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Short Communication

New loci of *Lychnophora ericoides* and transferability to *Lychnophora pinaster*, endangered medicinal species from Brazil

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ABSTRACT. *Lychnophora ericoides* and *Lychnophora pinaster* are species used in popular medicine as analgesic or anti-inflammatory

agents to treat contusions, rheumatism, and insect bites. In this study, 21 simple sequence repeat loci of *L. ericoides* were developed and transferred to *L. pinaster*. Three populations of *L. ericoides* and 2 populations of *L. pinaster* were evaluated; they were collected in the State of Minas Gerais. Population parameters were estimated, and the mean values of observed and expected heterozygosity were 0.297 and 0.408 (*L. ericoides*) and 0.228 and 0.310 (*L. pinaster*), respectively. Greater genetic variability was observed within populations than between populations of *L. ericoides* (62 and 37%) and *L. pinaster* (97 and 2.8%). These results provide information for genetic conservation and taxonomic studies of these endangered species.

Key words: Microsatellite; Population genetics; Arnica; Medicinal plant

INTRODUCTION

Several species of the genus *Lychnophora* (Asteraceae) have aromatic and medicinal potential and are found in rock sites in the Brazilian states of Minas Gerais, Bahia, Goiás, and Distrito Federal (Semir et al., 2011). Predatory collection associated with the restricted distribution of the habitat, which includes small and isolated populations, has contributed to the decline of natural populations of the genus *Lychnophora*. The species *Lychnophora pinaster* Mart. and *Lychnophora ericoides* Mart. are on the list of endangered species of the State of Minas Gerais (COPAM, 2000), and the latter species is also on the list of endangered plants of IBAMA (IBAMA, 2008), highlighting the need to understand the ecology of these species and the contribution of new techniques to their preservation.

L. ericoides, which is popularly known as arnica or false arnica, occurs in the states of Minas Gerais, Goiás, and Distrito Federal. *L. pinaster*, which is known as arnica, is found in the State of Minas Gerais. The 2 species are very close, and the taxonomic separation between them is very complicated (Semir et al., 2011). The species are used in popular medicine as alcoholic extracts, such as anti-inflammatory, anesthetic, and scarring agents (dos Santos et al., 2005; Silveira et al., 2005).

MATERIAL AND METHODS

Total genomic DNA was extracted from freeze-dried leaves of *L. ericoides* using the cetyltrimethylammonium bromide protocol described by Doyle and Doyle (1990) with modifications. A library enriched in microsatellite regions was obtained through protocols adapted from Billotte et al. (1999). The total DNA of a genotype of *L. ericoides* was digested by the enzyme *Afa*I (Invitrogen, CA, USA) and enriched with fragments using probes with the motifs $(CT)_8$ and $(GT)_8$. The amplified fragments were cloned in the pGEM-T Easy Vector System (Promega, Madison, WI, USA) according to the protocol provided with the plasmid vector and transformed in XL1-Blue competent cells. Positive clones were selected using the β-galactosidase gene and kept overnight with ampicillin.

The sequencing reaction was performed with 2 µL Save Money buffer (5 mM $MgCl_2$, 200 mM Tris-HCl, pH 9), 5 pM SP6 initiator (5'-CATACGATTTAGGTGACACTATAG-3'), 2 µL plasmid extraction, and 0.4 µL BigDye Terminator v3.1 (Applied Biosystems, CA, USA)

Table 1. Characteristics of 8 microsatellite loci from *Lychnophora ericoides* and *Lychnophora pinaster*.

Locus	GenBank accession	Primer nucleotide sequence (5'-3')	Repeat motif	Size range (bp)	N_A	*L. ericoides*		*L. pinaster*		PIC	P value H-W proportions	
						H_O	H_E	H_O	H_E		*L. ericoides*	*L. pinaster*
Lye2	KC016419	R: AAAACAACCGAACTGGAGTCA F: TGAGATTTAGCCGGAGGAAG	$(TA)_5TG(CA)_6(CT)_{11}$	220-242	4	0.306	0.570	0.357	0.415	0.480	0.0000*	0.0613
Lye6	KC016420	R: TTCACCACAACCTACACTCTC F: GAAAAGCCACCCCAAACT	$(ATGT)_3$	220-274	5	0.542	0.608	0.378	0.394	0.536	0.0000*	0.0002
Lye9	KC016421	R: CATGAGCAGTAATTGAGGTTT F: TAGCATTTTAATTTTGGGAAGT	$(AAT)_4TT(AAT)$	130-142	4	0.377	0.381	0.031	0.201	0.301	0.0036	0.0000*
Lye11	KC016422	R: TGAAGTTGCTTGTGTAAGAGAG F: GAGAAAATAGTGTTGAGGGAAG	$(CA)_5$	310-330	2	0.726	0.493	0.543	0.400	0.355	0.0006*	0.0218
Lye12	KC016423	R: AGAGAACCTTCATTTTCAAAGC F: TTTTTGAGGGAATAAAGCTC	$(TGA)_3TCA(AT)_3$	190-198	2	0.076	0.381	0.226	0.203	0.266	0.0000*	1.0000
Lye13	KC016424	R: CATCAAACCAGCACAATAGAAC F: TTGGGGATAGATAATGGTGTAA	$(AT)_3C(AT)_3$	230-240	4	0.059	0.513	0.000	0.369	0.404	0.0000*	0.0000*
Lye14	KC016425	R: TTCTCTGGCGTTTCTTAGGTG F: GGTTGGTGGTGAGGAATCAT	$(TGG)_4$	160-170	2	0.106	0.102	0.000	0.000	0.054	1.0000	0.0000*
Lye15	KC016426	R: ATCCTCTGTCCCACCATTAT F: TTTTCCTCTCCCAAGCAGT	$(AC)_4(TC)_{10}$	266-280	6	0.188	0.212	0.286	0.496	0.304	0.0391	0.0118
AVERAGE						0.297	0.408	0.228	0.310			

F = forward primer; R = reverse primer; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphism information content. P value from exact test for Hardy-Weinberg proportions. *P ≤ 0.00125 with Bonferroni correction; 0.000 = monomorphic locus.

in a final volume of 10 µL. The sequencing reactions were performed in a thermocycler (MJ Research, BioRad, Hercules, California, USA), under the following conditions: 96°C for 2 min; 26 cycles at 96ºC for 45 s, 50°C for 30 s, and 60°C for 4 min. Polymerase chain reaction products were precipitated in isopropanol (65%), centrifuged, and washed with 70% alcohol. Ninety-six positive clones were sequenced in an ABI 3700 automatic sequencer (Applied Biosystems, CA, USA).

In total, 21 pairs of primers were designed using the Primer 3 v 0.4.0 software (Rozen and Skaletsky, 2000). The loci were assessed in 3 populations of *L. ericoides* and transferred to 2 populations of *L. pinaster*, which were both collected in the state of Minas Gerais, Brazil. Eight loci amplified well from the populations of *L. ericoides*, and 6 loci amplified well from the populations of *L. pinaster*. These loci were polymorphic and informative. The reactions included 1 ng DNA, 0.4 mM each primer, 0.2 mM dNTPs, and 2 mM $MgCl_2$. The amplification conditions were as follows: 94°C for 2 min; 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and extension at 72ºC for 10 min. The amplification products were separated by electrophoresis on 7% denaturing polyacrylamide gels and viewed by silver staining (Creste et al., 2001).

The adherence test (Fisher's exact test) for the balance proportions of Hardy-Weinberg was determined using the GenePop software (Raymond and Rousset, 1995). The allelic frequencies, the number of alleles per locus, the observed (H_O) and expected heterozygosity (H_E), and the polymorphic content were calculated using the MSTOOLS software (Park, 2008) (Table 1). Wright's F-statistics were estimated using the GDA software (Lewis and Zaykin, 2001), and the analysis of molecular variance was performed between and within populations using the Arlequin software (Schneider et al., 2007).

RESULTS AND DISCUSSION

The average H_O was smaller than the average H_E, 0.297 and 0.408 (*L. ericoides*) and 0.228 and 0.310 (*L. pinaster*), respectively (Table 1). Rabelo et al. (2011) found that the H_O (0.828) was greater than the H_E (0.797) when they analyzed natural populations of *L. ericoides*. For the species *L. pinaster*, Haber et al. (2009) found that the H_O was smaller than the H_E in the Antena (0.481 and 0.574) and Estrada Real (0.563 and 0.625) populations.

Greater genetic variability was observed within populations than between the populations of *L. ericoides* (62 and 37%) and *L. pinaster* (97 and 2.85%). The data obtained in this study showed a high variability between populations of *L. ericoides*, indicating that it is necessary to sample a large number of individuals in each population and to collect various populations to ensure the maintenance and conservation of this species. The low genetic variability found among populations of *L. pinaster* may be related to the geographical proximity among them, which allows high gene flow.

We may conclude that microsatellite markers for these species may be used in the creation of a germplasm database and contribute to the conservation, genetic diversity, and taxonomic studies of these endangered medicinal species.

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