Conserv Genet
DOI 10.1007/s10592-008-9514-4

TECHNICAL NOTE

Isolation and characterization of microsatellite loci in the Brazilian orchid *Epidendrum fulgens*

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Received: 7 January 2008 / Accepted: 8 January 2008

Abstract *Epidendrum fulgens* has a patchy distribution along the Atlantic Rainforest in the Brazilian coast, due to the destruction of its native habitat. Here, we report on both the development of nine new microsatellite markers isolated from this species and the characterization of their allele variability in two distant and unrelated populations. The number of alleles observed for each locus ranged from 2 to 17 with an average of 6.4 alleles per locus. These microsatellites should be valuable tools for studying the effect of habitat fragmentation on the genetic structure of *E. fulgens* populations.

Keywords *Epidendrum* · Orchidaceae · Microsatellites · Atlantic Rainforest · Cross-amplification

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Epidendrum fulgens is an endemic orchid of coastal Brazilian Atlantic Rainforest. In the past, this orchid had a wide geographic distribution between the Brazilian States of Rio de Janeiro and Rio Grande do Sul but, nowadays, its populations are small and fragmented due to habitat loss. The knowledge of patterns of genetic diversity and gene flow is essential to guide conservation management decisions and for understanding the genetic consequences of habitat loss in fragmented populations. Therefore, the aim of this study was to develop a set of polymorphic microsatellite (simple sequence repeat—SSR) markers for *E. fulgens* for describing the population genetic structure of this threatened species.

Total DNA was extracted from silica gel exsiccated leaves of *E. fulgens* following the protocol of Doyle and Doyle (1990). Marker isolation involved the construction of a genomic library partially enriched for $(CT)_n$ and $(GT)_n$ repeats by using biotinylated oligonucleotide sequences bound to Streptavidin-coated magnetic particles as described by Kijas et al. (1994) with modifications by Billote et al. (1999). Microsatellite-enriched DNA fragments were ligated into pGEM-T Easy vector (Promega) as described by supplier and used to transform XL1Blue competent *E. coli* cells (Stratagene). A total of 96 recombinant colonies were obtained and sequenced using the BigDye v3.1 terminator kit on the ABI PRISM 3130 Sequence Analyser (Applied Biosystems). For 25 clones, containing SSR motifs, forward and reverse sequences were aligned in SeqMan (DNASTAR package), and primers were designed for 16 loci using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

For each SSR, the forward primers were synthesized with a 19 bp long M13 tail (5′-CACGACGTTGTAAAACGAC-3′) following the amplification method of Schuelke (2000), which involved three primers: a forward

Table 1 Characteristics of microsatellite loci from *Epidendrum fulgens*, including locus name, primer sequences, repeat type (interrupted microsatellites are indicated by a (...) between repeats), no. of alleles (A), allele size range, observed (H_O) and expected (H_E) heterozygosity for each population, and test for departure from Hardy–Weinberg equilibrium (HWE)

Locus	Primer sequences[a]	Repeat	A	Size range (bp)	Guaratuba		Imbituba	
					H_O	H_E	H_O	H_E
EFF06	F: TCAAGCCTATCATAAGTGCTCCA	$(CA)_8$	4	364–370	0.500	0.567	0.500	0.633
	R: CCTTGTTGCAACTGGGTGTT							
EFF26	F: TGTCCTAAGTCAAGTGGGGTTT	$(GT)_{15}$	4	199–205	0.400	0.343	0.550	0.680
	R: TCCGAGTCTGTCGGTCTTTT							
EFF29	F: TCCGCTGATTTGAGTTTGCT	$(TC)_{33}$	17	185–229	0.900	0.910	0.700	0.915*
	R: CTGGTCCCGTAAGATCAATCAC							
EFF43	F: TGCCCCACAGACAATTAAGC	$(GA)_9$	6	148–160	0.100	0.097	0.700	0.584
	R: CCTCGATGGAACCCCATAAT							
EFF45	F: TTGGGTTTCGTCTCACATCA	$(CT)_{11}...(CT)_4$	4	288–294	0.450	0.514	0.550	0.670
	R: CCCTCAGTATCCGCCACTT							
EFF51	F: CTTGTCTACGTGAGGGCACTG	$(GT)_8$	5	369–377	0.700	0.744*	0.600	0.573
	R: TCAACAACGTGAAAAGCCATC							
EFF58	F: TGAATGCTTATACTCTCCCATCA	$(CA)_7$	2	210–212	0.200	0.184	0.200	0.430*
	R: AAGTGGCAAAGCACCATGTA							
EFF61	F: TGTCCCCTATATTCTGATGGTG	$(CA)_9$	2	264–266	0.050	0.050	0.350	0.357
	R: AGGGTTTTAGGTCAAAGTGCTC							
EFF70	F: CGCGAGATTGTTCCAAACC	$(AG)_{30}$	14	321–349	0.750	0.866*	0.700	0.896*
	R: GCTCCACGCAAAACCTTTTTA							

Genbank Accession nos. EU363791–EU363799

[a] All forward primers were M13-tailed at the 5′ end. Significant departures from HWE: $*P < 0.001$

Table 2 Cross-species and genera amplification of nine microsatellite primers from *Epidendrum fulgens* within the subtribe Laeliinae

Species	EFF6	EFF26	EFF29	EFF43	EFF45	EFF51	EFF58	EFF61	EFF70
Epidendrum xanthinum	−	+	W	+	+	−	+	W	+
Epidendrum secundum	−	+	W	+	+	+	+	+	++
Pseudolaelia cipoensis	−	+	W	+	−	−	W	W	−
Cattleya eldorado	−	−	W	+	+	−	W	+	−
Prosthechea vespa	−	+	W	+	−	−	W	+	−

Successful amplifications with a single band visualized (+), successful amplifications with more than one band visualized (++), weak amplifications (W) and failed amplifications (−) are indicated

SSR-specific primer with the M13 tail at its 5′ end, a reverse locus-specific primer, and a universal M13 primer labelled with a fluorescent dyes, 6-FAM or JOE (Applied Biosystems) respectively. All PCR amplifications were performed in a Applied Biosystems 2700 thermocycler in 10 µl reactions containing: 10 ng DNA template, 1× PCR buffer, 2 mM $MgCl_2$, 100 µM dNTPs, 1 pmol forward primer, 4 pmol reverse primer, 0.4 pmol universal M13 primer and 0.5 U *Taq* polymerase (Amersham Pharmacia Biotech). A 'touchdown' cycling program was used: 95°C for 3 min, then 10 cycles of 94°C for 30 s, 58°C decreasing to 48°C at 1°C per cycle for 30 s, 72°C for 30 s followed by 40 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 30 s, followed by a final extension of 20 min at 72°C. Microsatellite alleles were resolved on a 3130 DNA Sequence Analyser and were sized with LIZ (500) standard by using GENEMAPPER v3.7 software (Applied Biosystems).

A total of 40 individuals from two Brazilian populations of *E. fulgens* (Imbituba and Guaratuba) were analyzed to evaluate SSR polymorphism. ARLEQUIN 3.11 (Excoffier et al. 2005) software was used to calculate observed (H_O) and expected (H_E) heterozygosity, to test for departure from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium between all pairs of loci. MICRO-CHECKER (Van Oosterhout et al. 2004) software was used to quantify genotyping errors. Nine SSRs were polymorphic, with number of alleles per locus ranging from 2 to 17 with an average of 6.4 alleles per locus. The observed and expected heterozygosities (H_O and H_E) ranged from 0.10 to 0.90 and 0.05 to 0.91, with averages of 0.494 and 0.556, respectively (Table 1). Four loci showed a significant departure from Hardy–Weinberg equilibrium ($P < 0.001$), three due to heterozygote deficiency (EFF29, EFF58 and EFF70) in Imbituba population, and one due to heterozygote excess (EFF51) in the Guaratuba population. The small size of the Imbituba population and fragmentation history of the region may be the factors promoting the local observed low levels of heterozygosity. No linkage disequilibrium was detected among any pair of loci, and no genotyping errors due to presence of null alleles, short allele dominance or scoring of stutter peaks was detected.

Cross-species and cross-genera amplification were performed on *Epidendrum* species and allied genera, on single individuals by using the same amplification conditions used for *E. fulgens*. Several positive amplifications occurred across all tested species (Table 2).

The primers proved to be useful in revealing levels of diversity in *E. fulgens* and thus can be used to explore the genetic structure of scattered populations across its actual geographical range.

Acknowledgements We thank C. Palma-Silva and R. P. Romanini for technical support and helpful discussions on this manuscript. This work was supported by grants from FAPESP (06/54189-3) and CNPq (471929/2006-9) to F. Pinheiro and F. Barros, and by awards from CAPES to F. Pinheiro and CNPQ to F. Pinheiro, F. Barros, A. Salatino and A. P. Souza.

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