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Isolation and characterization of microsatellite loci in *Epidendrum puniceoluteum*, an endemic orchid from the Atlantic Rainforest

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Abstract

***Epidendrum puniceoluteum* is an endemic orchid of Atlantic Rainforest, restricted to few populations only due to the destruction and fragmentation of its native habitat. Here, we report on the development of 10 microsatellite markers isolated from this orchid species. Genetic variability was characterized in two distant populations from Brazil coast. The number of alleles observed for each locus ranged from two to 12 and with an average of 6.4 alleles per locus. These microsatellites should be valuable tools for studying both fine-scale genetic structure of scattered *E. puniceoluteum* population and patterns will be useful genetic markers for other closely related taxa.**

Keywords: Atlantic Rainforest, cross-amplification, *Epidendrum*, microsatellites, Orchidaceae

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The fragmentation of the Atlantic Rainforest, a worldwide biodiversity hotspot, represents a dramatic process with a strong impact on genetic diversity of local plant community (Cardoso *et al*. 2005). Knowledge about demographic events that shape the genetic patterns in fragmented populations can help conservation efforts to preserve threatened species. Among the several Neotropic orchids of the Atlantic Rainforest, *Epidendrum* is the largest genus showing great morphological diversity that has recently generated many taxonomic doubts about its generic classification and species delimitation (Hágsater 1984). *Epidendrum puniceoluteum* is a recently described orchid species (Pinheiro & Barros 2006) that had a wide distribution in the past, from São Paulo and Rio Grande do Sul States, Brazil, but is now restricted to few populations only due to the destruction of its native habitat. Here, we report on the development of a set of polymorphic microsatellite [simple sequence repeat (SSR)] markers for *E. puniceoluteum* that will be useful in addressing questions on the genetic structure of this endangered species.

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Total genomic DNA was extracted from silica gel-exsiccated leaves of *E. puniceoluteum* following the protocol of Doyle & Doyle (1990). Markers 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 the supplier and used to transform XL1-Blue competent *Escherichia coli* cells (Stratagene). A total of 96 recombinant colonies were obtained and sequenced using the BigDye version 3.1 terminator kit on the ABI PRISM 3130 Sequence Analyser (Applied Biosystems). For 21 clones, containing SSR motifs, forward and reverse sequences were aligned in SEQMAN (DNASTAR package), and primers were designed for 14 loci using the PRIMER 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Table 1 Characteristics of microsatellite loci from *Epidendrum puniceoluteum*, 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 the significance of the test for departure from Hardy–Weinberg equilibrium (HWE). Locus EPP49 was monomorphic for Imbituba population. GenBank Accession nos EU326290–EU326299

					Pontal		Imbituba	
Locus	Primer sequences†	Repeat	A	Size range (bp)	H_O	H_E	H_O	H_E
EPP08	F: TGTTCAAGAACAACATCGGACT R: TCTTGCTGGTTGGCATTATCT	$(GA)_9$	3	219–223	0.100	0.184	0	0.405*
EPP10	F: GGAGGCCAATGTGATGAAAC R: TCGAATAAGCTCCTGCATCC	$(GT)_5 \ldots (AG)_9 \ldots (AG)_{25}$	6	234–250	0.350	0.344	0.400	0.478
EPP12	F: GTCGGTGAGGGTCCAGAAA R: CACCATCTTCTCTCCCCTGAG	$(GA)_{21}$	9	177–197	0.600	0.715	0.400	0.457
EPP17	F: AGCACATCCGGGCCTAACTA R: TGCCTGGCATCCATAATGAC	$(TC)_{13}T(TC)_9$	10	203–223	0.700	0.750	0.250	0.708*
EPP18	F: TGCATACGTAACAACTGGAGGT R: GGAAGGTCATTCTAACCAGGAA	$(AG)_{24}$	12	288–324	0.350	0.321	0.400	0.503
EPP49	F: GCAAAGGGAGACGATTTGAG R: AGCATTTTTCGCCCTTAACA	$(GA)_{17}$	2	182–186	0.150	0.142	mono	mono
EPP56	F: ACGCTCTTTGGCTGGAACT R: CTCACATGCCTTTAGCCTCAC	$(TC)_{16}$	2	136–144	0.150	0.142	0.300	0.492
EPP86	F: CAGCCTTTAGGCATTCTTGG R: GCTCATTGGCCTTAGTGACC	$(GA)_{14}$	11	215–239	0.550	0.650	0.950	0.846
EPP89	F: TTCTTGTTGTCGCCTTCGAT R: TCAGAGAGCTCGTCCGACA	$(GA)_3AA(GA)_3 \ldots (GA)_{10}$ GG$(GA)_5$	4	284–290	0.300	0.328	0.350	0.314
EPP96	F: TCTAACATGCGAAGGCAAAA R: TTTGGTTGTTAAGCCCCATT	$(AG)_{12}$	5	291–299	0.650	0.544	0.600	0.635

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

For each SSR, the forward primers were synthesized with a 19-bp long 5′ M13 tail (5′-CACGACGTTGTAAAACGAC-3′) following the amplification method of Schuelke (2000). All polymerase chain reaction (PCR) amplifications were performed in an Applied Biosystems 2700 thermocycler in 10-μL reactions containing: 10 ng template, 1× PCR buffer (Amersham Pharmacia Biotech), 2 mM $MgCl_2$, 100 μM dNTPs, 1 pmol forward primer, 4 pmol reverse primer, 0.4 pmol label (6-FAM or JOE: Applied Biosystems) M13 primer and 0.5 U *Taq* polymerase (Amersham Pharmacia Biotech). A 'touchdown' cycling programme 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. PCR products were resolved on a 3130 DNA Sequence Analyser and were sized with Genescan 500 LIZ size standard using GENEMAPPER version 3.7 software (Applied Biosystems).

A total of 40 individuals from two Brazilian populations of *E. puniceoluteum* (Imbituba and Pontal do Paraná) were analysed to evaluate SSR polymorphism. ARLEQUIN 3.11 (Excoffier *et al*. 2005) software was used to calculate observed and expected heterozygosities, and to test for departure from Hardy–Weinberg equilibrium and for linkage disequilibrium between all pairs of loci. Ten SSRs were polymorphic, with the number of observed alleles per locus ranging from two to 12 with an average of 6.4 alleles per locus. The observed heterozygosity for the polymorphic loci ranged between zero and 0.95 with an average of 0.377 (Table 1). We found an absence of polymorphism at locus EPP49 in the Imbituba population. In the same population, two loci (EPP8 and EPP17) showed a significant departure from Hardy–Weinberg equilibrium ($P < 0.001$), due to heterozygote deficiency. The small size of the Imbituba population and fragmentation history of the region may be causing the observed low levels of diversity and the Hardy–Weinberg disequilibrium for some loci in this population. No linkage disequilibrium between any pair of loci was detected.

The primers proved to be useful in revealing levels of diversity in both populations and thus can be used to explore the genetic structure of fragmented populations of *E. puniceoluteum* across its actual geographical range. Historical demographic patterns such as bottlenecks and range contraction will be compared with information on reproductive success and seed dispersal ability in order to identify the evolutionary processes that are shaping their actual genetic patterns of populations.

Table 2 Cross-species and genera amplification of 10 microsatellite primers from *Epidendrum puniceoluteum* within the subtribe Laeliinae. Successful amplification with a single band visualized with expected allele size (+), successful amplification with more than one band visualized, with at least one band with the expected allele size (++), weak amplification of a band with the expected allele size (W) and failed amplification (–) are indicated

Species	EPP8	EPP10	EPP12	EPP17	EPP18	EPP49	EPP56	EPP86	EPP89	EPP96
Epidendrum xanthinum	+	W	+	+	+	+	++	++	+	+
Epidendrum secundum	+	W	+	–	++	++	++	+	+	+
Pseudolaelia cipoensis	+	–	+	+	W	+	–	++	+	W
Cattleya eldorado	W	–	W	–	W	+	+	+	+	W
Prosthechea vespa	+	–	+	–	+ +	+	++	+	+	W

Cross-species and cross-genera amplification of the microsatellite primers were performed on *Epidendrum* species and related members from subtribe Laeliinae (Table 2). Amplification was performed on single individual of each tested species using the same amplification conditions used for *E. puniceoluteum*. Several positive amplifications of PCR products with the expected allele sizes occurred across all tested species. These primers therefore should be useful for population studies both in *E. puniceoluteum* and in related species and genera, contributing to the knowledge about diversification processes and conservation in South American orchids.

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