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Development of a novel set of microsatellite markers for *Lippia alba* (Verbenaceae)

D.S. Rocha[1], C.P. Santos[2], M.M. Bajay[3], J.B. Campos[1], A.F. Blank[2], J.B. Pinheiro[3] and M.I. Zucchi[1]

[1]Laboratório de Biologia Molecular,
Agência Paulista de Tecnologia dos Agronegócios, Piracicaba, SP, Brasil
[2]Laboratório de Cultura de Tecidos e Melhoramento Vegetal,
Departamento de Engenharia Agronômica, Universidade Federal de Sergipe,
São Cristóvão, SE, Brasil
[3]Laboratório de Diversidade Genética e Melhoramento,
Departamento de Genética, Escola Superior de Agricultura "Luiz de Queiroz",
Universidade de São Paulo, Piracicaba, SP, Brasil

Corresponding author: M.I. Zucchi
E-mail: mizucchi@apta.sp.gov.br

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ABSTRACT. Microsatellite primers were developed and optimized for *Lippia alba* to characterize the *L. alba* germplasm bank of Universidade de São Paulo. A genomic library enabled the design of 9 microsatellite primers. Six of the 9 primers yielded polymorphic products, which defined 2 groups in the bank. The data provide support to characterize germplasm banks, genetic breeding programs for *L. alba*, and other genetic diversity studies and classifications of species in the genus *Lippia*.

Key words: *Lippia alba*; Microsatellite; Genetic diversity; Essential oils; Germplasm bank

INTRODUCTION

Brazil has a wide diversity of medicinal plants that play an important role in traditional medicine. *Lippia alba* is a medicinal and aromatic plant of the Verbenaceae family, and it is spread around Central and South America. It is a shrub that produces essential oils with aromatizing, biological, and pharmacological properties. Essential oils from vegetal material of *L. alba* has potential uses in cosmetic industries, and it also has analgesic (Viana et al., 1998), anticonvulsant (Viana et al., 2000), sedative (Zétola et al., 2002), and antifungal (Shukla et al., 2009) activity. Accessions of *L. alba* may be defined in chemicals groups based on their major chemical components.

Information about the diversity and genetic structure in germplasm accessions is very important to allow the effective maintenance of different accessions with reduced costs (Gupta and Varshney, 2000). Microsatellites markers stand out despite the availability of other markers because they are multi-allelic and co-dominant, and they have a relatively high abundance and extensive genome coverage (Gupta and Varshney, 2000).

Despite these advantages, only 8 polymorphic microsatellite markers were previously published by Santos et al. (2012), indicating the necessity to increase the available set of these highly informative genetic markers for efficient management and improvement of *L. alba* germplasm resources. In this study, we describe 9 novel simple sequence repeat (SSR) markers for *L. alba* that were developed from a microsatellite-enriched library.

MATERIAL AND METHODS

The genomic DNA was extracted from young leaves of the *L. alba* accessions of the germplasm bank of Universidade de São Paulo. Leaves were collected from 90 accessions of the germplasm bank, instantly frozen with liquid nitrogen and stored at -80°C. The plant material was lyophilized, and DNA was extracted by the cetyltrimethylammonium bromide protocol described by Doyle and Doyle (1990). The DNA was quantified on a 1% agarose gel stained with SYBR safe using different concentrations of lambda phage DNA.

A microsatellite-enriched library was constructed following adapted protocols from Billotte et al. (1999). Genomic DNA from one genotype of *L. alba* was digested with *Afa*I (Invitrogen, Carlsbad, CA, USA) and enriched in microsatellite fragments using the $(CT)_8$ and $(GT)_8$ motifs. Microsatellite-enriched DNA fragments were ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and used to transform XL1-blue supercompetent cells. The positive clones were selected using the β-galactosidase gene and then were grown overnight with ampicillin. A total of 96 clones were sequenced in an ABI3700 automated sequencer (Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems).

The WebSat software (http://wsmartins.net/websat/) was used to identify the microsatellite-containing motifs. Nine primer pairs were designed for SSR-flanking regions using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/), and they were tested in 90 accessions of *L. alba* the germplasm bank.

Polymerase chain reaction (PCR) amplifications were performed in a 20-µL volume consisting of 2.0 µL 10 ng/µL DNA, 2.0 µL 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.9), 1.0 µL 2.5 mM dNTPs, 0.2µL 1 U *Taq* DNA polymerase (Invitrogen), 1.6 µL 25 mM $MgCl_2$, 0.32 µL 10 µM forward primer synthesized with an M13 sequence on the 5'-end, 0.4

µL 10 µM reverse primer, 0.2 µL 10 µM IRDye-labeled M13 primer (LI-COR Biosciences, Lincoln, Nebraska, USA), and 10.88 µL H_2O.

The amplification program consisted of an initial denaturing step at 94°C for 5 min; 10 cycles touchdown at 94°C for 40 s (denaturation), annealing temperature of each primer for 40 s (-1°C per cycle), and 1 min at 72°C for fragment extension; 30 cycles at 94°C for 40 s, 40°C for 40 s, and 72°C for 1 min; a final extension cycle at 72°C for 10 min; and finally 15°C forever.

The amplified fragments were electrophoresed on a LI-COR Model 4300 automated DNA sequencer (LI-COR Biosciences). Genotyping was performed by the Saga^{MX} software (LI-COR Biosciences). The number of alleles and observed and expected heterozygosities were calculated using the MStools software (Park, 2001).

RESULTS AND DISCUSSION

Nine microsatellite primers were optimized and characterized for *L. alba*, and 6 were polymorphic (Table 1). The number of alleles per locus ranged from 1 to 7 alleles, with an average of 3.83 alleles per locus. All microsatellite loci were composed of dinucleotide repeats. The observed heterozygosity ranged from 0 to 0.494 (on average), and the expected heterozygosity ranged from 0.054 to 0.771 (on average).

Table 1. Microsatellite markers developed for *Lippia alba.*

Locus	GenBank No.	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Size range (bp)	N_A	H_O	H_E
LAB05	KF611774	F: TCCACCTCTTCTGCTTCACA R: CAGTTCGTGGCATCTGTGTT	$(AC)_{19}$	50	123-141	7	0.091	0.771
LAD03	KF611775	F: CGACCTAAGACACACCTAAGCA R: TGGAAATATGGGTTCACCTTG	$(CT)_7(AC)_6$	55	252-268	2	0.056	0.054
LAE03	KF611776	F: GCAGCTCCAAATCCAACAG R: GTTGATTGCCAAAGCGTCTA	$(CA)_9$	56	254-260	4	0.000	0.583
LAE09	KF611777	F: GCATGAAATAATAAAATAAAAGACTCC R: CCCCTAAACCCCAAACTCAT	$(AG)_7(GA)_5$	55	204	1	0.000	0.000
LAF04	KF611778	F: GGCCTTGTGGTAAGATCCTG R: ACCATGCTGGGTTTATGTCC	$(TA)_5(GT)_{10}$	55	165-177	3	0.124	0.542
LAG04	KF611779	F: TGGAATTGGCTAGGCATGAT R: GGGTTGACCAAAAAGTCACAA	$(TG)_8$	55	206-216	2	0.000	0.000
LAG05	KF611780	F: CGATTCTGGAAAATCTGGGTA R: TGTTCTTGATGTTCATAAACCCTA	$(CA)_6$	55	255	1	0.000	0.000
LAH06	KF611781	F: TACACCACCACAGCAGCAC R: ACAGGCTTTACGCACGAAGT	$(AG)_8$	55	174-180	2	0.494	0.385
LAH09	KF611782	F: CGTGTCTCGGGATATACGTG R: CCTCACAAGAAAGCATGTGG	$(GT)_7$	55	252-266	5	0.236	0.323

F = forward; R = reverse; Ta = optimal annealing temperature; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity. All values are based on 90 accessions belonging to the germplasm bank of the Universidade de São Paulo.

In this study, the expected heterozygosity was higher than the observed heterozygosity of *L. alba* because the genotypes were collected in many different locations and they were reproduced through stem cuttings. Therefore, the accessions are not in Hardy-Weinberg equilibrium.

The microsatellite markers that were developed in this study efficiently characterized the genetic diversity of *L. alba* germplasm accessions. These highly informative markers will

provide support to manage the germplasm bank and will be useful for breeding programs for *L. alba*. These markers may be an important tool for distinguishing among species in the genus *Lippia* and may help identify quantitative trait loci, associations with agronomic and economic traits, and marker-assisted selection.

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