DNA Analyser (Applied Biosystems) and GeneMapper version 3.7 analysis software (Applied Biosystems).

Tests for Hardy-Weinberg equilibrium and linkage disequilibrium were conducted using GenePop version 3.4 (Raymond & Rousset 1995) with a Bonferroni correction for multiple comparisons to a significance level of $P < 0.05$. Significant deviation from Hardy-Weinberg equilibrium was detected for one locus, HoA006, and no significant linkage association was found among the loci. Genetic diversity parameters were assessed using GENALEX 6 (Peakall & Smouse 2006) (Table 1) and showed moderate diversity. Expected heterozygosity ranged from 0.279 to 0.770 with a mean of 0.633, and observed heterozygosity ranged from 0.321 to 0.786 with a mean of 0.598. The number of alleles per locus ranged from 2.0 to 6.0 and averaged 4.5. Polymorphic information content was assessed using Cervus 2.0 (Marshall *et al*. 1998). The polymorphic information content of the loci was high and power of exclusion for detection of parentage was 0.998 over all 13 loci.

These microsatellite loci are being used to assess levels of diversity and patterns of pollen dispersal among populations of *H. oldfieldii* in the threatened ecological community restricted ironstone habitat in southwest Western Australia.

Acknowledgements

We thank Mark Todd at Genetic Identification Services for microsatellite library development.

References

Byrne M, Parrish TL, Moran GF (1998) Nuclear RFLP diversity in *Eucalyptus nitens*. *Heredity*, **81**, 225–232.

Byrne M, Macdonald B, Francki M (2001) Incorporation of sodium sulfite into extraction protocol minimizes degradation of *Acacia* DNA. *BioTechniques*, **30**, 742–748.

Gibson N, Keighery G, Keighery B (2000) Threatened plant communities of Western Australia. 1 The Ironstone communities of the Swan and Scott Coastal Plains. *Journal of the Royal Society of Western Australia*, **83**, 1–11.

Marshall T, Slate J, Kruuk L, Pemberton J (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639–655.

Peakall R, Smouse P (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.

Raymond M, Rousset F (1995) GenePop (version 3.4): population genetics software for exact test and ecumenicism. *Journal of Heredity*, **86**, 248–249.

doi: 10.1111/j.1755-0998.2009.02616.x

Isolation and characterization of 12 microsatellite loci for *Rhamnus alaternus* (Rhamnaceae)

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Abstract

Twelve polymorphic microsatellite loci were developed for *Rhamnus alaternus* using an enriched-library approach. We detected 69 alleles in 49 individuals genotyped (mean number of alleles per locus was 4.79) in two different populations. The values of observed and expected heterozygosities ranged from 0.045 to 0.963 and 0.089 to 0.873 respectively. Levels of polymorphism and the exclusionary power of the developed markers render them readily applicable for studies of contemporary pollen and seed gene flow through parentage analyses.

Keywords: Rhamnaceae, *Rhamnus alaternus*, seed dispersal

Received 19 December 2008; revision accepted 27 January 2009

The measurement of contemporary geneflow patterns is a powerful tool for ecological studies of pollen and seed dispersal in natural plant populations (Ouborg *et al.* 1999). In particular, recent advances allow direct estimation of dispersal distances based on assignment procedures that use the genotype of maternally derived seed endocarps and the genotype of candidate maternal trees (Godoy & Jordano 2001). A requirement of these techniques is the

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availability of microsatellite markers applicable to a variety of plant tissues (seed endocarps, leaf, embryo) sampled from seeds, seedlings or adult plants, and allowing robust exclusion of candidate source trees.

Rhamnus alaternus L. (Rhamnaceae) is a shrub or a small tree distributed throughout the Mediterranean Basin. The species is dioecious and insect pollinated. Its fleshy fruits are regularly consumed by birds and small mammals, and seeds are secondarily dispersed by ants (Gómez *et al.* 2003). Frugivores have been shown to select individual *R. alaternus* trees according to the chemical composition of their fruits (Tsahar *et al.* 2002). Hence, *R. alaternus* represents a suitable model to investigate how differences between individual plants translate into differential patterns of seed dispersal and ultimately spatial genetic structures. Such studies require highly polymorphic markers that allow individual genotyping and parentage analyses of DNA samples derived from leaf and seed tissue.

We developed microsatellite libraries following Jones *et al.* (2002). We extracted approximately 100 μg of genomic leaf tissue DNA of one tree using the QIAGEN DNeasy Plant Extraction kit. The DNA was partially restricted with seven blunt-end restriction enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I and *Eco*RV). Fragments (300–750 bp) were ligated with 20-bp oligonucleotides containing a *Hin*dIII site at the 5′ end, and subjected to magnetic bead capture. Four libraries were prepared in parallel using Biotin-CA_{15}, Biotin-GA_{15}, Biotin-ATG_{12} and Biotin-AAC_{12} as capture molecules (CPG Inc.). Captured molecules were amplified and restricted with *Hin*dIII to remove the adapters, and the resulting fragments were ligated into the *Hin*dIII site of pUC19 plasmid and introduced into *Escherichia coli* DH5α by electroporation. Recombinant clones ($N = 100$) were selected at random for sequencing and 72 of them contained a microsatellite sequence. Polymerase chain reaction (PCR) primer pairs were designed for 24 clones using DESIGNER PCR 1.03 (Research Genetics Inc.).

For primer testing, we isolated DNA from silica-dried leaves of 27 trees collected in a population located in Garganta del Medio and an additional sample of 22 trees from Tarifa (both samples from 'Los Alcornocales' Natural Park). We used a standard CTAB extraction method (Milligan 1998) with some minor modifications (tissue grinding in a MM301 Retsch™ mill and TLE resuspension).

Polymerase chain reaction reactions were performed in final volume of 20 μL containing 1X buffer [67 mM Tris–HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20], 2.5 mM $MgCl_2$, 0.01% BSA (Roche Diagnostics), 0.25 mM dNTP, 0.40 μM dye-labelled M13 primer, 0.25 μM tailed-reverse primer, 0.034 μM M13 tailed-forward primer, 0.5 U *Taq* DNA polymerase (Bioline) and 50 μL of genomic DNA. Samples were incubated in a 'touchdown' PCR in a BIO-RAD DNA Engine® Peltier Thermal Cycler, with an initial 2 min of denaturation at 94 °C; 17 cycles at 92 °C for 30 s, annealing at 60–44 °C for 30 s (1 °C decrease in each cycle) and extension at 72 °C for 30 s; 25 cycles at 92 °C for 30 s, 44 °C for 30 s and 72 °C for 30 s with a final extension for 5 min at 72 °C. Amplified fragments were analysed on an ABI 3130xl Genetic Analyser and sized using GeneMapper 4.0 (Applied Biosystems) and LIZ 500 size standard.

We also tested the feasibility of DNA isolation from seed endocarp tissues (Godoy & Jordano 2001). Seeds were split open and the endocarp was separated by hand from the embryo. We followed the DNA isolation protocol for leaves with two modifications: after tissue grinding, samples were homogenized in 400 μL of extraction buffer and the DNA pellet was resuspended in 85 μL TLE, with a reaction mix identical to that described earlier.

We tested a total of 24 primer pairs. Four of them failed to amplify or showed complex amplification, seven were monomorphic and 13 were polymorphic. We finally retained 12 primers after inspecting their observed and expected heterozygosities (Cervus 3.0; Kalinowski *et al.* 2007) and testing for deviations from Hardy–Weinberg equilibrium, gametic disequilibrium (GENEPOP 4.0; Rousset 2007) and the presence of null alleles (MICRO-CHECKER 2.2.3; Oosterhout *et al.* 2004). We used Bonferroni-corrected *P*-values to assess the significance.

Table 1 shows the characteristics of the 12 loci. We detected 69 alleles, corresponding to an average of 4.79 alleles per locus (range: 2–9). Two loci showed a significant deviation from HWE (Bonferroni-corrected $P < 0.05/12 = 0.004$) and evidence for the presence of null alleles ($P < 0.004$), RaA12 in the Medio population, and RaA101 in the Tarifa population. Moreover, in the Tarifa population, there was another locus (RaA7), which presented evidence for the presence of null alleles ($P < 0.004$). Gametic disequilibrium was detected for one pair of loci (RaA111 and RaA101; $P < 0.004$) in the Medio population. Mean observed and expected heterozygosities were 0.693–0.524 and 0.686–0.607, respectively, for each population. The combined nonexclusion probability across all 27 trees was 0.017 for the first parent and 0.0007 for the second parent in the Medio population, and 0.044 and 0.0030, respectively, for 22 trees in the Tarifa population. Polymorphism levels and exclusionary power of the markers are adequate for direct measurements of seed dispersal through parent assignment.

By comparing the endocarp genotype of seeds collected from known trees with the leaf-derived mother genotype, we could confirm the maternal derivation of the endocarp tissue in *R. alaternus* and therefore its suitability for assigning source trees to dispersed seeds.

The analysis of pollen- and seed-mediated gene flow patterns performed by different vectors will serve to investigate the spatial genetic structure and connectivity of *R. alaternus* populations across the chronically fragmented landscapes typical of lowland areas in Southwestern Spain (Albaladejo *et al.* 2008).

Table 1 Characteristics of 12 polymorphic microsatellite markers isolated from *Rhamnus alaternus* at Medio (MED) and Tarifa (TAR) (Cádiz, Spain)

Locus name (GenBank ID)	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	*n* MED	*K* MED	H_O MED	H_E MED	HW MED	*N* TAR	*K* TAR	H_O TAR	H_E TAR	HW TAR
RaC113 (FJ492949)	F: ACCCTCTGTTCCTTCTCTTGC R: GGTGGCTAAGCAGTGGTAGTG	$(CAA)_7$	113–122	27	2	0.704	0.498	0.996	22	2	0.091	0.089	1.000
RaD107 (FJ492950)	F: GCAGTTGTGTTGGGTATTTC R: GGGGAGAAGAGGTTGATAAG	$(TCA)_{10}$	170–179	27	4	0.519	0.607	0.246	22	3	0.545	0.571	0.374
RaA12 (FJ492951)	F: GCTCACTCAGTTAGAGGTATGC R: TGAGAAAGTGTAAGTGCATGTG	$(CT)_9(CA)_{24}$	259–298	27	5	0.481	0.667	0.002	22	5	0.364	0.424	0.239
RaB105 (FJ492952)	F: CAGTTGGCTTCCGACTAAA R: AGCATTCAGCAGTGTGACC	$(AG)_{16}$	133–157	27	6	0.741	0.813	0.207	22	5	0.682	0.730	0.289
RaA111 (FJ492953)	F: CTCCTCATCTCCTCCACCTC R: GGACGACAAGGTTTTTACACC	$(AC)_{17}(TC)_{20}$	165–186	27	5	0.630	0.624	0.029	22	7	0.727	0.809	0.146
RaA101 (FJ492954)	F: CCAGCATGGTAAATGGAAG R: AACCAGATGCAATACAGTTAGC	$(TG)_{13}(AG)_{15}$	260–315	27	5	0.577	0.606	0.430	22	4	0.045	0.678	0.000
RaB117 (FJ492955)	F: CAAAGCTAGTTGCTTGAGATG R: AAGGGCTAATGATCCATGTAT	$(TC)_{22}$	152–184	27	7	0.741	0.755	0.379	22	5	0.636	0.678	0.537
RaA7 (FJ492956)	F: CCAAGGCATGACACTAGC R: AACTCCAACCCACATGATC	$(CA)_{13}(TA)_3TC(TA)_3$	237–254	27	4	0.704	0.686	0.529	22	3	0.381	0.570	0.013
RaA118 (FJ492957)	F: TCTGGGTCTTCTTGCCTCT R: AATCTCCCGCCAAAAAGTA	$(TG)_{18–9}(AT)_8$	308–334	27	9	0.889	0.873	0.610	22	5	0.682	0.626	0.745
RaA102 (FJ492958)	F: CTTTCCTCAGAGGTCCTAGCAC R: GCCCAAGTCTCCAATCTAAAAG	$(TG)_{15}T(TG)_4$	154–175	27	4	0.741	0.660	0.839	22	5	0.636	0.664	0.210
RaC10 (FJ492959)	F: CTGCACAGAACCTCCTAAAGAC R: GGAAAGACAGCAATTTTCAATC	$(GTT)_8$	273–292	27	7	0.963	0.778	0.999	22	7	0.682	0.784	0.253
RaA117 (FJ492960)	F: ATTCAGAAGCAAGAAGAGTCTG R: AAGACAGCGTAAAGACAGTGAT	$(AC)_{12}(TC)_6$	298–302	27	3	0.630	0.660	0.343	22	3	0.818	0.660	0.935

n, number of individuals successfully genotyped; *k*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; HW, nominal *P*-values for the test of deviations from Hardy–Weinberg equilibrium.
PCR products were labelled using FAM, VIC, NED or PET (Applied Biosystems) dyes on an additional 19-bp M13 primer (5′-CACGACGTTGTAAAACGAC-3′) according to the methods of Boutin-Ganache *et al.* (2001). A palindromic sequence tail (5′-GTGTCTT-3′) was added to the 5′ end of the reverse primer to improve adenylation and facilitate genotyping.

Acknowledgements

We kindly thank the staff of the 'Los Alcornocales' Natural Park for permissions and logistical support in the area. Alfredo Valido helped with sampling and useful comments on the manuscript. The study was supported by the Spanish MEC (CGL2006-00373) and Junta de Andalucía (P07-RNM02824).

References

Albaladejo RG, Carrillo LF, Aparicio A, Fernández-Manjarrés FJ, González-Varo JP (2008) Population genetic structure in *Myrtus communis* L. in a chronically fragmented landscape in the Mediterranean: can gene flow counteract habitat perturbation? *Plant Biology*, doi: 10.1111/j.1438-8677.2008.00121.x.

Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *BioTechniques*, **31**, 24–27.

Godoy JA, Jordano P (2001) Seed dispersal by animals: exact identification of source trees with endocarp DNA microsatellites. *Molecular Ecology*, **10**, 2275–2283.

Gómez C, Pons P, Bas JM (2003) Effects of the Argentine ant *Linepithema humile* on seed dispersal and seedling emergence of *Rhamnus alaternus*. *Ecography*, **26**, 532–538.

Jones KC, Levine KF, Banks JD (2002) Characterization of 11 polymorphic tetranucleotide microsatellites for forensic applications in California elk (*Cervus elaphus canadensis*). *Molecular Ecology Notes*, **2**, 425–427.

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, **16**, 1099–1006.

Milligan BG (1998) Total DNA isolation. In: *Molecular Genetic Analysis of Populations: A Practical Approach*, 2nd edn (ed. Hoelzel AR), pp. 29–64. Oxford University Press, Oxford, UK.

Oosterhout CV, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Ouborg NJ, Piquot Y, van Groenendahl JM (1999) Population genetics, molecular markers and the study of dispersal in plants. *Journal of Ecology*, **87**, 551–558.

Rousset F (2007) GENEPOP'007: a complete re-implementation of the GENEPOP. *Molecular Ecology Resources*, **8**, 103–106.

Tsahar E, Friedman J, Izhaki I (2002) Impact on the fruit removal and seed predation of a secondary metabolite, emodin, in *Rhamnus alaternus* fruit pulp. *Oikos*, **99**, 290–299.

doi: 10.1111/j.1755-0998.2009.02657.x