Microsatellite Loci for Orthophytum ophiuroides (Bromelioideae, Bromeliaceae) Species Adapted to Neotropical Rock Outcrops

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Primer Note

Microsatellite loci for *Orthophytum ophiuroides* (Bromelioideae, Bromeliaceae) species adapted to neotropical rock outcrops[1]

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- *Premise of the study:* Microsatellite primers were developed for *Orthophytum ophiuroides*, a rupicolous bromeliad species endemic to neotropical rocky fields. These microsatellite loci will be used to investigate population differentiation and species cohesion in such fragmented environments. The loci were tested for cross-amplification in related bromeliad species.
- *Methods and Results:* Eleven polymorphic microsatellite markers were isolated and characterized from an enriched library of *O. ophiuroides*. The loci were tested on 42 individuals from two populations of this species. The number of alleles per locus ranged from three to nine and the expected and observed heterozygosities ranged from 0.167 to 0.870 and from 0.369 to 0.958, respectively. Seven loci successfully amplified in other related bromeliad species.
- *Conclusions:* Our results suggest that the microsatellite loci developed here will be useful to assess genetic diversity and gene flow in *O. ophiuroides* for the investigation of population differentiation and species cohesion in neotropical mountainous habitats.

Key words: Bromeliaceae; cross-amplification; gene flow; genetic diversity; *Orthophytum ophiuroides*; population genetics.

The Bromeliaceae are exclusive to the tropical and subtropical areas of the Americas (except for one African species) and show extreme adaptive radiation. Species of this typical neotropical family have evolved to fill a variety of niches in a highly heterogeneous habitat, with an amazing diversity of adaptations (Benzing, 2000). Such rapid processes of adaptation and speciation can be used as models to study and understand larger issues regarding the evolution of neotropical plants. Studies on bromeliad species adapted to neotropical rock outcrops have improved our understanding of the processes of speciation and species cohesion in naturally isolated environments (i.e., Barbará et al., 2009; Palma-Silva et al., 2011).

Orthophytum Beer species are rupicolous herbs exclusive to the northeastern and southeastern regions of Brazil (Louzada and Wanderley, 2010). Although $2n = 50$ chromosomes are common within the Bromeliaceae, many *Orthophytum* species are polyploids ($2n = 100$ and 150) (Cotias-de-Oliveira et al., 2000; Louzada et al., 2010). Species of this genus generally inhabit granitic-gneiss inselbergs and quartzitic-sandstone outcrops in the Brazilian campos rupestres ("rocky fields") along the Espinhaço Range (Louzada and Wanderley, 2008). Such environments are spatially and ecologically isolated, providing a barrier against dispersal and migration, and they possess elevated species diversity and high levels of endemism (Echternacht et al., 2011). In fact, most *Orthophytum* species are restricted to small geographic ranges and some of them are narrow endemics (Louzada and Wanderley, 2010). This genus offers an interesting model system for examining speciation processes and endemism in neotropical mountainous habitats.

Here, we report microsatellite loci isolation, characterization, and cross-amplification for *O. ophiuroides* Louzada & Wand., a species endemic to the Chapada Diamantina section of the Espinhaço Range in the state of Bahia, Brazil (Louzada and Wanderley, 2008). The microsatellite loci reported here will be used to access levels of gene flow to resolve forces operating during species radiations on naturally isolated habitats. The knowledge of patterns of diversity and gene flow will also be important for management of conservation strategies and for understanding the genetic consequences of variation in mating systems or pollination syndromes.

METHODS AND RESULTS

To characterize microsatellite loci, 42 individuals of *O. ophiuroides* located in two populations from Lençóis in the Brazilian Federal State of Bahia were

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TABLE 1. Primer sequences and characteristics of microsatellite loci from *Orthophytum ophiuroides*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)[a]	Allele size range (bp)	GenBank accession no.
Op13	F: CACCCATTGAGAAGGAAACG R: GATGAGTGGACTGGTAAAGACC	$(CA)_{12}$	TD-48	208–216	KF753870
Op30	F: CAACCCTTTTTGTGCCCACT R: CACTTGTGATGTTTCCTCGTC	$(CT)_{24}$	ST-54	140–185	KF753871
Op38	F: TGCCCACTGTGCTGCTATTA R: GAGCTCATCCATGGCGATT	$(TCA)_8N(GT)_9(GA)_{10}$	ST-54	162–182	KF753872
Op62	F: ATAAGGCCCTCTCGAATGCT R: GCATGTAAACCAGCCAACCT	$(CT)_{27}AT(CTAT)_6CT(GT)_9$	TD-48	190–233	KF753873
Op69	F: CCTCCCTTTTTCCCTCCTAC R: ACGCGCTCTACTACCACCAT	$(CT)_{20}$	TD-48	108–136	KF753874
Op73	F: ACCATCGTCCTGTTCACGTC R: CAGCTAAACTAAGAGAGGGTGGA	$(AC)_{16}$	ST-54	168–189	KF753875
Op77	F: CACAACAGAGGCTCGAAAGA R: GCCCGACTCCTCCAATAACC	$(AG)_{10}$	TD-48	151–168	KF753876
Op78	F: CCGGAAAAGCTGTAATGTTTC R: GCGTGGCTCTCTTTTATGGT	$(CT)_9A(CT)_8(CT)_{12}(CA)_{17}$	TD-48	263–299	KF753877
Op89	F: AACCCTAGTTCCACCGATCA R: CTCTTCACCCCCACAAATTC	$(CT)_{11}$	TD-48	135–139	KF753878
Op92	F: TCTTTCTCCGTTCAGCTCTCA R: CCGGTTTCCAGGATCAATAA	$(CT)_7CC(CT)_9CC(CT)_6$	TD-48	271–287	KF753879
Op93	F: TTATCGGGCAGGGGAAATTA R: ACCTTGTCACACACGCAAAG	$(CT)_{12}(GT)_8$	TD-48	161–178	KF753880

Note: T_a = annealing temperature.

[a]TD = touchdown program cycling as published by Palma-Silva et al. (2007); ST = standard program cycling: 95°C for 3 min, 40 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s, and a final elongation step at 72°C for 10 min.

collected and stored in silica gel. Genomic DNA was extracted from leaf tissue using the protocol described by Palma-Silva et al. (2007).

The microsatellite-enriched library of *O. ophiuroides* was constructed according to Billote et al. (1999). Genomic DNA was digested using the restriction enzyme *Rsa*I (Invitrogen, Carlsbad, California, USA). The library was then enriched for $(CT)_8$ and $(GT)_8$ repeats using biotinylated microsatellite probes, and the target fragments were captured by the use of streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). These microsatellite-enriched DNA fragments were cloned into pGEM-T Easy Vector (Promega Corporation), and competent XL1-blue *Escherichia coli* cells were transformed with the recombinant plasmids. The resulting recombinant clones were sequenced using the BigDye Terminator Cycle Sequencing Kit (version 3.1) on an ABI 3730 DNA Analyzer Sequencer (Applied Biosystems, Carlsbad, California, USA).

For the clones containing microsatellite motifs, forward and reverse sequences were aligned using the software Sequencher version 4.1.2 (Gene Codes Corporation, Ann Arbor, Michigan, USA), and primers were designed with Primer3 software (Rozen and Skaletsky, 2000). Forward primers were synthesized with a 19-bp M13 tail (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end to allow labeling with a tailed fluorescent dye M13 primer during amplification and genotyping procedures.

The microsatellite fragments were PCR amplified in a reaction volume of 10 µL containing ~20 ng DNA template, 5× GoTaq Master Mix (Promega Corporation), 5 pmol forward primer, 10 pmol reverse primer, and 1 pmol universal M13 primer tagged with distinct fluorochromes (FAM, VIC, PET, or NED). PCRs were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems) using different touchdown or standard cycling programs according to the annealing temperature of the primers (Table 1) as described by Palma-Silva et al. (2007). Loci were genotyped on an ABI 3730 DNA Analyzer Sequencer and sized against a GeneScan LIZ molecular size standard (Applied Biosystems) using GeneMarker software v.DEMO (SoftGenetics, State College, Pennsylvania, USA).

The levels of genetic diversity were evaluated through the following descriptive summary statistics: expected heterozygosity (H_e), observed heterozygosity (H_o), and number of alleles per locus (A). These parameters were calculated with the MSA program (Dieringer and Schlötterer, 2003). Tests for departure from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were calculated for all the polymorphic loci with GENEPOP version 3.5 (Raymond and Rousset, 1995). For population 2, which showed a tetraploid genotyping

TABLE 2. Characteristics of microsatellite loci from two populations of *Orthophytum ophiuroides*.[a]

	Population 1 (n = 26)					Population 2 (n = 18)				
Locus	Size range (bp)	A	H_e	H_o	f[b]	Size range (bp)	A	H_e	H_o	f[b]
Op13	208–216	5	0.190	0.524	0.642***	208–216	5	0.686	0.410	0.402***
Op30	140–185	9	0.583	0.832	0.304***	—	—	—	—	—
Op38	162–182	5	0.467	0.772	0.404**	—	—	—	—	—
Op62	190–233	8	0.870	0.854	–0.019	190–218	7	0.831	0.958	–0.153*
Op69	108–136	9	0.714	0.791	0.099	108–123	4	0.723	0.708	0.021**
Op73	168–189	6	0.400	0.677	0.415**	152–191	6	0.749	0.369	0.507***
Op77	151–168	6	0.571	0.762	0.255**	151–162	5	0.752	0.778	–0.034**
Op78	263–299	8	0.826	0.830	0.005**	265–303	9	0.848	0.912	–0.076*
Op89	135–139	5	0.167	0.708	0.770***	125–139	3	0.567	0.469	0.173***
Op92	271–287	7	0.750	0.849	0.119	268–293	7	0.806	0.885	–0.097***
Op93	161–178	5	0.286	0.608	0.540**	139–194	6	0.682	0.556	0.185***

Note: A = number of alleles; f = inbreeding coefficient; H_e = expected heterozygosity; H_o = observed heterozygosity.

[a]Geographic coordinates of the populations: Population 1 = 12°34′S, 41°22′W and Population 2 = 12°30′S, 40°25′W. Voucher information: Population 1 = Louzada & Wanderley 88 (SP) and Population 2 = Louzada 194 (UFP).

[b]Significant departure from Hardy–Weinberg equilibrium: *P < 0.05, **P < 0.01, ***P < 0.001.

Table 3. Cross-amplification of seven microsatellite loci isolated from *Orthophytum ophiuroides*.

Species (sample size)	Allele size range (bp)						
	Op13	Op30	Op62	Op69	Op77	Op89	Op93
Vriesea gigantea (2)	212–217	—	—	—	151–153–161	136–140	168
V. scalaris (2)	212	—	—	114	151–161	138–140	—
Pitcairnia albiflos (2)	212	140–150	—	—	151–153–161	136–140	168
P. corcovadensis (2)	212	—	196–206	—	151–153	136–140	168
P. flammea (2)	212	—	206	112–114	151–153	136–140	168
P. staminea (2)	212	138–152	206	114–136	151–159–161	136	168
Orthophytum disjunctum (1)	212	138–139	175–206	—	183	125–136	168
O. foliosum (1)	233	142–165	189–219	—	188–190	136	—
O. zanonii (1)	233	144–148	—	151	203	140–147	168

pattern, genetic diversity parameters were calculated with AUTOTET, specific to polyploid species (Thrall and Young, 2000). The same program was used to test for departure from HWE for the polyploid population.

Cross-amplification tests were performed for nine bromeliad species from three subfamilies (Appendix 1) using the same protocols established for each loci: *O. disjunctum* L. B. Sm., *O. foliosum* L. B. Sm., and *O. zanonii* Leme (Bromelioideae); *Vriesea gigantea* Mart. ex Schult. f. and *V. scalaris* E. Morren (Tillandsioideae); and *P. albiflos* Herb., *P. corcovadensis* Wawra, *P. flammea* Lindl., and *P. staminea* Lodd. (Pitcarnioideae).

From the 96 sequenced recombinant colonies, 78 contained microsatellite motifs and 29 of those presented proper flanking regions for primer design (Table 1). Eleven microsatellite loci amplified and were polymorphic for population 1 while nine loci amplified and were polymorphic for population 2 (Table 2). Loci that performed poorly in the amplification screening or were monomorphic are listed in Appendix S1. In the genotyping process, population 1 showed a diploid and codominant pattern, with no more than two bands per individual, while population 2 showed a tetraploid and codominant pattern, with up to four bands per individual. Overall, the 11 microsatellite loci were highly polymorphic. The number of alleles per locus ranged from five to nine in population 1, and from three to nine in population 2 (Table 2). H_e and H_o ranged from 0.167 to 0.870 and from 0.369 to 0.958, respectively. The inbreeding coefficient values were high for most of the loci, and eight of them showed significant departures from HWE in population 1, while all of the loci showed significant departures in population 2. LD was significant ($P < 0.05$) for 33 out of 55 pairwise locus comparisons. Such departures (HWE and LD) were consistent with inbreeding and/or Wahlund effects. Although null alleles cannot be ruled out, MICRO-CHECKER software (van Oosterhout et al., 2004) found no evidence for scoring error due to stuttering or large allele dropout. Because of the polyploidy of population 2, MICRO-CHECKER could only be used to estimate the null alleles in population 1. The differences in the number of alleles per locus between the two populations indicate that different cytotypes may occur within *O. ophiuroides* species, a phenomenon observed for other plants (Levin, 1980) including bromeliad species (i.e., *Tillandsia*: Till, 1992).

Seven of the markers showed satisfactory amplification within the expected allele size range across three Bromeliaceae subfamilies in the cross-amplification tests (Op13, Op30, Op62, Op69, Op77, Op89, and Op93) (Table 3). This indicates that this set of loci could be used in population genetic studies involving species cohesion and delimitation, phylogeography, and barriers to gene flow for other bromeliad species.

CONCLUSIONS

The data showed that the set of optimized and tested microsatellite markers will be useful for studying evolution and population genetics of *O. ophiuroides* and other species from the same genus. Furthermore, the markers can also be used for studying species within other genera of the Bromeliaceae family.

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Appendix 1. Sampling information, locality details, and voucher information for analyzed samples.

Species	Locality	Voucher (Herbarium)[a]
Vriesea gigantea Mart. ex Schult. f.	Itaimbezinho, RS, Brazil	Martinelli et al. 14897 (RB)
V. scalaris E. Morren	Santa Lucia, ES, Brazil	Coser 4070 (MBML)
Pitcairnia albiflos Herb.	Rio de Janeiro, RJ, Brazil	Wendt 593 (RB)
P. corcovadensis Wawra	Rio de Janeiro, RJ, Brazil	Wendt 18 (RB)
P. flammea Lindl.	Campina Grande do Sul, PR, Brazil	Gasper 300 (FURB)
P. staminea Lodd.	Rio de Janeiro, RJ, Brazil	Wendt 143 (RB)
Orthophytum disjunctum L. B. Sm.	Pesqueira, PE, Brazil	Louzada et al. 149 (SP)
O. foliosum L. B. Sm.	Santa Teresa, ES, Brazil	Louzada et al. 13 (SP)
O. ophiuroides Louzada & Wand.	Lençóis, BA, Brazil	Louzada & Wanderley 88 (SP)
O. zanonii Leme	Pancas, ES, Brazil	Louzada et al. 18 (SP)

Note: BA = Bahia; ES = Espírito Santo; PE = Pernambuco; PR = Paraná; RJ = Rio de Janeiro; RS = Rio Grande do Sul.

[a] Herbarium codes: FURB = Herbário Dr. Roberto Miguel Klein; MBML = Herbário Mello Leitão; RB = Herbário Dimitri Sucre Benjamin; SP = Herbário do Estado "Maria Eneyda P. Kaufmann Fidalgo".