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

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MICROSATELLITE LOCI FOR Orthophytum ophiuroides (Bromeliioideae, Bromeliaceae) SPECIES ADAPTED TO NEOTROPICAL ROCK OUTCROPS

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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 systems or pollination syndromes.

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.

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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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 *RsaI* (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’-CAGCAGCCTGTGAAACGAC-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 (HE), observed heterozygosity (HO), 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 GENEPopol version 3.5 (Raymond and Rousset, 1995). For population 2, which showed a tetraploid genotyping...
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 (Bromeliioideae); Vriesea gigantea Mart. ex Schult. f. and V. scalaris E. Morren (Tillandsioideae); and P. albifrons Herb., P. corcovadensis Wawra, P. flammae (Lindl.), and P. staminea Loddi. (Pitcairnioideae).

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 and 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 more than two bands per individual, while population 2 showed a tetralkyploid 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). H₁ and H₂ 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) 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 the 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.

### LITERATURE CITED


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

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

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

* 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”.

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