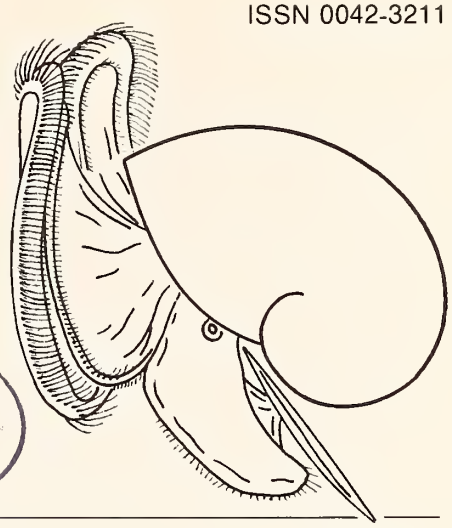
ISSN 0042-3211

THE VELIGER



A Quarterly published by
CALIFORNIA MALACOZOOLOGICAL SOCIETY, INC.
Berkeley, California
R. Stohler, Founding Editor

Volume 38 April 3, 1995 Number 2

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The Veliger (ISSN 0042-3211) is published quarterly in January, April, July, and October by the California Malacozoological Society, Inc., % Santa Barbara Museum of Natural History, 2559 Puesta del Sol Road, Santa Barbara, CA 93105. Second Class postage paid at Berkeley, CA and additional mailing offices. POSTMASTER: Send address changes to *The Veliger*, Santa Barbara Museum of Natural History, 2559 Puesta del Sol Road, Santa Barbara, CA 93105.

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The Veliger is an international, peer-reviewed scientific quarterly published by the California Malacozoological Society, a non-profit educational organization. *The Veliger* is open to original papers pertaining to any problem connected with mollusks. Manuscripts are considered on the understanding that their contents have not appeared, or will not appear, elsewhere in substantially the same or abbreviated form. Holotypes of new species must be deposited in a recognized public museum, with catalogue numbers provided. Even for non-taxonomic papers, placement of voucher specimens in a museum is strongly encouraged and may be required.

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The Veliger 38(2):85-91 (April 3, 1995)

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Genetic Heterogeneity in Populations of *Littorina brevicula* (Philippi) (Mollusca: Gastropoda) in the Northern Part of Peter the Great Bay (Sea of Japan)

by

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Abstract. Genetic differences among eight samples of *Littorina brevicula* in Peter the Great Bay in the far east of Russia were surveyed using five highly polymorphic allozyme loci as genetic markers. Geographic distances between samples ranged from a few meters to more than a hundred kilometers. The coefficient of relative gene differentiation was quite low, G_{ST} = 1.6%. Such a low level of differentiation was expected because *L. brevicula* has a planktonic larval stage lasting more than 10 days. Despite the low level of gene differentiation, there was statistically significant heterogeneity in allele frequencies among samples at three loci. Microgeographic heterogeneity (within a few dozen meters) among samples within continuous settlements was found to be significant in the loci *Pgi, Alpdh,* and *Fdh*. Differential natural selection, recruitment processes, and presence of a cryptic species are discussed as possible explanations for this observation.

INTRODUCTION

Most marine gastropod species have limited adult dispersal. One may expect that the level of gene flow among settlements of sedentary and poorly vagile species is largely determined by the mode of larval development (Scheltema, 1971). Species with a pelagic larval stage are characterized by a higher level of gene flow than those with a direct development (Ward, 1990). Undoubtedly, the extent of interpopulation differentiation should be related to the extent of gene flow if, at least, the genetic markers are neutral. However, it is interesting to evaluate to what extent the mode of larval development influences the level of genetic differentiation among populations.

Genetic-population structure has been described in a number of gastropod species both with a pelagic larval stage (Johnson & Black, 1982; Brown, 1991; Mitton et al., 1989; Campton et al., 1992) and with direct development (Grant & Utter, 1988; Day & Bayne, 1988; Day, 1990). Ward (1990) reviewed the biochemical genetics of *Littorina* species covering data for 10 species. The most intensively studied species are *Littorina saxatilis* (Olivi, 1792), *L. arcana* Hannaford Ellis, 1978 (Ward & Warwick, 1980; Janson & Ward, 1984; Knight et al., 1987; Janson, 1987a, b; Knight & Ward, 1991), and *L. littorea* (Linnaeus, 1758) (Janson, 1987b; Johannesson, 1992). The level of interpopulation genetic differentiation in species with a direct development was shown to be higher than in species with planktonic larvae. For example, the average genetic differentiation among populations (expressed in G_{ST} and d_m) in seven littorinid species with pelagic larvae was about one-third that of the species with crawling young (Ward, 1990). However, the variation in, for example, G_{ST}, is large within both groups. Thus, despite a pelagic larval development, both *L. scutulata* Gould, 1849, and *L. plena* Gould, 1849, exhibit quite high levels of interpopulation genetic differentiation (Mastro et al., 1982; Ward, 1990).

Most studies describing genetic differentiation and population structure in littorinids have been done on Atlantic species. There have also been a few studies of genetic differentiation among populations of Pacific periwinkles

from the American coast (Mastro et al., 1982), but examples of western Pacific littorinids are absent.

The goal of this study was to measure the genetic differentiation among *Littorina brevicula* (Philippi, 1844) settlements in the northern part of Peter the Great Bay in the Sea of Japan, using polymorphic enzymes as genetic markers.

Littorina brevicula is one of the most common periwinkles in the Asian coast of the Pacific. This species ranges from Taiwan in the south to Peter the Great Bay in the north. Development of *L. brevicula* is entirely pelagic. Females produce pelagic egg capsules, from which planktonic larvae hatch in 8–10 days and the larvae remain in the plankton for some additional time (Golikov, 1976).

In this study, I have evaluated genetic heterogeneity among settlements of *L. brevicula* on different geographic scales, from meters to a hundred kilometers, using five enzyme loci which were earlier found to be highly polymorphic in this species (Tatarenkov, 1992).

MATERIALS AND METHODS

A total of 330 periwinkles were collected from eight locations in Peter the Great Bay in the Sea of Japan (Figure 1) in the summer of 1991. The first six samples (1–6) were collected in a small inlet in Vostok Bay. The distances between these samples ranged from two meters to 1 km. The other two samples (7, 8) were collected in Ussyriysky Bay, which is more than 100 km from Vostok Bay. The distance between 7 and 8 was about 100 m. Each sample was collected within 25 cm^2 of the shore. Snails were approximately of the same size, 7 to 10 mm. Individuals of this size are 3–4 years old.

The animals were kept alive in a cooler at 4°C prior to electrophoresis. The details of electrophoresis and of gel staining, and also of allozyme variation in *L. brevicula,* have been described earlier (Tatarenkov, 1992). Five polymorphic enzymes were chosen as genetic markers to investigate the level of interpopulation differentiation. These enzymes were: inorganic pyrophosphatase (E.C. 3.6.1.1, locus *Ipp*), alanopine dehydrogenase (E.C. 1.5.1.17, *Alpdh*), phosphoglucose isomerase (E.C. 5.3.1.9, *Pgi*), formaldehyde dehydrogenase (E.C. 1.2.1.1, *Fdh*), and peptidase (substrate gly-leu, E.C. 3.4.*.*, *Pep-2*). Loci coding for these enzymes was characterized by high heterozygosity levels, while zymogram patterns were easy to score. Allozymes of *Ipp, Pgi,* and *Fdh* were separated using Tris-EDTA-Borate buffer (pH 8.6), whereas Tris-Citrate (pH 7.0) was used for *Alpdh* and *Pep-2*.

Genotype frequencies of each sample were analyzed for consistency with Hardy-Weinberg expectations. The conventional chi-square test for goodness of fit between observed genotype distribution and those expected for random mating population would not be applicable to some of the samples because of small expected numbers of rare genotypes. Therefore, I estimated the significance of deviations of observed genotype frequencies from those expected under Hardy-Weinberg equilibrium using a pseudo-probability test (Hernandez & Weir, 1989) provided by the program CHIHW (Zaykin & Pudovkin, 1993). The program estimates the probability of the null hypothesis (agreement with Hardy-Weinberg equilibrium) using Monte-Carlo simulations.

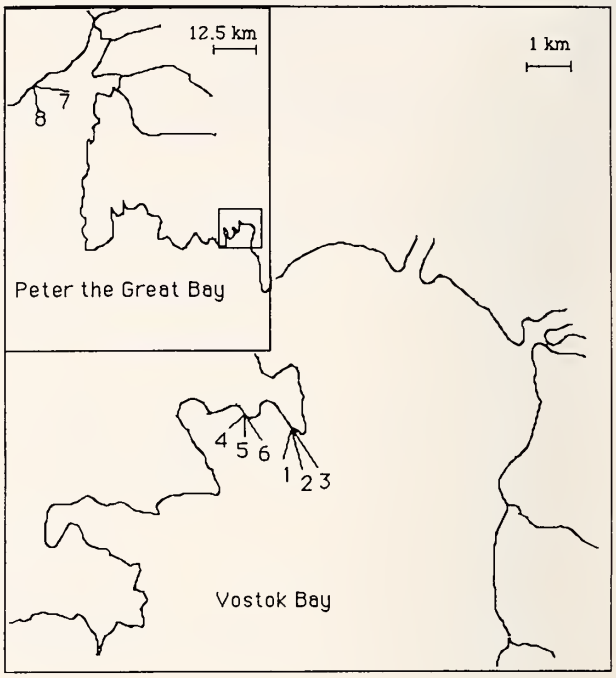

Figure 1

Locations of eight samples of *Littorina brevicula* in Peter the Great Bay. Samples 1–3 and 4–6 were collected in two continuous settlements of the species in Vostok Bay. Distances between the samples within the settlements ranged between two and 30 meters, while the distance between the settlements was about 1 km. Samples 7 and 8, with 100 meters in between, were from a continuous settlement in Ussuriysky Bay, which is about 100 km from Vostok Bay.

Tests for homogeneity of allele frequencies among samples were performed with chi-square statistics. To avoid small expected numbers, I pooled the frequencies of the rarest alleles, so that expectations in contingency tables should not be less than 4, and tested for allele homogeneity with pooled frequencies. As an alternative approach, I estimated the hypothesis of allele frequency homogeneity using the pseudo-probability test (the CHIRXC program by Zaykin & Pudovkin, 1993). The combining of probabilities obtained with this test was performed using the Fisher's approach as given by Sokal & Rohlf (1981:779–782). This approach is based on the fact that natural logarithm of probability (ln P) is distributed as $-0.5\chi^2_{[2]}$. Therefore, to evaluate the combined probability of null hypothesis, the $-2\ \Sigma_i^k \ln P_i$ should be compared to χ^2 with

Table 1

Allele frequencies at five polymorphic loci in samples of *Littorina brevicula* from Peter the Great Bay (see Figure 1).

Locus	Allele	Sample 1	2	3	4	5	6	7	8
Ipp	(*n*)	50	40	18	45	46	45	45	41
	A	0.100	0.038	0.056	0.144	0.130	0.133	0.044	0.098
	B	0.850	0.863	0.889	0.767	0.815	0.767	0.900	0.817
	C	0.050	0.100	0.056	0.089	0.054	0.100	0.056	0.085
Alpdh	(*n*)	49	40	18	45	46	44	44	41
	A	0.041	0.063	0.000	0.044	0.076	0.125	0.114	0.024
	B	0.806	0.875	0.944	0.844	0.859	0.852	0.841	0.890
	C	0.082	0.038	0.056	0.044	0.011	0.023	0.045	0.024
	D	0.071	0.025	0.000	0.067	0.054	0.000	0.000	0.061
Pgi	(*n*)	50	40	18	45	46	45	11	41
	A	0.080	0.013	0.000	0.022	0.011	0.011	0.000	0.000
	B	0.860	0.962	1.000	0.978	0.978	0.944	0.955	0.988
	C	0.060	0.025	0.000	0.000	0.011	0.044	0.045	0.012
Fdh	(*n*)	39	40	18	45	46	45	45	41
	A	0.103	0.112	0.056	0.089	0.087	0.044	0.133	0.146
	B	0.064	0.013	0.139	0.067	0.043	0.111	0.000	0.073
	C	0.833	0.875	0.806	0.844	0.870	0.844	0.867	0.780
Pep-2	(*n*)	15	40	17	43	46	45	44	41
	A	0.033	0.013	0.029	0.023	0.022	0.011	0.023	0.037
	B	0.433	0.363	0.412	0.337	0.478	0.389	0.341	0.463
	C	0.367	0.575	0.529	0.581	0.478	0.544	0.580	0.488
	D	0.167	0.050	0.029	0.058	0.022	0.056	0.057	0.012

2k degrees of freedom (k = the number of separate tests and probabilities).

When testing for conformity of genotype distribution to Hardy-Weinberg equilibrium or testing for homogeneity of allele frequencies among samples, one usually has to perform a number of separate tests (for individual loci and for different sets of samples). In such cases of multiple testing, the critical values of coefficients of significance for each individual test are not equal to the value in a single test. Indeed, according to statistical expectations, approximately five out of 100 tests performed are expected to result in *P*-values less than 0.05 by chance only. To avoid type I errors, I performed corrections for multiple tests using Sidak's multiplicative inequality for calculations of critical values of chi-square distribution (Sokal & Rohlf, 1981:728; Rohlf & Sokal, 1981:101). I used the program MULTTEST (Zaykin & Pudovkin, 1991) to find the critical values of χ^2 for each individual (replicate) test considering that it was a part of a group of analogous and independent tests.

The relative amount of genetic variation among populations was analyzed using Nei's (1973) measure of gene diversity, G_{ST}.

RESULTS

Allele frequencies with corresponding sample sizes for the five loci studied are given in Table 1. At *Ipp* the observed genotype ratios did not differ significantly from Hardy-Weinberg expected ratios. At the remaining four loci, significant deviations (pseudo-probability test, $P < 0.005$) were present in one or two samples. After correction for multiple testing for each locus separately, the deviations were still significant. When considering all five loci and eight samples and applying the corresponding Sidak correction for the whole set of tests, the deviations from Hardy-Weinberg expectations were significant in samples 1 and 2 for *Pgi* ($P < 0.05$), and in sample 5 for *Alpdh* ($P < 0.05$). These deviations were due to a deficiency of heterozygotes.

Statistically significant allele frequency heterogeneity among the eight samples of *L. brevicula* was present in *Alpdh, Pgi,* and *Fdh* (Table 2). Considering, however, these tests as a set of multiple tests and applying Sidak's correction, the heterogeneities in *Alpdh* and *Fdh* can be explained by a statistical type I error. However, heterogeneity in *Pgi* remained significant ($0.01 < P < 0.05$). The combined test for heterogeneity over all loci revealed significant differences among samples ($P < 0.001$); even disregarding data on *Pgi,* the combined probability over the four remaining loci was also significant ($P < 0.05$).

Samples 1–3, 4–6, and 7, 8 were collected in three different settlements. Within each settlement, the spatial distribution of mollusks was continuous. Settlements were disrupted by places where *L. brevicula* was not present (e.g., sand beaches). Within the settlements, samples were

Table 2

Conventional χ^2-test and pseudo-probability test for heterogeneity in allele frequencies of five polymorphic loci among eight samples of *Littorina brevicula*. Numbers of alleles, χ^2-values, and degrees of freedom are referred to χ^2-test. P and P_{pseudo}-probabilities of null hypothesis obtained with χ^2-test and with pseudo-probability test, respectively.

Locus	Number of alleles*	χ^2	d.f.*	P	P_{pseudo}
Ipp	3	16.04	14	0.311	0.301
Alpdh	3	28.50	14	0.012	0.012
Pgi	2	26.00	7	0.0005	0.005
Fdh	3	24.75	14	0.037	0.038
Pep-2	3	16.73	14	0.271	0.360
Total		112.02	63	0.0001	0.0007

* Rare alleles were pooled so that expected numbers should not be less than 4.

Table 3

Pseudo-probability test for heterogeneity among samples within continuous settlements of *Littorina brevicula*-test for microgeographic variation. P_{pseudo}-probability of null hypothesis that allele frequencies among samples do not differ.

Locus	Samples 1–3	4–6	7, 8	Total
	P_{pseudo}	P_{pseudo}	P_{pseudo}	P_{pseudo}
Ipp	0.359	0.818	0.313	0.569
Alpdh	0.211	0.082	0.003	0.003
Pgi	0.017	0.198	0.363	0.037
Fdh	0.075	0.343	0.015	0.015
Pep-2	0.231	0.535	0.169	0.258
Total	0.018	0.283	0.002	0.0008

taken at distances of between two meters (samples 2 and 3) to 100 meters (samples 7 and 8). It is likely, that, within the settlements, gene exchange may be due to movements of adult snails, in addition to exchange through pelagic larvae. The results of pseudo-probability tests for heterogeneity of samples within continuous settlements are shown in Table 3. Statistically significant heterogeneity of allele frequencies was found between samples 1–3 in *Pgi,* and between 7 and 8 in *Alpdh* and *Fdh*. Combining the probabilities over all three settlements, I concluded that samples within continuous settlements were significantly different in *Alpdh* ($P < 0.005$), *Pgi* ($P < 0.05$), and *Fdh* ($P < 0.05$). The totals over five loci for each settlement were significant for samples 1–3 ($P = 0.018$), and 7, 8 ($P = 0.002$). The combined probability over all loci and all groups of samples shows that samples within continuous settlements do differ in allele distributions ($P < 0.001$).

Table 4 outlines estimates of gene diversity among *L. brevicula* samples. The average coefficient of gene differentiation is quite low, G_{ST} being 1.6% only. Thus, almost all variation observed is explained by within sample component. However, at *Pgi* the between sample component of gene diversity is somewhat larger, $G_{ST} = 3.7\%$.

Considering the five polymorphic loci of this study, Nei's (1972) index of gene identity (I) ranged from 0.9860 between samples 1 and 4 to 0.9987 between 2 and 7, with an average of 0.9926.

DISCUSSION

Despite dozens of papers devoted to genetic-population structure in littorinids, data on Hardy-Weinberg equilibrium are quite poor. As Ward (1990) noted, this may be because most *Littorina* species show Hardy-Weinberg proportions. This is in contrast to the situation in bivalve mollusks in which many cases of deviations from Hardy-Weinberg equilibrium have been described (reviewed by Zouros & Foltz, 1984). Different kinds of selection models, assortative mating, Wahlund effects, and null-alleles have been suggested as explanations for these.

The majority of samples of *L. brevicula* were in good agreement with Hardy-Weinberg proportions at most loci. There was no general tendency for deficit or excess of heterozygotes as well. However, there were a few significant cases of departures from Hardy-Weinberg expectations in *Pgi* and *Alpdh*. It seems unlikely that the heterozygote deficiencies in *Pgi* or *Alpdh* of *L. brevicula* populations were caused by the presence of null-allele because I did not observe even a single case of no-enzyme activity on zymograms. Assuming that the deviations found in *Pgi* were due to a null-allele, the frequency of this allele should have been about 0.25, and a total of 19 null-allele homozygotes should have been expected in my data as blank tracks on the zymograms. As Richardson et al. (1986) pointed out, null-allele homozygotes may not be present if they are lethal or have a reduced fitness. It is doubtful, however, that such deleterious alleles could exist in population at such a high frequency of 0.25.

The Wahlund effect seems also to be an unlikely explanation for the heterozygote deficiency. Differences in allele frequencies among demes must be very large in this case to explain the heterozygote deficiency found. In fact, the variance of allele frequencies among the samples of this study may explain only about 3% of the mean heterozygote deficiency found in *Pgi,* and less than 1% of the deficiency in sample 1. However, as this study includes geographic variation in allele frequencies over a minor area of the species total distribution, a Wahlund effect may be caused by larval input from remote populations which could have substantially different allele frequencies. If this is the case, however, such larval influx from other areas must be very rare. Otherwise, it would be difficult to explain the required high geographical variation in allele

frequencies over the species area. Occasional influx of larvae from remote areas is known in the literature. Johannesson (1992) described a case of a mass occurrence of *Melarhaphe neritoides* (Linnaeus, 1758) in Sweden, where this species has previously been absent. However, no cases of substantial geographic variation in gastropods with planktonic larvae have been described. Instead, similarities of allele frequencies over vast areas have been described for littorinids *L. littorea* and *Melarhaphe neritoides* (Johannesson, 1992), the conch *Strombus gigas* Linnaeus (Mitton et al., 1989; Campton et al., 1992), the abalone *Haliotis rubra* (Brown, 1991), and an undescribed species of limpet of the genus *Siphonaria* Sowerby, 1823 (Johnson & Black, 1982). There are no reasons to believe that *L. brevicula* is an exception to these cases.

Assortative mating seems to be an unlikely explanation in relation to electrophoretic characters, unless these are tightly linked to a locus controlling visible characteristics, and in linkage disequilibrium with it (Richardson et al., 1986).

Of the remaining models which may explain the observed deviations from Hardy-Weinberg equilibrium, natural selection against heterozygotes or the presence of a reproductively isolated taxon, which I may have included by mistake together with *L. brevicula,* seem to be likely ones. However, without additional data, it is difficult to choose between these two hypotheses.

If the investigator is certain that he is dealing with only one taxon, microgeographic heterogeneity of allele frequencies in a species with a high level of gene flow may be substantial evidence for local natural selection. An example of variation due to selection at several loci on a small scale (dozens of meters) was found in the bivalve mollusk *Crenomytilus grayanus* (Dunker) with pelagic larvae (Pudovkin & Balakirev, 1985). Other examples of supposed selection based variation on microgeographic scale are in the limpet snail *Siphonaria* sp. (Johnson & Black, 1982) and in the copepod *Tigriopus californicus* (Baker, 1912) (Burton & Feldman, 1981). Assuming that *L. brevicula* is a taxonomically uniform group, the significant heterogeneity in allele frequencies among samples within some dozen meters that was found in *L. brevicula* is unexpected because the larvae of this species spend at least 10 days in plankton. Therefore, one might expect a reasonable shuffle of genes during that period within the bay plankton. Besides, there is an exchange of genes due to migration of adult snails in continuous settlements. Therefore, the microgeographic variation found could not be explained by stochastic drift of allele frequencies. The heterogeneity of allele frequencies among samples within continuous settlements, and also among settlements within an inlet, may be caused by differential natural selection. It is not necessary that this selection act directly on the allozyme loci. It might be selection on a closely linked locus or loci.

The other possible reasons might be the presence of unknown isolated taxon or genotype-dependent distribution of individuals along the shore. The latter explanation seems unlikely, as we deal with variation at allozyme loci. Until now very few examples of association between enzyme polymorphism and habitat selection behavior have been known (Byers, 1983). Furthermore, it is possible to give other explanations of association of enzyme polymorphism with habitat types instead of genotype-dependent habitat choice. Thus, in the case with *Tegula funebralis* (A. Adams, 1855) (Byers, 1983), it might be selection in favor of certain alleles at *Pgi* and *Pgm,* which is coupled with different habitat preferences of shore levels by individuals of different ages.

The possibility of the presence of a taxon morphologically similar to *L. brevicula* is difficult to reject. In Peter the Great Bay, some forms of *L. mandshurica* Schrenck, 1867, have shell morphology similar to *L. brevicula.* However, these species are distinct in drastic differences in *Pep-2, Alpdh,* and *Fdh,* which in combination make it impossible to mistake *L. mandshurica* for *L. brevicula.* However, it might be another taxon, which is at present unknown. I should stress here, that this presumed taxon must be genetically very close to *L. brevicula,* as it has the same alleles at five polymorphic loci. Furthermore, I did not observe any indication for the presence of reproductively isolated taxon when I previously explored *L. brevicula* for 39 loci (Tatarenkov, 1992). Still, the hypothesis of presence of unknown taxon deserves further investigation. Recently Takada (1992) discovered that in southern Japan in early winter, before copulation occurs, some snails of *Littorina brevicula* migrate down the shore as far as 20 meters, whereas the other snails remain in the upper intertidal zone. In early spring, presumably after copulation and releasing of veligers, snails begin to move upward again. In summer the distribution of individuals is unimodal. Later Takada put forward a hypothesis of the presence of two different species (personal communication). However, it is not yet known if this migration causes some kind of reproductive isolation. It is necessary to show that the very

Table 4

Analysis of gene diversity (Nei, 1973) based on five polymorhpic loci for eight samples of *Littorina brevicula.*

Locus	H_T	H_S	G_{SL}	G_{LT}	G_{ST}
Ipp	0.2903	0.2871	0.0055	0.0055	0.0110
Alpdh	0.2740	0.2426	0.0142	0.0036	0.0178
Pgi	0.0813	0.0783	0.0221	0.0148	0.0369
Fdh	0.2811	0.2774	0.0085	0.0046	0.0132
Pep-2	0.5664	0.5571	0.0157	0.0007	0.0164
Average	0.2932	0.2885	0.0123	0.0038	0.0161

H_T = total heterozygosity.
H_S = mean sample heterozygosity.
G_{SL} = relative gene differentiation among samples within continuous settlements.
G_{LT} = relative gene differentiation between continuous settlements.
G_{ST} = relative gene differentiation among samples.

same snails and their descendants show such migration, and that this does prevent gene flow between the two groups. Besides, it is not yet known if such partition of population is characteristic for the snails from Peter the Great Bay. Thus, in northern Japan not some but all adult snails showed downward migration in winter (Kojima, 1957, 1959). Summarizing, however, it is necessary to say that there might be some unknown peculiarities of biology leading to some kind of assortative mating and reproductive isolation, which might explain microgeographic heterogeneity in *L. brevicula*.

Despite the statistically significant heterogeneity in allele frequencies at some polymorphic loci, *L. brevicula* is characterized with low genetic differentiation in general. Thus, the average coefficient of gene differentiation (G_{ST}) in the species is only 1.6%. This is comparable to the value of 1–2% obtained for *L. littorea* which also has a pelagic larval stage (Janson, 1987b). In contrast, the differentiation among populations of ovoviviparous *Littorina saxatilis* over similar geographic scales ranges from 7 to 12% (Janson, 1987b; Ward & Warwick, 1980). In the likewise direct developing species *Littorina arcana* and *L. nigrolineata* Gray, 1839, although on a somewhat larger geographic scale, G_{ST} is in the range of 16% to 18% (Knight et al., 1987; Knight & Ward, 1991). This indicates that gene flow through planktonic dispersal in *L. brevicula* largely prevents the accumulation of genetic divergence (which may occur due to both genetic drift and differential selection).

The data of this paper demonstrate that investigation of microgeographic genetic-population structure of mollusks with planktonic dispersal poses many interesting questions. Usually species with pelagic larvae show little or no differentiation among populations over broad areas. However, sometimes this similarity of allele frequencies over broad areas is accompanied by patchy microgeographic variation. There is no universal explanation for such heterogeneity at present. Three main explanations for such patchiness are possible:

(1) Local natural selection. The cases of microgeographic variation in species with planktonic dispersal can be explained by natural selection, because the role of genetic drift in local settlements becomes negligible as a consequence of extensive gene exchange. The more substantial evidences of natural selection might be the cases of repeated correlations of certain environmental factors with allozyme variation. However, the probability of observing such cases is rare because one can study only a limited number of both environmental parameters and polymorphic loci.

(2) Presence of a cryptic taxon. It is always possible to assume that material at study is taxonomically heterogeneous. If a large number of loci were surveyed and no diagnostic loci were found, presence of a cryptic taxon becomes less likely, but still difficult to reject completely.

(3) Unknown peculiarities of larval dispersal. The assumption of widespread gene flow might be misleading. Discrete cohorts of larvae are possibly maintained due to the complex nature of sea currents or unknown peculiarities of larval behavior.

Further observations should be performed to choose between the hypotheses explaining microgeographic heterogeneity in *L. brevicula*.

ACKNOWLEDGMENTS

I am grateful to Dr. A. I. Pudovkin for the discussions, and to Dr. K. Johannesson for the valuable suggestions and comments on the manuscript. Dr. Thierry Backeljau and an anonymous reviewer provided helpful criticism and advice. This work was supported in part by grants from the Russian Governmental Scientific and Technical Project "Priority Directions of Genetics Development," and from the Youth Foundation (Russia, Novosibirsk). Financial assistance by the George Soros Scholarship from the International Science Foundation is thankfully appreciated.

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