American Journal of Botany

American Journal of Botany: e289–e291. 2012.

AJB Primer Notes & Protocols in the Plant Sciences

Microsatellite markers for *Vellozia gigantea* (Velloziaceae), a narrowly endemic species to the Brazilian campos rupestres[1]

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- *Premise of the study:* Microsatellite primers were developed for the first time in Velloziaceae, in the endangered species *Vellozia gigantea*.
- *Methods and Results:* Using two different protocols, seven primer sets were characterized in three populations of *V. gigantea*. The primers amplified di- and trinucleotide repeats with six to 12 alleles per locus. These revealed high levels of genetic variation, presenting an average observed heterozygosity of 0.508 in *V. gigantea*. The seven primers were tested for cross-amplification in three *Vellozia* species. All primers successfully amplified in *V. auriculata.* Six primers amplified in *V. compacta* and three in *V. hirsuta*.
- *Conclusions:* The new marker set described here will be useful for studies of population genetics of *V. gigantea.* The cross-amplification results indicate the utility of primers for studies in other *Vellozia* species.

Key words: cross-species amplification; endangered species; genetic diversity; microsatellites; *Vellozia gigantea*; Velloziaceae.

Vellozia gigantea N. L. Menezes & Mello-Silva (Velloziaceae) is a rupicolous monocot species reaching ca. 6 m tall, endemic to the campo rupestre (rocky field) vegetation in the Espinhaço Range, eastern Brazil (Mello-Silva and Menezes, 1999). There are only nine known populations with a narrow distribution in disjunct outcrops, and the species is ranked as endangered (Biodiversitas, 2005) because of disturbance and habitat loss caused by anthropic effects. There are no known microsatellite markers developed for Velloziaceae species. Therefore, we developed these markers for *V. gigantea* to further investigate the population structure and genetic diversity in the species. We also tested the transferability of these primers to other Brazilian endemic *Vellozia* Vand. species.

METHODS AND RESULTS

Two genomics libraries were constructed to obtain microsatellite primers for *V. gigantea*. Because the first library provided few microsatellite loci due to very high or very low percentages of GC and an excess of repetitive bases, a second library was added to obtain additional useful loci. The first library followed Yazbeck and Kalapothakis (2007) with some modifications. Genomic DNA from one individual was extracted from fresh leaves according to Doyle and Doyle (1987). Vouchers are deposited in the herbarium BHCB of the Universidade Federal de Minas Gerais (*V. gigantea*: *L. A. Soares 28*, *V. compacta*: *F. F. Carmo 1684*, *V. hirsuta*: *Mello-Silva 407*, *V. auriculata*: *N. F. O. Mota 726*). Total DNA (10 µg) was digested with S*au*3AI (Promega Corp., Madison, Wisconsin, USA), size-selected (>400 bp) and ligated into *Bam*H1, digested and dephosphorylated pUC18 (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA), and used to transform electrocompetent *Escherichia coli* XL1-blue (Phoneutria, Belo Horizonte, Minas Gerais, Brazil). After selection (ampicillin and IPTG/X-gal), plasmid purification was conducted by alkaline cell lysis. Plasmids were spotted on a nylon membrane and screened for simple sequence interactions with 5′-end-labeled 16-bp oligonucleotides (repeat motifs: GATA, TATC, GACA, GGAT, TATT, TACT, CGGA, GTCA, CCGT, GAGG, GATT, CATG, CCAG, CATG, CCAG, CTTG, CAGC, TATG, TAGT, AAGT, AAGT, CAT, CAC, GTA, GAA, AAT, CTT, CAC, CCG, CAG, CA, CT, and TA). Oligo labeling was carried out with radioactive (γ32P) ATP (10 mCi/mL) (GE Healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA) using T4 kinase (5′ Labeling kit, GE Healthcare Bio-Sciences Corp.). After prehybridization (6× SSC [0.9 M NaCl, 0.09 M sodium citrate], 0.1% sodium docecyl sulfate [SDS], and 5% BLOTO), membranes were incubated with hybridization buffer (6× SSC and 0.1% SDS) containing 5 ng/mL labeled probes for 14 h at 48°C. The membranes were then washed and exposed to autoradiography films Kodak T-Mat G/RA (Carestream Health, Rochester, New York, USA) at −80°C for 4–24 h. Positive clones were sequenced with universal primer M13 on ABI 3130 DNA sequencer (Applied Biosystems–Life Technologies, Foster City, California, USA).

The second library was a microsatellite-enriched library (Nunome et al., 2006). Total DNA was extracted from silica gel–dried leaves from one individual. Total DNA (5 µg) was digested with *Rsa*I (Promega Corp.), and the fragments ligated with *Rsa*I linkers (LinkerA: 5′-CTCTTGCTTACGCGTGGACTA-3′; LinkerB:

[1]Manuscript received 28 December 2011; revision accepted 1 March 2012.

The authors thank Kátia T. Ribeiro and Leonardo C. Ribeiro for help with population sampling. This work was funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Brazil. A.P.V.M. received a fellowship from CNPq. E.K., F.R.S., and E.L.B. are supported by productivity grants from CNPq.

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doi:10.3732/ajb.1100611

TABLE 1. Characteristics of seven microsatellite markers developed in *Vellozia gigantea.*

Primer	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
Vel01	F: TAATCTGGGCCAACTTCCAG R: TGGGACAGTCGCTGAACG	$(CTT)_4C(CTT)_2$	320–353	60.0	JQ286009
Vel02	F: CAGGTAATCTTCCTCGGTGGC R: ACCGGTCCTCTAAACACGTC	$(CT)_{19}$	221–249	67.0	JQ286010
Vel03	F: CCTAGCCTTGATATGTCGTCGG R: GGTCCGCCAGTTAGACTTTG	$(GT)_{18}$	140–161	60.2	JQ286011
Vel04	F: TCTGCCTCGACCAAATCCG R: CCACCCATCACTAACATCGTTCAC	$(CT)_{20}$	154–189	67.0	JQ286012
Vel05	F: CGAACTCCGTGGGATGTTG R: GCACGTTCGTCTACCATCATCG	$(CT)_9$	137–157	63.9	JQ286013
Vel06	F: GAGCTGGCGCTTCTTCCATTTAC R: CTGCCGTCGATGATGATGTCC	$(CT)_{16}$	104–132	67.0	JQ286014
Vel07	F: AGCCGGCCACACCAAATAC R: TGCATGTGGTATTGGGACAC	$(GT)_8$	145	61.0	JQ286015

Note: T_a = annealing temperature.

5′-PO_4-TAGTCCACGCGTAAGCAAGAGCACA-3′) with T4 DNA ligase for 2 h at 20°C. PCR was performed using LinkerA-specific primer, and amplicons (400–1600 bp) were then hybridized to 5′-biotin-labeled oligonucleotide probes $(CT)_8$, $(GT)_8$, $(GATA)_4$, and $(GACA)_4$. Enriched fragments were recovered using streptavidin-coated magnetic beads (Streptavidin MagneSphere; Promega Corp.). The enriched DNA was amplified using LinkerA as a primer, and the product was purified, ligated into pGEM-T Easy Vector (Promega Corp.), and transformed into the *E. coli* XL1-Blue competent cells. After selection (ampicillin and IPTG/X-gal), recombinant clones were diluted and lysed, and PCR-amplified using M13 forward and reverse primers. Amplicons (>400 bp) were purified and sequenced on an ABI 3730XL (Applied Biosystems–Life Technologies). Sequences containing microsatellite repeats were obtained using the Microsatellite Repeats Finder (http://www.biophp.org/minitools/microsatellite_repeats_finder/demo.php). Primers were designed using OLIGO Analyzer 3.1 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) and Primer3 (Rozen and Skaletsky, 2000) by setting product size ranges from 100 to 500 bp, primer size from 18 to 27 bp, GC% from 40 to 60, and annealing temperature from 57 to 63°C.

One hundred and sixty clones from the first library were sequenced, and 10 contained microsatellites ≥12 bp for primer design. However, only one locus (*Vel01*) showed good quality amplification. Its forward primer was labeled with the fluorescent dye HEX at its 5′-end. PCR was performed in 25-μL reactions containing 10–15 ng genomic DNA, 1× PCR buffer IB (100 mM Tris-HCl [pH 8.4], 500 mM KCl, 1% Triton, 15 mM $MgCl_2$), 0.1 mM of dNTPs, 0.2 μM each primer, and 1 U *Taq* DNA polymerase (Phoneutria, Belo Horizonte, Minas Gerais, Brazil). The PCR profile used to amplify the microsatellites was 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, 72°C for 30 s; and a final elongation step at 72°C for 3 min. From the second library, 229 clones were sequenced and 48 clones presented microsatellite inserts with GT or CT dinucleotide repeats. Primers were designed for 16 loci of simple and perfect microsatellites. An M13-tailed primer was added at the 5′-end of the forward primers (Schuelke, 2000). Good quality amplifications were obtained only in six loci (*Vel02* to *Vel07*). PCR was performed in a 25-μL reaction mixture containing 10 ng genomic DNA, 1× PCR buffer IVB 5× (1.5 $MgCl_2$), 0.25 mM of dNTPs, 0.04 μM of forward primer, 0.16 μM of reverse primer, 0.16 μM of 6-FAM.M13 (6-FAM fluorescent labeling), and 1 U *Taq* DNA polymerase (Phoneutria). We used 0.4 mg/mL of BSA for loci *Vel02*, *Vel04*, and *Vel05*. The PCR profile used to amplify the microsatellites was 94°C for 5 min; 20 cycles of denaturation at 94°C for 30 s, annealing temperature at T_a (Table 1) for 45 s, 72°C for 45 s, and 18 cycles of denaturation at 94°C for 30 s; melting at 50°C for 45 s (for annealing of 6-FAM.M13 primer), 72°C for 45 s; and a final extension step at 72°C for 30–60 min. PCR products were analyzed in an ABI 3730XL sequencer and visualized with Peak Scanner Software (Applied Biosystems–Life Technologies). The Autobin macro for Excel (http://www4.bordeaux-aquitaine.inra.fr/biogeco/Ressources/Logiciels/Autobin), which automatically analyses raw data generated with commercial software, was used for the nomination of the alleles. The number of samples and loci are automatically detected, alleles in raw sizes are sorted and plotted to detect relevant gaps in size, and alleles are binned with manual checking (Guichoux et al., 2011).

All microsatellite primers were tested for polymorphism in a primary sample of five individuals from five populations. Then, 24 individuals were genotyped from three populations (eight individuals/population) for estimation of number of alleles per locus and heterozygosity using GenAlEx 6.4 (Peakall and Smouse, 2006) (Table 2). We found high average observed (0.508) and expected (0.668) heterozygosity. We also tested cross-amplification for these markers in *V. auriculata* Mello-Silva & N. L. Menezes, *V. compacta* Mart. ex Schult. f., and *V. hirsuta* Goeth. & Henrard (eight individuals each), using the same protocol described above. All loci amplified in *V. auriculata*, and 86% and 43% of the markers amplified in *V. compacta* and *V. hirsuta*, respectively (Table 3). All loci are polymorphic in all the species, except for *Vel06*, which was fixed in *V. hirsuta*, and *Vel07*. The latter, while fixed in all four species, appeared as polymorphic between them (Table 3).

CONCLUSIONS

We present the first initiative of development of microsatellite markers for the Velloziaceae. We identified one monomorphic and six polymorphic loci for *V. gigantea*. The monomorphic locus presented interspecific polymorphism, so it may be useful in taxonomic, hybridization, and phylogeographic studies. The six polymorphic markers would be useful to further investigation on population genetic studies in *V. gigantea.* This study should be expanded to demonstrate the usefulness of these markers in the other species of the genus, but the high number of alleles found in the small samples used indicates a high potential for future population studies in the group. Such information should be helpful in developing plans for the management

TABLE 2. Results of initial primer screening in populations of *Vellozia gigantea.*[a,b]

	Alto Palácio			Leste da Estrutura			Salitreiro		
Primer	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e
Vel01	6	0.250	0.617	3	0.375	0.492	3	0.125	0.342
Vel02	7	1.000	0.892	6	0.750	0.833	6	0.875	0.833
Vel03	6	0.500	0.842	8	0.500	0.892	6	0.500	0.842
Vel04	8	0.625	0.900	6	0.750	0.783	7	0.875	0.850
Vell05	7	0.250	0.867	5	0.375	0.450	5	0.250	0.533
Vel06	6	0.750	0.817	9	1.000	0.933	6	0.750	0.833
Vel07	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

[a] Geographic coordinates of the populations: Alto Palácio (19°14′52.19″S, 43°30′37.10″W), Leste da Estrutura (19°16′25.7″S, 43°29′34.5″W), Salitreiro (19°16′37.9″S, 43°30′08.8″W).

[b] n = 8 for each population.

Table 3. Cross-amplification of primers that were originally developed in *Vellozia gigantea* in three other species of the genus.[a]

	V. auriculata			*V. hirsuta*			*V. compacta*		
Locus	*A*	Size range (bp)	T_a (°C)	*A*	Size range (bp)	T_a (°C)	*A*	Size range (bp)	T_a (°C)
Vel01	5	350–360	63	3	339–343	55	3	350–354	65
Vel02	5	227–235	59	2	232–234	59	3	227–233	65
Vel03	7	138–151	59	—	—	—	4	145–163	59
Vel04	4	142–168	65	—	—	—	6	142–173	61.4
Vel05	4	151–163	63.6	—	—	—	2	151–153	61.2
Vel06	4	105–117	63.6	1	103	57	4	115–152	57
Vel07	1	145	57	—	—	—	1	142	59

Note: — = no amplification product; *A* = allele number; T_a = annealing temperature.
[a] $n = 8$ for each species.

of this endangered group of species from the campo rupestre vegetation in the Espinhaço Range.

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