Gaca_208) exhibited significant ($P < 0.01$) linkage disequilibrium. The observed linkage disequilibrium is likely driven by population differentiation. Each population has only two or three of the four observed alleles for each locus and apparently elevated co-occurrence of those alleles.

The markers described in this study will be used to investigate the population structure, levels of genetic variability, and past demographic events for *G. catalinense* ssp. *acrispum*. Furthermore, these markers are intended for use throughout the genus *Galium* (Rubiaceae), which is composed of *c*. 400 species worldwide, including 18 North American species of conservation concern.

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References

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**, 24–28.

Hamilton MB, Pincus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques*, **27**, 500–507.

Junak SA, Wilken DH (1998) Sensitive plant status survey, NALF San Clemente Island, California. *Technical Report No. 1*. Santa Barbara Botanic Garden, Santa Barbara, California.

Raymond M, Rousset F (1995) GenePop (version-1.2): population-genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rozen S, Skaletsky HJ (2000) Primer 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols*, (eds Misener S, Krawetz SA), Humana Press, Totowa, New Jersey.

Van Oosterhout C, 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.

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Isolation and characterization of 16 polymorphic microsatellite loci for *Frangula alnus* (Rhamnaceae)

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Abstract

We report the first 16 polymorphic nuclear microsatellite markers developed for *Frangula alnus* (Rhamnaceae). Markers were tested on all three subspecies as well as on three local populations, including analyses of both leaf and seed endocarps. A total of 87 alleles were found (mean number of alleles per locus was 5.44) for 72 individuals genotyped. Observed and expected heterozygosities ranged from 0.097 to 0.792 and from 0.093 to 0.794, respectively. The levels of polymorphism and exclusionary power of the developed markers render them applicable for parentage analyses and measurements of seed dispersal through direct comparison of endocarps and adult tree genotypes.

Keywords: endocarp, *Frangula*, Rhamnaceae, seed dispersal, SSR

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Recent advances in seed dispersal studies allow the 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). These techniques require reliable sets of microsatellite markers allowing robust exclusion of candidate source trees and applicable to tissues of both seeds and established seedlings.

Frangula alnus Mill. (Rhamnaceae) is a shrub or small tree widely distributed over Europe and West Asia; most of the native range is occupied by *Frangula alnus alnus*, while *F. a.*

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baetica and *F. a. pontica* are endemic to the Southwestern Mediterranean and Anatolia, respectively (Hampe *et al*. 2003). The species lacks vegetative reproduction and therefore depends on its animal-dispersed seeds for regeneration. Hence, patterns of seed dispersal may greatly influence spatial patterns of regeneration and the resulting genetic structure. We developed microsatellite markers for *F. alnus*, since they have been successfully applied to parentage and relatedness testing and would allow genotyping of both leaf and endocarp tissues.

Microsatellite libraries were developed following Jones *et al*. (2002). Genomic DNA was extracted from leaves of a single 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. The resulting fragments were ligated into the *Hin*dIII site of pUC19 plasmid and introduced into *Escherichia coli* DH5α by electroporation. Recombinant clones were selected at random for sequencing. Seventy of them contained a microsatellite sequence. Polymerase chain reaction (PCR) primer pairs were designed for 36 clones using Designer PCR 1.03 (Research Genetics Inc.).

For primer testing, DNA was isolated from silica-dried leaves of 72 trees collected in three populations (Aljibe, Medio and Puerto Oscuro; 'Los Alcornocales' Natural Park, Cádiz, Spain). We used a standard cetyltrimethyl ammonium bromide (CTAB) extraction method (Milligan 1998) with minor modifications tissue grinding in a MM301 Retsch™ mill and TLE resuspension.

PCR was performed in 20 μL final volume containing 1× 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 tail-reverse primer, 0.034 μM M13 tailed-forward primer, 0.5 U *Taq* DNA polymerase (Bioline) and 5 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 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 DNA isolation and amplification from seed endocarps. For this purpose, 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. The reaction mix was identical to that described above.

All 36 primer pairs amplified products of appropriate size. Ten were monomorphic or showed complex amplification. Twenty-six were polymorphic, eight of them showing only two alleles and four having a high frequency of null alleles. We finally retained 16 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; van Oosterhout *et al*. 2004). We used Bonferroni-corrected *P* values to assess significance of the results obtained.

Table 1 summarizes the features of the 16 loci reported. We detected a total of 87 alleles (allele numbers per locus: 2–11; mean = 5.44). No locus showed deviations from Hardy–Weinberg equilibrium ($P > 0.1$ in all three populations). We detected however, some evidence of gametic disequilibrium (Bonferroni-corrected $P < 0.05/16 = 0.003$) in two primer combinations: FaB7/FaA7 in the Medio population, and FaA116/FaB8 in the Medio and Aljibe populations. The presence of null alleles was confirmed for two loci (Bonferroni corrected $P < 0.003$): FaA103 in the Aljibe site and FaA8 in Puerto Oscuro. The combined nonexclusion probability across all 72 trees was 0.041 for the first parent and 0.002 for the second parent (0.035–0.144 and 0.002–0.013, respectively, for each population separately). These levels of polymorphism and the exclusionary power of the markers render them readily applicable for direct measurements of seed dispersal through parent assignment.

Reliable genotypes were obtained from seed endocarps. By comparing the endocarp genotype of seeds collected from known trees with the leaf-derived genotype, we could confirm the maternal derivation of the endocarp tissue in *F. alnus* and therefore its suitability for assigning source trees to dispersed seeds (Godoy & Jordano 2001).

We also assessed the transferability of the 16 microsatellite loci by analysing material from 10 populations including all three subspecies; four *baetica* populations from Morocco (Jbel Bouhachem I and II; see Hampe *et al*. 2003 for population features) and southern Spain (Doñana and Huerta Vieja), five *alnus* populations (Coruña, Guara, Mondego, Nava del Barco and Pierroton) and one *pontica* population (Djarnali). Three loci revealed some problems: FaA116 did not amplify in *alnus* and *pontica* as well as in one *baetica* population (Doñana), FaA103 showed deficient amplification for some *alnus* and *pontica* samples, and FaB4 failed to amplify for the Guara population (ssp. *alnus*). The remaining 13 markers worked reliably for all tested populations. We observed a total of 128 new alleles, suggesting that analyses of additional material may result in a further noteworthy increase in polymorphism.

Table 1 Characteristics of 16 polymorphic microsatellite markers isolated from *Frangula alnus* populations at Aljibe (ALJ), Medio (MED), and Puerto Oscuro (PO) (Cádiz, Spain)

Locus name (GenBank ID)	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	*n* ALJ	*k* ALJ	H_O ALJ	H_E ALJ	HW ALJ	*n* MED	*k* MED	H_O MED	H_E MED	HW MED	*n* PO	*k* PO	H_O PO	H_E PO	HW PO
FaA103	F: CCGGATGTCCGAATAGTAG	$(AC)_{27}$	115–141	30	7	0.633	0.790	0.102	19	6	0.789	0.745	0.761	23	6	0.652	0.705	0.352
(FJ375935)	R: GTCAGCGATTATAGCAATATCC																	
FaA12	F: TCCTCCGAGTCTTCCTACC	$(AC)_{11}$	177–179	30	2	0.200	0.183	1.000	19	2	0.053	0.053	—	23	1	0.000	0.000	—
(FJ375936)	R: AAGCCAGATTCAAGCATTG																	
FaB102	F: TAAAGCTGTTTGCACAATCTC	$(CT)_{16}$	207–211	30	2	0.167	0.155	1.000	19	2	0.158	0.149	1.000	23	2	0.217	0.198	1.000
(FJ375937)	R: ACCATTTTATCTGTTGATCCAG																	
FaB101	F: TGGTGGAAAAGGTTTGTTG	$(TC)_6(CCTC)_3(TC)_{20}$	185–265	30	6	0.800	0.696	0.946	19	6	0.789	0.710	0.803	23	4	0.652	0.631	0.694
(FJ375938)	R: GCATCAAGGATTGTTGTCTC																	
FaA110	F: CAAAGTTAGCCAAAGTCAACTG	$(GT)_{18}$	296–320	30	3	0.533	0.603	0.160	19	4	0.526	0.514	0.619	23	4	0.348	0.345	0.624
(FJ375939)	R: CCAACATCAAACCTACTTGAAC																	
FaB7	F: ATGGAAGGGAGAAGACAGTC	$(TC)_{14}$	142–150	30	5	0.633	0.651	0.556	19	4	0.632	0.613	0.660	23	4	0.652	0.617	0.750
(FJ375940)	R: ATCTGAAACCAACAGGACAAC																	
FaA104	F: GGAGGAAGACACAGTTCTGG	$(TG)_{14}$	187–197	30	5	0.533	0.518	0.522	19	3	0.211	0.280	0.247	21	4	0.381	0.336	1.000
(FJ375941)	R: CTGGAAAGCAATACCAAGTTG																	
FaB106	F: GCACTTGATTGTTTCAGCAC	$(TC)_{24}$	226–236	30	5	0.600	0.654	0.390	19	4	0.526	0.559	0.423	23	4	0.304	0.277	1.000
(FJ375942)	R: AGAGGTGGGTTCCAATTATG																	
FaB4	F: TGCAATCACTTCTTTTGAGTTC	$(TC)_{33}$	269–321	30	10	0.933	0.851	0.961	19	7	0.842	0.792	0.788	23	5	0.565	0.470	1.000
(FJ375943)	R: ACCAGCATTTGCCACATT																	
FaA125	F: GAGCCGCTCAAATTAATGATCT	$(TG)_{14}(CG)_3$	117–138	30	4	0.467	0.581	0.183	19	4	0.684	0.627	0.676	23	3	0.609	0.478	0.969
(FJ375944)	R: CCTACCTAGCGCTATATGCAAAG																	
FaA7	F: CCTTCGTCAACTAAAAACCA	$(TG)_{17}$	173–191	30	5	0.633	0.698	0.265	19	6	0.789	0.671	0.968	23	4	0.696	0.664	0.860
(FJ375945)	R: GATGTTATAGCTGGACCTCAAC																	
FaA116	F: TGTTCCTCATGCTCTATGTAAC	$(GT)_{13}(AT)_2$	251–257	30	3	0.600	0.551	0.795	18	3	0.500	0.541	0.418	23	3	0.391	0.478	0.219
(FJ375946)	R: TTGGTGCTGGTAAGTAAACTAG																	
FaA3	F: TTCATTTTCTGTCCCCATGC	$(CA)_{12}(TA)_4$	302–314	30	4	0.533	0.584	0.088	19	4	0.684	0.696	0.344	23	4	0.652	0.598	0.715
(FJ375947)	R: TGTGAAGCAAACATGAACACC																	
FaA8	F: TGGAGAAGTTTGGTGTCTTG	$(CA)_6(CG)_5(CA)_{30}(TA)_7$	118–225	30	5	0.233	0.301	0.119	19	5	0.316	0.333	0.475	23	6	0.304	0.477	0.014
(FJ375948)	R: GTGGGAAGCGAATGAAAG																	
FaB8	F: CTCAAGAAGATGGGGAGTGTC	$(AG)_{17}$	257–261	30	3	0.267	0.242	1.000	19	3	0.421	0.432	0.190	23	3	0.261	0.240	1.000
(FJ375949)	R: ACATGGCATGAGTCACTACGT																	
FaB9	F: AGGGTCCAATGTATTTTAGTAG	$(CT)_{22}$	308–328	30	3	0.233	0.213	1.000	19	4	0.684	0.596	0.587	22	5	0.273	0.290	0.460
(FJ375950)	R: ACTGGCAAGCACTGTAAG																	

(*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). Moreover, a palindromic sequence tail (5′-GTGTCTT-3′) was added to the 5′ end of the reverse primer to improve adenylation and facilitate genotyping.

The reported markers will be used for directly estimating patterns of animal-mediated pollen and seed dispersal within and among a set of *F. alnus* populations in the 'Los Alcornocales' Natural Park. We will furthermore assess how the spatial genetic structure is affected by frequent secondary seed dispersal through water flow (Hampe 2004) as compared to animal-mediated dispersal. Finally, microsatellite data will be used to guide the selection of source populations for *ex-situ* conservation measures.

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References

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.

Hampe A (2004) Extensive hydrochory uncouples spatiotemporal patterns of seedfall and seedling recruitment in a 'bird-dispersed' riparian tree. *Journal of Ecology*, **92**, 797–807.

Hampe A, Arroyo J, Jordano P, Petit RJ (2003) Rangewide phylogeography of a bird-dispersed Eurasian shrub: contrasting mediterranean and temperate glacial refugia. *Molecular Ecology*, **12**, 3415–3426.

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. 43–44. Oxford University Press, Oxford, UK.

van 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.

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

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Isolation and characterization of microsatellite loci in *Symbiodinium* B1/B184, the dinoflagellate symbiont of the Caribbean sea fan coral, *Gorgonia ventalina*

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Abstract

Here we report primers targeting 10 microsatellite loci of dinoflagellates in the genus *Symbiodinium* (clade B1/B184) symbiotic with the Caribbean sea fan coral, *Gorgonia ventalina*. Primers were tested on 12 *Symbiodinium* B1/B184 cultures, as well as 40 genomic DNA extracts of *G. ventalina* tissue samples. All loci were polymorphic with allelic richness ranging from 4–16. Gene diversity ranged from 0.15 to 0.91. These primers provide powerful tools for examining the fine-scale population structure and dynamics of *Symbiodinium* within a single host species.

Keywords: *Gorgonia ventalina*, gorgonian coral, microsatellite, population genetics, *Symbiodinium*, zooxanthellae

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