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TECHNICAL NOTE

Characterization of polymorphic microsatellites for the rough periwinkle gastropod, *Littorina saxatilis* (Olivi, 1792) and their cross-amplification in four congeners

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Abstract Eight new microsatellite loci were characterized for *Littorina saxatilis* (Olivi, 1792) and tested for their cross-hybridization in congeners. All loci were polymorphic in Irish and Celtic Sea samples, with an average number of alleles per locus of 15 (range, 6–31). Observed and expected locus heterozygosities ranged from 26 to 85% and from 53 to 92%, respectively. Three loci showed excess homozygosity and significant departures from Hardy–Weinberg expectations in one sample, possibly due to null alleles, population structuring or inbreeding. No linkage disequilibrium was detected among loci within samples. A high degree of cross-hybridization was observed in closely related congeners and most loci were polymorphic. These markers will be useful for investigating population genetic diversity and connectivity in coastal populations, especially for marine reserve design.

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

The marine gastropod *Littorina saxatilis* (Olivi, 1792), a common member of intertidal communities in the eastern and western Atlantic (Reid 1996), is a useful model system for evolutionary studies (e.g., Johannesson 2003). As an oviviparious brooder, *L. saxatilis* lacks a planktonic larval phase. Gene flow within this widely distributed species is likely to be due to both short geographic scale active migration and long distance passive dispersal, which occurs predominantly by rafting. The effect of rafting has never been fully assessed. For the establishment of marine reserves, it is important to understand levels and patterns of gene flow of representative species, especially for ambiguous dispersers such as *L. saxatilis*. Here we characterize eight *L. saxatilis* microsatellites, and assess their usefulness for genetic studies in four congeners.

Microsatellites were isolated from genomic libraries constructed from the pooled DNA of five individuals. DNA was isolated using a modified phenol chloroform extraction (Taggart et al. 1992). Genomic libraries were enriched using biotinylated oligonucleotides according to Kijas et al. (1994) with modifications (details available upon request from P. Prödohl). Briefly, enriched DNA fragments were cloned into pBluescript II phagemid (Stratagene) and transformed into competent cells (XL2-Blue Ultracompetent cells, Stratagene). Approximately 1,000 colonies were screened for microsatellites 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). Phagemid DNA from 67 positive clones was bi-directionally sequenced (Macrogen Inc., Republic of Korea). A total of 25 PCR primer sets were designed using PrimerSelect™ (Lasergene 4.0, DNAStar Inc.) for clones that had sufficient flanking region. Following initial testing, eight PCR primer sets (Table 1) amplified in the correct size range and were further optimized. PCRs were undertaken in 12 µl reaction volumes containing 50 ng DNA, 100 µM dNTPs, 1.5 mM $MgCl_2$, 1× *Taq* DNA polymerase reaction buffer, 1 pmol of each primer and 0.5 U of *Taq* DNA

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Table 1 Summary description of microsatellite loci for *Littorina saxatilis* with PCR conditions and variability

Locus ID	Genbank Acc. no.	Cloned repeat motif	Primer sequence (5′–3′ end)	T_m (°C)	N_A	Clone length (bp)	Allele size range (bp)	H_O	H_E
*Lsax*17	FJ794781	$(GACA)_6GTCAGACCGTGA(CA)_2(GACA)_2$	F:AATATCCAACGCTCCGAATCAA	55	7	146	196–228	0.360	0.569
			R:ATCCAGCGTCTTACCACCAA[a]						
*Lsax*4	FJ794782	$(CAAA)_5CAGACTGACTTACAGAAAGAA$ $AAACAAATCCGA(CA)_4$	F:AGCCTTTGTCAGTTGGTCA	53	7	278	270–284	0.535	0.745
			R:CGTTGCCCTGCCCTCTTTT[a]						
*Lsax*20	FJ794783	$(GGAT)_8(GGAA)_2$	F:CGAAGCGTTGTAGCGGTTGA	55	9	288	275–303	0.668	0.715
			R:GCACGGGCGTAAGCAGT						
*Lsax*8	FJ794784	$(GA)_4TA(GA)_5TA(GA)_6TA(GA)_7CAGA$ $(GACA)_8GAAAGACA(GA)_4$	F:TGGTGTTGGTCAGATGGAGTG[a]	55	31	210	174–290	0.667	0.871
			R:TTGATGCTGAAGAATTGAAGGAC						
*Lsax*18	FJ794785	$(GAA)_{22}$	F:CTGACAGGCGCCGTTGTATT	55	24	156	133–262	0.852	0.917
			R:GATCTCACACGTAACCAGAAT[a]						
*Lsax*12[b]	FJ794786	$(GA)_5(CAGAGA)_4CA(GACA)_2(GA)_2GTGAGA$ $GGGGAGATAGAG(GA)_5CC(GACA)_7$	F:AAAATGCCGAAGCGACAGT	53	6	187	197–338	0.452	0.531
			R:TGCCGAAAACCAAACCA[a]						
*Lsax*13[b]	FJ794787	$(GAA)_{20}$	F:ACTCCGCGCCACTATAAAGAGCAC[a]	60	16	235	214–301	0.481	0.641
			R:GATCTGCAGCCCGTAGAAAT						
*Lsax*3	FJ794788	$(GAA)_5GAC(GAA)_8(GGAGAA)_6(GAA)_3$	F:CGTCAGCAGAGCAAGGAACAT[a]	53	16	186	296–554	0.261	0.600
			R:AAACGAGCTCACACGTAACCAG						

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 observed; H_O, observed heterozygosity; H_E, expected heterozygosity

[a] IRD labelled primer

[b] Touchdown PCR profile was used

polymerase (Invitrogen). PCR 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 formamide stop solution (95% formamide, 10 mM NaOH, 10 mM EDTA, 0.01% pararosaniline) was added and denatured PCRs were analyzed on 25 cm, 6% polyacrylamide gels mounted in a LI-COR 4200 automated system (LI-COR^{TM} Inc.). Gels were run for 2–3 h at a constant power of 40 W and temperature of about 50°C. A DNA size ladder (MicroStep-13b, Microzone) was ran adjacent to PCR products to accurately size allelic fragments. Genotyping was carried out with SAGA genotyping software (LI-COR^{TM} Inc.). All loci were screened for two samples, one from Cushendall (Irish Sea, Northern Ireland, $N = 46$) and one from League Point (Celtic Sea, Republic of Ireland, $N = 40$). The number of alleles per locus ranged from 6 to 31 with an average of 15. Observed and expected heterozygosities calculated in GENEPOP 3.4 (Raymond and Rousset 1995), ranged between 26–85% and 53–92%, respectively. Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium were tested within each sample in GENEPOP. No linkage disequilibrium was detected between loci pairs. In one sample, three loci (*Lsax*17, *Lsax*3, *Lsax*8) deviated significantly from HWE even after Bonferroni correction for multiple tests. Additional evaluation of these three loci and relevant sample involving testing for heterozygote deficiencies in Genepop, and genotyping errors in MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) showed no evidence for either scoring errors or large allele dropout. There was, however, evidence for both heterozygote deficiencies and the presence of null alleles, albeit at low frequency (<0.19). While an excess of homozygotes can result from null alleles, it can also be attributed to several factors including population structuring, inbreeding due to non-random mating and selection. Preliminary data analysis from an ongoing genetic population survey by the authors and other studies (Bell 2008) indicates that a considerable level of population structure appears to exist within this species, which is characterised by very large effective population sizes. Furthermore an effect of inbreeding due to non-random mating cannot be excluded, as this behaviour has been shown to occur in *L. saxatilis* (Conde-Padín et al. 2008). Additional studies involving larger sample sizes and additional populations are required to disentangle the effects of these possible factors. Null alleles were found to be relatively common for microsatellites previously developed for *L. saxatilis* (e.g., Panova et al. 2008). Even if null alleles are present in certain loci/samples, their presence can be accounted for in population genetic studies given new statistical approaches now implemented in a number of routinely used software (e.g., FreeNA, Structure, Geneland), which reduce the bias associated with them (e.g., Chapuis and Estoup 2007; Falush et al. 2007; Guillot et al. 2008).

We investigated the cross-hybridization of *L. saxatilis* microsatellites in four congeners: *L. fabalis* (Turton, 1825), *L. obtusata* (Linnaeus, 1758), *L. compressa* (Jeffreys, 1865) and *L. littorea* (Linnaeus, 1758). Our results suggest that these loci will be useful to estimate genetic diversity and population connectivity in these species (Table 2). The results are in agreement with known phylogenetic relationships among *Littorina* (Reid 1996). Successful cross-hybridization of loci was observed more in the nearest relatives, and loci proved highly polymorphic ($N_A = 1$–10). Seven loci cross-amplified in *L. saxatilis*' sister-species *L. compressa* and six loci cross-amplified in *L. fabalis* and *L. obtusata*. A lack of conservation of microsatellite primer binding sites tends to increase with increasing genetic distance (Panova et al. 2008; McInerney et al. 2009). Not

Table 2 Summary results of the cross-amplification experiments for the microsatellite marker loci developed for *Littorina saxatilis* tested on *L. compressa*, *L. fabalis*, *L. obtusata* and *L. littorea* ($N = 6$)

Locus ID	*Littorina littorea*	*Littorina compressa*	*Littorina fabalis*	*Littorina obtusata*
*Lsax*17	–	2 (204–212)	1 (218)	2 (196–204)
*Lsax*4	1 (276)	3 (280–284)	–	5 (270–280)
*Lsax*20	9 (184–228)	4 (295–311)	3 (268–280)	–
*Lsax*8	–	3 (178–210)	5 (194–212)	8 (200–236)
*Lsax*18	–	1 (197)	10 (103–261)	5 (247–303)
*Lsax*12	3 (304–316)	–	2 (304–316)	–
*Lsax*13	–	7 (224–322)	3 (238–256)	6 (198–276)
*Lsax*3	–	3 (514–551)	–	3 (503–557)
Total loci amplified	3	7	6	6

The number of alleles is listed in successful amplification and their size range described in parenthesis

'–' indicates no scorable PCR product

surprisingly then given larger genetic differences, just three loci cross-amplified in *L. littorea*, nonetheless they were polymorphic ($N_A = 9$).

The development of these eight highly polymorphic microsatellite loci increases the number of microsatellite markers currently available for genetic and evolutionary studies in *L. saxatilis* (Sokolov et al. 2002). These loci are at present being used to investigate patterns of gene flow between marine nature reserves in Ireland and surrounding areas to improve marine reserve design.

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