ISOLATION AND CHARACTERIZATION OF NINE POLYMORPHIC MICROSATellite LOCi IN Piper solmsianum (Piperaceae)¹

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PRIMER NOTE

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 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 trypanomastigote 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.

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 (Itaitia National Park, Itaitia, 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-1516 [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 Itaitia (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 Rsal and ligated to the adapters Rsa21 (5′-TTGCTTCTCAAGCTACGTGGA-3′) and Rsa25 (5′-TAGTCCAAAGACCGTACGAGA-3′). The product was amplified by PCR, and the enrichment process and selection of fragments

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

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containing microsatellite sequences (simple sequence repeats [SSRs]) were performed by hybridization of oligonucleotides (CTTs) and (GTs), 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 Rsa21, 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 for 21 h.

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 | Repeat motif | GenBank accession no. | Primer sequences (5′–3′) | T_\text{a} (°C) | Size range in bp (no. of alleles) |
|-------|--------------|-----------------------|---------------------------|----------------|----------------------------------|
| Psol1 | (GT)_7       | JQ924476              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 167 (no. of alleles) |
| Psol2 | (GT)_8       | JQ924477              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 230 (no. of alleles) |
| Psol3 | (TG)_12      | JQ924478              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 226–278 (no. of alleles) |
| Psol4 | (GT)_8(GA)_12| JQ924479              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | NA (no. of alleles) |
| Psol5 | (CA)_15(AC)_3| JQ924480              | ′F: GACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 130 (no. of alleles) |
| Psol6 | (CT)_5(CA)_4 | JQ924481              | ′F: GACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 254–266 (no. of alleles) |
| Psol7 | (TG)_10      | JQ924482              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 260 (no. of alleles) |
| Psol8 | (GT)_7       | JQ924483              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 228 (no. of alleles) |
| Psol9 | (AC)_3       | JQ924484              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 190 (no. of alleles) |
| Psol10| (TG)_9       | JQ924485              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 240 (no. of alleles) |
| Psol11| (TG)_10      | JQ924486              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 223–288 (no. of alleles) |
| Psol12| (GA)_10      | JQ924487              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 116–188 (no. of alleles) |
| Psol13| (TC)_7       | JQ924488              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 190 (no. of alleles) |
| Psol14| (GT)_7       | JQ924489              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 190 (no. of alleles) |
| Psol15| (GT)_8       | JQ924490              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 145–151 (no. of alleles) |
| Psol16| (GT)_7       | JQ924491              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 166–214 (no. of alleles) |
| Psol17| (CA)_3       | JQ924492              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 392–430 (no. of alleles) |
| Psol18| (GT)_7       | JQ924493              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 152–166 (no. of alleles) |
| Psol19| (CA)_7       | JQ924494              | ′F: CACGACGTGTGAACACGCAGTCTAACAGGCTTGGAA R: GAGTGGTGTGTGAAGTGT′                  | 58             | 305 (no. of alleles) |

Note: NA = no amplification; T_\text{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.

Tag 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™ (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 average number of alleles per locus (A) and the polymorphism information content (PIC).
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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