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The adaptive role of *Phosphoglucomutase* and other allozymes in a marine snail across the vertical rocky-shore gradient

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Natural selection can play an important role in the maintenance of genetic polymorphisms, despite ongoing gene flow. In the present study, we use previously analysed allozymic loci and perform an F_{ST} outlier-based analysis to detect the signatures of divergent selection between sympatric ecotypes of the marine snail *Littorina saxatilis* at different localities. The results obtained show that different allozyme polymorphisms are affected (directly or indirectly) by selection at distinct geographical regions. The *Phosmogluco mutase-2* locus was the best candidate for adaptation and further biochemical analyses were performed. The kinetic properties of the three more common genotypes of *Pgm-2* were studied. The results obtained are concordant with two alternative hypotheses: (1) natural selection is acting directly on this locus or, more probably, (2) selection is affecting a genomic region tightly linked to the enzyme locus. In both cases, the known existence of a parallel and partially independent origin of these ecotypes would explain why different candidate loci were detected in different localities. *Biological Journal of the Linnean Society*, 2009, **98**, 225–233.

ADDITIONAL KEYWORDS: candidate loci – divergent natural selection – F_{ST} – kinetic properties – outliers.

INTRODUCTION

Biologists have been traditionally impressed with the existence of natural polymorphisms because they can be used as models to study the evolutionary forces underlying the maintenance of the genetic variation in the wild (Endler, 1986). There is a large controversy about the causal mechanisms explaining natural variation in allozymes. Some meta-analyses have shown that, as a whole, allozymic polymorphisms behave more like neutral than selective variation (Kimura, 1991; Merilä & Crnokrak, 2001). However, a large number of studies have also found that some of the alleles segregating in various allozymic loci and species were adaptive (Hedrick, Ginevan & Ewing, 1976; Nevo, Shimony & Libni, 1977; Kohen, Zera & Hall, 1983; Johannesson, Johannesson & Lundgren, 1995; Powers & Schulte, 1998; Panova & Johannesson, 2004). It is possible, however, that natural selection is affecting genomic regions tightly linked to the allozyme locus under study (Endler, 1986), giving the wrong impression that it directly affects the allozyme. Moreover, the observed kinetic differences of a particular metabolic allele/genotype in laboratory conditions could be irrelevant *in vivo*; for example, if the enzyme lacks of any regulatory property on its metabolic pathway (Hochachka & Somero, 2002; Somero, 2004). In summary, there is some consensus that allozymic polymorphism can be directly affected by natural selection in certain cases, although the problem can be to find convincing evidence, as well as to unravel the mechanism responsible. Moreover, even if natural selection is acting on allozymic polymorphisms directly, it can be produced by means of three different mechanisms (Somero, 2004): by (1) changes in the amino acid sequence of

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the protein producing adaptive variation; (2) changes in gene expression and protein turnover; and (3) changes in the cellular microenvironment where the protein functions. The latter can be produced if two different alleles are expressed on different genetic pools or on different environments.

Organisms living in habitats showing strong environmental gradients such as the rocky intertidal are, a priori, appropriate for finding cases where natural selection is responsible for the maintenance of some allozymic polymorphisms (Raffaelli & Hawkins, 1996). *Littorina saxatilis* is a widespread intertidal marine snail. It is highly polymorphic and ecotypic variation can be found within a few meters. This is partly a result of its nonplanktonic development and the low mobility of the adults (Reid, 1996; Rolán-Alvarez, 2007). Two ecotypes of *L. saxatilis* are found in the exposed rocky shores of Northwest Spain, which are adapted to different shore levels (Rolán-Alvarez, 2007). The RB ecotype inhabits the upper shore. The SU ecotype, smaller in size and thin-shelled, is adapted to the heavily wave-exposed lower shore. The mid-shore represents an intermediate environment and both ecotypes are found in sympatry; at this level, there is ongoing gene flow via hybridization, despite partial assortative mating. Divergent natural selection is responsible for the maintenance of the RB-SU polymorphism (Rolán-Alvarez, 2007) and this is reflected at the genomic level (Galindo, Morán & Rolán-Alvarez, 2009). In the present study, we reanalyse previously made available allozymic data (Rolán-Alvarez *et al.*, 2004) with the aim of detecting signatures of selection within the studied allozyme loci by means of an F_{ST} outlier-based analysis. Then, we performed a biochemical study with *phosphoglucomutase-2*, which showed the strongest divergence between the ecotypes, aiming to unravel the kinetic properties of the different genotypes of *Pgm-2*.

MATERIAL AND METHODS

Experimental design and reanalysis of allozyme variation

We studied three different shores (*Corrubedo*; *Silleiro*, 52 km south of Corrubedo; *Cetarea*, 25 km south of Silleiro) from Northwest Spain. Four samples were collected within locality RB upper, RB mid, SU mid and SU lower (for further details, see Rolán-Alvarez *et al.*, 2004: fig. 1). This reanalysis of the 2003 allozyme data set was performed on 624 snails (22–30 per sample) analysed for six allozymic loci: leucine aminopeptidase (*Lap*; EC 3.4.1.1), phosphoglucose isomerase (*Pgi*; EC 5.3.1.9), aspartate aminotransferase-1 (*Aat-1*; EC 2.6.1.1), arginine kinase (*Ark*; EC 2.7.3.3), purine nucleoside phosphorilase (*Pnp*; EC 2.4.2.1), and phosphoglucomutase-2 (*Pgm-2*; EC 5.3.1.9). Allele frequencies were calculated using GENEPOP, version 3.4 (Raymond & Rousset, 1995).

Detection of outlier loci

We used an F_{ST} outlier-based analysis (Beaumont & Nichols, 1996; Beaumont, 2005) for detecting selection in our survey of allozymic polymorphism. Analyses were carried out in FDIST2 (http://www.rubic.reading.ac.uk/~mab/software.html), which generates a neutral null distribution of genetic differentiation (F_{ST}), conditional on heterozygosity (H_E), with mean F_{ST} similar to the empirical F_{ST}. This is a key parameter in the analysis because it is representative of the level of gene flow for neutral loci. This empirical F_{ST} was obtained from amplified fragment length polymorphism (AFLP) loci analysed for the same samples (Galindo *et al.*, 2009) because the number of allozyme loci was too low to have an unbiased non-selective measure of population differentiation. As suggested by Caballero, Quesada & Rolán-Alvarez (2008), a trimmed mean F_{ST} was used (Galindo *et al.*, 2009). Neutral F_{ST} distributions were obtained after simulating 50 000 loci. Allozyme loci significantly ($P < 0.01$) higher than the neutral null distribution are considered as outlier loci (i.e. candidate loci of adaptation). Two different types of pairwise comparisons were performed within each locality: 'between ecotypes', pooling transects and comparing upper versus lower and mid-RB versus mid-SU (these comparisons can be considered pseudoreplicates) and 'within ecotype', comparing transects within level (four levels: upper, mid-RB, mid-SU, and lower). We assume that outliers are more likely to be affected by divergent selection (directly or indirectly) when pseudoreplicated comparisons between ecotypes assign the same candidate loci. Although other interpretations are possible, outliers could be false positives (with an a priori expectation similar to the significance level used). We performed, a posteriori, an FDR multitest adjustment (Benjamini & Hochberg, 1995) using the SGoF software (http://webs.uvigo.es/acraaj/SGoF.htm).

Sampling and extracts preparation of *Pgm-2* genotypes

We collected new samples from our Silleiro site to study the *Pgm-2* activity because this locus showed the strongest differentiation between the ecotypes (Rolán-Alvarez *et al.*, 2004). We collected 104 RB specimens from upper shore and 94 SU from the lower shore. Each individual was genotyped for

Pgm-2 from a small piece of tissue (2 mm^2), the rest of the snail was maintained at –85 °C for further kinetic analysis of the phosphoglucomutase activity.

Conventional protein electrophoresis in horizontal starch gels was used for the allozyme analysis of *Pgm-2* (Rolán-Alvarez *et al.*, 2004). Six genotypes, the homozygotes of the three alleles (*Pgm-2*^{100}, *Pgm-2*^{110}, and *Pgm-2*^{115}) and their heterozygotes were present in the RB ecotype. However, only one genotype, *Pgm-2*$^{100/100}$, was found in the SU ecotype. Those RB individuals genotyped as *Pgm-2*$^{110/100}$ and *Pgm-2*$^{110/110}$, and those SU with genotype *Pgm-2*$^{100/100}$, were used for the enzyme activity analysis. Samples within genotype were pooled and the same amount of tissue (0.5 g), but with a different number of individuals because of differences in size between the ecotypes (30 RB individuals for *Pgm-2*$^{110/100}$ and *Pgm-2*$^{110/110}$ and 90 SU for *Pgm-2*$^{100/100}$), was used for each pool to measure the enzyme activity under the same conditions.

Kinetic characterization of *Pgm-2* genotypes

For the kinetic study of *Pgm*, the pooled tissues of each genotype were homogenated in five volumes (w/v) of ice-cold Tris-acetate buffer (40 mM; pH 7.0), containing imidazole, ethylenediaminetetraacetic acid and β-mercaptoethanol in a concentration of 5 mM, 10 mM NaF and 0.12 M KCl. The homogenate was centrifuged for 30 min at 15 400 ***g*** and 4 °C. The supernatants (enzyme extracts) were stored at –85 °C until used. Before the measurements of enzyme activity, the enzyme extracts were dialysed against 30 volumes of extraction buffer without KCl. The enzyme activity was assayed spectrophotometrically coupling the glucose-6-phosphate (G6P) production to the $NADP^+$ reduction, via G6P dehydrogenase (G6PDH) and measuring the increase of optic density at 340 nm because of NADPH formation (King, 1974). The reaction mixture contained 100 mM Tris-acetate buffer (pH 7.5), 0.1 M $MgCl_2$, 10 mM glucose-1-phosphate (G1P), 10 mM $NADP^+$, 50 μM glucose-1,6-diphosphate, 0.5 IU of G6PDH and the enzyme extract in a total volume of 1 mL. The unit of activity (IU) was defined as the amount of enzyme catalysing the formation of 1 μmol G6P in 1 min at 20 °C. A negative control without the substrate (G1P) was used to discard unspecific activities.

For each genotype, the kinetic parameters and optimum of pH and temperature were calculated. The apparent K_m and V_{max} values were estimated using Lineweaver–Burk double-reciprocal plots. Optimum pH was determined by *Pgm* activity at various pH values in the range 6.8–9.0 at 20 °C. Optimum temperature of enzyme was determined by *Pgm* activity at temperatures in the range 5–55 °C at pH 7.5. These data allow us also to calculate the energy of activation (E_a) from the Arrhenius plot of log (enzyme activity) versus $1/T$ and expressed as kJ/mol (Whitaker, 1972).

To determine enzyme thermal stability, the enzyme activity was assayed at 20 °C and pH 7.5 after incubation of enzymatic extract for 1 h to different temperatures in the range 5–55 °C. The rate of the *Pgm* stability response to 10 °C changes was quantified by calculating the temperature coefficient or Q_{10}. The temperature coefficient (Q_{10}) has been related to the degree of adaptation to temperature on its habitat (Hochachka & Somero, 1984, 2002); $Q_{10} > 2.0$ is considered to reveal evidence of important adaptative change; $Q_{10} = 1.0$ indicates an interval of thermal insensitive or temperature independence; whereas $Q_{10} < 1.0$ indicates lethal effects of high temperatures and an irreversible damage and loss of function (Hochachka & Somero, 2002).

Each data value corresponds to the mean of three determinations. Regression coefficients of Lineweaver–Burk and Arrhenius plots were in the range 0.95–0.99. Results are given as the mean ± SD. A sign test was used to test the differences between the continuous distributions of the variables assayed.

RESULTS

Outlier detection and clines in allele frequencies

The results of the outlier analysis with FDIST2 are summarized in Figure 1 showing the F_{ST}/H_E plots for between and within ecotype comparisons and for each one of the localities (for further results of the outlier analysis, see Supporting information, Appendix S1). Comparisons 'within ecotype' did not detect outliers, as expected from samples collected in similar habitats. However, for the comparisons 'between ecotypes', three loci (*Lap*, *Aat-1*, and *Pgm-2*; Fig. 1) fell outside the neutral expectations ($P < 0.01$) in both pseudoreplicates, and after FDR multitest correction. In Corrubedo, *Lap* (average $F_{ST} = 0.206$) was detected as outlier. *Aat-1* was detected in Silleiro (0.183) and *Pgm-2* in Silleiro (0.443) and Cetarea (0.256). *Lap* was also significant in Cetarea in one of the comparisons (mid-RB versus mid-SU), but the F_{ST} value was low (0.044) and this locus was located in the lower tail of the F_{ST}/H_E distribution, where the method loses accuracy. The consistency observed between the pseudoreplicates for the detection of outliers provides additional support for a role of natural selection in the maintenance of these polymorphisms. The clines for the alleles involved in the divergence between ecotypes for each one of the outlier loci (*Lap*, *Aat-1*, and *Pgm-2*) are presented in Figure 2. The steepest cline was observed for *Pgm-2* which also showed a

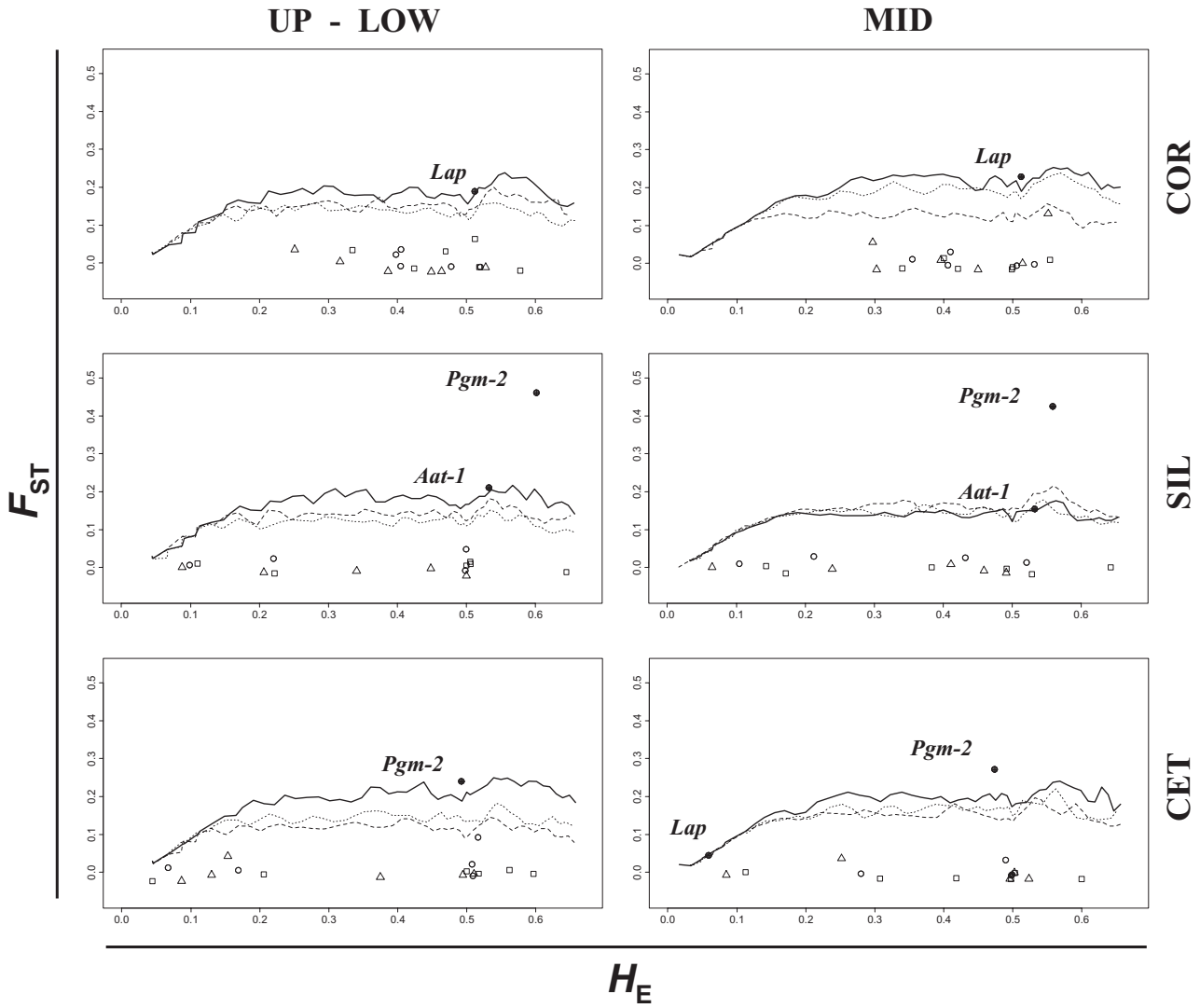

Figure 1. Summary of the F_{ST} outlier analysis showing the F_{ST}/H_E plots with the empirical allozyme data (points) and the 99 percentile of the neutral distribution (lines) for each level [upper versus lower and mid-RB (inhabits upper shore) versus mid-SU (smaller in size and thin-shelled, inhabits wave-exposed lower shore)] and locality (COR, Corrubedo; SIL, Silleiro; CET, Cetarea). Solid lines and circles represent the comparison between ecotypes (RB versus SU), dotted lines and squares comparisons within RB (transect 1 versus transect 2) and dashed lines and triangles comparisons within SU. Loci detected as outliers ($P < 0.01$; see Material and Methods) are represented by filled symbols. RB ecotype inhabits the upper shore.

significant ecotype differentiation in four of the six pairwise comparisons. This confirms that *Pgm-2* was the best choice for a detailed biochemical study of its genotypes.

KINETIC PARAMETERS OF *PGM-2* GENOTYPES

The kinetic parameters (K_m, V_{max}, and E_a) are presented in Table 1. *Pgm-2*$^{100/100}$ showed significant higher phosphoglucomutase activity (V_{max}) and affinity for the substrate (lower K_m) than *Pgm-2*$^{110/110}$ ($P < 0.01$). The heterozygote *Pgm-2*$^{110/100}$ showed intermediate values between both homozygotes in these parameters. Accordingly, we also observed significant differences for activation energy (E_a) between the homozygotes ($P < 0.01$). The lower value of E_a, estimated in the range 10–40 °C, was found in *Pgm-2*$^{100/100}$, with both *Pgm-2*$^{110/110}$ and the heterozygote showing very similar values (Table 1). On the other hand, the three genotypes studied did not differ significantly in their pH and temperature optimum (Fig. 3A, B), with values in the range pH 7.5–7.8 and 45–50 °C,

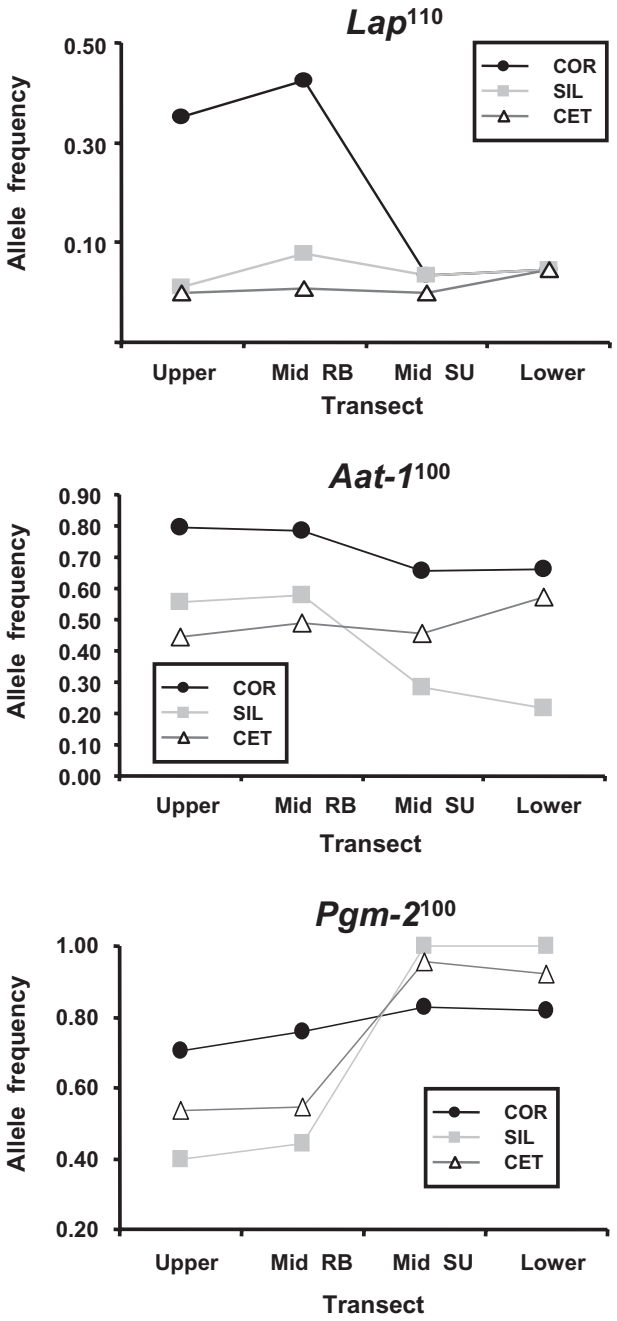

Figure 2. Alleles showing clines in frequency across the vertical shore gradient (upper, mid and lower-shore) for outlier loci (Lap^{110}, $Aat\text{-}^{1100}$, and $Pgm\text{-}^{2100}$) in the three studied localities (Corrubedo, Silleiro, and Cetarea). Mid-RB (inhabits upper shore) and mid-SU (smaller in size and thin-shelled, inhabits wave-exposed lower shore) represent sympatric populations and they were collected in the same shore level (mid-shore). COR, Corrubedo; SIL, Silleiro; CET, Cetarea.

Table 1. Kinetic parameters and energy of activation (E_a) of the different *Pgm-2* genotypes studied for *Littorina saxatilis*

Genotype	K_m (mM)	V_{max} (IU mL^{-1})	E_a (kJ mol^{-1})
110/110	0.050 ± 0.001	0.447 ± 0.002	13.61 ± 0.034
110/100	0.040 ± 0.003	0.645 ± 0.005	12.82 ± 0.030
100/100	0.032 ± 0.012	0.685 ± 0.011	10.33 ± 0.005

Values represent the mean ± SD over three determinations. K_m and V_{max} relative to substrate (glucose-1-phosphate); E_a calculated from Arrhenius equation in the range 10–40 °C (see Material and Methods).

respectively. Similarly, the thermal stability of the protein structure (Fig. 3C) was not significantly different between the studied genotypes ($P > 0.05$). However, there was a trend suggesting that $Pgm\text{-}2^{110/110}$ and $Pgm\text{-}2^{110/100}$ presented higher thermal stability than $Pgm\text{-}2^{100/100}$. The Q_{10} values corroborate this trend (Table 2): the genotype $Pgm\text{-}2^{100/100}$ showed values below 1 at temperatures higher than 25 °C, whereas $Pgm\text{-}2^{110/100}$ and $Pgm\text{-}2^{110/110}$ showed values near 1 until 30 and 35 °C, respectively. These results suggest that the allele $Pgm\text{-}2^{110}$, present in the upper shore, has a higher thermal stability and lower activity, which could be an adaptation to the environmental stresses present in this habitat (i.e. desiccation) because of the reduction in the metabolic rates.

DISCUSSION

Littorina saxatilis ecotype variation (i.e. RB and SU ecotypes in Spain, H and M ecotypes in the UK and E and S in Sweden) is becoming a key model system in evolutionary biology because of its implications in the study of local adaptation and speciation (Johannesson, 2003; Quesada *et al.*, 2007; Rolán-Alvarez, 2007). In the present study, we reanalyse allozymic data from Rolán-Alvarez *et al.* (2004) with the aim of detecting possible signatures of natural selection in this data set. Previous publications have already reported steep adaptive clines in allele frequencies for different allozyme loci in other *L. saxatilis* populations (Johannesson & Johannesson, 1989; Johannesson *et al.*, 1995; Johannesson & Tatarenkov, 1997; Panova & Johannesson, 2004) or in other organisms (Koehn, Hall & Zera, 1980; Anderson, McDonald & Santos, 1981; Watt, Donohue & Carter, 1996; Giokas, Pafilis & Valakos, 2005), although these authors did not use F_{ST} outlier-based analysis. We show that *Lap*, *Aat-1*, and *Pgm-2* could be potentially affected (i.e. directly or indirectly) by divergent natural selection in *L. saxatilis* populations from Northwest Spain. A similar result has been reported in *Aat* for this

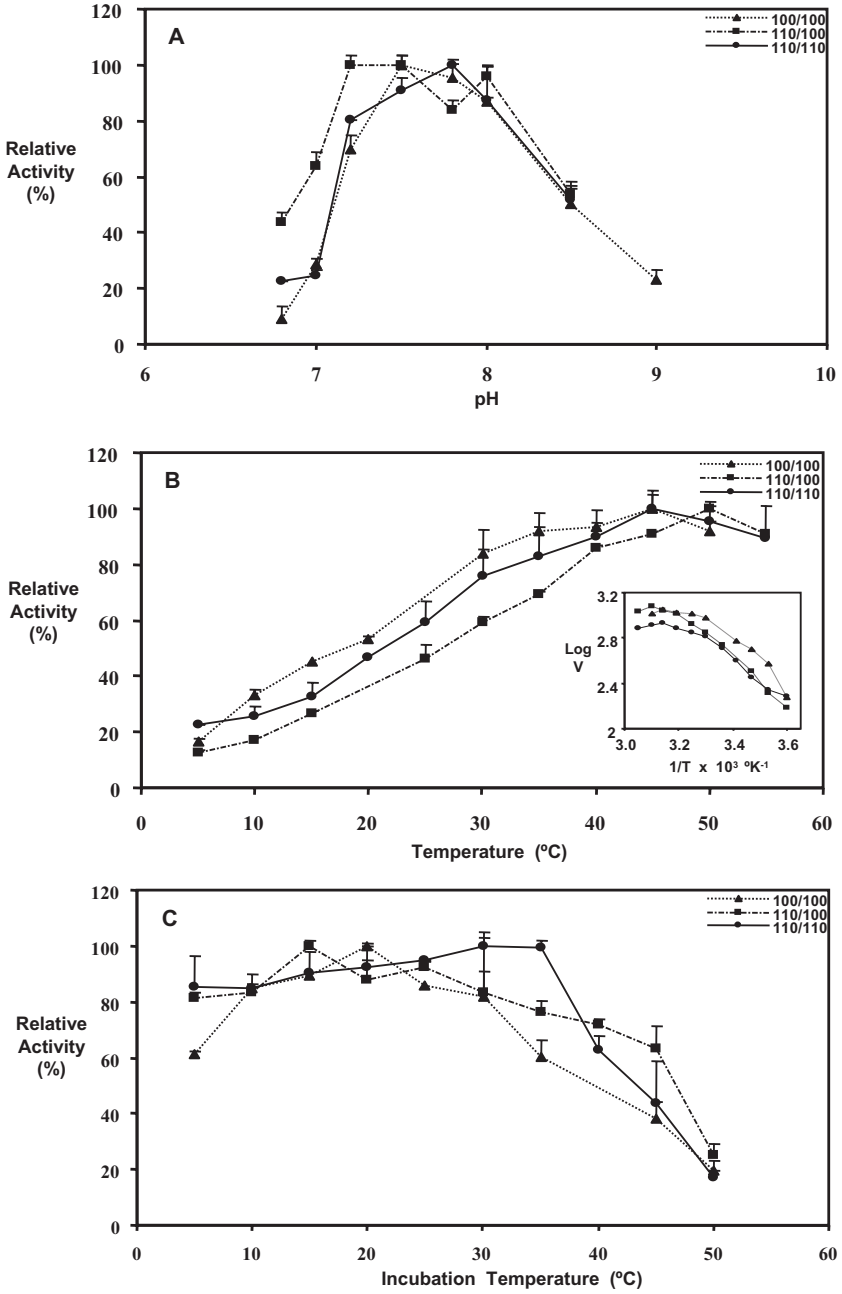

Figure 3. Effects of pH (A) and temperature (B) on the phosphoglucomutase activity and phosphoglucomutase stability (C) from all three *Pgm-2* genotypes studied from *Littorina saxatilis*. Data points represent the mean of triplicate determinations ± SEM.

species in populations from Sweden: the allele Aat^{100} is present preferentially on the lower shore, whereas the allele Aat^{120} is preferentially present on the mid shore (Johannesson & Johannesson, 1989; Johannesson *et al.*, 1995), although we found the opposite pattern in the present study (Fig. 2). Further investigations will be necessary to determine the causes of this different pattern. The detection of these three outlier loci is interesting for unravelling the mechanisms maintaining this polymorphism, but additional interest stems from the fact that different allozyme loci are being affected by natural selection in different localities. The same pattern has been already reported in the same populations by means of outlier AFLP loci (Galindo *et al.*, 2009), where geographically distant localities presented different outlier loci, whereas closer localities (i.e. Silleiro and Cetarea) presented a greater proportion of overlapping outli-

Table 2. Q_{10} values of the studied genotypes for *Pgm-2* in *Littorina saxatilis* populations

Temperature (°C)	Genotype		
	110/110	110/100	100/100
5–15	1.06 ± 0.006	1.23 ± 0.044	1.45 ± 0.049
10–20	1.09 ± 0.006	1.05 ± 0.005	1.18 ± 0.005
15–25	1.05 ± 0.014	1.00 ± 0.010	0.96 ± 0.024
20–30	1.08 ± 0.015	0.98 ± 0.022	0.82 ± 0.029
25–35	1.04 ± 0.009	0.85 ± 0.017	0.70 ± 0.027
30–40	0.86 ± 0.010	0.63 ± 0.007	0.50 ± 0.004
35–45	0.63 ± 0.005	0.61 ± 0.010	0.33 ± 0.003
40–50	0.35 ± 0.005	0.25 ± 0.002	–

Measures performed within a temperature range of 5–50 °C and values represent the mean ± SD over three determinations.

ers, as in the present study. This pattern supports the interpretation that natural selection could be working partially independently at the most distant localities. Quesada *et al.* (2007) already proposed a local origin of these ecotypes and, in parallel, at distant localities after performing an mitochondrial DNA phylogeny of RB and SU populations from a similar geographical area. The results of the present study, in addition to the AFLP outliers (Galindo *et al.*, 2009), strengthen support for the hypothesis of independent and parallel divergence of the ecotypes.

A different problem is how natural selection can produce a pattern of allelic variation such as this. One possibility would be that natural selection is acting directly over the allozyme locus but a similar adaptation is achieved through different loci in different localities as a result of the local effect of natural selection. The second possibility is that the allozyme locus is not the target of selection but it is tightly linked to the real target (a different gene). In this case, it is difficult to determine whether the locus under selection is the same or different between localities because both hypothesis can give similar results. The greatest differentiation between ecotypes was observed for *Pgm-2*, and therefore we compared the enzymatic properties of the different genotypes aiming to shed light on the evolutionary mechanisms maintaining such differentiation in sympatry.

Activation energy of the three genotypes studied for *Pgm* in *L. saxatilis* was relatively low compared to the same enzyme in other organisms (Harsman *et al.*, 1965; Hashimoto *et al.*, 1967; Dawson & Jaegers, 1970), suggesting a high efficiency in this organism. However, we also found significant differences between the three genotypes in relation to this parameter (E_a), which could be sufficient to justify the differences observed in K_m (affinity for its substrate) and V_{max}. On the other hand, the genotype characteristic from the lower shore (homozygote *Pgm-2*$^{100/100}$) showed a higher catalytic efficiency (minor E_a and K_m and higher V_{max}) but lower thermal stability in comparison with the homozygote *Pgm-2*$^{110/110}$. This minor catalytic efficiency but higher thermal stability of the genotypes found in the upper shore (*Pgm-2*$^{110/110}$ and *Pgm-2*$^{110/100}$) could be interpreted as an enzymatic adaptive strategy to the stresses (i.e. high temperatures) that are characteristic of this habitat. This result is similar to that reported by Panova & Johannesson (2004) for *Aat*, and as previously noted by McMahon, Russell-Hunter & Aldridge (1995), a reduction in the metabolism is found in gastropods during emergence stress to avoid water-loss and save energy and resources.

The temperature coefficient (Q_{10}) has been related to the degree of adaptation to temperature on its habitat (values > 1 suggesting an increase of efficiency, whereas values < 1 indicate inefficiency of function; Hochachka & Somero, 1984, 2002). The Q_{10} values of the genotypes *Pgm-2*$^{110/100}$ and *Pgm-2*$^{110/110}$ showed their stability at temperatures up until 30–35 °C, where the genotype *Pgm-2*$^{100/100}$ showed an important loss of its function. This may suggest a certain contribution to adaptation to high temperature across the vertical environmental gradient, as described in other poiquilothermous intertidal molluscs under temperature conditions higher than 38 °C (His, Robert & Dinet, 1989).

The action of natural selection on allozymic polymorphisms can be produced by three distinct mechanisms (Somero, 2004): by affecting the protein sequence, the protein expression, or the cellular microenvironment. In the present study, we have detected how changes in the amino acid sequence (i.e. alleles with different electrophoretic mobility) can produce differences of activity in the laboratory, which could have an adaptive interpretation *in vivo*. This will require of future attention and perhaps suggests, at least for *Pgm-2*, the relative importance of the first mechanism suggested by Somero during adaptation. Another complication, in the case of *Pgm*, is the lack of a key regulatory function on its metabolic pathway (Hochachka & Somero, 2002; Somero, 2004), making less likely a primary role of this locus in adaptation. Taking into account all these arguments, perhaps the high F_{ST} values obtained for this locus in two localities could be partially explained by its physical linkage to another unknown candidate locus. Future directions in the study of *Pgm* in these populations of *L. saxatilis* could focus on the sequence variation at this locus and the surrounding genomic regions. This may reveal new insights into the molecular basis of ecological adaptation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Results of the F_{ST} outlier analysis between ecotypes of *Littorina saxatilis* using FDIST2. Analysis per locality and level (upper versus lower and mid-RB versus mid-SU). F_{STemp}, trimmed mean F_{ST} obtained from amplified fragment length polymorphisms (see Material and Methods), an estimation of the empirical neutral differentiation. F_{STsim}, mean F_{ST} obtained from the simulations. $F_{STallozymes}$, genetic differentiation calculated from six allozyme loci. F_{ST} values per allozyme locus are shown.