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ALLOZYME VARIATION IN A SNAIL (*LITTORINA SAXATILIS*)—DECONFOUNDING THE EFFECTS OF MICROHABITAT AND GENE FLOW

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Abstract.—It is commonly observed that a restricted gene flow among populations of a species generates genetic differentiation in, for example, allozyme markers. However, recent studies suggest that microhabitat-specific variation may contribute to the total differentiation. To appreciate the relative contributions of geographic variation and habitat-specific variation, we sampled 42 subpopulations of the intertidal snail *Littorina saxatilis* from three different microhabitats (boulders, low and high rocky intertidal) on five small islands within a distance of 15 km. We used a modified orthogonal version of Nei's gene diversity analysis with a modified analysis of variance (ANOVA) that estimated the significance of habitat and geographic separation and the interaction between them. Between subpopulation differentiation (G_{ST}) was usually in the range of 5% to 10% but was exceptionally high in one locus (*Aat*; 53%). Genetic differentiation attributable to different habitats accounted for 10% to 81% (mean 35%) of the between subpopulation variation and was significant ($P < 0.05$) in six loci. Differentiation due to geographic separation accounted for 11% to 61% (mean 36%) and was significant ($P < 0.05$) in seven loci. Furthermore, three loci showed interactions between habitat and island, suggesting varying effects of habitat in different islands. Microhabitat-specific variation, probably through spatially varying fitness, seems particularly likely in *Aat* and *Pgm*-2. Moderate levels of habitat associated variation added to the observed differentiation due to gene flow in *Pgi*, *Pnp*, and *Pgm*-1, whereas in the remaining three loci either the habitat effect was confused by strong habitat-island interaction (*Ark*) or was virtually absent (*Pep* and *Mpi*).

Key words.—Analysis of variance, balancing selection, gene diversity analysis, genetic differentiation, habitat-specific variation, heterogeneous environment, neutral variation.

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Numerous morphological characters of species show habitat-specific variation that is most likely maintained through spatially varying selection in heterogeneous habitats (Endler 1986). This is particularly true for direct developing intertidal snails, in which extensive habitat-associated morphological variation has confused taxonomists for a century (review by Reid 1996). In contrast, neutral or near neutral variation is still mostly the null hypothesis when patterns of allozyme polymorphisms are interpreted (Avise 1994, p. 28). Indeed some authors suggest nonneutral variation plays but an insignificant role in generating and maintaining the patterns of allozyme variation found in nature (e.g., Thorpe and Solecava 1994; Eanes 1987; Kimura 1991). Others, however, emphasize the potential role of selection and warn against the use of strict neutral variation as a general assumption (Avise 1994, p. 231; Hedrick et al. 1976; Hedrick 1986; Kreitman and Akashi 1995).

Earlier studies suggest gene flow as the main determinant of genetic differentiation among populations of a species at various scales (e.g., Janson 1987; Waples 1987; Hellberg 1995, 1996) although historic changes in gene flow may sometimes be important (Hellberg 1994). However, studies of intraspecific genetic variation over heterogeneous habitats suggest habitat-specific variation at local or even microscales (Hickey and McLean 1980; Nevo et al. 1986; Johannesson and Johannesson 1989; Carvalho 1989; Day 1990; Nevo et al. 1994; Tatarenkov and Johannesson 1994; Prentice et al. 1995).

A dilemma is to separate the effects of habitat and gene flow. *F*-statistics and Nei's gene diversity analysis are obviously suitable for the analysis of either factor separately. If, however, effects of habitat and geographic separation are acting simultaneously, it will not be possible to estimate any of the two factors properly by the conventional analyses. If, for example, samples are taken from different geographic areas and in each area from different habitat types, geographic separation and habitat are confounded factors. A hierarchical design is not appropriate in this case because different habitats will be nested under each island, and differences between habitats within islands will add to a total variation at this level even if they are of opposite directions.

A possible way to escape from this dilemma is to run two separate hierarchical analyses, one analyzing the geographical separation and the other emphasizing habitat variation (see e.g., Johannesson and Johannesson 1990). With this design, however, the two factors (habitat and geographical separation) are not maximally resolved because interactions between the two factors cannot be unveiled.

To solve this problem, we used an orthogonal modification of Nei's gene diversity analysis, which separates effects of habitat-specific variation and gene flow. With this method, we were also able to evaluate the interactions between these two factors. Our model organism is a common Atlantic species of snail, *Littorina saxatilis*. This intertidal species experiences a range of different microhabitats from salt marches, boulder fields, and smooth rocky shores (Reid 1996).

In this study, we analyzed the genetic structure of eight highly polymorphic allozyme loci in populations from five small islands of the same area. On each island, we sampled snails living in three different habitats. The aim of our study was to test the hypothesis that habitat-specific variation con-

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TABLE 1. Distribution of samples from five islands (in order from northwest to southeast) and three habitats in each island (B = boulder, L = low rocky intertidal, H = high rocky intertidal). All sites except URS boulder are from shores exposed to moderate to strong wave action. All distances are in meters.

Island	No. of replicates B + L + H	Mean distance between replicates of same habitat	Mean distance between B and L/H	Mean distance between L and H	Minimum distance between L and H
Jutholmen (JUT)	2 + 2 + 2	36	80	24	6
Burholmen (BUR)	2 + 2 + 2	13	25	7	2.5
Ursholmen (URS)	2 + 3 + 3	50	>300	43	10
Arsklovet (ARS)	2 + 2 + 3	43	>50	30	6
Svangen (SVA)	2 + 2 + 2	100	100	72	6

tributed significantly to the local genetic structure found in this species, and to resolve the relative contributions of microhabitat and gene flow and their interaction. Indeed, we found significant effects of microhabitat in a number of the analyzed loci, as well as effects of gene flow and interactions between habitat and gene flow. Thus, we conclude that both factors are very important in this species, at least in the study area, and therefore the orthogonal analysis seems particularly relevant in this case.

MATERIALS AND METHODS

Sampling Design

We sampled *L. saxatilis* from five islands within 15 km of each other in the Koster-Tjärnö archipelago on the west coast of Sweden. (The islands, except Arsklovet, are all in the map of Tatarenkov and Johannesson 1994, Arsklovet is 3.5 km north Svangen.) On each island, we sampled two to three replicate samples of three different habitats (boulders, low rocky intertidal and high rocky intertidal). Samples from boulders were all from mean tidal level and, except in one island (Ursholmen), from boulder shores exposed to wave action. In rocky shores, we sampled at about mean tidal level ("low rocky intertidal"), and at the uppermost level of the intertidal zone ("high rocky intertidal"). The tidal range in Sweden is no more than 0.3 m, and the level of the upper intertidal is set by a combination of topography and wave action. The sampled rocky shores were all exposed to strong waves; and, therefore, the high intertidal sites were at vertical levels of one to four meters above the low sites. The samples of the same islands were all from continuous populations. The distances between habitats and between replicates of the same habitat ranged between 2.5 to 300 meters (Table 1).

In two of these five islands we added samples from rock pools (unreplicated) in the high intertidal at vertical levels of three to four meters. We discuss the allozyme frequencies of these pools in the text, but they were not included in the analysis of gene diversity.

Electrophoresis

Snails were kept at −70°C until electrophoresis. We used horizontal starch-gel electrophoresis (methods as described in Tatarenkov and Johannesson 1994) to reveal variation in eight polymorphic loci (arginine kinase, *Ark*, 2.7.3.3; aspartate aminotransferase, *Aat*, 2.6.1.1; mannose-6-phosphate isomerase, *Mpi*, 5.3.1.8; peptidase (gly-leu), *Pep*, 3.4.—.—; phosphoglucomutase, *Pgm*-1 and *Pgm*-2, 5.4.2.2; phosphoglucose isomerase, *Pgi*, 5.3.1.9; purine-nucleoside phosphorylase, *Pnp*, 2.4.2.1). These loci were chosen because they are among the most variable ones from earlier allozyme studies of *L. saxatilis* (Janson and Ward 1984; Ward et al. 1991), and this was confirmed in a pilot study.

Statistics

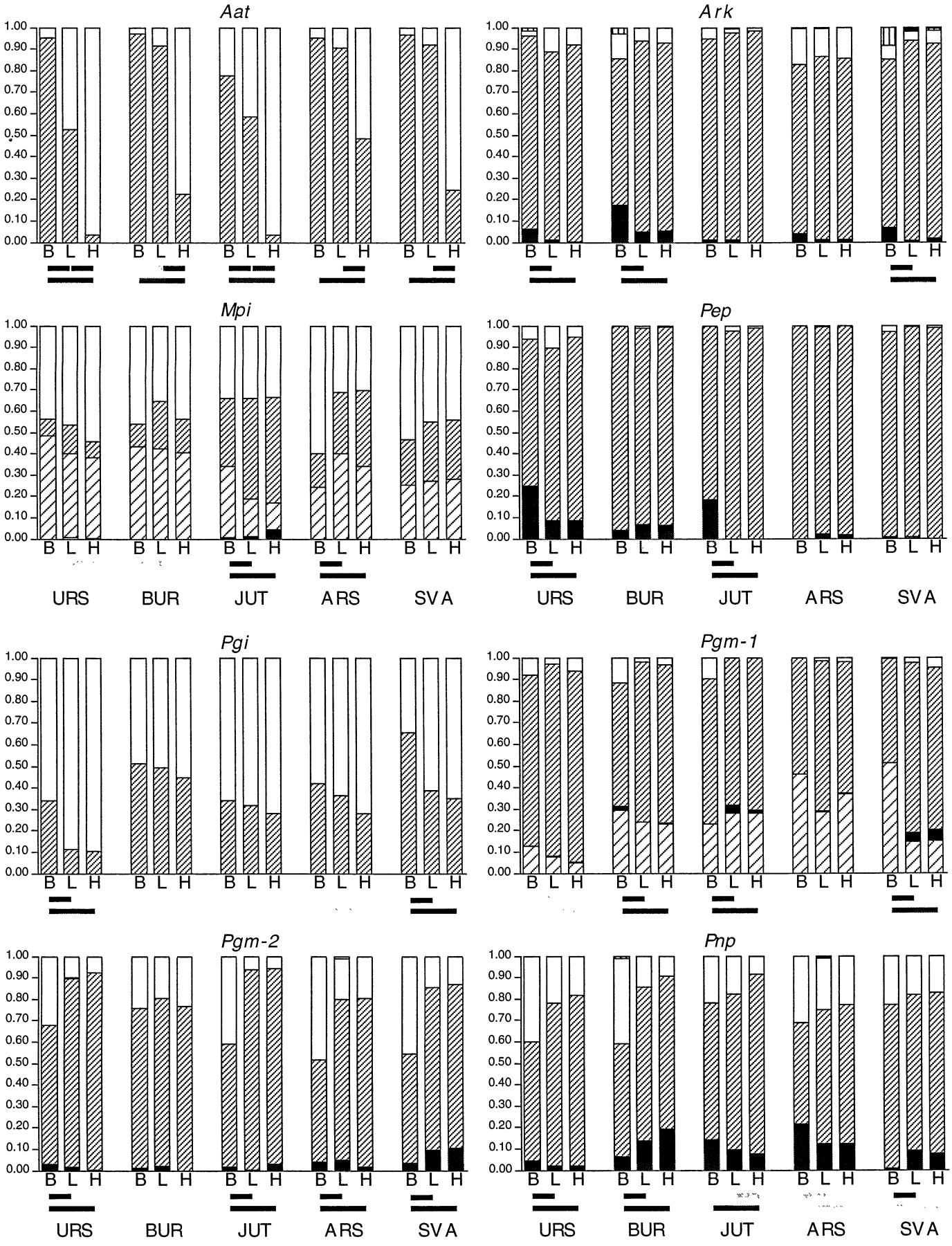
In each sample, genotype distributions were analyzed for consistency with Hardy-Weinberg expectations. We used conventional χ^2 tests with pooling of rare alleles when expected numbers of a genotype were less than four. In addition, we ran a pseudoprobability test (Hernández and Weir 1989) with the program CHIHW (Zaykin and Pudovkin 1993). To compensate for the high number of tests performed, we corrected the obtained probability estimates for multiple testing using Sidák's multiplicative inequality (Sokal and Rohlf 1981, p. 728) with the program MULTTEST (Zaykin and Pudovkin 1991).

We looked for associations between loci using a χ^2 contingency test with pooled alleles and a pseudoprobability test with unpooled alleles. Expected numbers of genotype combinations were derived from marginal totals (Spiess 1977, p. 128). This test shows whether the observed frequencies of the di-locus genotype combinations correspond to those expected from the single-locus genotype frequencies (rather than if they fit to gamete frequency equilibrium). We chose this method because it is not confused by deviation from Hardy-Weinberg equilibrium in one or both of the contributing loci.

Gene diversity analysis as originally designed (Nei 1973; Chakraborty 1980) has a hierarchical structure and thus total diversity may be decomposed in differentiation among regions, among localities within regions, among sites within localities, and so forth. With a nested sampling scheme this analysis is fully adequate. Nested sampling cannot, however, be used for the simultaneous study of habitat and isolation effects (see introduction). This requires an orthogonal design (e.g., Underwood 1981). Our sampling scheme included different islands, different habitats within each island, and replicate samples of each habitat. We developed a modified version of the original gene diversity analysis, analogous to an orthogonal ANOVA model (see Appendix 1),

$$H_T = H_S + D_{IT} + D_{HT} + D_{H\times I} + D_{SHI} \qquad (1)$$

in which H_T is total variation, H_S variation within samples, D_{IT} differentiation among islands, D_{HT} differentiation among habitats, $D_{H\times I}$ is an interaction term, which will be significant if there are differences among habitats of islands, but these differences are of different magnitudes or of opposite directions. D_{SHI} is differentiation among replicate samples of the same habitat and of the same island. The D-values are algebraically related to the sum of squares of all alleles for a given factor or interaction of factors (or for a corresponding level of subdivision)


Aat
Ark
Mpi
Pep
Pgi
Pgm-1
Pgm-2
Pnp
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
B L H
URS
BUR
JUT
ARS
SVA

$$D = \left(\sum_{i=1}^{a} SS_i\right)/n, \quad (2)$$

where D is the coefficient of gene differentiation (at any level of subdivision, factor, or interaction of factors), SS_i is the sum of squares of the ith allele at the corresponding level, a is the number of alleles, and n is the number of samples. We derived this relation algebraically for the two-allele case, but we believe it holds for any number of alleles as numerical comparisons of D with $\Sigma\ SS_i$ generated exactly this relation.

Due to this simple relationship, it seems indeed possible to test the significance of the components of the gene diversity analysis by an ordinary ANOVA test using D-values instead of SS-values (see Appendix 1 for details). We used this possibility for testing the significance of the habitat and island components and for the interaction, in each loci. The significance of G_{ST} and G_{SHI} were, however, tested using the χ^2 distribution, from

$$\chi^2 = 2NG_x,$$

in which N is the total number of individuals at the corresponding level (e.g., Hedrick 1985, p. 294).

One potential problem is the binomial distribution of the allele frequency data. ANOVA is designed for normally distributed data. However, the binomial distribution tends to a normal distribution with increasing numbers of individuals, and may be approximated as normal if the number of individuals multiplied by the proportion of a character (e.g., an allele) is greater than 15 (Snedecor and Cochran 1967, p. 223). In our study sample sizes were generally 40 to 50 and seldom fewer than 30, and thus alleles of frequencies 0.25 to 0.75 may be considered normally distributed. Although rare alleles may be a problem, their contribution to $\Sigma\ SS_i$ will be quite small, and they would probably not change the MS estimate much, unless there is a number of infrequent alleles in a loci. If this is the case, pooling may be advisable, and the allele frequencies may also be arcsine transformed (e.g., Underwood 1981). In analyzing our data, we pooled multiple alleles for the ANOVA. When we repeated the analysis with arcsine-transformed pooled frequencies it gave an identical result. We also analyzed the unpooled data, which gave slightly different results in one of the loci.

Results

Variation within Samples and Di-Locus Associations

In each locus, 1 to 4 samples of a total of 35 departed from Hardy-Weinberg equilibrium ($P < 0.05$). These deviations, however, showed no consistent trend (heterozygote and homozygote deficiencies were about equally common) and were all nonsignificant when corrected for multiple testing. This indicates that the deviations were most probably type-I errors, and the populations sampled were all, at least approximately, equilibrium populations.

In any of the 35 samples, no more than four di-locus associations (of 28 possible combinations) deviated significantly ($P < 0.05$) from the expected genotype frequencies. Indeed these deviations all proved to be nonsignificant when corrected for multiple testing, and we thus got no indication of genetic linkage between any two of the analyzed loci.

Variation among Islands and Habitats

Allele frequencies varied substantially among islands and within islands among the three different habitat types (see Fig. 1). All loci were highly variable with total heterozygosities (H_T) of 0.14 to 0.49. Much of the variation (6%–53%) was attributable to differentiation among samples (G_{ST}, highly significant in all eight loci, Table 2) although only one locus (*Aat*) revealed significant differences among replicate samples from the same habitat and island (G_{SHI}, Table 2). Thus, most of the among-sample variation was caused by differentiation among habitats (on average 35%) and differentiation among islands reflecting a restricted gene flow (on average 36%). The proportions of habitat and island variation differed, however, substantially between loci. All loci, except perhaps *Mpi* (but see note of Table 2) revealed significant differentiation due to island separation, but this part of the total variation among samples (G_{IT}/G_{ST}) ranged widely (11%–61%, Table 2). Likewise, the habitat-specific part of the variation differed substantially over loci. The most habitat dependent locus was *Aat* in which 81% of the among sample variation were attributable to differences among habitats. Five additional loci (*Pgm*-2, *Pgi*, *Pgm*-1, *Pnp* ,and *Ark*) revealed habitat components that were significant with 22% to 59% of the differentiation among samples being habitat specific (G_{HT}/G_{ST} in Table 2). In *Ark*, *Pep*, and *Pgm*-2, there were significant effects of the interaction between habitat and island, implying that the habitat effects were different in different islands.

The conspicuous habitat variation in *Aat* was mostly due to differences between the low and the high rocky shore samples (Fig. 1). This despite the fact that samples from these two habitats were sometimes only meters apart (Table 1). In contrast, allele frequencies in *Aat*, especially from the boulder habitat, were surprisingly stable over islands (Fig. 1). In *Ark* and *Pgm*-1, the most common allele was in general less frequent in the boulder habitats. However, both the interaction and the island effects were predominant in *Ark*, which suggested that the allele frequency distribution reflected the combined effect of different factors (Table 2). Island separation was significant in *Pep*: in particular two islands (ARS and SVA) were less variable than the others (Fig. 1). Three of the remaining loci (*Pgi*, *Pgm*-2, and *Pnp*) all revealed within-

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FIG. 1. Allele frequency distributions of eight polymorphic loci in *Littorina saxatilis* from three different habitats (B, L and H, replicate samples pooled) of five different islands (URS, BUR, JUT, ARS and SVA; abbreviations as in Table 1). Alleles are given in order of increasing mobility (faster alleles placed on top of slower in the bar plot). Horizontal bars below the X-axis indicate significant differences between samples within islands. Filled bars show differences which are significant ($P < 0.05$) after correction for multiple testing within each locus (15 tests per locus). Stippled bars are differences which were significant ($P < 0.05$) before but not after correction for multiple testing.

TABLE 2. Orthogonal analysis of gene diversity (see text and Appendix 1) for 30 samples distributed over five islands and three habitats within each island.† We used pooled alleles‡ in each locus (all except the most common allele pooled). χ^2 were used to test the significance of among sample differentiation (G_{ST}) and variation among replicate samples of the same habitat and island (G_{SHI}). We used ANOVA to test the significances of the genetic variation over habitats (G_{HT}), over islands (G_{IT}), and the habitat-island interaction ($G_{H\times I}$).

Locus	H_T	H_S	G_S	G_{ST}	G_{SHI}	G_{HT}	G_{IT}	$G_{H\times I}$
Aat	0.465	0.216	0.466	0.534***	0.025*	0.431***	0.058***	0.020
Ark	0.217	0.205	0.943	0.057***	0.004	0.018*	0.023***	0.012**
Mpi	0.489	0.465	0.952	0.048***	0.019	0.006	0.010	0.013
Pep	0.141	0.125	0.887	0.113***	0.008	0.011	0.069***	0.025**
Pgi	0.462	0.422	0.914	0.086***	0.010	0.019*	0.047***	0.010
Pgm-1	0.418	0.389	0.929	0.071***	0.009	0.021*	0.032***	0.010
Pgm-2	0.380	0.340	0.895	0.105***	0.007	0.062***	0.022***	0.014*
Pnp	0.433	0.408	0.942	0.058***	0.010	0.020*	0.019**	0.010

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

† We balanced the test by using two samples from each habitat in all islands.

‡ We produced a similar analysis with arcsine-transformed pooled alleles and one with unpooled alleles (without transformation). The arcsine transformation gave identical results as above and using unpooled alleles gave similar results in all loci except in *Mpi,* in which total variation (H_T) increased to 0.654 and differentiation due to island separation (G_{IT}) increased to 0.035, which was significant ($P < 0.001$).

island allele frequency clines (boulder—low rock—high rock). In contrast to *Aat*, these loci revealed more pronounced differences between boulders and rocks than between low and high rocks. Noticeably in *Pgi*, allele frequencies of similar habitats from different islands sometimes differed markedly, despite congruent clinal patterns (Fig. 1).

We found two alleles that were unique to a particular island. Mpi^{75} was found in five of six samples of Jutholmen and Ark^{130} in three of seven samples from Svangen.

In two of the islands (ARS and SVA), we sampled rock pools close to the high shore samples. These samples were similar to the high shore samples in all loci, except *Aat* (Table 3). In *Aat*, the rock-pool frequencies were markedly different from nearby high shore samples and were instead more similar to the low rock samples (Table 3).

DISCUSSION

The allozyme variation at local scales in *L. saxatilis* seems largely structured by two factors: first, a restricted gene flow due to the physical separation of populations on different islands; and second, habitat-related differentiation. However, the impact of these two factors vary substantially over the eight loci of our study. All loci were significantly differentiated among islands (*Mpi* became significant without pooling), and all except *Mpi* and *Pep* revealed habitat-related variation (Table 2). The relative importance of island separation and habitat varied, however, among loci. Thus, in *Pep*, *Pgi*, and *Mpi* differentiation among islands were the most important factor, whereas in *Aat* and *Pgm*-2 habitat effects were dominating. In *Ark*, *Pnp*, and *Pgm*-1, the two effects were about similarly important. Most important, we also found interactions between island and habitat effects in three of the loci (*Ark*, *Pep*, and *Pgm*-2), which implies varying effects of habitat in different islands. In conclusion, individual loci responded differently on the two structuring factors, and there seemed to be no point of generalizing effects over all the studied loci.

TABLE 3. Frequencies of allele Aat^{100} in samples from high-shore rock pools and mean frequencies of adjacent high and low rocky samples from the same shores.

	Rock pool		High rocky		Low rocky	
Island	*N*	Aat^{100}	*N*	Aat^{100}	*N*	Aat^{100}
ARS	40	0.81	120	0.48	74	0.91
SVA	35	0.77	80	0.24	80	0.92
URS	>100	0.52*	151	0.03	149	0.52

* Average of four samples from the same pool from Johannesson et al. (1995).

The habitat-specific part of the variation in allozyme frequencies may potentially be explained by spatially varying selection acting directly on the loci in question. From recent studies of perturbed natural populations of *L. saxatilis* in Ursholmen, it is evident that the variation between high and low rocky populations in *Aat* is intraspecific and due to strong selection favoring different alleles in different microhabitats (Johannesson et al. 1995). The actual selective factors are not yet known but one clue is given by the observation that rock-pool subpopulations at high-shore levels are dominated by the low-shore allele (Aat^{100}). This was earlier reported from one rock pool at Ursholmen (Johannesson et al. 1995), and now we have confirmed that this is a general trend (Table 3). Rock-pool and low-shore snails live mainly submerged, whereas high-shore snails not inhabiting rock pools live emerged. This suggests that some factor related to dampness, temperature, or perhaps salinity is involved. A remarkable observation is the sharp genetic clines found. Thus, for example, over distances of a few meters either between rock pools and high-shore samples or between low-shore and high-shore samples dominance of one allele is replaced by a complete dominance of the other (Table 3).

The habitat-specific parts of the variation in *Pgm*-2, as well as in *Pgi*, *Pgm*-1, and *Pnp*, may likewise be due to natural selection. The best model seems to be that of balancing selection in which different alleles of the habitat-dependent loci have different fitnesses in different habitats. Possibly, the targets of the balancing selection are not these loci but are tightly linked loci. Because we found each locus to segregate independently, this requires tight linkage between our allozyme loci and the putative targets of selection.

Although a selection model seems by far the most likely explanation, some of the habitat-specific variation may perhaps be neutral and might be explained by past or present levels of gene flow. This assumes that there is (or has been)

a relatively higher gene flow among subpopulations of similar habitats than among subpopulations of contrasting habitats. Because *L. saxatilis* lacks an effective dispersal stage, the ability to exchange genes among populations of similar habitats inhabiting different islands is very low and probably not large enough to counteract the effect of genetic drift ($Nm \approx 0.07$, recalculated from direct estimates of migration in Johannesson and Johannesson 1995). Our conclusion is that it is unlikely that current patterns of gene flow would explain the habitat-related variation found in some of the allozymes of this study. On the other hand, we cannot rule out an explanation based on discrete distributions of, for example, the boulder and rocky-shore snails during some historical period.

Strong habitat-related variation may obviously suggest populations of separate gene pools (sibling species), each confined to a different habitat. However, different loci show incongruent patterns of variation (Fig. 1), and this is not predicted by a two-species model. Furthermore, different habitats graded into each other and samples of, for example, low and high rocky shores were often taken only meters apart in dense continuous distributions without signs of Wahlund effects. We believe it is unlikely that the samples include different species, and indeed morphological (Janson and Sundberg 1983) as well as behavioral (Erlandsson, pers. comm., 1996) studies support this conclusion. We cannot, however, reject the possibility of a somewhat impeded gene flow among subpopulations of different habitats at a local scale. Indeed partial reproductive barriers have been found in Spanish (Johannesson et al. 1995) and English populations (Hull et al. 1996), although Swedish populations of *L. saxatilis* do not appear to show such barriers (Erlandsson, pers. comm., 1996).

In some earlier studies of allozyme variation in *L. saxatilis* of this area, we used many of the same allozymes and sampled similar habitat types as in the present study. In the study by Janson and Ward (1984) (Janson is the earlier name of one of us, KJ), 11 subpopulations were sampled along a continuous island population over alternatively boulder and rocky pieces of shore. We considered the variation due to habitat differences (three habitat types; boulder, intermediate, and rock) by comparing the differentiation among habitats and within habitats by Nei's gene diversity analysis. We found that differentiation among habitat types on average contributed with about half the among sample variation. Some loci were more habitat dependent than others, for example, *Odh* (octanol dehydrogenase) and *Pgm*-2 in which 94% and 82%, respectively, of the among sample differentiation was habitat dependent. In *Aat*-1 (*Aat* of this study), 66% of the among population variation was due to different habitats. This is somewhat less than in the present study but is likely explained by the fact that only low rocky and boulder samples were used in Janson and Ward (1984), and in the present study a large part of the habitat-related variation in *Aat* was generated between low and high rocky sites (Fig. 1). Of the remaining 12 polymorphic loci of Janson and Ward's (1984) study, six had habitat effects explaining 25% to 50% of the among population variation (table 4 in Janson and Ward 1984). Thus, even if in Janson and Ward (1984) we did not emphasize the importance of habitat-specific variation the results are fully consistent with the results of the present study.

In two other studies, we compared low and high rocky populations of *L. saxatilis* at the island Ursholmen, and found variation in the four loci *Pnp*, *Pgm*-1, *Pgi*, and *Mpi* that were consistent with the neutral model (Johannesson and Johannesson 1989; Johannesson et al. 1995). The lack of habitat differences between high and low rocks in these loci is fully consistent with the present study as the habitat effects of *Pgi*, *Pgm*-1, and *Pnp* found in the present study were due to differences between boulder and rocky habitats (Fig. 1). Moreover, the allozyme frequencies of these three loci and of *Mpi* were stable over five years (Johannesson et al. 1995). Both studies, however, revealed steep clinal variation in *Aat* from low to high rocks in Ursholmen. Furthermore, a natural pertubation made it possible to estimate an approximate fitness of 0.6 for the $Aat^{120/Aat120}$ homozygote in the low-shore zone relative to 1.0 for the $Aat^{100/Aat100}$ homozygote in this zone (Johannesson et al. 1995), which support a model of strong selection gradients.

In summary, it seems as if the genetic structure of *L. saxatilis* in the studied area is shaped by the combining effects of two microevolutionary mechanisms. The fragmented distribution with barriers of water effectively restricts dispersal among island populations and certainly enhance the effects of stochastic forces. Indeed, Johnson and Black (1995) found barriers of water to be much more effective than similar distances of continuous habitats in isolating populations of a direct developing Australian littorinid. Our finding of a few rare alleles in *L. saxatilis*, each unique to a particular island, suggests that water barriers probably promote genetic drift more effectively than do isolation by distance over continuous habitats at similar scales also in this species (but see Janson 1987 for isolation over long distances).

The comprehensive effects of genetic drift, at various scales, is truly expected in a direct developing species in contrast to species with effective dispersal larval stages (Janson 1987; Hellberg 1996). However, the conclusion from our data that six out of eight polymorphic loci to varying degrees are affected by microscale habitat differences is an important complement. Although the strong habitat-related variation in *Aat* is thoroughly documented (Johannesson and Johannesson 1989; Johannesson et al. 1995), no other loci except *Odh* (Janson and Ward 1984) has before explicitly been suggested as habitat dependent in *L. saxatilis*.

Habitat-associated allozyme changes at local scales are found in grasses (Nevo et al. 1986; Nevo et al. 1994; Prentice et al. 1995), and invertebrates (Day 1990; Hickey and McLean 1980; Carvalho 1989; Tatarenkov and Johannesson 1994). Together with the results of the present study their studies accent the need to consider microscale variation in general and habitat associated variation in particular in the study of allozyme variation of natural populations.

This is not to say that habitat-specific variation is always present. For example, Johnson and Black (1996) address geographic versus habitat-related variation among island populations of the direct developing littorinid *Bembicium vittatum* in Australia. Interestingly, they did not find any habitat-associated variation in the 14 allozyme loci studied, and their main conclusion is that the allozyme variation reflects the patterns of past and present gene flow. Thus, conclusions about allozyme structure are not easily generalized among

species, even not between related species with similar life-history characters inhabiting corresponding types of habitats.

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Appendix

Consider samples distributed over several islands (I), and in each island different localities (L) are sampled with replicate samples (S) then

$$H_T = H_S + D_{SL} + D_{LI} + D_{IT}, \quad \text{(A1)}$$

where H_T is total variation, H_S variation within samples, D_{SL} differentiation among samples within localities, D_{LI} differentiation among

localities within islands, and D_{IT} differentiation among islands in an ordinary nested way. If, however, the different localities of an island represent different habitats we may evaluate the effect of habitat by another design:

$$H_T = H_S + D_{SL} + D_{LH} + D_{HT}, \quad \text{(A2)}$$

where D_{LH} is differentiation among localities within habitats, and D_{HT} is variation over habitats. If combining (A1) and (A2) we have $D_{LI} + D_{IT} = D_{LH} + D_{HT}$, and as $D_{LH} = H_H - H_L$ and $D_{IT} = H_T - H_I$ we may rearrange this into $D_{LI} = D_{HT} + (H_H + H_I - H_L - H_T)$ and put it back into (A1): $H_T = H_S + D_{SL} + D_{HT} + (H_H + H_I - H_L - H_T) + D_{IT}$. The term $(H_H + H_I - H_L - H_T)$ may be considered an interaction term, $D_{H\times I}$, and thus the final equation will be

$$H_T = H_S + D_{SL} + D_{HT} + D_{H\times I} + D_{IT}. \quad \text{(A3)}$$

We used a slightly altered designation of the term D_{SL} in our study, as replicate samples of the same habitat and island were not always from the same locality, that is, they were sometime more dispersed than were samples of different habitats (Table 1). Thus, we use D_{SHI} (and G_{SHI}), read as differentiation among samples of the same habitat and island, instead of D_{SL}. If equations (A2) and (A3) are compared, it is clear that D_{LH} is the sum of the differentiation among island (D_{IT}) and the interaction of habitats and islands ($D_{H\times I}$). If there is no interaction, differentiation among islands equals differentiation among localities of similar habitats. This must be so because in our model each island had only one locality of each type of habitat. The last equation (A3) is analogous to an ANOVA model with two orthogonal factors, and this suggests that the D estimates can readily be put into an ANOVA analysis replacing the mean square estimates. Assuming that island is a random factor and habitat a fixed factor the sums of squares, degrees of freedom, mean square estimates and F-ratios may be estimated as follows:

Source	SS	df	MS	F-ratio
Islands	D_{IT}	$n_i - 1$	D_{IT}/df	MS_{IT}/MS_{SL}
Habitats	D_{HT}	$n_h - 1$	D_{HT}/df	$MS_{HT}/MS_{H\times I}$
Habitats × islands	$D_{H\times I}$	$(n_i - 1)(n_h - 1)$	$D_{H\times I}$/df	$MS_{H\times I}/MS_{SL}$
Within localities	D_{SL}	$n_i n_h(n_{sl} - 1)$	D_{SL}/df	

n_i, number of islands; n_h, number of habitats; n_{sl}, number of replicate samples in one locality.