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Environmental heterogeneity and balancing selection in the acorn barnacle *Semibalanus balanoides*

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The northern acorn barnacle *Semibalanus balanoides* occupies several intertidal microhabitats which vary greatly in their degree of physical stress. This environmental heterogeneity creates distinct selection regimes which can maintain genetic variation in natural populations. Despite considerable attention placed on the link between spatial variation in fitness and balancing selection at specific loci, experimental manipulations and fitness estimates for molecular polymorphisms have rarely been conducted in the wild. The aim of this transplant experiment was to manipulate the level of physical stress experienced by a cohort of barnacles in the field and then investigate the spatial variation in fitness for genotypes at three loci: two candidate allozymes and the mitochondrial DNA control region. The viability of mannose-6-phosphate isomerase (*Mpi*) genotypes was dependent on the level of physical stress experienced in the various treatments; alternative homozygotes were favoured in alternative high stress–low stress environments. In contrast, the fitness of genotypes at other loci was equivalent among treatments and unaffected by the manipulation. Evaluated in the light of balancing selection models, these data indicate that the presence of multiple environmental niches is sufficient to promote a stable *Mpi* polymorphism in barnacle populations and that allelic variation at this locus reflects the process of adaptation to the heterogeneous intertidal landscape.

Keywords: balancing selection; *Mpi*; *Semibalanus balanoides*

1. INTRODUCTION

The role of molecular polymorphisms in the adaptation of organisms to heterogeneous environments remains a fundamental issue in evolutionary biology. Theoretical models of balancing selection outline how genetic variation may be adaptively maintained when organisms occupy more than one environmental niche (e.g. Levene 1953; Maynard Smith 1962; Gillespie 1976). The original multiple niche model of balancing selection proposed by Levene (1953) considers two alleles at a single locus in a diploid organism. If selection pressures vary between niches such that the alternative homozygous genotypes are of higher fitness in alternative environments, both alleles may be maintained in the population by natural selection. Although the amount of parameter space in which the Levene (1953) model allows a stable polymorphism is quite restricted (Maynard Smith & Hoekstra 1980), the likelihood of achieving a stable polymorphism can be greatly increased by certain natural history attributes such as assortative mating (Strobeck 1974), limited gene flow between niches (Karlin 1982) or habitat selection (Maynard Smith 1966). However, in the absence of empirical studies which validate such models the relationship between the spatial variation in genotypic fitness and existing levels of genetic variation in natural populations has not been resolved.

Detecting fitness differences between genotypes in the field or laboratory presents a major obstacle in documenting the occurrence and mechanisms of balancing selection, as such differences may be minute yet evolutionarily significant (Lewontin 1974). In recent years, comparative sequencing studies and neutrality tests have been used to distinguish between the historical action of selection and neutral evolutionary processes at specific loci (e.g. Hudson *et al.* 1987; Tajima 1989; McDonald & Kreitman 1991). While the patterns of nucleotide variation within and between species have indicated that balancing selection is an important factor in maintaining genetic variation (Kreitman & Hudson 1991; Takano *et al.* 1993; Eanes *et al.* 1996; Katz & Harrison 1997), such studies may not have addressed how or why selection presently operates in natural populations. For this reason, systems in which balancing selection can be directly examined provide a valuable complement to inferences regarding the cumulative evolutionary history of particular genes.

Semibalanus balanoides populations are common in the intertidal zone on rocky shorelines in the north-east Pacific and north-west and north-east Atlantic. Individuals are simultaneous hermaphrodites but cannot self-fertilize (Barnes & Crisp 1956). When mating occurs in autumn, individuals must mate with neighbours in order to reproduce. Larvae are brooded in the mantle cavity and, when released into the water column in the spring, progress through six naupliar stages over a period of three to five weeks (Lucus *et al.* 1979). After metamorphosing into non-feeding cypris larvae, individuals are able to settle and commence a sessile existence. Once a barnacle larva recruits, it experiences only the environmental conditions of its settlement position; the environment is discrete and coarse grained. At any given site, an annual cohort recruits to distinct intertidal microhabitats which may vary greatly in the environmental conditions which affect fitness. In particular, the effects of thermal and/or desiccation stress on *S. balanoides* survivorship have been extensively documented (e.g. Barnes 1958; Southward 1958; Wethey 1984). The increased physical

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stress commonly associated with the upper reaches of the intertidal zone can be alleviated when barnacles are associated with either ephemeral algae (Minchinton & Scheibling 1993) or a canopy of the brown alga *Ascophyllum nodosum* (Leonard 1999; Bertness *et al.* 1999). A consistent and finely adjusted association between genotypes at the mannose-6-phosphate isomerase (*Mpi*, E.C. 5.3.1.8) locus and the degree of environmental stress has been previously documented in this species (Schmidt & Rand 1999). The data suggest that variation in the fitness of *Mpi* genotypes among intertidal habitats may result in the active maintenance of both common *Mpi* alleles in barnacle populations.

If some form of environmentally mediated balancing selection is operating at the *Mpi* locus, two basic predictions can be made. First, the fitness of genotypes should vary consistently between intertidal habitats in a predictable manner. Second, the empirically derived fitness matrix for *Mpi* genotypes in various habitats should predict the maintenance of a stable or protected polymorphism. Changes in genotype frequencies over time may be used to estimate the viability component of fitness, provided the end-point frequencies are gathered after selection is completed (Prout 1965). However, documenting temporal change in genotype frequencies at a single locus cannot distinguish between selection acting at that or a linked locus, or on a suite of traits which differ between genetically isolated populations. Genetic differentiation due to population subdivision should be evident across the genome and the spatial variation in genotype frequencies should not be specific to one locus. Thus, comparisons between patterns of variation at putatively selected loci and others that are evolving in a manner consistent with neutrality provides a simple method of testing the prediction that selection operates at a specific linkage group (Karl & Avise 1992; Berry & Kreitman 1993; Podgson *et al.* 1995). In *S. balanoides*, a 401 bp fragment of the mitochondrial DNA (mtDNA) control region exhibits patterns of nucleotide variation which are consistent with neutrality (Brown 1995). The presence of a presumably neutral DNA marker which can be used in comparisons with candidate allozyme loci further strengthens the use of *S. balanoides* as an ecological model system in which to evaluate the action of balancing selection.

In this study, newly metamorphosed barnacles from a common source were transplanted into six experimental treatment combinations which were designed to subject barnacles to a natural range of environmental stress levels. In each treatment, viability was estimated for genotypes at the glucose-6-phosphate isomerase (*Gpi*, E.C. 5.3.1.9) and *Mpi* allozyme loci as well as for the mtDNA control region haplotypes. To determine whether the variation in fitness among treatments would be predicted to result in a stable polymorphism, fitness estimates were entered into the Levene (1953) multiple-niche model of balancing selection. These results were then compared with those obtained using genotypic fitness estimates from natural habitats in two preceding generations. Together, these data provide a direct examination of the potential importance of the spatial variation in selection pressures in maintaining genetic variation in nature.

2. MATERIAL AND METHODS

(a) *Experimental design*

Prior to the onset of larval settlement, 60 granite settlement plates were anchored to the substrate at the mean low tide level at a site chosen to minimize larval mortality (Glidden Ledge, Damariscotta River Estuary, Maine, USA; Leonard *et al.* 1998). At the end of the settlement period, the plates were then transplanted to a site where the temperature and/or desiccation differences between the intertidal microhabitats are extensive and have been previously characterized (the west shore of Hodgson Island, Damariscotta River Estuary; Schmidt & Rand 1999). Each settlement plate was randomly assigned to a tidal height (high or low) and an experimental treatment (exposed, artificial shade or algal canopy) for a total of ten replicates in each of six treatment combinations. In the exposed (X) treatment, the plates were drilled to bare areas of the substrate where the barnacles had no protection from environmental stress. The plates in the algal (A) treatment were placed beneath a surrounding cover of *A. nodosum*, which acted as a buffer against thermal and desiccation stress. In the shade (S) treatment, each plate was anchored beneath a steel cage–marine culture cloth enclosure. The experimental shades were designed to subject the barnacles to an intermediate level of thermal stress.

The treatments in the high intertidal zone (H) were positioned towards the upper limit of barnacle distribution (*ca.* 2.5 m above the mean low tide) and the treatments in the low intertidal zone (L) were similarly placed near the lower distributional limit (*ca.* 0.1 m above the mean low tide). All treatments were randomly spread over a 50 m transect at each tidal position. Temperature data loggers (Onset Computer Corporation, Pocasset, MA, USA) were used to quantify the substrate temperature four times an hour for 30 days in two replicates of each treatment combination. Natural patterns of mortality occurred over the experimental period, from 20 April to 2 July 1996.

(b) *Genotyping and fitness estimates*

Immediately prior to transplantation, ten replicate samples of 100 barnacles were randomly sampled from the plates to determine their initial genotype frequencies. At the end of the experiment, 75 barnacles from each settlement plate were collected to determine their post-transplant frequencies. For both the pre- and post-transplant samples, each individual's genotype was determined for the *Mpi* and *Gpi* loci and the mtDNA control region according to the methodology outlined in Schmidt & Rand (1999). For each genotype in each treatment combination, viability was calculated from the mean change in frequency over the experimental period (Spiess 1989). A two-way analysis of variance was used to evaluate the effects of tidal height, treatment and the interaction term on the pre- to post-transplant change in the arcsine square-root-transformed genotype frequencies. Separate analyses were performed for each locus.

For the natural habitats sampled in the 1994 and 1995 generations, viability was calculated as above. The genotype frequencies in larval samples (less than one day old) collected from the various habitat types were used as pre-selection estimates. The post-selection genotype frequencies have been published elsewhere (Schmidt & Rand 1999).

(c) *Maintenance of polymorphism*

The estimated fitness values for the genotypes in the six treatment combinations were used to determine whether a stable

Table 1. *Observed genotype frequencies*

(Mean frequencies and the standard deviations are given for the ten replicates in each treatment combination.)

			Mpi				*Gpi*				mtDNA		
sample	tidal height	treatment	*n*	SS	SF	FF	*n*	SS	SF	FF	*n*	A	B
pre-transplant	—	—	741	0.158	0.479	0.363	762	0.507	0.420	0.074	509	0.529	0.471
pre-selection 1994	—	—	542	0.161	0.461	0.378	571	0.543	0.382	0.075	322	0.556	0.444
pre-selection 1995	—	—	562	0.155	0.475	0.370	540	0.502	0.422	0.076	291	0.519	0.481
transplant	H	X	671	0.075	0.357	0.568	677	0.527	0.429	0.046	504	0.533	0.467
			—	0.020	0.028	0.041	—	0.048	0.043	0.018	—	0.024	0.024
	H	S	629	0.160	0.455	0.386	738	0.513	0.419	0.068	516	0.508	0.492
			—	0.043	0.079	0.097	—	0.053	0.058	0.024	—	0.055	0.055
	H	A	622	0.164	0.430	0.406	654	0.530	0.368	0.103	542	0.554	0.446
			—	0.049	0.067	0.061	—	0.068	0.073	0.040	—	0.040	0.040
	L	X	675	0.144	0.432	0.424	721	0.544	0.378	0.079	523	0.535	0.465
			—	0.033	0.071	0.066	—	0.040	0.068	0.055	—	0.028	0.028
	L	S	702	0.156	0.452	0.392	732	0.524	0.417	0.058	563	0.524	0.476
			—	0.038	0.063	0.069	—	0.050	0.044	0.034	—	0.018	0.018
	L	A	721	0.192	0.450	0.359	766	0.527	0.402	0.071	520	0.571	0.429
			—	0.031	0.051	0.059	—	0.058	0.052	0.026	—	0.063	0.063

Table 2. *Two-way ANOVA for the pre- to post-transplant change in arcsine square-root genotype frequency*

		Mpi-SS		*Mpi*-FF		*Gpi*-SS		*Gpi*-FF		mtDNA-A	
source	d.f.	SS	*F*	SS	*F*	SS	*F*	SS	*F*	SS	*F*
tidal height	1	0.036	13.62^{***}	0.059	12.18^{***}	0.001	0.31	0.001	0.12	0.001	1.32
treatment	2	0.111	21.10^{***}	0.168	17.45^{***}	0.003	0.45	0.027	2.74	0.001	0.05
tidal height × treatment	2	0.035	6.64^{*}	0.060	6.18^{*}	0.001	0.16	0.031	3.17	0.001	0.52

$^{*}p<0.05$, $^{***}p<0.001$.

polymorphism would be predicted by the Levene (1953) model of balancing selection. Assuming that the genotypic fitnesses were constant within and variable between niches (treatment combinations or habitats), the net fitness of the population was calculated as the geometric mean of fitnesses over k niches:

$$\overline{W} = (\overline{W}_1)^{c_1}(\overline{W}_2)^{c_2}\ldots(\overline{W}_k)^{c_k}, \quad (1)$$

where $\overline{W}_k$ is the average fitness in niche k and c_k is the proportion of the population which exists in niche k. The equilibrium allele frequency (or frequencies) were determined by solving

$$0 = \sum c_i \frac{(ps_1 - qs_2)_i}{\overline{W}_i}, \quad (2)$$

where $s_1 = 1 - W_1$, $s_2 = 1 - W_3$, W_1 is the niche-specific fitness of the AA homozygote standardized by the fitness of the heterozygote, W_2 is the standardized fitness of the Aa heterozygote (1.0), W_3 is the standardized fitness of the aa homozygote, p and q are the allele frequencies, $\overline{W}_i$ is the average fitness of the population in the ith niche and c_i is defined as the proportional reproductive output of the population occupying the ith niche (Spiess 1989). For a given set of habitat proportions, the stability of any calculated equilibrium allele frequency was evaluated by plotting the net fitness of the population over all niches as a function of the frequency of a specified allele (Li 1955). If the equilibrium point represented a local fitness maximum, such that selection would restore any deviation in allele frequencies, the polymorphism was considered stable.

3. RESULTS

(a) *Fitness variation between treatments*

The mean genotype frequencies for the three loci in each treatment combination are given in table 1. The mtDNA haplotype frequencies were essentially homogeneous between treatments at each tidal height and did not change over the course of the experiment (table 2). A similar pattern can be seen for *Gpi* (tables 1 and 2).

As predicted, the *Mpi* genotypes demonstrated a strong response to the experimental manipulation (table 2). The significant interaction between tidal height and treatment on the frequencies of the two *Mpi* homozygous genotypes can be seen in figure 1: increasing environmental stress was associated with an increase in the frequency of the *Mpi*-FF homozygote but a decrease in the frequency of the *Mpi*-SS homozygote. Of particular importance are the direction and degree of change in the two treatment combinations which represent the low stress–high stress ends of the environmental gradient (LA and HX, respectively). The frequency of the *Mpi*-SS genotype increased in the LA treatment ($p<0.01$, one-tailed test), but decreased in the HX treatment ($p<0.001$, one-tailed test). However, the frequency of the alternative homozygote (*Mpi*-FF) did not

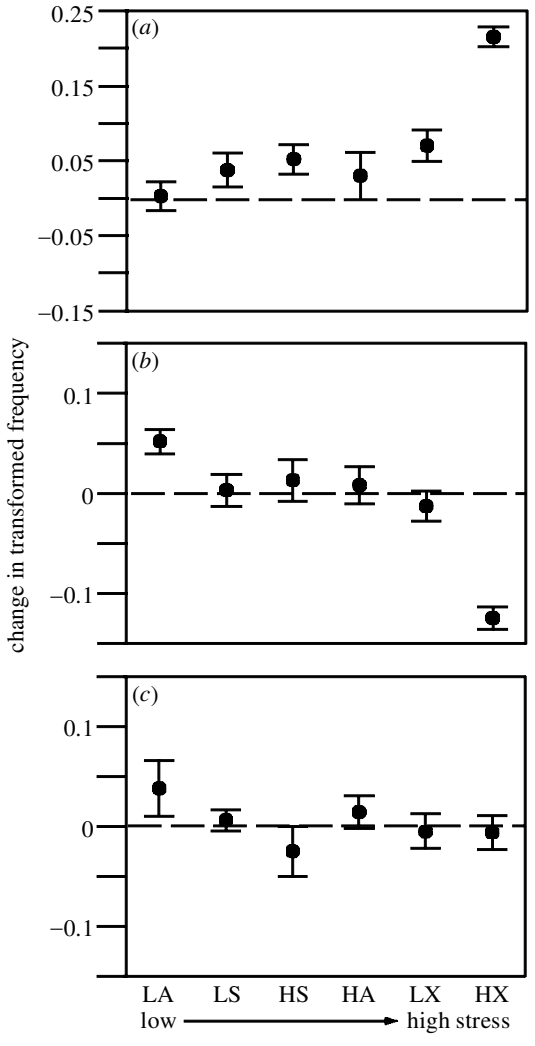

Figure 1. Mean (±s.e.) change in the arcsine square-root-transformed frequencies for the (*a*) *Mpi*-FF, (*b*) *Mpi*-SS and (*c*) mtDNA-A genotypes in the six treatment combinations. The first letter on the *x*-axis refers to tidal height (H or L) and the second to treatment (X, A or L). The *x*-axis is ordered by increasing level of thermal and/or desiccation stress, as estimated from the temperature profiles of each treatment combination.

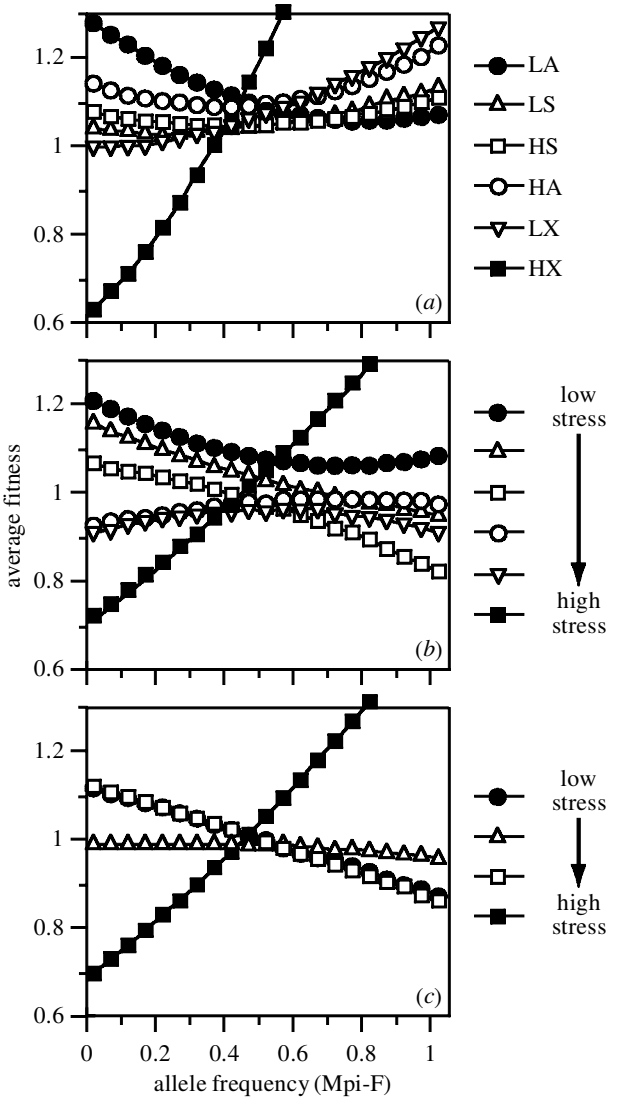

Figure 2. Average fitness ($\overline{W}$) as a function of the *Mpi*-F allele frequency in (*a*) the six treatment combinations in the transplant, (*b*) the six habitat types sampled in 1994 and (*c*) the four habitat types sampled in 1995. In all panels, the treatments and/or habitats are ordered by increasing level of temperature and/or desiccation stress.

change in the LA treatment ($p > 0.10$, one-tailed test), but increased in the HX treatment ($p < 0.001$, one-tailed test). Thus, the LA and HX treatments had very different effects on the survivorship of the two homozygous *Mpi* genotypes.

(b) *Evaluation of the Levene (1953) model*

The average fitness curves for the experimental treatments are given in figure 2*a* and the viability of the *Mpi*-SS and *Mpi*-FF genotypes in each treatment can be inferred from the average fitness value when the frequencies of the *Mpi*-F allele are 0 and 1, respectively. A general pattern of underdominance is evident, with one homozygote or the other being the highest fitness genotype in each of the six treatments. Due to selection for alternative homozygotes in alternative environments, the average fitness is least variable between environments when the allele frequencies are at intermediate values. The question of interest is whether the net fitness across all environments is maximized when both the *Mpi*-S and *Mpi*-F alleles are present. For the transplant data, there are many combinations of habitat proportions which result in a non-trivial predicted equilibrium point ($0 < q < 1$), such that the selective pressures in the various treatments are perfectly balanced. However, despite the habitat-specific variation in selection pressures none of the equilibria are stable. If the calculated viabilities were constant over time, one allele or the other would be predicted to sweep to fixation.

The viability of the *Mpi* genotypes appears to vary temporally as well as spatially, as indicated by the differences between the fitness curves for the treatments in the transplant and for the natural habitats sampled in the two preceding generations (figure 2). In addition, the general pattern of underdominance evident in the experimental treatments is less frequent in the natural habitats. However, the patterns of genotypic fitness variation between the sampled habitats, which exhibited similar

variation in level of environmental stress, are similar to those evident in the experimental treatments. Unlike the transplant data, the Levene (1953) model predicts several stable equilibria for the preceding two generations whose specific values depend on the particular combinations of habitat proportions used. Taking the simplest scenario in which the reproductive output of each habitat is equal, the predicted equilibrium allele frequency represents a local fitness maximum in both generations. Given the large selective differences between habitats, the equilibria are stable over a relatively wide (but still very restrictive) range of habitat proportions. Thus, in at least some portion of the entire parameter space, the variation in selective regimes between intertidal habitats predicts maintenance of allelic variation at the *Mpi* locus.

4. DISCUSSION

The selection pressures in the intertidal zone may vary over a scale of a few metres, resulting in locus-specific genetic differentiation between habitats (Johannesson *et al.* 1995; Schmidt & Rand 1999). The different responses exhibited in this experiment by *Mpi* and the mtDNA marker, which is assumed to be neutral, strongly suggest that selection is operating specifically at the *Mpi* linkage group. Based on previous results (Schmidt & Rand 1999), it was predicted that the effects on the *Mpi* genotype frequencies would be graded by the degree of thermal and/or desiccation stress experienced by the barnacles in the various treatment combinations. The experimental results are consistent with this prediction, although many environmental factors certainly covaried with the level of physical stress (e.g. ultraviolet exposure and the presence or absence of other members of the intertidal community whose distributions are similarly affected).

The neutral patterns evidenced by the *Gpi* genotypes and non-neutral patterns of the *Mpi* genotypes are provocative, considering that the enzymatic products of both loci are glycolytic isomerases which share fructose-6-phosphate as a substrate. Although the present data does not demonstrate that the *Mpi* locus itself is the true target of selection, a potential mechanism of selection at this locus may result from the impact of physiological stress on mannose metabolism. Mannose and related compounds are present at relatively high levels in the various algal groups represented in the plankton (e.g. Kreger 1962; Craigie 1974) and studies of mannose metabolism in other organisms have demonstrated the effects of mannose-6-phosphate isomerase activity on energy pools and survivorship when mannose is an appreciable dietary component (Arnold *et al.* 1974; De la Fuente *et al.* 1986; Hernandez & De la Fuente 1989).

The results presented here show a remarkable fit to the Levene (1953) model of balancing selection. Barnacles recruit throughout the intertidal zone on an annual basis, suggesting that any existing genetic differentiation between habitats is reset and re-established in each cohort. If both *Mpi* alleles are present in the population, the data indicate that each homozygous genotype will experience the highest relative survivorship in a substantial proportion of the intertidal environment. Given the extremely restrictive nature of the Levene (1953) model (Maynard Smith & Hoekstra 1980), the demonstration of predicted stable equilibrium allele frequencies indicates that spatial variation in fitness may be essential in maintaining the *Mpi* polymorphism in this species.

Because *S. balanoides* individuals must mate with neighbours, mating is assortative by habitat. The differential survivorship of genotypes occurs before the time of first reproduction and, thus, mating is also effectively assortative by *Mpi* genotype. Although the reproductive output of all habitats is assumed to be randomized in the plankton, assortative mating increases the likelihood of achieving a balanced polymorphism (Maynard Smith 1966), as do large selective differences between genotypes and a finely partitioned and coarse-grained environment (Maynard Smith & Hoekstra 1980).

Habitat heterogeneity at larger spatial scales, such as between coastal and estuarine populations or northern and southern biogeographical regions, may also contribute to the maintenance of polymorphism by natural selection in this system. *Mpi* is strongly polymorphic in every barnacle species investigated, a pattern which applies to crustaceans in general (Hedgecock *et al.* 1982). Habitat-specific selection on *Mpi* genotypes has also been documented in another barnacle (Hedgecock 1986) and nine amphipod species (Siegismund 1985; McDonald 1987, 1991), suggesting that polymorphism at the *Mpi* locus may be adaptive in many marine environments.

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