An analysis of allozyme, mitochondrial DNA and morphological variation in mussel (*Mytilus galloprovincialis*) populations from Greece

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Abstract. Three populations of *M. galloprovincialis* from northern Greece were investigated using isozyme analysis, discriminant analysis of morphological characteristics and analysis of restriction fragments of mtDNA. For all three types of analysis significant intra- and interpopulation differentiation was found. This differentiation is very noticeable at the mtDNA genotype frequencies. Furthermore, the restriction patterns of mtDNA were different from those reported for Atlantic populations of this species.
Key words. Allozyme; mitochondrial DNA; morphology; *Mytilus galloprovincialis*; Greece.

In recent years has been a shift in the study of evolution and systematics from the more classical methods, such as morphological analysis, to molecular methods. There is an increasing interest in the use of mtDNA for such studies. The maternal inheritance and the faster rate of change of mtDNA, compared with the nuclear genome, make it useful for the study of phylogenetic relationships, or of population differentiation within species[1-3]. However, the specific forces underlying the evolutionary changes of mtDNA (mutation, selection, founder effect and genetic drift) could be quite distinct from those acting on the nuclear genome. Thus patterns of mtDNA differentiation will not necessarily coincide with patterns of morphological and nuclear differentiation.

Mytilus galloprovincialis Lmk, a Mediterranean mussel, is widespread along Mediterranean coasts and also occurs on the Atlantic coasts of western Europe, as does the blue mussel *M. edulis* L. The external fertilisation and the extended larval period provide the opportunity for extensive homogenisation of population variation at the micro- and macrogeographic level. Furthermore, the two species of *Mytilus* hybridise and introgress in parts of Britain and Ireland[4]. According to Gosling[5], *M. galloprovincialis* could be regarded as a distinct subspecies of *M. edulis* which originated allopatrically in the Mediterranean. The Atlantic populations of the two species have been extensively investigated at the allozymic[6-9] and mtDNA level[10-12]. Until recently only a few studies concerning the genetic structure of Mediterranean populations have been published[7,8,13].

M. galloprovincialis predominates on the coasts of Northern Greece especially in the eutrophic gulfs, where food is readily available. This is the first report of the genetic structure of populations of *M. galloprovincialis* from Greece using morphological, allozymic and mtDNA analysis. The results provide evidence of significant population differentiation for all three types of analysis. Differentiation of mtDNA genotype frequencies is particularly marked suggesting the utility of this technique for analyses of population structure and stock differentiation.

Materials and methods

Mussels were collected from three areas/sites, two from the Thermaikos gulf (Kalohori, Agia Triada) and one from the Strymonikos gulf (Stavros). The two gulfs are separated by the Khalkidiki peninsula.

The hepatopancreas and the posterior adductor muscle were used for the isozyme analysis. Ten enzymic systems were investigated using starch gel electrophoresis. The buffers and electrophoretic conditions used are those given by Ahmad et al.[6]. Nei's[15] genetic identity was calculated based on the allele frequencies. The mtDNA was extracted from ripe gonads of male and female individuals using the method of Edwards and Skibinski[12]. Three restriction endonucleases were used for the mtDNA analysis: *BstEII*, *EcoRI* and *PvuII*. The restriction fragments obtained were separated in 1% agarose gels which were stained with ethidium bromide. In some cases, to clarify the restriction patterns, fragments were blotted onto nitrocellulose membrane, hybridised with a probe containing the whole *M. galloprovincialis* mtDNA genome[11]. The labelling of probe, hybridisation and visualisation of mtDNA bands were carried out using the Boehringer digoxygenin system, using the protocols suggested by the manufacturer.

For the shell morphological analysis six measurements were taken: shell length, width, height, the length of the hinge plate and the lengths of the posterior and anterior adductor muscle scars. All the measurements were divided by shell length and a stepwise discriminant analysis performed using SPSSX routines. The Mahalanobis distance between the three populations was also calculated using the morphological characteristics.

Results and discussion

The ten enzyme systems examined are represented by 14 gene loci. Eight of these loci were found to be polymor-

Table 1. Allele frequencies at the polymorphic loci for the three populations examined. D = Ho-He, where Ho is observed frequency of heterozygotes and He is frequency of heterozygotes according to Hardy-Weinberg expectations. χ^2 = Chi-test for Hardy-Weinberg equilibrium. χ^2_a = heterogeneity test for allele frequencies, N = sample size

Locus	Allele	Kalohori N = 30	Agia Triada N = 30	Stavros N = 30	χ^2_a
Aat-2	100	0.966	0.983	1.00	
	60	0.017	0.017		
	40	0.017			3.03
	D	0.009	0.000		
	χ^2	0.018	0.000		
Est-1	100	1.00	0.983	1.00	
	75		0.017		5.05
	D		0.00		
	χ^2		0.00		
Est-2	100	0.95	0.867	0.817	
	85	0.05	0.133	0.183	2.01
	D	0.035	−0.716	−0.891	
	χ^2	0.055	17.34**	25.82**	
Est-D	150	0.083			
	100	0.850	0.983	0.983	
	80	0.067	0.017	0.017	12.95**
	D	−0.630	0.00	0.00	
	χ^2	31.47**	0.00	0.00	
Tdh-1	100	0.9	0.933	0.967	
	80	0.1	0.067	0.033	2.14
	D	−0.272	−0.473	0.017	
	χ^2	2.616	8.816*	0.018	
Me-1	100	0.85	0.933	0.85	
	75	0.15	0.067	0.15	2.59
	D	−0.357	0.054	−0.357	
	χ^2	4.248	0.113	4.24*	
Pgi	110	0.233	0.017	0.1	
	100	0.750	0.983	0.667	
	95	0.017		0.233	42.73**
	D	−0.743	0.00	−0.867	
	χ^2	21.308	0.00	56.47**	
6Pgdh-1	220		0.17		
	190	0.017			
	100	0.966	0,983	1.00	
	60	0.017			6.03
	D	0.009	0.00		
	χ^2	0.018	0.00		

Idh-2, Odh-1, Sod-1, Sod-2, Mdh-1, Mdh-2 are monomorphic, *, significant at the 0.05 level; **, significant at the 0.01 level.

phic in the three populations. The allelic frequencies are given in table 1.

The allelic frequencies at two loci (*Est-D* and *Pgi*) show significant heterogeneity among populations (table 1). Pairwise comparisons revealed that all population samples are significant different from each other, at these loci (table 2). They represent either distinct gene pools or distinct mixtures from different gene pools. Also some significant deviations from the Hardy-Weinberg equilibrium were detected (table 1). In all these cases a significant heterozygote deficiency was observed (table 1). This phenomenon is common to bivalves and could be the result of selection acting differentially during the life history of this organism[14]. But this pattern is perhaps

Table 2. Above diagonal genetic identity from isozymic analysis, in parentheses Mahalanobis distance. Below diagonal, χ^2heterogeneity test for pairwise comparison of the populations; **, significant at the 0.01 level

Population	Kalohori	Agia Triada	Stavros
Kalohori	–	0.995 (3.4)	0.996 (4.0)
Agia Triada	34.36**	–	0.994 (4.7)
Stavros	57.89**	30.65**	–

more likely to be due to the Wahlund effect (which is the heterozygote deficiency due to pooling samples from random mating populations with different gene frequencies) as indicated by the large values of D (table 1) and the existence of significant allelic frequency heterogeneity at *Est-D* and *Pgi* loci. Other loci have large and significant D values for some populations (e.g. *Est-2* at Agia Triada and Stavros). This suggests that these population samples also have contributions from other populations differing in allele frequencies from the populations studied here.

The significant differences in allele frequencies between these three *M. galloprovincialis* populations contrast with other studies of *Mytilus* which suggest little geographic differentiation within species. For example, *Mytilus edulis* exhibits little differentiation in allozyme frequencies throughout the whole of the British Islands. This was attributed to the high dispersal ability of the species. In regions of Britain where *M. edulis* occurs sympatrically and hybridises with *M. galloprovincialis*, geographic differentiation is much more marked. It is possible that

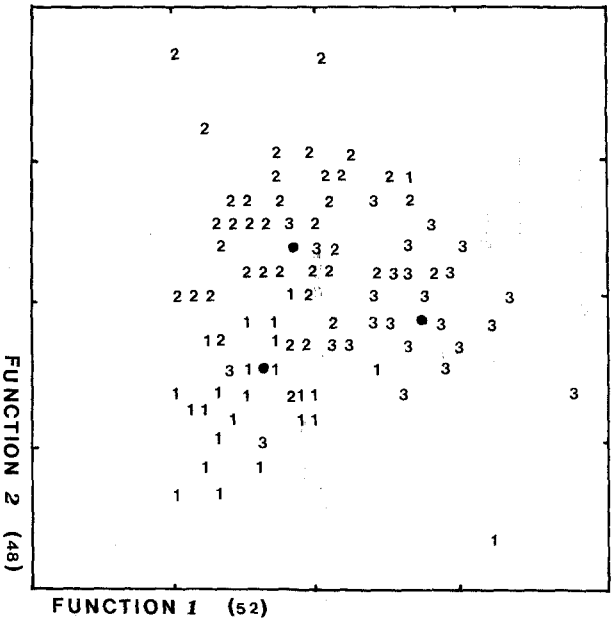

Figure 1. Results of the discriminant analysis. In parentheses the percentage of variation expressed in each function. 1 = Agia Triada, 2 = Kalohori, 3 = Stavros.

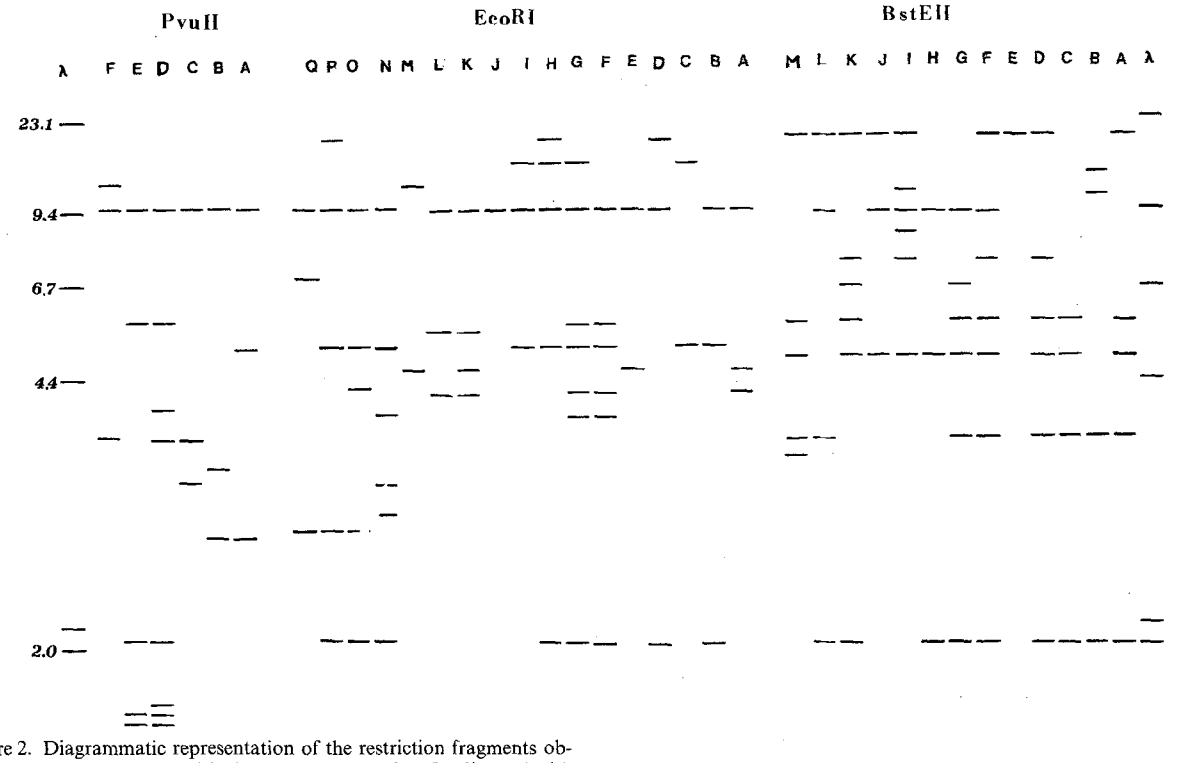

Figure 2. Diagrammatic representation of the restriction fragments obtained from three enzymes. λ is the marker (phage λ DNA digested with *HindIII*).

dispersal ability is more restricted around the Greek coast, forming population differentiation. Another possibility is that the results for the Greek populations reflect cryptic or incipient speciation.

The results of the discriminant analysis of the morphological characteristics indicate that the three populations are clearly distinct (fig. 1). The main characteristics discriminating the populations are the posterior adductor scar for the first discriminant function and the length of the hinge plate for the second discriminant function. The analysis indicates that 75% of individuals could be correctly assigned to the three populations, using the measured characteristics.

The mtDNA analysis revealed many different patterns in the populations examined. The observed digestion patterns for *BstEII*, *EcoRI* and *PvuII* are named and shown diagrammatically in figure 2. The size of *Mytilus* mtDNA is about 17 kb[11, 12]. As indicated in figure 2, many patterns were found to have a greater size. Density differences in the bands and stoichiometric analysis indicated that heteroplasmy could exist in some of the mussels examined (fig. 3). A high frequency of heteroplasmy exists in mussels, perhaps resulting from the limited biparental mitochondrial inheritance which occurs in these species[16, 17].

The same patterns were found in the three populations examined, but in significantly different frequencies. Also, many rare patterns were found in each population. However, some of the most common patterns, e.g. *EcoRI* C and B, can be interconverted by a single mutation. Significant differences in the frequencies of the patterns were found for *EcoRI* enzyme ($\chi^2 = 101.578$, $df = 66$, $p < 0.01$)[18]. Table 3 gives the frequencies of the *EcoRI-PvuII-BstEII* multiple genotypes for the three popula-

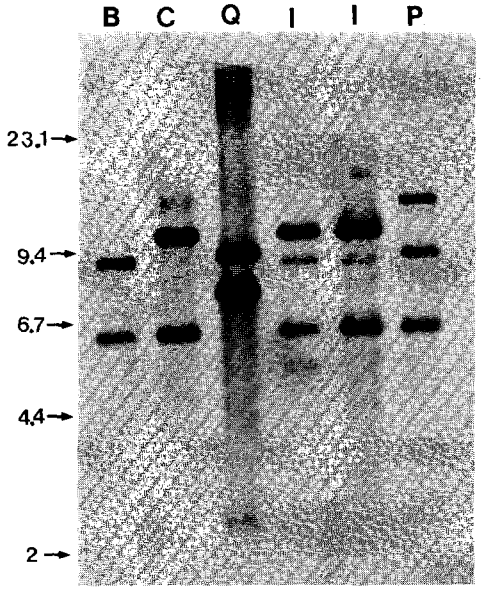

Figure 3. Different patterns of mtDNA from mussels revealed after digestion with *EcoRI* enzyme and Southern blotting. Density differences indicates the existence of two types of molecule in pattern I.

Table 3. Frequencies of *EcoRi-PvuII-BstEII* multiple genotypes in three populations. N = sample size

Genotype	Kalohori	Agia Triada	Stavros
GAA	0.05	–	–
BDE	0.05	–	–
HAA	0.05	–	–
CAE	0.05	–	–
ABL	0.05	–	–
IBC	0.05	–	–
IAA	0.05	–	–
JBC	0.05	–	–
CAC	0.12	0.5	–
KCJ	0.05	–	–
LCJ	0.05	–	–
GAF	0.05	–	–
BAC	0.18	–	–
FCJ	0.05	–	–
KCD	0.05	–	–
BBB	–	0.1	–
AAA	–	0.1	–
CH	–	0.1	–
CCC	–	0.1	–
PCG	–	–	0.07
NAC	0.05	–	0.2
BCG	–	–	0.07
CBJ	–	–	0.07
CFC	–	–	0.07
ABJ	–	–	0.12
PAA	–	–	0.07
BCH	–	–	0.07
BCD	–	–	0.07
BCA	–	–	0.12
N	19	10	15

tions. Significant differences were also found between the populations ($\chi^2 = 469.15$, $df = 391.5$, $p < 0.01$). The significant interpopulation differences for the mtDNA multilocus genotypes, as opposed to the mtDNA single locus, indicates that the rate of evolution probably varies between different regions of the molecule[19]. The three populations have very different frequencies but the Kalohori and the Agia Triada population, which are close geographically, are more similar to each other than to the Stavros population.

The differentiation between the populations, as measured by the degree of genetic identity (table 2) obtained from the allozymes, is much lower than that measured from mtDNA genotypes frequencies (table 3), with one population, Stavros, having no genotypes in common with Agia Triada, and only a single genotype in common with Kalohori. It has been suggested previously that mtDNA analysis might have higher resolution at the molecular level than enzyme electrophoresis[21]. The results of this analysis are in agreement with this hypothesis. The greater overall level of mtDNA differentiation might also be the result of a lower effective population size for mtDNA than nuclear genes. The mean time of fixation (or loss) of any new mutation will be approximately twice as long for the nuclear than the mitochondrial genome. Populations can thus be distinct for mtDNA under circumstances in which gene flow is high enough to homogenate nuclear gene frequencies geographically. Whatever the difference between allozymes and mtDNA, this study demonstrates that from the point of view of stock discrimination in this species, mtDNA analysis is likely to be more useful than allozyme analysis. The three populations studied also appear to be distinct morphologically. However, this might be the result of immediate environmental factors, as it is known that local environments can influence shell morphology substantially[20]. Experiments involving transfer of spat between these sites would be required to assess the relative importance of genetic and environmental factors on morphology.

The patterns revealed in this study for the *BstEII* and *EcoRI* restriction enzymes differ from those reported by Edwards and Skibinski[12] for *M. galloprovincialis* populations from Britain. Some of the most common patterns in the Atlantic populations exist in the populations examined in this study, but at very low frequencies. Geographic isolation and subsequent genetic drift, different selective forces acting on mtDNA genotypes of the Mediterranean and the Atlantic populations of this species, or introgression of mtDNA from *M. edulis* could account for this difference. Further studies of mtDNA variation in western Europe might help to shed light on and distinguish between these hypotheses.

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