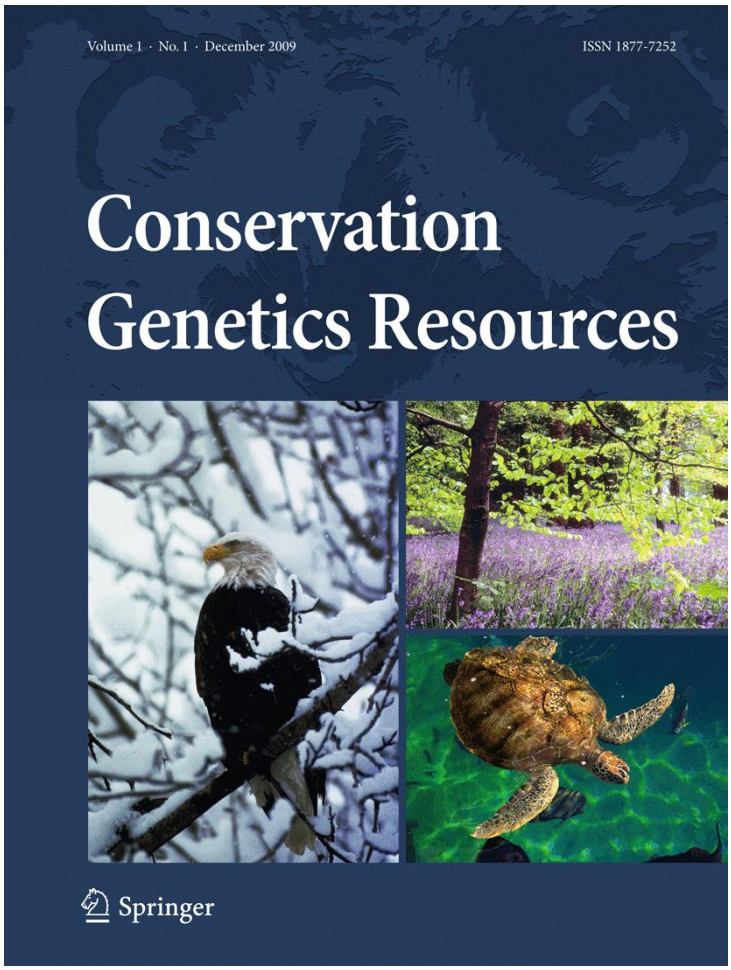
ISSN 1877-7252, Volume 1, Number 1



Conservation Genet Resour (2009) 1:505–511
DOI 10.1007/s12686-009-9121-9

TECHNICAL NOTE

Chloroplast microsatellite markers for the Neotropical orchid genus *Epidendrum*, and cross-amplification in other Laeliinae species (Orchidaceae)

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Received: 2 October 2009 / Accepted: 4 October 2009 / Published online: 25 October 2009

Abstract One of the most significant challenges confronting orchid researchers is the lack of specific molecular markers, mainly for species in the Neotropics. Here we report the first set of specific chloroplast microsatellite primers (cpSSR) developed for Neotropical orchids. In total, nine polymorphic cpSSR loci were isolated and characterized in four species occurring in the Brazilian Atlantic Rainforest: *Epidendrum cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum*. Levels of intraspecific polymorphism were characterized using two populations for each species, with 13–20 individuals each. Allele numbers varied from two to three per locus, while the number of haplotypes ranged from three to six per species. Extensive differentiation among the taxa was detected. All markers were successfully cross-amplified in eight other different genera. These cpSSRs markers will enable novel insights into the evolution of this important Neotropical genus.

Keywords Orchidaceae · *Epidendrum* · Marker development · Chloroplast microsatellites · Cross-amplification · Populations genetics

Electronic supplementary material The online version of this article (doi:10.1007/s12686-009-9121-9) contains supplementary material, which is available to authorized users.

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Noncoding chloroplast (cp) DNA markers are a valuable resource in plant phylogenetics and evolution. The uniparentally inherited nature of the cpDNA genome is particularly useful to detect historical demographic processes, such as range contractions, expansions, and fragmentation (Cozzolino et al. 2003; Hedrén et al. 2008). Of particular interest for population level studies are chloroplast microsatellites or simple sequence repeats (cpSSR), also known as chloroplast simple sequence repeats (Provan et al. 2001) due to the high amounts of polymorphisms recovered. When genus-specific cpSSR primers are not available, de novo sequencing of noncoding chloroplast regions is the most effective and efficient way to discover chloroplast microsatellites in wild species (Ebert and Peakall 2009).

One of the most significant challenges confronting orchid researchers is the lack of specific molecular markers (Peakall 2007). Specific cpSSRs for orchid species belonging mainly to temperate regions have been developed in the last few years (Fay and Cowan 2001; Hedrén et al. 2008; Ebert et al. 2009). On the other hand, there is a

Table 1 Description of chloroplast universal primers used to search for microsatellite regions in *Epidendrum cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum*

Primer pairs (reference)	T_a (°C)	*E. cinnabarinum*		*E. denticulatum*		*E. fulgens*		*E. puniceoluteum*		Genbank accession no.[a]
		pb	SSR	pb	SSR	pb	SSR	pb	SSR	
trnH^{GUG} (1)–*psbA* (2)	55	837	T_9	839	T_{11}	839	T_{11}	839	T_{11}	GQ890570–GQ890573
3′rps16 (3)–*5′trnK*^{UUU} (3)	55	513	(C_{11}) (T_{12})	512	(C_{11}) (T_{12})	512	(C_{10}) (T_{11})	523	(C_{11}) (T_9)	GQ890574–GQ890577
trnS^{UGA} (4)–*trnfM*^{CAU} (4)	48	931	T_{13}	930	T_{12}	929	T_{11}	930	T_{12}	GQ890578–GQ890581
*rpS4*R2 (5)–*trnT*^{UGU}R (5)	50	476	A_{10}	531	A_9	520	A_9	531	A_9	GQ890582–GQ890585
trnT^{UGU}A (6)–*trnL*^{UAA}B (6)	48	549	T_{12}	475	T_9	551	T_{10}	496	T_9	GQ890586–GQ890589
ndhJ (3)–*trnL*^{UAA}E (6)	60	589	T_9	603	T_9	516	T_{10}	618	T_9	GQ890590–GQ890593
trnL^{UAA}C (6)–*trnF*^{GAA}F (6)	55	1017	(A_9) (A_{10})	1018	(A_9) (A_9)	1019	(A_9) (A_9)	1002	(A_9) (A_9)	GQ890594–GQ890597
ndhF (3)–*rpl32*R (3)	48–58	mu								
trnD^{GUC}F (4)–*trnT*^{GGU} (4)	48–58	mu								
psaI (3)–*accD* (3)	58	516	–	728	–	727	–	728	–	GQ890598–GQ890601
*psbM*F (5)–*trnD*^{GUC}R (4)	48–58	mu								
*rpl32*F (3)–*trnL*^{UAG} (3)	58	805	–	801	–	791	–	801	–	GQ890602–GQ890605
trnC^{GCA}F (4)–*psbM*R (5)	48–58	mu								
rpL16F71 (7)–*rpL16R1516* (7)	48–58	mu								
atpI (3)–*atpH* (3)	48–58	na								

Annealing temperature (T_a), size of the amplified product (base pairs) and microsatellite motif (SSR) are included

References 1, Tate and Simpson (2003); 2, Sang et al. (1997); 3, Shaw et al. (2007); 4, Demesure et al. (1995); 5, Shaw et al. (2005); 6, Taberlet et al. (1991); 7, Small et al. (1998)

-, microsatellite region absent; mu, region not sequenced due to amplification with multiple bands; na, amplification failed

Table 2 Characteristics of chloroplast microsatellite loci in *Epidendrum cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum*, including locus name and region from which it was isolated, primer sequences, repeat type (SSR), allele size range, no. of alleles (A) and expected heterozygosity (*H*E) for each population

Locus (region)	Primer sequences[a]	Species (population code)	SSR	Size	*A*	H_E
Epcp-01 (*trnH–psbA*)	*F:* TTTTGAACATAGAAAGCAATCC	*E. cinnabarinum* (PI)	T_6–T_{10}	150–154	2	0.282
	R: GATTCGGATAGAGAAGCAAAA	*E. cinnabarinum* (RR)	T_{10}	154	1	0.000
		E. denticulatum (AL)	T_{10}–T_{11}	154–155	2	0.458
		E. denticulatum (PC)	T_{11}–T_{13}	155–157	2	0.439
		E. fulgens (IT)	T_{11}–T_{12}	155–156	2	0.133
		E. fulgens (ST)	T_{11}	155	1	0.000
		E. puniceoluteum (CO)	T_{10}–T_{11}	154–155	2	0.198
		E. puniceoluteum (GU)	T_{11}	155	1	0.000
Epcp-02 (*rps16–trnK*)	*F:* TTCTTGCTTCTTTTTGTGGA	*E. cinnabarinum* (PI)	C_8–C_{11}	267–270	3	0.666
	R: ATTTGTTTGATACGCCATTG	*E. cinnabarinum* (RR)	C_8	267	1	0.000
		E. denticulatum (AL)	C_{11}	270	1	0.000
		E. denticulatum (PC)	C_{10}	269	1	0.000
		E. fulgens (IT)	C_8–C_{10}	267–269	2	0.133
		E. fulgens (ST)	C_8	267	1	0.000
		E. puniceoluteum (CO)	C_8–C_{11}	267–270	3	0.444
		E. puniceoluteum (GU)	C_{11}	270	1	0.000
Epcp-03 (*rps16–trnK*)	*F:* GTGCTAATTCAACGCAAA	*E. cinnabarinum* (PI)	T_{12}	218	1	0.000
	R: TTAAAAGCCGAGTACTCTACC	*E. cinnabarinum* (RR)	T_9	215	1	0.000
		E. denticulatum (AL)	T_{12}	218	1	0.000
		E. denticulatum (PC)	T_9	215	1	0.000
		E. fulgens (IT)	T_{10}–T_{11}	216–217	2	0.133
		E. fulgens (ST)	T_{11}	217	1	0.000
		E. puniceoluteum (CO)	T_9–T_{11}	215–217	2	0.105
		E. puniceoluteum (GU)	T_9	215	1	0.000
Epcp-04 (*trnS–trnfM*)	*F:* TGCATCATGAAGGGATTTGA	*E. cinnabarinum* (PI)	T_{13}	113	1	0.000
	R: ACATGTCGACTCCATGTCCA	*E. cinnabarinum* (RR)	T_{13}	113	1	0.000
		E. denticulatum (AL)	T_{12}	112	1	0.000
		E. denticulatum (PC)	T_{12}–T_{13}	112–113	2	0.439
		E. fulgens (IT)	T_{11}	111	1	0.000
		E. fulgens (ST)	T_{11}	111	1	0.000
		E. puniceoluteum (CO)	T_{11}–T_{12}	111–112	2	0.280
		E. puniceoluteum (GU)	T_{12}	112	1	0.000

Table 2 continued

Locus (region)	Primer sequences[a]	Species (population code)	SSR	Size	*A*	H_E
Epcp-05 (*rpS4–trnT*)	*F:* TGTCGGGTCACCGATCTATT	*E. cinnabarinum* (PI)	A_{11}	141	1	0.000
	R: GGAACTTCAAGCGAAGTTTACG	*E. cinnabarinum* (RR)	A_{10}	140	1	0.000
		E. denticulatum (AL)	A_9	139	1	0.000
		E. denticulatum (PC)	A_9	139	1	0.000
		E. fulgens (IT)	A_9	139	1	0.000
		E. fulgens (ST)	A_9	139	1	0.000
		E. puniceoluteum (CO)	A_9	139	1	0.000
		E. puniceoluteum (GU)	A_9	139	1	0.000
Epcp-06 (*trnL–trnF*)	*F:* CCTAGCCCCTGAATTTCTTAG	*E. cinnabarinum* (PI)	A_{10}	191	1	0.000
	R: CTTCCAATCCAATCTCATTTG	*E. cinnabarinum* (RR)	A_9	190	1	0.000
		E. denticulatum (AL)	A_9	190	1	0.000
		E. denticulatum (PC)	A_9	190	1	0.000
		E. fulgens (IT)	A_9–A_{10}	190–191	2	0.133
		E. fulgens (ST)	A_9	190	1	0.000
		E. puniceoluteum (CO)	A_9	190	1	0.000
		E. puniceoluteum (GU)	A_9	190	1	0.000
Epcp-07 (*trnL–trnF*)	*F:* TGAGATTGGATTGGAAGAAGA	*E. cinnabarinum* (PI)	A_9	220	1	0.000
	R: TGAGGGTTCAAGTCCCTCTA	*E. cinnabarinum* (RR)	A_9–A_{10}	220–221	2	0.133
		E. denticulatum (AL)	A_9	220	1	0.000
		E. denticulatum (PC)	A_9	220	1	0.000
		E. fulgens (IT)	A_9	220	1	0.000
		E. fulgens (ST)	A_9	220	1	0.000
		E. puniceoluteum (CO)	A_9	220	1	0.000
		E. puniceoluteum (GU)	A_9	220	1	0.000
Epcp-08 (*trnT–trnL*)	*F:* AGTGCATCTTTGAATAGTGGA	*E. cinnabarinum* (PI)	T_{12}	90	1	0.000
	R: TCAATGAAATGAGAATTCAAAA	*E. cinnabarinum* (RR)	T_9	87	1	0.000
		E. denticulatum (AL)	T_9	87	1	0.000
		E. denticulatum (PC)	T_9	87	1	0.000
		E. fulgens (IT)	T_9–T_{10}	87–88	2	0.133
		E. fulgens (ST)	T_{10}	88	1	0.000
		E. puniceoluteum (CO)	T_9–T_{10}	87–88	2	0.280
		E. puniceoluteum (GU)	T_9	87	1	0.000

Table 2 continued

Locus (region)	Primer sequences[a]	Species (population code)	SSR	Size	*A*	H_E
Epcp-09 (*ndhJ–trnL*)	*F:* TAGGATGATGCACGGGAAA	*E. cinnabarinum* (PI)	T_9–T_{10}	241–242	2	0.384
	R: GGGGGTTTTTATCATTGAGGA	*E. cinnabarinum* (RR)	T_9–T_{10}	241–242	2	0.505
		E. denticulatum (AL)	T_9	241	1	0.000
		E. denticulatum (PC)	T_9–T_{10}	241–242	2	0.439
		E. fulgens (IT)	T_{11}	243	1	0.000
		E. fulgens (ST)	T_{10}–T_{11}	242–243	2	0.400
		E. puniceoluteum (CO)	T_9–T_{10}	241–242	2	0.280
		E. puniceoluteum (GU)	T_9	241	1	0.000

Locus Epcp-05 and Epcp-07 were monomorphic for all populations tested. Genbank accession n°s GQ890606–GQ890614

complete lack of specific cpSSRs for species-rich Neotropical orchid groups, limiting the options for population level research on those taxa.

The target group for the present study is the genus *Epidendrum,* the largest (1500 species) and most widespread (South United States to North Argentina) Neotropical orchid genus (Hágsater and Soto Arenas 2005). *Epidendrum* is famous for its taxonomic uncertainties regarding taxa delimitation in many species complexes, as many of those taxa show an impressive morphological diversification. Studies in *Epidendrum* are mainly limited to the description of new species, and the evolutionary processes involved in species radiation of this genus are poorly understood.

Here, we report on the development of a set of polymorphic chloroplast microsatellite markers for *Epidendrum cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum* that will be useful in addressing questions on evolutionary processes shaping the phylogeographic and genetic structure of these species, thus serving evolutionary and conservation purposes.

Total genomic DNA was extracted from silica gel-exsiccated leaves from four *Epidendrum* target species following the protocol of Pinheiro et al. (2008). cpSSR regions were isolated from *E. cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum* by sequencing 15 noncoding regions of chloroplast DNA, based on polymorphism levels described in Shaw et al. (2005, 2007). Briefly, the chloroplast DNA fragments of each species were amplified by polymerase chain reaction (PCR) using universal chloroplast primer pairs described in Table 1. All PCR were carried out in a total volume of 20 µl containing: 10 ng template, 1× Bioline PCR buffer, 2 mM Bioline $MgCl_2$, 100 µM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, and 2U *Taq* polymerase (Bioline, London, UK). Reactions were performed in a PE Applied Biosystems 9700 thermocycler by using a standard cycling program: 95°C for 3 min, 38 cycles of 94°C for 30 s, T_a (annealing temperature—Table 1) for 30 s, 72°C for 30 s and a final elongation step at 72°C for 10 min. Products were purified (QIAquick, West Sussex, UK) and sequenced using the BigDye terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a 3730 DNA Analyzer (Applied Biosystems). Forward and reverse sequences were edited in SeqMan 5.01 software (Lasergene 7.0, DNASTAR Inc.) and multiple sequence alignments were generated with MegAlign software (Lasergene 7.0, DNASTAR Inc.) using the ClustalW option. Based on sequence alignments, specific primers were designed to match regions conserved across the four *Epidendrum* species, flanking cpSSRs with nine or more uninterrupted mononucleotide repeats using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

To analyze the polymorphisms of the isolated cpSSR loci, a total of 130 samples were collected from eight natural populations (two for each species) distributed along the Brazilian Atlantic Rainforest (Table 2). Genomic DNA was extracted as described before. For each SSR, the forward primers were synthesized with a 19-bp long 5M13 tail (5′-CACGACGTTGTAAAACGAC-3′) following Schuelke (2000). PCR amplifications, genotyping and allele scoring were performed according to Pinheiro et al. (2008). To ascertain the basis of the observed polymorphism, we sequenced a large proportion of the different length variants for each locus. This allowed us to separately analyze mononucleotide repeat variation from insertion-deletion (indel) variation.

Each locus was characterized for levels of diversity using the number of alleles detected and the gene diversity (H_E) according to Nei (1978). CpSSR length variation was combined to define the haplotype of each individual, and levels of diversity of each population were characterized using the number of haplotypes and the analysis of molecular variance (AMOVA), using the software Arlequin 3.01 (Excoffier et al. 2005). Furthermore, cross-genera amplification tests were performed with these loci on eight related genera from subtribe Laeliinae (Table S2), using the same amplification conditions described above.

Seven regions out of the 15 universal chloroplast regions tested contained microsatellite loci (Table 1). The *rps16*/*trnK*^{UUU} and *trnL*^{UAA}/*trnF*^{GAA} regions contained two microsatellite loci each. In total, primers were designed for nine loci (Table 2), and all of them were polymorphic within and/or among species. Between one and three alleles were detected per polymorphic locus, and genetic diversity ranged between 0 and 0.66 (Table 2). The sequencing of polymorphic alleles revealed that the polymorphisms were, indeed, restricted to length variation occurring in the mononucleotide repeats. The number of haplotypes ranged from three to six per species. Unique haplotypes were found for different species and populations (Fig. S1). The analysis of molecular variance (AMOVA) across all populations and species revealed extensive and significant ($P < 0.001$) differentiation among the four species (32.5%), among populations within species (51.6%) and within populations (15.9%). All loci were successfully amplified in the related genera tested for their cross-amplification potential, showing PCR products with the expected allele sizes across all tested species (Table S2).

The results showed that these loci provide cpSSR markers with polymorphisms at different levels, useful in species delimitation, inter and intraspecific phylogeographic studies and for characterization of historical demographic processes. The extensive difference in chromosome numbers and hybridization events reported for many *Epidendrum* species (Hágsater and Soto Arenas 2005) imposes many challenges for molecular marker-based research on this genus. In such groups, results obtained with nuclear markers alone are often difficult to interpret due to independent events of polyploidization and hybridization (Hedrén et al. 2008). Differences in ploidy levels and chromosome numbers do not affect results obtained with organellar markers. Furthermore, when results from nuclear marker loci are combined with results from chloroplast markers, the direction and extension of introgression can be measured (Lexer et al. 2005), facilitating the depiction of complex scenarios of hybridization and species radiation. Chloroplast markers are tools that can overcome these challenging characteristics of *Epidendrum*, and the loci described and characterized here should be useful for population studies both in *Epidendrum* species and in related genera, thus contributing to the knowledge on diversification processes and conservation strategies in South American orchids.

Acknowledgments We thank Felipe N. M. de Assis for help during field work, Robyn S. Cowan, Dion Devey and Edith Kapinos for helping in the laboratory. This research was funded by grants from the Prance Fellowship in Neotropical Botany under the Kew Latin American Research Fellowship Programme (KLARF) to FP, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq—471929/2006-9) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP—06/54189-3) to FB. FP received fellowship from CNPq.

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