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Short Communication

Cross-amplification and characterization of microsatellite loci for the Neotropical orchid genus *Epidendrum*

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Abstract

In this study we tested the cross-amplification of 33 microsatellite loci previously developed for two closely related Neotropical orchid genera (*Epidendrum* and *Laelia*). A set of ten loci were polymorphic across five examined species (20 individuals each) with 2 to 15 alleles per locus. The mean expected and observed heterozygosity (average across species) ranged from 0.34 to 0.82 and from 0.27 to 0.85, respectively. In addition we tested all loci in 35 species representative of the genus *Epidendrum*. Of these, 26 loci showed successful amplification. Cross-application of these loci represent a potential source of co-dominant markers for evolutionary, ecological and conservation studies in this important orchid genus.

Key words: Epidendrum, Orchidaceae, short tandem repeat, cross-amplification.

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Epidendrum L. is the largest of the orchid genera in the Neotropical region, with approximately 1500 species (Hágsater and Arenas, 2005). Species of this genus display extensive variation in morphological features and growth habits (epiphytic, lithophytic and terrestrial). Moreover, they offer an interesting opportunity for exploring the influence of human activities on natural environments, since they occur within several types of threatened vegetation (Amazon and Atlantic Rainforests, savannas, coastal sand dunes, 'tepuis', and 'páramos').

Microsatellite markers are the current choice for most studies on evolution, ecology and conservation, due to their high levels of polymorphism and high reproducibility. Studies increasingly aim at comparing genetic, demographic, behavioural, and breeding system parameters among related species. To address these questions, researchers require 'universal' genetic markers that can easily be transferred between species (Barbara *et al.*, 2007). The capacity to transfer and apply the same set of microsatellite loci in different species can significantly facilitate studies among closely related and endemic taxa. This is important where resources for undertaking conservational genetic studies are limited, thus making it less cost-effective to develop specific microsatellite loci for many species of the same taxa.

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Although, positive cross-amplification of some microsatellite loci have been previously reported for a few species of *Epidendrum* (Cortés-Palomec *et al.*, 2008; Pinheiro *et al.*, 2008a, 2008b), to date, there is no precise and standardized information about amplification-efficiency or polymorphism in these loci. The aim of this study is to report the potential of cross-species transferability of microsatellite markers across the genus *Epidendrum* in order to identify a set of polymorphic loci available for inquiries assessing the effect of landscape fragmentation on gene flow, species delimitation, origin and the maintenance of reproductive barriers among species of this genus.

Total genomic DNA was extracted from silica gel-exsiccated leaves according to the Pinheiro *et al.* (2008a) protocol. We tested three sets of microsatellite markers previously described for *Epidendrum fulgens* (nine loci - Pinheiro *et al.*, 2008a), *E. puniceoluteum* (ten loci - Pinheiro *et al.*, 2008b) and *Laelia speciosa* (14 loci - Cortés-Palomec *et al.*, 2008), the latter a closely related genus belonging to the subtribe Laeliinae, the same subtribe as that of *Epidendrum* (Hágsater and Arenas, 2005). Altogether, we tested 33 microsatellite loci (Table S4) for cross-amplification in 35 species belonging to different sections of genus *Epidendrum*.

For each microsatellite locus, the forward primers were synthesized with a 5'-M13 tail according to the Schuelke (2000) method, involving three primer polymerase chain reactions (PCRs), including a universal M13 primer labelled with a fluorescent dye, 6-FAM (Applied Biosystems). All PCR amplifications were performed in an Applied Biosystems 2700 thermocycler according to Pinheiro *et al.* (2008a, 2008b). The conditions were maintained constant for all loci so as to maximize standardization. Microsatellite alleles were resolved on a 3130 Genetic Analyzer (Applied Biosystems) and sized in accordance with LIZ (500) standard by using GENEMAPPER v. 3,7 software (Applied Biosystems).

We initially tested the potential of cross-amplification for all loci with one sample from each of the 35 *Epidendrum* species. Furthermore, we focused our effort on five of those species belonging to different phyletic sections of the genus *Epidendrum* (Hágsater and Arenas, 2005): *E. denticulatum, E. secundum, E. campestre, E. densiflorum* and *E. rigidum*. We sampled 20 individuals from each species of a single population (Table S1). GENEPOP software (Raymond and Rousset, 1995; web version 3.4) was used to calculate observed (H_O) and expected (H_E) heterozygosity, and to test for departure from Hardy - Weinberg equilibrium (HWE) as well as for link-

Table 1 - Size range of the PCR products, number of observed alleles (A), expected and observed heterozygosity (H_E/H_O), and the significance of the test for departure from Hardy-Weinberg equilibrium, for the ten selected microsatellite loci (indicated by rows) in each of the five *Epidendrum* species. The size range of the original alleles described by the authors is indicated in parentheses below each locus.

Locus	Species	size range	A	H_E/H_O
EPP08	*E. campestre*	211-219	3	0.19/0.20
(219-223)	*E. densiflorum*	211-229	5	0.71/0.36
	E. denticulatum	211-213	2	0.51/1.00*
	E. rigidum	201-215	4	0.64/0.05*
	E. secundum	213-221	4	0.28/0.30
	Mean		3.6	0.47/0.38
EPP18	*E. campestre*	274-314	11	0.88/0.89
(288-324)	*E. densiflorum*	284-290	3	0.68/0.70
	E. denticulatum	288-328	15	0.92/0.84
	E. rigidum	284-312	4	0.64/0.55
	E. secundum	284	monomorphic	-
	Mean		8.3	0.62/0.60
EPP49	*E. campestre*	no amplification	-	-
(182-186)	*E. densiflorum*	162-187	9	0.79/0.32*
	E. denticulatum	176-190	6	0.78/0.70
	E. rigidum	170-186	3	0.46/0.10
	E. secundum	176-186	6	0.83/0.63
	Mean		6	0.71/0.44
EPP56	*E. campestre*	136-154	3	0.14/0.10
(136-144)	*E. densiflorum*	148-152	2	0.10/0.10
	E. denticulatum	132-166	10	0.87/0.85
	E. rigidum	152-156	2	0.51/0.00*
	E. secundum	122-162	9	0.78/0.28*
	Mean		5.2	0.48/0.27
EPP86	*E. campestre*	217-231	8	0.83/0.85
(215-239)	*E. densiflorum*	217-227	6	0.81/0.80
	E. denticulatum	217-223	4	0.71/0.60
	E. rigidum	217-235	7	0.83/1.00*
	E. secundum	215-229	8	0.85/1.00
	Mean		6.6	0.81/0.85

Locus	Species	size range	A	He/Ho
EFF26	*E. campestre*	190-204	8	0.76/0.84
(199-205)	*E. densiflorum*	196-202	4	0.66/0.95
	E. denticulatum	164-202	5	0.64/0.65
	E. rigidum	196-204	5	0.66/0.75
	E. secundum	192-204	6	0.77/0.88
	Mean		5.6	0.70/0.81
EFF45	*E. campestre*	280-284	3	0.50/0.35
(288-294)	*E. densiflorum*	288-294	3	0.49/0.53
	E. denticulatum	278-294	5	0.32/0.25
	E. rigidum	288-340	5	0.81/0.35*
	E. secundum	288-294	4	0.67/0.55
	Mean		4	0.56/0.41
EFF58	*E. campestre*	210-212	2	0.46/0.68
(210-212)	*E. densiflorum*	210-216	4	0.66/0.95
	E. denticulatum	212	monomorphic	-
	E. rigidum	210-212	2	0.51/1.00
	E. secundum	212	monomorphic	-
	Mean		2.7	0.34/0.54
Lspe-1	*E. campestre*	219-221	2	0.36/0.35
(350-390)	*E. densiflorum*	215-225	4	0.49/0.35
	E. denticulatum	471-493	3	0.34/0.28
	E. rigidum	225-233	2	0.49/0.00*
	E. secundum	462-488	9	0.85/0.85
	Mean		4	0.51/0.37
Lspe-3	*E. campestre*	250-266	8	0.83/0.79
(224-250)	*E. densiflorum*	250-288	12	0.88/0.40*
	E. denticulatum	262-304	15	0.93/1.00
	E. rigidum	268-286	5	0.78/0.25*
	E. secundum	244-260	6	0.69/0.90
	Mean		9.2	0.82/0.67

Significant departures from HWE: *p < 0.05.

age disequilibrium at each locus, by applying the Bonferroni correction to account for multiple comparisons.

Among the 33 loci tested, 26 showed positive amplification and PCR products with the expected allele sizes throughout most of the 35 species tested (Table S2). The percentage of cross-amplification was 78% on an average, thus higher than the mean value reported for monocot species (60% - Barbara *et al.*, 2007).

A total of ten polymorphic loci exhibited the features so desired for use as co-dominant molecular markers in the five examined species (Table 1), with the number of alleles per locus ranging from two to 15 (overall mean 5.6 alleles) Expected and observed heterozygosity ranged from 0.34 to 0.82 and 0.27 to 0.85, respectively (an average of 0.60 and 0.53, respectively) (Table 1). For each sampled population of the five species, we found sporadic cases of departure from HW equilibrium ($p < 0.05$): for loci EPP8 (in *E. denticulatum* and *E. rigidum*), EPP49 (in *E. densiflorum*), EPP56 (in *E. rigidum* and *E. secundum*), EPP86 (in *E. rigidum*), EFF45 (in *E. rigidum*), Lspe-1 (in *E. rigidum*) and Lspe-3 (in *E. rigidum*). Interestingly, six out of ten loci in *E. rigidum* departed significantly from HW equilibrium due to heterozygotic deficiency. Such deviations could be caused by inbreeding and/or Wahlund effects arising from secondary population subdivision. Although null alleles cannot be ruled out, there was no evidence of scoring error due to 'stuttering' or 'large allele dropout', when using MICRO-CHECKER software (van Oosterhout *et al.*, 2004. Three loci in *E. campestre*, five loci in *E. denticulatum,* five loci in *E. rigidum* and seven in *E. secundum* exhibited linkage disequilibrium ($p < 0.001$). Loci that were monomorphic or not amplified in most of the five *Epidendrum* species are listed in Table S3.

This study unveiled evidence that cross-transferability of developed microsatellite loci can increase the availability of markers to address both ecological and evolutionary questions in *Epidendrum*. The markers tested here showed to be of great potential for the use in comparing multiple co-occurring *Epidendrum* species in different ecological communities, thus contributing to knowledge on diversification processes and conservation among neotropical orchids.

Acknowledgments

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Supplementary Material

The following online material is available for this article:

Table S1 - Geographical region, sample size and Biome of species sampled.

Table S2 - Cross-species amplification of 26 loci tested for 30 additional *Epidendrum* species. Size range of the PCR products and unsuccessful amplifications are indicated (-).

Table S3 - Size range of the PCR products, number of observed alleles (A), expected heterozygosity (He), observed heterozigosity (Ho), and the significance of the test for departure from Hardy - Weinberg equilibrium (HWE - Significant departures from HWE: $p < 0{,}001$), for the microsatellite loci (indicated by rows) that were not detected as polymorphic (monomorphic), or not amplified (na) in most of the five *Epidendrum* species. The size range of the original alleles described by the authors is indicated in parentheses on the bottom of each locus.

Table S4 - Primer names, sequences and Genbank Accession numbers of 33 orchid species SSR loci.

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Table S1. Geographical region, sample size and Biome of species sampled.

Species	**N**	**Population**	**Biome**
E. denticulatum	20	Araruama, Rio de Janeiro, RJ	Atlantic Rainforest
E. densiflorum	20	Ilha do Cardoso, Cananéia, SP	Atlantic Rainforest
E. rigidum	20	Ilha do Cardoso, Cananéia, SP	Atlantic Rainforest
E. secundum	20	Serra do Cipó, Conceição do Mato Dentro, MG	Cerrado
E. campestre	20	Serra do Cipó, Conceição do Mato Dentro, MG	Cerrado

Table S2. Cross-species amplification of 26 loci tested for 30 additional *Epidendrum* species. Size range of the PCR products and failed amplifications are indicated (-).

Locus	**epp_8§**	**epp_10§**	**epp_18§**	**epp_86§**	**epp_49§**	**epp_56§**	**epp_89§**	**epp_96§**	**eff_06Ψ**	**eff_26Ψ**	**eff_29Ψ**	**eff_43Ψ**	**eff_45Ψ**
species													
E. xanthinum	213	250-264	306-308	221-227	170-179	-	291	292-294	372	200-202	193	150	288
E. cattilus	214-216	306	280-290	219-241	156-165	119-146	-	-	-	200-202	198	150	286
E. calanthum	215	-	290-331	217-223	156-165	119	-	-	372	-	205	150	-
E. funkii	215-219	252-266	278	223-241	157-166	159	-	-	-	200-202	-	150	282-286
E. myrmecophorum	213-219	278	284-286	219-225	152-161	144-154	-	-	-	200-202	-	150	288
E. purpureum	213	268	284-300	241	-	141-153	289	300	370	198-	203	150	288
E. ibaguense	215	-	290-304	221-223	156-165	-	-	-	375	200-202	-	150	-
E. radicans	215	246	288-290	223-225	168-178	129-139	-	-	368	200-202	179-181	150	288
E. incisum	215	245	288-290	217-219	-	-	-	-	377	200-202	191	150	289
E. cinnabarinum	210	221	286	221-227	-	-	281-285	294-298	366-370	196-200	193-191	150	289
E. martianum	213-219	-	289-291	225	-	140-151	-	-	378	200-202	-	150	278
E. flexuosum	219	223	290	223	161-169	-	-	-	368	200-202	-	150	279
E. ramosum	213-219	250-254	290	219-223	152-160	140-151	-	-	-	200-202	-	150	288
E. saxatile	219	290	290	242	169-172	144-147	-	-	-	200-202	-	150	305-316
E. cristatum	215-219	236	284-286	219-225	-	133-144	-	-	372	200-202	-	150	289-291
E. purpurascens	219	254-261	304-306	225-227	169-172	-	-	-	-	200-202	-	150	-
E. ciliare	212-219	303,69	290-308	217-223	-	141-152	-	-	-	200-202	-	150	-
E. nocturnum	215-219	-	295-321	219-225	-	141-152	-	-	391	200-202	-	150	319-321
E. cooperianum	219	-	290	215-219	159-161	141-152	-	-	-	200-202	-	150	-
E. warasii	219	261	297	219-221	156-166	139-150	-	-	377	200-202	-	150	286
E. avicola	213-221	250-252	290-308	217-219	-	-	-	-	330	200-202	-	150	-
E. schlechterianum	212-219	290	290-308	219-227	167-169	241-246	-	-	-	200-202	-	150	-
E. coronatum	220-222	-	289-307	219	161-164	-	-	-	373	200	-	150	279
E. filicaule	219	-	290-306	225-229	139-149	-	-	-	377	200-202	-	150	-
E. chlorinum	219	-	290-306	217-219	-	-	259	-	-	200-202	-	150	321-323
E. tridactylum	212-219	-	290-306	-	188-198	-	-	-	-	200-202	-	150	-
E. vesicatum	215	-	290-308	227-229	166-168	-	-	-	-	200-202	-	150	295-301
E. latilabre	219-221	261	313	221-223	152-161	-	-	-	-	200-202	-	150	-
E. fulgens	210	265-275	286-308	219-243	152-160	-	284	-	366	199-203	201-211	150-152	291-295
E. puniceoluteum	211-219	271-273	290-310	221-227	160-184	136-144	288	294	370	197	219-225	150	289-291

§Markers isolated by Pinheiro et al (2008b); ΨMarkers isolated by Pinheiro et al (2008a); *Markers isolatated by Cortés-Palomec et al (2008).

Table S2. Continue

Locus	eff_58Ψ	eff_61Ψ	eff_70Ψ	eff_51Ψ	LS_1*	LS_3*	LS_4*	LS_6*	LS_8*	LS_9*	LS_10*	LS_11*	LS_14*
species													
E. xanthinum	212	265-271	329	335	218	249	214	172-175	221-247	219-229	-	205	254
E. cattilus	210	264	322-328	369-371	218-220	258-260	214	172-175	221	231-	-	205	254
E. calanthum	208	265	-	376-383	229	253	214	172-175	221	223-231	172-197	205	253
E. funkii	210	265	325-329	-	220	247	214	172-175	242	229-235	-	205	255
E. myrmecophorum	212-216	265	327-329	375-378	-	237-242	214	172-175	207-211	229-231	-	205	254
E. purpureum	212	265-270	328-341	375-377	215-218	233-237	214	172-175	207-211	232	172	205	254
E. ibaguense	208	265	300	-	218-220	269-271	214	172-175	208-210	231	172	205	254
E. radicans	210	265-272	308	374-376	221-227	258-260	214	172-175	243	231	-	205	252
E. incisum	210	266-275	329-331	376-382	242	241	214	172-175	221-247	229-233	-	205	254
E. cinnabarinum	210-212	266-270	325	376	-	-	214	172-175	244-246	232	-	205	252
E. martianum	210-215	262-265	320-325	-	218	260	214	172-175	226-230	229-231	197	205	258
E. flexuosum	208	265	336	-	225	250-252	214	172-175	-	231	275	205	253
E. ramosum	210-212	264-266	327-330	-	220	242	213-214	172-175	246	232	-	205	246
E. saxatile	210	266	339	-	226	234-236	214	172-175	203-205	229-231	196	205	209
E. cristatum	212	266	324-326	370	218-220	258	213	172-175	207	231	-	205	253
E. purpurascens	210	266	323-336	-	218	248-252	213-220	172-175	207-209	226-231	-	205	253
E. ciliare	222	264-266	325-329	339-340	218	259	214	172-175	214-218	227-232	219	205	259
E. nocturnum	211	264-266	329-336	372-380	217	239-242	214	172-175	220	227-233	-	205	253
E. cooperianum	216	-	323-326	373	219-222	261-263	213	172-175	237	231	-	205	253
E. warasii	216	264-266	318-330	372	218	264-272	214-218	172-175	219	231-237	196	205	246-253
E. avicola	210-213	264-266	323-325	272	-	253	213	172-175	220	232	-	205	254
E. schlechterianum	210	264-266	328	375	228-234	248	213-214	172-175	220	232	-	205	253
E. coronatum	210	262-266	318-323	373	226-228	242	213	172-175	238	231	152-172	205	249-256
E. filicaule	219	264-266	329-330	335	218	264-274	214	172-175	221	233-239	172	205	253
E. chlorinum	215	264-266	323-325	-	218	251	214	172-175	218-220	229-232	-	205	254
E. tridactylum	210-213	270-277	321-329	335	218-224	236	213	172-175	192	231-238	197-219	205	253
E. vesicatum	216	264-266	-	335	218-220	260-266	213	172-175	219-226	231	120-172	205	254
E. latilabre	210	264-266	328-338	374	216-226	268-270	213	172-175	247	231	-	205-209	253
E. fulgens	210-212	266	343-345	371-375	215	256-266	214	172-175	221-247	232	142	205	253
E. puniceoluteum	210-212	264-266	333-347	373-377	215-218	251-272	214	172-175	247	232	-	205	253

§Markers isolated by Pinheiro et al (2008b); ΨMarkers isolated by Pinheiro et al (2008a); *Markers isolatated by Cortés-Palomec et al (2008).

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Table S3. Size range of the PCR products, number of observed alleles (A), expected heterozygosity (He), observed heterozigosity (Ho) and the significance of the test for departure from Hardy–Weinberg equilibrium (HWE - Significant departures from HWE: P< 0,001), for the microsatellite loci (indicated by rows) that were not successful polymorphic (monomorphic), or not amplified (na) in most of the five *Epidendrum* species. The size range of the original alleles described for the authors is indicated in parentheses on the bottom of each locus.

Locus	***Species***	**size range**	**A**	**He**	**Ho**	**HWE**
EPP10	*E. campestre*	na	-	-	-	-
(234–250)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	252-274	9	0.836	0.900	ns
	E. rigidum	na	-	-	-	-
	E. secundum	246-278	10	0.903	0.833	***
EPP89	*E. campestre*	na	-	-	-	-
(284-290)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	279-291	6	0.200	0.432	***
	E. rigidum	na	-	-	-	-
	E. secundum	na	-	-	-	-
EPP96	*E. campestre*	286-302	5	0.643	0.765	ns
(291-299)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	282-310	9	0.849	0.500	***
	E. rigidum	na	-	-	-	-
	E. secundum	286-308	8	0.795	0.450	***
EFF29	*E. campestre*	na	-	-	-	-
(185–229)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	193-225	13	0.920	0.850	***
	E. rigidum	na	-	-	-	-
	E. secundum	na	-	-	-	-
EFF43	*E. campestre*	150	monomorphic	-	-	-
(148–160)	*E. densiflorum*	150	monomorphic	-	-	-
	E. denticulatum	150-154	3	0.405	0.400	***
	E. rigidum	150	monomorphic	-	-	-
	E. secundum	150	monomorphic	-	-	-
EFF70	*E. campestre*	353-369	7	0.800	0.800	ns
(321–349)	*E. densiflorum*	na	-	-	-	
	E. denticulatum	na	-	-	-	
	E. rigidum	na	-	-	-	
	E. secundum	na	-	-	-	

Significant departures from HWE: ns – not significant; ***$P < 0.05$.

Table S3. Continued.

Locus	*Species*	size range	No. of alleles	He	Ho	HWE
Lspe-4	*E. campestre*	213	monomorphic	-	-	-
(176–189)	*E. densiflorum*	212	monomorphic	-	-	-
	E. denticulatum	213	monomorphic	-	-	-
	E. rigidum	na	-	-	-	-
	E. secundum	213	monomorphic	-	-	-
Lspe-6	*E. campestre*	na	-	-	-	-
(176–185)	*E. densiflorum*	172	monomorphic	-	-	-
	E. denticulatum	172	monomorphic	-	-	-
	E. rigidum	173-175	2	0.097	0.100	ns
	E. secundum	172	monomorphic	-	-	-
Lspe-8	*E. campestre*	na	-	-	-	-
(222–239)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	247-251	5	0.773	0.150	***
	E. rigidum	246	monomorphic	-	-	-
	E. secundum	na	-	-	-	-
Lspe-9	*E. campestre*	229	monomorphic	-	-	-
(190–206)	*E. densiflorum*	219-235	6	0.767	0.400	-
	E. denticulatum	na	-	-	-	-
	E. rigidum	na	-	-	-	-
	E. secundum	na	-	-	-	-
Lspe-11	*E. campestre*	204	monomorphic	-	-	-
(183–184)	*E. densiflorum*	204	monomorphic	-	-	-
	E. denticulatum	204	monomorphic	-	-	-
	E. rigidum	204	monomorphic	-	-	-
	E. secundum	204	monomorphic	-	-	-
Lspe-14	*E. campestre*	208	monomorphic	-	-	-
(221–233)	*E. densiflorum*	243-251	5	0.792	0.368	***
	E. denticulatum	252	monomorphic	-	-	-
	E. rigidum	259	monomorphic	-	-	-
	E. secundum	252	monomorphic	-	-	-

Significant departures from HWE: ns – not significant; ***$P < 0.05$.