, SP
Rustia formosa (Cham. & Schltdl.) Klotzsch	HRCB 59785	Rio Claro, SP
Coussarea accedens Müll. Arg.	HRCB 59788	Rio Claro, SP
Rudgea jasminoides (Cham.) Müll. Arg.	IAC 49279	Campinas, SP

Note: HRCB = Herbário Rioclarense, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil; IAC = Herbarium of the Instituto Agronômico de Campinas, Campinas, São Paulo, Brazil; SP = São Paulo.

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