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Development and across-species transferability of microsatellite markers in the genus *Limonium* (*Plumbaginaceae*)

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The aim of this work was the development of microsatellite markers for the species *L. narbonense*, a sexual tetraploid, and to test their transferability to other *Limonium* species. The genus *Limonium* (*Plumbaginaceae*) has a worldwide distribution, with the largest number of species found in the Western Mediterranean basin (Erben 1993). It includes species with a wide range of ploidies (mostly di-, tri-, and tetraploids) and reproductive systems (sexual and asexual through apomyxis), and a high proportion of hybrid taxa. Many of these species inhabit salt-rich soils, and due to human pressure their range has been reduced, resulting in increased isolation. Several *Limonium* species endemic to the Iberian Peninsula have been declared as endangered (Laguna et al. 1994). We are involved in the conservation effort of different *Limonium* species through the analysis of genetic variation and population structure, and in the study of the evolutionary relationships among species in this genus. The use of microsatellite markers provides an ideal tool to investigate these questions due to their codominant, multiallelic and highly polymorphic nature (e.g., Morgante and Olivieri 1993). However, given the long, expensive process of SSRs isolation and the relatively low frequency of SSRs in plants (Powell et al. 1996), it is desirable to extend the use of primer sequences identified in one species into others.

A size-selected (200–600 bp) genomic DNA library of *L. narbonense* was constructed in Lambda-ZapII after digestion with EcoRI. The library was screened with a mixture of digoxigenin (Boëhringer-Mannheim) end-labeled synthetic oligonucleotides, combined in two groups with similar optimum hybridization temperatures, one with $(CT)_{10}$, $(GT)_{10}$, $(GCT)_8$, $(CTC)_8$, $(CAC)_8$, $(ACTG)_6$, $(ATCC)_6$, and $(CCTT)_6$, and the second with $(ACT)_{10}$, $(ATC)_{10}$, $(AAC)_{10}$, $(CTT)_{10}$, $(AGAT)_6$, and $(CTTT)_6$. Clones scored as positive after a second screening by hybridization to PCR-amplified inserts were sequenced and primers were designed using the program Right Primer 1.2 (Biodisk, San Francisco, USA).

Primers were tested in three different sample sets. The first one included 30 *L. narbonense* individuals from 5 different populations, representative of the species range in the East Mediterranean Spanish coast. The second was designed to test the across-species transferability of those primers (see species list and sample sizes in Table 1). Most species belong to section *Limonium* (which also includes *L. narbonense*), whereas *L. sinuatum*, *L. caesium* and *L. echioides* belong to different sections in the genus (see Palacios et al. (2000) for detailed information on chromosome numbers and reproductive system). Finally, a third sample consisted of 101 individuals from four populations of *L. rigualii*.

PCR reactions consisted of 1–20 ng of genomic DNA, 0.2–0.4 μM each primer, 200 μM each dNTP, 1–2 mM $MgCl_2$, 1 × PCR buffer (Pharmacia) and 0.5 units of Taq DNA polymerase (Pharmacia) in 25 μL volume. An initial denaturation step of 5 min at 94 °C was followed by 30 cycles of 40 s at 94 °C, 50 s at the appropriate annealing temperature (see Table 1) and 50 s at 72 °C, and a final extension of 7 min at 72 °C. DNA fragments produced by these amplifications were separated by electrophoresis in agarose gels stained with EtBr or polyacrylamide gels visualized by silver staining.

Table 1. Primer sequences and features of *Limonium narbonense* microsatellite loci. The last column refers to the species in which each primer amplifies: **A**, *L. narbonense* (*L.n.*, sampled individuals N = 30), **B**, *L. n.* and *L. vulgare* (*L.v.*, N = 1), **C**, *L. n. L. v., L echioides* (N = 1) and *L. sinuatum* (N = 1) and **D**, in the previous and all remaining species tested: *L. caesium* (N = 1), *L. girardianum* (N = 1), *L. cavanillesii* (N = 5), *L. delicatulum* (N = 3), *L. interjectum* (N = 1), *L. gymnesicum* (N = 1), *L. virgatum* (N = 1), *L. camposanum* (N = 1), *L. angustebracteatum* (N = 1), *L. furfuraceum* (N = 1), *L. dichotomum* (N = 1), *L. dufourii* (N = 5), *L. tenuicaule* (N = 2), *L. rigualii* (N = 101), *L. sinuatum* (N = 1)

Locus	Repeat motif	Primer sequences (5′-3′)	Temp. (°C)	Fragment size (bp)	Species
Ln3	$(CAC)_9$	AATTCAGTGGAGCATGGTAGTG CTTTACGAACGGCCGCTG	50	64	D
Ln36	$(CT)_{11}$	CAGGCCACGTGCTTAATTACTTAGTGGTCGTT GCCTTTACAACTGGGGGACGGTAG	65	228	D
Ln39	$(CAC)_6$	TGAGCCAATTAGGGCCGCTACCGAG TCAAGACCCAATGGCTCTGCAGCAACAAAA	65	151	D
Ln41	$(CAG)_2CAA(CAG)_4$	AATTCAGAGTAGCAGCAGCAACAG CTCTGCTGAGCTCTGCCCAAGT	58	97	B
Ln44	$(CTT)_6$	CGGCCCTGGTTTTGACTCCGTACGTAA CGTGAGTTCAGCGGGACCGGTAA	60	216	B
Ln45	$(AGC)_6$	AATTCTCATGACAGAATTGGAGAAGG CGACGTCTTTATGGTCACGGT	54	345	B
Ln52	$(CAC)_4(GAC)_5$	TCCTCTGCTACAAAATCTACTGCGGCGA GTTGGTCTGTTTGGGAGAGGATCCGGT	65	252	B
Ln68	$(TG)_2T(TG)_7$	AGGTCAACATTGTACATCATAGCAACTCAG CTGGTTGCTTCGTTTTGAGGTTAC	59	211	D
Ln115	$(CAC)_6$	CGGATCCAGAAACTAGATCCTGATAACGCC GCTGCATGGAGAGTGGGAGTGAGCGATTG	65	259	D
Ln122	$(GT)_8$	CGTGTACCGTGTATCTTCATGTAA AATTCGATTCTTTCAAACCCTC	60	260	B
Ln138	$(CTT)_9$	CGTCAGTTCTCTCGTCGTTGTTT ACCAATCTGAGAAAAAGAGCTTGGG	57	90	B
Ln141	$(CTT)_{29}$	AATTCGATTGCTGCCGAACTG AATTCATGGACAAGAAGAAGAAGAAGAAGA	65	189	B
Ln146	$(CTT)_{33}$	CCCGTTCTTCTCTTCCTCCCTTTG CCATGGATAGATCCCCGCAATTAGCC	62	196	A
Ln149	$(TGA)_6$	GAGTGGAAATTAACCGACGGA CTCCATCCCATCATTATTGTACTCATTGTG	55	90	C
Ln152	$(CTT)_{19}$	CAGCACTTTCTATACTAAAACATCGTCGCC AATTCGCTGGTGAGCCAACCCTATT	65	370	D
Ln154	$(GAT)G(GAT)_2GT$ $(GAT)_3GGT(GAT)_2$	GAATTCGCCGATGGATGATGTG CGATTTTCTCCACGCTCGTACGAC	60	87	B
Ln162	$(CTT)_{10}$	CCGCTCATTTTCAATCTCTTCTAGCAC CAACATCAATGAAGCCACTACGTGGG	65	86	B

Table 2. Summary of the results obtained in the amplification of 16 polymorphic microsatellite loci described in Table 1 on 30 *L. narbonense* individuals. H_{min} and H_{max} represent, respectively, the lower and upper limits of heterozygosity of each locus

Locus	Number of alleles	Size range	H_{min}	H_{max}
Ln3	8	52–73	0.577	0.724
Ln36	11	228–250	0.729	0.814
Ln39	4	142–157	0.552	0.661
Ln41	4	91–100	0.677	0.736
Ln44	16	185–240	0.889	0.909
Ln45	10	342–379	0.399	0.579
Ln52	14	194–271	0.737	0.784
Ln68	9	181–231	0.741	0.801
Ln115	5	249–261	0.553	0.691
Ln122	9	254–280	0.582	0.709
Ln141	11	118–170	0.788	0.814
Ln146	15	50–400	0.854	0.866
Ln149	4	84–102	0.220	0.387
Ln152	10	210-396	0.633	0.752
Ln154	3	75–90	0.443	0.575
Ln162	13	50–125	0.745	0.791

Approximately 125,000 clones were screened, 155 (0.12%) of which were positive. The second screening eliminated 118 (76%) clones as false positives or with too large inserts. From the remaining, 27 clones contained real microsatellites: 22 had a trinucleotide repeat sequence, one clone presented a tetranucleotide repeat, and the 4 remaining clones had a dimer repeat. Twenty-one of the sequences were suitable for primer design. Seventeen primer sets (Table 1) amplified microsatellite loci in the 30 individuals sample of *L. narbonense*, and 16 were polymorphic (Table 2). Locus Ln138 was monomorphic In tetraploids some genotypes cannot be established without analyzing the progeny of test crosses, therefore heterozygosity estimates shown in Table 2 represent the upper and lower limits.

While cross-species amplification was possible (Table 1), no polymorphism was detected in other species. Annealing temperatures for across-species amplification were similar, up to 5 °C lower, to those for *L. narbonense*.

We further tested the 6 primers that amplified in most *Limonium* species in a sample of 101 individuals from 4 populations of *L. rigualii*, another endangered species from section *Limonium*. In this case, three primers (Ln39, Ln115, and Ln152) amplified in all the individuals but did not present any variation. The other three primers did not amplify consistently in all the individuals.

From the across species transferability studies, we can conclude that microsatellite loci were more likely to be amplified in closely related species, although no polymorphism was detected. Simultaneous studies in our lab to ascertain phylogenetic relationships in section *Limonium* have shown that *L. narbonense* and *L. vulgare* are very closely related, at 0.011 nucleotide substitutions/site – with Jukes-Cantor correction – in the rDNA ITS region (Palacios et al. 2000). These species form a monophyletic group separate from the rest of the section, and less related to them than outgroup species included in this study (*L. echioides*, 0.122 nucl.subs./site on average). The remaining species from section *Limonium*, including *L. rigualii*, conform a monophyletic group, separate to that formed by *L. narbonense* and *L. vulgare*, at an average of 0.156 nucl.subs./site. This relatively large evolutionary distance may explain the low success rate for the across species transferability of microsatellite primers from *L. narbonense* to most other species from section Limonium. These results corroborate previous findings about the feasibility of across-species transfer of primers (Kresovich et al. 1995; Steinkellner et al. 1997).

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