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PRIMER NOTE

CHARACTERIZATION OF MICROSATELLITE MARKERS FOR *BACCHARIS DRACUNCULIFOLIA* (ASTERACEAE)[1]

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- *Premise of the study: Baccharis dracunculifolia* (Asteraceae) is a native plant of the Atlantic Forest that is used for the production of essential oil. Microsatellite markers were developed for this species to investigate the genetic diversity of three natural populations.
- *Methods and Results:* Seventeen out of 27 microsatellite loci identified in a genomic library used for the characterization of 315 individuals derived from three natural populations of *B. dracunculifolia* resulted in successful amplifications. Eleven polymorphic loci, ranging from two to seven alleles per locus, were obtained with expected and observed heterozygosity values ranging between 0.068 and 0.775 and 0.046 and 0.667, respectively.
- *Conclusions:* The microsatellite loci described in this study are tools that can be used for further studies of population genetics of *B. dracunculifolia* with a focus on deforested areas and conservation of natural populations.

Key words: altitude; Asteraceae; *Baccharis dracunculifolia*; microsatellites; population genetics.

The genus *Baccharis* L., a member of the Asteraceae family, is extremely widespread and comprises about 500 species of herbs, shrubs, and trees. Approximately 120 of these species, including *B. dracunculifolia* DC., have been identified in the deforested areas of the Atlantic Forest and scrubland, occurring in the southeastern, southern, and midwestern regions of Brazil. The species has vegetative apices that are used as raw material by honeybees (*Apis mellifera*) for the production of green propolis, which is a resinous substance that serves as a protective barrier against fungi and bacteria in beehives. Aside from its use for this purpose, *B. dracunculifolia* contains an essential oil that is extracted from its leaves and that has an immense commercial value in the perfume industry. The essential oil, with *trans*-nerolidol as its main component, has been regulated by the Food and Drug Administration (FDA) and used commonly as a flavoring agent in the food industry (Heywood, 1993; Park et al., 2004; Arruda et al., 2005; Gilberti, 2012; Sforcin et al., 2012).

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Microsatellite markers are especially desirable for plant conservation studies as they are codominant in nature, multiallelic, and widely distributed in the genome (Alves et al., 2014). Here we developed and characterized 11 microsatellites of *B. dracunculifolia*, which will be used as a tool for studying the conservation genetics of this species.

METHODS AND RESULTS

In March 2012, 315 samples of *B. dracunculifolia* were collected from three natural populations in the Atlantic Forest region, at an altitudinal gradient in São Paulo State, Brazil: Campos do Jordão municipality (22°25′S, 45°20′W; 1620 m), Campinas municipality (22°31′S, 47°20′W; 680 m), and Ubatuba municipality (23°15′S, 45°20′W; 2 m). Voucher specimens were deposited at the Herbarium of the Instituto Agronômico de Campinas (IAC; accession numbers: Campos do Jordão IAC 54.955, Ubatuba IAC 54.956, and Campinas IAC 54.957). The genomic DNA was extracted from leaves, following the protocol described by Risterucci et al. (2000). A genomic microsatellite library was constructed using the protocol adapted from Billotte et al. (1999). The genomic DNA digestion from one individual of *B. dracunculifolia* collected in Campinas was performed with *Afa*I (Invitrogen, Carlsbad, California, USA) and enriched with microsatellite fragments with $(CT)_8$ and $(GT)_8$ motifs. Microsatellite-enriched DNA fragments were linked into pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA) and used to transform Epicurean Coli XL1-Blue *Escherichia coli* competent cells (Promega Corporation). The sequencing reaction with final volumes of 10 µL contained 200 ng of plasmid DNA, 0.5 pmol SP6 primer, 0.4 µL of BigDye Terminator mix version 3.1 (Applied Biosystems, Foster City, California, USA), 1 mM $MgCl_2$, and 40 mM Tris-HCl (pH 9.0).

From 96 clones sequenced with an ABI 3700 automated DNA sequencer (Applied Biosystems), we identified microsatellites in 40 sequences considering at least six repeats for dinucleotide and four repeats for tri- and tetranucleotide motifs, resulting in an enrichment index of 47.06%. Twenty-seven primer pairs were designed with Primer3 (Rozen and Skaletsky, 1999) and analyzed with Gene Runner (Spruyt and Buquicchio, 1994). The main parameters considered for primer design were: GC content of PCR products with amplification ranging from 50% to 60% and ranging from 150 to 250 bp; primer annealing temperatures varying from 55°C to 70°C; maximum difference in annealing temperature between the primer pairs was 3°C and the 5′ forward end of each primer pair was labeled with M13 fluorescence (5′-CACGACGTTGTAAAACGAC-3′) (LI-COR Biosciences, Lincoln, Nebraska, USA).

Nine samples of *B. dracunculifolia,* three from each population, were used during optimization of the PCR conditions, resulting in amplicons for the 11 primer pairs presented in Table 1. The PCR reactions contained 0.5 ng of DNA, 0.10 µL of forward primer (10 µM), 0.10 µL of reverse primer (10 µM), 0.1 µL of fluorochrome-labeled M13(–29) primer (10 µM), 0.4 µL of dNTP mix (2.5 mM), 0.75 µL of 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.9]), 0.8 µL of bovine serum albumin (BSA, 2.5 µM), 0.3 µL of $MgCl_2$ (50 mM; with the exception of 0.6 µL of $MgCl_2$ [50 mM] for primer Bd 09), and 0.4 µL of *Taq* DNA polymerase, with final volumes of 10 µL. For the primers Bd 06, Bd 13, Bd 16, Bd 17, and Bd 26, a touchdown cycling program was used before the regular cycling: 94°C for 4 min, followed by 10 cycles of 94°C for 45 s, 55°C decreasing to 53°C at 0.5°C per cycle for 1 min, and 72°C for 1 min 15 s. Afterward, we performed 24 cycles of 94°C for 45 s, with an annealing temperature of each primer for 45 s, and finally, 72°C for 1 min 15 s was performed prior to a final extension at 72°C for 5 min. For the primers Bd 09, Bd 14, Bd 17, Bd 19, Bd 27, Bd 01, and Bd 04, the fixed temperature of 55°C was used. Polymorphisms were detected in 5% (v/v) polyacrylamide gels in an automated sequencer (LI-COR 4300S DNA Analysis System; LI-COR Biosciences). The loci were genotyped using Saga^{GT} software (LI-COR Biosciences), and the allele sizes were determined with the aid of the IRDye-700 and IRDye-800 sizing standards (LI-COR Biosciences).

Based on all samples (n = 105) for three populations, 11 loci were polymorphic and six were monomorphic. In the three populations of *B. dracunculifolia*, we detected two to seven alleles per locus and the average number of alleles per locus was 3.33. The observed and expected heterozygosity ranged from 0.046 to 0.667 and from 0.068 to 0.775, respectively (Table 2). The linkage disequilibrium and other statistics were estimated with GENEPOP (Raymond and Rousset, 1995) and hierfstat package (Goudet, 2005) developed for R program (R Core Team, 2015). No linkage disequilibrium was observed between the loci pairs after the Bonferroni correction (Weir and Cockerham, 1984).

CONCLUSIONS

This is the first set of microsatellite markers developed for *B. dracunculifolia*, a species that needs to be conserved, especially within deforested areas. We have identified 17 SSR markers, with 11 being polymorphic in three different populations. The set of 11 polymorphic SSR markers is a molecular tool that will be useful for *Baccharis* researchers interested in population genetics studies and conservation, particularly for those researchers performing phylogenetic studies on closely related species.

TABLE 1. Characteristics of the 17 microsatellite markers developed for *Baccharis dracunculifolia.*

Locus	Primer sequences (5′–3′)[a]	Repeat motif	T_a (°C)	Expected allele size (bp)	GenBank accession no.
Bd 06	F: TGATCGGATGTGTGTGATGA	$(TG)_6(CT)_6$	53	187	KR781489
	R: GGCTTAGAACGTGGAGTGGT				
Bd 09	F: CGCGTGGACTAACCTGTATG	$(GT)_7$	53	158	KR781490
	R: AAATGGAAGTTGGGAACACG				
Bd 13	F: CGTGGACTACCCACCTTCTC	$(CA)_7(CA)_7$	53	191	KR781491
	R: TCGTATCAACCTCCTAGCTGTG				
Bd 14	F: TGCGTTACACACATTGCTCA	$(AC)_{10}$	53	154	KR781492
	R: TCTTGCTTACGCGTGGACTA				
Bd 16	F: TCTGCATAGGGCATTTTGTG	$(CT)_6(CT)_{10}$	53	181	KR781493
	R: GCGAGGAAGAGAGAGATGGA				
Bd 17	F: AGGGTGTACCAACGGCTAAC	$(AC)_6$	53	233	KR781494
	R: TGCATAGTGATTCCGATAGATG				
Bd 19	F: TTTTTAGGATCGCTCCACCA	$(TG)_6$	53	189	KR781495
	R: CTCAAGCTATGCATCCAACG				
Bd 26	F: CTTCCCCTATTTGATGATGACA	$(AC)_{21}(CA)_9$	53	238	KR781496
	R: CGCGTGGACTAACTTGTCTTT				
Bd 27	F: CCCGTGGTTGTTTCTTACA	$(TCC)_4(CTT)_4$	56	165	KR815901
	R: TCCGATACAGTTTATGGCTGT				
Bd 01	F: GCTGTCAATGATGCCCACTA	$(AC)_7$	56	221	KR815902
	R: GCCAGACTTGAACCTTGTCC				
Bd 04	F: TCATGGTTCATTGGTCTTGA	$(TC)_{17}$	56	224	KR815903
	R: CGAACAATTTGCCCATTAAC				
Bd 02*	F: CTCTTGCTTACGCGTGGACT	$(AC)_{16}$	53	151	KT321673
	R: TTCGTTCGCAGGGAACTATT				
Bd 03*	F: GCACTGCCATAATCACAAGG	$(AC)_{17}$	53	246	KT321674
	R: GGATGGGTCCTCATAATCAAA				
Bd 10*	F: GCCTGGGTGGTACATATCATT	$(TG)_6$	53	195	KT321675
	R: GTCACAAGACGACCCCAAAT				
Bd 11*	F: GAGGCTCGCTGTTAGGATTG	$(CA)_7$	53	222	KT321676
	R: GCATAATCGTTGATCGGAAA				
Bd 21*	F: CTTACGCGTGGACTACATACG	$(AC)_9$	53	223	KT321677
	R: GGCGCGAATGAATGTAAAAT				
Bd 24*	F: TGGAATGGACTTTTGGGAAG	$(AC)_7$	53	237	KT321678
	R: CGCGTGGACTAACCTGTATG				

Note: T_a = annealing temperature.

[a]The 5′ forward end was labeled with an M13(–29) tail (5′-CACGACGTTGTAAAACGAC-3′).

*Monomorphic loci.

TABLE 2. Estimates of genetic diversity for three populations of *Baccharis dracunculifolia* based on 11 polymorphic microsatellite markers.[a]

	Campos do Jordão				Campinas				Ubatuba			
Locus	*A*	H_e	H_o	F_{IS}	*A*	H_e	H_o	F_{IS}	*A*	H_e	H_o	F_{IS}
Bd 06*	4	0.585	0.296	0.494	1	0	0	NA	1	0	0	NA
Bd 09*	3	0.459	0.046	0.899	4	0.673	0.564	0.162	4	0.381	0.388	−0.019
Bd 13	2	0.503	0.365	0.274	2	0.363	0.269	0.257	2	0.492	0.247	0.498
Bd 14*	2	0.165	0.180	−0.091	2	0.316	0.392	−0.239	2	0.068	0.070	−0.031
Bd 16*	2	0.406	0.562	−0.385	1	0	0	NA	1	0	0	NA
Bd 17	2	0.413	0.579	−0.402	1	0	0	NA	1	0	0	NA
Bd 19	2	0.447	0.667	−0.493	1	0	0	NA	1	0	0	NA
Bd 26*	1	0	0	NA	2	0.497	0	1	1	0	0	NA
Bd 27*	3	0.668	0.487	0.272	1	0	0	NA	1	0	0	NA
Bd 01*	3	0.535	0.347	0.351	2	0.096	0.100	−0.048	2	0.079	0.082	−0.037
Bd 04*	7	0.775	0.463	0.403	7	0.708	0.633	0.106	7	0.721	0.519	0.281

Note: *A* = number of alleles per locus; F_{IS} = fixation indices; H_e = expected heterozygosity; H_o = observed heterozygosity; NA = not applicable (i.e., monomorphic loci).

[a]All values are based on 315 samples from three populations ($N = 105$ for each) representing the altitudinal gradient in São Paulo State, Brazil: Campos do Jordão (22°25′S, 45°20′W; 1620 m), Campinas (22°31′S, 47°20′W; 680 m), and Ubatuba (23°15′S, 45°20′W; 2 m).

* Significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction ($P = 0.004$).

LITERATURE CITED

ALVES, F. M., M. I. ZUCCHI, A. M. G. AZEVEDO-TOZZI, A. L. B. SARTORI, AND A. P. SOUZA. 2014. Characterization of microsatellite markers developed from *Prosopis rubriflora* and *Prosopis ruscifolia* (Leguminosae–Mimosoideae), legume species that are used as models for genetic diversity studies in Chaquenian areas under anthropization in South America. *BMC Research Notes* 7: 375.

ARRUDA, D. C., F. L. D'ALEXANDRI, A. M. KATZIN, AND S. R. B. ULIANA. 2005. Antileishmanial activity of the terpene nerolidol. *Antimicrobial Agents and Chemotherapy* 49: 1679–1687.

BILLOTTE, N., P. J. L. LAGODA, A. M. RISTERUCCI, AND F. C. BAURENS. 1999. Microsatellite-enriched libraries: Applied methodology for the development of SSR markers in tropical crops. *Fruits* 54: 277–288.

GILBERTI, L. H. 2012. Potencial para o uso da espécie nativa, *Baccharis dracunculifolia* DC. (Asteraceae) na fitorremediação de áreas contaminadas por arsênio, 77. Universidade Federal de Minas Gerais, Minas Gerais, Brazil.

GOUDET, J. 2005. Hierfstat, a package for R to compute and test hierarchical *F*-statistics. *Molecular Ecology Notes* 5: 184–186.

HEYWOOD, V. H. 1993. Flowering plants of the world. B. T. Bastford Ltd., London, United Kingdom.

PARK, Y. K., J. F. PAREDES-GUZMAN, C. L. AGUIAR, S. M. ALENCAR, AND F. Y. FUJIWARA. 2004. Chemical constituents in *Baccharis dracunculifolia* as the main botanical origin of southeastern Brazilian propolis. *Journal of Agricultural and Food Chemistry* 52: 1100–1103.

R CORE TEAM. 2015. Writing R extensions, version 3.2.1, 167. R Foundation for Statistical Computing, Vienna, Austria.

RAYMOND, M., AND F. ROUSSET. 1995. GENEPOP, version 1.2: Population genetics software for exact tests and ecumenism. *Journal of Heredity* 86: 248–249.

RISTERUCCI, A. M., L. GRIVET, J. A. K. N'GORAN, I. PIERETTI, M. H. FLAMENT, AND C. LANAUD. 2000. A high-density linkage map of *Theobroma cacao* L. *Theoretical and Applied Genetics* 101: 948–955.

ROZEN, S., AND H. SKALETSKY. 1999. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

SFORCIN, J. M., J. P. B. SOUSA, A. A. FILHO, J. K. BASTOS, M. C. BÚFALO, AND L. R. S. TONUCI. 2012. *Baccharis dracunculifolia*: Uma das principais fontes vegetais da própolis brasileira. Fundação Editora da UNESP, São Paulo, Brazil.

SPRUYT, M., AND F. BUQUICCHIO. 1994. Gene Runner version 3.05. Website http://www.generunner.net/ [accessed 10 April 2015].

WEIR, B. S., AND C. C. COCKERHAM. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358–1370.