 doi: 10.1111/j.1755-0998.2009.02523.x

PERMANENT GENETIC RESOURCES

Development and characterization of nine polymorphic microsatellite markers in the Chilean kelp *Lessonia nigrescens*

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Abstract

A total of nine microsatellite loci were isolated and characterized in the Chilean kelp *Lessonia nigrescens* Bory. Using two different enriched libraries, we observed 1–14 alleles per locus in two samples of 21 kelp individuals each. The observed heterozygosities ranged from 0.05 to 0.80 and all loci are in Hardy–Weinberg equilibrium for one or both samples. Seventeen samples collected from different sites showed high allele diversity along the species distribution. The variation detected at these markers is currently being used for the study of populations of *Lessonia nigrescens* at different geographical scales.

Keywords: genetic variability, kelp, *Lessonia nigrescens*, microsatellite markers

Received 28 August 2008; revision accepted 26 October 2008

Lessonia nigrescens Bory is an intertidal kelp inhabiting the southeast Pacific coasts, from Cape Horn to southern Peru. It is a keystone species and a bioengineer of the intertidal communities. In order to establish kelp stocks, estimate some important demographic parameters and design effective conservation plans, the study of the distribution of the genetic diversity became a main concern. However, the progress of such research was severely limited by the poor quality and reduced statistical power of the random amplified polymorphic DNA markers used so far (Martínez *et al*. 2003; Faugeron *et al*. 2005), highlighting the need for highly polymorphic, codominant and reliable molecular markers. Microsatellite markers have been developed in only one kelp species, *Laminaria digitata* (Billot *et al*. 1998), and cross-amplification in different kelp species within the order Laminariales has been unsuccessful, in particular with *L. nigrescens* (Martínez *et al*. 2005). For all these reasons, species-specific microsatellites markers were developed.

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We describe here the isolation and characterization of nine polymorphic microsatellites markers for *L. nigrescens* using two enriched libraries. Genomic DNA was extracted from a bulk of immature and healthy fronds of *L. nigrescens* using the phenol-chloroform method described by Sambrook *et al*. (1989). A first genomic library enriched for CA_n, GA_n and CAA_n microsatellite motifs was produced according to the protocol previously described (Billotte *et al*. 1999) with the following modifications: total DNA of *L. nigrescens* was restricted with the endonuclease *Rsa*I; the selection of microsatellite sequences was performed using two biotin-labelled oligoprobes in a mix for simultaneous selection of CA, GA and CAA repeats, 5′-I*IIIITCTCTCT-CTCTCTCTC-3′ and 5′-I*IIIITGTGTGTGTGTGTG-3′ where I* is a biotinylated Inosine. After ligation, transformation was made in Epicurian XL1-blue *Escherichia coli* strain. A total of 192 clones were transferred to microplates containing 150 μL of LB/Ampicilin solution. Insert sizes were estimated by agarose gel electrophoresis of insert PCR products using M13 universal primers. Gels were alkaline Southern transferred to Hybond N + nylon membrane (Amersham) and hybridized with a ^{32}P radiolabelled

Table 1 Characteristics of the nine polymorphic microsatellite markers, including their polymorphism in two populations (PAz, Pan de Azucar, 26°07′S; 70°39′W and LC, Las Cruces, 33°30′S, 71°38′W) and for a sample of individuals from most of the species range (SR). T_m, annealing temperature; N, number of analysed individuals; N_A, number of alleles; $^{**}P < 0.01$ for significant departures from HWE, tested after 1000 permutation in Genetix (Belkhir *et al*. 1996)

Locus	Repeat motif (cloned allele)	Primer sequence (5′–3′)	T_m (°C)	GenBank Accession no.	Sample	N	Size range (bp)	N_A	H_O/H_E
First Library									
LESS1T3	$(GCA)_8$	F: CTCTAAACCAATGGGAAACA	55	AY787003	SR	17	106–169	14	—
		R : TTTTGGTTCGTCCCTCTC			PAz	21	115	1	—
					LC	20	106–124	5	0.60/0.53
LESS1D4	$(CT)_{29}$	F : ACTTTGTGAAGCGGCATA	55	AY787004	SR	17	160–200	11	—
		R : CGTGCATCATTATGTGTCC			PAz	20	168–200	5	0.40/0.35
					LC	21	166–168	2	0.05/0.13
LESS1T9	$(AAC)_{18}AAA(AAC)_7$	F : TATTCATGCCCGCATCTA	55	AY787009	SR	17	221–335	19	—
		R : ATTCCCACTACATACCACCA			PAz	20	239–263	8	0.80/0.75
					LC	20	270–306	7	0.80/0.71
LESS1T11	$(CAA)_2N_{29}(CAA)_7$	F : TAGGGCTAGTCGCCCACT	58	AY787011	SR	11	152–164	5	—
		R : TAGTTCGTTGGCGTGTGTT			PAz	20	158–164	3	0.25/0.37
					LC	21	—	—	—
LESS1D17	$(CAA)_2AAA(CAA)_2N_{20}$	F: TTGACCACCAAACATGTAAA	55	AY787019	SR	15	281–451	16	—
	$(CAA)_9A(CAA)_2N_{15}$	R : CGATAGATGCTTTGCTCTTC			PAz	20	281–317	9	0.75/0.76
	$(CAA)_5CAG\ (CAA)_5$				LC	21	302	1	—
Second Library									
LESS2D1	$(TC)_5TT(TC)_2CC(TC)_2$	F: TTGTTTCACACCCTGGGATG	54	FJ161703	SR	17	259–317	13	—
	$TT(TC)_5CC(TC)_{15}$	R: CGGGGATAGGCGATGTTC			PAz	21	259–317	11	0.71/0.78
					LC	21	287	1	—
LESS2D22	$(GA)_{14}N_6(GA)_3AA(GA)_3$	F: TCCGATACGGGAGTCATC	51	FJ161704	SR	17	101–127	13	—
		R: AATTTGTTTTCGTCGTTGC			PAz	21	105–121	7	0.57/0.63
					LC	21	103	1	—
LESS2D25	$(GA)_{28}$	F: TCCGCAAGGCAAGATTCG	54	FJ161705	SR	17	218–279	17	—
		R: GGCGTAGATTTGGTGGGC			PAz	21	220–266	14	0.67/0.88**
					LC	20	220–256	6	0.45/0.42
LESS2D26	$(GA)_{12}AA(GA)_5$	F: AGACCCTCGCATCACCAC	52	FJ161706	SR	17	126–158	8	—
		R: AGCAGTTTTAGACCCATAGC			PAz	21	134–138	3	0.50/0.46
					LC	21	132–136	3	0.43/0.52

$(GA)_{15}$, $(GT)_{15}$ and $(CAA)_{10}$ probes. About 71% (136) of the clones contained a microsatellite region. From these, 42 were correctly sequenced, 17 presented a truly unique sequence, with motifs longer than 11 bp and flanking regions longer than 20 bp. Seventeen primer pairs were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Oligonucleotides were synthesized with an M-13 tail at the 5′-end of the forward primers. Of these 17 loci, only six gave reliable amplifications and five were polymorphic for 17 sampled across the species' range, with 5–19 alleles per locus (Table 1).

In order to increase the number of available loci, a second library was developed by ATG Genetics (Vancouver, BC, Canada). Template DNA was digested by the restriction enzyme *Hae*III and the microsatellite enrichment by biotin capture of dinucleotide (TC_n and GA_n) microsatellites. The first 36 plasmid clones containing a dinucleotide motif were purified and amplified by PCR. Twelve primer pairs were designed using Primer3 and tested in our laboratory, of which four gave reliable amplifications and polymorphic markers with 13–17 alleles per locus (Table 1).

Polymerase chain reaction (PCR) amplification mixtures (12.5 μL) contained 25 ng of template DNA, 0.2 μM of each primer (Table 1), 100 μM of each dNTP, 2 mM of $MgCl_2$, 1.25 μL 10× PCR buffer and 0.5 U *Taq* DNA polymerase (Invitrogen). Cycling conditions consisted of an initial denaturing step of 5 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at the specific temperature (Table 1), 2 min at 72 °C, and a final elongation step at 72 °C for 5 min. PCR products were run in ABI-PRISM 310 (Perkin Elmer) using 500 ROX Size Standard (Applied Biosystems) for allele identification with GeneScan (Applied Biosystems).

Intrapopulation analyses based on 42 individuals from two different sites separated by approximately 1000 km revealed 1–14 alleles per locus and population, showing observed heterozygosities from 0.05 to 0.80 at the polymorphic loci. No departure from Hardy–Weinberg equilibrium was observed in all but one case (Table 1), based on permutation tests implemented in Genetix software (Belkhir *et al*. 1996). However, several loci showed a pattern of diversity dependent of the geographical origin of the samples:

LESS1T11 could not amplify samples from sites located at the south of 30°S, while LESS1D17, LESS2D1 and LESS2D22 were monomorphic in Las Cruces, also located south of 30°S. Finally, linkage equilibrium was rejected for only 2 out of 72 comparisons after Bonferroni correction (Rice 1989), indicating that each loci could be considered as independent markers. These preliminary results indicate that those loci will be useful for investigating the population genetic structure of *L. nigrescens*.

Acknowledgements

The present study is a joint effort under the framework of the LIA-DIAMS. It was supported by FONDECYT Grant 1060493. This work was also carried out as part of ECOS-CONICYT C02B02, FONDAP-FONDECYT 1501-0001 Program 7; Financiamiento Basal Contrato PFB 023 Conicyt Chile and Contrato ICM-P05-002. We thank Claire Daguin for thorough help in primer design and polymorphism assessment of loci from the second bank.

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