American Journal of Botany

American Journal of Botany: e26–e30. 2010.

AJB Primer Notes & Protocols in the Plant Sciences

Isolation and characterization of 20 microsatellite loci for laurel species (*Laurus*, Lauraceae)[1]

Juan M. Arroyo[2], Cristina Rigueiro[2], Rocío Rodríguez[2], Arndt Hampe[2], Alfredo Valido[2], Francisco Rodríguez-Sánchez[3], and Pedro Jordano[2,4]

[2]Department of Integrative Ecology, Estación Biológica de Doñana (CSIC), Avda. Américo Vespucio, s/n, E-41092 Sevilla, Spain; [3]Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Apdo.1095, E-41080 Sevilla, Spain

- *Premise of the study:* Microsatellite primers were developed for the evergreen tree *Laurus* to investigate population genetic structure and patterns of gene flow via animal-dispersed pollen and seeds.
- *Methods and Results:* Twenty polymorphic nuclear microsatellite markers were developed using CA, GA, AAC, and ATG n-enriched genomic libraries. Given the tetraploidy of the sampled populations, we analyzed our data both as dominant loci and as codominant genotypic data to calculate allele frequencies and genetic diversity. A total of 196 and 222 alleles were found in 37 Mediterranean (*L. nobilis*) and 26 Macaronesian islands (*L. azorica*) individuals, respectively.
- *Conclusions:* Levels of polymorphism of the reported markers are adequate for studies of diversity and parentage in natural populations of this Tertiary relict tree.

Key words: genetic diversity; *Laurus azorica*; *Laurus nobilis*; polyploidy; SSR.

The genus *Laurus* (Lauraceae) includes relict evergreen trees of the Tethyan flora that covered southern Europe and northern Africa during the mid Tertiary but is currently restricted to isolated refugia in the southern Black Sea area, Mediterranean Basin, Morocco, and the Macaronesian archipelagoes of Azores, Madeira, and Canaries (Rodríguez-Sánchez et al., 2009). Two species, *L. nobilis* L. and *L. azorica* (Seub.) Franco (Tutin, 1993), have been recognized, although recent molecular data do not support their distinction (see Rodríguez-Sánchez et al., 2009). Different ploidy levels have been described in *Laurus* (e.g., Ehrendorfer et al., 1968), with tetraploidy ($2n = 4x = 48$) being the most frequent karyotype.

Laurels are dioecious, insect-pollinated, and vertebrate-dispersed species. To investigate population genetic structure and patterns of gene flow via pollen and seeds, we isolated and characterized nuclear microsatellite markers. This marker type has been successfully applied to describe spatial patterns of genetic structure and diversity, perform parentage analyses, and assess sexual vs. vegetative reproduction (Selkoe and Toonen, 2006).

METHODS AND RESULTS

A microsatellite library was developed following Jones et al. (2002). DNA was extracted from one *Laurus nobilis* leaf sample using the Qiagen DNeasy Plant Mini kit and digested with seven blunt-end restriction enzymes (*Rsa*I, *Hae*III, *Bsr* B1, *Pvu*II, *Stu*I, *Sca*I, *Eco* RV; New England Biolabs, Ipswich, MA). Four libraries were prepared using Biotin-CA_{15}, Biotin-GA_{15}, Biotin-ATG_{12}, and Biotin-AAC_{12} as capture molecules (CPG, Lincoln Park, NJ). Seventy-four positive clones contained a microsatellite sequence, and primers were designed from 44 of them using Designer PCR 1.03 (Research Genetics, Huntsville, AL). For primer testing, DNA was isolated from silica-dried leaves using a modified CTAB extraction method (Milligan, 1998): tissue grinding in a MM301 Retsch™ and TLE resuspension (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). We sampled a total of 37 *L. nobilis* individuals from three natural populations located in the "Los Alcornocales" Natural Park, Cádiz, southern Spain (Jarda, Zapato, and Fuente de los Caños; see Appendix 1 for details). We also sampled 30 *L. azorica* trees from the islands of Madeira, Tenerife, Gran Canaria, La Gomera, La Palma, and El Hierro, as well as from Morocco.

PCR amplifications were performed in a 20 μL final volume containing 1× buffer [67 mM Tris-HCL pH 8.8, 16 mM $(NH4)_2SO_4$, 0.01% Tween-20], 2.5 mM $MgCl_2$ (1.5 mM for LnB121), 0.01% BSA (Roche Diagnostics, Rotkreuz, Switzerland), 0.25 mM dNTP, 0.40 μM dye-labeled M13 primer (Table 1), 0.25 μM "pig-tailed" reverse primer, 0.034 μM M13-tailed forward primer, 0.5 U *Taq* DNA polymerase (Bioline, London, UK) and 50 ng genomic DNA. Reactions were undertaken in a "touchdown" PCR in a Bio-Rad DNA Engine^R 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; and final extension of 5 min at 72°C. Amplified fragments were analyzed on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA), and sized using GeneMapper 4.0 (Applied Biosystems) and LIZ 500 size standard. So far, no multiplexing was attempted.

We tested a total of 44 primer pairs: 3 of them failed to amplify, 4 were monomorphic, 14 showed complex amplification, and 3 showed high frequencies of null alleles. Therefore, we finally retained 20 loci (Table 1), which produced a total of 196 alleles for our *L. nobilis* sample. All loci also amplified well in *L. azorica,* although scoring was difficult in five of them due to the existence of one-base peaks and nonspecific amplifications (Table 1). The remaining 15 loci produced a total of 222 alleles.

SSR marker scoring represents some additional problems in polyploid species, because it usually is very difficult to assess which allele(s) occur in more than one copy. Although some techniques have been developed for this purpose (Esselink et al., 2004), we adopted a more conservative and commonly used approach by treating data as dominant markers with phenotypic banding patterns recorded in a presence/absence matrix. Based on these data, and following Andreakis et al. (2009), we calculated allele frequencies and diversity statistics distinguishing between amplification variants (AV) for each allele within a

[1] Manuscript received 18 February 2010; revision accepted 11 March 2010.

The authors thank Mark Todd, Nikos Andreakis, and Patrick Meirmans for helpful discussions and clarifications. This study was supported by grants CGL2006-00373 and Junta de Andalucía P07-RNM02824 to PJ.

[4] Author for correspondence: jordano@ebd.csic.es

doi:10.3732/ajb.1000069

TABLE 1. Characteristics of 20 microsatellite markers isolated from populations *of Laurus nobilis* and *L. azorica*.

Locus name (GenBank ID)	Primer sequences (5′-3′)	Repeat motif	T_a [a] (°C)	Dye	*L. nobilis* Size Range (bp)	*n*	K_{Ln}	*Ho*	$H_E(Ce)$	$F(Ce)$	*L. azorica* Size Range (bp)	*n*	K_{La}	*Ho*	$H_E(Ce)$	$F(Ce)$
LnB119 (GU344686)	F: GGTAAGCAACAGAGCACATC R: AGGAAAACCAGTCAATAACTCC	$(TC)_{27}$	57	FAM	185-241	37	9	0.950	0.848	−0.121[b]	185–235	26	15	0.712	0.865	0.177[b]
LnA2 (GU344687)	F: TGCCCAAAAATGGTGTAG R: CGTGGTCTTAGCCTTAGTAGTC	$(GT)_8GC(GT)_{11}$	57	VIC	260–299	37	15	0.914	0.903	−0.012	256–313	24	26	0.958	0.938	−0.021
LnA115[c] (GU344688)	F: CATGCAAACAGTAACAACATGG R: GGTGACCTTCCTATCACACATC	$(CT)_{17}(CA)_{14}$	58	VIC	248–276	37	12	0.964	0.881	−0.094[b]	244–291	–	–	–	–	–
LnB121 (GU344689)	F: TCTCCCTCTCCATGCTCAC R: CTCCTTCTCCCCGTCTCTT	$(TC)_{17}(TG)_{10}$	58	VIC	310–364	37	17	0.964	0.923	−0.044	304–359	26	23	0.923	0.943	0.021
LnD109 (GU344690)	F: GCTGCTTATTGACACAACCAC R: GAAGGGAAACTGTAGGGCATA	$(ATG)_7$	58	FAM	281–287	37	3	0.833	0.645	−0.292[b]	275–287	26	4	0.641	0.691	0.072[b]
LnD106 (GU344691)	F: TGCTCTACGTTTTGTGAAGATC R: CATTGGAGGGAACTTCTTTTAC	$(ATC)_8$	56	NED	152–161	37	3	0.770	0.560	−0.376[b]	152–167	26	4	0.667	0.593	−0.124[b]
LnD5 (GU344692)	F: CGTTAGCACTGTCCCATCTG R: CCGAAATCACCACCTTTTTC	$(TGA)_8$	55	FAM	109–124	37	4	0.739	0.729	−0.013	115–130	26	5	0.853	0.782	−0.091[b]
LnB2 (GU344693)	F: TATTTGAAGGTTTCCTCTCAGA R: ATAAAGCGTGTCATTGTGAAC	$(GA)_{24}$	57	PET	244–279	37	15	0.995	0.900	−0.106[b]	242–293	26	24	0.994	0.938	−0.059[b]
LnD102[c] (GU344694)	F: TGATTCTCTTCGGGTGATC R: CCCAATACTTATCAAAGGTGAC	$(ATC)_{11}$	56	NED	197–237	37	6	0.766	0.762	−0.004	185–249	–	–	–	–	–
LnB116 (GU344695)	F: GCTTTCTCTTCCTCCCTGTC R: ACCCTCTCAATAATGGTTTGG	$(TC)_{17}$	57	PET	184–218	37	16	0.905	0.916	0.012	175–221	26	26	0.949	0.929	−0.021
LnD101 (GU344696)	F: TTTTCCTTACTCCATAGACACG R: TGGCTCAAGGTAGACTAGAATG	$(TCA)_8$	57	PET	251–263	37	2	0.608	0.500	−0.217[b]	241–263	26	4	0.615	0.747	0.176[b]
LnA106 (GU344697)	F: CAAATGATTTCAAGGACCAC R: AGGGGTCTTACTTCTATGAAGG	$(AC)_{12}$	56	VIC	157–165	37	2	0.599	0.493	−0.215[b]	157–167	26	6	0.872	0.766	−0.139[b]
LnB124 (GU344698)	F: TGGAATGTATGGCTCTGAACTC R: CCAATCACAACCAGAAAGACAG	$(CT)_{16}$	58	PET	225–273	37	11	0.964	0.882	−0.093[b]	223–285	26	18	0.923	0.890	−0.037
LnB10 (GU344699)	F: TTAGCCCCAAAAAATGTCAC R: AGCCGAAACAACTACAATCC	$(CT)_{14}(CGCT)_4$	57	VIC	185–231	37	8	0.896	0.842	−0.064	164–249	25	20	0.873	0.939	0.070[b]
LnB118	F: ATCCAGTGAGGTAACAGTCAGG	$(GA)_{19}$	57	NED	191–230	37	10	0.973	0.853	−0.141[b]	191–230	26	19	0.962	0.903	−0.064[b]

TABLE 1. Continued.

Locus name (GenBank ID)	Primer sequences (5′-3′)	Repeat motif	T_a [a] (°C)	Dye	*L. nobilis* Size Range (bp)	*n*	K_{Ln}	*Ho*	$H_E(Ce)$	$F(Ce)$	*L. azorica* Size Range (bp)	*n*	K_{La}	*Ho*	$H_E(Ce)$	$F(Ce)$
(GU344700)	R: ATGAACAGCACAGAGCAAGAC															
LnB106[c]	F: CATCGAAGGTAAATGTGAAATG	$(CT)_{16}$	57	FAM	246–281	37	18	0.982	0.922	−0.065[b]	242–294	–	–	–	–	–
(GU344701)	R: TAACCCCCATAAAGTCAAGAAG															
LnA103[c]	F: CCAGGTGGGTCTAAAGACATT	$(TG)_{12}C(GT)_5$ $(GA)_{15}N_{11}$ $(ACAT)_5$	58	NED	275–315	37	17	0.955	0.901	−0.060	255–351	–	–	–	–	–
(GU344702)	R: TTGTTGTTCTGTTGCAGTCAC															
LnD10	F: TAGCCAACCCAACATAATGG	$(TGA)_7$	57	VIC	212–234	37	7	0.937	0.805	−0.164[b]	219–239	26	11	0.936	0.858	−0.090[b]
(GU344703)	R: CTTTGCTCTTTGCTGGATTG															
LnD8	F: CCTCTTCTTTGTCCTTGTCC	$(CAT)_4$ $(CCTCAT)_3$ $(CAT)_8$	57	FAM	218–257	37	7	0.856	0.815	−0.050[b]	218–287	26	17	0.949	0.907	−0.046
(GU344704)	R: TCCCTTATTTCTCACATCCC															
LnA101[c]	F: TCACCTCCACACAGTAAGTCA	$(CA)_{12}$	57	NED	253–286	37	14	0.950	0.888	−0.070[b]	247–281	–	–	–	–	–
(GU344705)	R: ATTCGTATCAACTCCAATGAGA															
		Mean						0.876	0.798	−0.097				0.855	0.846	−0.011
		SD						0.120	0.141	0.058				0.129	0.107	0.078
		Total					196						222			

n, Number of individuals successfully genotyped; K_{Ln}, number of alleles for *L. nobilis*; K_{La}, number of alleles for *L. azorica;* H_O, observed heterozygosity; $H_E(Ce)$, expected heterozygosity under chromosome segregation; $F(Ce)$, fixation index under chromosome segregation.

[a] Annealing temperature (T_a) is given for nontailed primers.

[b] Locus showed significant deviation from Hardy–Weinberg equilibrium (Bonferroni-corrected) between observed and expected genotype frequencies according to χ^2 goodness of fit test.

[c] Locus discarded for *L. azorica* because of scoring problems (one-base peaks and nonspecific amplifications).

PCR products were labeled 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.

TABLE 2. Statistics for 20 microsatellites (treated as dominant markers) in tetraploid *Laurus nobilis* and *L. azorica*.

	Laurus nobilis					*Laurus azorica*				
Locus	**AV**	**BP**	C_j	D_j	D_L	**AV**	**BP**	C_j	D_j	D_L
LnB119	9	20	0.035	0.965	0.939	15	15	0.083	0.917	0.882
LnA2	15	29	0.017	0.983	0.957	26	24	0.000	1.000	0.958
LnA115[a]	12	29	0.020	0.980	0.954	–	–	–	–	–
LnB121	17	35	0.003	0.997	0.970	23	26	0.000	1.000	0.962
LnD109	3	5	0.620	0.380	0.370	4	5	0.302	0.698	0.672
LnD106	3	5	0.521	0.479	0.466	4	5	0.379	0.621	0.598
LnD5	3[b]	6	0.333	0.667	0.649	5	11	0.114	0.886	0.852
LnB2	15	30	0.011	0.989	0.963	24	25	0.003	0.997	0.959
LnD102[a]	6	10	0.173	0.827	0.805	–	–	–	–	–
LnB116	16	32	0.009	0.991	0.964	26	26	0.000	1.000	0.962
LnD101	2	3	0.514	0.486	0.473	4	5	0.299	0.701	0.675
LnA106	1[b]	2	0.571	0.429	0.418	6	12	0.139	0.861	0.828
LnB124	11	28	0.020	0.980	0.954	18	22	0.015	0.985	0.947
LnB10	8	19	0.080	0.920	0.896	20	24	0.003	0.997	0.957
LnB118	10	24	0.035	0.965	0.939	19	23	0.009	0.991	0.953
LnB106[a]	18	34	0.006	0.994	0.967	–	–	–	–	–
LnA103[a]	17	28	0.020	0.980	0.954	–	–	–	–	–
LnD10	7	16	0.111	0.889	0.865	11	20	0.031	0.969	0.932
LnD8	7	18	0.044	0.956	0.931	17	23	0.009	0.991	0.953
LnA101[a]	14	26	0.042	0.958	0.932	–	–	–	–	–
Average	9.700	19.950	0.159	0.841	0.818	14.800	17.733	0.092	0.908	0.872
Total	194	399				222	266			

AV, number of distinct single amplification variants (bands); BP, number of distinct banding patterns of each single locus; C_j, confusion probability (probability that two randomly chosen individuals from a successfully amplified sample have identical banding patterns); D_j, discriminating power (1- C_j); D_L, limit of D_j as N tends toward infinity.

[a] Locus discarded for *L. azorica* because of scoring problems (one-base peaks and nonspecific amplifications).

[b] Locus with one additional AV (not counted) present in all individuals.

given locus and banding patterns (BP) for each locus. A BP represents the combination of AVs that a single locus produces for a given individual. We first built a dataset assuming a dominant marker system for 37 *L. nobilis* and the 26 *L. azorica* island samples. For the full set of markers, the discrimination power (1-P_G, where P_G denotes the probability that two randomly drawn multilocus genotypes are identical) was very close to 1.0 (*L. nobilis*: $P_{G=}$ 0.9537 × 10^{-20}; *L. azorica*: $P_{G=}$ 0.1157 × 10^{-15}). Similarly, the confusion probability for a given locus (C_j: the probability that two randomly drawn individuals from a given sample have an identical BP) was low for all except the five less polymorphic markers (Table 2).

We used the software AUTOTET (Thrall & Young, 2000), designed for autotetraploid species, to derive estimates of heterozygosity and genetic diversity with data scored as codominant markers (Table 1). χ^2 goodness of fit tests comparing the observed and expected genotype frequencies under chromosomal segregation (Ce; random assortment of homologous chromosomes into gametes) showed significant deviation from Hardy-Weinberg equilibrium (HWE) for 13 loci in *L. nobilis* (Bonferroni-corrected $P < 0.05/20 = 0.0025$) and ten loci in *L. azorica* (Bonferroni-corrected $P < 0.05/15 = 0.0033$; see Table 1). H_E and F under chromatid segregation (Cd; random assortment of chromatids into gametes) are not reported as their values, calculated assuming maximum double reduction, were not significantly different from those for Ce. These results were not unexpected and could largely stem from our sampling scheme, designed to maximize the genetic differentiation of individuals, and the pooling of populations and islands. Species characteristics (e.g., relict status, dioecy, animal-mediated pollen and seed dispersal) could also contribute to HWE deviations. When separate analyses were run for each of the three *L. nobilis* populations, significant deviations from HWE appeared only in 7 to 11 loci. In particular, loci LnB119, LnB2, and LnB118 showed deviations in all three populations whereas LnD5, LnD102, LnB116, LnB10, and LnA101 showed none.

CONCLUSIONS

Observed levels of polymorphism and genetic diversity suggest that the reported markers are fully adequate for characterizing local and regional-scale levels of genetic variation and studying patterns of pollen- and seed-mediated gene flow (Selkoe and Toonen, 2006). Therefore, they should represent a useful tool to inform effective protection and management strategies for this emblematic and potentially threatened relict genus (Rodríguez-Sánchez et al., 2009).

LITERATURE CITED

ANDREAKIS, N., W. H. C. F. KOOISTRA, AND G. PROCACCINI. 2009. High genetic diversity and connectivity in the polyploid invasive seaweed *Asparagopsis taxiformis* (Bonnemaisoniales) in the Mediterranean, explored with microsatellite alleles and multilocus genotypes. *Molecular Ecology* 18: 212–226.

BOUTIN-GANACHE, I., M. RAPOSO, M. RAYMOND, AND C. F. DESCHEPPER. 2001. M13-Tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *BioTechniques* 31: 24–27.

EHRENDORFER, F., F. KRENDL, E. HABELER, AND W. SAUER. 1968. Chromosome numbers and evolution in primitive angiosperms. *Taxon* 17: 337–353.

ESSELINK, G. D., H. NYBOM, AND B. VOSMAN. 2004. Assignment of allelic configuration in polyploids using the MAC-PR (microsatellite DNA allele counting—peak ratios) method. *Theoretical and Applied Genetics* 109: 402–408.

JONES, K. C., K. F. LEVINE, AND J. D. BANKS. 2002. Characterization of 11 polymorphic tetranucleotide microsatellites for forensic applications in California elk (*Cervus elaphus canadensis*). *Molecular Ecology Notes* 2: 425–427.

MILLIGAN, B. G. 1998. Total DNA isolation. *In* A. R. Hoelzel [ed.], Molecular genetic analysis of populations: a practical approach, 2nd ed., 43–44. Oxford University Press, Oxford, UK.

RODRÍGUEZ-SÁNCHEZ, F., B. GUZMÁN, A. VALIDO, P. VARGAS, AND J. ARROYO. 2009. Late Neogene history of the laurel tree (*Laurus* L.,

Lauraceae) based on phylogeographical analyses of Mediterranean and Macaronesian populations. *Journal of Biogeography* 36: 1270–1281.

SELKOE, K. A., AND R. J. TOONEN. 2006. Microsatellites for ecologists: A practical guide to using and evaluating microsatellite markers. *Ecology Letters* 9: 615–629.

THRALL, P. H., AND A. YOUNG. 2000. AUTOTET: A program for analysis of autotetraploid genotypic data. *The Journal of Heredity* 91: 348–349.

TUTIN, T. G. 1993. *Laurus* L. *In* T. Tutin, N. Burges, A. Chater, J. Edmondson, V. Heywood, D. Moore, D. Valentine, S. Walters, and D. Webb [eds.], Flora Europaea, vol. 1, 2nd ed., 296–297. Cambridge University Press, Cambridge, UK.

APPENDIX 1. Geographical locations of *Laurus nobilis* and *L. azorica* populations and number of individuals sampled.

Species	Place / Island	Population	Latitude (N)	Longitude (W)	No. of individuals
L. nobilis	S Spain	Jarda	36.5691	5.5922	17
	S Spain	Zapato	36.4786	5.6242	8
	S Spain	Fuente de los Caños	36.4371	5.5889	12
L. azorica	Gran Canaria	Barranco Los Tilos de Moya	28.0871	15.5945	4
	Tenerife	Vueltas de Taganana	28.5445	16.2264	3
	Tenerife	Monte del Agua	28.3299	16.8249	3
	La Gomera	Bosque de El Cedro	28.1311	17.2202	4
	La Palma	Barranco de Los Tiles	28.7891	17.8022	4
	El Hierro	Fuente de Tinco	27.7606	17.9837	4
	Madeira	Ribeiro Frío	32.7415	16.8855	4
	C Morocco	Jbel Ksiba	32.5011	6.0011	4