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TECHNICAL NOTE

Characterization of polymorphic microsatellites for the periwinkle gastropod, *Littorina littorea* (Linnaeus, 1758) and their cross-amplification in four congeners

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Abstract Eight polymorphic microsatellite loci are described for *Littorina littorea* (Linnaeus, 1758). Data on allelic variation in Irish and Celtic Sea samples are reported. The average number of alleles per locus was 11 (range 4–29), and observed and expected heterozygosities ranged from 6.9 to 84.3% and from 9.4 to 95.2%, respectively. Loci did not deviate from Hardy–Weinberg equilibrium and no linkage disequilibrium between loci pairs was detected. Microsatellites were not highly conserved in the congeners, *L. fabalis*, *L. saxatilis*, *L. compressa* and *L. obtusata* as evidenced by a low rate of cross-amplification. These microsatellites should prove useful in population genetic studies.

Keywords Enrichment · Microsatellite · Gastropod · Littorina · Population genetics · *Littorina littorea*

The periwinkle *Littorina littorea* (Linnaeus, 1758) is a key abundant species in intertidal communities. It is widely distributed in both the eastern and western Atlantic Ocean, and has been the subject of many biological investigations (Reid 1996). *L. littorea* has a planktonic veliger larva with a lengthy duration (4–7 weeks) in the water column (Graham 1988). Thus, larvae are most likely transported between widely separated areas via ocean currents, potentially promoting high levels of gene flow and consequently low levels of population structure. An understanding of the spatiotemporal dynamics and connectivity of populations through larval dispersal is fundamental for effective design of marine reserves and their subsequent management (Sala et al. 2002). Intertidal molluscs with an almost continuous distribution, such as *L. littorea*, are ideal model organisms for population genetic investigations to further our understanding of the relationship between dispersal potential and gene flow in the marine environment. While microsatellite markers are particularly suited to examine geographical patterns and levels of gene flow among populations, to date no such markers are available for *L. littorea*. Although microsatellites have been developed for the congeners, *L. striata* King and Broderip, 1832 (Winnepenninckx and Backeljau 1998), *L. subrotundata* (Carpenter, 1864); (Tie et al. 2000) and *L. saxatilis* (Olivi, 1792) (Sokolov et al. 2002; McInerney pers. comm.), their utilization in *L. littorea* is limited due to a lack of conservation of microsatellite flanking regions and a high incidence of null alleles (McInerney pers. comm.). We describe here the development of polymorphic microsatellite loci designed to *L. littorea* DNA, and the results of cross-species testing in four co-occurring Northeast Atlantic congeners.

Microsatellites were isolated from enriched genomic libraries prepared according to Kijas et al. (1994) using biotinylated oligonucleotides with modifications (details available upon request from P. Prodöhl). Genomic libraries were constructed from the DNA of five individuals. DNA was isolated from 50 mg of the foot muscle using a modified chloroform phenol extraction (Taggart et al. 1992). Briefly, enriched DNA fragments were cloned into pBluescript II phagemid (Stratagene) and transformed into competent cells (XL2-Blue Ultracompetent cells, Stratagene). Some 1,800

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Table 1 Description of microsatellite loci for *Littorina littorea* with PCR conditions and variability

Locus / GenBank Acc. no.	Cloned repeat motif	Primer sequence (5′–3′)	T_m (°C)	N_A	Clone length (bp)	Allele size range (bp)	H_O	H_E
*Llit*1/FJ480214	$(GAA)_8GCAGATAACGACGAAGAGGAACAA(GA)_2G(GA)_2ATCTA(GGA)_4$	[a]F:AGTCTACTCTGTGCCTCCCTCTT R:CAGCTCTAATTGTTGACCTTCCTT	58	10	227	223–250	0.733	0.771
*Llit*57/FJ480215	$(GAA)_4AAAGAAAAAGAAGCA(GAA)_{14}$	[a]F:GATCCGTGGTCAAGCAGGTCA R:ATGGCGGGGTAAAAATCGTCAT	56	16	241	233–281	0.586	0.627
*Llit*61/FJ480216	$(GACA)_2AACA(GACA)_5AAAA(GACA)_5(GA)_4$	F:ACCCGGAGTGCGAAAAT [a]R:CCTTGACGGGATACTGG	51	6	205	197–217	0.228	0.242
*Llit*16/FJ480217	$(GACA)_5CACAGGCAGAAA(GACA)_2AACAGA(GACA)_5CTGA(GACA)_3$	F:GACGTTGGGACTATCGGAACA [a]R:CTGATAGTCCCAATGTCTACACTT	58	10	244	223–249	0.701	0.705
*Llit*55/FJ480218	$(GAA)_2GGC(GGA)_2AGAA(GA)_2TCGAA(GGA)_2AGA(GGA)_2AGAATAA(GGA)_3$	[a]F:CACGGGTACAGAAGCTGGTGAG R:GCCAAATCAGGACCCCTTCC	59	5	260	241–268	0.071	0.093
*Llit*2/FJ480219[b]	$(GGAT)_6CGATGGATCGATGTTT(GGAT)_3GATTAGAT(GGAT)_{10}$	[a]F:GATCCATTGGTCCCGTCTG R:CTGGCTCAGCTGGACCTCA	57	6	213	195–223	0.613	0.722
*Llit*17/FJ480220[b]	$(GA)_{21}(GACA)_{10}$	[a]F:ATGCATATAAGATCAGAGTAGTC R:ACATTAAGTCACGCAAAAC	51	29	209	211–275	0.843	0.952
*Llit*50/FJ480221	$(GGA)_3GGG(GGA)_2GTA(GGA)_2GAAGATGGCACGACAA(GA)_2CAGGGAAG(GA)_5$	[a]F:GCGCTGGCTGCCTACCTGA R:GATCCACACGCCGCTAAGT	56	4	277	255–275	0.069	0.094

GenBank Acc. no., GenBank Accession numbers for the cloned sequences from which the markers were derived

T_m°C annealing temperature

N_A number of alleles

H_O observed heterozygosity

H_E expected heterozygosity

[a] Indicates which of the primer pair is IRD labelled

[b] Indicates a touchdown PCR profile was used

recombinant clones for tri- and tetra-nucleotide microsatellites were screened by southern blot hybridisation with the [P^{32}] endlabelled oligonucleotide probes $(AAT)_5$, $(GGA)_5$, $(AGC)_5$, $(GAA)_5$, $(GACA)_4$ and $(GGAT)_5$ following the protocol described in Prodöhl et al. (1996). A total of 113 positive clones were isolated, purified (Purelink^{TM} HQ mini, Invitrogen), and commercially sequenced (Macrogen Inc., Republic of Korea) in both orientations using T3 and T7 universal primers. Sequences contained microsatellites in 94% ($N = 106$) of cases. Of these, 37 sequences were found to contain suitable flanking regions for primer design. PCR primer sets were designed using PrimerSelect^{TM} (Lasergene 4.0, DNAStar Inc.). Following initial testing, eight PCR primer sets (Table 1) were found to amplify products in the correct size range, and were further optimized for automated genetic screening on a LI-COR 4200 dual-laser automated system (LI-COR Inc., Lincoln, NE) using IRD fluorescently labelled primers. PCRs were undertaken in 12 µl reaction volumes containing 50 ng DNA, 100 µM of dNTPs, 1.5 mM $MgCl_2$, 1× *Taq* DNA polymerase reaction buffer, 1 pMol of each primer and 0.5 U of *Taq* DNA polymerase (Invitrogen). PCR amplifications were performed using a MWG Primus PCR thermal cycler and cycling conditions were as follows: an initial denaturation (94°C for 5 min), followed by 25 cycles of 1 min at 94°C, annealing for 1 min at locus-specific temperature (Table 1) and 1 min at 72°C. Following amplification, 6 µl of stop solution (95% formamide, 10 mM NaOH, 10 mM EDTA, 0.01% pararosaniline) were added to PCR products. PCRs were denatured (94°C for 5 min) and 0.3 µl was loaded and separated on 25 cm, 6% polyacrylamide gels. A commercially available size ladder for the LI-COR system (MicroStep-13b, Microzone) was run adjacent to the samples to size allelic fragments. Gels were run on the LI-COR system at a constant power of 40 W and at a temperature of about 50°C for 2–3 h. Genotyping was carried out with SAGA automated genotyping software (LI-COR Inc., Lincoln, NE).

The eight microsatellite loci were screened against 86 samples collected from Ballyhornan (Irish Sea, Northern Ireland) and League Point (Celtic Sea, Republic of Ireland). The number of alleles per locus for the polymorphic loci ranged from 4 to 29 (average, 11). Expected and observed heterozygosities were calculated in GENETIX 4.03 (Belkhir et al. 2004) and ranged from 0.069 to 0.843 and from 0.094 to 0.952, respectively. Testing for Hardy–Weinberg equilibrium and genotypic disequilibria were carried out using exact tests as implemented in GENEPOP 3.4 (Raymond and Rousset 1995). No loci were found to significantly deviate from Hardy–Weinberg equilibrium within each sample. Similarly there was no evidence of linkage disequilibrium between loci pairs. The majority of the microsatellite repeat motifs were complex (i.e., repeated regions interspaced with short unique nucleotides) (Table 1). Only one of the eight microsatellites developed here consisted of a compound (two adjacent distinct repeat motifs) microsatellite (*Llit*17). Sequence information for an additional monomorphic locus (*Llit*52) was deposited in GenBank (Accession number FJ480222).

We investigated the cross-species amplification of *L. littorea* microsatellite primer sets in four congeners: *L. fabalis* (Turton 1825), *L. obtusata* (Linnaeus 1758), *L. compressa* Jeffreys, 1865 and *L. saxatilis* (Olivi 1972). Results (presented in Table 2) are in agreement with known phylogenetic relationships among *Littorina* (Reid 1996). Thus, no loci amplified in *L. saxatilis*, which is the species least closely related to *L. littorea*. For more closely related species, however, cross-amplification was observed for 1–2 loci in each case (Table 2). Despite the small sample size in this evaluation, more than one allele was observed in two cases suggesting the potential usefulness of these markers.

Table 2 Results of the cross-amplification experiments for the *Littorina littorea* microsatellites tested on *L. saxatilis*, *L. compressa*, *L. fabalis* and *L. obtusata* ($N = 6$)

Locus ID	*Littorina saxatilis*	*Littorina compressa*	*Littorina fabalis*	*Littorina obtusata*
*Llit*1	–	–	–	–
*Llit*57	–	1 (226)	1 (226)	2 (226–229)
*Llit*61	–	–	–	–
*Llit*16	–	–	–	–
*Llit*55	–	–	–	–
*Llit*2	–	–	–	–
*Llit*17	–	3 (203–217)	–	–
*Llit*50	–	–	–	–
Total loci amplified	0	2	1	1

The number of alleles is listed in successful amplification and their size range described in parenthesis. '–' indicates no scorable PCR product

These loci represent the first microsatellites isolated for the model species *L. littorea* and should prove useful in population genetic studies especially to measure varying degrees of gene flow. These marker loci are being implemented in a high-throughput genetic screening study of Irish and Celtic Sea *L. littorea* samples to investigate patterns of gene flow between marine nature reserves and surrounding areas to improve marine reserve design.

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