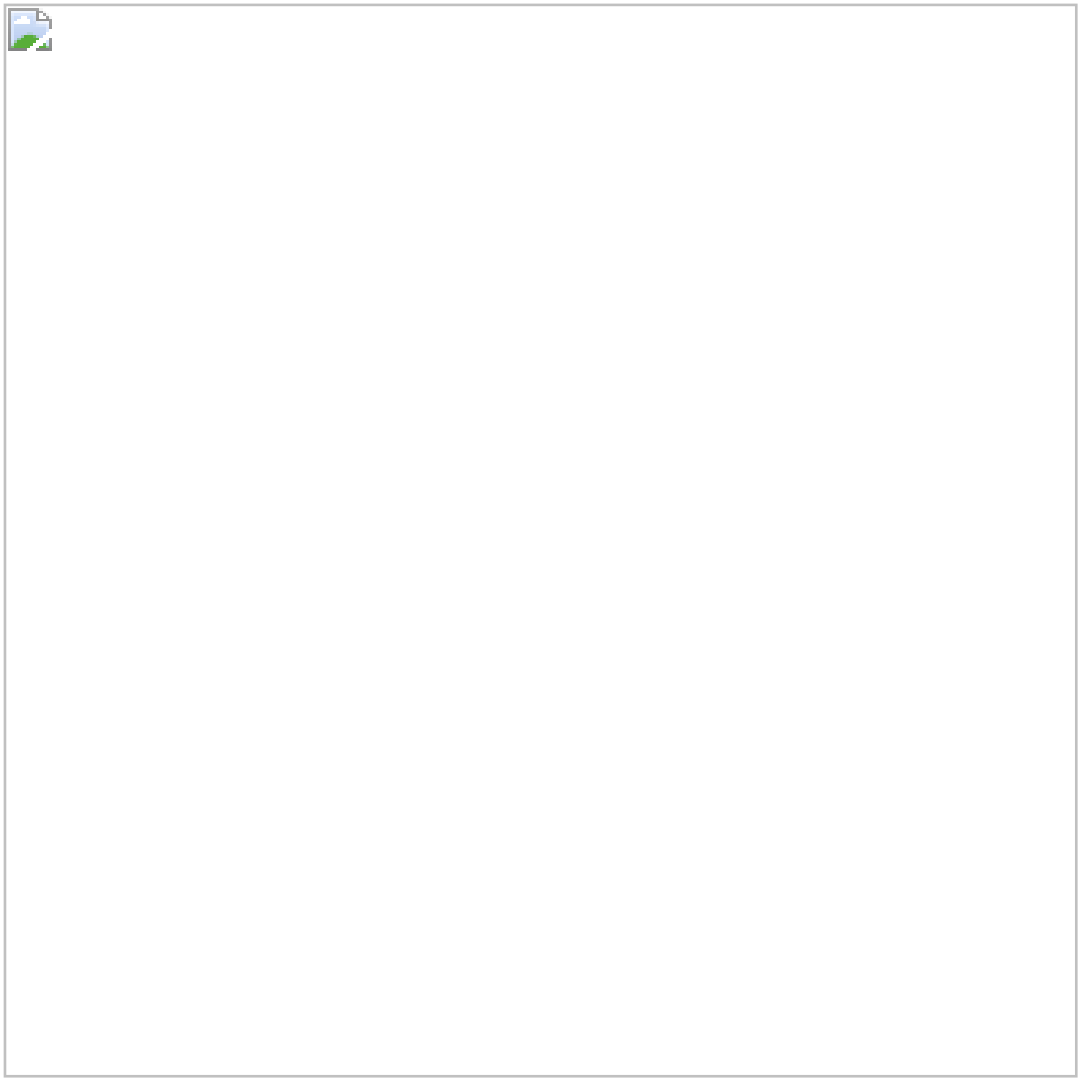

Isolation and Characterization of Nine Polymorphic Microsatellite Loci in Piper solmsianum (Piperaceae)

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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF NINE POLYMORPHIC MICROSATELLITE LOCI IN *PIPER SOLMSIANUM* (PIPERACEAE)[1]

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- *Premise of the study:* Nine microsatellite (simple sequence repeat [SSR]) loci were characterized for natural populations of *Piper solmsianum*, a potential source of bioactive secondary metabolites, and analyzed to assess the levels of genetic diversity in this species.
- *Methods and Results:* Based on an enriched library using the oligonucleotides $(CT)_8$ and $(GT)_8$, a total of 19 pairs of SSR primers were designed and nine of them were highly polymorphic after screening of 37 specimens from two populations. The number of alleles per locus ranged from one to six while the observed heterozygosity for polymorphic loci ranged from 0.000 to 0.875.
- *Conclusions:* The SSR regions characterized were informative, and the genetic markers will be useful to assess the genetic diversity and gene flow in populations of *P. solmsianum*.

Key words: allelic polymorphism; genetic diversity; medicinal plant; *Piper solmsianum*; Piperaceae; simple sequence repeat (SSR).

Piper L. is one of the largest genera of the Piperaceae family and abundant in the Neotropics, the South Pacific, and Asia (Jaramillo et al., 2008). Some species are well known for their medicinal and commercial uses, such as *P. nigrum* L. (black pepper) and *P. methysticum* G. Forst. (kava-kava). *Piper solmsianum* C. DC. is a medium-sized shrub and allogamous species endemic to the Atlantic Forest of Brazil. Individuals of *P. solmsianum* sampled in São Paulo State were characterized by the presence of tetrahydrofuran lignans such as (−)-grandisin. (−)-Grandisin has significant toxicity against trypomastigote forms of *Trypanosoma cruzi* (Martins et al., 2003) and against larvae of *Aedes aegypti* (Leite et al., 2012)—vectors of Chagas disease and dengue fever, respectively. Samples of the same species collected in Rio de Janeiro uniquely yielded benzofuran neolignans such as conocarpan and eupomatenoid-5, which have antifungal, antibacterial, and antinociceptive activity (Campos et al., 2005, 2007; Silva et al., 2010). Such a divergence in chemistry from distinct populations of the same species motivated genetic and chemical studies of *P. solmsianum*. Here we describe the development of genetic markers that will serve in future work with the species.

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METHODS AND RESULTS

To analyze the genetic variability of *P. solmsianum*, 37 individuals from two distinct populations were sampled in the Atlantic Forest in Cidade Universitária Armando de Salles Oliveira–Universidade de São Paulo (CUASO-USP) Forest Reserve, São Paulo (SP), about 300 km distant from the other sampling site (Itatiaia National Park, Itatiaia, Rio de Janeiro [RJ]) (Table 1). The sampling of plant species was carried out under a permit from Instituto Florestal (Secretaria do Meio Ambiente [SMA] no. 40.272/2006) and Sisbio/Ministério do Meio Ambiente (MMA) (no. 15780-2). The individuals were sampled with a minimum distance of 50 m from each other. The botanical classification was carried out by Dr. Elsie Franklin Guimarães (Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Rio de Janeiro, Brazil), and the vouchers were deposited in that institution's herbarium (Herbarium RB; K-1384 [SP] and K-1316 [RJ]). The assessment of chemical composition in sampling from the two populations showed large variability in chromatographic profiles (unpublished data), with samples from CUASO (SP) showing tetrahydrofuran lignans and phenylpropanoids as the major components in their chemical profiles and samples from Itatiaia (RJ) showing benzofuran neolignans as the main metabolites, indicating a possible genetic variation.

The microsatellite-enriched library was constructed according to the protocol described by Billotte et al. (1999) with modifications. Approximately 6 µg of total DNA from a single genotype of *P. solmsianum* were digested with *Rsa*I and ligated to the adapters *Rsa*21 (5′-TTGCTTCTCACGCTACGTGGA-3′) and *Rsa*25 (5′-TAGTCCAAGACGCGTCACCAAGAG-3′). The product was amplified by PCR, and the enrichment process and selection of fragments

TABLE 1. Microsatellite markers developed for *Piper solmsianum*. All values are based on 37 samples representing two populations from the Atlantic Forest in CUASO-USP Forest Reserve, São Paulo (SP) ($N = 24$), and in Itatiaia National Park, Itatiaia (RJ) ($N = 13$), Brazil.

Locus[a]	Repeat motif	GenBank accession no.	Primer sequences (5′–3′)[b]	T_a (°C)	Size range in bp (no. of alleles)
Psol1	$(GT)_7$	JQ924476	F: **CACGACGTTGTAAAACGAC**AGTCCTAACGGACCTGTGAA R: GAGGTGTTGTTGATGTGAGC	58	167
Psol2	$(GT)_8$	JQ924477	F: **CACGACGTTGTAAAACGAC**CCTAACGGACCTGTGAGGTA R: TCGATTTACGTGCCGAGT	58	230
Psol3	$(TG)_{12}$	JQ924478	F: **CACGACGTTGTAAAACGAC**GCGGATCTTACCAGAATCAG R: GAGTAGCCTTTGGTTGTTGC	56	256–272 (8)
Psol4	$(GT)_9(GA)_{12}$	JQ924479	F: **CACGACGTTGTAAAACGAC**GCTTGTTCTTGTGGGAAACC R: CAGGAAAACATACCGTCGTC	58	NA
Psol5	$(CA)_{13}(AC)_8$	JQ924480	F: **CACGACGTTGTAAAACGAC**ACCTCTGTCATTTCACCTTG R: GGGTGTGTTTTTAGAATGGA	58	130
Psol6	$(CT)_9(CA)_8$	JQ924481	F: **CACGACGTTGTAAAACGAC**CTCTTGGCAAAAGTCACCTG R: ATCCCATACCGATCTCCTTC	56	254–266 (6)
Psol7	$(TG)_{10}$	JQ924482	F: **CACGACGTTGTAAAACGAC**TTGCTTACGCCTGGACTAAC R: CAAGATCTGAGTCCCCAGTG	56	260
Psol8	$(GT)_7$	JQ924483	F: **CACGACGTTGTAAAACGAC**GGCAGATCAAGCTGAGAGAA R: GGATTTGGTGGTGGAGTGT	56	228
Psol9	$(AC)_8$	JQ924484	F: **CACGACGTTGTAAAACGAC**GGAACCCACGAGTTTCTTG R: GGGGTCCTTTTTACGTTGAG	56	180–202 (6)
Psol10	$(TG)_9$	JQ924485	F: **CACGACGTTGTAAAACGAC**CAGACGGATTCCCACTGAT R: GGACTTGTAACCCATCGAGA	56	228–238 (5)
Psol11	$(TG)_{10}$	JQ924486	F: **CACGACGTTGTAAAACGAC**TTATTTGGTTGGAGCTGTGTG R: CCACGGTGGGTTATCACAC	58	106–118 (6)
Psol12	$(GA)_{10}$	JQ924487	F: **CACGACGTTGTAAAACGAC**CCCTCGAACGAGAGAGAAG R: ATGGCCAGGGGAGTAAGATA	58	190
Psol13	$(TC)_7$	JQ924488	F: **CACGACGTTGTAAAACGAC**ACGCAAAGTATTGCCTTAGC R: TTTAACCATCTTTCGGTCCA	56	240
Psol14	$(GT)_7$	JQ924489	F: **CACGACGTTGTAAAACGAC**GCTCAACTCCGGAATCTACA R: GTATGCGTGCCGAGTGTTTA	56	190
Psol15	$(GT)_9$	JQ924490	F: **CACGACGTTGTAAAACGAC**CGCGGACTAACCAGAGTTAC R: GCCACAAAAACCCACTCA	56	145–151 (2)
Psol16	$(GT)_7$	JQ924491	F: **CACGACGTTGTAAAACGAC**GAAGTCCTAACGGACCTGTG R: GAGGTGTTGTTGATGTGAGC	56	166–214 (3)
Psol17	$(CA)_7$	JQ924492	F: **CACGACGTTGTAAAACGAC**TATTCCCATGCGAGATGC R: CGGCATAACCACTAAACCAC	48	392–430 (7)
Psol18	$(GT)_7$	JQ924493	F: **CACGACGTTGTAAAACGAC**ACTGTTGTGGACCTTGTTGC R: TGTATTAGGCCCCATCGAC	58	152–166 (2)
Psol19	$(CA)_7$	JQ924494	F: **CACGACGTTGTAAAACGAC**CGCGTGATGCATGCTTAT R: GCTCAACTCCGGAATCTACA	58	305

Note: NA = no amplification; T_a = annealing temperature.

[a] Fluorescent dyes: IRDye700 for Psol1–Psol9; IRDye800 for Psol10–Psol19.

[b] A 5′ M13 tail (identified in boldfaced text) is attached at the beginning of each forward primer sequence.

containing microsatellite sequences (simple sequence repeats [SSRs]) were performed by hybridization of oligonucleotides $(CT)_8$ and $(GT)_8$ conjugated to biotins and recovered by magnetic beads connected to streptavidin. The selected fragments were submitted to amplification by PCR using a primer complementary to the adapter *Rsa*21, cloned in a pGEM-T vector (Promega Corporation, Madison, Wisconsin, USA), and subsequently transformed into TOP10 *Escherichia coli* competent cells. Sequencing reactions for the fragments containing SSRs were performed using a T7 primer. For extraction of plasmid DNA, 192 recombinant colonies were transferred to Luria–Bertani broth containing ampicillin and maintained at 37°C with agitation (300 rpm) for 22 h.

Of the 192 sequences analyzed, 53 (28%) had at least one microsatellite region. After exclusion of redundant sequences, 41 high-quality sequences were selected for microsatellite primer design using the program Primer3 (Rozen and Skaletsky, 2000). A total of 19 pairs of SSR primers were designed from 41 sequences obtained from *P. solmsianum* (Table 1). Nine primer pairs with a satisfactory amplification profile and high polymorphism were evaluated for their use in assessing genetic variability. The amplification reactions were performed in 20-μL reaction volumes containing 10 mM Tris-HCl and 50 mM KCl buffer with 30 ng of DNA, 2 mM $MgCl_2$, 0.25 μM of each dNTP, 0.5 $\mu g \cdot \mu L^{-1}$ bovine serum albumin (BSA), 0.16 μM of the forward primer (F), 0.2 μM of the reverse primer (R), 0.3 μM of the M13 primer IR700 or IR800, and 1 unit of *Taq* DNA polymerase. The total cycle amplification was performed in a PTC-100 thermal cycler (MJ Research, St. Bruno, Quebec, Canada) programmed to hold at 94°C for 5 min and then run 10 cycles at 94°C for 1 min, 58°C for 1 min (with a decrease of 1°C per cycle), 72°C for 1 min; with an additional 30 cycles at 94°C for 40 s, 48°C for 40 s, 72°C for 1 min; and a final extension at 72°C for 10 min.

The amplification products (alleles) were separated using the 4300S DNA Analyzer (LI-COR, Lincoln, Nebraska, USA) in the electrophoresis system with 6.5% polyacrylamide gels. Using the computer program Saga^{MX} (LI-COR), the sizes of the alleles were obtained using 50–700-bp IRDye700 and IRDye800 (LI-COR) as standard molecular comparison. Of the 19 SSR loci derived from the genomic enriched library, 14 were optimized (65%) and nine loci were highly polymorphic. The polymorphism in the loci was evaluated by the average number of alleles per locus (A) and the polymorphism information content (PIC).

The observed average number of alleles per locus was 3.3, with a maximum of six (Psol3) and a minimum of one (Psol17). The mean of PIC values was 0.693, and the values ranged from 0.375 (for the loci Psol15 and Psol18) to 0.861 (Psol3), with Psol3 and Psol17 being the most polymorphic loci. The variation in the observed heterozygosity (H_o) ranged from 0.000 for the Psol17 locus to 0.875 for the Psol9 locus. The variation in expected heterozygosity (H_e) ranged from 0.000 for Psol17 to 0.749 for Psol18 (Table 2). Allele frequencies, A, H_o, and H_e

TABLE 2. Results of screening of microsatellite markers developed for *Piper solmsianum*. Voucher specimens K-1384 (SP) and K-1316 (RJ) are deposited in the herbarium of the Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Brazil.

	São Paulo, SP (N = 24)			Itatiaia, RJ (N = 13)		
Locus	A	H_e	H_o	A	H_e	H_o
Psol3	6	0.716	0.542	5	0.508	0.077
Psol6	5	0.706	0.739	3	0.507	0.167
Psol9	3	0.542	0.875	4	0.664	0.538
Psol10	4	0.662	0.565	3	0.658	0.727
Psol11	2	0.042	0.042	2	0.212	0.231
Psol15	2	0.311	0.375	2	0.443	0.000
Psol16	4	0.625	0.647	4	0.697	0.545
Psol17	1	0.000	0.000	2	0.391	0.167
Psol18	3	0.611	0.150	5	0.749	0.273

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size for each population; RJ = Rio de Janeiro; SP = São Paulo.

were estimated using the program GDA (Lewis and Zaykin, 2006). Values of the peaks measured were moderate to high, indicating that the loci characterized in this work gave a high level of information for genetic studies.

CONCLUSIONS

The SSR regions characterized in this study were found to serve as reliable indicators of the genetic variability in wild populations of *P. solmsianum*. These regions both document interpopulation genetic variation and serve as reliable markers for identifying the sources of *P. solmsianum* individuals. This will help to develop natural population management strategies, will assist in the selection of plants containing the target metabolites, and will also support future phytochemical studies exploring chemical variation between populations.

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