

probably due to supercoiling phenomena of the DNA double helix.

We conclude that for samples critical for DNA extraction, drowning must not be used as a method of relaxation and that cooling the samples during drowning does not improve the conditions. For studies that require material for both histological/dissection and molecular purposes, we suggest that a tiny piece of tissue is cut from the animals prior to the drowning and placed in 10–20 vols of alcohol or is frozen. For molecular studies only very little tissue is usually needed (1–3 mm<sup>3</sup>).

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## Microscale genetic differentiation along the vertical shore gradient in White Sea snails *Littorina saxatilis* (Olivi) assessed by microsatellite markers

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The intertidal zone represents a unique type of habitat, where several important environmental characteristics (especially temperature and degree of aerial exposure) change concordantly and drastically over a small spatial scale along the vertical shore gradient. Studies on a model gastropod, *Littorina saxatilis* (Olivi), have demonstrated considerable morphological, physiological and genetic differentiation in populations of this species along this gradient.<sup>1–7</sup> Selection has been suggested to play an important role in genetic differentiation of *L. saxatilis*, leading to significant differences at a few (presumably selectively important) loci between high and low shore subpopulations despite high genetic homogeneity for most other (presumably neutral) loci.<sup>3,4,8,9</sup> Stable physiological<sup>5–7</sup> and heritable morphological differences<sup>10,11</sup> between high and low shore subpopulations of *L. saxatilis* are consistent with adaptative responses to differential selection at different shore levels. In some populations, partial reproductive isolation has been observed between high and low-shore ecotypes of *L. saxatilis*, suggesting possible speciation *in statu nascendi*.<sup>12,13</sup> All these findings have firmly established high and low-shore ecotypes of *L. saxatilis* as a valuable model-system for the study of the roles of selection and gene flow in phenotypic differentiation and microevolution. Study of fine-scale metapopulation structure and microevolution in this species requires further development of high-resolution genetic markers, which might be useful in revealing the genetic differentiation between closely related groups such as subpopulations of *L. saxatilis*.<sup>14</sup> In this study, we use microsatellite DNA markers for the first time in this model organism. Using this technique, we examined microscale genetic differentiation along the vertical gradient of the intertidal zone in a White Sea

population of *L. saxatilis*. In order to test whether genetic differentiation along the vertical shore gradient is higher than would be expected from the distance alone, we assayed the variability in allele frequencies for five microsatellite loci in *L. saxatilis* from high and low shore levels, and between equidistant subpopulations within the same shore level.

Animals were collected in the intertidal zone of the Kandalaksha Bay of the White Sea (66°20'N 33°39'E) in September 1999 from two contrasting shore levels: (1) low shore, from small stones and gravel patches in the low intertidal, within the zone of brown macrophytes (*Ascophyllum nodosum* and *Fucus vesiculosus*), at the lower limit of distribution of *L. saxatilis*; (2) high shore, in the narrow (50 cm) belt along the upper limit of distribution of *L. saxatilis* on large isolated rocks. From each shore level, two samples were collected, such that the four studied groups (2 shore levels × 2 replicates) were separated by a distance of 12–17 m from each other. It should be noted that this distance is potentially within the migration range of *L. saxatilis*.<sup>1,5</sup> For the sake of clarity, the two studied sites, each including a high and a low shore group, will be referred to as localities A and B, and the minimum sampling unit (a sample from a given shore level at a given locality) as a subpopulation. The distance of 12–17 m was chosen because this was close to the maximum distance (15 m) between the upper and lower limits of the distribution of *L. saxatilis* in the intertidal zone at the study site. This sampling design allowed us to discriminate between the effects of distance *per se* and shore level on microscale genetic differentiation in this species.

For each sample, animals were collected from a patch of 0.75 m<sup>2</sup> (50 × 150 cm). Only adult animals (4–6 years old,<sup>15</sup> shell diameter 5–7 mm) were used in the subsequent analysis. In the laboratory, snails were dissected and checked for trematode

infestation under the binocular microscope. Infested snails were discarded and the foot muscles of uninfested snails (20–50 mg) were cut into small pieces (*c.* 1 mm<sup>3</sup>), fixed individually in 1 ml of 20% dimethyl sulfoxide and 0.25 M EDTA in saturated NaCl solution<sup>16</sup> and stored at 4°C for 1–3 months until DNA extraction. We avoided snails infected with trematodes in this study because some trematode species are known to change the behaviour of *L. saxatilis*, leading to the concentration of infested individuals on open stone and rock surfaces<sup>15</sup>. Hence, some of the infested individuals found on the open rock surfaces on high shore levels might not be resident in the high shore subpopulations, but rather migrants from the low shore. Besides, irreversible castration of *L. saxatilis* by most trematode species found at the study site excludes infected specimen from reproduction, so that they do not contribute to gene flow.<sup>15</sup> Due to the low population density and high infection rates of *L. saxatilis* in the high intertidal levels, the maximum sample size was 25 individuals.

DNA was extracted and purified according to the protocol described elsewhere.<sup>17</sup> Individual genotypes were assessed using five microsatellite loci: Lx-12, Lx-14, Lx-18, Lx-23 and Lx20CAA.<sup>14</sup> All five studied loci are trinucleotide tandem-repeats with CAA repeat units.

Amplification of microsatellites was performed in a final reaction volume of 15 µl, containing 10 mM Tris-HCl (pH = 8.3 at room temperature), 50 mM KCl, 0.01% (w/v) gelatine, 0.5 U of the Taq DNA polymerase (Life Technologies, Karlsruhe, Germany), 1.5 mM MgCl<sub>2</sub>, 50 µM of each dNTP, 0.02 µM of each primer and 20–40 ng of genomic DNA. The forward primer was end-labelled with [<sup>32</sup>P]-ATP. Cycling parameters were: 5 min at 95°C, followed by 35 cycles of 40 s at 95°C, 1 min at the optimal annealing temperature for each pair of primers<sup>14</sup> and 35 s at 72°C, and finally, a 5-min extension step at 72°C. PCR products were resolved on 6% polyacrylamide gels under denaturing conditions and visualized by autoradiography. Allele sizes were determined by comparison with a sequencing size-standard obtained from the bacteriophage M13mp18 (Amersham Pharmacia Biotech, USA).

The studied microsatellite loci were moderately to highly polymorphic, with 17–27 alleles per locus. Three of the five studied loci (Lx20CAA, Lx-14 and Lx-23) displayed high variability in allele length, with most alleles being 150–300 bp long and a few rare alleles up to 500–750 bp long. The long alleles (>500 bp) differing by less than 20 bp were combined as a single ‘allele’, due to the difficulties with the assessment of their exact length. At the Lx-23 locus, singular alleles were found, which differed from others by uneven numbers of repeats, presumably due to point mutations. These rare alleles were combined with the nearest ‘even’ allele.

For each subpopulation group, we quantified allelic frequencies, the mean number of alleles per locus ( $n_a$ ), the observed heterozygosity ( $H_{obs}$ ) and gene diversity ( $H_e$ ).<sup>18</sup> Tests for linkage disequilibria were calculated using Arlequin version 2.000 software<sup>19</sup> by means of the permutation test with 5000 permutations and 100 initial conditions for the Expectation-Maximization (EM) algorithm. Deviations from Hardy-Weinberg expectations at each locus were tested using Fisher’s exact test.<sup>20</sup> Obtained probability levels for linkage disequilibria and Hardy-Weinberg expectations were corrected for multiple comparisons using the sequential Bonferroni procedure.<sup>20</sup>

The genetic differentiation among subpopulations was analysed by two complementary approaches: (1) a genetic distance  $\rho$ , which is an unbiased estimator of Slatkin’s  $R_{st}$ , which assumes applicability of the SMM mutation model and is calculated based on the squared differences in allele size;<sup>21</sup> (2)  $F_{ST}$ , which considers all alleles as mutationally equidistant from each other (IAM mutation model) and is calculated based on the number of different alleles.<sup>19</sup> There is an ongoing debate con-

cerning which model better describes the evolution of microsatellites in animal populations, and simulations and case studies suggest that performance of different estimators of genetic differentiation strongly depend on the sample size, the evolutionary time of divergence and the demographic history of the populations.<sup>22–25</sup> Slatkin’s  $R_{st}$  was estimated using standardized allele lengths,<sup>21</sup> where the allele lengths were expressed in terms of standard deviations from the global mean, rather than repeat unit number, in order to eliminate the bias due to the presence of loci with different variability.<sup>21</sup> Estimates of gene flow ( $Nm$ ) were calculated as described by Slatkin.<sup>26</sup> Due to the small number of subpopulations, AMOVA could not be used to test genetic differentiation between different shore levels. Instead, pairwise comparisons of  $F_{ST}$  and  $R_{st}$  between subpopulations were performed.<sup>19</sup> Because the gametic phase was unknown in our samples and genotype frequencies deviated from Hardy-Weinberg equilibrium (making maximum-likelihood estimates of haplotype frequencies implausible), probabilities of  $F_{ST}$  and  $R_{st}$  were tested by permuting individual genotypes, rather than haplotypes among subpopulations.<sup>19</sup>

Our study showed that all analysed microsatellite loci were moderately to highly polymorphic in the studied population of *L. saxatilis*. The mean number of alleles per locus varied between 11.0 and 14.0, and did not differ significantly in the four studied subpopulations ( $P > 0.05$ ). The average gene diversity across the five studied loci was 0.60–0.76 in high-shore subpopulations and 0.78–0.79 in the low-shore ones. The expected heterozygosity ( $H_e$ ) for different loci varied between 0.54 and 0.93 (Table 1). Two loci (Lx-14 and Lx-23) showed a close agreement with Hardy-Weinberg expectations with respect to heterozygosity, whereas in the three remaining loci a significant heterozygote deficiency was observed in at least some of the compared groups (Table 1).

**Table 1.** Observed and expected heterozygosities for five studied microsatellite loci in White Sea *Littorina saxatilis* from different shore levels.

Locus	Subpopulation	$H_e$	$H_{obs}$	$P$
Lx-18	High A	0.7008	0.2632	0.0217
	Low A	0.5485	0.1579	0.0382
	High B	0.5368	0.2800	0.0741
Lx-12	Low B	0.6053	0.0526	0.0011*
	High A	0.8824	0.3529	0.039
	Low A	0.9113	0.4000	0.0022*
	High B	0.9304	0.5200	0.036
Lx-14	Low B	0.8767	0.3684	0.0019*
	High A	0.9180	0.8750	0.6129
	Low A	0.9100	0.9474	1.0000
	High B	0.8878	0.3810	0.0036
Lx-23	Low B	0.8740	0.8421	1.0000
	High A	0.8643	0.8421	1.0000
	Low A	0.8725	0.7500	0.6948
	High B	0.8848	0.7600	0.4635
Lx20CAA	Low B	0.8843	0.7778	0.6581
	High A	0.8542	0.5000	0.1930
	Low A	0.7922	0.2632	0.0029*
	High B	0.8922	0.3478	0.0007*
	Low B	0.8633	0.2941	0.0013*

$H_e$ , expected heterozygosity for each locus that was calculated from the observed allele frequencies under the assumption of the Hardy-Weinberg equilibrium;  $H_{obs}$ , observed heterozygosity calculated as the frequency of heterozygotes at each locus.

Probabilities ( $P$ ) for  $H_e = H_{obs}$  were calculated using the Fisher exact test. Asterisks mark the probabilities when deviations from the Hardy-Weinberg expectations were significant at the 5% level after Bonferroni corrections.  $N = 19–25$  for different subpopulations.

The permutation test using the EM algorithm showed that in low-shore subpopulations Low A and Low B, all five studied loci were in the linkage equilibrium ( $P > 0.05$ ). In contrast, in the high shore groups High A and High B a significant linkage disequilibrium was found. In the High A subpopulation, locus Lx-12 was at a linkage disequilibrium with loci Lx-14 and Lsax20CAA ( $P < 0.001$ ). In the High B group, a significant linkage disequilibrium was observed between locus Lsax20CAA and all other loci, as well as between loci Lx-18 and Lx-23 ( $P < 0.001$ ).

Estimates of genetic distance  $\rho^{21}$  based on the SMM model revealed a higher degree of genetic differentiation between subpopulations from different shore levels (0.023–0.048) as compared to subpopulations from different localities within shore levels (0.007–0.008) (Table 2). Significant genetic differentiation was observed between high- and low-shore subpopulations at locality B, but not at locality A, probably due to the small sample size and associated low power of analysis (Table 2). Estimates of genetic distance based on the IAM model ( $F_{ST}$  in Table 2) gave qualitatively similar results. Gene flow ( $Nm$ ) estimates were 3–6 times higher for subpopulations from the same shore level (31.7–37.5) as compared to subpopulations from different shore levels from the same locality ( $Nm = 4.9$ –10.4; Table 3).

Heterozygote deficiency, such as that observed for some microsatellite loci in the studied population of *L. saxatilis*, is a well-known phenomenon of genetic structure of marine bivalves and freshwater gastropods, the origin of which remains unknown.<sup>27–31</sup> The possible explanations for this phenomenon generally fall into four categories, including a selective advantage of homozygotes, a non-detection of the part of polymorphism due to aneuploidy or null alleles, inbreeding and the Wahlund effect. In the present study, the Wahlund effect seems to be a less plausible explanation, as the animals from each group (subpopulation) were collected from a small area (0.75 m<sup>2</sup>) and were thus unlikely to belong to different panmictic units. However, our samples included animals of different age classes between 4 and 6 years of age; hence, a temporal Wahlund effect (mixing of successive genetically different cohorts) cannot be excluded.<sup>32</sup> In a species with an ovoviparous mode

of reproduction and limited dispersal, such as *L. saxatilis*, inbreeding might explain the excess of homozygous individuals. The effects of inbreeding may be especially pronounced, as the samples collected over the small area are likely to include close relatives. However, inbreeding effects should have affected all the neutral loci equally, whereas in the present study only three of the five microsatellite loci demonstrated significant excess of homozygotes. Alternatively, heterozygote deficiency for some microsatellite loci in *L. saxatilis* may have resulted from the presence of null alleles at those loci. A test for the possibility of null alleles would involve controlled mating experiments to allow pedigree analysis. In an extensive study of 24 microsatellite loci of *M. edulis* it has been shown that 16 loci contained null alleles and that null alleles segregated in the offspring according to Mendelian expectations.<sup>33</sup> Using the formula<sup>34</sup>  $r = D / (2 - D)$ , where  $r$  is the estimated frequency of the null allele and  $D$  is heterozygote deficiency, we obtain expected frequency of null alleles in the range of 0.25–0.39 for the loci demonstrating significant heterozygote deficiency in our samples. This would imply expected frequencies of homozygotes for null alleles of 0.07–0.16. However, in our study no homozygotes for null alleles (i.e. failed amplifications) were observed, suggesting that the presence of null alleles could not be the only explanation for the observed deviation from Hardy-Weinberg equilibrium in the studied subpopulations of *L. saxatilis*. In general, the factors resulting in the excess of homozygotes for some microsatellite loci in the studied *L. saxatilis* population require further investigation.

It is interesting to note that, while none of the five studied loci were associated in the low-shore subpopulations of *L. saxatilis*, there was a significant linkage disequilibrium between at least some of the microsatellite loci in the high-shore groups. Epistatic interactions in fitness between the linked microsatellite loci (or the closely linked loci upon which the microsatellite loci ‘hitch-hiked’) are an unlikely explanation for the observed associations, because different loci are at disequilibrium in different subpopulations. Possibly, linkage disequilibrium in the high-shore subpopulations of *L. saxatilis* may be a result of frequent bottleneck events, which occur due to unpredictable and severe environmental conditions at high-shore levels. Thus, field observations have indicated that in White Sea populations of *L. saxatilis*, high mortality (up to 36%) occurs during summer neap tides at high-shore levels<sup>5</sup> suggesting that high-shore subpopulations may frequently experience severe declines. Repopulation of high-shore levels either due to migration from low shore or due to reproduction of the few local survivors would result in linkage disequilibrium between some loci.<sup>35,36</sup>

Our data showed that the genetic structure of *L. saxatilis* populations was heterogeneous over distances as small as 12–17 m. On this small spatial scale, the genetic distance was considerably higher between subpopulations of *L. saxatilis* from different shore levels as compared to groups within the same shore level and did not correlate with the physical distance between the studied groups. Gene flow between different subpopulations of *L. saxatilis* from the same shore level was very high ( $Nm$  of *c.* 30–37) indicating genetic homogeneity of these groups, and was 3–6 times higher than between shore levels. Although the distance of 12–17 m is considered to be within an average migration range of *L. saxatilis* as estimated by direct mark-recapture methods,<sup>1</sup> behavioural experiments have demonstrated that while snails move readily within their shore levels, migration between shore levels is restricted.<sup>37</sup> Moreover, experimental transfers of *L. saxatilis* between shore levels have shown a directional migration of the snails towards their original shore level, so that they regain their vertical position in the intertidal within a few days after the experimental transfer.<sup>37</sup> Our genetic data agree with these behavioural observations and indicate that gene flow may be restricted between different shore levels in

**Table 2.** Pairwise genetic distances  $R_{st}$  and  $F_{ST}$  between and within habitats (= shore levels) in White Sea *Littorina saxatilis*.

	High A	Low A	High B	Low B
High A		–0.00030	0.01607	0.01208
Low A	0.02339		0.03623*	0.01508
High B	0.00662	0.04927*		0.02665*
Low B	0.02516	0.00783	0.04854*	

The values of  $R_{st}$  are given below the diagonal of the table, and the estimates of  $F_{ST}$  above the diagonal.  $R_{st}$  estimates are based on the standardized allele lengths. Significant values ( $P < 0.05$ ) are marked with asterisks.

**Table 3.** Estimates of gene flow ( $Nm$ ) between and within habitats (= shore levels) in White Sea *Littorina saxatilis*.

	Low A	High B	Low B
High A	10.437	37.511	9.688
Low A		4.824*	31.689
High B			4.901*

Estimates are based on the standardized allele lengths.  $Nm$  values associated with significant genetic differentiation as estimated by  $R_{st}$  and  $F_{ST}$  ( $P < 0.05$ ) are marked with asterisks.

## RESEARCH NOTES

*L. saxatilis* populations, thus meeting a prerequisite for local genetic adaptation in different microhabitats. Interestingly, restricted gene flow due to the assorted mating of high- and low- or middle-shore morphotypes has previously been demonstrated in some *L. saxatilis* populations in Spain<sup>12</sup> and Britain.<sup>13</sup>

As a corollary, our data on a White Sea population of *L. saxatilis* demonstrate the utility of highly variable microsatellite markers in the analysis of fine metapopulation structure and population genetic processes on small spatial scales, such as between different shore levels. In this population of *L. saxatilis*, significant genetic differentiation was observed between high- and low-shore subpopulations. We interpret this differentiation as a result of the restricted gene flow between subpopulations from different shore levels, based on behavioural studies in this population<sup>37</sup> and selective neutrality of most variation in microsatellite loci studied so far.<sup>38</sup> However, further research is required to test the latter assumption and to establish unequivocally the value of the studied microsatellite loci for estimation of gene flow in *L. saxatilis*.

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