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Isolation and characterization of microsatellite markers for the endangered *Comanthera elegans* (Eriocaulaceae) and cross-species amplification within the family

Bárbara Simões Santos Leal [a], Michelle Pires Tannure [a], Fabrício Rodrigues dos Santos [a], Maria Bernadete Lovato [a], Renata Acácio Ribeiro [b,*]

[a] *Departamento de Biologia Geral, Universidade Federal de Minas Gerais (UFMG), Caixa Postal 486, Belo Horizonte, Minas Gerais 31270-901, Brazil*
[b] *Departamento de Ciências Biológicas, Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM), Campus JK, Rodovia MGT 367, Km. 583, n. 5.000, Diamantina, Minas Gerais 39100-000, Brazil*

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1. Introduction

Brazilian "campos rupestres" vegetation (rupestrian field or rocky field) occurs in mountains above 900 m altitude, in shallow and sandy soils with a high degree of rocky outcropping. This vegetation type is notable for its species richness and for its high number of endemic species (Joly, 1970; Giulietti and Pirani, 1988). Most of the endemic taxa are restricted to isolated mountains or smaller ranges that make up part of the Espinhaço Range (Alves and Kolbek, 1994), a mountain chain extending approximately 1000 km in a North–South direction in eastern Brazil.

The Eriocaulaceae family is typical of the "campos rupestres" of the Espinhaço Range, where ca. 500 species occur (Giulietti et al., 2005, 2012). Some Eriocaulaceae species have considerable economic importance as ornamental plants, particularly those of the *Syngonanthus* Ruhland genus and the recently reestablished *Comanthera* L.B. Sm. genus (Parra et al., 2010; Giulietti et al., 2012). Plants of both genera are collected and sun-dried for sale in Brazil, and are also often exported to

* Corresponding author. Tel.: +55 38 3532 1200.
E-mail addresses: reacacio@hotmail.com, reacacio@ufvjm.edu.br (R.A. Ribeiro).

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different countries as "everlasting plants" ("sempre-vivas") (Giulietti et al., 1988, 1996). In the regions where these plants occur naturally, they are an important source of income and employment (Giulietti et al., 1988).

In Brazil, the most economically important species of Eriocaulaceae is *Comanthera elegans* (Bong.) L.R. Parra & Giul. (*Syngonanthus elegans* (Bong. Ruhland), commonly known as "sempre-viva-pé-de-ouro" or "sempre-viva-maxi" (Parra et al., 2010). Although this species has a restricted distribution in the mountains of Minas Gerais, it is the main "everlasting" traded species with exports of up to 40,000 kg a year (Giulietti et al., 1988, 1996). Indiscriminate inflorescences harvesting, which occurs mainly before seed production and maturation, and the consequent reduction in populations size, justified the inclusion of *C. elegans* in the Minas Gerais Red List of Threatened Species in the category of critically endangered (Mendonça and Lins, 2000). To develop appropriate conservation management plans, it is essential to develop tools that allow us to infer levels and patterns of genetic diversity of the species.

The aims of the present study were to isolate and characterize a set of microsatellite markers for *C. elegans* and to test the cross-amplification of these markers in nine species of Eriocaulaceae. Despite the increasing efforts in the past years to characterize the genetic diversity in plants of the "campos rupestres" vegetation (e.g., Borba et al., 2001; Jesus et al., 2001; Lambert et al., 2006; Azevedo et al., 2007; Silva et al., 2007; Lousada et al., 2011, 2013), Eriocaulaceae species are still poorly studied (but see Pereira et al., 2007), and there are no microsatellite markers described for the family.

2. Material and methods

2.1. Plant material and DNA extraction

To construct a microsatellite-enriched library, genomic DNA was extracted from silica gel-dried leaves of one individual of *C. elegans* sampled in Diamantina, Minas Gerais state, southeastern Brazil, following the procedure of Doyle and Doyle (1987). To characterize the microsatellite markers, we also extracted DNA from silica gel-dried leaves of 50 individuals of *C. elegans* from two natural populations, named SVN and SVS, both located in the Parque Nacional das Sempre-Vivas, Minas Gerais, Brazil (Appendix A). Furthermore, DNA from leaf samples of two individuals of each of other nine species of Eriocaulaceae was extracted according to the same protocol to perform a cross-amplification test (see below). All vouchers are deposited in the DIAM – UFVJM herbarium (Appendix A).

2.2. Microsatellite-enriched library and primer design

A microsatellite-enriched library was developed according to the protocol described by Billote et al. (1999). Approximately 5 μg of DNA was cut using the *Rsa*I restriction enzyme (Promega), and the fragments were linked to *Rsa21* (5′-CTCTTGCTTACGCGTGGACTA-3′) and *Rsa25* (5′-TAGTCCACGCGTAAGCAA GAGCACA-3′) adapters. Resulting fragments were PCR-amplified using *Rsa21*-specific primers, and amplicons were hybridized to 5′-biotinylated oligonucleotide probes $(CT)_8$, $(GT)_8$, $(GACA)_4$, and $(GATA)_4$. Microsatellite-enriched fragments were captured using magnetic beads coated with streptavidin (Promega), cloned into pGEM-T Easy Vector (Promega), and subsequently transformed into XL1-blue *Escherichia coli* competent cells. The recombinant clones were selected on LB agar medium containing ampicillin and IPTG/X-galactosidase. Positive (white) colonies were diluted and lysed to be added as a template in a PCR using M13 forward and reverse universal primers. Amplification products were purified and sequenced using M13 universal primers and the DYEnamic ET-terminator Kit (GE Healthcare) on an automated MegaBACE 1000 sequencer (GE Healthcare).

The sequences obtained were screened for the existence of SSR repeats using the Gramene Project's SSR identification tool (www.gramene.org/db/markers/ssrtool). Clones containing SSR motifs were bidirectionally sequenced using an automated ABI 3730xl sequencer (Applied Biosystems) and consensus sequences were generated using the PHRED/PHRAP/CONSED package (Gordon et al., 1998). Nucleotide sequences belonging to the cloning vector were carefully identified and discarded using the BLAST VecScreen option (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and primers were designed with Primer3 software (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky, 2000). An M13 sequence tail (5′-TTTTCCCAGTCACGAC-3′) was added to forward primers.

2.3. PCR amplification and genotyping

PCR reactions were performed according to the single-reaction nested-PCR method developed by Schuelke (2000). Amplifications were performed in a total volume of 13 μL containing 15 ng of genomic DNA, 1 × PCR buffer I0 [100 mM Tris–HCl (pH 8.4), 500 mM KCl, 1% Triton (Phoneutria)], 1.5 mM of $MgCl_2$ (except for reactions of the loci *Ce02* and *Ce11* that contained 2 mM of $MgCl_2$ and of the loci *Ce04*, *Ce12*, and *Ce14* that contained 1 mM of $MgCl_2$), 0.2 mM of each dNTP, 0.04 μM of forward primer, 0.16 μM of reverse primer, 0.16 μM of fluorescent-labeled (6-FAM or HEX) M13 primer, and 1 U Taq polymerase (Phoneutria). An additional 0.2 mg/mL of bovine serum albumin (BSA) was used in loci *Ce11* and *Ce17*, whereas 2% DMSO was added to loci *Ce02* and *Ce04*. PCRs were performed using a Mastercycler thermocycler (Eppendorf) and consisted of an initial 5-min denaturing step at 94 °C; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60–63 °C (see Table 1) for 45 s, and extension at 72 °C for 45 s; followed by eight cycles at 94 °C for 30 s, 50 °C for 45 s and 72 °C for 45 s; and a final extension at 72 °C for 60 min. We used a long final extension to avoid genotyping errors resulting from an incomplete A nucleotide addition to the 3′ end of the amplified fragments (–A or split peaks). PCR products were electrophoretically

Table 1
Characteristics of ten polymorphic microsatellite markers of *Comanthera elegans*, analyzed in 50 individuals.

Locus	Primer sequence (5′–3′)	Repeat motif	Ta (°C)	Allele size range (bp)	A	PIC	GenBank accession
Ce02	F: **M13-**TTTAACACGACCCGTTCTCAC R: GGTGCACCATGACATCAGTT	$(GA)_{21}$	60	247–289	8	0.703	KJ476530
Ce03	F: **M13-**CCTCGAAGCAAGAATTCACC R: TGTTTTTAAACTGTTTGAGCTTCTC	$(GT)_9$	63	287–295	4	0.442	KJ476531
Ce04	F: **M13-**CAGGACCCGTCTCAGCTACT R: GGAGACCTCCGACAGAGTGA	$(GT)_9(GA)_{19}$	62	164–224	22	0.909	KJ476532
Ce06	F: **M13-**AGGAGGACTGCACCTTCGTA R: TGGCAAGCATCGAAATTTTA	$(AG)_9A(AG)_{15}$	60	222–270	17	0.883	KJ476534
Ce07	F: **M13-**ACTCCGGGCCACTATTCAC R: ACTCTCTGGCTCGGATTCAC	$(AG)_5A(AG)_4$	60	183–219	10	0.576	KJ476535
Ce11	F: **M13-**TCCGACAATAAGGTGGAAGC R: AACGTGAAGATTGGGCTGAC	$(CT)_{20}$	61	170–222	16	0.820	KJ476536
Ce12	F: **M13-**CCTTCGTCGGTCTTACTTCG R: GCCATGACCGTTGTTCATTT	$(GA)_9GCTA(GA)_{17}$	60	171–187	7	0.711	KJ476537
Ce14	F: **M13-**AAACCGACCCACTAAAGCTG R: GGCTGCATTTCTTTGCTTTC	$(AG)_{18}$	61	257–299	17	0.896	KJ476538
Ce16	F: **M13-**TTTGGTCCTCCACATTCTTTG R: TGCAGTTGGTTCAACTTTCG	$(AG)_{27}$	60	210–248	11	0.856	KJ476539
Ce17	F: **M13-**TACACATGTTCGGTCGGAAA R: TCTGCTATCAACCGGAGGTC	$(GA)_6AGC(GA)_3$	61	174–194	9	0.696	KJ476540

Annealing temperature (Ta), number of alleles per locus (*A*) and polymorphic information content (PIC).
M13 tail: 5′ TTTTCCCAGTCACGAC 3′.

separated using a MegaBACE 1000 automated sequencer (GE Healthcare) and sized in accordance with the GeneTab 500 ROX standard (GENE ID) by using MegaBACE Fragment Profiler version 1.2 software (GE Healthcare). Raw alleles sizes were automated binned into discrete classes using FlexiBin macro for Excel (Amos et al., 2007).

2.4. Data analysis

The Micro-Checker 2.2.3 program (Van Oosterhout et al., 2004) was used to assess genotyping errors due to the presence of null alleles, stuttering, and allele dropout. For each population and locus, we estimated the number of alleles (A), and observed (H_O) and expected (H_E) heterozygosities according to the Hardy–Weinberg equilibrium, using GenAlEx 6.5 (Peakall and Smouse, 2012), and within-population inbreeding coefficient (Fis) using FSTAT (Goudet, 1995). Deviations from Hardy–Weinberg equilibrium (HWE) were evaluated using exact tests, as implemented in GENEPOP on the Web (Raymond and Rousset, 1995). Linkage disequilibrium between all pairs of loci in each population was tested in FSTAT. The statistical significance was adjusted for multiple testing using a sequential Bonferroni correction (Rice, 1989). Cervus 3.0 (Kalinowski et al., 2007) was employed to estimate the polymorphism information content (PIC) for each locus and the probabilities of paternity exclusion for each population and locus when the genotype of the other parent is unknown [Pr(Ex1)] and when the genotype of the other parent is known [Pr(Ex2)].

2.5. Cross amplification test

The ability of the developed microsatellite markers to cross-amplify in other species was tested in seven congeneric species (*Comanthera acyphilla*, *Comanthera bisulcata*, *Comanthera centauroides*, *Comanthera elegantula*, *Comanthera magnifica*, *Comanthera rupprechtiana*, and *Comanthera suberosa*), as well as in another two Eriocaulaceae species, *Actinocephalus incanus* and *Syngonanthus nitens*. PCR and cycling conditions were as previously described, except for the lower annealing temperature (−2 °C) and higher $MgCl_2$ concentration (+0.5 mM) used for almost all combinations of loci and species. The amplification products were analyzed by vertical electrophoresis on polyacrylamide gels, followed by silver staining. The loci were considered successfully amplified when at least one band of the expected size was clearly visualized in at least one individual of each species.

3. Results and discussion

The enrichment protocol resulted in a low frequency of microsatellite-containing sequences of 672 sequenced clones, only 17 contained at least one microsatellite with a motif equal to or greater than eight dinucleotide repeats, six trinucleotide repeats, or six tetranucleotide repeats. Ten of these 17 loci were polymorphic and showed consistent amplification following optimization (Table 1). The number of alleles per locus ranged from four to 22 and PIC values ranged from 0.442 to 0.909 (Table 1). One additional locus (*Ce05*) was monomorphic in our initial genotyping tests including 14 individuals (Primers sequence = F: 5′-AGAAGGATGATTCCGACAACAC-3′; R: 5′-AGTGAGGAACGAGCAAAGTGA-3′; motif repeat = $(TGA)_6$; expected

Table 2
Results of initial primer screening in two natural populations of *Comanthera elegans*.

Locus	SVN population ($N = 25$)						SVS population ($N = 25$)					
	A	H_O	H_E	F_{IS}	Pr(Ex1)	Pr(Ex2)	A	H_O	H_E	F_{IS}	Pr(Ex1)	Pr(Ex2)
Ce02	4	0.095	0.328	0.721*	0.345	0.523	6	0.320	0.713	0.565*	0.306	0.484
Ce03	2	0.000	0.080	1.000	0.148	0.249	3	0.292	0.320	0.110	0.051	0.163
Ce04	17	0.680	0.872	0.240*	0.710	0.830	7	0.800	0.818	0.047*	0.465	0.640
Ce06	12	0.680	0.849	0.218	0.648	0.786	10	0.680	0.739	0.100	0.367	0.554
Ce07	7	0.520	0.627	0.191	0.220	0.410	6	0.400	0.537	0.274	0.166	0.342
Ce11	9	0.304	0.619	0.525*	0.526	0.693	14	0.545	0.881	0.401*	0.616	0.763
Ce12	6	0.542	0.683	0.227	0.350	0.529	4	0.417	0.734	0.449	0.310	0.483
Ce14	14	0.875	0.852	−0.005	0.675	0.806	11	0.760	0.831	0.106	0.512	0.681
Ce16	6	0.480	0.782	0.404*	0.581	0.737	9	0.640	0.743	0.159	0.353	0.532
Ce17	7	0.174	0.654	0.744*	0.333	0.510	4	0.250	0.600	0.597*	0.186	0.332
Mean (SE)	8.40(1.47)	0.44(0.09)	0.64(0.08)	0.334*	–	–	7.40(1.12)	0.51(0.06)	0.69(0.05)	0.282*	–	–
Combined	–	–	–	–	0.998	0.999	–	–	–	–	0.987	0.999

Number of individuals sampled (N), number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E), within inbreeding coefficient (F_{IS}), probability of excluding a false candidate parent knowing only the genotypes of the offspring (Pr(Ex1)), and probability of excluding a false candidate parent knowing the genotype of one parent (Pr(Ex2)).
Significant departure from Hardy–Weinberg equilibrium ($P < 0.001$).

size = 284 bp; GenBank accession: KJ476533) and thus it was not analyzed in the remaining sample. The remaining loci were discarded due to non-specific amplification patterns.

Table 2 summarizes the genetic diversity of two populations of *C. elegans* based on the analysis of 10 polymorphic microsatellite markers. The H_O ranged from 0 to 0.875 (mean = 0.44) in the SVN population and from 0.250 to 0.800 (mean = 0.51) in the SVS population. The H_E ranged from 0.080 to 0.872 (mean = 0.64) in the SVN population and from 0.320 to 0.881 (mean = 0.64) in the SVS population (Table 2). Among the 10 analyzed loci, five loci (*Ce02*, *Ce04*, *Ce11*, *Ce16*, and *Ce17*) showed significant deviation from Hardy–Weinberg equilibrium ($P < 0.001$) in the SVN population, and four loci (*Ce02*, *Ce04*, *Ce11*, and *Ce17*) in the SVS population (Table 2). Heterozygous deficiency can be explained by the occurrence of null alleles at these loci, as evidenced by Microchecker. The multilocus exclusion probabilities of SVN and SVS populations were >0.98 (Table 2), indicating a high information content for parentage-based analyses. In both populations, no linkage disequilibrium was observed between any two loci.

All loci successfully cross-amplified in at least two of the tested species, with the exception of *Ce16* (Table 3). Of the 10 isolated markers, nine showed successful amplification in *C. bisulcata* and eight in *Comanthera ruppretchiana* (Table 3), suggesting its potential application in these species. Despite the development of microsatellites is becoming increasingly fast and cheap after the advent of next-generation sequencing (NSG) technologies, transferability is still a cost-effective alternative to microsatellite development and can also facilitate comparative studies among closely related taxa (Barbará et al., 2007).

In this study, we isolated and evaluated 10 microsatellite loci for *C. elegans*, an endemic and threatened species of Brazilian "campos rupestres" (rupestrian field) vegetation. To our knowledge, this is the first set of markers for an Eriocaulaceae species. These primers will provide essential information on the genetic consequences of extractivism on *C. elegans* populations, including assessment of genetic diversity, inbreeding and fine- and large-scale structure that may guide the sustainable management and conservation plans for the species. Furthermore, these loci represent promising tools for conservation genetics studies in other related species. Some of these [e.g., *C. bisulcata* ("sempre-viva-chapadeira"), *Comanthera aciphylla* ("mini-saia"), *C. centauroides*, *C. suberosa* ("margarida"), and particularly *C. magnifica* ("sempre-viva gigante"] are also threatened by human exploitation, being traded individually or mixed with *C. elegans* (Mendonça and Lins, 2000; Parra et al., 2010).

Table 3
Cross-amplification of ten microsatellite loci of *Comanthera elegans* in nine Eriocaulaceae species.

Species	Microsatellite loci									
	Ce02	Ce03	Ce04	Ce06	Ce07	Ce11	Ce12	Ce14	Ce16	Ce17
Comanthera aciphylla	–	–	++	–	–	+	++	–	–	–
Comanthera bisulcata	++	++	++	++	++	++	++	++	–	++
Comanthera centauroides	++	–	++	–	++	+	++	++	–	++
Comanthera elegantula	++	++	++	–	++	++	++	++	–	–
Comanthera magnifica	+	++	++	–	–	+	++	++	–	++
Comanthera rupprechtiana	++	++	++	–	++	++	++	++	–	++
Comanthera suberosa	–	–	++	–	–	++	++	–	–	–
Actinocephalus incanus	–	–	++	–	+	+	++	+	–	++
Syngonanthus nitens	++	++	–	+	–	++	++	+	–	++

'++' indicates successful amplification; '+' indicate successful, but weak, amplification and '–' indicates unsuccessful amplification.

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Appendix A. Information on voucher specimens deposited in DIAM – UFVJM herbarium.

Species	Population	Locality (state), country	Coordinates	Voucher number
Comanthera elegans (Bong.) L.R.Parra & Giul.	SVN	Bocaiúva (MG), Brazil	17°37'17.8"S, 43°46'01.9'W	4234
Comanthera elegans (Bong.) L.R.Parra & Giul.	SVS	Diamantina (MG), Brazil	17°54'58.7'S, 43°47'01.4'W	4233
Comanthera aciphylla (Bong.) L.R.Parra & Giul.	–	Diamantina (MG), Brazil	18°17'42.8"S, 43°44'15" W	4093
Comanthera bisulcata (Körn.) L.R.Parra & Giul.	–	Diamantina (MG), Brazil	18°54'55"S, 43°47'15.2" W	4095
Comanthera centauroides (Bong.) L.R.Parra & Giul.	–	Diamantina (MG), Brazil	18°17'42.8"S, 43°44' 15"W	4091
Comanthera elegantula (Ruhland) L.R.Parra & Giul.	–	Serro (MG), Brazil	18°29'20.6"S, 43°28'18.5"W	1998
Comanthera magnifica (Giul.) L.R.Parra & Giul.	–	Rio Vermelho (MG), Brazil	18°08'33.4"S, 43°01'24.9"W	3550
Comanthera rupprechtiana (Körn.) L.R.Parra & Giul.	–	Diamantina (MG), Brazil	18°54'55"S, 43°47'15.2"W	4094
Comanthera suberosa (Giul.) L.R.Parra & Giul.	–	Rio Vermelho (MG), Brazil	18°08'2.4"S, 43°01'2.3"W	3564
Actinocephalus incanus (Bong.) Costa	–	Diamantina (MG), Brazil	18°16'34.4"S. 43°38'09.6"W	2909
Syngonanthus nitens (Bong.) Ruhland	–	Diamantina (MG), Brazil	18°17'42.8"S, 43°44'15"W	4092

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