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Simple-sequence repeat markers of *Cattleya coccinea* (Orchidaceae), an endangered species of the Brazilian Atlantic Forest

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ABSTRACT. Microsatellite markers were developed for the endangered Brazilian orchid species *Cattleya coccinea* to describe its genetic diversity and structure and to support conservation studies. Nine microsatellite loci were isolated and characterized using an enriched genomic library. All loci are polymorphic at least in the 2 populations sampled, except for loci Cac05 and Cac09 for the Petrópolis population. The mean number of alleles per locus was 8.8 between populations. The mean values of the observed and expected heterozygosities were 0.541 (ranging from 0 to 1) and 0.639 (ranging from 0 to 0.9), respectively. Cross-amplifications were performed in 7 additional Epidendroideae species, and at least 2 loci were successful in 3 additional *Cattleya* species, *Epidendrum secundum*, and *Brasiliorchis gracilis*. All markers described herein will be useful in further studies evaluating the genetic diversity, population dynamics,

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and conservation genetics of *C. coccinea* and related species.

Key words: *Cattleya*; Epidendroideae; Laeliinae; Microsatellite markers; Orchidaceae; *Sophronitis*

INTRODUCTION

Orchidaceae is one of the largest plant families of angiosperms, with approximately 26,000 species (WCSP, 2012). This enormous diversity is concentrated in the Neotropics, and more than 2000 species are found in Brazil (Barros et al., 2012). *Cattleya coccinea* Lindl., with its bright, scarlet flowers, is a well-known ornamental orchid found at medium to high elevations in the Atlantic Forest in southeastern Brazil (Fowlie, 1987). Although the Atlantic Forest biome has been reduced to less than 8% of its original area, deforestation and indiscriminate collection continue to be decisive factors in the loss of genetic diversity of natural populations (Morellato and Haddad, 2000).

Microsatellite markers are ideal for population genetic studies because they are highly polymorphic owing to expression and co-dominant multiallelism. Thus, these markers are important tools for the assessment of the effects of habitat fragmentation on the diversity, demography, and genetic structure of populations, providing crucial information to guide conservation strategies.

In this study, we report the isolation and characterization of 9 microsatellite loci for the species *C. coccinea*. These markers are the first simple-sequence repeat primers developed for this genus, which is the most ornamental among Brazilian orchids. Additionally, the developed markers were tested for cross-transferability within the subtribe Laeliinae as well as in other species of the subtribe Epidendroideae.

MATERIAL AND METHODS

Forty individuals from 2 natural populations of *C. coccinea* were sampled: 20 individuals from Petrópolis (Rio de Janeiro State, Brazil, S22°26'39", W43°13'41") and 20 individuals from São José do Barreiro (São Paulo State, Brazil, S22°46'30" W44°39'63"). Voucher specimens for all species/populations sampled were deposited at the herbarium of Escola Superior de Agricultura Luiz de Queiroz, University of São Paulo (Piracicaba, SP, Brazil) (Table 1).

Table 1. DNA vouchers for specimens tested for transferability in this study.

Taxon	Voucher specimen	Collection origin	Herbarium registration number
Cattleya coccinea Lindl.	Rodrigues 761	Petrópolis/RJ	ESA120066
C. coccinea Lindl.	Koehler et al. sn (1615)*	São José do Barreiro/SP	ESA118314
C. cernua (Lindl.) Van den Berg	Bicalho sn (31219)*	Jeriquara/SP	ESA119236
C. dayana (Rchb.f.) Van den Berg	Brólio sn (5908)*	Nova Friburgo/RJ	ESA118317
C. pumila (Hook.) Van den Berg	Bicalho sn (5169)*	Santana do Riacho/MG	ESA118318
Brasiliorchis gracilis (Lodd.) R.B. Singer, S. Koehler, Carnevali	Bicalho sn (114)*	Embu-Guaçu/SP	ESA118310
Epidendrum secundum Jacq.	Pinheiro 637	Ubatuba/SP	ESA119239
Oncidium longipes Lindl.	Bicalho sn (6338)*	Dourados/MS	ESA118313
Prosthechea vespa (Vell.) W.E. Higgins	Silva sn (15861)*	Porto Velho/RO	ESA118311

*Registration number of specimen in cultivation at the orchid nursery of Instituto de Botânica de São Paulo, SP, Brazil.

A microsatellite-enriched genomic library was constructed using a procedure published by Billotte et al. (1999), with modifications. Genomic DNA was extracted from fresh leaves of *C.*

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coccinea using a modified cetyltrimethylammonium bromide method (Doyle and Doyle, 1987). Total DNA was digested with enzyme *Rsa*I, and the resulting fragments were ligated to *Rsa*21 and *Rsa*25 adapters. For enrichment, we used $(CT)_n$ and $(GT)_n$ biotinylated oligonucleotide sequences linked to streptavidin-coated magnetic particles. The enriched DNA fragments were amplified and cloned using the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into XL1-BLUE *Escherichia coli* competent cells (Stratagene, La Jolla, CA, USA).

A total of 192 positive clones were sequenced using the universal T7 and SP6 primer combination and a BigDye v3.1 terminator kit on an ABI3730 DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA); 66 clones contained simple sequence repeats. Of these, 15 regions with sufficient quality were selected for primer design. Sequences were assembled using the SeqMan software (DNASTAR, Madison, WI, USA). The selection of sequences containing microsatellites was performed using WebSat (Martins et al., 2009). Primers were designed using PRIMER 3 (Rozen and Skaletsky, 2000).

The amplification followed the protocol of Schuelke (2000), which involves a forward primer synthesized with a 19-bp M13 tail (5'-CACGACGTTGTAAAACGAC-3'), a reverse locus-specific primer, and a universal M13 primer labeled with the fluorescent dyes FAM or HEX (Applied Biosystems). All polymerase chain reaction (PCR) amplifications were performed in 30 µL volumes containing 10X PCR buffer, 1-2 mM $MgCl_2$, 2.5 deoxyribonucleotide triphosphates, 0.2 pmol forward primer, 1 pmol reverse primer, 1 pmol universal M13 tail, 1 U *Taq* DNA polymerase, and 1 µL DNA template (10-20 ng). A touchdown cycling program was used with thermal conditions as follows: 95°C for 3 min; 10 cycles at 94°C for 30 s, 58°C decreasing to 48°C at 1°C per cycle for 30 s, and 72°C for 30s; 40 cycles at 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 20 min. For some primers, the annealing temperature was optimized to minimize the amplification of unspecific bands. In these cases, we used the following thermal conditions: 95°C for 3 min; 40 cycles at 94°C for 30 s, variable annealing temperature (Table 2) for 30 s, and 72°C for 30 s; and a final extension at 72°C for 10 min.

Amplified PCR products were checked using electrophoresis on 1.5% agarose gel stained with ethidium bromide, purified with a QIAquick® PCR Purification Kit (Invitrogen, Life Technologies, São Paulo, SP, Brazil), and sequenced. Allele sizes were estimated using GeneMarker v.1.95 (Softgenetics, State College, PA, USA). The number of alleles and observed and expected heterozygosities were calculated using Genetic Data Analysis (Lewis and Zaykin, 2001).

We also checked the transferability of the designed primers by performing cross-amplifications in 7 orchid species. We sampled 3 congeneric specimens, *C. cernua* (Lindl.) Van den Berg, *C. dayana* (Rchb.f). Van den Berg, and *C. pumila* (Hook) Van den Berg; 2 species belonging to the same subtribe Laeliinae, *Epidendrum secundum* Jacq. and *Prosthechea vespa* (Vell.) W.E. Higgins, and 2 of the same subfamily Epidendroideae, *Brasiliorchis gracilis* (Lodd.) R.B. Singer, S. Koehler and Carnevali, and *Oncidium longipes* Lindl. A touchdown cycling program was used for these tests following the program described above.

RESULTS AND DISCUSSION

Of the 15 designed primers, 9 were successfully amplified and used to characterize 2 natural populations of *C. coccinea*. The 6 remaining primer pairs amplified multiple bands and were excluded from further analysis. All 9 loci were polymorphic among populations, with a mean number of alleles per locus of 8.8 between populations. The number of alleles within

populations ranged from 1 to 14 in Petrópolis and 2 to 12 in São José do Barreiro. The mean values of the observed and expected heterozygosities were 0.541 (ranging from 0 to 1) and 0.639 (ranging from 0 to 0.9), respectively (see Table 2). Low levels of heterozygosity were observed for most primers within populations, probably owing to intense habitat fragmentation. Isolation likely influenced the fixation of alleles for loci Cac08 and Cac10 in both populations analyzed.

Table 2. Characteristics of 9 microsatellite primers developed for *Cattleya coccinea* Lindl.

Locus	Sequence	Repeat	Range size (bp)	Ta (°C)	GeneBank	Petrópolis			São José do Barreiro		
						N_A	H_O	H_E	N_A	H_O	H_E
Cac01	F: TACAACGCCCAATTTGAATG R: CCATCATTTGCCTTTTCACA	$(GA)_{17}$	108	TD	JQ994289	8	0.750	0.735	9	0.250	0.838
Cac02	F: CAGGATTTCTCCTCGTGCTC R: GCAGAGCGGAACAAGGATAG	$(AG)_{18}$	173	52	JQ994290	10	0.600	0.864	7	0.150	0.784
Cac05	F: GTGCGTATTGTGAGTGGATGT R: TCACGCATGCATAAGTTCAA	$(TG)_{10}$	278	TD	JQ928870	1	0.000	0.000	3	0.000	0.477
Cac08	F: AGCATGCACTCACGATACAAA R: GTGCATGAGTGCTTGTGTGA	$(CA)_{10}$	186	TD	JQ994291	2	1.000	0.513	3	1.000	0.627
Cac09	F: GGGAGGGAGGAATAGGAAGA R: TGAGCTGCGATATCAAAGGA	$(AG)_8$	179	TD	JQ994292	1	0.000	0.050	4	0.200	0.583
Cac10	F: GCATGAGTGCTTGTGTGAAG R: TAAGCATGCACTCACGATACA	$(TG)_9$	184	TD	JQ994293	2	1.000	0.513	2	1.000	0.513
Cac11	F: TCAAGGCCTGCACATAGAGA R: AAGAGGAAGGCTTCGTTGC	$(AG)_8$	167	TD	JQ994294	8	0.750	0.794	9	0.700	0.820
Cac16	F: AACAGGCATTTGGAGCTTTT R: CCTCATTTCTCTCACCCTCTTT	$(AG)_{23}$	250	TD	JQ994295	14	0.850	0.888	8	0.650	0.823
Cac18	F: CTGGTGAGGGAGAAGAAAAACA R: CCCTCTCCCTCTCTTTTCCA	$(GA)_{11}N(AG)_{26}$	224	60	JQ996245	9	0.150	0.788	12	0.650	0.904

Ta = annealing temperature; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; TD = touchdown PCR program with a temperature from 58°C decrease of 1°C per cycle until 48°C.

Eight loci had positive cross-amplification for at least 1 of the other 7 species tested. One locus (Cac09) failed to amplify in any of the additional taxa tested. Two species (*O. longipes* and *P. vespa*) resulted in no positive amplifications for any of the loci tested (Table 3). The results indicated that the 9 microsatellite reported in this study are potentially useful tools for the evaluation of genetic variation and population structure in Epidendroideae orchids.

Table 3. Results of cross-amplifications of the 9 primers developed for *Cattleya coccinea* Lindl. in 7 Epidendroideae orchid species.

Species	Loci								
	Cac01	Cac02	Cac05	Cac08	Cac09	Cac10	Cac11	Cac16	Cac18
Cattleya pumila	+	+	-	+	-	+	+	-	+
C. dayana	+	+	+	+	-	+	+	+	-
C. cernua	+	+	-	-	-	+	-	-	-
Oncidium longipes	-	-	-	-	-	-	-	-	-
Prosthechea vespa	-	-	-	-	-	-	-	-	-
Brasiliorchis gracilis	-	+	+	-	-	-	-	-	-
Epidendrum secundum	-	+	+	-	-	-	-	-	-

+ = single product; - = no product.

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