Development and Characterization of Microsatellite Primers in the Endangered Mediterranean Shrub Ziziphus lotus (Rhamnaceae)

Authors: González-Robles, Ana, Manzaneda, Antonio J., Bastida, Jesús M., Harvey, Nick, Jaime, Rafael, et al.

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Primer Note

Development and characterization of microsatellite primers in the endangered Mediterranean shrub *Ziziphus lotus* (Rhamnaceae)[1]

Ana González-Robles[2], Antonio J. Manzaneda[2], Jesús M. Bastida[2], Nick Harvey[3], Rafael Jaime[2], Teresa Salido[2], Luisa M. Martínez[2], Ana Fernández-Ocaña[2], Julio M. Alcántara[2], and Pedro J. Rey[2,4]

[2]Departamento de Biología Animal, Biología Vegetal y Ecología, Universidad de Jaén, E-23071 Jaén, Spain; and [3]Genetic Marker Services, Brighton, United Kingdom

- *Premise of the study:* Microsatellite primers were developed to characterize and evaluate patterns of genetic diversity and structure in the endangered Mediterranean shrub *Ziziphus lotus* (Rhamnaceae).
- *Methods and Results:* Twenty microsatellite primers were developed for *Z. lotus*, of which 14 were polymorphic. We evaluated microsatellite polymorphism in 97 specimens from 18 Spanish and seven Moroccan populations. Between two and eight alleles were found per locus, and the average number of alleles was 5.54. Observed heterozygosity and expected heterozygosity ranged from 0.08 to 0.90 and from 0.08 to 0.82, respectively. Nine of these primers also amplified microsatellite loci in *Z. jujuba*.
- *Conclusions:* The microsatellite markers described here will be useful in studies on genetic variation, population genetic structure, and gene flow in the fragmented habitat of this species. These markers are a valuable resource for designing appropriate conservation measures for the species in the Mediterranean range.

Key words: habitat fragmentation; keystone species; microsatellites; Rhamnaceae; southeastern Spain; *Ziziphus lotus*.

Ziziphus Mill. (Rhamnaceae) is a pantropical and paraphyletic genus comprising approximately 170 species, 150 of them native to tropical and subtropical regions of Europe, the Middle East, Africa, India, and Asia (Islam and Simmons, 2006). The genus includes two economically important tree species, *Z. jujuba* Mill. (Chinese jujube) and *Z. mauritiana* Lam. (Indian jujube), that are cultivated for their fruit (Huang et al., 2015). *Ziziphus lotus* (L.) Lam. is a diploid ($2n = 20$; Pérez-Latorre and Cabezudo, 2009), hermaphrodite, sclerophyllous thorny shrub species occurring across the Mediterranean Basin, North Africa and the Sahara, and the Arabian Peninsula. In Europe, *Z. lotus* is restricted to some semiarid localities in the southeast of the Iberian Peninsula (Pérez-Latorre and Cabezudo, 2009) and the island of Sicily. *Ziziphus lotus* blooms from May to July, and flowers are pollinated primarily by bees. The fruit ripening period occurs in September, and fruits (drupes) are dispersed by foxes and other mammals. It is a keystone species in those semiarid ecosystems (Tirado, 2009). Since 1992, *Z. lotus* habitats have been included in the Habitats Directive of the European Commission (Council Directive 92/43/EEC 1992, namely Arborescent "matorral" with *Ziziphus*: habitat 5220; Council of the European Union, 2007), which lists Europe's most endangered and vulnerable habitats. Population size ranges from 10 to thousands (typically less than 100) of individuals depending on the alteration status. In fact, European *Z. lotus* populations are seriously threatened by severe habitat destruction and fragmentation due to agriculture intensification and land-use change (Mota et al., 1996; Tirado, 2009; Mendoza-Fernández et al., 2015).

Microsatellite (simple sequence repeat [SSR]) markers have been recently developed for *Z. jujuba* (Huang et al., 2015); however, transferability of jujube SSR primers to *Z. lotus* has not been shown. Here, we characterized 20 microsatellite markers (14 polymorphic) developed specifically for *Z. lotus*, which will be subsequently used to evaluate the impact of land-use change and fragmentation on the genetic diversity of the species. We also amplified polymorphic markers in 10 North African individuals of *Z. lotus* (from Morocco) to assess genetic variation, diversity levels, and population genetic structure across the region for conservation purposes. Finally, cross-amplification was tested in *Z. jujuba* samples, the other *Ziziphus* species with a presence in the Iberian Peninsula.

METHODS AND RESULTS

Total genomic DNA was extracted from frozen young leaves following a slightly modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle, 1987). We used 0.7 volumes of cold isopropanol to precipitate nucleic acid, a

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[4]Author for correspondence: prey@ujaen.es

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wash buffer with 70% EtOH (without ammonium acetate), and mixed RNase with distilled water to resuspend the nucleic acid pellet; the samples were then incubated for 60 min at 37°C. The last dilution step was removed from the protocol. Leaves were collected from seven individuals across seven distinct populations covering the range of distribution of *Z. lotus* in southern Spain. Microsatellite isolation was performed by Genetic Marker Services (Brighton, United Kingdom). Briefly, microsatellite isolation was based on the production of an enriched library, using a hybridization capture protocol. Enrichment involved incubating adapter-ligated, restricted DNA, with filter-bonded synthetic repeat motifs: $(AG)_{17}$, $(AC)_{17}$, $(AAC)_{10}$, $(CCG)_{10}$, $(CTG)_{10}$, and $(AAT)_{10}$. The library was transformed into *Escherichia coli* JM109 and plated onto Luria–Bertani agar/ampicillin plates. The motif-positive clones were screened, isolated, and sequenced. Primers were designed using the online primer design software Primer3 (Rozen and Skaletsky, 1999). The amplifying products were 100–250 bp long, to help minimize later multiloading overlap ambiguities during sequencer genotyping. The GC content of the designed primers is given in Table 1. To test the effectiveness of primer amplification, we used a touchdown PCR protocol. PCR amplification was performed in a 25-µL reaction volume that contained 7 pmol of each primer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTPs, 1× PCR buffer, 0.8 µg/µL bovine serum albumin (BSA), 0.5 units of *Taq* polymerase (AmpliTaq Gold polymerase; Applied Biosystems, Carlsbad, California, USA), and 1.5 µL of DNA 1 : 20 diluted. PCR amplification of the template was performed according to the following protocol (32 cycles): 95°C for 60 s for initial denaturation; annealing for 60 s as two cycles each 64–59°C, 10 cycles 58°C, 10 cycles 57°C; elongation at 72°C for 60 s; and a final extension at 72°C for 5 min using a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). All PCR products were checked for specificity, active polymorphism, and null alleles on high-resolution agarose gels (4% MetaPhor; Lonza, Basel, Switzerland) in a TAE buffer system. Fourteen out of 20 loci showed clear and specific bands displaying size variation among the seven individuals assayed. These 14 loci were then selected for fluorescent labeling (Table 1).

We tested the efficiency and functionality of the 14 selected microsatellites in 97 samples from 18 Iberian and seven Moroccan populations corresponding to the three main population centers of this species in the western Mediterranean (Appendix 1). The larger populations are located in Almeria; the populations in Murcia and Morocco are basically relicts where scattered individuals occur, often growing at the margin of cultivated fields.

Multiplex PCRs were performed in 11.11-µL volumes containing 7 pmol of each primer (labeled with the fluorescent dye 5-HEX or 56-FAM; Table 1), 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs mix, 1× PCR buffer, 0.5 units Ampli*Taq* Gold polymerase (Applied Biosystems), and 10 ng/µL of DNA. Touchdown PCR conditions (32 cycles) comprised an initial heat step at 95°C for 4 min; followed by 10 cycles at 95°C for denaturation for 1 min, annealing at 64°C for 1 min (decreasing 1°C for each of two cycles), elongation at 72°C for 1 min; followed by 11 cycles with denaturation at 95°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for 1 min; followed by 11 cycles with denaturation at

TABLE 1. Characteristics of 20 microsatellite loci developed in *Ziziphus lotus*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent dye	Mix groups	GenBank accession no.
zlo60§	F: GTTCATCAATTTCACCATGC	$(CT)_{15}$	205–234	50	—	—	KU530111
	R: CCTATGGATTACTCCTATAAGCTG						
zlo61§	F: GCAACTTTCCCAACCAAGAA	$(CT)_{24}$	364–411	53.5	—	—	KU255442
	R: CCAAATTTTCGTTACACCATGT						
zlo64*	F: TTGGATAAAAAGAGGTTAGCACA	$(CT)_{15}$	61–90	52	5-HEX	A	KU530112
	R: CCAAACAGCTTAGGGACCAA						
zlo65*	F: ATTGTCCCACCTTCCAACAC	$(TC)_{10}$/ACTCC/$(CT)_5$	332–366	55	5-HEX	A	KU255443
	R: AACTCCACCTGAAACCTTGC						
zlo66*	F: TAAAGAGCGTAAGCGGGAAA	$(AG)_{15}$	321–350	54	56-FAM	E	KU255444
	R: GCGCGCAGTAGATAGACACC						
zlo67*	F: GATCTGAACACCGTGCGTTT	$(CT)_{19}$	139–176	53	5-HEX	D	KU255445
	R: AAACCAACGAAACCAACGAA						
zlo68*	F: TGACGTTAGGAAAGAAGATTCAAA	$(TG)_9(AG)_{14}$	289–334	52.5	5-HEX	E	KU255446
	R: GGCATCCTTAATGCAAGAGG						
zlo69§	F: AGCTTGGTCTCTCCAACGAA	$(CT)_6$/CC/$(CT)_{11}$	54–89	56	—	—	KU255447
	R: GCAAAACCCTAGGTGGCTTA						
zlo71*	F: CCGAGGTGAGGTATGTCTTT	$(CT)_{20}$	57–96	51.5	56-FAM	C	KU255448
	R: TCCCCAAGAAGCAAATATCA						
zlo73*	F: GGACTGTGTATTTCAGCAAGG	$(TC)_{21}$	429–470	51	56-FAM	A	KU255449
	R: AAAGTTGTGCCAATCTTGTTT						
zlo76*	F: ACAACGTTTGCTGTCCTCTC	$(CT)_{16}$	108–139	55	56-FAM	C	KU255450
	R: TAAACCCACAAGAGGGAATG						
zlo77*	F: CGCCATGTAAAATGGTGTTG	$(GA)_{12}$	217–240	52.5	56-FAM	D	KU235551
	R: CCACTTCCCAGGAGATCCAT						
zlo79*	F: TATAATCTCTTTCCAGATTCCTAGAA	$(CT)_{19}$	311–348	51	56-FAM	B	KU235552
	R: CTTGTGTCATCCCAAAACCA						
zlo80*	F: TTTCACGGTATTGCAACCAG	$(GT)_{10}$	377–396	53.5	5-HEX	C	KU255451
	R: CACCTGAGGCACAACAAAGTT						
zlo84§	F: CGTTTTCTGTTTGGAGACCA	$(AG)_{18}$	216–251	53	—	—	KU255452
	R: AATCAATTCCTTCCCAAGCA						
zlo85§	F: TGGCATTTAATTGTGTCATCAGA	$(AG)_{14}$	136–163	53	—	—	KU255453
	R: CCATACTGTCCCTTTCCAGTC						
zlo86*	F: TTTGAGATTGATGGCTCCTT	$(AC)_{10}$	233–252	51.5	56-FAM	E	KU255454
	R: GCAGTTTCCGTTGTTGTTTT						
zlo87*	F: CAGCTCGTCCTCTTCGAGAT	$(TC)_{16}$	134–164	56	56-FAM	B	KU255455
	R: GGACAATTTGGAATTTCAGTTTC						
zlo88*	F: TTCATAACAGCACGTCAAACT	$(AG)_{21}$	409–450	52.5	56-FAM	B	KU255456
	R: TATGGAGTGGGTTCATTTCC						
zlo89§	F: TGGTCTTAATTCACCAAGCTCA	$(GA)_{14}$/GG/$(GA)_{10}$	158–207	54	—	—	KU255457
	R: GCTTGTCAGTTGTATGTGAAGTGA						

Note: T_a = annealing temperature.
*Tested for polymorphism.
§Monomorphic markers.

Table 2. Genetic characterization of 14 newly developed polymorphic microsatellites of *Ziziphus lotus*.[a]

	Almeria, Spain (n = 50)				Murcia, Spain (n = 37)				Morocco (n = 10)			
Locus	A	H_o	H_e	HWE	A	H_o	H_e	HWE	A	H_o	H_e	HWE
zlo64[§]	8	0.72	0.79	0.011	8	0.62	0.78	0.006	5	0.20	0.72	0.000*
zlo65	8	0.74	0.74	0.005	6	0.62	0.72	0.015	7	0.80	0.80	0.346
zlo66	6	0.76	0.72	0.784	6	0.30	0.39	0.016	6	0.60	0.73	0.172
zlo67	6	0.58	0.67	0.231	4	0.43	0.44	0.199	8	0.70	0.80	0.021
zlo68	5	0.66	0.67	0.872	6	0.73	0.73	0.520	4	0.40	0.68	0.066
zlo71[§]	6	0.54	0.57	0.228	5	0.41	0.59	0.000*	7	0.20	0.70	0.000*
zlo73[§]	4	0.46	0.62	0.014	5	0.51	0.65	0.020	5	0.40	0.74	0.015
zlo76[§]	5	0.44	0.56	0.046	3	0.43	0.46	0.098	6	0.60	0.76	0.424
zlo77	3	0.18	0.17	1.000	2	0.08	0.08	1.000	3	0.60	0.55	0.587
zlo79[§]	6	0.74	0.72	0.249	5	0.49	0.65	0.046	7	0.70	0.82	0.079
zlo80[§]	6	0.68	0.72	0.203	6	0.59	0.74	0.020	4	0.50	0.66	0.131
zlo86	5	0.50	0.50	0.562	6	0.57	0.66	0.000*	8	0.60	0.71	0.011
zlo87	5	0.56	0.56	0.161	5	0.38	0.47	0.030	5	0.90	0.78	0.082
zlo88	7	0.50	0.55	0.078	6	0.46	0.55	0.015	5	0.50	0.72	0.238
Mean	5.71	0.58	0.61	—	5.21	0.47	0.57	—	5.71	0.55	0.73	—

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = P values of the exact test of Hardy–Weinberg equilibrium; n = number of individuals sampled.

[a] See Appendix 1 for geographic locations of all populations sampled.

* Locus showed significant deviations from Hardy–Weinberg equilibrium, after Bonferroni correction ($P < 0.001$).

[§] Significant presence of null alleles (zlo73 and zlo76 from Almeria; zlo64, zlo71, zlo73, zlo79, and zlo80 from Murcia; zlo64, zlo71, and zlo73 from Morocco).

95°C for 1 min, annealing at 57°C for 1 min, elongation at 72°C for 1 min; with a final extension of 5 min at 72°C. Mix A (Table 1) was best amplified and optimized with a common PCR protocol for SSR (Ghaffari and Hasnaoui, 2013), which comprised an initial heat step at 95°C for 3 min, followed by 40 cycles with denaturation at 95°C for 20 s, annealing at 55°C for 1 min, elongation at 72°C for 1 min, and a final extension at 72°C for 6 min. Fluorescently labeled PCR products were analyzed on an ABI 3500 Genetic Analyzer sequencer (Applied Biosystems) using GeneScan 600 LIZ Size Standard (Applied Biosystems) in the automated genotyping. GeneMapper software version 4.1 (Applied Biosystems) was used for the assignment of alleles and fragment analysis.

We used the package *pegas* (Paradis, 2010) of R software version 3.2.2 (R Core Team, 2015) to estimate the number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), and Hardy–Weinberg equilibrium (HWE). The presence of null alleles was checked using MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004), and their statistical significance was assessed using Bonferroni corrected P values. Linkage disequilibrium was estimated using GENEPOP software (Rousset, 2008).

The number of alleles ranged from two to eight per locus, depending on the population (Table 2). H_o varied from 0.08 to 0.90, and H_e varied from 0.08 to 0.82. Overall, it is shown that H_o is lower than H_e in the three study areas (Almeria, Murcia, and Morocco; Table 2), likely as a result of nonrandom mating and/or genetic drift. Two loci (zlo71 and zlo86) showed a significant deviation from HWE in Murcia populations, while two other loci (zlo64 and zlo71) showed deviation from HWE in Moroccan populations. Null alleles were present in six loci (Table 2) concordant in some cases with deviation from HWE, which may be caused by the intensive fragmentation of *Z. lotus* habitat in those populations. The presence of microsatellite null alleles is reported frequently in PCR primer characterization, and it should be taken into account when estimating population differentiation (Chapuis and Estoup, 2007). Significant linkage disequilibrium was detected only for zlo76/zlo80 loci after pairwise Bonferroni correction. Cross-amplification in *Z. jujuba* showed fragments of the expected size in nine of the 14 microsatellite loci (Table 3).

Table 3. Genetic properties of the microsatellite loci developed for *Ziziphus lotus* in single populations[a] of *Z. jujuba* (n = 5).

Locus	A	Allele size range (bp)	H_o	H_e
zlo64	—	—	—	—
zlo65	—	—	—	—
zlo66	3	172–178	0.4	0.62
zlo67	1	156	0	0
zlo68	5	158–176	1	0.74
zlo71	—	—	—	—
zlo73	—	—	—	—
zlo76	1	125	0	0
zlo77	1	136	0	0
zlo79	1	142	0	0
zlo80	2	142–155	1	0.5
zlo86	1	112	0	0
zlo87	1	114	0	0
zlo88	—	—	—	—

Note: — = not amplified; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

[a] See Appendix 1 for geographic locations of all populations sampled.

CONCLUSIONS

These 20 microsatellite markers are the first markers developed specifically for *Z. lotus*, and will be a useful tool for studies on genetic variation, diversity, population genetic structure, and gene flow in the fragmented habitat of this species. These markers are thus a valuable resource for designing appropriate conservation measures for the species in the Mediterranean range.

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Appendix 1. Voucher and location information for *Ziziphus lotus* and *Z. jujuba* populations used in this study.

Species	Voucher[a]	Population	Locality	Country of origin	Geographic coordinates[b]	*n*
Ziziphus lotus (L.) Lam.	GDA62533	El Ejido	Almería	Spain	36°45′40″N, 2°47′59″W	5
	GDA62534	Santa María del Águila	Almería	Spain	36°47′38″N, 2°46′16″W	5
	GDA62535	Sierra Alhamilla	Almería	Spain	36°56′55″N, 2°24′34″W	5
	GDA62541	El Toyo	Almería	Spain	36°50′51″N, 2°19′58″W	5
	GDA62540	Torregarcia	Almería	Spain	36°49′37″N, 2°17′22″W	5
	GDA62539	El Playazo	Almería	Spain	36°51′10″N, 2°01′05″W	5
	GDA62538	Campohermoso	Almería	Spain	36°56′44″N, 2°08′30″W	5
	GDA62537	Nijar	Almería	Spain	36°56′20″N, 2°13′10″W	5
	GDA62536	Autovia	Almería	Spain	36°52′11″N, 2°15′48″W	5
	GDA62542	Guazamara	Almería	Spain	37°20′53″N, 1°46′24″W	5
	GDA62543	Cantera Puerto Lumbreras	Murcia	Spain	37°31′51″N, 1°48′01″W	5
	GDA62544	Bejar-Torrecilla	Murcia	Spain	37°36′51″N, 1°45′17″W	5
	GDA62545	Purias	Murcia	Spain	37°35′28″N, 1°39′27″W	5
	GDA62546	Tercia	Murcia	Spain	37°42′57″N, 1°35′14″W	5
	GDA62548	Peñas Blancas	Murcia	Spain	37°40′52″N, 1°08′52″W	5
	GDA62549	Palas	Murcia	Spain	37°40′28″N, 1°13′48″W	5
	GDA62550	Los Garres	Murcia	Spain	37°56′14″N, 1°07′42″W	4
	GDA62547	Fuente Álamo	Murcia	Spain	37°42′12″N, 1°09′16″W	3
	GDA62551	Agadir	Agadir	Morocco	30°23′43″N, 9°34′52″W	2
	GDA62557	Ouazzane	Ouazzane	Morocco	34°34′48″N, 5°32′01″W	1
	GDA62556	Taza	Taza	Morocco	34°06′23″N, 4°27′13″W	2
	GDA62555	Fez	Fez	Morocco	33°56′21″N, 5°00′05″W	2
	GDA62552	Tiznit	Tiznit	Morocco	29°39′45″N, 9°51′53″W	1
	GDA62554	Zag	Assa-Zag	Morocco	27°58′47″N, 9°43′0″W	1
	GDA62553	Tistguezzemtz	Tistguezzemtz	Morocco	28°24′36″N, 9°10′48″W	1
Ziziphus jujuba Mill.	GDA62558	Pinos del Valle	Granada	Spain	36°53′29″N, 3°32′29″W	1
	GDA62559	Puente Tablas	Jaén	Spain	37°47′31″N, 3°45′11″W	1
	GDA62560	Las Yucas	Jaén	Spain	37°45′15″N, 3°41′02″W	2
	GDA62561	Algeciras	Cádiz	Spain	36°09′08″N, 5°27′20″W	1

Note: *n* = number of individuals (samples) per locality.

[a] Voucher specimens deposited at the Universidad de Granada Herbarium (GDA), Universidad de Granada, Granada, Spain.

[b] Datum: World Geodetic System 1984 (WGS84).