

Small-scale clinal variation, genetic diversity and environmental heterogeneity in the marine gobies *Pomatoschistus minutus* and *P. lozanoi* (Gobiidae, Teleostei)

ES Gysels, V Leentjes, and FAM Volckaert

Laboratory of Aquatic Ecology, Katholieke Universiteit Leuven, Ch. de Bériotstraat 32, B-3000 Leuven, Belgium

Genetic variation was assayed at 14 allozyme loci in estuarine, coastal and offshore samples of lozano's goby, *Pomatoschistus lozanoi* and the sand goby, *P. minutus*. Samples were taken from locations on the Belgian Continental Shelf and in the Schelde estuary with a range of environmental heterogeneity. We evaluate whether any differences in (1) the degree of genetic variation and (2) allele frequencies at the various loci exist within samples occurring in various habitats on the BCS and in the Schelde estuary. No significant differences in levels of genetic

diversity were recorded between estuarine, coastal and offshore samples in either species. A temporally stable clinal gradient in allele frequencies at the two-allele locus *GPI-A** was observed in *P. lozanoi*, differentiating the samples in an estuarine, coastal and offshore group. We suggest that these differences might be maintained by balancing selection at locus *GPI-A**.

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Introduction

The adaptive significance of allozymatic polymorphisms has been the subject of a heated debate for years, until it waned in the early 1990s with the advance of DNA techniques (Hey, 1999). Allozyme-based population surveys had revealed high levels of polymorphism with no detectable effects on the phenotype of the organisms, suggesting that the majority of the variation found at the molecular level was selectively neutral (Kimura, 1979). DNA data at first seemed to confirm this hypothesis, with noncoding DNA evolving much faster and containing a higher amount of variability than coding regions. But while this neutral theory of evolution continues to provide a useful null hypothesis against which to test population genetic differentiation, genetic studies of natural populations have yielded convincing evidence that a number of polymorphisms are probably maintained by natural selection at the level of allozymes (Eanes, 2002 and references herein), as well as DNA markers (Ballard and Kreitman, 1995). Both large- and small-scale gradients in allele frequencies have been reported for a wide range of organisms (eg Koehn *et al.*, 1983 and references herein), suggesting environmental selection pressure, with variability in temperature and (for marine species) salinity being considered as main selective agents.

Gobies of the genus *Pomatoschistus* (Gill) are among the most abundant fish species along the northeastern Atlantic coasts and have been widely used in ecological, evolutionary and behavioural studies (Fonds, 1973; Pampoulie *et al.*, 1999; Jones *et al.*, 2001). An electrophoretic survey of Atlantic and Mediterranean members of the genus *Pomatoschistus* occurring in habitats with a different degree of heterogeneity showed a positive correlation between the level of genetic variation within species and the degree of heterogeneity of the habitat in which each species preferentially occurs (Wallis and Beardmore, 1984a). In this study, we present the results of an allozyme survey of two of these species, the sand goby, *Pomatoschistus minutus* (Pallas, 1770) and lozano's goby, *P. lozanoi* (de Buen, 1923), carried out on the Belgian Continental Shelf (BCS) and in the Schelde estuary. These two gobies are closely related and show a similar mode of reproduction. During the breeding season males of either species display nuptial colours, territorial behaviour, nestbuilding and nest-guarding behaviour. After hatching the larvae are swept out of the nest and drift in the plankton for about a month before settling down for a demersal lifestyle (Fonds, 1973). *P. minutus* is more of a generalist in its habitat and food choice than *P. lozanoi* (Hamerlynck and Cattrijsse, 1994), and also has the broadest distribution, occurring in estuaries, coastal areas and offshore. (Miller, 1986). *P. lozanoi* is more neritic (Fonds, 1973), although at times large numbers occur in estuaries as well (Claridge *et al.*, 1985; Maes *et al.*, 1998). Wallis and Beardmore (1984a) reported significantly lower levels of heterozygosity in *P. lozanoi* than in its estuarine–marine relative *P. minutus*,

Correspondence: ES Gysels, Laboratory of Aquatic Ecology, Katholieke Universiteit Leuven, Ch. de Bériotstraat 32, B-3000 Leuven, Belgium.

E-mail: els.gysels@bio.kuleuven.ac.be

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but it was unclear whether this could be explained by either heterozygote advantage or else diversifying selection. If habitat heterogeneity promotes overall levels of polymorphism, then we expect populations of a species occurring in a heterogeneous environment to show higher levels of genetic diversity than those inhabiting more stable environments (Powell, 1971). Alternatively, a single locus under selection might show differences in allele frequencies as expected under the multiple niche model of balancing selection (Levene, 1953), when comparing populations across a heterogeneous environment subjected to differential selection pressure. Such a heterogeneous environment exists on the BCS, situated in the Southern Bight of the North Sea. The BCS is characterised by a system of several groups of sand banks alternating with gullies, which are continuously swept by strong tidal currents. A long-shore and inshore/offshore gradient under the influence of the highly turbid and heterogeneous Schelde estuary is reflected in a number of abiotic factors such as temperature, salinity, turbidity and nutrient concentrations (Nihoul and Hecq, 1984).

Hence, our aim is to assess possible effects of natural selection on the maintenance of genetic diversity and allele frequencies within two *Pomatoschistus* species occurring in a gradient of environmental heterogeneity. We evaluate whether any differences in (1) the degree of genetic variation and (2) allele frequencies at the various loci exist within samples occurring in various habitats on the BCS and in the Schelde estuary.

Materials and methods

Sampling

Sampling on the BCS was carried out with R/V 'Belgica' along an inshore/offshore gradient on the Coastal Banks (Stroombank), Flemish Banks (Kwintebank) as well as offshore banks (Bligh Bank, Noordhinder and Oosthinder). In addition, a number of governmental monitoring stations (MUMM stations) on the BCS were trawled. Fish were caught with a beam trawl (width: 3 m) and an outer net mesh size of 20 mm stretched and a cod end mesh size of 12 mm. The samples from the Schelde estuary were taken in Doel near the city of Antwerp at the cooling water intake of a nuclear power plant (Figure 1). Mesh size of the nets used was 4 mm. For comparison a sample of *Pomatoschistus lozanoi* and *P. minutus* caught at the Frisian Front (North of the Wadden Sea) was subjected to allozyme electrophoresis as well.

Allozyme electrophoresis

The samples were frozen on dry ice or liquid nitrogen immediately after capture and kept in a -80°C freezer until analysis. Gobies were identified morphologically on the basis of the dermal papillae of the head according to Miller (1986) and biochemically according to Wallis and Beardmore (1984b). As trawling in winter and spring yielded fewer gobies than during autumn, almost all fish from the winter samples were analysed, while a random subsample of *Pomatoschistus* sp caught in autumn was used. Liver, eye and muscle tissues were dissected and ground in 50–150 μl of distilled water depending on the amount of tissue. The samples were



Figure 1 Sampling locations and codes of the sampling sites of *P. minutus* and *P. lozanoi* on the BCS and in the Schelde estuary. Triangles: offshore samples; Squares: coastal samples; Circle: estuarine sample. For the abbreviations, see Table 2.

subjected to cellulose acetate allozyme electrophoresis (Richardson *et al*, 1986). Two buffer systems were used: Tris-maleate (pH = 7.8) and Tris-glycine (pH = 8.8) as described by Hebert and Beaton (1989) (Table 1). Loci were stained according to recipes described by Hebert and Beaton (1989) and Richardson *et al* (1986). The slowest migrating locus was designated 1 or A according to the nomenclature of Shaklee *et al* (1990). The most common allele in *P. minutus* at each locus was called '100' and all the other alleles were classified according to their mobility relative to allele 100 of the locus under study.

Data analysis

Genetic diversity was estimated as observed and unbiased expected heterozygosity (Nei, 1978) and calculated in the GENETIX version 4.0. (Belkhir *et al*, 2001). A locus was considered polymorphic when the frequency of its most common allele did not exceed 0.99 in at least one sample. Samples were tested for linkage disequilibrium and deviations from Hardy–Weinberg proportions in the GENEPOP version 3.1 (Raymond and Rousset, 1995). F_{ST} values (Weir and Cockerham, 1984) were calculated in GENETIX and significance was assessed with permutation tests (1000 replicates). Multidimensional scaling analysis (MDSA) was applied on a matrix of pairwise F_{ST} values for both species in order to assess whether any geographical clustering of the samples could be found. Individual heterozygosity was computed as the number of heterozygous loci per individual. Samples were grouped into 'coastal', 'estuarine' and 'offshore' habitats (Figure 1, Table 2) to assess whether any significant differences in the level of heterozygosity could be found between habitats. A Kruskal–Wallis ANOVA was performed with the number of hetero-

Table 1 *P. minutus* and *P. lozanoi*: summary of the 15 loci examined including the enzyme code (E.C.), the tissue collected and the buffer used to separate the alleles at the different loci

Name	E.C. nr	Locus	Tissue	Buffer
Lactate dehydrogenase	1.1.1.27	LDH-A*	Eye, muscle	TM/TG
		LDH-B*	Eye	TM/TG
		LDH-C*	Eye	TM/TG
Malate dehydrogenase	1.1.1.37	MDH-A*	Muscle	TM
		MDH-B*	Muscle	TM
Phosphoglucosmutase	2.7.5.1	PGM-1*	Eye, muscle	TM/TG
		PGM-2*	Eye, muscle	TM
Phosphoglucose isomerase	5.3.1.9	GPI-A*	Eye, muscle	TM/TG
		GPI-B*	Eye, muscle	TM/TG
		IDHP-1*	Muscle	TM
Aspartate aminotransferase	2.6.1.1	AAT*	Muscle	TM
Adenlyate kinase	2.7.4.3	AK*	Muscle	TM
Creatine kinase	2.7.3.2	CK-A*	Muscle	TG
Fumarate hydratase	4.2.1.2	FH*	Muscle	TM

TM = Tris-Maleate (pH = 7.8) and TG = Tris-glycine (pH = 8.8).

Table 2 *P. minutus* and *P. lozanoi* sampling sites on the sand banks of the BCS including code, period, coordinates and numbers of fish examined.

Habitat	Sampling site	Code	Period	Longitude/latitude	Numbers of <i>P. minutus</i>	Numbers of <i>P. lozanoi</i>
Estuary	Doel	D3	March 1998	51° 19.30 N 04° 16.00 E	—	34
	Doel	D10	October 1998	51° 19.30 N 04° 16.00 E	71	—
Coast	Kwintebank	Ki2	February 1997	51° 17.61 N 02° 38.92 E	68	—
				51° 13.80 N 02° 52.42 E	83	—
	Stroombank	Sb	February 1997	51° 15.36 N 02° 38.34 E	161	42
	Kwintebank	Ki10	October 1997	51° 28.05 N 03° 03.69 E	50	—
	780 (Raan)	Ra	March 1997	51° 22.63 N 03° 18.68 E	—	78
	702 (Zeebrugge)	Ze	October 1997	51° 16.87 N 02° 51.13 E	52	50
	120	Htw	October 1998	51° 11.10 N 02° 47.07 E	35	—
	GC1	Gc	February 1999	51° 34.40 N 03° 24.80 E	—	23
	250	Gc	February 1999	51° 31.00 N 03° 19.00 E	—	13
	51° 30.30 N 02° 38.32 E	51	31			
Off-shore	Oosthinder	Oh	October 1997	51° 33.37 N 02° 44.16 E	28	61
	Bligh Bank	Bli	February 1998	51° 39.50 N 02° 36.14 E	—	46
Wadden Sea	Noordhinder	Nh	October 1998	53° 30 N 4° 00 E	38	31
	Frisian Front	FF	December 1998			

zygous loci as the dependent variable and the sampling group as the independent variable.

Results

The total number of *Pomatoschistus lozanoi* and *P. minutus* analysed amounts to 409 and 637 specimens respectively (Table 2). A total of 14 loci were found suitable for cellulose acetate allozyme electrophoresis. The allele frequencies have been compiled in Appendix A (*P. minutus*) and Appendix B (*P. lozanoi*).

An *a priori* exact test for allelic homogeneity (Raymond and Rousset, 1995) revealed no differentiation between *P. lozanoi* caught at the adjacent sampling stations GC-1 and 250 (Figure 1). They were subsequently pooled to increase the sample size and recoded as Gc.

Genetic diversity

The following loci were polymorphic in *P. lozanoi*: AAT*, GPI-A*, GPI-B*, LDH-B*, LDH-C*, MDH-B*, PGM-1* and PGM-2* (Appendix A). No samples of *P. lozanoi* deviated from Hardy–Weinberg proportions. In *P. minutus*, the loci

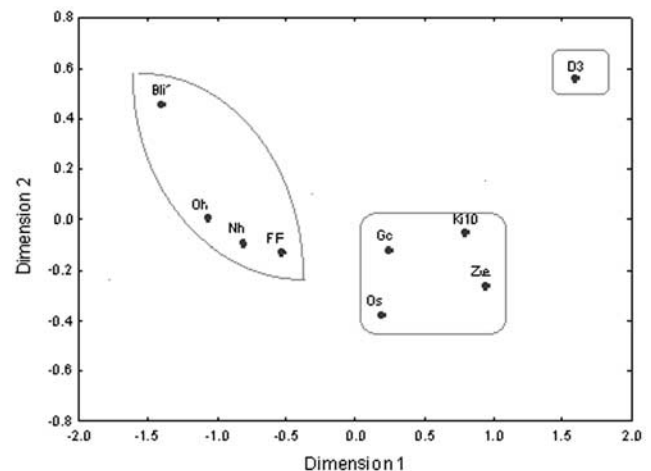
Table 3 Genetic diversity of (a) *P. lozanoi* and (b) *P. minutus* expressed as unbiased expected heterozygosity (H_e), observed heterozygosity (H_o), percentage of polymorphic loci with the frequency of the most common allele not exceeding 0.99 ($P(0.99)$) and the average number of alleles per locus (AVG)

Season	Sampling site	H_e	H_o	$P(0.99)$	AVG
(a) <i>P. lozanoi</i>					
Winter/spring	D3	0.0977 (0.1452)	0.0735 (0.1282)	0.533	1.733
	Gc	0.1037 (0.2185)	0.0708 (0.1651)	0.400	1.800
	Bli	0.0925 (0.1971)	0.0761 (0.1628)	0.400	1.733
Frisian Front Summer/autumn	Ff	0.0506 (0.1242)	0.0367 (0.0920)	0.286	1.357
	Ze	0.0784 (0.1726)	0.0664 (0.1442)	0.400	1.667
	Ki10	0.0888 (0.1880)	0.0711 (0.1322)	0.400	1.600
	Os	0.0768 (0.1798)	0.0540 (0.1163)	0.333	1.533
	Nh	0.0895 (0.2003)	0.0710 (0.1506)	0.467	1.667
Oh	0.0851 (0.2066)	0.0692 (0.1641)	0.200	1.333	
(b) <i>P. minutus</i>					
Winter/spring	Bli	0.1020 (0.1830)	0.0666 (0.1101)	0.357	1.64
	Ki2	0.0996 (0.2098)	0.0862 (0.1792)	0.214	1.64
	Sb	0.1017 (0.2041)	0.1042 (0.2204)	0.286	1.50
	Ra	0.1099 (0.2075)	0.0955 (0.1885)	0.429	1.71
	FrF	0.1083 (0.2120)	0.0639 (0.1134)	0.357	1.64
Autumn	D10	0.0995 (0.1845)	0.0841 (0.1599)	0.357	2.07
	Ki10	0.1104 (0.2103)	0.0929 (0.1679)	0.286	2.14
	Oh	0.1100 (0.2079)	0.0838 (0.1683)	0.429	1.86
	Htw	0.0844 (0.1670)	0.0649 (0.1204)	0.357	1.57
	Os	0.1114 (0.2193)	0.0861 (0.1700)	0.286	1.79

Table 4 Single-locus F_{ST} values for *P. minutus* and *P. lozanoi* across all samples

Locus	<i>P. minutus</i>	<i>P. lozanoi</i>
AAT*	-0.0003	-0.002
LDH-A*	-0.004	—
LDH-B*	0.0071	-0.010
LDH-C*	0.026	0.006
GPI-A*	0.006	0.042
GPI-B*	-0.001	-0.006
MDH-A*	0.009	—
MDH-B*	—	-0.005
PGM-1*	-0.004	0.003
PGM-2*	0.009	-0.014
Multi locus F_{ST}	0.012 (all loci)	0.024 (all loci)
Multi locus F_{ST}	0.005 (without LDH-C*)	-0.003 (without GPI-A*)

AAT*, GPI-A*, GPI-B*, LDH-A*, LDH-B*, LDH-C*, MDH-A*, PGM-1*, PGM-2* were polymorphic (Appendix B). Locus LDH-C* in *P. minutus* showed a significant heterozygote deficit across all samples with the exception of the samples from the Stroombank and 120. The other loci did not deviate significantly from Hardy–Weinberg proportions in any sample. In *P. lozanoi*, four cases of linkage disequilibrium were noticed and in *P. minutus* five cases were recorded out of a total of 91 comparisons, involving mainly the enzyme systems LDH, PGM and GPI. Observed and expected heterozygosity across all loci per sampling were similar across all samples within each species, with a high standard deviation (Table 3a and b). *P. minutus* had a higher degree of heterozygosity than *P. lozanoi*. *P. lozanoi* and *P. minutus* showed no significant differences in heterozygosity between sites along the Belgian coast (Kruskal–Wallis ANOVA: *P. lozanoi*: $P = 0.457$; *P. minutus*: $P = 0.900$, NS).

**Figure 2** *P. lozanoi*: MDSA based on pairwise F_{ST} values (Weir and Cockherham, 1984) at locus GPI-A*. Stress value = 0.0085.

Genetic differentiation

P. minutus was differentiated between sampling sites at LDH-C*, but this locus was excluded from the overall F_{ST} analysis due to the large deviations from Hardy–Weinberg proportions in most samples. No gradient in allele frequencies was observed at this locus. Only the pairwise F_{ST} value between Doel and the Frisian Front turned out to be significant. The multilocus F_{ST} value across all populations and loci (except LDH-C*) for *P. minutus* amounted to 0.005 and was not significant (Table 4). MDSA did not reveal any geographical clustering, with or without LDH-C* included.

P. lozanoi showed significant pairwise differences in allele frequencies at the GPI-A* locus, differentiating the samples into three groups: an estuarine, a coastal and an offshore group (Figure 2). The Frisian Front grouped

with the offshore samples from the Belgian coast. Single-locus F_{ST} values were significant for $GPI-A^*$ only (Table 4). The multilocus F_{ST} value across all samples and all loci amounted to 0.024. When the $GPI-A^*$ locus was excluded from the analysis, no significant differentiation between the samples was observed anymore (multilocus $F_{ST} = -0.003$).

Clinal variation at locus $GPI-A^*$ of the alleles $^{100}GPI-A^*$ and $^{113}GPI-A^*$ in *P. lozanoi*

Locus $GPI-A^*$ has three alleles that occur in both species, namely $^{90}GPI-A^*$, $^{100}GPI-A^*$ and $^{113}GPI-A^*$ (Appendices A and B). Allele $^{90}GPI-A^*$ was rarely recorded in either species. *P. minutus* shows limited polymorphism at $GPI-A^*$, with a frequency of $^{100}GPI-A^*$ above 0.95. *P. lozanoi* on the contrary shows a much larger degree of polymorphism at this locus and a clinal change in allele frequencies of $^{100}GPI-A^*$ and $^{113}GPI-A^*$. The frequency of allele $^{100}GPI-A^*$ in *P. lozanoi* increases with distance from the shore, with the lowest frequencies in Doel and the highest at the Hinderbanken (Figure 3). A Spearman rank correlation showed a significant relationship between coastal distance and the frequency of $^{100}GPI-A^*$ ($P < 0.05$). In Doel, no $^{100}GPI-A^*/^{100}GPI-A^*$ homozygotes and only few $^{100}GPI-A^*/^{113}GPI-A^*$ heterozygotes were recorded. In *P. minutus*, this locus showed minimal polymorphism and no gradient in allele frequencies was observed.

Discussion

Lack of small-scale genetic variation in relation to environmental heterogeneity

Our study did not find any differences in the level of genetic variation in *P. minutus* and *P. lozanoi*, which could be linked to the degree of heterogeneity of the habitats where the samples were collected. We can confirm the lower degree of genetic variation in *P. lozanoi* compared to *P. minutus*. The fact that the level of heterozygosity is lower in all *P. lozanoi* samples than in *P. minutus* has been linked to the degree of heterogeneity of the environment in which both species prefer to live (Wallis and

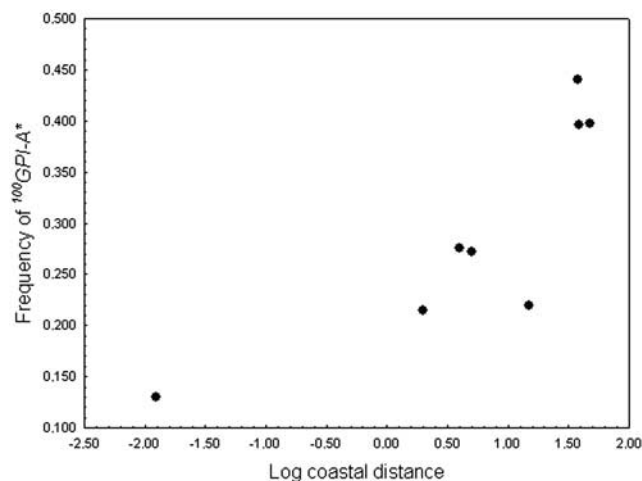


Figure 3 *P. lozanoi*: frequency of $^{100}GPI-A^*$ in function of coastal distance.

Beardmore, 1984b). However, we cannot support the hypothesis that this difference may be due to their occurrence in habitats subject to a different degree of heterogeneity, because *P. minutus* and *P. lozanoi* occur sympatrically throughout the whole sampling range. Sorice and Caputo (1999) noticed that highly polymorphic Mediterranean goby species were also karyologically highly variable and suggested genomic constraints in chromosome variation as an alternative hypothesis for explaining different levels of polymorphism between goby species. We found no evidence for increased levels of heterozygosity for individuals occurring in estuarine (heterogeneous) vs marine (more stable) habitats in either species.

Small-scale population differentiation in *Pomatoschistus* sp or natural selection on specific loci?

$GPI-A^*$ in *P. lozanoi*: The differentiation between the coastal, estuarine and offshore samples in *P. lozanoi* (Figure 2) is entirely due to the differences at a single locus, $GPI-A^*$. Genetic drift and gene flow should affect all polymorphic loci similarly, while natural selection acts on specific loci. The complete lack of genetic differentiation between samples at all other loci, together with the extended pelagic larval phase in *Pomatoschistus* sp, suggests extensive gene flow throughout the area. This is confirmed by Pampoulie et al (2004), who found little genetic differentiation among sand goby samples from the BCS, suggesting one single breeding unit off the Belgian coast, and another breeding unit in the Oosterschelde. Hence, the genetic differences between the *P. lozanoi* samples are probably maintained by some form of natural selection rather than by limited gene flow. The fact that the reference sample from the Frisian Front, an offshore location, groups together with the offshore Belgian samples based on its allele frequencies at locus $GPI-A^*$ (Figure 2) supports the hypothesis that some environmental factor might be responsible for the observed allele frequencies. Christiansen and Frydenberg (1974) reported a similar cline in allele frequencies at two unlinked loci in eelpout and suggested that these clines were maintained by selective pressure. Levene's model (1953) of balancing selection at a two-allele locus states that both alleles may be maintained in the population by natural selection when selection pressure varies between niches, resulting in a higher fitness of the alternative homozygote genotypes in the various habitats. If we apply this to the lozano gobies investigated on the BCS and the Schelde estuary, this would mean that those fish carrying allele $^{100}GPI-A^*$ may perform less well in estuarine conditions, whereas individuals possessing allele $^{113}GPI-A^*$ might be less adapted to the marine environment.

Although a correlation between allele frequencies at the GPI^* loci and temperature in fish (including gobies) and other organisms has been reported (Al-Hassan et al, 1987; Zera, 1987), there was no such evidence in this study. The sites were sampled in different seasons (Table 2), yet the samples from the respective habitats (estuarine, coastal and marine) grouped together in the MDSA. Oxygen concentration at the sampling sites (data not shown) did not reveal any trend that might be linked

to the differences at *GPI-A**. Salinity, which increases with increasing distance from the Schelde estuary, yielded a weak but significantly positive linear correlation with the frequency of ¹⁰⁰*GPI-A** ($R^2 = 0.52$, $P < 0.04$). Koehn *et al* (1980) demonstrated an association between allele frequencies at the *LAP** locus in *Mytilus edulis* and a salinity gradient. It is tempting to point to the salinity gradient for explaining the observed differences at *GPI-A** but any other factor correlating with distance to the coast would show a similar correlation. For example, the Schelde estuary is heavily polluted while the level of pollution decreases towards the offshore areas; the water flowing through the English Channel towards the Southern Bight represents relatively clean Atlantic water. De Wolf *et al* (2001) reported allozymatic differences at the esterase locus between samples of *Littorina littorea* in the Schelde estuary and adjacent areas, and suggested either salinity or anthropogenic effects as agents responsible for the differentiation. A number of studies have revealed differential mortality of distinct genotypes when exposed to heavy metals, suggesting they could be useful markers for assessing levels of pollution (Ben-Shlomo and Nevo, 1988). Nevo *et al* (1984) and Newman and Jagoe (1998) found evidence for an allele shift at the *GPI** loci in shrimps and fish respectively, when exposed to mercury. However, in order to prove an association between the observed *GPI-A** genotypes and the level of pollution, detailed biochemical experiments would be required.

***LDH-C** in *P. minutus*:** *LDH-C** showed a consistent heterozygote deficit across almost all *P. minutus* samples. Such a deficit might be attributed to scoring errors, null alleles, selection against heterozygotes, assortative mating or the Wahlund effect. Scoring error is ruled out because a very distinct three-allele-polymorphism was recorded at this locus using CAGE, contrary to starch gel electrophoresis (Wallis and Beardmore, 1984a, b; Stefanni *et al*, 2003). If the heterozygote deficit were due to the mixing of subpopulations, we would have expected a similar effect at the other polymorphic loci; this is not the case. Null alleles may be another explanation (Richardson *et al*, 1986), but this should be obvious from the pattern and the intensity of the bands, and we found no evidence for it. Selective mortality of heterozygotes has often been invoked for the heterozygote deficit observed in marine organisms (Zouros and Foltz, 1984; Kotoulas *et al*, 1995). Evidence exists that alleles of the *LDH** loci are subject to natural selection (Mitton and Koehn, 1975; DiMichele *et al*, 1991). However, detailed biochemical experiments would be needed to ascertain whether the products of the various alleles perform differently.

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