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Polymorphic microsatellite loci for *Littorina plena* show no population structure between the eastern and western coasts of Vancouver Island, Canada

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Marine snails of the genus *Littorina* are found on rocky intertidal shores throughout the north Atlantic and north Pacific oceans and comprise 19 species (Reid, 1996). This genus is commonly used for population genetic studies; *Littorina* species typically have separate sexes, moderately short generation times, low adult dispersal rates and large intertidal populations, making them convenient for comparative genetic studies (Berger, 1973; Kyle & Boulding, 2000; Lee & Boulding, 2009).

Microsatellite markers have been developed for several *Littorina* species (Tie, Boulding & Naish, 2000; Sokolov *et al*., 2003; McInerney *et al*., 2009), but none have been developed for *L. plena* (Gould, 1849) or its sister species, *L. scutulata* (Gould, 1849). In this study, primers for microsatellite loci were initially developed from a genomic library for *L. plena* and were subsequently tested for cross species amplification in six other *Littorina* species: *L. scutulata*, *L. littorea* (Linnaeus, 1758), *L. sitkana* Philippi, 1846, *L. subrotundata* (Carpenter, 1864), *L. obtusata* (Linnaeus, 1758) and *L. saxatilis* (Olivi, 1792). Amplification success in related species was expected to decrease with increasing evolutionary distance (Primmer *et al*., 2005; Panova *et al*., 2008). Therefore we expected that *L. plena* microsatellite primers would also amplify loci in its sister species *L. scutulata* (Reid, Rumbak & Thomas, 1996) and possibly in *Littorina* species closely related to that clade.

Population genetic theory predicts that organisms with high dispersal rates will show less population structure or less differenti ation than those with low dispersal rates (Wright, 1943; Hartl & Clark, 1989; Bossart & Prowell, 1998). Several comparative popu lation genetic studies on *Littorina* species with and without a planktotrophic larval stage, using a variety of molecular markers, have supported this theory (Berger, 1973; Snyder & Gooch, 1973; Janson, 1987; Johannesson, Johannesson & Rolán Alvarez, 1993; Kyle & Boulding, 2000; Lee & Boulding, 2009). Like its sister species, *L. scutulata* (Buckland Nicks, Chia & Behrens, 1973), *L. plena* has a planktonic egg capsule and free swimming plankto trophic larval stage of four to six weeks (Hohenlohe, 2002). *Littorina plena* has a larger potential dispersal distance compared with direct developing *Littorina* species (*L. subrotundata*, *L. sitkana*, *L. saxatilis* and *L. obtusata*) that lay benthic egg masses from which hatch crawl away juveniles (reviewed by Reid, 1996).

The purpose of this research was to determine if there is one panmictic population of *L. plena* along the coast of Vancouver Island, British Columbia, or if the coastline is composed of mul tiple populations with little or no gene flow. Kyle & Boulding (2000) detected weak population structure between three sites on southwestern Vancouver Island using mtDNA sequences. On the other hand, Lee & Boulding (2009) did not find population structure among the same three sites using both a single mtDNA sequence and sequences from two nuclear genes. We expected that there might be population structure between the opposite coasts of Vancouver Island, due to the greater distance involved.

Primers were developed by extracting DNA from five live *L. plena* individuals from 5 to 10 mg of tissue from the side of the foot. One individual was extracted using an E.Z.N.A Mollusc DNA extraction kit (Omega Bio tek) according to the manufac turer's directions, and the other four individuals were extracted using the MCIA Extraction Protocol (see Kyle & Boulding, 1998). The DNA was digested using restriction enzyme MboI to provide blunt ends for cloning, and size selected for 250 1,000 base pairs before being purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) according to the manufacturer's directions. *c*. 30 ng/ul DNA was then ligated to an OligoA adapter (Promega); 2 μl of DNA adapter was added to a 20 μl PCR reaction containing 0.625 μM of primer targeting the adapter sequence, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 1× PCR buffer and 0.125 U/μl *Taq* DNA polymerase. PCR was run with an initial denaturing step for 5 min at 95 °C, followed by 30 cycles of denaturing, annealing and polymeriza tion (45 s at 95 °C, 1 min at 58 °C and 1 min at 72 °C, respect ively) with a final extension at 72 °C for 10 min.

600 ng of DNA was hybridized to biotin labelled probe, which targets microsatellite repeats in the genome using Streptavidin MagneSphere Paramagnetic Particles kit (Promega) according to the manufacturer's directions, except for the incubation tempera tures and duration. The mixture was incubated at 37 °C overnight after the addition of the probe to the DNA and, after the addition of the mixture to the bead solution, it was incubated at 43 °C for 3 h.

PCR amplification was again performed using 10 μl of DNA solution and OligoA adapter primer, using the same thermal regime described above. The PCR product was then ligated into P GEM T Easy vector (Promega) and transformed into JM 109 competent cells (Promega) using the manufacturer's Quick Protocol. This procedure was conducted three times, the first

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two enriched for $(GATA)_7$ repeats and the third enriched for $(AC)_8$ repeats.

In order to screen the primers, the primer sequences were extracted from the *Escherichia coli* by PCR using $(GATA)_7$ and oligonucleotide primers for the first two libraries with an anneal ing temperature of 58 °C. The third library was extracted using M13 primers for the PCR with an annealing temperature of 45 °C. SANGER sequencing reactions were performed using the BigDye^{TM} Direct Cycle Sequencing kit (Life Technologies) and the reaction products were then sequenced using a 3730 DNA Analyzer (Applied Biosystems). CLC Workbench 5 (CLC Bio) was used to design primers of lengths 21 27 bp for the flanking regions (Table 1). PCRs were performed to test for primer per formance, using an annealing temperature 2 °C lower than the theoretical melting temperature of the primer. Variability in the microsatellites was tested across several individuals using 5% non denaturing polyacrylamide gel electrophoresis. Of the *c*. 30 devel oped primers, only 5 were polymorphic and scorable in *L. plena*.

Cross species amplification was performed on the six addition al *Littorina* species. DNA was extracted from species that had not been previously extracted for other studies using the Chelex protocol outlined by Lee & Boulding (2009). PCR reactions with a 25 μl final volume were performed with a final concentra tion of 0.5 μM each of forward and reverse primers (Invitrogen) (Table 1), a concentration range of 1.5 2.5 mM of $MgCl_2$, 0.3 mM of each dNTP (Invitrogen), 1X concentration of supplied PCR buffer, 0.125 U/μl *Taq* DNA polymerase (Invitrogen) and 2 μl of DNA template. The thermal regime was the same for each locus, but the annealing temperatures differed between loci and even between individuals of the same species (Table 1). PCR products were run on 5% nondenaturing polyacrylamide gels and gels were visualized using silver staining methods (Naish & Skibinski, 1998). The resulting bands representing the various alleles were scored by comparing with focal individuals and recorded for all individuals at all loci.

To determine population structure of the focal species, *L. plena* was sampled from two sites, each of four locations, on Vancouver Island (August 2011): Willow Point A and Willow Point B (500 m distance) on the east coast (Campbell River) and Nudibranch Point and Prasiola Point (1 km distance) on the west coast (Bamfield) (Fig. 1). At the time of sampling, and also in the laboratory, *L. plena* snails were distinguished from sympatric *Littorina* species using shell shape, penis morphology and tentacle pigmentation patterns (Reid, 1996; Hohenlohe & Boulding, 2001). DNA was extracted and amplified from at least 40 individuals from each of the four location populations using the methods previously described. Of the five primers mentioned above, four were consistently scorable and were used to test for population structure.

GenePop v. 4.1 (Raymond & Rousset, 1995) was used to determine the estimated frequency of null alleles at each of the loci for each population ($\alpha = 0.025$ with a 95% CI). The soft ware Micro Checker (van Oosterhout *et al*., 2004) was used to assess the null allele frequency and to correct the dataset. Observed and expected heterozygosities were calculated with the exact test using the Markov chain methodology in Arlequin v. 3.5 (Excoffier, Laval & Schneider 2005). The F_{SC} and F_{CT} were then calculated using an analysis of molecular variance (AMOVA) in Arlequin. In addition, Arlequin was used to perform an exact test of population differentiation. As this test utilizes genotype frequencies rather than allele frequencies it is useful when loci are not in Hardy Weinberg equilibrium (HWE) (see Supplementary material, Methodology).

All five microsatellite loci that consistently amplified in *L. plena* also amplified in its sister species *L. scutulata* (Table 2). Due to the high amplification success in *L. scutulata*, both in number of alleles amplified and number of individuals ampli fied, expected and observed heterozygosity could be calculated

Table 1. Characteristics of five new di- and tetra-microsatellite loci derived from *Littorina plena*

Locus	NCB accession numbers	Repeat motif	Primer sequence (5′–3′)	T_a	No of alleles	H_E *L plena*	H_E *L scutulata*	Expected size (bp)	Size (bp)
Hunt_2 2 3E	JX000471	$(AG)_4N_2(AG)_3N_5(AG)_3N_{22}(AG)_4$	F TGAATCTTTTGCGTATCGG R CGTGGGTTGTGTATATCTGT	46	11	0 602	0 81*	244	275–300
Hunt_1 2 11Bv2	JX000470	$(GATA)_{11}N_4(GATA)_7$	F ATTGTAATTGCTACTTTGCC R CTATATGAAGCCGAATGTGA	46	27	0 911*	0 91*	156	175–200
Hunt_3 2 2H	JX000473	$(CA)_{21}N_2(CA)_6$	F TAGAAAAGCTATGTGTGGTT R TTCCCTTGTCTTCAGCTAGT	46	21	0 917*	0 93	220	200–225
Hunt_3 2 3H	JX000474	$(CA)_7(CG)_3(CA)_9(TG)_2(CA)_{10}N_{55}(CA)_8$	F TTCCCTTGTCTTCAGCTAGT R TCAGACAGAGACAACAGGTA	46	3	0 540*	0 53	175	165–175
Hunt_3 1 5Bv2	JX000472	$(GT)_3N_2(GT)_{14}$	F CACGTTATATGTCAAAGCAGCA R ACACGTAGATTACCACACTC	45	11	0 822*	0 81*	192	115–145

H_E is the expected heterozygosity estimated from the *L plena* samples collected in August 2011 at Prasiola Point for all loci except for locus 3 2 3H which was estimated form samples collected in August 2007 at Pachena Point and the *L scutulata* samples collected August 2007 at Pachena Point All primers developed by D Hunt

Abbreviations F forward R reverse T_a annealing temperature (°C)

*Significant ($P < 0\ 05$) difference between expected and observed heterozygosities

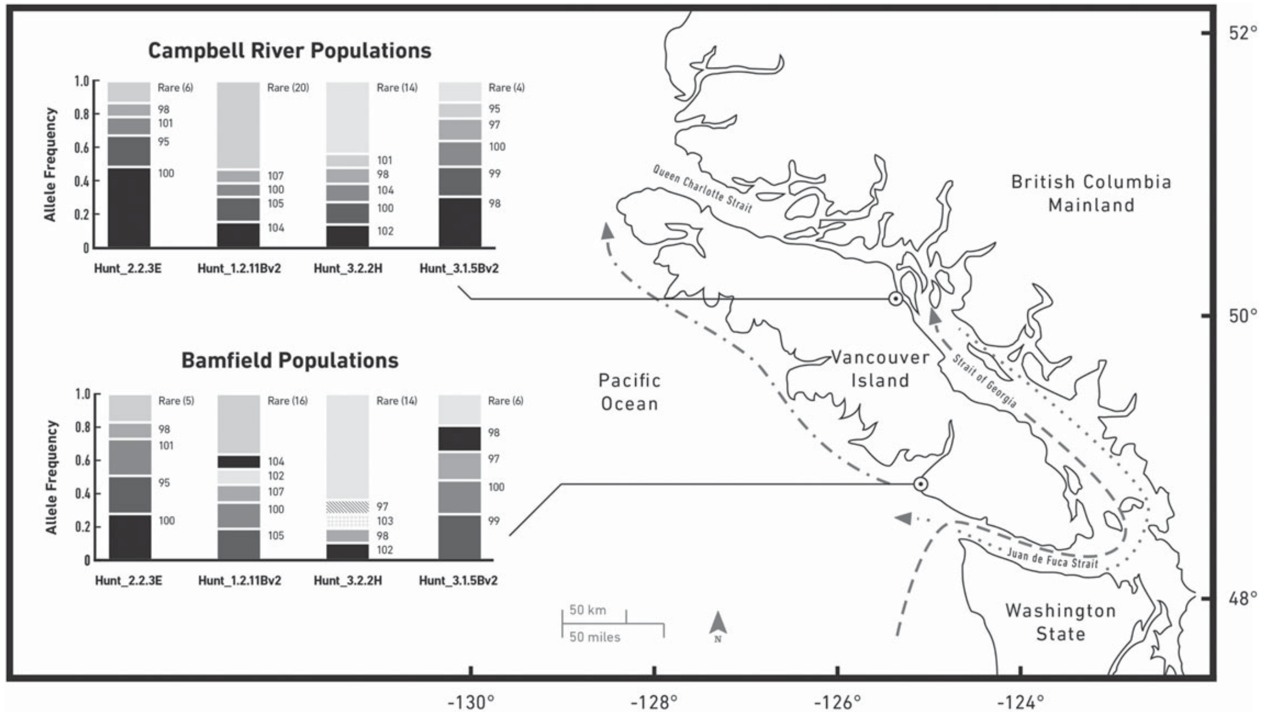

Figure 1. Sampling locations of *Littorina plena* on Vancouver Island, British Columbia, and the allele frequencies of the loci (rare allele cutoff 0.08 or lower). The dashed line represents the incoming tidal current and the dotted line the retreating tidal current. The dotted and dashed line represents the Vancouver Island Coastal Current, which is powered by the freshwater outflow from the Juan de Fuca Strait from mainland rivers such as the Fraser River. There was no structure among populations within the two sites ($F_{SC} = 0.00471$, $P = 0.249$) and no structure between the two populations ($F_{CT} = 0.01389$, $P = 0.328$). Overall there was no genetic difference among the four sites ($F_{ST} = 0.0185$).

(Table 1). Three loci showed statistically significant differences between the expected and observed heterozygosity, suggesting either the presence of null alleles or of scoring errors. Loci 3.2.3H and 3.2.2H did not show deviations from HWE. Additionally, the number of alleles amplified for each locus in *L. scutulata* was similar to that in *L. plena* (Table 1). Three loci amplified in *L. subrotundata* and *L. sitkana*, which are both Pacific species like *L. plena*. Amplification in the Atlantic species had a low success rate and was observed for two polymorphic loci in *L. saxatilis* (Table 2), but only for one monomorphic locus in *L. obtusata* and one monomorphic locus and one polymorphic locus in *L. littorea* (Supplementary material, Table S1).

The results of the cross species amplification study show that the primers developed for *L. plena* can also be used for *L. scutulata*. Heterozygote deficiency is an indicator of the presence of partial null alleles (O'Connell & Wright, 1997), which was observed in *L. scutulata*. Additionally, Panova *et al.* (2008) determined, by using segregation analysis in half sib families, that the perceived heterozygote deficiency in *L. saxatilis* was due to null alleles at the microsatellite loci. There was some amplification success with *L. sitkana* and *L. subrotundata*, both Pacific species, as both species amplified at three of the five microsatellite loci, but this would benefit from further optimization. This supports the idea that relative success should be related to phylogenetic distance.

There were totals of 42 *L. plena* from Prasiola Point, 37 from Nudibranch Point, 49 from Willow Point site A and 38 from Willow Point site B, in which amplification was seen in at least two of the four primers used to determine the presence or absence of population structure (see Supplementary material, Table S2 for allele statistics). Although there was a difference in the most prevalent allele between the populations, there was no large difference in the allele size composition at each locus (Fig. 1). Several of the loci showed significant deviations from HWE (Table 2). In the other three populations this locus deviated from HWE so it is likely with increased sampling that this population would also deviate from HWE. Null Allele Analysis in GenePop v. 4.1 estimated that two of the four loci (1.2.11Bv2 and 3.1.5Bv2) had frequencies of null alleles greater than 0.10. One locus 2.2.3E showed no evidence of null alleles for any of the four populations. Micro Checker found no evidence for scoring error due to stuttering or large allele dropout.

The results of the AMOVA indicated that there were no significant differences between the allele frequencies of the two sites at Bamfield on the western coast or between the two sites at Campbell River on the eastern coast of Vancouver Island ($F_{SC} = 0.00471$, $P = 0.249$). Additionally, there was no evidence of metapopulation structure between the opposite coasts ($F_{CT} = 0.01389$, $P = 0.328$). The pairwise F_{ST} was 0.0185. Reanalysis of the dataset after adjusting allele frequencies for null alleles indicated the same results ($F_{CT} = 0.01144$, $P = 0.737$). For the exact test, only individuals without missing data for any locus were used (as was done by Riquet *et al.*, 2013), lowering the number of individuals in each population to between 21 and 34. For the Willow Point site B population, the number of individuals was too low to include in subsequent analysis ($n = 4$), so it was removed. We think that degraded DNA template was the cause of the high rate of missing data in this population (Dakin & Avise, 2004). The exact test found that there was no pairwise population differentiation between the remaining three populations ($P = 1.0$).

This study found no population genetic structure between the two populations at Campbell River and between the two populations at Bamfield. This was predicted, because the populations are very close, only 500 m apart in the case of Prasiola Point and Nudibranch Point at Bamfield, while 1 km separates Willow Point sites A and B at Campbell River. There was also no population structure between the groups of *L. plena* populations on the eastern and western coasts of Vancouver Island, and there is

Table 2. Percentage of successful amplification for five of the seven *Littorina* species, and the approximate allele size-range in base pairs

Species	Location	Locus D				
		Hunt_2 2 3E	Hunt_1 2 11Bv2	Hunt_3 2 3H	Hunt_3 2 2H	Hunt_3 1 5Bv2
L plena[*,†] ($n = 26$)	Pachena Point 2007	81% (~250–275)	85% (~125–250)	70% (~165–175)	58% (~195–270)	31% (~125–150)
L plena[*,†] ($n = 166$)	4 sites Vancouver sland 2011	85% (~250–275)	92% (~125–250)	n/a	64% (~195–270)	93% (~125–150)
L scutulata[*,†] ($n = 26–40$)	Pachena Point 2007	53% (~240–280)	43% (~125–195)	61% (~160–170)	43% (~200–260)	65% (~120–145)
L sitkana[*,‡] ($n = 20–26$)	Pachena Point 2007	–	60% (~175–220)	46% (~165–170)	–	80% (~125–135)
L subrotundata[*,‡] ($n = 20$)	Pachena Point 2007	–	20% (~185–220)	35% (~160–175)	–	60% (~125–130)
L saxatilis[‡,§] ($n = 8$)	Passamaquoddy Bay 2010	–	–	100% (~250–265)	–	12 5% (~130)

Where the PCR failed it was attempted again by lowering the annealing temperature 2–5 °C and/or by increasing the $MgCl_2$ concentration
Symbols – no amplification
[*]Planktotrophic species
[†]Pacific species
[‡]Direct-developing species
[§]Atlantic species

in fact significant gene flow between these two sites. This is most likely due to the planktotrophic, free swimming larval life history of the species. The F_{ST} value of 0.0185 indicates that only about 2% of the total genetic variation is distributed among the subpopulations, leaving almost 98% of the variation within the subpopulations (Hedrick, 1999). These preliminary results suggest that even with more statistical power from larger sample sizes, more sampling sites and more microsatellite loci, that no significant population structure between these two coasts would be detected. Even if null alleles are present, the allele fre quencies can still be used to determine population structure (Miller & Withler, 1998).

Currents could play a large role in the dispersal of larvae, and therefore genes, between the two coasts (Davenne & Masson, 2001) (Fig. 1). Advection within the fast flow of the currents between Vancouver Island and the mainland would ensure the surface transport of pelagic larvae from the Juan de Fuca Strait into the Strait of Georgia. This tidal current could likely transport larvae from the Bamfield populations to the Campbell River populations as the tides rise and the current moves through the straits, and transport the larvae from Campbell River to Bamfield as the tides move out. Therefore, the most likely reason for the findings of this study is that there is gene flow between the two coasts of Vancouver Island, due to present day tidal and surface currents acting on the pelagic larvae of *L. plena*.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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