# Development and Characterization of Microsatellite Markers for the Medicinal Plant Smilax brasiliensis (Smilacaceae) and Related Species

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Primer Note

# Development and characterization of microsatellite markers for the medicinal plant *Smilax brasiliensis* (Smilacaceae) and related species[1]

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- *Premise of the study:* A new set of microsatellite or simple sequence repeat (SSR) markers were developed for *Smilax brasiliensis*, which is popularly known as sarsaparilla and used in folk medicine as a tonic, antirheumatic, and antisyphilitic. *Smilax brasiliensis* is sold in Brazilian pharmacies, and its origin and effectiveness are not subject to quality control.
- *Methods and Results:* Using a protocol for genomic library enrichment, primer pairs were developed for 26 microsatellite loci and validated in 17 accessions of *S. brasiliensis*. Thirteen loci were polymorphic and four were monomorphic. The primers successfully amplified alleles in the congeners *S. campestris*, *S. cissoides*, *S. fluminensis*, *S. goyazana*, *S. polyantha*, *S. quinquenervia*, *S. rufescens*, *S. subsessiliflora*, and *S. syphilitica*.
- *Conclusions:* The new SSR markers described herein are informative tools for genetic diversity and gene flow studies in *S. brasiliensis* and several congeners.

**Key words:** medicinal plant; microsatellites; sarsaparilla; *Smilax*; transferability.

The Smilacaceae is grouped within the Monocotyledoneae of the Liliales and has only two genera: *Smilax* L., with 300 species, and *Heterosmilax* Kunth, with 15 species (Angiosperm Phylogeny Group III, 2009). The family is distributed worldwide and is composed mainly of herbaceous vines and shrubs, and rarely of subshrubs and dioecious species. In Brazil, *Smilax* comprises 31 species, 14 of which are exclusively Brazilian (Andreata, 1997). *Smilax* species, which are popularly known as sarsaparilla, are used in folk medicine as tonics, antirheumatics, and antisyphilitics and are sold in Brazilian pharmacies without any quality control over their origin and effectiveness (Andreata, 1997). The quality control of herbal drugs should be more stringent, and molecular markers may be useful tools for the identification of species sold in pharmacies. Thus, the aim of the current study was to isolate and characterize microsatellite markers to identify *Smilax* species.

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## METHODS AND RESULTS

Genomic DNA was extracted from fresh leaves of *S. brasiliensis* Spreng., *S. campestris* Griseb., *S. cissoides* Mart. ex Griseb., *S. fluminensis* Steud., *S. goyazana* A. DC., *S. polyantha* Griseb., *S. quinquenervia* Vell., *S. rufescens* Griseb., *S. subsessiliflora* Duhamel, and *S. syphilitica* Humb. & Bompl. ex Willd. using the cetyltrimethylammonium bromide (CTAB) protocol described by Doyle and Doyle (1990) with modifications. The plant samples were registered (Appendix 1) and added to the plant collection of the Herbarium of the Escola Superior de Agricultura "Luiz de Queiroz" (ESA) of the Universidade de São Paulo, Brazil, and the Herbarium "Coleção de Plantas Medicinais e Aromáticas" (CPMA) of the Universidade Estadual de Campinas, Brazil.

A microsatellite-enriched library was obtained using protocols adapted from Billotte et al. (1999). Genomic DNA from one individual of *S. brasiliensis* (Campina Verde, Minas Gerais) was digested with *Afa*I (Invitrogen, Carlsbad, California, USA) and enriched in microsatellite fragments using $(CT)_8$ and $(GT)_8$ motifs. Microsatellite-enriched DNA fragments were ligated into pGEM-T Easy Vectors (Promega Corporation, Madison, Wisconsin, USA), which were used to transform Epicurian Coli XL1-Blue *Escherichia coli* competent cells (Promega Corporation). Positive clones were selected using the β-galactosidase gene and grown overnight with ampicillin. The sequencing reactions (10 μL) contained 200 ng of plasmid DNA, 0.5 pmol SP6 primer, 0.4 μL of BigDye Terminator mix (version 3.1; Applied Biosystems, Foster City, California, USA), 1 mM $MgCl_2$, and 40 mM Tris-HCl (pH 9.0). The sequencing reactions were performed in a thermal cycler (MJ Research, BioRad, Hercules, California, USA) under the following conditions: 2 min at 96°C for the first denaturation followed by 26 cycles of 45 s at 96°C, 30 s at 50°C, and 4 min at 60°C. The PCR products were precipitated with isopropanol (65%), centrifuged, and washed with 70% ethanol. Ninety-six positive clones were sequenced on an ABI 3700 automated sequencer (Applied Biosystems).

TABLE 1. Sequences and characteristics of primer pairs designed for *Smilax brasiliensis* that amplified microsatellite loci.

| Locus | Primer sequences (5′–3′) | Repeat motif | Size range (bp) | $T_a$ (°C) | GenBank accession no. |
|---|---|---|---|---|---|
| Sbr01 | F: AGTCTGCATGAGTTGGTGGA | $(GA)_7(GT)_9$ | 229–241 | 52 | JX070058 |
| | R: AATGGTTCTACTCCTGCCATGT | | | | |
| Sbr02 | F: CCAAGAGCAGGGTAGGAGAG | $(AG)_{14}$ | 179–217 | Touchdown | JX070059 |
| | R: AGGGTCAGAATCGTAGCTGTT | | | | |
| Sbr03 | F: TGTTCTCTGGCGGAGTTTCT | $(TCT)_5$ | 256–259 | 52 | JX070060 |
| | R: AGGACAATCAGCTCGGAAGT | | | | |
| Sbr04 | F: GTTACCTCCATGCTGCTGTG | $(AG)_{34}(TAGC)_5$ | 139–179 | 60 | JX070061 |
| | R: CCACCTCTGCCTCTCTCCTA | | | | |
| Sbr05 | F: TGCGGATCTGTAACACACTTG | $(TC)_5$ | 209 | 52 | JX070062 |
| | R: AAGTCTTGAGGCATGGATGA | | | | |
| Sbr06 | F: CCCTACGCACGGTAACACAT | $(TC)_5$ | 249–251 | Touchdown | JX070063 |
| | R: TCCAAGCTCTCCCTCTATTCC | | | | |
| Sbr07 | F: GGGGGTGCTTTACAAGATCA | $(TC)_5(AC)_8$ | 161–179 | 54 | JX070064 |
| | R: CGTCTGACCACTCCTTTCCT | | | | |
| Sbr08 | F: GGAGTCGGATCACAGAGGAG | $(AG)_6(AG)_4$ | 180–182 | Touchdown | JX070065 |
| | R: TGTGTGAGAGGTGTTGAGTGC | | | | |
| Sbr09 | F: CGATATGGGCCTCATTCAGA | $(TC)_{11}$ | 178–230 | 52 | JX070066 |
| | R: ACATGCCTTTCCCCTACCAC | | | | |
| Sbr10 | F: CGATGGATTCAGGTGATTGA | $(GA)_{24}$ | 169–209 | Touchdown | JX070067 |
| | R: TCAACCCATCAGACCAGTGA | | | | |
| Sbr11 | F: CCAGGGAATCTGTGAAATCC | $(CT)_{13}$ | 217–249 | 55 | JX070068 |
| | R: TTCTTCAAAACGTGCTGCAT | | | | |
| Sbr12 | F: GCCAAATGCATGAGACTCG | $(GA)_9$ | 237–245 | Touchdown | JX070069 |
| | R: GCTTCGTCAACAATCCATCA | | | | |
| Sbr13 | F: CCCATCTACACGTCCAAGAG | $(CT)_5(CT)_{18}$ | 171–195 | 60 | JX070070 |
| | R: GTCACTTGCTTCCCAACCAT | | | | |
| Sbr14 | F: TGATTTCAACCACCCATTACC | $(AGA)_8$ | 204–243 | Touchdown | JX070071 |
| | R: TGGGGCCAAAGCTACTAAAT | | | | |
| Sbr15 | F: GCCACAACTAGGTCGAGCA | $(AG)_{21}$ | 149–193 | 52 | JX070072 |
| | R: TGATTTTGCGTGTTGGACA | | | | |
| Sbr16 | F: GTTTTGGAAGCCGAGAT | $(TTC)_5$ | 259 | 52 | JX070073 |
| | R: GAGCCTTTTTCTTTTTCTCTCC | | | | |
| Sbr17 | F: CTGATTGGAAACCCGAACC | $(AG)_{21}$ | 204–240 | 56 | JX070074 |
| | R: AACAAGGGAGATGCAGCAGT | | | | |

*Note*: $T_a$ = annealing temperature.

A total of 26 primer pairs were designed against simple sequence repeat (SSR) flanking regions using Primer3 software (Rozen and Skaletsky, 2000) and tested in DNA extracted from leaves of *S. brasiliensis* (two specimens collected from Minas Gerais State, Brazil, and 30 specimens from a germplasm bank at the University of Campinas, Brazil [Appendix 1]). Primer sequences, repeat motifs, GenBank accession numbers, optimal annealing temperatures, and allele size ranges are provided in Table 1.

PCR was performed in a 20-μL reaction mixture containing 30 ng of DNA, 0.24 μL of forward primer (10 μM), 0.30 μL of reverse primer (10 μM), 0.45 μL of fluorochrome-labeled primer (10 μM), 1.2 μL of dNTP mix (2.5 mM), 1.5 μL of 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.9]), 0.6 μL of bovine serum albumin (BSA, 2.5 μM), 0.6 μL of $MgCl_2$ (3 mM), and 1 U of *Taq* DNA polymerase (Thermo Scientific, Vilnius, Lithuania). The PCR program consisted of an initial denaturation step at 95°C for 5 min followed by 30 cycles of amplification (94°C for 30 s, 40 s at the specific annealing temperature of each primer pair, and 72°C for 1 min), and a final elongation step at 60°C for 10 min (Table 1). The following touchdown cycling program was used for certain primers: an initial denaturation at 94°C for 5 min followed by 10 cycles of 94°C for 1 min, 65°C decreasing to 55°C at 1°C per cycle for 40 s, and 72°C for 1 min. Subsequently, 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 1 min were performed prior to a final extension at 72°C for 10 min. The amplification products were separated under denaturing conditions on a 5% (v/v) polyacrylamide gel containing 8 M of urea and 1× TBE (0.045 M Tris-borate and 1 mM EDTA) in an automatic sequencer (LI-COR 4300S DNA Analysis System; LI-COR Biosciences, Lincoln, Nebraska, USA) for approximately 2 h at 70 W. The loci were genotyped using Saga software (LI-COR Biosciences).

From the 26 loci tested, 17 successfully amplified in *S. brasiliensis* including 13 polymorphic and four monomorphic loci (Sbr05, Sbr06, Sbr08, and Sbr016). The number of alleles per locus, the allele size range, and the observed ($H_o$) and expected ($H_e$) heterozygosities under Hardy–Weinberg equilibrium (HWE) were determined for the polymorphic loci (Table 2). Each locus was tested for deviations from HWE expectations using exact tests, and the gametic disequilibrium between pairs of loci was calculated using GENEPOP (Raymond and Rousset, 1995). The sequential Bonferroni correction was used to correct multiple applications of the same test (Weir, 1996). The presence of null alleles was determined using MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004). In the *S. brasiliensis* population, the number of alleles per locus in the remaining 13 loci ranged from four to 11, and the mean number of alleles per locus was 7.4, whereas the $H_o$ and $H_e$ varied from 0.20 to 1.00 and from

TABLE 2. Estimates of the genetic diversity indices of *Smilax brasiliensis* accessions based on 13 microsatellite markers.

| Locus | *N* | *A* | PIC | $H_o$ | $H_e$ |
|---|---|---|---|---|---|
| Sbr01 | 19 | 7.00 | 0.57 | 0.74 | 0.66 |
| Sbr02 | 14 | 7.00 | 0.81 | 1.00 | 0.86 |
| Sbr03 | 20 | 2.00 | 0.38 | 1.00 | 0.51 |
| Sbr04 | 15 | 8.00 | 0.81 | 0.53 | 0.86 |
| Sbr07 | 20 | 6.00 | 0.69 | 0.50 | 0.75 |
| Sbr09 | 21 | 9.00 | 0.85 | 0.48 | 0.89 |
| Sbr10 | 21 | 9.00 | 0.82 | 0.71 | 0.86 |
| Sbr11 | 17 | 11.00 | 0.82 | 0.59 | 0.86 |
| Sbr12 | 14 | 4.00 | 0.57 | 0.29 | 0.66 |
| Sbr13 | 20 | 8.00 | 0.71 | 0.20 | 0.76 |
| Sbr14 | 17 | 11.00 | 0.84 | 0.65 | 0.88 |
| Sbr15 | 19 | 8.00 | 0.75 | 0.63 | 0.80 |
| Sbr17 | 19 | 10.00 | 0.64 | 0.63 | 0.68 |
| Mean | 19 | 7.46 | 0.74 | 0.61 | 0.77 |

*Note*: *A* = number of alleles; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; *N* = number of accessions analyzed; PIC = polymorphic information content.

Table 3. Transferability of loci designed for *Smilax brasiliensis* and tested in nine other *Smilax* species with their respective allele size ranges (in base pairs).

| Locus | *S. campestris* (*n* = 2) | *S. cissoides* (*n* = 2) | *S. fluminensis* (*n* = 2) | *S. goyazana* (*n* = 2) | *S. polyantha* (*n* = 2) | *S. quinquenervia* (*n* = 1) | *S. rufescens* (*n* = 2) | *S. subsessiliflora* (*n* = 1) | *S. syphilitica* (*n* = 1) |
|---|---|---|---|---|---|---|---|---|---|
| Sbr01 | 231–235 | 229–235 | — | 231–237 | 231–237 | — | 229 | — | 231 |
| Sbr02 | 191–217 | 193–213 | — | 191–213 | 179–203 | — | 179–209 | — | — |
| Sbr03 | 256–259 | 256–259 | 256–259 | 256–259 | 256–259 | — | 256–259 | — | 256 |
| Sbr04 | 147–163 | 165 | — | 145–179 | 145–155 | — | 145–167 | — | 145–159 |
| Sbr05 | 209 | 209 | — | 209 | 209 | 209 | 209 | 209 | — |
| Sbr06 | 249 | 249 | 251 | 249 | 249 | 251 | 249 | 249 | 251 |
| Sbr07 | 175–179 | 161–169 | 163–165 | 161–167 | — | 165 | 167 | — | — |
| Sbr08 | 182 | 182 | 180 | 182 | 182 | — | 182 | — | 182 |
| Sbr09 | 184–200 | 178–230 | 188–206 | 182–210 | 188–190 | — | 180–192 | — | — |
| Sbr10 | 169–189 | 179–195 | 169 | 175–205 | 173–185 | — | 179–193 | — | 169–185 |
| Sbr11 | 221–243 | 223–241 | — | 219–247 | 237–249 | — | 225–239 | — | — |
| Sbr12 | 237 | 237–245 | 245 | 241–245 | 237–243 | — | 245 | — | 237 |
| Sbr13 | 171 | 171 | 173 | 177 | 171–185 | 189 | 177–189 | — | — |
| Sbr14 | 207–231 | 204–231 | 219–231 | 213–234 | 207–231 | — | 204–213 | — | 222 |
| Sbr15 | 167–179 | 163–183 | 149–159 | 163–193 | 171 | 149–159 | 175–189 | 163–179 | — |
| Sbr16 | 259 | 259 | 259 | 259 | 259 | — | 259 | — | 259 |
| Sbr17 | 206–224 | 220–230 | 206–224 | 212–226 | 208 | — | 204–212 | — | 204 |

*Note*: — = unsuccessful amplification.

0.51 to 0.89, respectively (Table 2). The total number of alleles for 13 loci was 97 with an average of 7.46 alleles per locus.

The mean polymorphism information content (PIC) for all the loci was high (PIC > 0.7), suggesting that these microsatellite markers could be useful for population genetics and diversity studies. No linkage disequilibrium was detected between pairs of loci after Bonferroni correction for multiple tests ($P = 0.0038$).

Of the polymorphic loci described in this study, between three and 13 were successfully amplified in the other nine *Smilax* species studied (*S. campestris*, *S. cissoides*, *S. fluminensis*, *S. goyazana*, *S. polyantha*, *S. quinquenervia*, *S. rufescens*, *S. subsessiliflora*, and *S. syphilitica*) (Table 3).

## CONCLUSIONS

The development of microsatellite markers for *S. brasiliensis* will facilitate research focused on germplasm diversity, conservation, and taxonomic studies of the *Smilax* genus. The described primers represent a useful tool for population genetics in *S. brasiliensis* as well as in the other nine species of the genus in this study, allowing for enhanced quality control of this group of medicinal plants and avoiding adulterations in the products derived from these plants.

## LITERATURE CITED

Andreata, R. H. P. 1997. Revisão das espécies brasileiras do gênero *Smilax* Linnaeus (Smilacaceae). *Pesquisas: Botânica* 47: 7–244.

Angiosperm Phylogeny Group III. 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. *Botanical Journal of the Linnean Society* 161: 105–121.

Billotte, N., P. J. L. Lagoda, A. M. Risterucci, and F. C. Baurens. 1999. Microsatellite-enriched libraries: Applied methodology for the development of SSR markers in tropical crops. *Fruits* 54: 277–288.

Doyle, J. J., and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. *Focus (San Francisco, Calif.)* 12: 13–15.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenism. *Journal of Heredity* 86: 248–249.

Rozen, S., and H. J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Resources* 4: 535–538.

Weir, B. S. 1996. Genetic Data Analysis II. Sinauer, Sunderland, Massachusetts, USA.

Appendix 1. Voucher information for plant materials of nine *Smilax* species collected from different regions of Brazil.

| Species | Location (Geographical coordinates) | Voucher no. |
|---|---|---|
| *S. brasiliensis* | Minas Gerais/Campina Verde (19°32′55″S, 49°26′41.1″W) | ESA 107638 |
| *S. brasiliensis* | São Paulo/Paulínia (22°45′40″S, 47°09′15″W) | CPMA 736 |
| *S. campestris* | Rio Grande do Sul/Porto Alegre (30°04′07.5″S, 51°07′10.1″W) | ESA 107657 |
| *S. cissoides* | Bahia/Feira de Santana (12°12′05.5″S, 38°58′07.0″W) | ESA 107659 |
| *S. fluminensis* | São Paulo/Itirapina (22°13′22.6″S, 47°54′2.9″W) | ESA 107633 |
| *S. goyazana* | Goiás/Alto Paraíso de Goiás(14°10′22.8″S, 47°49′31.7″W) | ESA 107645 |
| *S. polyantha* | São Paulo/Pratânia (22°48′54.4″S, 48°44′35.8″W) | ESA 10764) |
| *S. quinquenervia* | São Paulo/Mogi-Guaçu (22°15′930″S, 47°08′804″W) | CPMA 2020 |
| *S. rufescens* | São Paulo/Cananéia (25°03′58.7″S, 47°54′58.1″W) | ESA 107648 |
| *S. subsessiliflora* | São Paulo/Ilha Bela (23°46′41″S, 45°21′29″W) | CPMA 1843 |
| *S. syphilitica* | Espírito Santo/Itarana (19°53′29.3″S, 40°49′09.4″W) | ESA 107665 |

*Note*: CPMA = Herbarium "Coleção de Plantas Medicinais e Aromáticas," Universidade Estadual de Campinas, Brazil; ESA = Herbarium of the Escola Superior de Agricultura "Luiz de Queiroz," Universidade de São Paulo, Brazil.