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Short Communication

Development and characterization of microsatellite primers in *Pogostemon cablin* (Lamiaceae)

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ABSTRACT. Microsatellite primers were developed and optimized for patchouli (*Pogostemon cablin*) to characterize the patchouli Active Germplasm Bank of Universidade Federal de Sergipe. Creation of a genomic library for patchouli enabled the design of 12 microsatellite primers. Six of these microsatellites were polymorphic, revealing two well-defined groups of individuals that possess exclusive alleles. The data allowed us to characterize the patchouli active Germplasm Bank, identify its genetic diversity, and provide new information for researching this species.

Key words: Genetic diversity; Lamiaceae; *Pogostemon cablin*; SSR

INTRODUCTION

Patchouli [*Pogostemon cablin* (Blanco) Benth.] is an aromatic, herbaceous plant that belongs to the Lamiaceae family, and is native to Southeast Asia. Its leaves produce an essential oil that is highly regarded in the perfume and cosmetic industries (Swamy et al., 2010). Because of the importance of patchouli essential oil, research groups around the world studied the production of sesquiterpenes that compose the oil in order to create more profitable and higher quality products (Paul et al., 2010).

A patchouli collection from Universidade Federal de Sergipe (UFS) has been chemically and morpho-agronomically characterized (Blank et al., 2011). However, for complete characterization of a germplasm bank, molecular markers are an efficient alternative. 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). However, because SSR primers have not yet been found in species closely related to patchouli, it was necessary to construct a genomic library and design oligonucleotide initiators. In this study, we constructed an enriched genomic library and designed microsatellite primers for patchouli.

MATERIAL AND METHODS

Young leaves of the patchouli accessions of the Active Germplasm Bank of the UFS were collected. There are 10 genotypes in the Active Germplasm Bank, and efforts are being made to collect a larger number of divergent materials all over the world (Table S1). The plant material was stored in liquid nitrogen for lyophilization. After lyophilization, the plant material was ground, and the resulting powder was used for the subsequent steps. The CTAB protocol, described by Doyle and Doyle (1990), was used to extract the genomic DNA. DNA was quantified on 1% agarose gel stained with SYBR safe, using lambda phage DNA at different concentrations.

A microsatellite-enriched library was obtained using adapted protocols from Billotte et al. (1999), with modifications. Genomic DNA from one genotype of patchouli was digested with *Afa*I (Invitrogen), and enriched in microsatellite fragments using $(CT)_8$ and $(GT)_8$ motifs. Microsatellite-enriched DNA fragments were ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA) and were used to transform Epicurian Coli XL1-Blue *Escherichia coli* competent cells (Promega). The positive clones were selected using the β-galactosidase gene and then were grown overnight with ampicillin. Sequencing reactions (10 µL) contained 200 ng plasmid DNA, 0.5 pmol SP6 primer, 0.4 µL BigDye Terminator mix (version 3.1; Applied Biosystems, Foster City, CA, USA), 1 mM $MgCl_2$, and 40 mM Tris-HCl, pH 9.0. Sequencing reactions were performed in a thermal cycler (MJ Research, BioRad, Hercules, CA, 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. Polymerase chain reaction (PCR) products were precipitated with isopropanol (65%), centrifuged, and washed with 70% alcohol. Ninety-six positive clones were sequenced on an ABI 3700 automated sequencer (Applied Biosystems).

The WebSat software (http://wsmartins.net/websat/) was used to identify the microsatellite-containing regions using at least 12 bp. A total of 12 primer pairs were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/). The Gene Runner v. 3.05 (http://www.generunner.net/) program was used to validate the parameter values that were estab-

lished for primer design and showed the probability of secondary structure formation that prevented primer usage.

The amplification reaction used 3.0 µL 5 ng/µL DNA, 0.5 µL 10 µM forward primer, 0.5 µL 10 µM reverse primer, 1.6 µL 2.5 mM dNTPs, 1.0 µL 1 U/µL *Taq* DNA polymerase, 2.0 µL 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.9), 0.8 µL 25 mM $MgCl_2$, and 10.6 µL H_2O. The amplification program consisted of a denaturation step at 94°C for 5 min followed by 33 amplification cycles (94°C for 1 min, followed by the specific annealing temperature for each primer pair (Table 1), and 72°C for 1 min), and a final elongation step at 72°C for 10 min. To amplify the *Pca3*, *Pca6*, and *Pca8* primer pairs, a touch-down thermal profile was used, which was similar to the program used above, except that the annealing temperature was initially reduced in steps of 2°C every 7 cycles, from 67° to 55°C, followed by 30 cycles at 53°C. The PCR-amplified fragments were run on a 7% denaturing polyacrylamide electrophoresis gel and stained with silver nitrate (Creste et al., 2001).

RESULTS AND DISCUSSION

The 6 polymorphic microsatellite primers are shown in Table 1. The primer *Pca1* had the most alleles (4), and all other loci had 2 alleles, resulting in an average of 2.33 alleles per polymorphic locus. Of the microsatellite loci that were polymorphic, 5 were composed of dinucleotide repeats, and only one had a trinucleotide repeat. The largest expected heterozygosity (H_E) for the locus *Pca1* was 0.678. For the other loci (*Pca3*, *Pca4*, *Pca6*, *Pca7*, and *Pca8*), the H_E was 0.356 (Table 1). The H_E observed for all loci was less than the observed heterozygosity (H_O), indicating an excess of heterozygotes compared to expectations under Hardy-Weinberg equilibrium (HWE). The high H_O and H_E averages may be due, in part, to

Table 1. Characteristics of eleven microsatellite loci of *Pogostemon cablin*.

Locus	GenBank No.	Primer sequence (5'- 3')	Repeat motif	Ta (°C)	Size range (bp)	N_A	H_O	H_E
Pca2	GF111966	F: GTCGAAGGTTCAGCCTCTTG R: TCGGAACATCAGCAATGAG	$CAATG_{(3)}$	58	125-130	1	0.000	0.000
Pca5	GF111967	F: CCCTTTACAATAACCTCGACA R: ATCAACAGCACACCGTAGAGA	$TATT_{(3)}$	61	130-134	1	0.000	0.000
Pca11	GF111968	F: TTCCCCTTAGATTGTGCAAA R: AGACAAAGTGAGGCATCTGATT	$TTGA_{(3)}$	61	232-238	1	0.000	0.000
Pca12	GF111969	F: AAAAAGCTCACGCCCTTCT R: CTCGTTATCAATGGGATCAGG	$AACC_{(3)}$	65	250-254	1	0.000	0.000
Pca1	GF111970	F: ACACACTCCCCCACCATAC R: CCACCTGTTTCTTTCACTTCC	$GA_{(16)}$	57	228-240	4	0.167	0.678
Pca3	GF111971	F: CCATTTCGTCACCTCTC R: AAACAGGCAAGTGAAAGT	$CA_{(8)}$	53	164-168	2	0.167	0.356
Pca4	GF111972	F: AGGGAAGCAGGGAAAACATT R: CTTGCGGCATTTAGGGATAC	$AG_{(14)}$	65	258-266	2	0.167	0.356
Pca6	GF111973	F: ACAAAGGGTTGACGATTG R: GTGATGAAACTGTCTCTCCTG	$TG_{(4)}$.TC.$TG_{(5)}$ $AG_{(4)}$...$TGTT_{(3)}$	53	180-216	2	0.167	0.356
Pca7	GF111974	F: AAGCGTGATTGCCCTTAAT R: GCCGTATTTGAGAAATGCTT	$TG_{(6)}$.AGTC.$TA_{(4)}$	57	186-220	2	0.167	0.356
Pca8	GF111975	F: ACCTGACGCCACTACTCCTC R: TCACTTTGAATGCGAACCA	$CAT_{(5)}$	53	207-210	2	0.167	0.356
Pca10	GF111976	F: TCAAGATGAACTGGAGGAAACA R: CGCTAACGCCAACTATGCTA	$TCA_{(4)}$	63	215-218	1	0.000	0.000

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 10 accessions deposited at the active germplasm bank of Universidade Federal de Sergipe.

the low number of evaluated individuals. However, results were obtained from a germplasm collection, which may not be in HWE. Indeed, there is absolutely no reason to expect HWE in germplasm collections since genotypes are collected in different populations and since no further breeding occurred from the moment of sampling; HWE is expected in panmictic populations. The presence of a large number of homozygotes can also be explained by the fact that patchouli is reproduced through stem cuttings.

The production of a patchouli genomic library enabled the development of 12 microsatellite primers, 6 of which were polymorphic in the evaluated genotypes. The development of these novel primers for patchouli will facilitate research on the conservation and diversity of the germplasm. The developed markers will also be essential for ongoing studies on the variability of the bank and other populations of this important aromatic species.

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Supplementary material

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