

PERMANENT GENETIC RESOURCES

Development and characterization of eight polymorphic microsatellite loci from *Pistacia lentiscus* L. (Anacardiaceae)

RAFAEL G. ALBALADEJO,* F. SEBASTIANI,† A. APARICIO,* A. BUONAMICI,

† S. C. GONZÁLEZ-MARTÍNEZ‡ and G. G. VENDRAMIN§

*Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Profesor García González 2, 41012 Seville, Spain, †Dipartimento di Biotecnologie Agrarie, Genexpress, Università degli Studi di Firenze, Via della Lastruccia 14, 50019 Sesto Fiorentino (Firenze), Italy,

‡Department of Forest Systems and Resources, CIFOR-INIA, Carretera de La Coruña km 7.5, 28040 Madrid, Spain, §Istituto di Genetica Vegetale, Consiglio Nazionale delle Ricerche, Via Madonna del Piano 10, 50019 Sesto Fiorentino (Firenze), Italy

Abstract

We have developed a set of eight polymorphic nuclear microsatellite markers for the Mediterranean shrub *Pistacia lentiscus* by means of an enriched library method. Characterization for the eight loci was carried out on 42 individuals from two populations sampled in southern Spain. The overall number of alleles detected was 59, ranging from three to 13 per locus. Expected heterozygosity per locus and population ranged from 0.139 to 0.895. Two loci albeit only in one population (Seville) departed significantly from Hardy–Weinberg equilibrium expectations and no linkage disequilibrium between pairs of loci was detected. These markers will be used in studies of gene flow across a fragmented landscape.

Keywords: gene flow, nuclear microsatellites, *Pistacia*

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Pistacia lentiscus L. (Anacardiaceae) is a common sclerophyllous shrub up to 3–4 m in height distributed throughout the Mediterranean region. The plants are dioecious, the female flowers are wind pollinated, and the one-seeded drupes are actively dispersed by birds (Herrera 1984; Jordano 1988). As many other plant species, populations of *P. lentiscus* naturally occurring in the agricultural landscape of the Guadalquivir River Valley (southwestern Spain) have been subjected to intense habitat management and chronic deforestation. In fact, the degree of deterioration is so severe in this area that today only about 1% of natural or seminatural habitat remains, with an extremely low degree of connectivity (Aparicio 2008). Therefore, there is an urgent need to gather information about the impact of such extreme habitat patchiness on the pollination biology and seed dispersal of the species, *P. lentiscus* included, to elaborate efficient conservation action plans. Under the frame of a comprehensive project that aims to evaluate the consequences of chronic habitat fragmentation in Mediterranean forests, a set of highly polymorphic nuclear microsatellites [SSR (simple sequence repeat)] markers has been developed

to carry out gene flow and population structure studies in *P. lentiscus*. Although a set of 14 SSR primer pairs has been recently published for *Pistacia vera* L. (Ahmad *et al.* 2004), the transferability across species resulted to be poor because of lack of amplification or low polymorphism (our unpublished results).

For the isolation of the microsatellite markers, we followed the protocol of Edwards *et al.* (1996). Briefly, we extracted *c.* 2 µg of total genomic DNA from a single *P. lentiscus* individual with the DNeasy Plant Kit (QIAGEN) following the manufacturer's protocol. Genomic DNA was digested with the *RsaI* enzyme, ligated to the *MluI* adaptor (5'-CTCTTGCTTACGCGTGGACTA-3') and amplified through polymerase chain reaction (PCR). Amplified PCR products were hybridized twice with di-(GA, GT, AT, GC), tri-(CAA, ATT, GCC) and tetranucleotide (GATA, CATA, ATAG) repeats bounded to Hybond N⁺ filters (GE Healthcare). Enriched fragments were then amplified and cloned using the TOPO TA Cloning Kit (Invitrogen). Randomly chosen clones (163) were sequenced in the forward direction with the M13 universal primer and using the DYEnamic ET dye Terminator Kit (GE Healthcare), and run on a GE Healthcare MegaBACE 1000 automatic sequencer. Microsatellite motifs were detected in 75 (46%) of the sequences,

Correspondence: Rafael G. Albaladejo, Fax: +34 954233765;

E-mail: albaladejo@us.es

Table 1 Characteristics of the *Pistacia lentiscus* microsatellites markers

Locus name (GenBank Accession no.)	Primer sequence 5'–3'	Dye (forward primer)	Repeated motif	PCR profile†	Allele size range (bp)	Total no. of alleles
Pislen 20B (EU263299)	F: ACACAACCTCAAGCAACAACA R: AAAGAATCCTACGCATGAAA	HEX	(CA) ₁₈ (TA) ₅	B	237–261	7
Pislen 21 (EU263300)	F: GGAAGTGGGTTAGGAATTA R: GGGTGGTTACAATTAGGTCA	FAM	(CT) ₂₃	B	245–281	12
Pislen 114 (EU263301)	F: GTGACTTTGGTTGGTGTTTT R: CTGCTTTGACTGGATTTGAT	HEX	(GTT) ₄ GCTGTTGCT (GTT) ₅ GCTGTTGCT(GTT) ₅	A	183–207	6
Pislen 333 (EU263302)	F: TTTGATAAGAAGCTCGCTTCC R: TTTCTGCCTTTGCTTTACTC	TAMRA	(TA) ₃ (CA) ₂₁ (TA) ₄	A	181–217	13
Pislen 501 (EU263303)	F: TTCAACTCAACAAATATGCAA R: ATTGTATTGGCGAAACCTAA	FAM	(GA) ₂₈	A	185–211	9
Pislen 510 (EU263304)	F: TGGTGGAGTCTTACTTTGCT R: TGACAATCAATATGCCTTCA	HEX	(AC) ₂₀	A	216–234	6
Pislen 526 (EU263305)	F: CAGTGAGGGTAAAAATGGAA R: ATTACCATTTTGAGGGAACC	HEX	(GGT) ₂ GCT(GGC) ₆	A	142–148	3
Pislen R05 (EU263306)	F: GGAATTTCTCTACCACTCC R: GAAAACGAGGTTATTGGTCA	FAM	(CCG) ₅ CTG(CCG) ₂	A	188–212	3

†PCR profiles contain 10 touchdown cycles with annealing temperatures of 60–50 °C (–1 °C/cycle; profile A), and 60–55 °C (–0.5 °C/cycle; profile B) (see text for details).

among which 40 were discarded because of too short SSR motifs, SSR motifs too close to the vector for primer designing, or because the clones showed high sequence homology with each other. We sequenced the reverse direction of the selected clones and 21 primer pairs were designed using PRIMER 3 software (Rozen & Skaletsky 2000). After careful examination of 16 individuals from several populations, eight out of the 21 primers displayed consistent and polymorphic patterns, whereas the others were discarded because they failed to amplify, produced multibanding patterns, showed too pronounced stuttering or were monomorphic.

To characterize the selected eight SSR markers, we analysed 21 plants in each of two populations from the Guadalquivir River Valley located *c.* 100 km apart: Seville (Utrera, UTM 29S X248002 Y4122941) and Cádiz (Chiclana de la Frontera, UTM 29S X222013 Y4030524). Genomic DNA was isolated from dried young leaves using the QIAGEN Dneasy Plant Kit, and amplification reactions were carried out in a final volume of 10 µL containing *c.* 30 ng of template DNA, 1× PCR buffer (Promega), 0.2 mM of each dNTP (GE Healthcare), 1 U *Taq* polymerase (*GoTaq*, Promega), 1.5 mM of MgCl₂, 0.5% of BSA and 0.2 µM of each primer (forward primers were fluorescence labelled with FAM, HEX or TAMRA in the 5' end; see Table 1). Two PCR profiles were employed for the successful amplification of the microsatellite loci: (i) denaturation at 94 °C for 4 min followed by 10 touchdown cycles at 94 °C (30 s), 60 °C (30 s; –1 °C/cycle), 72 °C (1 min), 30 cycles at 94 °C (30 s), 50 °C (30 s), 72 °C (1 min), and a final extension step at

72 °C 7 min; (ii) 94 °C for 4 min followed by 10 touchdown cycles at 94 °C (30 s), 60 °C (30 s; –0.5 °C/cycle), 72 °C (1 min), 30 cycles at 94 °C (30 s), 55 °C (30 s), 72 °C (1 min), and a final extension step at 72 °C for 7 min (Table 1). Amplification reactions were carried out in a GeneAmp PCR system 9700 (PE Biosystems). Fluorescence-labelled amplified products were run in a MegaBACE 1000 (GE Healthcare) automatic sequencer. Allele sizes were assessed according to the ET400-R size standards (GE Healthcare) using MegaBACE FRAGMENT PROFILER version 1.2 (GE Healthcare). Standard genetic diversity parameters and departure from Hardy–Weinberg equilibrium (HWE) were estimated using GENALEX version 6 (Peakall & Smouse 2006). Frequency of null alleles, following Dempster *et al.* (1977), and linkage disequilibrium between pairs of loci was estimated using GENEPOP'007 version 4 (Rousset 2007). Bonferroni corrections were applied to account for multiple testing in HWE and linkage disequilibrium tests.

The number of alleles per locus ranged from three to 13, with a total of 59 alleles scored, suggesting high information content potential for these loci in population studies. Expected heterozygosity ranged from 0.291 to 0.848 and from 0.139 to 0.895 in Seville and Cádiz, respectively. Observed heterozygosity for the eight resolved loci ranged from 0.143 to 0.762 in Seville, and from 0.150 to 0.905 in Cádiz (Table 2). Pislen R05 and Pislen 20B loci showed significant departures from HWE in Seville population (excess of homozygotes) while none of the assayed loci displayed a significant heterozygote deficiency in Cádiz. The existence of local genetic structure and/or the small

Table 2 Genetic diversity estimates for two populations (Seville and Cádiz) using eight newly developed polymorphic microsatellite markers in *Pistacia lentiscus*

	No. of alleles	H_O	H_E	F_{IS}^\dagger	Null allele frequency
Seville					
<i>Pislen R05</i>	3	0.143	0.291	0.510*	0.24
<i>Pislen 20B</i>	4	0.158	0.688	0.771***	0.31
<i>Pislen 21</i>	8	0.762	0.777	0.019NS	0
<i>Pislen 114</i>	4	0.714	0.690	-0.034NS	0.14
<i>Pislen 333</i>	10	0.714	0.848	0.158NS	0.26
<i>Pislen 501</i>	8	0.600	0.804	0.253NS	0.15
<i>Pislen 510</i>	5	0.500	0.621	0.195NS	0.06
<i>Pislen 526</i>	3	0.267	0.407	0.344NS	0.78
Cádiz					
<i>Pislen R05</i>	2	0.150	0.139	-0.081NS	0
<i>Pislen 20B</i>	7	0.438	0.793	0.448NS	0.24
<i>Pislen 21</i>	11	0.524	0.811	0.354NS	0.17
<i>Pislen 114</i>	6	0.714	0.707	-0.010NS	0.22
<i>Pislen 333</i>	11	0.905	0.895	-0.011NS	0.12
<i>Pislen 501</i>	9	0.857	0.817	-0.049NS	0.02
<i>Pislen 510</i>	5	0.500	0.625	0.200NS	0.07
<i>Pislen 526</i>	2	0.700	0.495	-0.414NS	0.45

H_O , observed heterozygosity; H_E , expected heterozygosity; F_{IS} , inbreeding coefficient. †Test for Hardy-Weinberg equilibrium after Bonferroni correction; NS, not significant ($P > 0.05$); * $P < 0.05$; *** $P < 0.001$.

effective population size of the Seville population might account for the significant excess of homozygotes detected at these two loci. No significant linkage disequilibrium was detected between locus pairs.

The eight newly developed polymorphic markers provide enough variation (Exclusion Probability > 99%) to carry out studies to infer patterns of gene flow (via pollen and seeds) in *P. lentiscus* across the chronically fragmented landscape of the Guadalquivir River Valley. This information will be useful for conservation and restoration efforts for this species.

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