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Isolation and characterization of simple sequence repeat markers for the herbaceous species *Phyla scaberrima* (Verbenaceae)

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ABSTRACT. *Phyla scaberrima* (Verbenaceae) is a herbaceous species distributed from Mexico to Panama. Because of its well-known sweet properties and other medicinal uses, this species is cultivated in South America and the Caribbean. *Phyla scaberrima* has been arbitrarily extracted from nature, resulting in a severe reduction in its gene pool. In this study, we developed and characterized 11 simple sequence repeat markers for *P. scaberrima* to determine the genetic variability and patterns of population structure of the species. Fifty-six alleles were detected in a sample of 48 individuals belonging to 3 different populations. The average number of alleles per locus was 5.09, while the polymorphic information content ranged from 0.000-0.587. The observed and expected heterozygosities varied from 0.000-0.543 and from 0.000-0.651, respectively. Two loci exhibited significant deviation of the expected Hardy-Weinberg proportion. The 11 primer pairs were

also tested for cross-amplification to 6 species of the related genus *Lippia.* The transferability rate ranged from 4 loci in *Lippia florida* and *L. rotundifolia* to 6 loci in *L. corymbosa* and *L. microcephala.* The 11 primer sets were shown to be valuable tools for population genetic studies in *P. scaberrima* and in species of the genus *Lippia* in which primer transferability was detected.

Key words: Aztec sweet herb; Medicinal species; Microsatellites markers; Primers

INTRODUCTION

Phyla scaberrima (Juss. ex Pers.) Moldenke, formerly known as *Lippia dulcis* and *Phyla dulcis*, is an herbaceous perennial species that belongs to the Verbenaceae family (Plant List, 2013; Tropicos, 2014). This species is naturally distributed from Mexico to Panama and is cultivated in South America, the Andes, Antilhas (Pascual et al., 2001), and the Caribbean. *P. scaberrima* grows in forest edges, river banks, open fields, and pastures at altitudes ranging from the sea level up to 1800 m (Cáceres, 1999, 2006).

P. scaberrima is also known by the common names of Aztec sweet herb or Orozul because of its sweet taste that is approximately 1000 times higher than sugar from *Saccharum officinalis*. The sweet property of *P. scaberrima* is attributed to the presence of hernandulcin and 4β-hidroxihernandulcin. This species also shows anti-inflammatory, antispasmodic, and antibiotic properties (Souto-Bachiller et al., 1997), as well as inhibits murine melanoma, gastric adenocarcinoma, and uterine carcinoma (Fumiko et al., 2002).

Medicinal plants are very important in developing countries, as they are often included in social health programs (Soler, 2005; Hoeffel et al., 2011). Thus, *P. scaberrima* has been exploited by a very long time for use in natural medicine to treat infections, particularly those related to the respiratory tract (Diseldorff, 2009). The main consequence of this indiscriminate extraction has been a loss of genetic variability because of the drastic reduction in natural populations.

Simple sequence repeat (SSR) markers are commonly used in studies of genetic variability at the population level because of their codominant pattern, high levels of polymorphism, and high levels of reproducibility (Ferreira and Gattapaglia, 1998). The main goal of this study was to develop and characterize microsatellite loci for *P. scaberrima* for future population genetic studies and to guide strategies for conserving the genetic resources of this species.

MATERIAL AND METHODS

Genomic DNA from *P. scaberrima* was extracted using the cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). An enriched microsatellite library was developed for 1 individual of *P. scaberrima* using the hybridization method with biotin-labeled $(CT)_8$ and $(GT)_8$ (Billotte et al., 1999). Approximately 5 μg genomic DNA was digested with *Rsa*I (Promega, Madison, WI, USA), and blunt-ended fragments were linked to adapters (*Rsa*21 and *Rsa*25). Fragments containing repeats were selected by hybridization with the bio-

tinylated oligonucleotides and recovered using streptavidin-coated magnetic beads (Invitrogen, Carlsbad, CA, USA). Microsatellite-rich fragments were amplified by polymerase chain reaction (PCR) using the *Rsa*21 adapter as a primer, cloned into the pGEM-T Easy vector (Promega), and transformed into *Escherichia coli* XL1Blue MRF supercompetent cells (Agilent Technologies, Stratagene Products Division, La Jolla, CA, USA).

Plasmids were isolated and sequencing reactions were performed through subsequent purifications. Sequences were determined using a 3500XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using the program Gramene (Ware et al., 2002) to identify regions containing microsatellites and primers were designed using the program PRIMER3 version 0.4.0 (Rozen and Skaletsky, 2000). A total of 16 primer pairs were designed and tested for amplification. Amplification failed with 5 primers, while 11 primers showed clear and consistent amplification patterns and were used for further analysis.

PCR amplification and the consistency of each primer pair were tested in a sample of 48 individuals from 3 populations of *P. scaberrima.* Reactions contained 4.5 µL Go*Taq* Green Master Mix (Promega), 0.08 µL M13 tailed forward primer, 0.32 µL reverse primer (Table 1), and M13 primer labeled with fluorophors, and DNA samples consisted of 10 ng PdulD12 and PdulE08 primers, 25 ng PdulG11, and PdulG12 primers, 50 ng Pdul4, Pdul8, Pdul10, PdulF12, and PdulG08 primers, 30 ng PdulC12 primer, and 75 ng PdulB12 primer; the final reaction volume was adjusted to 10 µL with nuclease-free water.

PCR profiles consisted of a touchdown program with the initial annealing temperature at 65°C for each primer and PCR cycling parameters of: 1 cycle of 94°C for 4 min, followed of 12 cycles at 94°C for 30 s, 65°C for 30 s with a linear decrease of 1°C per cycle for the annealing temperature until reaching 55°C, followed by 29 additional cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; a cycle of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s; 7 cycles at 94°C for 30 s, 53°C 45 s, and 72°C for 45 s; final extension at 60°C for 40 min.

PCR products were separated in an automatic sequencer 3500xl (Applied Biosystems). Genetic analysis of the fragments was carried out using the GeneMapper 4.0 software (Applied Biosystems). For data analysis, we applied standard population genetic statistics using Cervus version 3.0.3 (Kalinowski et al., 2007) and Fstat version 2.9.3.2 (Goudet, 2001) to test for linkage disequilibrium using Fisher's method ($\alpha = 0.05$) with 1100 permutations. We also determined the presence of null alleles, which generally cause deviations from Hardy-Weinberg equilibrium at microsatellite loci (Pemberton et al., 1995), applying the MicroChecker software (van Oosterhout et al., 2004).

RESULTS AND DISCUSSION

To characterize the 11 loci selected, we genotyped 48 individuals of *P. scaberrima* representing native populations of Colombia and Mexico. Samples consisted of 15 individuals of 2 populations from Mexico (Atzalan 19°55'32"N, 97°12'36"W, altitude of 600 m and Misantla 19°58'00"N, 96°52'48"W, altitude of 120 m), and 33 individuals from Colombian populations (Cali 3°26'48.75"N, 76°37'46.39"W, altitude of 1525 m; Dagua 3°35'5.04"N, 76°38'58.25"W, altitude of 1468 m; and Pereira 4°45'34.00"N, 75°37'46.67"W, altitude of 1302 m). The sequences of the 11 microsatellite loci were submitted to Genbank and registered under the accession numbers cited in Table 1.

Table 1. Characterization of 11 polymorphic microsatellite loci genotyped in 48 individuals from 3 populations of *Phyla scaberrima*.

Loci/GenBank No.	Primer sequence (5'-3')	Repeat motif	Allele size	Ta	K	H_O	H_E	PIC
Pd4/KF546846	F: AGGTGTGGGGATCAAACAAA R: TGCCGTGAATTGAATCCATA	$(CA)_2CG(CA)_4...(CA)_4...(CA)_6...(CA)_4$	160-212	60	4	0.043	0.063	0.062
Pd8/KF546847	F: CGACTCACTATAGGGCGAATTG R: TGCAAGATTCAAAGGTGTTCA	$(AC)_4GA(AC)_2...(AC)_4TC\ (AC)G(AC)_7$	100-237	60	18	0.489	0.551	0.535
Pd10/KF546845	F: GCGGGAATTCGATTCTCTT R: TGTTGATACGTGTGTGCGAGT	$(AC)_4TA(AC)_4AT(AC)R\ (AC)_3GC(AC)_3$	107-212	60	13	0.239**	0.651	0.587
PdB12/KF546840	F: TGTGCTCTTGCTTGTCTTCC R: TGGGCGGAGAATAGTCATG	$(TG)_{10}$	210-220	60	2	0.043	0.043	0.042
PdC12/KF546837	F: GGTCCCACACCTTTGACTCC R: TCGGATACCTGGAGGAAGTG	$(TC)_4...(TC)_5...(TC)_6...(TC)_5...(TC)_7$	181-210	63	3	0.543	0.479	0.371
PdD12/KF546838	F: ACTCGGATTTATGGGGACCT R: ACTCGTTTGGGCTCAAGGTG	$(CTT)_3$	156-226	61	4	0.149	0.181	0.172
PdE8/KF546839	F: TGGGATGTTCCTCAAGCACT R: TTCCTTGAAGCAAGATCAGC	$(TA)_4$	225	59	1	0.000	0.000	0.000
PdF12/KF546842	F: TCTCTTGCTTACGCGTGGAC R: AGGCAATTCCCCATCTCCTG	$(AAT)_3$	184-223	62	5	0.021**	0.650	0.571
PdG8/KF546843	F: TGCTCGAGAAGGTGAAGATG R: TTGTTGAAGGGTTGGGACAT	$(GT)_7$	222-223	59	2	0.000	0.357	0.291
PdG11/KF546844	F: ACAGTTCAATGGGCTCAAAG R: CAGAGCTCTCCTCGATTTGC	$(CT)_9$	204-205	60	2	0.000	0.308	0.258
PdG12/KF546841	F: GGCACGGGTTTAGGAGATAA R: CGCCTGTTGAGTAAGCCTTC	$(TA)_3CA(TA)_3...(CA)_7$	223-224	61	2	0.000	0.374	0.301

Allele size indicates the range of observed alleles in bp; Ta = annealing temperature (°C); K = number of alleles; H_O and H_E = observed and expected heterozygosities, respectively; PIC = polymorphic information content. **Significant deviation from Hardy-Weinberg equilibrium with $P \leq 0.001$.

Genotyping of 48 individuals of *P. scaberrima* identified a total of 56 alleles and a moderate polymorphism level. The number of alleles ranged from 1 (PdE8) to 18 (Pd8), with an average of 5.09 alleles per locus (Table 1) and average polymorphic information content ranging from 0.000-0.587. The observed and expected heterozygosities for each locus ranged from 0.000-0.543 and from 0.000-0.651, with mean values of 0.139 and 0.332, respectively (Table 1). Pairwise comparisons for multiple tests showed no significant linkage disequilibrium between loci, while the null allele was observed for locus PdE8.

The 11 selected primers were tested for cross-amplification in 6 species of the related genus *Lippia* (Table 2). The primer pairs PdB12, PdC12, and PdG12 showed inconsistent amplification for all species tested, while the locus PdF12 showed amplification only in *Lippia*

Table 2. Eleven microsatellite loci isolated for *Phyla scaberrima* and tested for cross-amplification in 6 species of the related genus *Lippia*.

Species	*Lippia alba*	*Lippia corymbosa*	*Lippia florida*	*Lippia microcephala*	*Lippia rosella*	*Lippia rotundifolia*
Locus						
Pd4	-	+	-	-	-	+
Pd8	-	+	+	+	+	+
Pd10	-	+	-	+	-	+
PdB12	-	-	-	-	-	-
PdC12	-	-	-	-	-	-
PdD12	-	-	+	+	-	-
PdE8	-	+	+	+	+	+
PdF12	-	-	-	-	+	-
PdG8	-	+	+	+	+	+
PdG11	-	+	-	+	-	-
PdG12	-	-	-	-	-	-

(+) = clear amplification; (-) = no amplification.

rosella Moldenke. Loci Pd4, PdD12, and PdG11 were amplified in 2 species and locus Pd10 was amplified in 3 species, while loci Pd8, PdE8, and PdG8 showed consistent amplification in 5 species. *Lippia alba* (Mill.) N.E.Br. ex Britton & P.Wilson showed no amplification for all loci tested. Species showing higher levels of amplification included *Lippia microcephala* Cham. and *P. corymbosa* Cham. with 6 loci.

The development of SSR markers is an important step in understanding the consequences of predatory extraction that have affected the genetic variability of *P. scaberrima*. Cross-amplification for species of the genus *Lippia* showed the potential of these markers to be utilized for population genetics studies of these species.

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