

Genetic heterogeneity among intertidal habitats in the flat periwinkle, *Littorina obtusata*

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Abstract

Comparisons among patterns exhibited by functionally distinct genetic markers have been widely used to infer the impacts of demography and selection in structuring genetic variation in natural populations. However, such multilocus comparisons remain an indirect evaluation of selection at particular candidate loci; ideally, the identification of a candidate gene by comparative genetic methodologies should be complemented by functional analyses and experimental manipulations of genotypes in the laboratory or field. We examined genotype frequency variation among replicated intertidal habitats at two spatial scales in the grazing snail *Littorina obtusata*. Both of the candidate allozyme markers varied predictably with environment, and these patterns were consistent at both spatial scales. Three of four reference loci were spatially homogeneous, but one microsatellite exhibited significant structure at both geographical and mesoscales. To initiate a direct examination of whether the observed genotype frequency variation at one of the candidate markers, mannose-6-phosphate isomerase (MPI), was impacted by differential survivorship of genotypes, we conducted a series of laboratory-based thermal stress assays using snails from two geographically disparate source populations. When snails were exposed to bouts of thermal/desiccation stress, patterns of mortality were nonrandom with respect to MPI genotype. Furthermore, patterns of mortality in the laboratory manipulation coincided with the observed distribution of genotypes in the field. The data suggest the operation of selection at the *Mpi* or a linked locus, but functional studies and further experimentation are required to establish the relationship between MPI genotype and fitness across heterogeneous intertidal environments.

Keywords: ARK, cline, dispersal, *Littorina*, microsatellite, MPI

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Introduction

Environmental heterogeneity can have widespread impacts on the dynamics of particular polymorphic loci in natural populations (e.g. Hedrick 1986). The intertidal zone on rocky shores has been extensively characterized as a highly variable environment; at multiple spatial scales, there exist pronounced gradients for such factors as temperature and desiccation stress, salinity, and wave exposure. This variation in specific environmental parameters among intertidal habitats has been linked to genotype frequency variation in a variety of taxa (e.g. Koehn *et al.* 1980;

Johannesson *et al.* 1995; Schmidt & Rand 1999). While a pattern such as an allele or genotype frequency cline may be generated by selection, it may also simply reflect demography and population history (e.g. Adams *et al.* 2006). Given the observation that a particular genetic marker varies among habitats, the effects of genetic drift and selection may be evaluated in a number of ways. For example, genotype frequency variation may be assessed across replicate, independent populations (e.g. Oakeshott *et al.* 1982) or at multiple reference loci that are more likely to exhibit selective neutrality, such as synonymous sites within a coding sequence (Berry & Kreitman 1993; Sezgin *et al.* 2004). Variation among populations for the reference markers can then be used to generate a null distribution against which variation for candidate markers is assessed (McDonald 1994; Storz 2002; Beaumont & Balding 2004).

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However, reference nuclear loci may yet exhibit significant variation among habitats even when gene flow appears sufficient to preclude differentiation solely due to drift (Gockel *et al.* 2001; Johannesson *et al.* 2004). Such a pattern may be of particular relevance to taxa for which a large number of traits and alleles vary across habitats or along an environmental gradient, as has been extensively documented in *Drosophila melanogaster*. Despite evidence for widespread gene flow among *D. melanogaster* populations (e.g. Coyne & Milstead 1987; Kennington *et al.* 2003), latitudinal clines have been documented for a number of nuclear loci as well as a variety of quantitative traits (e.g. Boulétreau-Merle *et al.* 1982; Oakeshott *et al.* 1982; Capy *et al.* 1993; Karan *et al.* 1998; Gockel *et al.* 2001; Hoffmann *et al.* 2001, 2002; Verrelli & Eanes 2001; Frydenberg *et al.* 2003; Sezgin *et al.* 2004; Schmidt *et al.* 2005). If a significant proportion of reference markers can vary systematically among habitats (e.g. Wilding *et al.* 2001; Grahame *et al.* 2006), complex interactions among selection, gene flow, and colonization history may limit the inferential power of multilocus comparisons in systems for which a limited number of genetic markers are available.

This emphasizes the importance of additional methods that may complement inferences based on comparisons among genetic markers. The experimental manipulation of genotypes in the field or laboratory can provide a direct evaluation of competing hypotheses, but may be of insufficient power to detect small but meaningful selection coefficients. When coupled with information regarding functional differences between alleles or genotypes, experimental manipulations can also differentiate between selection acting directly at a polymorphic locus rather than at an unidentified target in linkage disequilibrium (e.g. Clarke 1975). Regardless, in order to assess the dynamics of candidate polymorphisms in natural populations, a comprehensive approach should be utilized (Clarke 1975; Avise 1994).

Here, we examine patterns of variation among habitats for a series of markers in a grazing marine snail *Littorina obtusata*. Two allozymes [MPI (mannose-6-phosphate isomerase), E.C. 5.3.1.8; ARK (arginine kinase), E.C. 2.3.7.7] were selected a priori as candidates for significant associations between genotype frequencies and particular environmental factors that vary among habitats. The selection of ARK and MPI as candidates was based on (i) an identified association between the function of the enzyme and organismal performance under a specific form of environmental stress, and (ii) the observation that each allozyme locus exhibits non-neutral patterns of variation, associated with a particular environmental parameter, in other intertidal taxa (Tatarenkov & Johannesson 1994, 1999; Schmidt & Rand 1999, 2001). These candidates were compared to four reference loci, comprising one additional polymorphic allozyme and three microsatellite loci. As a

complement to these analyses, the thermal environment experienced by snails was manipulated in the laboratory to examine patterns of relative survivorship. Our results indicate that for one of the candidate allozymes, the association between genotype and environment observed in the field results from the differential fitness of genotypes among habitats.

Materials and methods

Species description and sampling regime

Littorina obtusata is a common species of the mid-eulittoral zone on rocky shores in the North Atlantic, ranging from the White Sea to Portugal in Europe, and Greenland to Long Island Sound in North America. Individuals are highly variable for various aspects of shell morphology, including shape, size, colouration and banding pattern; the degree of body pigmentation is also variable (Reid 1996). Snails are almost exclusively associated with the algal canopy, principally *Ascophyllum nodosum* and *Fucus vesiculosus* in the sites sampled here. Females deposit spawn masses on algae, where the embryos develop within individual capsules until hatching as juveniles. There are two aspects of natural history for *L. obtusata* that are of particular importance for the current study. First, *L. obtusata* is associated with macrophytes that can act as an environmental buffer (e.g. Bertness *et al.* 1999) and whose distribution and abundance can vary among habitats: much of the relevant environmental variation exists among sites and there is relatively less microhabitat structuring within sites as can occur for species associated with exposed rocky substrates (e.g. Johannesson & Johannesson 1989; Sokolova & Pörtner 2001). Second, the absence of planktotrophic dispersal suggests that among-population gene flow may be limited, resulting in isolation by distance and significant substructure (Bohonak 1999). Thus, the expectation was that genetic markers would be differentiated among populations in a manner consistent with the action of genetic drift.

Population sampling was designed to assess genetic variation associated with two aspects of environment, thermal regime and degree of wave exposure. At the broad geographical scale (approximately 450 km), populations were sampled from three regions in the Gulf of Maine: south (Manchester, Massachusetts; 42.561°N, 70.763°W), mid (New Harbor, Maine; 43.861°N, 69.580°W), and north (Swans Island, ME; 44.109°N, 68.468°W). Within each region, approximately 100 snails were sampled from each of two replicate wave exposed and two wave-sheltered sites. Across this latitudinal gradient, maximum substrate and water temperature vary predictably and have been shown to impact snail growth rates (e.g. Trussell 2000). At a smaller spatial scale (approximately 20 km), populations were sampled from two replicate coastal, two midriver,

and two upriver sites along the Damariscotta River Estuary, Maine, USA. These collections were independent of those made at the larger spatial scale. The south (coastal) to north (upriver) orientation of the Damariscotta is associated with gradients for a number of environmental factors, including wave exposure and flow rates (Leonard *et al.* 1998) as well as thermal regime (Schmidt & Rand 1999). Wave exposure and thermal regime are tightly coupled, as high wave energy coastal habitats are more buffered from thermal stress than are the wave-sheltered, low-flow habitats at the upriver locations. Approximately 100 snails were haphazardly sampled from macrophytes along 50-m transects in the mid-intertidal zone at each of the six sites. Transects were utilized in an attempt to avoid the sampling of related individuals within microsites.

Genotype determination

Snails were frozen in liquid nitrogen in the field and subsequently transferred to a -80°C freezer in the laboratory. Prior to genetic analysis, each individual was scored for size class, shell colour, and body pigmentation. Soft tissue was then dissected and partitioned into two samples; a portion of the foot was used for extraction of genomic DNA, and the remainder was ground in 200 μL of 0.1 M Tris-HCl, pH 8.0. Genotype at three allozyme loci [MPI, ARK, and GPI (glucose-6-phosphate isomerase), E.C. 5.3.1.9] was determined simultaneously for each snail by cellulose acetate electrophoresis according to Hebert & Beaton (1989). Total genomic DNA was extracted using DNeasy Blood & Tissue kits (QIAGEN). Three microsatellite loci (Lsub08, Lsub32, Lsub62) were amplified by polymerase chain reaction (PCR) according to Tie *et al.* (2000). Each locus was amplified separately using a different fluorescent tag; samples were then multiplexed and multilocus genotype for each individual was determined by fragment analysis on an ABI 3100 capillary sequencer.

Statistical analysis

For the allozymes, polytomous logistic regression in JMP version 5 (SAS Institute) was used to model the effects of predictor variables on the observed genotype counts. For each allozyme, two alleles were common and labelled according to relative mobility (S for slow, F for fast); two additional rare alleles were observed for each allozyme and their pooled frequency was less than 1% (7/1912 for MPI; 11/1896 for GPI; 16/1760 for ARK). All rare alleles occurred as heterozygotes with a common allele and were scored as heterozygous genotypes in the analyses. For each marker, counts were modelled for the three genotypes; the heterozygous genotype was used as the reference genotype for all analyses. The regressions modelled two logits, the log odds of sampling an FF genotype relative to the SF

heterozygote, and the log odds of sampling an SS genotype relative to the SF heterozygote. The logistic regressions in JMP version 5 utilize a deviation from the mean in testing the significance of parameter estimates. Two analyses were performed for each of the allozyme loci. The first evaluated the effects of geographical region (south, mid, north), habitat type (wave-exposed vs. wave-sheltered), and their interaction on the observed genotype counts in these coastal populations. The second analysis was conducted on independent samples of populations along the Damariscotta River, and modelled the impacts of site (coast, midriver, upriver), shell colour (dark vs. light), and the interaction term. For the analyses of allozyme genotype frequency variation, critical P values were adjusted for multiple comparisons across three loci ($\alpha = 0.0166$; Rice 1989).

The microsatellite data were analysed with MICROSATELLITE ANALYSER (MSA version 3, Dieringer *et al.* 2003). The proportion of shared alleles (Bowcock *et al.* 1994), variance in repeat number, and the unbiased estimator of F_{ST} (Weir & Cockerham 1984) were calculated using MSA version 3. Significance levels for F_{ST} estimates were generated by 10 000 random permutations of genotypes among population comparisons, and critical P values adjusted for multiple tests across three microsatellite loci (Rice 1989). Given the small number of microsatellite loci analysed, we did not conduct further analyses of population structure using a Bayesian approach. GENEPOP web version 3.4 was used to test for linkage disequilibrium among allozyme and microsatellite loci, as well as to test the assumption of Hardy-Weinberg equilibrium for each marker.

Laboratory manipulations

Approximately 150 adult snails of a specific size class (8–12 mm maximum shell length, Trussell 1997) were collected from both a northern (Sand Cove, Damariscotta River, Maine) and a southern (Gloucester Point, Gloucester, Massachusetts) site. Animals were kept with clippings of *Ascophyllum nodosum* during transport to the Marine Science Center, Northeastern University, Nahant, Massachusetts, and were allowed to recover for 24 h in a flowing seawater system. No mortality occurred during transport or laboratory acclimation. Two shell colour morphs, citrina (light, yellow) and fusca (dark, black) were then used in a thermal stress assay. Snails were removed from the tanks, towel dried, and placed on a 0.5 m \times 0.6 m granite slab. Individuals were randomly placed on the granite into evenly spaced rows and columns with a 10-cm buffer around each edge. This array was then taken outdoors on a clear day and experimental snails were exposed to ambient solar radiation. After exposure for 30 min, snails were immersed in a flowing seawater tank for a recovery period of 10 min and mortality was assessed by lack of response to a blunt probe. This procedure was repeated until mortality was

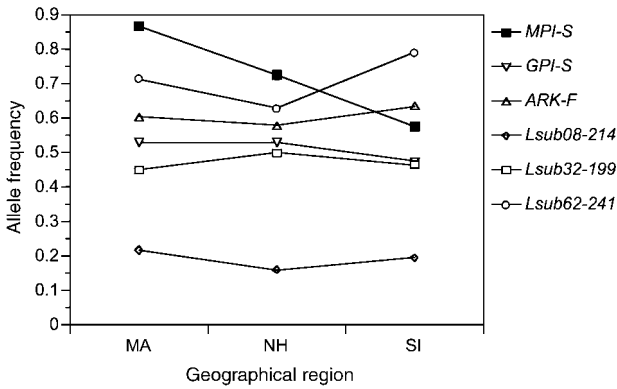


Fig. 1 The frequency of the most common allele at the six assayed markers as a function of the geographical origin of the source populations (MA, Nahant and Manchester, Massachusetts; NH, New Harbor, Maine; SI, Swans Island, Maine). Only the MPI allozyme exhibited a significant allele frequency cline.

approximately 50%. Live and dead individuals were sorted, labelled, and stored at -80°C until genetic analysis. During the experiment, maximum shell temperature was measured for a random subset of snails using a noncontact infrared thermometer (Raynger ST80 Pro Plus, Raytek Corporation). Each snail was briefly shaded and temperature measured at a fixed distance of 30 cm (distance to spot ratio of 50:1) with a standard and constant emissivity of 95%.

Results

Variation among geographical regions

Allele frequencies for MPI were clinal in the Gulf of Maine, whereas the frequency of the most common allele was geographically uniform for the five other assayed markers (Fig. 1). The observed allele frequency cline reflects the

Table 1 Parameter estimates from polytomous logistic regression of MPI genotype counts among the sampled geographical regions

Term	Estimate	SE	χ^2	Odds ratio
log odds MPI-FF/MPI-SF				
Region (DRE)	-0.199	0.255	0.61	0.67
Region (MA)	-0.074	0.289	0.07	0.86
Habitat (exposed)	-0.383	0.183	4.37*	0.47+
Region \times habitat (DRE \times exposed)	0.040	0.255	0.02	1.08
Region \times habitat (MA \times exposed)	-0.278	0.289	0.93	0.57
log odds MPI-SS/MPI-SF				
Region (DRE)	-0.171	0.128	1.79	0.71
Region (MA)	0.994	0.138	52.17***	7.30+
Habitat (exposed)	-0.050	0.096	0.27	0.91
Region \times habitat (DRE \times exposed)	-0.095	0.128	0.55	0.83
Region \times habitat (MA \times exposed)	-0.084	0.138	0.37	0.85

* $0.016 < P < 0.05$; *** $P < 0.001$; +95% confidence intervals do not include 1.0.

substantial and predictable variation in the frequency of all three MPI genotypes among the three sampled regions (Fig. 2). In particular, the MPI-SS homozygote greatly increased in frequency from north to south; across all samples, the odds of sampling an MPI-SS homozygote relative to the reference genotype were more than seven times higher in the southern populations (Table 1). In contrast to patterns exhibited by MPI, ARK genotype frequencies did not vary systematically with geographical origin but were consistently distinct between wave-exposed and wave-protected habitats (Fig. 2). The odds of sampling an ARK-FF genotype were significantly reduced on

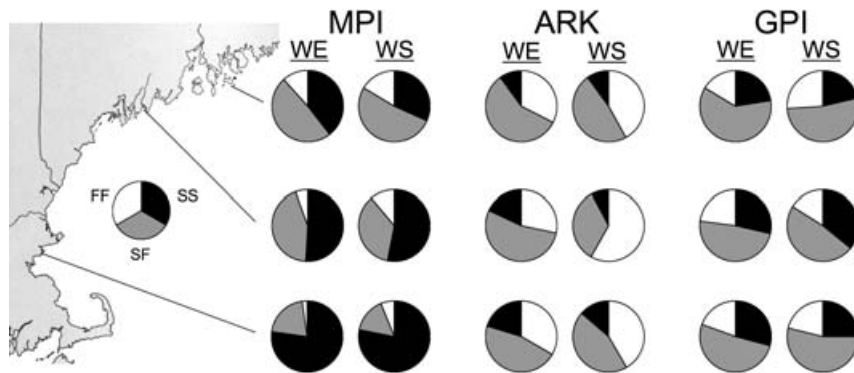


Fig. 2 Genotype frequency variation for the three allozyme loci between wave-exposed (WE) and wave-sheltered (WS) habitats in each of the three sampled geographical regions. Each diagram represents pooled genotype frequencies for approximately 100 individuals across two replicate sites. For MPI, only the predictor variable for geographical region was observed to impact genotype frequencies (Wald $\chi^2 = 69.39$, 4 d.f., $P < 0.0001$). ARK genotype frequencies varied between habitats ($\chi^2 = 6.28$, 2 d.f., $P < 0.05$; nonsignificant after Bonferroni correction) and among habitat by region combinations ($\chi^2 = 17.22$, 4 d.f., $P < 0.001$). None of the predictor variables (region, habitat, and the interaction term) had a significant effect on GPI genotype distributions.

Table 2 Parameter estimates for polytomous logistic regression of ARK genotype counts among the sampled geographical regions

Term	Estimate	SE	χ^2	Odds ratio
log odds ARK-FF/ARK-SF				
Region (DRE)	0.141	0.114	1.51	1.32
Region (MA)	0.008	0.126	0.01	1.01
Habitat (exposed)	-0.224	0.085	6.87**	0.64†
Region \times habitat (DRE \times exposed)	-0.358	0.114	9.77**	0.49†
Region \times habitat (MA \times exposed)	0.352	0.126	7.85**	2.02†
log odds ARK-SS/ARK-SF				
Region (DRE)	0.142	0.163	0.76	1.33
Region (MA)	0.250	0.173	2.09	1.65
Habitat (exposed)	-0.080	0.128	0.40	0.85
Region \times habitat (DRE \times exposed)	0.113	0.163	0.49	1.25
Region \times habitat (MA \times exposed)	-0.098	0.173	0.32	0.82

** $P < 0.01$; †95% confidence intervals do not include 1.0.

Table 3 Analysis of three polymorphic microsatellites in the sampled populations of *Littorina obtusata*

Locus	Fragment size (bp)	No. of alleles	Gulf of Maine F_{ST}	Damariscotta River F_{ST}
Lsub08	199–241	14	0.0038	-0.0035
Lsub32	193–229	10	0.0395***	0.0868***
Lsub62	226–253	10	0.0143	-0.0039

*** $P < 0.001$.

wave-exposed shores, and the magnitude of the difference in genotype frequencies between habitats varied among regions (Table 2). In the analysis of genotype frequency variation for GPI, none of the predictor variables had a significant effect (statistics not shown). As with the allozymes, the three reference microsatellite loci did not generate a consistent signal of variation among populations. Global F_{ST} values for two of the microsatellites, Lsub08 and Lsub62, were not statistically different from zero whereas one (Lsub32) exhibited significant heterogeneity among regions (Table 3). All loci were observed to be in Hardy-Weinberg equilibrium and no disequilibrium between any two markers was evident in any pairwise comparison.

Variation among sites within the Damariscotta River

An additional factor, shell colour, was included in the examination of genetic heterogeneity among river sites. North of the Damariscotta River, shell colour variation declines in richness and the lighter shell colours are either

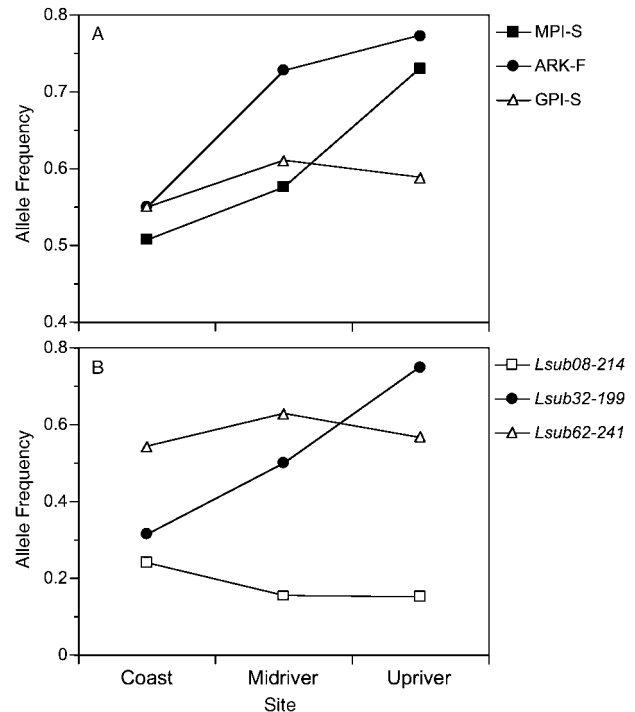


Fig. 3 The frequency of the most common allele for the three allozyme loci (panel A) and three microsatellite loci (panel B) across the environmental gradient in the Damariscotta River (ME). The MPI and ARK allozymes, as well as the Lsub32 microsatellite, demonstrated significant allele frequency clines.

at very low frequency or entirely absent; at the sites sampled on Swans Island, snails were all dark in colouration, predominantly of either the fusca (dark brown/black) or olivacea (dark green) shell colour morphs. The paucity of light coloured specimens in the northern regions precluded an analysis of genotype frequency variation among colour morphs at the regional scale.

Each of the six markers exhibited the same qualitative patterns of variation (i.e. homo- or heterogeneity among samples) across the 20-km environmental gradient within the Damariscotta River as they did at the larger spatial scale in the Gulf of Maine. Both MPI and ARK exhibited a significant allele frequency cline, whereas allele frequencies for GPI were homogeneous across sites (Fig. 3). Analysis of genotype frequency data showed that site had a significant effect for both MPI and ARK, and that either shell colour (MPI) or the interaction between shell colour and site (ARK) were also of significance; again there was no effect of any predictor variable on GPI genotype frequencies (Fig. 4). For the MPI allozyme, there are two focal observations. First, the frequency of the MPI-SS genotype substantially increased in frequency across the environmental gradient in the river whereas the frequency of both the heterozygous genotype and the MPI-FF homozygote decreased in frequency (Fig. 4, Table 4). This pattern mirrors the one

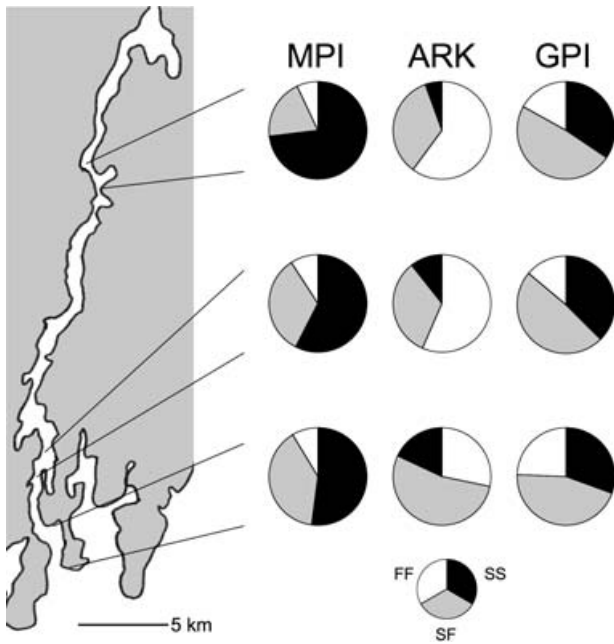


Fig. 4 Locations of sampled sites and genotype frequency variation for the three allozyme loci. Each diagram represents genotype frequencies in samples of approximately 180 snails pooled across two replicate sites at each location in the Damariscotta River. For both the MPI and ARK allozymes, genotype frequencies varied among sites sampled (MPI: $\chi^2 = 22.53$, 4 d.f., $P < 0.001$; ARK: $\chi^2 = 40.56$, 4 d.f., $P < 0.0001$). MPI genotype frequencies also varied between snails with light and dark shell colours ($\chi^2 = 7.29$, 2 d.f., $P < 0.03$; nonsignificant after Bonferroni correction). While shell colour did not have a significant effect for ARK, the difference in genotype frequencies between shell colours varied across the sites sampled ($\chi^2 = 10.62$, 4 d.f., $P < 0.03$, nonsignificant after correction for multiple tests). As with the regional analysis, GPI genotype frequencies were homogeneous across sites and between shell colours (statistics not shown).

previously observed at the regional spatial scale. Second, MPI-FF genotypes were less frequently observed in snails with dark shells, although this pattern was less pronounced at the midriver sites (Table 4). Genotype frequency variation for ARK in the river also closely matched the observed patterns at the larger spatial scale: the frequency of the ARK-FF genotype increased linearly from the wave-exposed, thermally buffered coastal sites to the low-flow, high-temperature stress upriver locations (Fig. 4, Table 5).

Both the Lsub08 and Lsub62 microsatellite loci exhibited negative F_{ST} values across the Damariscotta River samples. The Lsub32 locus, which was heterogeneous at the regional scale, was also differentiated among sites in the river (Table 3). Furthermore, the most common allele at Lsub32 exhibited a 45% cline across the river that was substantially steeper than that observed for either of the two candidate allozymes (Fig. 3). Across all loci, however, there was no indication of a significant relationship

Table 4 Parameter estimates from polytomous logistic regression of MPI genotype counts among replicate populations along the Damariscotta River

Term	Estimate	SE	χ^2	Odds ratio
log odds MPI-FF/MPI-SF				
Site (midriver)	0.236	0.230	1.05	1.60
Site (upriver)	0.026	0.276	0.01	1.05
Shell color (dark)	-0.483	0.179	7.28**	0.38†
Site \times shell color (midriver \times dark)	0.655	0.230	8.10**	3.71†
Site \times shell color (upriver \times dark)	-0.423	0.276	2.34	0.43
log odds MPI-SS/MPI-SF				
Site (midriver)	-0.227	0.138	2.70	0.64
Site (upriver)	0.610	0.145	17.62***	3.38†
Shell color (dark)	-0.093	0.010	0.87	0.83
Site \times shell color (midriver \times dark)	0.296	0.138	4.58*	1.81†
Site \times shell color (upriver \times dark)	-0.176	0.145	1.47	0.70

* $0.016 < P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; † 95% confidence intervals do not include 1.0.

Table 5 Parameter estimates from polytomous logistic regression of ARK genotype counts among replicate populations along the Damariscotta River

Term	Estimate	SE	χ^2	Odds ratio
log odds ARK-FF/ARK-SF				
Site (midriver)	0.371	0.138	7.20**	2.10†
Site (upriver)	0.413	0.132	9.77**	2.28†
Shell color (dark)	-0.080	0.099	0.66	0.85
Site \times shell color (midriver \times dark)	0.164	0.138	1.42	1.39
Site \times shell color (upriver \times dark)	-0.122	0.132	0.85	0.78
log odds ARK-SS/ARK-SF				
Site (midriver)	0.052	0.269	0.04	1.11
Site (upriver)	-0.449	0.276	2.65	0.41
Shell color (dark)	-0.084	0.182	0.21	0.85
Site \times shell color (midriver \times dark)	-0.668	0.269	6.18*	0.26†
Site \times shell color (upriver \times dark)	0.472	0.276	2.93	2.57

* $0.016 < P < 0.05$; ** $P < 0.01$; †95% confidence intervals do not include 1.0.

among measures of genetic and geographical distance (not shown). Pairwise F_{ST} between all sampled populations indicates that the differentiation between populations was primarily associated with the Lsub32 locus, particularly at the smaller spatial scale.

Table 6 Nominal logistic regression of mortality events in thermal stress assays, modelling the log odds (mortality/survivorship)

Term	Estimate	SE	χ^2	P	Odds ratio
Population (MA)	0.019	0.19	0.01	0.920	1.04
Shell color (dark)	0.722	0.145	24.87	0.00001	4.24*
Body pigment (pigmented)	0.494	0.209	5.58	0.018	2.69*
Genotype (MPI-FF)	0.935	0.457	4.18	0.041	6.49*
Genotype (MPI-SS)	-0.587	0.275	4.54	0.033	0.31*

*95% confidence intervals do not include 1.0.

Mortality assay

In the thermal stress assay in the laboratory ($N = 248$ snails), the mean maximum shell temperature following exposure to ambient conditions was 37.53°C ($SD = 2.12$, $N = 161$ measurements). Exposure to repeated bouts of thermal stress resulted in substantial mortality ($N = 105$ dead, 143 alive). Patterns of survivorship were significantly impacted by shell colour, degree of body pigmentation, and MPI genotype. Snails with dark coloured shells exhibited greater than a fourfold increase in mortality relative to snails with light-coloured shells. Similarly, individuals that possessed body pigmentation were more than two times more likely to experience a mortality event than were snails with unpigmented bodies. Both factors suggest a strong association between pigmentation, body temperature, and resistance to thermal stress. Patterns of mortality were also nonrandom with respect to MPI genotype. Odds of mortality for the MPI-SS homozygote, which was at high relative frequency both in southern populations and in the thermally stressful upriver sites, were significantly reduced (Table 6). In contrast, the MPI-FF genotype decreased in frequency along the environmental gradient in the Damariscotta River as well as along the north to south gradient in the Gulf of Maine; the odds of a mortality event were more than six times higher for this genotype in reference to others. The interaction between shell colour and MPI genotype on patterns of mortality is depicted in Fig. 5. Interestingly, the only MPI-FF genotypes that survived the stress assay were in light shell colour backgrounds. The MPI-SS genotype exhibited the highest survivorship, and this was more pronounced in the light shell colour background. The heterozygous genotype was intermediate between the two homozygotes with respect to patterns of survivorship, and the magnitude of the difference between light and dark shell colours was relatively less.

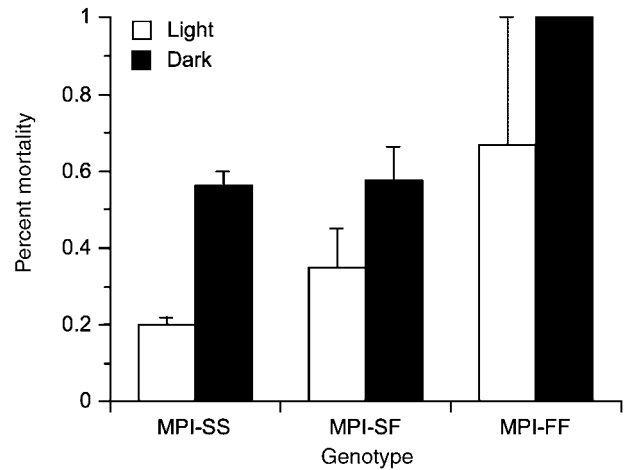


Fig. 5 Mean (\pm SE) percent mortality of the three MPI genotypes in light and dark shell colour backgrounds in the laboratory thermal stress assay.

Discussion

Littorina obtusata females deposit spawn masses on algae, and the retention of individuals within sites is predicted to be high (Reid 1996). The low level of adult dispersal would, in turn, predict low levels of among population gene flow and subsequent isolation by distance (e.g. Hellberg 1994; Bohonak 1999). Historical long-distance dispersal is evident in this taxon, as North American rocky shores were recolonized from Europe following the last glacial maximum (Wares & Cunningham 2001). It has been suggested for *Littorina fabalis*, a closely related species restricted to the eastern North Atlantic, that among-population gene flow may be achieved by the rafting of gravid females on displaced algal mats (Tatarenkov & Johannesson 1994). At coastal sites on the Damariscotta River, both viable adults and spawn masses have been observed on displaced algae following storm events (P. Schmidt, personal observation), indicating that routine patterns of adult movement may not accurately predict genetic structure at various spatial scales. Our data suggest the possibility of sufficient gene flow among populations to preclude genome-wide differentiation due to genetic drift, but cannot reject the hypothesis of significant genetic structuring of *L. obtusata* populations within the Damariscotta River or in the Gulf of Maine. Three of the four reference loci exhibit no detectable structure at any spatial scale, and regional effects are only evident at the MPI allozyme and the Lsub32 loci. Clearly, no consistent patterns of variation among populations are evident across all assayed markers, and one of the reference microsatellites (Lsub32) was heterogeneous across sites in both the Gulf of Maine and within the Damariscotta River.

A more comprehensive set of markers is required to generate a null distribution against which patterns of variation for Lsub32 can be compared; this would allow an

evaluation of whether or not the observed pattern is consistent with variance in demographic parameters (e.g. Wilding *et al.* 2001; Grahame *et al.* 2006). However, two observations suggest that differentiation at *Lsub32* is not indicative of the accumulation of among-population differentiation due to drift. First, this marker evidences a consistent pattern of allele frequency heterogeneity at two independent spatial scales. Second, allele frequency variation at *Lsub32* is much more pronounced at the small (20 km) than it is at the larger spatial scale (400 km); rather than allele frequencies being randomly divergent among populations, the two common alleles at *Lsub32* exhibit a striking cline from coastal to upriver sites. Although *Lsub32* was not observed to be in disequilibrium with any other marker (MPI, ARK) or trait (shell colour) that varies clinally in the Damariscotta River, it is possible that it is in disequilibrium with another locus that varies nonrandomly among habitats (e.g. Dufresne *et al.* 2002; Johannesson & Mikhailova 2004).

Wave exposure and non-neutral patterns at ARK

Although the series of putatively neutral reference loci did not generate a consistent signal against which variation in ARK genotype frequencies could be evaluated, several lines of indirect evidence suggest that the patterns of variation observed for ARK are inconsistent with strict neutrality. First, ARK genotypes are consistently differentiated between wave-exposed and wave-sheltered habitats across three geographical regions in the Gulf of Maine. Such a replicated pattern would not be predicted to result from demography alone. Second, ARK genotype frequencies vary predictably with environment across two independent gradients in wave exposure. In the Damariscotta River, ARK genotype frequencies vary linearly across the environmental gradient, and these patterns precisely match those observed between wave-exposed and wave-protected coastal habitats at the regional scale. Third, ARK genotype frequencies were sharply differentiated between wave-exposed and wave-protected habitats in European populations of *L. fabalis* (= *Littorina mariae*, Reid 1996) (Tatarenkov & Johannesson 1994). *Littorina fabalis* is the sister taxon to *L. obtusata* (Williams *et al.* 2003), and coincident patterns of genotype frequency variation over a specific environmental gradient in closely related taxa also implicate selection rather than demography (e.g. Harrison 1977; McDonald 1991).

Our data suggest that ARK genotype frequencies exhibit non-neutral patterns of variation in natural populations, but cannot differentiate between selection directly at *Ark* or at a linked locus; nor do the data identify a mechanism of selection, or the specific environmental variable that may generate the observed patterns. However, there is an association between the function of arginine kinase and

organismal performance in habitats that differ in degree of wave exposure. The dislodgement of individuals due to forces experienced during wave events (e.g. lift, drag) can have an obvious and detrimental impact on organismal fitness. Attachment to the substrate is achieved via the muscular foot, and foot area demonstrates a robust association with the degree of wave exposure in a given habitat (Trussell 1997). Wave events stimulate a coordinated burst of muscular activity that can place severe demands on cellular energy levels. The buffering of adenosine 5-triphosphate (ATP) levels in invertebrate cells that experience sporadic and intensive activity (e.g. muscle) is achieved by the activity of arginine kinase, which catalyses the transfer of a phosphoryl group between ATP and arginine (Walliman *et al.* 1992). Phosphoarginine then serves as a high-energy phosphagen reservoir for the rapid replenishment of depleted cellular ATP. Arginine kinase is homologous to the vertebrate creatine kinase, and these phosphagen kinases have been extensively characterized as mechanisms for cellular ATP homeostasis and as models for bimolecular reactions (e.g. Stroud 1996; Zhou *et al.* 1998). The patterns of ARK genotype frequency variation observed here are suggestive only, but may serve as a point of departure for subsequent characterizations of the functional properties of ARK alleles and their potential impact on fitness (e.g. Clarke 1975). The hypothesis that ARK activity may impact energy balance, while speculative, makes concrete predictions regarding relative enzymatic activities of ARK genotypes that could be readily tested (e.g. Panova & Johannesson 2004).

Thermal habitat and non-neutral patterns for MPI

In marine invertebrates, the function of MPI may also be associated with energy balance during periods of stress exposure. MPI catalyses the isomerization of mannose-6-phosphate to fructose-6-phosphate, which can then be shuttled through the glycolytic pathway to generate ATP. MPI genotype frequencies are associated with intertidal habitat in a variety of taxa (e.g. Siegmund 1985; McDonald 1991; but see Johannesson & Tatarenkov 1997 for a counter example), and it was hypothesized that this enzyme locus may be a generalized target of selection in grazing or filter feeding marine invertebrates for which mannose and mannose-containing compounds are a common dietary constituent (McDonald 1987). This was supported by a series of studies with the intertidal barnacle *Semibalanus balanoides*. In this taxon, MPI genotypes demonstrate a fine-grained association with habitat at several spatial scales (Holm & Bourget 1994; Schmidt & Rand 1999; Veliz *et al.* 2004) that results from the differential viability of genotypes in distinct environments (Schmidt *et al.* 2000; Schmidt & Rand 2001). The differential fitness of MPI genotypes was only realized when barnacles were exposed to both dietary mannose and temperature/desiccation

stress, and patterns of survivorship were associated with functional differences between MPI genotypes (Schmidt 2001; Rand *et al.* 2002; but see Veliz *et al.* 2006).

The data presented here suggest that a similar phenomenon may occur in populations of the marine snail *L. obtusata* in the Gulf of Maine. As with arginine kinase, patterns of MPI genotype frequency variation provide indirect evidence that this or a linked locus is subject to selection. In the sampling of replicate populations across independent thermal gradients at two spatial scales, MPI genotype frequencies varied in a concerted and predictable manner. Such replicated clines provide evidence that the observed patterns are not generated solely by genetic drift and isolation by distance. However, a wealth of environmental parameters covary with temperature at both the regional scale and along the gradient in the Damariscotta River; with an indirect evaluation of neutrality by the sampling of genotype frequency variation, it is not possible to identify the specific selective agent(s) that may generate the observed pattern. We hypothesized that temperature and desiccation stress may be the most relevant environmental parameters for structuring MPI genotype frequencies in natural populations of *L. obtusata*. Although snails are associated with an algal canopy that may buffer both temperature and desiccation stress (e.g. Bertness *et al.* 1999), canopy density and resulting substrate temperature vary substantially among sites. In the periwinkle *Littorina saxatilis*, exposure of snails to mild thermal stress results in an energy deficit, as measured by the levels of phospho-arginine and ATP, and results in a shift to anaerobic metabolism (Sokolova & Pörtner 2003). A previous study (Sokolova & Pörtner 2001) suggested that physiological parameters of *L. saxatilis* may reflect adaptation to a high-shore existence, and that *L. obtusata*, with a primarily mid- to low-shore distribution, may be more susceptible to heat and desiccation stress. In comparison to other species in the same genus, *L. obtusata* exhibits heat coma at similar temperatures but the LT50 temperature was observed to be the lowest of any assayed species (Clarke *et al.* 2000a). The temperature at which heat coma is induced is also highly variable among *L. obtusata* populations sampled from different geographical regions (Clarke *et al.* 2000b).

Differential survivorship of MPI genotypes

The examination of the impact of wave exposure on ARK genotype frequency variation would entail field transplants and longitudinal sampling, but the hypothesis that MPI genotype frequencies are impacted by thermal environment could be initially addressed by simple manipulations in a laboratory environment. The temperatures experienced by snails in the stress assay may have been sufficient to induce heat coma, but were below the lethal limit observed in European populations (Clarke *et al.* 2000b). In the habitats

sampled to produce experimental material for the stress assay, substrate temperatures routinely exceed 40 °C (M. Phifer-Rixey, unpublished data); however, the actual tissue temperatures of *L. obtusata* in these habitats remain unknown. The stress assay was designed as a relative measure of mortality in response to pronounced temperature/desiccation stress, and the relevance of the experimental conditions to those experienced in stressful habitats in the northwestern Atlantic cannot be addressed by the current study. In the laboratory assay, exposure of snails to ambient temperatures and solar radiation resulted in the differential mortality of MPI genotypes. These observed patterns of mortality precisely coincide with the distribution of genotype frequencies in distinct thermal environments at two spatial scales. The hypothesis of temperature-mediated selection is further supported by the impact of body pigmentation, snail shell colour, and the interaction between shell colour and MPI genotype on viability. Shell colour has a direct and predictable impact on snail body temperature (M. Phifer-Rixey, unpublished data); the role of variable thermal stress in the adaptive maintenance of shell colour variation in this taxon is addressed in a companion study (M. Phifer-Rixey, M. Heckman, G. C. Trussell & P. S. Schmidt, unpublished data).

Here, the survivorship of MPI genotypes was directly impacted by snail shell colour: all genotypes exhibited higher survivorship in light shell colour backgrounds, and the magnitude of the difference in survivorship between light and dark shells was heterogeneous across MPI genotypes. The MPI-SS homozygote was observed to be at greatly elevated frequency both in southern regions and in the thermally stressful upriver sites in the Damariscotta River, and this genotype demonstrated the highest survivorship in the laboratory manipulation. In contrast, the MPI-FF genotype was at very low frequency in thermally stressful locales, and suffered the highest mortality. Furthermore, in natural populations, this genotype was observed to be very infrequent in the more thermally susceptible dark shell colours, and no snails of the MPI-FF genotype/dark shell colour combination survived the thermal assay. Together, the distribution of MPI genotypes among thermal environments, the differential viability during periods of stress exposure, and the interaction between shell colour and genotype all suggest that the observed distribution of MPI genotypes in the field results from differential fitness among intertidal habitats that vary in degree of physiological stress. However, as the present study was confined to a single river system and three regions within the Gulf of Maine, it is unknown whether these results reflect a general pattern or idiosyncrasies associated with the populations sampled. It should also be noted that the effects of temperature and desiccation stress could not be separated in the laboratory manipulation, as elevated body temperatures also reduce oxygen availability (Sokolova & Pörtner 2003).

Summary

Six surveyed genetic markers yielded conflicting patterns of among-population and between-habitat variation in the Gulf of Maine. The allele frequency variation at putatively neutral microsatellite loci suggest that among-population gene flow may be higher than previously expected in this taxon, but this remains to be comprehensively addressed. Three of the assayed loci were homogeneous across samples, and three exhibited some form of a pattern (e.g. allele frequency cline) consistent with isolation by distance or selection. Examination of replicate population samples indicated that both ARK and MPI genotype frequency variation among habitats were not solely the result of genetic drift. The thermal stress assay was informative with regard to specific selective agents that may influence MPI genotype frequencies in natural populations, but in the absence of functional data cannot distinguish between selection at the *Mpi* or at a linked locus. Arguably, one aspect of studies exploring gene–environment interactions that has been difficult to establish is a general prediction across disparate taxa as to which genes are a priori candidates for exhibiting adaptive variation among environments. The data presented here support the hypothesis that the *Mpi* locus may be a generalized target of selection in a variety of invertebrate taxa because of the shared constraints imposed by an intertidal existence.

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