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# Development and characterization of microsatellite markers for *Lychnophora pinaster*: a study for the conservation of a native medicinal plant

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## Abstract

***Lychnophora pinaster*** **Mart. (Asteraceae) is a Brazilian medicinal plant, extensively employed in popular medicine as an anti-inflammatory, analgesic and healing agent. Thirteen polymorphic microsatellite markers were developed and optimized for *L. pinaster* from an enriched genomic library. The markers were used to analyse 37 plants from two native populations, generating an average number of 6.6 alleles per polymorphic locus. These loci are important tools for future studies of population genetics.**

*Keywords*: *Lychnophora pinaster*, diversity, native populations, primers, genetic structure, medicinal plant

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*Lychnophora pinaster* Mart. (Asteraceae), popularly known as 'arnica', is a remarkably microendemic species from the rocky fields of the Brazilian savannah. Its aromatic leaves and flowers are extensively used in traditional medicine due to its anti-inflammatory, analgesic and healing action. It has also cosmetic uses in soaps, to prevent rashes, dryness and to smooth out bruises and contusions (Souza 2003). Due to its properties, the essential oil of *L. pinaster* is potentially of great interest for the pharmaceutical and cosmetics industries.

Despite the intensive use of *L. pinaster* by native populations, there are no reports of its cultivation, being that all of the plant materials found in the market and in domestic use were obtained from predatory and indiscriminate harvesting in natural populations. This has led to the inclusion of the species in the category of 'vulnerable' plants, i.e. taxa whose populations are in decline as a consequence of excessive exploitation, habitat destruction or other environmental change, and whose long-term survival is not assured.

Therefore, due to its pharmacological significance, its potential for industrial use and the fact that *L. pinaster* is threatened by extinction, we have considered it relevant to investigate its genetic diversity in order to provide information to the conservation of native populations and to assist genotype selection for commercial cultivation and genetic breeding programmes aimed at the production of aromatic and medicinal compounds in commercial scale.

We have developed microsatellite markers according to Billotte *et al*. (1999) with modifications, using enriched genomic libraries. In short, DNA from a randomly chosen individual of *L. pinaster* was digested by the enzyme *Rsa*I (Biolabs) and the fragments were ligated to the adaptors generated by annealing *Rsa*21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and *Rsa*25 (5′-TAGTCCACGCGTAAGCAAGAGCACA-3′). The adaptor-ligated genomic fragments were amplified by polymerase chain reaction (PCR) and selected by biotin-labelled streptavidin-associated magnetic beads with the probes $(TTC)_{10}$, $(CG)_{10}$, and $(GT)_{10}$. The selected fragments were PCR-amplified by using the primer *Rsa*21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and cloned into pGEM-T vectors (Promega) that were subsequently transformed into competent XL1-Blue cells. Sequencing reactions were carried out using a T7 primer and Big Dye 3.1 (Applied Biosystems) in a PTC-200 (MJ Research) thermal cycler. Sequences were run on an automatic sequencer ABI PRISM 3730 (Applied Biosystems). Complementary primers to the (simple sequence repeat) SSR-flanking sequences were designed using the TROLL software (Castelo *et al*. 2002).

We sequenced 178 inserts; 152 (85.3%) contained at least a single microsatellite region. Microsatellites were aligned and complementary and/or overlapping ones were excluded. Seventeen primer were designed and 13 (76.4%) were optimized and used for loci amplification to genetically characterize and study the structure of the native population.

Plant material was obtained from young leaves of 37 individual plants found in two native populations of *L. pinaster*, named Antena (18 individuals) and Estrada Real (19 individuals), occurring naturally in Lavras and Carrancas cities, respectively, Minas Gerais State, Brazil. The sampled material was finely ground in liquid nitrogen and stored at –80 °C. DNA extraction was performed as described by Doyle & Doyle (1990) with modifications.

PCR amplifications were carried out in a final volume of 25 μL containing 150 μM of each dNTP, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 U of *Taq* DNA polymerase (Invitrogen), and 50 ng of DNA. $MgCl_2$ concentration ranged from 0.75 to 3 mM, whereas primer concentration was optimized for each locus and ranged from 0.4 to 1.6 μM. Primer and amplification data for each locus are shown in Table 1.

PCRs were carried out in a PT-100 thermal cycler (MJ Research) programmed to initiate the cycles at 94 °C for 2 min; followed by 32 cycles consisting of a initial denaturation step of 1 min at 94 °C, 1 min at the primer-specific annealing temperature and 1 min of primer extension at 72 °C, with a final extension step at 72 °C, for 10 min. Amplification products, along with molecular weight markers 10 bp ladders, were resolved in denaturing conditions in 4% (v/v) polyacrylamide gels containing 8 M of urea and 5× TBE (Tris-base; $H_3BO_3$; $Na_2EDTA \cdot 2H_2O$), run in 1× TBE at 60 V for 4 h. The gels were vertically run and silver-stained.

We have identified 89 alleles distributed along the 13 analysed loci from the two populations, at an average allele number of 6.6 alleles per polymorphic locus, and ranging from two alleles (Lpin3 and Lpin17) to 21 alleles (Lpin8). Loci Lpin17 (0.869) and Lpin5 (0.130) were the most and the least heterozygous loci, respectively, whereas the highest and lowest expected heterozygosity was found for loci Lpin8 (0.918) and Lpin5 (0.123). For certain loci, it has been demonstrated that the observed heterozygosity was higher than the expected (loci Lpin4, Lpin6, Lpin8, Lpin9, Lpin11 and Lpin13). The excessive observed heterozygosity may be due to small sample sizes or a selective advantage for heterozygotes.

The expected heterozygosity ranged from 0.0000 (Lpin3) to 0.8983 (Lpin13), and the observed heterozygosity ranged from 0.0000 (Lpin3) to 0.8333 (Lpin14 and Lpin16) in the Antena population. In the Estrada Real population, the expected heterozygosity ranged from 0.1977 (Lpin4) to 0.8705 (Lpin13), and the observed heterozygosity ranged from 0.2105 (Lpin4) to 0.9473 (Lpin1; Table 1).

Tests of Hardy–Weinberg equilibrium (HWE) was calculated for the 13 loci and for the populations using FSTAT

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**Table 1** Characteristics of the 13 microsatellite markers of *Lychnophora pinaster* Mart.

| Locus name | GenBank Accession no. | Repeat motif | Primers sequence | $T_a$ (°C) | $MgCl_2$ (mM) | Primer (µM) | Allele size (bp) | PIC | $N_a$ | $N_a$ | | $H_O$ | | $H_E$ | | $P$-HWE | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Ant | Ere | Ant | Ere | Ant | Ere | Ant | Ere |
| Lpin 1 | BV722918 | $(AG)_{26}$ | F: CAAATGTCACATCGATTCGC<br>R: GCAGAGGAGGAGTAGGGGAT | 54 | 0.375 | 0.8 | 280–310 | 0.634 | 6 | 4 | 6 | 0.611 | 0.947 | 0.617 | 0.750 | 1.0000$^{NS}$ | 0.0602$^{NS}$ |
| Lpin 3 | BV722919 | $(CA)_{16}$ | F: TGTCATCACCAAACCATTCAA<br>R: AAATCCCTGCTTGATGATGG | 54 | 0.375 | 0.4 | 230–232 | 0.095 | 2 | 1 | 2 | 0.000 | 0.316 | 0.000 | 0.273 | — | 1.0000$^{NS}$ |
| Lpin 4 | BV722920 | $(AC)_5(CA)_{16}(AG)_{20}$ | F: TCTGTCAACACCCCTCCCTA<br>R: GTGCAAGCTGGCGATAAGAT | 54 | 0.375 | 0.4 | 118–136 | 0.308 | 4 | 3 | 3 | 0.556 | 0.211 | 0.446 | 0.198 | 0.1237$^{NS}$ | 1.0000$^{NS}$ |
| Lpin 5 | BV722921 | $(CA)_{13}(AT)_5$ | F: CGGGTCACATATTACTCGCC<br>R: ATAATGGGCTCCACGAAACA | 50 | 1.500 | 0.8 | 162–192 | 0.777 | 8 | 7 | 7 | 0.389 | 0.474 | 0.657 | 0.797 | 0.0010* | 0.0201$^{NS}$ |
| Lpin 6 | BV722922 | $(TA)_5(AC)_{16}$ | F: AGTTCAGCTGTTTAGGGGCA<br>R: GCTGGAACCCGTTATTGCTA | 50 | 0.375 | 0.8 | 290–330 | 0.589 | 5 | 4 | 5 | 0.167 | 0.632 | 0.410 | 0.657 | 0.0325$^{NS}$ | 1.0000$^{NS}$ |
| Lpin 8 | BV722923 | $(TA)_{20}(TAA)_4$<br>$(ATT)_4(AC)_5(CA)_6$ | F: ATCAAAGGGGTTCTCTCGGT<br>R: AAAAAGCCATTGCCAAACAC | 54 | 0.375 | 0.4 | 150–254 | 0.911 | 21 | 14 | 11 | 0.444 | 0.526 | 0.810 | 0.859 | 0.0108$^{NS}$ | 0.0273$^{NS}$ |
| Lpin 9 | BV722924 | $(GA)_{14}$ | F: GGTTGGCGAAAGACAGAGAA<br>R: CACAATCCTAGCCCAACCAC | 54 | 0.375 | 0.8 | 266–280 | 0.639 | 4 | 4 | 3 | 0.722 | 0.526 | 0.662 | 0.542 | 1.0000$^{NS}$ | 0.6427$^{NS}$ |
| Lpin 10 | BV722925 | $(ATAA)_5(GA)_{12}$ | F: TCATGAGAGAAGGACGCTCA<br>R: GGAAATACAGTGGCAGGGTC | 50 | 0.375 | 0.8 | 242–250 | 0.687 | 5 | 5 | 3 | 0.278 | 0.158 | 0.754 | 0.633 | 0.0410$^{NS}$ | 0.0042$^{NS}$ |
| Lpin 11 | BV722926 | $(TC)_{11}$ | F: GCACGGCTGGTCACTAATCT<br>R: GAAGTTCCATGGCCTACCAA | 54 | 0.375 | 1.6 | 228–330 | 0.777 | 11 | 7 | 9 | 0.333 | 0.579 | 0.549 | 0.859 | 0.0413$^{NS}$ | 0.6069$^{NS}$ |
| Lpin 13 | BV722927 | $(TC)_{19}$ | F: TTGAAGGCTCAAGGTTTTGG<br>R: CCCATCGCACCGAATAATAA | 54 | 0.375 | 1.6 | 168–212 | 0.867 | 13 | 10 | 8 | 0.412 | 0.421 | 0.898 | 0.871 | 0.1774$^{NS}$ | 0.0096$^{NS}$ |
| Lpin 14 | BV722928 | $(CT)_{22}$ | F: TAGTGCCAAAGAGCAGCAAA<br>R: CAAAAATGGGTCTCTTTTCATTTC | 54 | 0.375 | 1.6 | 158–176 | 0.525 | 5 | 5 | 4 | 0.833 | 0.789 | 0.678 | 0.636 | 0.0548$^{NS}$ | 0.0749$^{NS}$ |
| Lpin 16 | BV722929 | $(TC)_7(TTG)_5(TTG)_4$ | F: CGGTTTGGACAAAAAGGTGG<br>R: GATTGGGAGATTTGGAAGCA | 54 | 0.375 | 1.6 | 176–182 | 0.429 | 3 | 3 | 3 | 0.833 | 0.895 | 0.522 | 0.550 | 0.0049$^{NS}$ | 0.0008* |
| Lpin 17 | BV722930 | $(AC)_{14}$ | F: AGAACGAATCATACGGGTGCGG<br>R: CCAAAGAGCAGAAGGATTGC | 54 | 0.375 | 1.6 | 202–208 | 0.350 | 2 | 2 | 2 | 0.667 | 0.842 | 0.457 | 0.501 | 0.1021$^{NS}$ | 0.0035* |

F, forward sequence; R, reverse sequence; $T_a$, optimized annealing temperature; $MgCl_2$, optimized $MgCl_2$ concentration; Primer, optimized primer concentration; PIC, polymorphism information content; $N_a$, number of observed alleles per locus; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; Ant, Antena population; Ere, Estrada Real population; $P$-HWE, $P$ values for the HWE test, significance threshold adjusted using Bonferroni correction: $P$ (5%) ≤ 0.0038; $^{NS}$, not significant. Numbers of individuals successfully genotyped are 18 (Ant) and 19 (Ere).

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software (Goudet 1995) and results were Bonferroni-corrected according to Rice (1989). One locus in the Antena population and two loci in Estrada Real population showed significant deviation from HWE after Bonferroni correction (Table 1). The observed departures from HWE can be due to population substructuring (Wahlund effect), mating system, small sample size and/or to the presence of null alleles. Null alleles were suggested by the software MICRO-CHECKER 2.2.3 (van Oosterhout *et al*. 2004) to occur over six loci [Lpin5 (0.2290), Lpin6 (0.0977), Lpin8 (0.2031), Lpin10 (0.3483), Lpin11 (0.1786) and Lpin13 (0.2576)]. All loci were tested for linkage disequilibrium using the GDA program (Lewis & Zaykin 2002) and no linkage was found ($P < 0.001$) using chi-squared test.

The described primers represent a useful tool for population genetics studies in *L. pinaster*. They will be used to verify the population dynamics of this species, allowing the development of a strategy for its conservation, management and breeding programmes.

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# Development of microsatellite and amplicon length polymorphism markers for *Camellia japonica* L. from tea plant (*Camellia sinensis*) expressed sequence tags

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## Abstract

**Simple sequence repeats and amplicon length polymorphism markers for *Camellia japonica* were developed, based on *Camellia sinensis* sequences in the National Center for Biotechnology Information database. In total, 2495 gene sequences were used to design 216 primer pairs. To identify amplicon length polymorphism markers, 61 gene loci in 16 *Camellia* individuals were re-sequenced. In total, 10 markers (three expressed sequence tags–simple sequence repeats and seven amplicon length polymorphisms) yielded polymerase chain reaction products with clear polymorphic patterns and were used for genotyping 22 *C. japonica* individuals from a population. Numbers of alleles and expected heterozygosity ranged from two to 13 and from 0.28 to 0.90, respectively.**

*Keywords*: *Camellia japonica*, *Camellia rusticana*, EST, re-sequencing, Theaceae

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