Microsatellite Markers for Studies with the Carnivorous Plant Philcoxia minensis (Plantaginaceae)

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PRIMER NOTE

MICROSATELLITE MARKERS FOR STUDIES WITH THE CARNIVOROUS PLANT *PHILCOXIA MINENSIS* (PLANTAGINACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed for the critically endangered carnivorous species *Philcoxia minensis* (Plantaginaceae) for further population genetic studies aiming at its conservation.
- *Methods and Results:* We identified 29 clones containing 40 microsatellites from a genomic enriched library. A total of 27 primer pairs were developed and evaluated in 30 individuals of a natural *P. minensis* population. Seventeen markers successfully presented amplification products within the expected size range, of which 12 were polymorphic. The expected and observed heterozygosities ranged from 0.03 to 0.65 and from 0.00 to 0.77, respectively. Positive transferability with the related species *P. bahiensis* was observed for the same 17 markers.
- *Conclusions:* The 12 polymorphic microsatellite markers are suitable for studies in genetic diversity and structure, mating system, and gene flow in *P. minensis* and also may be useful for similar issues regarding the related species *P. bahiensis.*

Key words: conservation genetics; Espinhaço Range; Gratioleae; *Philcoxia minensis*; Plantaginaceae; simple sequence repeat (SSR).

Philcoxia P. Taylor & V. C. Souza is a carnivorous plant genus, endemic to the Brazilian cerrado and caatinga biomes (Pereira et al., 2012; Carvalho and Queiroz, 2014). The genus is characterized by terrestrial herbs with underground stems and leaves under or on the soil surface. It comprises five species: *P. bahiensis* V. C. Souza & Harley and *P. tuberosa* M. L. S. Carvalho & L. P. Queiroz, from Bahia; *P. goiasensis* P. Taylor, from Goiás; *P. minensis* V. C. Souza & Giul., from Minas Gerais (Taylor et al., 2000); and a newly described species (Scatigna et al., unpublished), also from Minas Gerais. Only a small population of *P. minensis* is known from a sand patch at the Serra do Cabral State Park, in Minas Gerais State (Souza and Giulietti, 2009). Due to its rarity and the vulnerability of the formation where it occurs, this species could be treated as critically endangered. In addition, very little is known about the general biology of *P. minensis.* One important step for studies supporting its conservation is the characterization of its genetic diversity and structure, gene flow, breeding ratio of populations, and mating system. The use of microsatellite markers for population genetics and biodiversity conservation has increased in the past decade (Ouborg et al., 2010) and is now a well-established technique. Therefore, the purpose of this study was to develop microsatellite markers or simple sequence repeats (SSR), aiming to elucidate questions concerning aspects of *P. minensis* biology, such as its genetic structure, variability, and mating system.

METHODS AND RESULTS

A silica gel–dried inflorescence of a single individual of *P. minensis* was used in the initial DNA extraction, using the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. A voucher specimen of that individual (*Scatigna, A. V. 42*) was deposited at the UEC herbarium at the Universidade Estadual de Campinas, Brazil. The inflorescence of *Philcoxia* individuals is used for DNA extraction to (1) avoid killing the plant, as the leaves are situated on or below the soil surface; and (2) prevent contamination of the template DNA by the abundant nematodes stuck on the leaf surfaces.

A microsatellite-enriched genomic library was constructed following the protocol proposed by Billotte et al. (1999), with slight modifications. Genomic DNA was digested with *Afa*I enzyme (Invitrogen, Carlsbad, California, USA) for 3 h incubation at 37°C and ligated to the double-stranded adapters 5′-CTCTTGCTTACGCGTGGACTA-3′ and 5′-TAGTCCACGCGTAAGCAAGAGCACA-3′ for 2 h incubation at 20°C. Enrichment was performed using a hybridization-based capture with $(CT)_8$ and $(GT)_8$ biotin-linked probes and streptavidin-coated magnetic beads (MagneSphere Magnetic Separation Products; Promega Corporation, Madison, Wisconsin, USA). The enriched fragments were amplified by PCR, and the amplification products were cloned into pGEM-T Easy Vector (Promega Corporation). Competent XL1-Blue *Escherichia coli* (Stratagene, Agilent Technologies, Santa Clara, California, USA) were transformed with the recombinant plasmids and cultivated on agar medium containing ampicillin and 100 µg/mL of X-galactosidase. Eighty recombinant colonies were selected using blue/white screening and sequenced in an automated ABI 3500xL Genetic Analyzer (Perkin Elmer–Applied Biosystems,

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Foster City, California, USA) using T7 and SP6 primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Perkin Elmer–Applied Biosystems). Sequences containing microsatellites were identified using the SSR Identification Tool (SSRIT; Temnykh et al., 2001). As a criterion for the SSR selection, sequences that showed at least five dinucleotide repeats; four trinucleotide repeats; and three tetra-, penta-, and hexanucleotide repeats were selected. A total of 40 SSRs were identified in 29 clones, representing a 36.25% rate of enrichment success. The most abundant motifs in the sequences of microsatellites were dinucleotide (70%), followed by tetranucleotide (17.5%), trinucleotide and pentanucleotide (5% each), and hexanucleotide (2.5%). Most of the SSRs found were classified as perfect (67.5%). Twenty-seven primer pairs, complementary to the genomic sequences flanking the microsatellite region, were designed using Primer3Plus (Untergasser et al., 2007). PCR was conducted with a final volume of 15 µL containing 2.5 ng of template DNA, 1× PCR buffer (20 mM Tris HCl [pH 8.4] and 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 mg/mL bovine serum albumin (BSA), 0.5 mM of each primer, and 1 unit of *Taq* DNA polymerase (Invitrogen). The PCR program for all loci amplification consisted of an initial denaturation at 94°C for 2 min 30 s, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at specific temperature for 1 min (Table 1), extension at 72°C for 1 min, and a final extension at 72°C for 8 min. Amplification products were checked through 3% agarose gels prior to vertical electrophoresis using 6% denaturing polyacrylamide gels for genotyping through silver-staining (Creste et al., 2001). The product sizes were determined using a 10-bp DNA ladder (Invitrogen).

For polymorphism evaluation in *P. minensis*, we sampled a total of 30 specimens from the only known population, in Serra do Cabral (voucher: *Scatigna, A. V. 43* [UEC]), Municipality of Joaquim Felicio, Minas Gerais State, Brazil (17°42′23″S, 44°11′39″W). Furthermore, four specimens of *P. bahiensis* from a population in Chapada Diamantina (voucher: *Scatigna, A. V. 107* [UEC]), near the municipality of Piatã, Bahia State, Brazil (13°02′S, 41°57′W), were used to evaluate the transferability of the markers. Individuals were collected at a distance of at least 1 m to avoid sampling of clones. We ran the identity analysis test in CERVUS v. 3.0.7 (Kalinowski et al., 2007), allowing one mismatch to check if there were clones sampled. Statistical analyses were performed using Microsatellite Toolkit v.3.1.1 (Park, 2001) to calculate the number of alleles per locus (A), expected heterozygosity (H_e), observed heterozygosity (H_o), and polymorphism information content (PIC). FreeNA (Chapuis and Estoup, 2007) was used to identify the possible occurrence of null alleles (F_{NULL}). Linkage disequilibrium (LD) testing was performed between all loci pairs using a G-test. For Hardy–Weinberg equilibrium (HWE), we followed the probability test approach (Guo and Thompson, 1992) using GENEPOP v. 4.2 (Raymond and Rousset, 1995). The unbiased estimator of Wright's inbreeding coefficient (F_{IS}) was calculated according to Weir and Cockerham (1984) using GENEPOP v. 4.2. Of 27 markers tested, 17 were successfully amplified, of which 12 loci were polymorphic, five were monomorphic, and 10 did not amplify after PCR optimization (Table 1). The same 17 primer pairs successfully amplified with *P. bahiensis* samples.

No matching genotypes (clones) were found within the samples. A ranged from two to five, with an average of 3.5; H_o and H_e ranged from 0.00 to 0.77 and from 0.03 to 0.65, respectively (Table 2). The PIC values ranged from 0.032 to 0.577, showing medium levels of polymorphism (Table 2). Five loci showed significant deviation from HWE (Table 2). No significant LD between loci was detected when applying Bonferroni correction (P value for 5% = 0.000758), but null alleles were observed for two loci (null allele frequency estimate ≥0.2; Table 2). The significant and negative F_{IS} values for Pm21 are due to an excess of observed heterozygote genotypes. It is likely that the significant and positive F_{IS} estimates for the loci Pm10, Pm13, Pm15, and Pm16 (Table 2) can be explained by its proportions of null allele frequencies. These results were consistent with our expectations, because the population is apparently small and isolated, and the individuals propagate vegetatively.

Table 1. Characteristics of 17 microsatellite loci that successfully amplified in *Philcoxia minensis* samples.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
Pm01*	F: CCAACAGAAAAGCCCTAACG R: GCCCACAGTCCCTTGAGTAT	$(GA)_{17}$	234	60	KR019066
Pm02	F: AAGACGTTTGCCAGCCTTA R: TGCATTTATCCCCACAGACA	$(TAG)_4(TGTA)_3$	173	60	KR019067
Pm03	F: CCCAGCTTCACCTCACTCTC R: CGTTAGGGCTTTTCTGTTGG	$(TCC)_5$	161	60	KR019068
Pm04	F: AGTTGCCTTCCATCATCGTT R: TGGCTGAGAATCACTGACAAA	$(GT)_5(CT)_8$	156	60	KR019069
Pm08*	F: TGAAAACCCCAATCCCATAA R: CACAGGCGTATCAAGGAAGA	$(GT)_8$	232	60	KR019070
Pm09	F: TTGCTTTCCTCTCGGTGTTT R: CATTACTGCACCAAGCCTGA	$(CT)_{19}$	214	60	KR019071
Pm10	F: TGGCCCTTCTCAGATCTTTC R: ACATAGACCATTCGGGGAGA	$(AC)_9$	239	60	KR019072
Pm11	F: CATTTTTCGTTGTTCCCACA R: TACATGCGTTCCAAGCCTAA	$(CA)_{11}(TC)_7$	183	55	KR019073
Pm13	F: GCGTTCCTTTTTCGATCTGT R: AGCCATGGATATGTTCTCACC	$(CA)_{10}$	213	60	KR019074
Pm15	F: TCCTAATTGCTTCACGAGCAT R: AGCTTTGTCAGGCAGCTCA	$(AC)_5(GA)_5$	216	60	KR019075
Pm16	F: CCAAAGCTACACACCCAACA R: TCATATGCTGGGCATACCTG	$(AC)_5(ACAAA)_3$	244	60	KR019076
Pm18*	F: AGTCACTGCTTTCAGCCTGTC R: ATGAGATTGCTGAGCCTCGT	$(AG)_{26}$	161	60	KR019077
Pm20	F: ACTCATAGAGGACACACACAC R: TTATTTAGGGGACGGAGAG	$(AG)_{10}(AC)_{10}$	199	60	KR019078
Pm21	F: ATCGCTTCCTCCTCCTCTTC R: AACAAAGCTCGCTCGTTCTC	$(CA)_{10}$	181	60	KR019079
Pm22*	F: TGATCGACTTCATGGACACC R: CGGGATTCTCTTGCTTAACG	$(TC)_{10}(CA)_9$	181	60	KR019080
Pm26*	F: CTGGCGCTATCCTTCTCAAT R: TGGAGGATGTCTGTTTGTCG	$(AC)_{10}$	246	60	KR019081
Pm27	F: TTTTTCCAAGAGTCTCTGCAC R: GAGTGAAAGGGTGGTTTGTGA	$(CAAGC)_4$	225	60	KR019082

Note: T_a = specific annealing temperature.
* Monomorphic loci.

TABLE 2. Results of initial polymorphic microsatellite marker screening in the population of *Philcoxia minensis* ($N = 30$).

Locus	A	Allele size range (bp)	H_o	H_e	PIC	F_{IS}[a]	F_{NULL}
Pm02	3	163–181	0.3	0.264	0.233	−0.14	−0.08
Pm03	2	163–165	0.033	0.033	0.032	0	0
Pm04	4	146–166	0.5	0.417	0.379	−0.2	−0.13
Pm09	4	192–220	0.64	0.634	0.552	−0.01	−0.02
Pm10	4	239–269	0.067	0.098	0.095	0.33*	0.30[b]
Pm11	3	177–185	0.448	0.402	0.361	−0.12	−0.06
Pm13	4	109–215	0.367	0.434	0.389	0.16**	0.15
Pm15	4	214–220	0.364	0.518	0.418	0.30**	0.17
Pm16	2	242–244	0	0.089	0.083	1.00*	0.71[b]
Pm20	4	189–201	0.552	0.591	0.527	0.07	0.01
Pm21	5	155–185	0.773	0.655	0.577	−0.18*	−0.12
Pm27	3	221–227	0.455	0.502	0.391	0.1	0.05

Note: A = number of alleles sampled; F_{IS} = inbreeding coefficient index; F_{NULL} = occurrence of null alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; PIC = polymorphism information content.

[a] Significant deviation from Hardy–Weinberg equilibrium: $*P < 0.05$ and $**P < 0.01$.

[b] Markers with the probability of occurrence of null alleles.

CONCLUSIONS

The SSR markers herein described are the first developed for *P. minensis*. These microsatellites are important tools for genetic studies in *P. minensis* and may be used to evaluate the genetic variability of the related species *P. bahiensis*, aiming to elucidate questions regarding genetic diversity, spatial genetic structure, mating system, and gene flow. Data from such studies may contribute to conservation and management plans for *Philcoxia* species.

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