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Development and characterization of novel microsatellite markers in *Hyptis pectinata* (Lamiaceae)

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ABSTRACT. A microsatellite-enriched library was constructed and a set of 19 SSR markers were developed to characterize a germplasm collection of *Hyptis pectinata* (L.) Poit., maintained at the Universidade Federal de Sergipe (UFS). Fifteen markers of 19 ranged from moderately to highly polymorphic. A total of 113 alleles were identified, with a mean of 7.52 alleles per locus. The mean H_O and H_E were 0.582 and 0.657, respectively. The primers developed were efficient tools for accessing the genetic diversity of the germplasm collection analyzed and may also be useful for other studies involving this species and other species in the genus *Hyptis*.

Key words: Genetic diversity; Lamiaceae; Molecular markers; Simple sequence repeat

INTRODUCTION

Hyptis pectinata (L.) Poit (Lamiaceae) is an important aromatic species commonly known as "sambacaitá" or "canudinho" in the states of Sergipe and Alagoas; it is widely used in folk medicine for the treatment of inflammation and bacterial infections (Arrigoni-Blank et al., 2005). The anti-inflammatory and antinociceptive activities of *Hyptis pectinata* (L.) essential oil have been confirmed by Raymundo et al. (2011).

Due to the continuous loss of genetic diversity of many genetic resources, such as *Hyptis pectinata*, a Germplasm Bank of *H. pectinata* was created at the Universidade Federal de Sergipe (UFS); however, there is no information on the genetic variability of this germplasm collection, so far. Microsatellite markers, or simple sequence repeats (SSR), are used for a variety of applications in plant genetics and breeding because they are reproducible, multi-allelic, codominant, relatively abundant, and have good genomic coverage (Remya et al., 2010). In this study, we constructed an enriched genomic library and designed microsatellite primers for *H. pectinata*.

MATERIAL AND METHODS

Genomic DNA of *H. pectinata* was extracted from the leaf tissues of 56 accessions according to Doyle and Doyle (1990). The plant material collected was immediately stored in liquid nitrogen for lyophilization, ground, and stored at -20°C for DNA extraction.

A microsatellite-enriched genomic library was constructed following the adapted protocol from Billotte et al. (1999). High quality genomic DNA from a single individual of *H. pectinata* was digested with *Afa*I (Invitrogen).

The fragments obtained were ligated to *Afa*21 (5'-CTCTTGCTTACGCGTGGACTA-3') and *Afa*25 (5'-TAGTCCACGCGTAAGCAAGAGCACA-3') adapters and then amplified via polymerase chain reaction (PCR). Microsatellite-enriched DNA fragments were selected using the $(CT)_8$ and $(GT)_8$ motifs using magnetic beads connected to streptavidin (Promega). The fragments selected were ligated into the pGEM-T Easy Vector (Promega) and used to transform Epicurian Coli XL1-Blue *Escherichia coli* competent cells (Promega). The positive clones were selected using the β-galactosidase gene and then grown overnight with ampicillin. A total of 96 clones were sequenced in an ABI 3770 automated sequencer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). The WebSat software (http://wsmartins.net/websat/) was used to identify the microsatellite-containing regions. Twenty-four fragments were identified and contained at least 5 microsatellite motifs. A set of 19 SSR markers were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/). Subsequently, the primer pairs were synthesized and tested in 56 half-sib accessions from the germplasm collection of the UFS.

The PCR was performed in a final volume of 10 µL containing 1.0 µL genomic DNA 5.0 ng, 6.65 µL ultrapure water, 1.0 µL buffer 10X *Taq* DNA Polymerase [75 mM Tris-HCl; 20 mM $(NH_4)_2SO_4$, pH 8.8; 0.01% (v/v) Tween 20], 0.8 µL $MgCl_2$ 25 mM, 0.1 µL 2.5 U/µL *Taq* DNA Polymerase (Fermentas), 0.5 µL dNTPs (2.5 mM), 0.2 µL of each primer (forward + reverse 10 µM), and 0.1 µL (10 µM) labeled tag with the fluorophore IRDye700 or IRDye800 (LI-COR Biosciences). The PCR amplification consisted of a touchdown program, an initial cycle at 94°C for 5 min; 10 cycles of touchdown at 94°C for 40 s for denaturation, annealing temperature of -1°C for each primer for 40 s, 72°C for 1 min for fragments extension; 30 cycles at 94°C for 40 s, 40°C for 40 s, 72°C for 1 min; and a final extension cycle at 72°C for 10 min and 15°C forever.

The reaction product was electrophoresed on an automated DNA sequencer LI-COR Model 4300 (LI-COR Biosciences) equipped with 2 infrared lasers, with the ability to simultaneously read 2 wavelengths (700-800 nm) using the standard fragments (50-350 bp) labeled with fluorescence IRDye700 and IRDye800 (LI-COR Biosciences). The exact sizes of the fragments were determined using the SAGA^{MX} software v3.3 (LI-COR Biosciences). N_A, H_O, and H_E were estimated using the Mstools software (Park, 2001). The deviation from Hardy-Weinberg equilibrium and linkage disequilibrium were verified by using the GENEPOP (Rousset, 2008).

RESULTS AND DISCUSSION

All 19 loci amplified specific bands, with sizes ranging from 140 to 309 bp; their sequences were deposited at the National Center for Biotechnology Information (NCBI) and can be accessed using the GenBank accession Nos. provided in Table 1. Fifteen loci exhibited moderate to high levels of polymorphism, varying from 2 to 20 alleles. A total of 113 alleles were identified, with a mean of 7.52 alleles per locus. The H_O and H_E ranged from 0.000 to 1.000 and 0.071 to 0.937, with means of 0.582 and 0.657, respectively.

Table 1. Characteristics of 19 microsatellite loci developed for *Hyptis pectinata* from Sergipe State, northeast Brazil.

Locus GenBank No.	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Size range (bp)	N_A	H_O	H_E	P value
Hpe01 KF781265	F: *CCTCCATTCACACTCCCCTA R: GAAACACTCACAGCGAGAGC	$(CT)_{16}$	50	162-184	6	0.925	0.636	0.0000
Hpe02 KF781266	F: *TGAATCCCAATCCGACATC R: GCGACTGCTCCTACAACTC	$(GA)_{15}$	50	174-208	10	0.189	0.872	0.0000
Hpe03 KF781267	F: *GCTTCATCCCACGGACTA R: TGTGGGCTGGTGATAGATGT	$(CA)_7$	50	228-244	5	0.889	0.617	0.0000
Hpe04 KF781268	F: *TCTCGGATCAACATGAGCTG R: AGACGTTATTTGGAGCAGCA	$(AC)_{11}$	50	274-284	6	0.712	0.785	0.0000
Hpe05 KF781269	F: *AGCGACTGTTGGGTTTCTC R: GCACCTCCCTTCTTTCAAC	$(TG)_8(AT)_6$	50	165-213	20	0.830	0.937	0.0000
Hpe06 KF781270	F: *CACTGCTCCTGCAATCCCAC R: CCAACTCCAAAGACTTCTAGGC	$(CT)_5(CA)_7$	50	287-309	11	1.000	0.830	0.0000
Hpe07 KF781271	F: *AGCACAGTTTGGGACTTCA R: GCTTGTTTTCATCCTCATGC	$(AC)_7$	50	193-201	4	0.982	0.556	0.0000
Hpe08 KF781272	F: *CAGTTCAGCTCACCCTCCT R: GCTTTGATCCTGTGTTCCT	$(CA)_7$	50	178-192	5	0.080	0.770	0.0000
Hpe09 KF781273	F: *CAAAAATTGATAAGTTCGGTGAA R: TGTGCAGAATCTCAAACTTCAAA	$(GT)_9(AG)_6$	50	219-233	6	1.000	0.680	0.0000
Hpe10 KF781274	F: *CAACCTTCCTAACCAAGATATGG R: AAAGTGACAAGCTCTGCAAGG	$(AC)_8$	50	205-211	3	0.000	0.138	0.0000
Hpe11 KF781275	F: *GGACTAACCACTTAGGTCTCCAAA R: ATGGTAGGGTCGTCGGTAGA	$(CA)_7$	50	168-170	2	0.000	0.071	0.0000
Hpe12 KF781276	F: *ATGTCTGCTGCCAAATTGC R: GCCCATTCAATCAACCTACA	$(AC)_8(ATG)_4$	50	247-275	12	0.982	0.875	0.0090
Hpe13 KF781277	F: *CCCGCTGTTTTTAATAGGTCA R: CGTGTCTTTTGCTCATTGTTTC	$(TG)_9$	50	237-245	3	0.113	0.414	0.0000
Hpe14 KF781278	F: *CGTTGGAAATTCAAGGCTTC R: GGGATCAAAATGGTCGAGAA	$(GAT)_8$	50	268-301	11	0.944	0.861	0.0000
Hpe15 KF781279	F: *CCAATTTATGTTTGCCGTGA R: GCAGTCATCATGTTTCAAAGC	$CA)_5(AG)_{30}$	50	209-243	9	0.089	0.824	0.0000
Hpe16 KF781280	F: *TGCTACTTCATGTCTTGCACA R: CATTGAGTTTTAGAGGATTGC	$(AC)_6$	50	191	1	0.000	0.000	-
Hpe17 KF781281	F: *CAAGAATTTGGTTGGTAGC R: TCCACGTAAAGTCCCATCT	$(CA)_6(CATA)_3$	50	140	1	0.000	0.000	-
Hpe18 KF781282	F: *CGAAAGGTGAGGAAGAACA R: CAAACAAAACCAAGGCATGA	$(GT)_8$	50	149	1	0.000	0.000	-
Hpe19 KF781283	F: *CATGTCTCCAACCCCAACT R: CGATGTCCAACTCAAGTGG	$(CA)_5$	50	231	1	0.000	0.000	-

Ta = annealing temperature; P value = probability of deviation from Hardy-Weinberg equilibrium. *M13 tag (5'-CACGACGTTGTAAAACGAC-3') label.

After Bonferroni's correction, all the loci, except Hpe12, significantly departed from Hardy-Weinberg Equilibrium, which is common for germplasm banks (Sandes et al., 2013). The SSR markers developed were efficient in accessing the high genetic variability present in the germplasm collection of *H. pectinata* maintained at the UFS. The information generated will be useful for the conservation and selection of the most representative genotypes. The microsatellite markers developed may be used for genetic studies in other germplasm banks, population structure analyses, and interspecific genetic studies within the genus *Hyptis*.

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