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Genetic characterization and evaluation of anthropogenic impacts on genetic patterns in cultured and wild populations of mussels (*Mytilus galloprovincialis*) from Greece

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ABSTRACT. Despite the great commercial and economic importance of mussels, *Mytilus galloprovincialis* (Bivalvia, Mollusca) in Greece, little information is available concerning their population genetic structure. We used RAPD markers to examine genetic differentiation and potential impact of aquaculture practices and other anthropogenic activities on the genetic structure of two cultivated and eight wild mussel populations collected from one Turkish and nine Greek coast sites. Five random decamer primers were chosen, among 34 tested, for the analysis of 433 individuals. Eighty-eight bands (genetic loci) were scored, all of which were polymorphic. No indication of reduced genetic variability was observed in the cultured populations. In contrast, a loss in genetic diversity was observed in populations from two localities (Canakkale and Kalochori) that are heavily polluted by chemical contaminants. F_{ST}

analyses and exact tests revealed significant heterogeneity among *M. galloprovincialis* population samples, although their genetic divergence seemed to be independent of geographic distances. Anthropogenic activities, i.e., marine pollution and transplantation of mussels, appear to have played an important role in shaping patterns of genetic diversity and differentiation among Greek *M. galloprovincialis* populations.

Key words: *Mytilus galloprovincialis*; Genetic diversity; Pollution; Population structure; RAPD; Anthropogenic impact

INTRODUCTION

The Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) is the indigenous species of mussel of the Eastern Mediterranean coasts. Its biological traits, such as fast growth, immunity to parasites and disease, stress tolerance, and high survivorship (Branch and Steffani, 2004), in combination with its preferred environmental conditions prevailing in Greece, have greatly contributed to the large development of Greek mussel populations over the past decades (Smaal, 2002), which have become a very important resource for the national economy. The annual mussel production in Greece is approximately 40,000 tons, of which more than 80% comes from the Thermaikos Gulf (Mouratidou et al., 2006). The Thermaikos Gulf is a semi-closed bay in the Northwestern Aegean Sea, which is plentiful in organic material due to a delta formed by the deposition of the 4 rivers within it. Hence, it exhibits excellent conditions for mussel farming, particularly at the area of Chalastra that is located at the outflow of 3 rivers. At the neighboring coasts of Turkey, although there are currently only 2 mussel farms in Marmara and the Aegean Sea producing 1100 tons/year, there is a high potential for further spread of these mussel populations (Karayücel et al., 2010).

In important fisheries and aquaculture resources such as marine bivalves, knowledge of population structure is critical and can aid in refining existing management models (Graves, 1998). It is widely accepted that population genetics is an essential and efficient tool for the management and conservation of wild and adaptive populations (Gray, 1996). Several types of genetic markers have been frequently applied in population genetic studies of aquatic organisms. In mussels of the genus *Mytilus*, previous studies dealt with species and hybrid identification (Beaumont et al., 2008) and with their population structure in Western Europe (Daguin and Borsa, 1999) and America (Addison et al., 2008). However, despite its utility for aquaculture purposes, few reports have been published regarding the population genetic structure of *M. galloprovincialis* along the Greek and Turkish coastlines. Thus, the only available information comes from 2 studies of mitochondrial DNA (mtDNA) and allozyme polymorphisms performed on a few populations from the North Aegean Sea (Karakousis and Skibinski, 1992; Kravva et al., 2000, respectively). Nevertheless, the utility of allozyme markers in aquaculture genetics has become limited recently (Liu and Cordes, 2004), as the unusual manner of mtDNA inheritance in mussels obscures inferences about the population structure of the species. Therefore, a study conducted at the genomic DNA level to monitor the genetic diversity of *M. galloprovincialis* populations from the East Mediterranean Sea is still missing.

The random amplified polymorphic DNA (RAPD) technique has been demonstrated to be a helpful methodology for estimating genetic variations at the molecular level in marine

bivalves (Joaquim et al., 2010). RAPD is a low cost, rapid method that can be performed without any prior nucleotide sequence knowledge of the target DNA. The latter advantage has made the RAPD technique a very useful tool for studying the genetics of non-model species, in which little genome information is available (Klinbunga et al., 2010). Its simplicity and rapidity is mainly due to the use of a single, short and arbitrary oligonucleotide primer for amplification of the template DNA, thus avoiding the need for expensive and sophisticated equipment (Bardakci, 2001). It should also be noted that RAPD usually reveals great polymorphism levels and it can be applied to entire genomes.

The present study was focused on the investigation of the genetic differentiation of natural and cultured *M. galloprovincialis* populations from the Aegean and Ionian coasts. Furthermore, we attempted to examine the possible anthropogenic impact (aquaculture practices, water pollution, or other aspects) on the genetic structure of mussel populations. We believe that the results of this study will contribute to the rational management of resident mussel populations, especially in a long-term survival situation.

MATERIAL AND METHODS

Sampling

A total of 450 *M. galloprovincialis* individuals were collected by hand, outside of the water (due to the phenomenon of the tide) or by diving, from 9 localities in Greek waters (8 from the North, East, and West Aegean Sea and 1 from the Ionian Sea) and 1 in Turkey (near Canakkale, in the middle of Dardanelles, a narrow strait that connects the Aegean Sea with the Sea of Marmara) (Figure 1). Two of the collected samples (Chalastra and Epanomi) came from cultured populations, while all the rest were wild. Sampling took place between January and April 2009. After collection, the mussels were kept alive until their transportation to the laboratory for the DNA extraction.

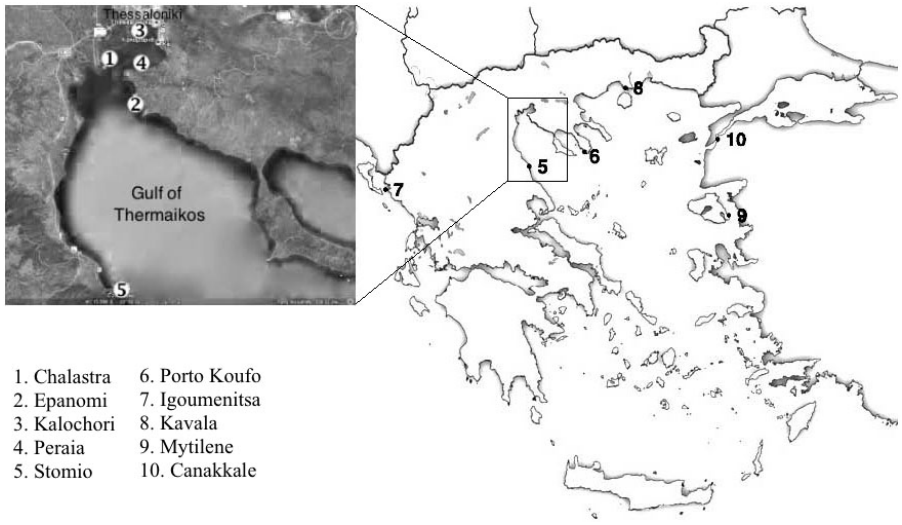

Figure 1. Map showing sampling locations of the 10 *Mytilus galloprovincialis* populations analyzed in this study.

Isolation of genomic DNA and RAPD-PCR amplification

Total DNA was isolated according to the protocol of Hillis et al. (1996). Thirty-four decamer primers, all purchased from Operon Technologies (Alameda, CA, USA), were screened on 3 to 5 individuals of each population. Five primers (Table 1), exhibiting easy scoring results and high polymorphism and reproducibility, were chosen for further genetic analysis of the populations. RAPD-PCRs were performed in a 15-µL reaction volume containing 50 ng genomic DNA, 1 U *Taq* DNA polymerase (HyTest Ltd., Finland), 1.5 µL 10X Taq DNA polymerase buffer (670 mM Tris-HCl, pH 8.8, 166 mM $(NH_4)_2SO_4$), 3 mM $MgCl_2$, 0.25 mM of each dNTP, and 20 pmoL primer. Amplification reactions were carried out in an Eppendorf Thermocycler programmed for the following reaction: initial denaturation for 4 min at 94°C, 35 cycles of denaturation (40 s at 94°C), annealing (1 min at 38°C), and extension (75 s at 72°C), followed by a final extension at 72°C for 7 min. One negative control without template DNA was also included to avoid any possible contamination. RAPD-PCR products were separated by gel electrophoresis with 1.5% agarose (w/v) in 1X TBE (0.89 M Tris, 0.89 M boric acid, 0.11 M Na_2EDTA, pH 8.3) buffer containing ethidium bromide, compared with a GeneRuler™ 100-bp DNA ladder (Fermentas Life Sciences, Canada), visualized, and photographed under an ultraviolet Vilber Lourmat, France trans-illuminator. To overcome limitations of the technique mainly related to reproducibility due to its sensitivity to changes in reaction conditions (Bardakci, 2001), 2 RAPD-PCR replications were performed for every sample and every primer. Bands that were reproduced after the 2 replications were considered reliable. Moreover, RAPD patterns were analyzed by 2 independent researchers.

Data analysis

RAPD-PCR products are dominant markers, thus heterozygotes are indistinguishable from homozygotes. Allelic information was therefore based on the assumption that genotype frequencies are in Hardy-Weinberg equilibrium and alleles in homozygous recessive individuals, as well as in dominant ones, are in identical states (Zhou et al., 2000). Hence, a binary matrix was made in which every band was scored as "1" for its presence or as a "0" for its absence. This matrix was used for statistical analysis to calculate the effective allele number (N_E), Shannon index (I), mean heterozygosity (H) at species and population level, and the values of genetic distance (D) (Nei, 1978) between the populations using the POPGENE version 1.31 software (Yeh et al., 1999). Based on these values, an unweighted pair group method with arithmetic mean (UPGMA) dendrogram was constructed with 1000 bootstrap replicates.

Population genetic differentiation was examined using exact tests in all possible pairwise comparisons. Moreover, F_{ST} values were estimated using the RAPDFST program (Black, 1995) on various groups of populations, i.e., 1) all samples, 2) all Aegean samples, and 3) all Thermaikos Gulf samples. The gene flow among populations was estimated as the number of individuals per generation that migrate from a population to another. Many authors have suggested that estimation of gene flow according to F_{ST} values is more preferable than using methods of allele frequency data (Zhou et al., 2000). Here, it was calculated by the F_{ST} value (RAPDFST software; Black, 1995) using the equation $N_m = (1 - F_{ST}) / 4F_{ST}$. Finally, the Mantel test was carried out (Mantel, 1967) to examine the correlation between genetic and coastal geographic distances using GENALEX version 6.41 (Peakall and Smouse, 2006).

RESULTS

RAPD polymorphism

The 5 selected primers were successfully amplified in 433 *M. galloprovincialis* individuals from 10 populations, generating a total of 88 clear, stable, and reproducible fragments (genetic loci), with a size range from 150 to 1900 bp. The number of scored bands per primer in all populations ranged from 15 to 21 (Table 1). All amplified fragments were polymorphic at species level; however, all primers failed to yield a diagnostic band, i.e., present in all individuals of 1 population and at the same time absent from the others, which could lead to the identification of the various geographic populations.

Table 1. Attributes of the five primers used for RAPD analysis from 433 individuals of *Mytilus galloprovincialis* sampled from 10 populations.

Primer code	Sequence (5'-3')	Number of bands scored
OPF 03	CCTGATCACC	21
OPA 04	AATCGGGCTG	17
OPA 15	TTCCGAACCC	19
OPA 16	AGCCAGCGAA	15
OPA 18	AGGTGACCGT	16

The values of the basic genetic parameters for each population are presented in Table 2. The average N_E was 1.312 at the population and 1.350 at species level. *I* was 0.309 and 0.3623 at population and species level respectively, while (assuming Hardy-Weinberg equilibrium) the average *H* was estimated to be 0.1969 within populations and 0.2231 at the species level (Table 2).

Table 2. Basic genetic variability parameters at the ten populations detected by random amplified polymorphic DNAs.

Population	*N*	P (%)	N_E	*I*	*H*
Chalastra	44	80.68	1.3187	0.3230	0.2042
Epanomi	42	75	1.3055	0.3063	0.1961
Kalochori	44	78.41	1.3010	0.2984	0.1764
Peraia	44	79.55	1.3196	0.3195	0.2016
Stomio	44	72.73	1.3135	0.3059	0.1975
Porto Koufo	43	79.55	1.3032	0.3095	0.1961
Igoumenitsa	43	76.14	1.3108	0.3018	0.1934
Kavala	42	71.59	1.3508	0.3199	0.2142
Mytilene	43	81.82	1.3297	0.3262	0.2106
Canakkale	44	72.73	1.2680	0.2801	0.1785
Species level		76.81	1.3500	0.3623	0.2231

N = sample size; P (%) = percentage of polymorphic loci; N_E = effective number of alleles per locus; *I* = Shannon's diversity index; *H* = heterozygosity.

Genetic distances and genetic differentiation

Values of Nei's (1978) pairwise genetic distance among the different geographic populations ranged from 0.0056 (between the 2 cultured samples of Chalastra and Epanomi) to 0.0731 (between Canakkale and Stomio), with an average $D = 0.0386$ (Table 3). The UPGMA

tree shown in Figure 2 illustrates the genetic relationships among the 10 populations studied. The bootstrap values were highly significant (100%) only for the branch of the 2 cultured populations, while all of the remaining values were less than 75%. The Mantel test did not reveal any correlation between geographic and genetic distances either at the macrogeographical scale when all samples were included (r = 0.06, P = 0.30), or within the restricted area of the Thermaikos Gulf (r = -0.02, P = 0.45). Pairwise exact tests revealed that with the exception of the 2 cultured populations (Chalastra and Epanomi), all remaining population pairs showed significant levels of genetic heterogeneity. This was further supported by the results of F_{ST} analyses; the genetic differentiation among the 10 studied populations was F_{ST} = 0.105 (P < 0.001) and translated to 2.1 migrants per generation (N_m). Genetic population subdivision was less evident, but still significant (P < 0.001), among the Aegean populations (omitting the samples of Igoumenitsa and Canakkale) and among the 4 populations of the Thermaikos Gulf (F_{ST} = 0.099 and 0.062, respectively).

Table 3. Pairwise genetic distance (Nei, 1978) among *Mytilus galloprovincialis* populations.

Population	Chalastra	Epanomi	Kalochori	Peraia	Stomio	Porto Koufo	Igoumenitsa	Kavala	Mytilene	Canakkale
Chalastra	***									
Epanomi	0.0056	***								
Kalochori	0.0207	0.0160	***							
Peraia	0.0149	0.0170	0.0191	***						
Stomio	0.0312	0.0348	0.0413	0.0391	***					
Porto Koufo	0.0253	0.0249	0.0293	0.0272	0.0422	***				
Igoumenitsa	0.0340	0.0336	0.0233	0.0339	0.0610	0.0408	***			
Kavala	0.0508	0.0588	0.0673	0.0490	0.0558	0.0543	0.0413	***		
Mytilene	0.0323	0.0368	0.0369	0.0309	0.0660	0.0447	0.0558	0.0278	***	
Canakkale	0.0480	0.0450	0.0367	0.0451	0.0731	0.0201	0.0367	0.0635	0.0352	***

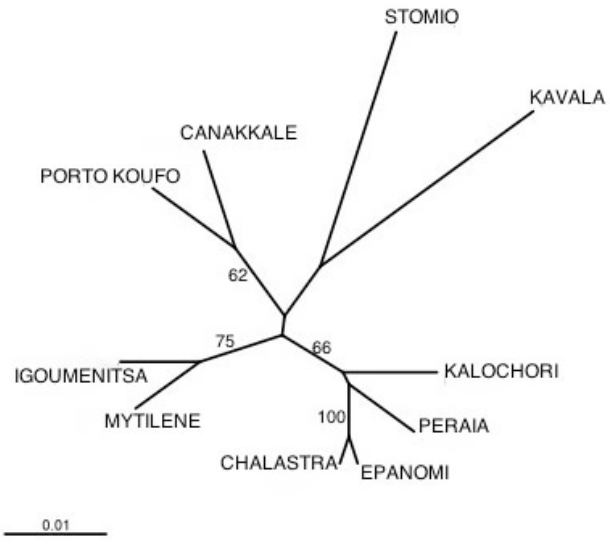

Figure 2. UPGMA tree illustrating the genetic relationships among all *Mytilus galloprovincialis* populations analyzed based on Nei's genetic distance. Branch lengths reflect genetic distance according to scale.

DISCUSSION

RAPD markers have been successfully applied in a range of phylogenetic, taxonomic, and genetic diversity studies (Bardakci, 2001; Hammad and Qari, 2010). In the present study, the RAPD technique was very useful, thus providing important information about the genetic pattern of *M. galloprovincialis* populations. The limitation of yielding a diagnostic marker band to discriminate one population from another was not unforeseen. Such diagnostic bands are usually obtained by the RAPD technique in freshwater species (Jug et al., 2004; Apostolidis et al., 2011), but are rarely seen in marine species, due to the absence of barriers to migration and gene flow (Mamuris et al., 1998).

Genetic diversity

Since all of the RAPD-generated bands were found to be polymorphic at species level, the frequency of polymorphism was 100%. However, it was not the same among the 10 populations that were studied, varying between 71.59 and 81.82% for the populations from Kavala and Mytilene, respectively (Table 2). Nevertheless, the proportion of polymorphic loci is not regarded as a good method to describe genetic variation, while more appropriate measures like effective number of alleles and heterozygosity are more efficient estimators (Elmaci et al., 2007; Tiira and Primmer, 2009). Especially for RAPD data, *I* is considerably suitable since it remains unaffected by the method's inability to distinguish heterozygous individuals but also does not require the presumption that populations are in Hardy-Weinberg equilibrium (Dawson et al., 1995).

As shown in Table 2, all 3 diversity measures mentioned above did not reveal any loss in genetic variability of the 2 cultured populations (Chalastra and Epanomi). Reduced genetic variability caused by inbreeding is a frequently occurring phenomenon with several possible deleterious effects in cultured fish and shellfish populations originating from hatchery stocks (Kong and Li, 2007). Nevertheless, 2 systems of mussel culture are applied in Greece, bouchot mussel culture and the long-line system, neither of which includes artificial fertilization of eggs (Gosling, 2003). Instead, spat is collected from traps (ropes or wooden stakes) involving large numbers of mating individuals, which are not closely related. It seems that the applied aquaculture practices in Greek mussel farms (at least according to the results from the two studied samples) have not affected their genetic variability.

In contrast, intensely reduced values of genetic diversity appeared in the wild populations of Canakkale and Kalochori (Table 2). Apart from stochastic sampling errors, a possible explanation of this phenomenon could be the heavy environmental pollution in these areas. Indeed, the levels of water pollution in the marine area of Canakkale exceed the limit values according to the World Health Organization (WHO) and the Environmental Protection Agency (EPA), mainly due to the deposition of approximately 280 tons cadmium and 4500 tons lead reaching the Dardanelles from the Danube River (Yilmaz and Sadikoglu, 2011). Similarly, approximately 120,000-150,000 tons of municipal wastewater are deposited daily in the marine area of Kalochori via a pipe (Karvelas et al., 2003). Violintzis et al. (2009) revealed that total organic carbon, acid volatile sulfur, and some toxic elements (Pb, Zn, Cu, As) are in very high concentrations in this area, thus presenting a significant risk to the local biota.

Although earlier approaches of environmental pollution influence on aquatic organisms were focused on mechanisms of damage on test organisms exposed in laboratory

settings to calculate biomass shifts and mortality proportion, recent studies emphasize the genetic impact as determined by a wide variety of DNA techniques (Belfiore and Anderson, 2001). RAPD markers, in particular, have proven to be not only useful to infer genotoxic-related population genetic effects, but also capable to preliminarily evaluate the different routes in which toxic exposure may influence the genetic patterns of natural populations (De Wolf et al., 2004). Toxicants may increase the genetic diversity of a population by affecting the mutation rate, or decrease it through selection of more tolerant genotypes, bottleneck effects, or alteration of migration rates (Maes et al., 2005). In any case, the consequences of such change could be catastrophic for the long-term survival of the population and also for the entire biotope.

Population genetic structure

Exact tests and F_{ST} values showed significant genetic structuring among populations, whereas, as revealed by the UPGMA tree (Figure 2) and the Mantel test, the genetic divergence among populations was independent of the geographic distances among collecting sites. Such results are common in *Mytilus* species and have been reported in a number of studies, including those for populations of *M. chilensis* along Chilean coasts using RAPD markers (Toro et al., 2004) and for *M. californianus* from Californian coasts using allozyme, mtDNA, and single-copy nuclear DNA markers (Addison et al., 2008).

The total average effective number of N_m was calculated to be 2.1 for all populations sampled and 2.3 for the 8 Aegean populations. These values of N_m are higher than 1 migrant per generation needed to prevent differentiation by random genetic drift, while at the same time small enough to allow significant genetic structuring. Low levels of gene flow were evident even at the restricted area of the Thermaikos Gulf (Table 4), reinforcing further the restricted dispersal ability around Greek coasts. Setting aside limitations of the RAPD technique (dominant inheritance and questionable reproducibility), biological parameters coupled with oceanographic conditions prevailing in the studied area and random transport via shipping are some of the reasons that might explain the observed genetic structuring. The Aegean Sea is characterized by a complex hydrography and is influenced by a low-salinity outflow from Dardanelles. The latter forms large and strong sea streams (Olson et al., 2007), which might either contribute to great mobility of gametes and adult mussels or act as barriers to this movement. On a microgeographical scale, the cyclonic flow of the Thermaikos Gulf (Kravva et al., 2000) may be partly responsible for the weak congruence between genetic and geographic distances among local populations (Figure 2).

However, particularly for the Greek mussel populations, repeated transplantations may

Table 4. Pairwise genetic differentiation (F_{ST}) and number of migrants (N_m) per generation among *Mytilus galloprovincialis* samples of Thermaikos Gulf.

Geographic level	F_{ST}	N_m
Epanomi - Chalastra	0.018	13.5
Epanomi - Kalochori	0.031	7.8
Epanomi - Peraia	0.035	6.9
Peraia - Kalochori	0.036	6.7
Peraia - Chalastra	0.032	7.5
Kalochori - Chalastra	0.037	6.6

have also played an important role in the absence of geographic structure. Indeed, numerous mussel farms have been developed along Greek coasts in the past decades and there are unrecorded reports that at least their initial spat originated from distant locations, mainly from Chalastra. In such a case, Chalastra, which was the first place in Greece where mussel culture took place, could be characterized as the natural source of mussel spat. Our results reinforce these reports, as this seems to be the case for the 2 cultured populations that we studied (Chalastra and Epanomi), which were found to be genetically homogenized, presenting double levels of gene flow in comparison with the rest populations of the Gulf (Table 4) and also sharing the smallest genetic distance ($D = 0.0056$) and the highest bootstrap values among all populations (Figure 2). However, before conclusions are drawn, further research using more DNA markers (such as microsatellites) and more populations from a broader geographical range is needed to investigate population genetic structure and differentiation between sampling localities of the species.

This report describes the first comprehensive genetic research study of Greek mussel populations, which was based on nuclear DNA markers. Our results suggest that anthropogenic activities may affect the genetic pattern of an organism in many different ways. Even though aquaculture practices did not seem to influence the genetic diversity of cultured mussels, marine pollution and human-mediated transport of mussels have possibly affected natural populations, with unresolved economic or environmental consequences.

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