Imsiridou A, et al. J Nutr Food Lipid Sci 2019(1): 54-83.

OCIMUM

Journal of Nutrition, Food and Lipid Science

Original Research

Can the Population Structure of Three Greek Marine Species (*Sardina pilchardus, Penaeus kerathurus, Mullus barbatus*) Become a Tool for their Future Characterization as PGI Products?

Anastasia Imsiridou[1*], Maria Papapetrou[1], Alexandros Tilikidis[1], Dimitrios Loukovitis[2], George Minos[3], Evangelia Gouva[4], Achilleas Chatzopoulos[4], Ioannis Skoufos[4] and Ioannis Paschos[4]

[1]Food Analysis, Quality & Safety Control Laboratory, Department of Food Technology, School of Agricultural Technology, Food Technology and Nutrition, Alexander Technological Educational Institute of Thessaloniki, Sindos, Thessaloniki, Greece
[2]Research laboratory of Agro Biotechnology and Inspection of Agricultural Products, Department of Agricultural Technology, School of Agricultural Technology, Food Technology and Nutrition, Alexander Technological Educational Institute of Thessaloniki, Sindos, Thessaloniki, Greece
[3]Laboratory of Biology & Histology, Microscopy & Image Analysis, Systematics & Biometry, Department of Nursing, School of Health and Medical Care, Alexander Technological Educational Institute of Thessaloniki, Sindos, Thessaloniki, Greece
[4]Department of Agricultural Technology, School of Agricultural Technology, Food Technology and Nutrition, Technological Education Institute of Epirus, Arta, Greece

Received: 27 January, 2019
Accepted: 06 March 2019
Version of Record Online: 15 March 2019

Citation

Imsiridou A, Papapetrou M, Tilikidis A, Minos G, Loukovitis D, et al. (2019) Can the Population Structure of Three Greek Marine Species (*Sardina pilchardus, Penaeus kerathurus, Mullus barbatus*) Become a Tool for their Future Characterization as PGI Products? J Nutr Food Lipid Sci 2019(1): 54-83.

Correspondence should be addressed to
Anastasia Imsiridou, Greece
E-mail: imsiri@otenet.gr

Copyright

Abstract

European Commission has established among others the Protected Geographical Indication (PGI) protection label in order to identify an agricultural product, raw or processed, of which quality, reputation or other characteristics are linked to its geographical origin. One hundred and twenty-four Greek products have been recognized as Protected Designation of Origin (PDO)/Protected Geographical Indication (PGI). In the present study the PCR and Sanger sequencing analysis of three mitochondrial segments (*COI, cytb, D-loop*) were applied, to examine the possibility that the population structure of three marine species *Sardina pilchardus*, *Penaeus kerathurus, Mullus barbatus* can be used as a tool for their further PGI determination. Three populations of each species were collected from Western and North Eastern Greece. *P. kerathurus* revealed to be the most structured species and all the populations were significantly differentiated, with the Thracian Sea population proposed as a PGI one. For *S. pilchardus* and *M. barbatus* a much lower differentiation was estimated among the populations, with the ones from Ionian Sea revealing a weak but significant genetic heterogeneity for both species. These results, if combined with further mitochondrial DNA (mtDNA) or microsatellite analyses from more locations in the Ionian basin, could shed light on a possible definition of these stocks as PGI products. Biological factors, hydrographic and ecological conditions coupled with evolutionary aspects were utilized for the interpretation of each species' population structure.

Keywords

Geographical Indication Products; Mitochondrial DNA; *Mullus barbatus; Penaeus kerathurus*; Population Structure; Protected; *Sardina pilchardus*

Introduction

In order to satisfy the increasing number of consumers demanding for edible products of certified origin, the European Commission established three protection labels: the "Protected Designation of Origin (PDO)" assigned to a product or a foodstuff produced, processed and prepared in a given area; the Protected Geographical Indication (PGI) that indicates a link of the product with the area where at least one of the stages of production, processing or preparation possesses a specific quality, reputation or other characteristics attributable to that geographical origin; the Traditional Specialities Guaranteed (TSG) for traditional products with specific characteristics (Council Regulations 509/2006 and 510/2006). One hundred and twenty-four Greek products have been recognized as Protected Designation of Origin (PDO)/Protected Geographical Indication (PGI) while there is only one marine product among them, Botargo of Messolonghi (PDO - Council Regulations 1107/96 and 1263/96).

Sardine (*Sardina pilchardus*, Walbaum 1792) is a small pelagic fish species of great interest to fisheries in central-eastern and north-eastern Atlantic Ocean. This cupleiform is found from the North Sea to Senegal, as well as in the Mediterranean Sea, Sea of Marmara, and Black Sea [1]. Northern and southern limits seem to be related to the average water temperature, being located within 10 and 20°C isotherm [2]. Nevertheless, several authors have hypothesized that sardine distribution and abundance are dependent on the hydrological regime [3]. Like other marine pelagic fishes, sardines show schooling and migratory behaviour, as well as great dispersal capabilities both at the larval and adult stages. Sardine supports important fisheries in the northeast Atlantic and in the Mediterranean Sea, with approximately 130,000 tons fished on the European coast, 660,000 tons fished on the African coast and 80,000 tons fished in the Mediterranean area [4-6]. In particular, Spain and Morocco are the countries with the largest captures (representing about the 77% of the total annual catch of sardines). In Greece it is one of the two most commercially important fish, with a total catch of 10,890.5t in 2016, representing 14.6% of the total catch of marine species [7].

The caramote prawn *Penaeus kerathurus* (Forskäl 1775) is an ecologically and economically important penaeid species. It is widespread in the Mediterranean and it also ranges from the south coast of England to Angola in the Eastern Atlantic. The benthic adults inhabit nearshore and offshore waters, to a depth of about 80 m, and prefer muddy or sandy-mud flats. In summer, adults migrate to reproduce in coastal areas and spawn in offshore waters. After a planktonic larval stage (about 4 weeks) post-larvae move into shallow waters, where they enter the juvenile stage until they reach 5-8 cm in length, and then join the adult population [8]. This species is extensively fished; the annual global capture is around 6,000 tons [5]. In Greece the total catch for 2016 was 1,404.1t, representing almost 2% of the total catch of marine species [7].

The red mullet *Mullus barbatus Linnaeus*, 1758 belongs to the Mullidae family and it is distributed in the eastern Atlantic, along the European and African coasts from the British Isles to Senegal, as well as in the Mediterranean Sea [9]. It is a gregarious benthic species living on sandy and muddy bottoms, usually between 10 and 100 m, occasionally in deeper waters up to 270 m [10]. It feeds on small benthic invertebrates such as crustaceans, worms, mollusks. The reproduction period extends from April to August at depths between 10 and 55 m on sandy or muddy bottoms and the postlarvae is pelagic until 28 mm SL [9]. Two subspecies are present, *Mullus barbatus barbatus and Mullus barbatus ponticus* which occur in the Black Sea and Azov Sea [9]. It is caught mainly by trawling fleet [11] and thus subject to intense fishing pressure; red mullet stocks are composed mainly of young fish [11]. The red mullet is one of the most commercial fish resources in the Mediterranean. In Greece it ranks among the most important demersal fish, with a total catch of 1,758.7t for 2016 (2.4% of the total catch of marine species) [7].

DNA-based methods like microsatellite genotyping [12,13], Internal Transcribed Spacer analysis (ITSs) [14,15], Single Nucleotide Polymorphisms (SNPs) [16] and High-Resolution Melting (HRM) analysis of mitochondrial markers [17] offer the possibility to identify individuals, breeds or populations along the food chain, in order to define them as PGI products. Once DNA is extracted from a biological sample (blood, muscle or even processed food), it is analyzed by molecular markers to assess variations among individuals or among populations [18].

The mitochondrial genome has a higher rate of mutation compared to the nuclear genome, is maternally inherited, has less hybridization and has a high copy number, which facilitates PCR amplification and sequence recovery from degraded tissue [19,20]. Furthermore, the mitochondrial genome lacks introns, pseudogenes and repetitive sequences, which makes sequence alignments of the amplified genes easier. Finally, complete mitochondrial DNA (mtDNA) genome sequences are available on public databases; primers can therefore be designed to amplify and sequence any species that has a published mtDNA genome [21-24].

Population genetic structure has been described for numerous species, using parts of the mitochondrial genome. A primary advantage of mtDNA is the inheritance pattern: clonal inheritance through the maternal line allows tracing of oceanographic events lasting multiple generations [25]. The

inheritance pattern of mtDNA involves that male dispersal does not participate in the homogenization of the population. Gene flow caused by male dispersal will not affect spatial patterns of variation in mtDNA [26], so, in general, mtDNA genes show more population structure than do nuclear genes [27]. This makes them particularly appropriate indicators of the population genetic differentiation of marine organisms, which are generally high gene-flow species.

Most population genetic studies of marine organisms have focused on coastal species, including sessile species with planktonic larvae. Significant population genetic structure at scales of hundreds of kilometres and smaller has been observed for a number of marine fish [28-33] and marine invertebrate species [34-39]. Two general principles emerge from many studies: first, that both marine fish and invertebrates are quite variable at the molecular level, and second, that this variability may determine genetically distinguishable, geographical populations in some of the species [40,25].

The aim of this research was to assess the potential of using the population differentiation of three Greek marine species (*S. pilchardus, P. kerathurus, M. barbatus*) as a tool for their future PGI definition. For this purpose, the PCR and further Sanger sequencing analysis of three mitochondrial segments (Cytochrome Oxidase subunit I - *COI*, cytochrome b - *cytb*, control region - *D-loop*) were applied. These mitochondrial markers have been repeatedly used for population differentiation analyses in all three species.

Materials and Methods

Sampling

Specimens of the three species were collected by professional fishermen from different localities: *S. pilchardus* from Amvrakikos Gulf (39°00' N, 20°48' W), Ionian Sea (39°33' N, 20°06' W), Kalloni Bay (39°09' N, 26°11' W) (July 2017); *P. kerathurus* from Amvrakikos Gulf (38°57' N, 20°53' W), Ionian Sea (39°37' N, 20°09' W), Thracian Sea (40°59' N, 25°04' W) (September 2016 - May 2017); *M. barbatus* from Amvrakikos Gulf (39°00' N, 20°46' W), Ionian Sea (39°37' N, 20°07' W), Kalloni Bay (39°10' N, 26°12' W) (July 2016 - May 2017); twenty individuals per population per species (180 individuals in total) (Figure 1). All specimens were preserved in ice, transferred to the laboratory and stored at

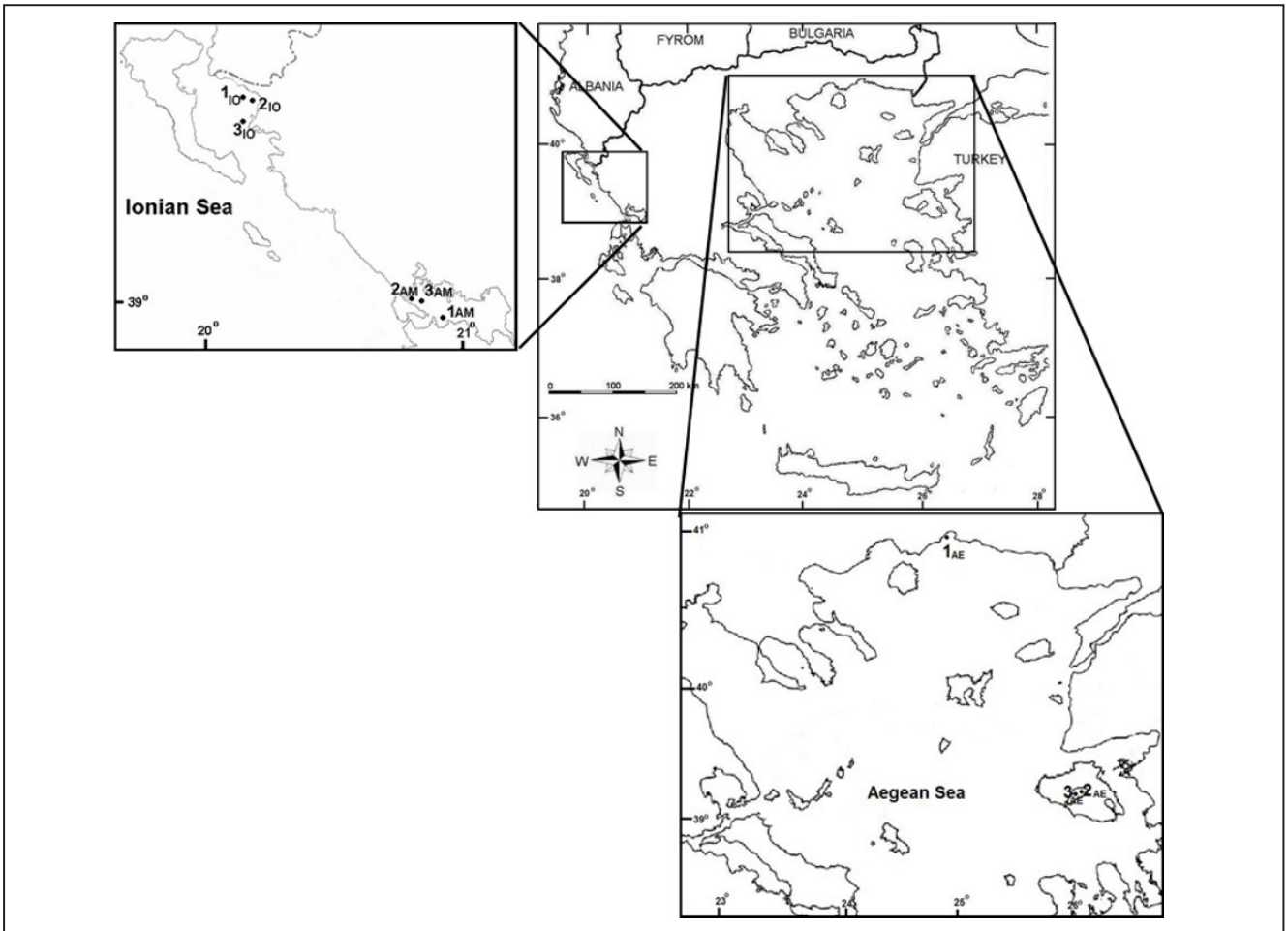


Figure 1: Sampling locations for *Penaeus kerathurus* (1_{IO}: Ionian Sea, 1_{AM}: Amvrakikos Gulf, 1_{AE}: Aegean Sea (Thracian sea); *Mullus barbatus* (2_{IO}: Ionian Sea, 2_{AM}: Amvrakikos Gulf, 2_{AE}: Aegean Sea (Kalloni bay) and *Sardina pilchardus* (3_{IO}: Ionian Sea, 3_{AM}: Amvrakikos Gulf, 3_{AE}: Aegean Sea (Kalloni bay).

-20°C until DNA preparation. In some cases, tissue samples were immediately extracted and preserved in absolute ethanol (Applichem Panreac, Germany).

DNA extraction

Total genomic DNA was extracted from muscle, with the UltraClean™ Tissue and Cells DNA Isolation Kit (MoBio, Germany), following the manufacturer's recommendations, and with the CTAB methodology [41]. The DNA concentration was determined by using the NanoDrop Spectrophotometer (ND-2000 Thermo Fisher Scientific, USA).

PCR amplification and sequencing analysis

Three mitochondrial segments were screened as potential markers for population differentiation analysis in this study: Cytochrome Oxidase subunit I (*COI*), cytochrome b (*cytb*) and the control region (*D-loop*). Double stranded DNA was amplified in a total reaction volume of 25μl containing 1 unit of Taq polymerase (Biolabs, USA), 2.5μl of 10 × reaction buffer (Biolabs, USA), 2.5 mM $MgSO_4$ (Biolabs, USA), 0.24 mM of each dNTP (Biolabs, USA), 1 pmole/μl of each primer (IDT, USA) and approximately 50-100 ng of DNA.

PCR amplification conditions were as follows

For *COI* gene: one preliminary denaturation step at 95°C (2 min), followed by strand denaturation at 94°C (30 sec), annealing at 52°C (30 sec) (for *P. kerathurus* 49.5°C, 1 min) and primer extension at 72°C (1 min) repeated for 35 cycles; and a final extension at 72°C (10 min).

For *cytb* gene: one preliminary denaturation step at 95°C (2 min), followed by strand denaturation at 94°C (1 min), annealing at 54.5°C (1.5 min) (for *M. barbatus* 52°C, 30 sec) and primer extension at 72°C (3 min) (for *M. barbatus* 1.5 min) repeated for 35 cycles; and a final extension at 72°C (10 min).

For *D-loop* region: one preliminary denaturation step at 94°C (3 min), followed by strand denaturation at 94°C (15 sec), annealing at 48.8°C (20 sec) (for *S. pilchardus* 52°C, 30 sec) and primer extension at 72°C (40 sec) (for *S. pilchardus* 1.5 min) repeated for 35 cycles; and a final extension at 72°C (5 min). The primers used for each mitochondrial segment per species are given in table 1SM. For primers used for the amplification of the COI gene in *P. kerathurus*, the numbers 1490 and 2198 refer to the position of the *Drosophila yakuba* 5' nucleotide. For primers used for the amplification of the *cytb* gene in *S. pilchardus* and *P. kerathurus*, the numbers 14841 and 15149 refer to the position of the 3' base of the primer in the complete human mtDNA sequence. For the amplification of the *D-loop* region in *P. kerathurus*, the amplified fragment (target size, 611 bp) corresponds to positions 14968 to 15578 of the complete mitochondrial genome sequence of *Penaeus monodon* (GenBank accession no. NC_002184), representing about 60% of the control region at the 5' end (Table 1SM).

Electrophoresis of 3μl of the PCR product was performed in 1 × TBE buffer (Applichem Panreac, Germany) for 1h at 150V, in 1.5% agarose gel (Applichem Panreac, Germany) containing 0.5μg/ml Midori Green (Neppon Genetics, Germany). The size of the PCR products was checked against a 100bp DNA ladder (GeneOn, Germany). After the end of the electrophoresis, the resulting DNA fragments were visualized by UV transillumination and photographed.

PCR products were purified using MOBIO Ultra Clean PCR Clean-Up Kit (MoBio, Germany), following the manufacturer's instructions. In order to avoid false positive polymorphisms and to double check mutations, sequencing was carried out in both directions on a ABI 3500 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA). Furthermore, for all the ambiguous sites the PCR and sequencing reactions were repeated. Both strands of each sequence were aligned with CLUSTAL W algorithm [42] as implemented in the software BioEdit v.7.0.5.3 [43] and visually confirmed. All sequences were confirmed as those of the corresponding genes by BLAST searches on GenBank except for *P. kerathurus*, the *cytb* and *D-loop* sequences of which were the first DNA sequences of the species submitted to any Genetic Database.

Statistical analysis

Different haplotypes for each mitochondrial segment were detected in all three populations of each species with the DAMBE6 software package [44]. Subsequently, haplotype frequencies were estimated for each population. In order to examine relationships among individuals, the maximum likelihood approach was applied, using MEGA7 software [45] and the complete nucleotide data set (*COI, cytb, D-loop*) of each analyzed sample. The best-fit substitution model was provided by the MEGA7 software [46]. Non-uniformity of evolutionary rates among sites were modelled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Branch support was assessed by 1,000 replicates and sites with missing data were removed only when needed.

Genetic distances among the three populations per species were estimated based on both the nucleotide sequences of each segment and the total nucleotide sequences (*COI, cytb, D-loop*), using the 2-parameter Kimura distance model [47]. Phylogenetic relationships among them were estimated with

the MEGA7 software selecting three different algorithms: Neighbour Joining [48], UPGMA [49] and Minimum Evolution [50], using both the nucleotide sequences of each segment and the complete nucleotide dataset.

For population differentiation estimates the total nucleotide sequences (*COI, cytb, D-loop*) for each individual were used. Population differentiation analyses were conducted with Arlequin 3.5.2.2. [51], and *P*-values were estimated. Differences were considered statistically significant at $P < 0.05$. To determine the amount of genetic variability partitioned within and among populations, an Analysis of Molecular Variance (AMOVA) was performed in Arlequin 3.5.2.2., and the significances of both the F statistics and variance components were assessed with 1,000 permutations. The geographic distances between sampling sites were measured in km, using Google Earth.

Results

S. pilchardus

The size of the PCR products was approximately 700 base pairs (bp) for *COI* gene, 340 bp for *cytb* gene and 1600 bp for the *D-loop* region. In total, 641 bp at the 5′ end of the *COI* gene, 294 bp at the 5′ end of the *cytb* gene and 473 bp at the 5′ end of the *D-loop* region were sequenced for the majority of the individuals.

Fifty-eight individuals were sequenced for *COI* gene; namely, 19 individuals from Amvrakikos Gulf population, 20 individuals from Ionian Sea and 19 individuals from Kalloni Bay population. Nine samples were replicated and the good quality sequences were successfully obtained with the second sequencing effort. Thirty-three haplotypes were detected for *COI* gene in the three *S. pilchardus* populations (Table 2SM). The most common Haplotype (H1) was found in 20 individuals of all three populations and two haplotypes were found in two populations. The high frequency and wide geographical distribution of haplotype 1 indicate that this is probably the ancestral haplotype. All the other haplotypes were detected in only one population.

Forty-nine individuals were sequenced for *cytb* gene; namely, 16 individuals from Amvrakikos Gulf population, 17 individuals from Ionian Sea and 16 individuals from Kalloni Bay population. Twelve samples had to be replicated. Twenty-five haplotypes were detected for *cytb* gene and the most common Haplotype (H1) was found in 23 individuals of all three populations (ancestral haplotype) (Table 3SM). All the other haplotypes were detected in only one population.

Finally, 55 individuals were sequenced for *D-loop* region; namely, 16 individuals from Amvrakikos Gulf population, 20 individuals from Ionian Sea and 19 individuals from Kalloni Bay population. Only five samples were repeated and the good quality sequences were successfully obtained with the second sequencing effort. Fifty-three haplotypes were found for the *D-loop* region (Table 4SM). All these haplotypes were detected in only one population, with the exception of H2, which was detected in two out of three populations. All haplotypes were deposited to GeneBank under the accession numbers MH141137-MH141169 (*COI* haplotypes), MH127862-MH127879 (*cytb* haplotypes) and MH141170-MH141222 (*D-loop* haplotypes).

The complete nucleotide dataset included 46 sequences with a total length of 1408 bp. The maximum likelihood topology estimated with the complete nucleotide dataset of each individual (Figure 2) revealed no clustering of the samples in relation to the population they are coming from. Besides, most of the bootstrap values were lower than 40%.

The Neighbour Joining phylogenetic trees, constructed with the nucleotide sequences of each mitochondrial segment and with the complete nucleotide dataset (Figure 3), revealed the same structure: populations from Amvrakikos Gulf and Kalloni Bay were grouped together, whereas the population from Ionian Sea formed a separate clade. The most significant outcome of the phylogenetic analysis was that both the UPGMA and the Minimum Evolution trees (based on the nucleotide sequences of each segment and on the total sequences) had identical topology: the first cluster included populations from Amvrakikos Gulf and Kalloni Bay while the second one was formed by the Ionian Sea population.

For population differentiation estimates, the total nucleotide sequences (*COI, cytb, D-loop*) for each individual were used. The AMOVA analysis showed that most of the genetic variation was present within samples (98.77%) and 1.23% of genetic heterogeneity was apportioned among them (Table 1). The total F_{st} value was $F_{st} = 0.01232$. F_{st} and statistical P values among all the studied population samples are shown in Table 2. The highest F_{st} value occurred between the populations from Ionian Sea and Amvrakikos Gulf. It is also obvious that the Ionian Sea population was clearly differentiated from the Amvrakikos Gulf population ($P < 0.05$), while there was no significant differentiation among the other pairs of populations.

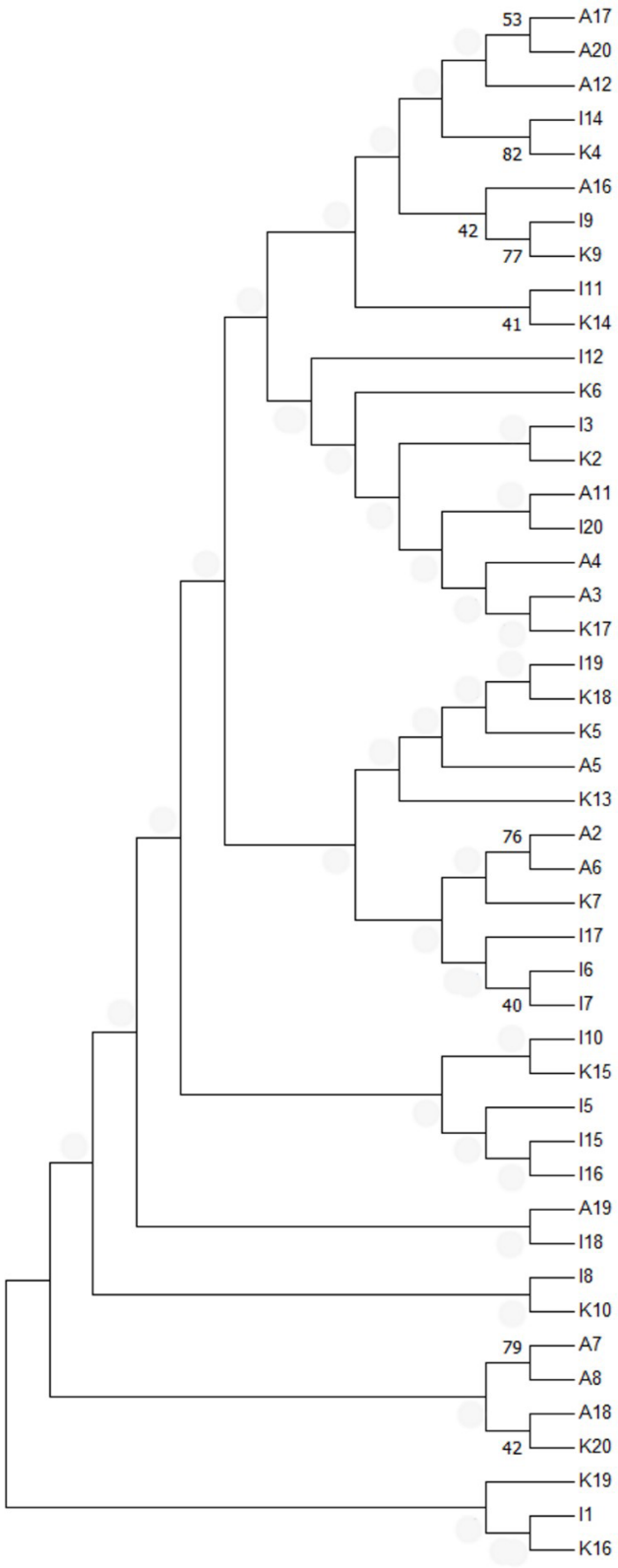


Figure 2: Maximum likelihood tree from the complete nucleotide dataset (*COI, cytb, D-loop*), illustrating the genetic relationships between *Sardina pilchardus* individuals. The model used was the Kimura 2-parameter model. Numbers above branches indicate bootstrap values among 1,000 replicates. Branches without bootstrap numbers mean that the bootstrap values are below 40%.

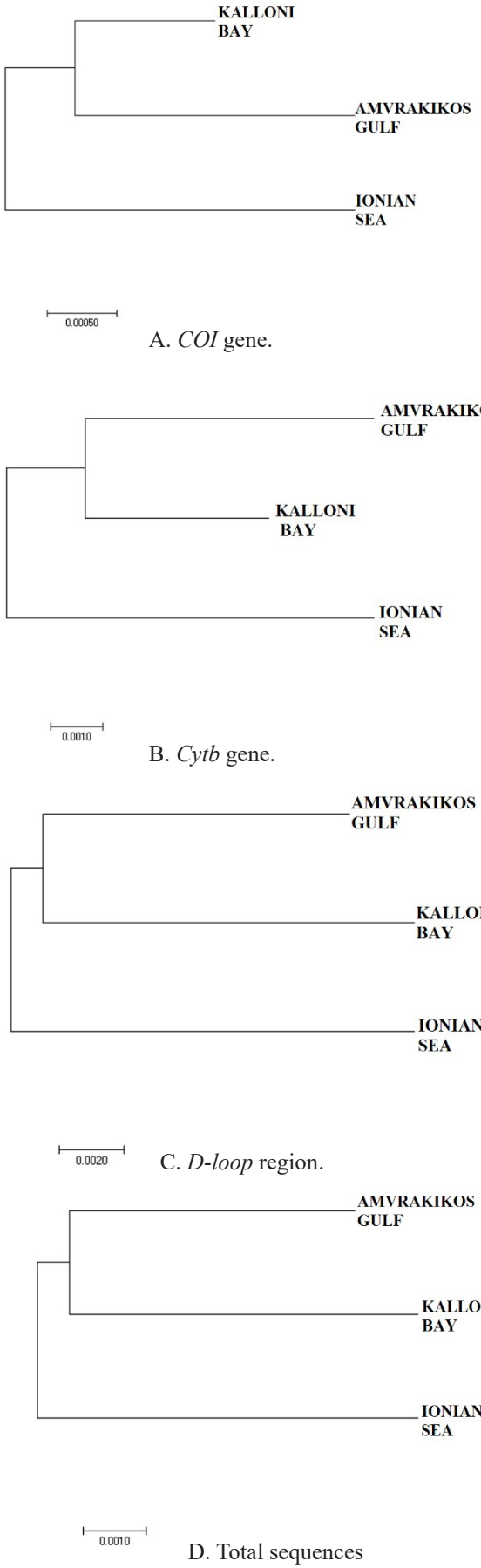


Figure 3: Neighbour Joining phylogenetic trees among the three populations of Sardina pilchardus, based on the nucleotide sequences of each mitochondrial segment and on the complete nucleotide dataset.

Sardina pilchardus				
Source of variation	d.f.[1]	Sum of squares	Variance components	Percentage of variation
Among populations	2	18.164	0.09515 Va	1.23
Within populations	43	327.989	7.62764 Vb	98.77
Total	45	346.152	7.72279	
Fixation Index	F_{st} = 0.01232			
Penaeus kerathurus				
Source of variation	d. f.[1]	Sum of squares	Variance components	Percentage of variation
Among populations	2	99.044	2.11354 Va	12.1
Within populations	46	706.568	15.36018 Vb	87.9
Total	48	805.612	17.47372	
Fixation Index	F_{st} = 0.12096			
Mullus barbatus				
Source of variation	d. f.[1]	Sum of squares	Variance components	Percentage of variation
Among populations	2	11.39	0.11656 Va	3.15
Within populations	52	186.21	3.58864 Vb	96.85
Total	54	198	3.70521	
Fixation Index	F_{st} = 0.03146			

Table 1: Measures of population differentiation for the three species, based on analysis of molecular variance approach with the complete nucleotide dataset: *COI, cytb, D-loop* for *Sardina pilchardus* and *Penaeus kerathurus, COI, cytb* for *Mullus barbatus*.

[1]$P < 0.05$

	Amvrakikos Gulf	Ionian Sea	Kalloni Bay
Amvrakikos Gulf		**[0.01802*]**	[0.27928]
Ionian Sea	0.03119		[0.45045]
Kalloni Bay	0.00687	-0.00126	

Table 2: F_{st} values among the *Sardina pilchardus* population samples, based on total sequences (*COI, cytb, D-loop*). Statistical *P* values are shown in brackets. (*) indicates the statistically significant values ($P < 0.05$).

P. kerathurus

The size of the PCR products was approximately 700 base pairs (bp) for *COI* gene, 350 bp for *cytb* gene and 600 bp for the *D-loop* region. In total, 632 bp at the 5′ end of the *COI* gene, 316 bp at the 5′ end of the *cytb* gene and 552 bp at the 5′ end of the *D-loop* region were sequenced for the majority of the individuals.

Fifty-nine individuals were sequenced for *COI* gene; namely, 20 individuals from Amvrakikos Gulf population, 20 individuals from Ionian Sea and 19 individuals from Thracian Sea population; only two samples had to be replicated. Thirty haplotypes were detected for *COI* gene in all three populations of *P. kerathurus*, and only Haplotype 1 (H1) was found in 14 individuals of both Amvrakikos Gulf and Ionian Sea populations (Table 5SM). All the other haplotypes were detected in only one population.

Fifty-seven individuals were sequenced for *cytb* gene; namely, 20 individuals from Amvrakikos Gulf population, 19 individuals from Ionian Sea and 18 individuals from Thracian Sea population. Overall, 15 samples were replicated and the good quality sequences were successfully obtained with the second sequencing effort. Forty-one haplotypes were determined for *cytb* gene while the most common Haplotype (H1) was found in 12 individuals of all three populations (ancestral haplotype) (Table 6SM). All the other haplotypes were detected in only one population.

Fifty-one individuals were sequenced for *D-loop* region; namely, 18 individuals from Amvrakikos Gulf population, 19 individuals from Ionian Sea and 14 individuals from Thracian Sea population. Overall, five samples were re-sequenced and fifty-one haplotypes were found for the *D-loop* region, none of which was common among the populations: 18 haplotypes appeared in Amvrakikos Gulf population, 19 haplotypes were detected in Ionian Sea population and 14 haplotypes were found in the population from Thracian Sea (Table 7SM). All haplotypes were deposited to Gene Bank under the accession numbers MF939109-MF939138 (*COI*

haplotypes), MF966418-MF966457 (*cytb* haplotypes) and MF796552-MF796602 (*D-loop* haplotypes).

The complete nucleotide dataset included 49 sequences with a total length of 1500 bp. The maximum likelihood topology estimated with the complete nucleotide dataset of each sample (Figure 4) showed that most of the individuals from Thracian Sea population were grouped separately, with high bootstrap values (66%, 96% and 98%).

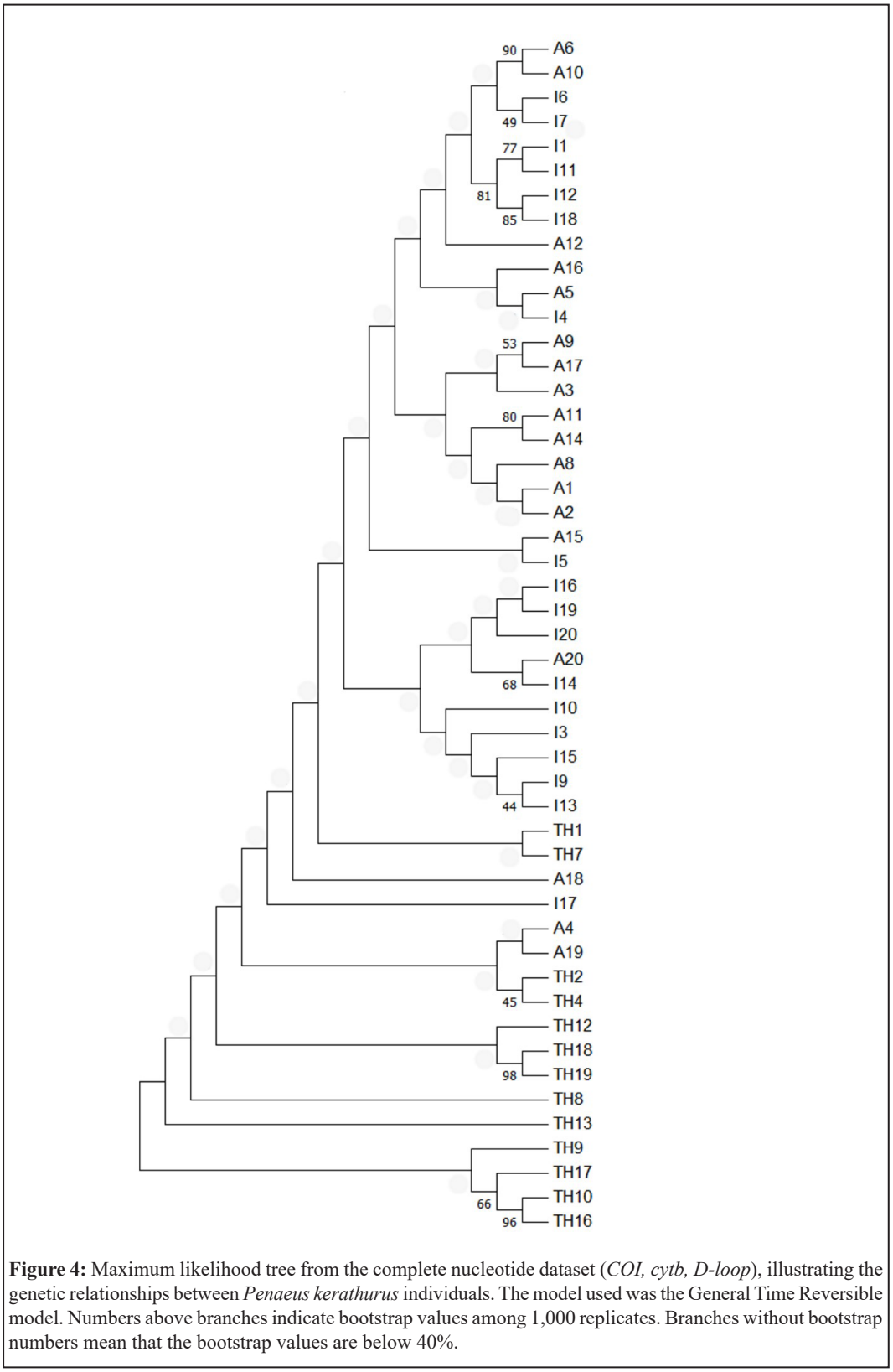
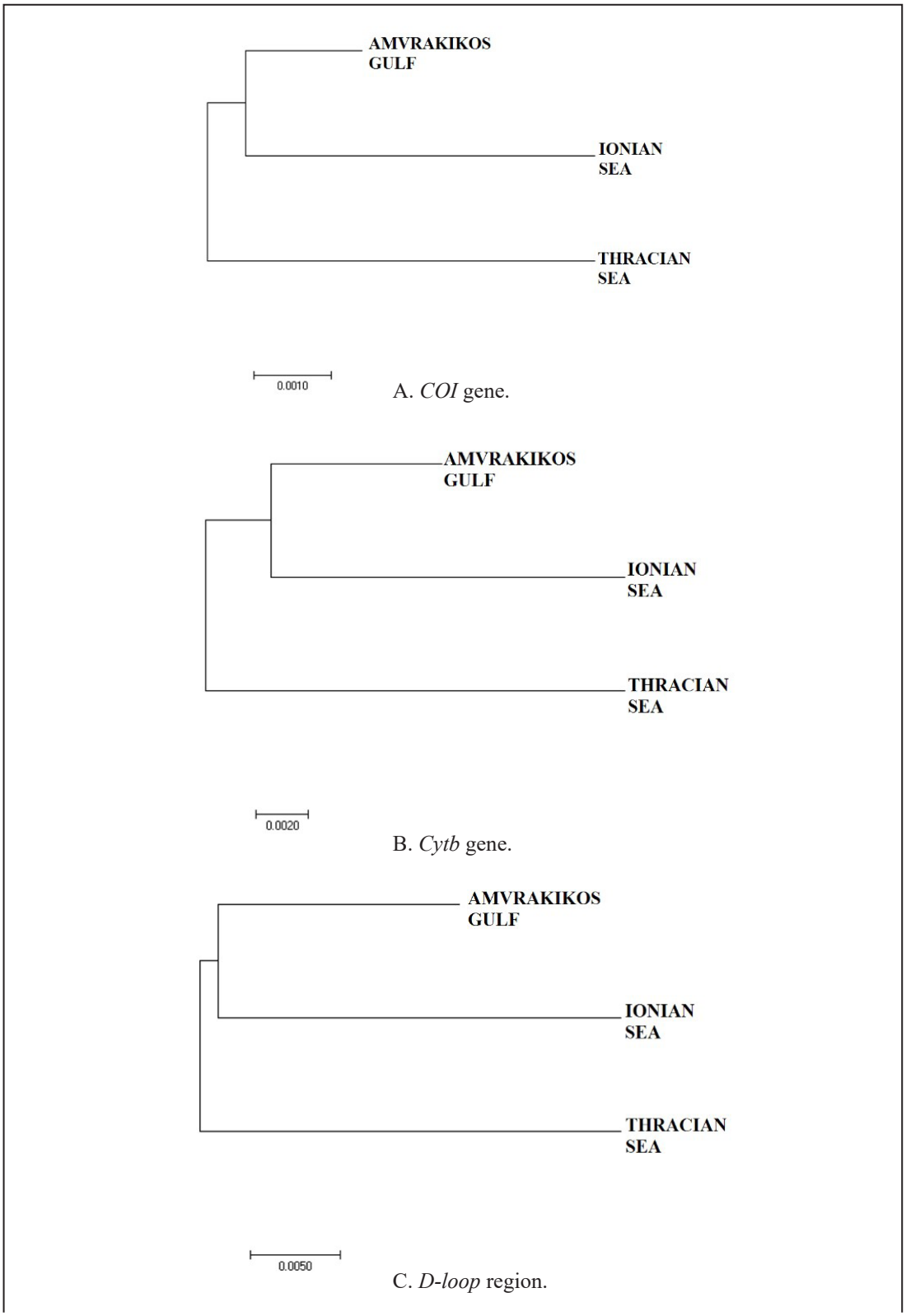


Figure 4: Maximum likelihood tree from the complete nucleotide dataset (*COI, cytb, D-loop*), illustrating the genetic relationships between *Penaeus kerathurus* individuals. The model used was the General Time Reversible model. Numbers above branches indicate bootstrap values among 1,000 replicates. Branches without bootstrap numbers mean that the bootstrap values are below 40%.

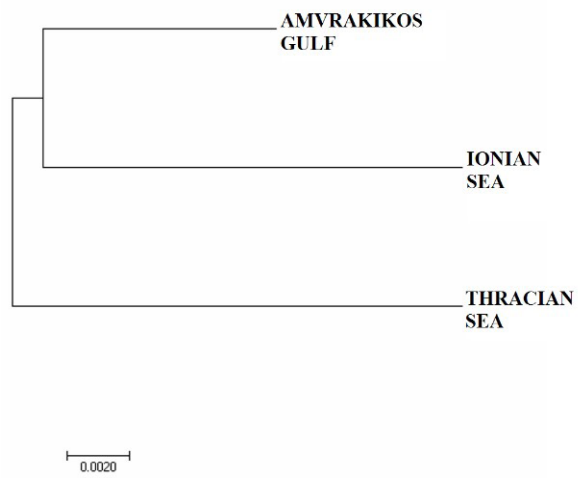
The Neighbour Joining phylogenetic trees, constructed with the nucleotide sequences of each mitochondrial segment and with the complete nucleotide dataset (Figure 5), revealed the same structure: populations from Amvrakikos Gulf and Ionian Sea were grouped together whereas the population from Thracian Sea formed a separate clade. The most remarkable outcome of the phylogenetic analysis was that both the UPGMA and the Minimum Evolution trees (based on the nucleotide sequences of each segment and on the total sequences) had the same structure: the first cluster included populations from Amvrakikos Gulf and Ionian Sea while the second one consisted of the Thracian Sea population.



A. *COI* gene.

B. *Cytb* gene.

C. *D-loop* region.



D. Total sequences.

Figure 5: Neighbour Joining phylogenetic trees among the three populations of *Penaeus kerathurus*, based on the nucleotide sequences of each mitochondrial segment and on the complete nucleotide dataset.

For population differentiation estimates total nucleotide sequences (*COI, cytb, D-loop*) for each individual were used. The AMOVA analysis showed that 87.90% of the genetic variation was present within samples and 12.10% of genetic heterogeneity was found among them (Table 1). The total F_{st} value was F_{st} = 0.12096. F_{st} and statistical *P* values among all the studied populations are shown in table 3. The highest F_{st} value occurred between the populations from Amvrakikos Gulf and Thracian Sea (F_{st} = 0.16438). There was also statistically significant genetic differentiation among all the pairs of populations (*P* = 0.0000).

	Amvrakikos Gulf	Ionian Sea	Thracian Sea
Amvrakikos Gulf		**[0.0000*]**	**[0.0000*]**
Ionian Sea	0.09187		**[0.0000*]**
Thracian Sea	0.16438	0.11833	

Table 3: F_{st} values among the *Penaeus kerathurus* population samples, based on total sequences (*COI, cytb, D-loop*). Statistical *P* values are shown in brackets. (*) indicates the statistically significant values ($P < 0.05$).

M. barbatus

The size of the PCR products was approximately 700 base pairs (bp) for *COI* gene and 620 bp for *cytb* gene. For the amplification of the *D-loop* region, previous universal primers were tested [52]. The resulted PCR product had many bands but there was no band with the expected size. Therefore, the mitochondrial *D-loop* segment was not used for further analysis. In total, 637 bp at the 5′ end of the *COI* gene and 576 bp at the 5′ end of the *cytb* gene were sequenced for all the individuals.

Fifty-five individuals were sequenced for *COI* gene; namely, 20 individuals from Amvrakikos Gulf population, 21 individuals from Ionian Sea and 14 individuals from Kalloni Bay population. Nine samples were re-sequenced and thirty-two haplotypes were detected for *COI* gene in the three *M. barbatus* populations (Table 8SM). The most common Haplotype (H1) was found in 14 individuals of all three populations. The high frequency and wide geographical distribution of haplotype 1 indicate that this is probably the ancestral haplotype. Two other Haplotypes (H3 and H4) were common for individuals of the three populations but in lower frequencies. Two haplotypes were detected in two out of three populations and all the other haplotypes were found in only one population (Table 8SM).

Altogether, fifty-five individuals were sequenced for *cytb* gene; namely, 20 individuals from Amvrakikos Gulf population, 21 individuals from Ionian Sea and 14 individuals from Kalloni Bay population. Overall, ten samples were replicated and the good quality sequences were successfully obtained with the second sequencing effort. Nineteen haplotypes were found for *cytb* gene (Table 9SM). Three haplotypes were common among the three populations and two haplotypes were detected

in two out of three populations. All the other haplotypes appeared in only one population. All haplotypes were deposited to Gene Bank under the accession numbers MG969169-MG969200 (COI haplotypes) and MG969201-MG969219 (*cytb* haplotypes).

The complete nucleotide dataset included 55 sequences with a total length of 1213 bp. The maximum likelihood topology estimated with the complete nucleotide dataset of each individual (Figure 6) revealed no clustering of the samples in relation to the population they are coming from. In addition, most of the bootstrap values were lower than 40%.

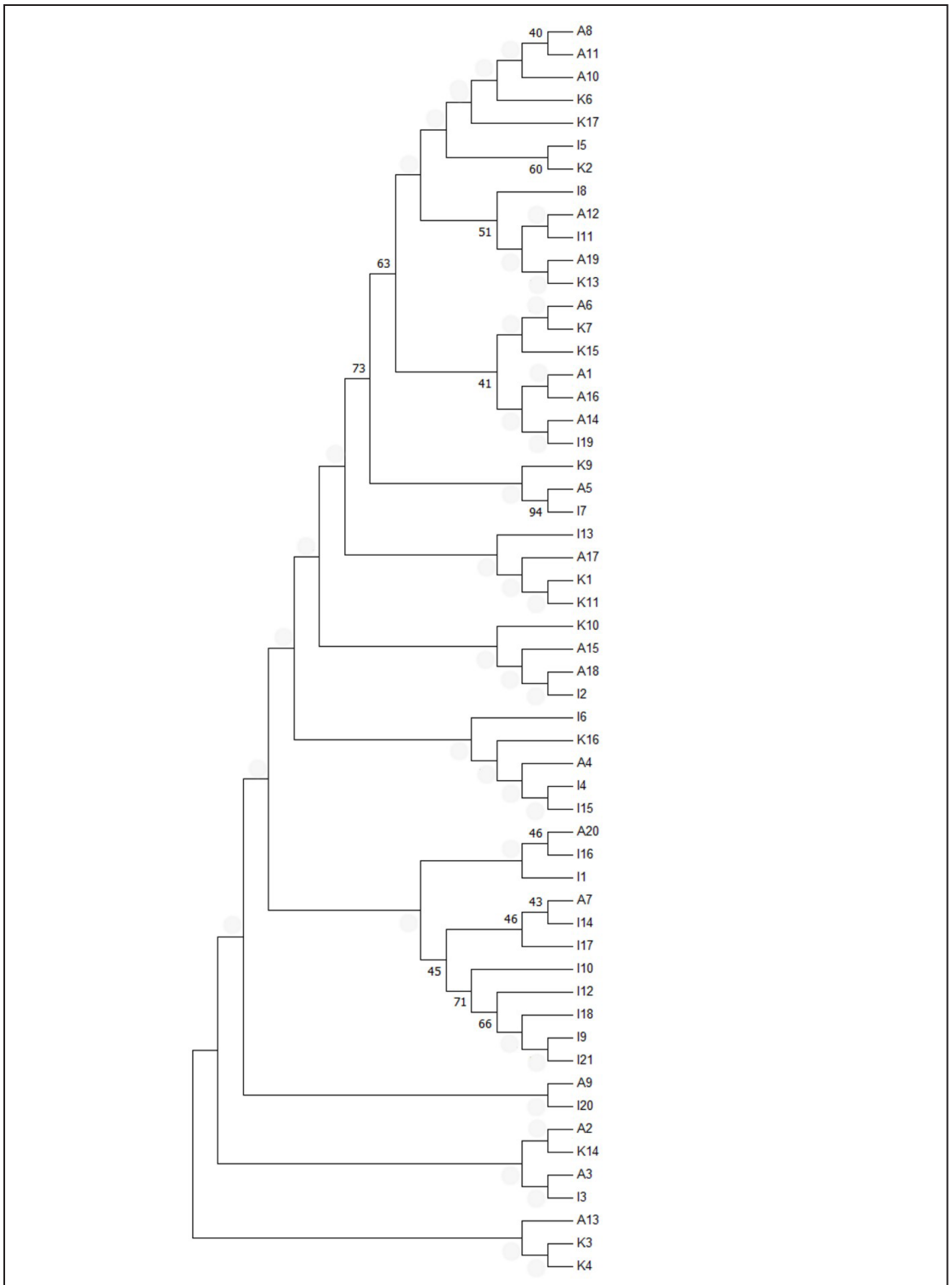


Figure 6: Maximum likelihood tree from the complete nucleotide dataset (*COI, cytb*), illustrating the genetic relationships between *Mullus barbatus* individuals. The model used was the Hasegawa-Kishino-Yano model. Numbers above branches indicate bootstrap values among 1,000 replicates. Branches without bootstrap numbers mean that the bootstrap values are below 40%.

The Neighbour- Joining phylogenetic trees constructed with both the nucleotide sequences of each mitochondrial segment and with the complete nucleotide dataset (Figure 7) could not differentiate any of the populations. Both the UPGMA and the Minimum Evolution trees (based on the nucleotide sequences of each segment and on the complete nucleotide dataset) had similar structure: there was no obvious differentiation among the *M. barbatus* populations.

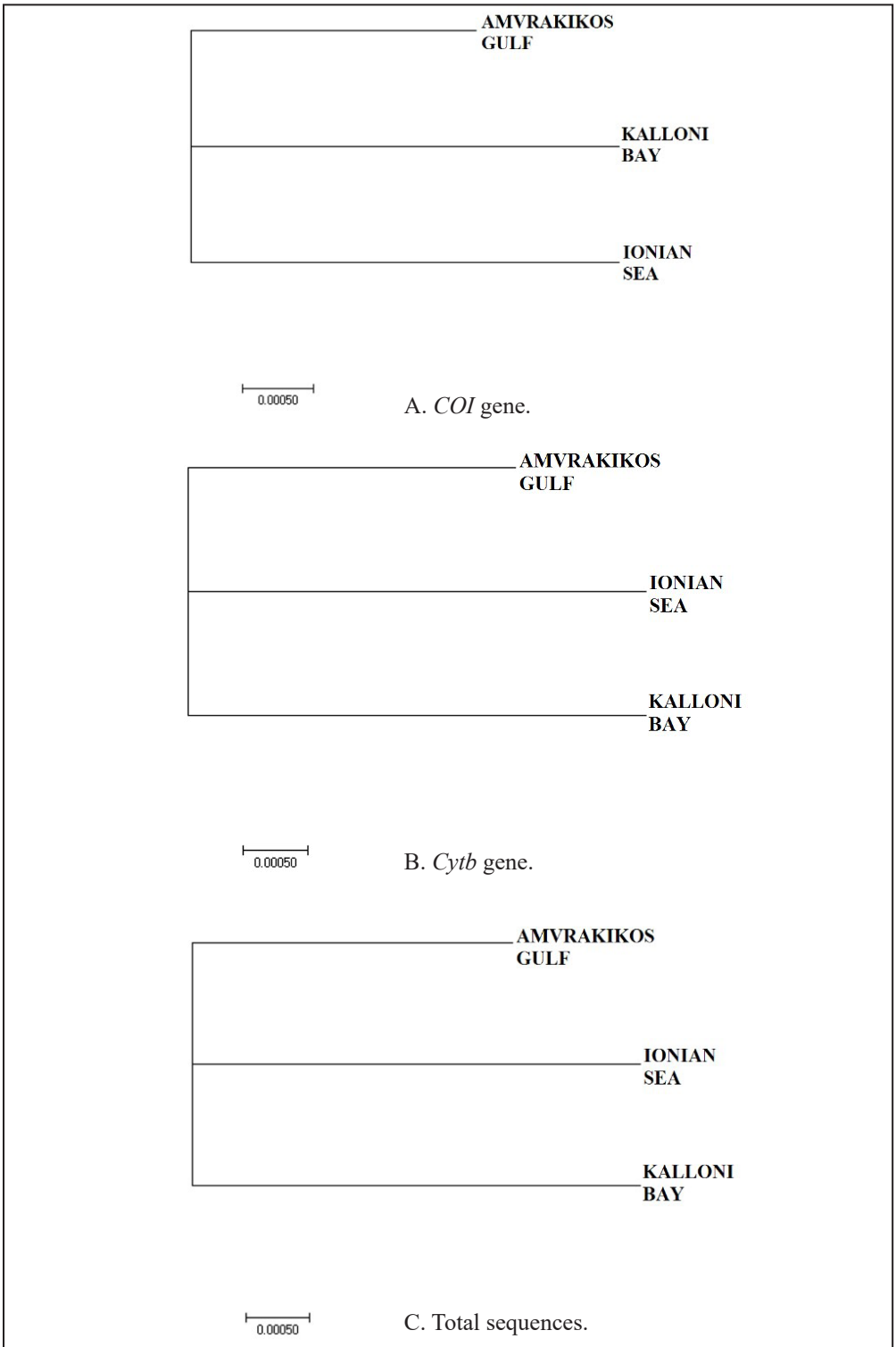


Figure 7: Neighbour Joining phylogenetic trees among the three populations of *Mullus barbatus*, based on the nucleotide sequences of each mitochondrial segment and on the complete nucleotide dataset.

For population differentiation estimates total nucleotide sequences (*COI, cytb*) for each individual were used.

The AMOVA analysis showed that most of the genetic variation was present within samples (96.85%) and 3.15% of genetic heterogeneity was apportioned among them (Table 1). The total F_{st} value was F_{st} = 0.03146. F_{st} and statistical P values among all the studied population samples are shown in table 4. It is obvious that only population from Kalloni Bay was differentiated from the Ionian Sea population ($P < 0.05$), while there was no significant differentiation among the other pairs of population.

	Amvrakikos Gulf	Ionian Sea	Kalloni Bay
Amvrakikos Gulf		[0.07207]	[0.79279]
Ionian Sea	0.05791		**[0.04505*]**
Kalloni Bay	-0.02741	0.04768	

Table 4: F_{st} values among the *Mullus barbatus* population samples, based on total sequences (*COI, cytb*). Statistical *P* values are shown in brackets. (*) indicates the statistically significant values ($P < 0.05$).

Discussion

Populations constitute interbreeding units with more or less autonomous dynamics. Population genetic structure is determined by the level of connectivity or exchange between individuals, and dispersal potential is a key factor in shaping the structure of populations [53]. In terrestrial and freshwater environments, populations are often well defined and distinct from each other, often physically separated by barriers to mixing and interbreeding [54]. Fish populations can be described from a biological perspective, which implies some level of reproductive isolation, or from a fishery perspective, which concerns a practical description of a group of fish exploited in a specific area [55].

The genetic stocks are intraspecific groups of randomly mating individuals with temporal and spatial integrity that are at some extent reproductively isolated from each other, and thus will react independently to exploitation [56]. Within marine populations it can be difficult to apply the concept of structural subdivisions, as many marine ecosystems lack obvious barriers [57]. The continuous water represents a potential means for dispersal, favouring intermixing of individuals over the species range. In practice, it is not easy to identify stocks, as the delimitation of adjacent populations involves many issues, especially in the sea where there are no clear geographical barriers. Tides and ocean currents may further act to mix passively drifting organisms, primarily eggs and larvae, over appreciable distances [58]. In marine species it is generally assumed that a high capacity for dispersal in the early life history stages results in reduced intra-specific differentiation over smaller spatial scales [59] and less genetic structure. Moreover, the effect of genetic drift in promoting differentiation is diminishing due to the very large population sizes [60]. In fact, marine populations often consist of localised sub-populations that are relatively independent and have distinct ecological and genetic properties [61].

Despite the general observation of relatively little geographic variation among populations of high dispersal marine species, many cases of divergence have been observed. Several evolutionary forces affect the amount and distribution of genetic variation among marine populations and thereby population differentiation [62]. Geographic distance and physical barriers enhance reproductive isolation by limiting the migration, and increase genetic differentiation between populations [63]. In cases where divergence among populations has been observed in an otherwise seemingly highly dispersal species, natural selection has usually been invoked to explain the pattern of genetic variation. The rationale for this argument is straightforward: when larval dispersal is large, there is virtually no capacity for populations to diverge by random processes, so significant genetic divergence among populations must be driven by selection [64]. The use of genetic markers for population description requires a detectable level of genetic differentiation, and this has presented problems in studies of many marine organisms [65]. In the marine environment many studies have failed to detect statistically significant population structuring because of low differentiation, especially over small geographical distances [66].

The different haplotypes inside a population represent the various nucleotide sequences having its individuals. Genetic distance among individuals or among populations is a measure of the genetic divergence between them [67], and arises primarily from the separate nucleotide haplotypes. The genetic differentiation of populations is the result of uneven spatial distribution of genetic variation in a species. All these genetic parameters constitute the population structure which is mainly based on the individuality of specimens, an item that is necessary for the PGI designation. For these reasons the population structure of a species could be used as a reliable marker for its future PGI definition.

S. pilchardus

Morphological studies based on gill raker counts and head

length [1] reported enough phenotypic variation to differentiate two subspecies, specifically the *S. p. pilchardus* (Eastern Atlantic Ocean, from the North Sea to Southern Portugal) and the *S. p. sardina* (Mediterranean Sea and Northwest African coast) [1]. Although no private mitochondrial control region sequence haplotype could be found for each proposed subspecies, they were suggested to be genetically distinct based on significant pairwise haplotype frequency differences [68].

There have been many studies addressing the genetic structure of sardine in different parts of its global distribution, using different molecular markers such as allozymes [69-74], mtDNA [68,75-78], microsatellites [78-80] and intron polymorphism [81]. The results of these studies, though not completely congruent, suggest a very weak genetic structure and low levels of genetic differentiation. In populations of pelagic fish, little genetic structure is expected, because adults often disperse over large distances. Populations of pelagic fish are therefore unfragmented over large areas, because groups of fish can migrate thousands of kilometres [73], usually leading to weak genetic differentiation [65].

In the present study the common haplotypes revealed in all three sardine populations for *COI* and *cytb* segments, resulted in similar findings with low levels of genetic structuring among populations. Furthermore, most of the genetic variation detected within samples and the total F_{st} value was F_{st} = 0.01232, which means that only 1.23% of genetic heterogeneity was apportioned among populations. The mean F_{st} revealed from the present work has a higher/lower value compared with previous findings (Table 10SM).

According to a previous study [69], the genetic distances based on electrophoretic variation and multivariate analysis of several morphometric and meristic characters, suggest that populations of sardine from the Aegean and Ionian seas do not form one panmictic population. The comparison of the two basins is highly significant, accounting for 90% of the total variance. These findings do not support the idea of the pure or discrete stock concept, and suggest a fair amount of gene exchange between seas, but not enough to homogenize the populations completely. Instead, they provide evidence for the dynamic population structure model according to which physical (e.g., hydrographic) or biological (e.g., predation or behaviour) factors impose a population structure. The existence of well-defined geographic and hydrographic boundaries in the Greek peninsula and related hydrographic conditions, can partially structure populations of this pelagic fish inhabiting adjacent areas [69].

Our findings do not seem to support completely the hypothesis of the population genetic structuring among the two basins, as there is no genetic differentiation between the Ionian Sea and the Kalloni Bay populations (F_{st} = -0.00126; P = 0.45045). Nevertheless, the Ionian Sea population formed a separate cluster in all four dendrograms. In addition, the highest F_{st} value was revealed between the populations from Ionian Sea and Amvrakikos Gulf (F_{st} = 0.03119) and it was significant (P = 0.01802). A value of F_{st} > 0.02 for marine fish means that there is a genetic population structure [65]. These findings imply a subtle but significant genetic structuring of the Ionian Sea sample. Such a pattern could be explained by the passive dispersion of larvae with the marine currents, rather than active migration of adult individuals [28], or by recent colonization events. Furthermore, the weak but significant differentiation between the two populations may be the result of genetic drift, which is likely maintained by the deep waters of the Ionian Sea (the Ionian Sea is the deepest sea of the Mediterranean basin) that separate this population from the shallow (maximum depth: 60 m), semi-enclosed and protected Amvrakikos Gulf, with different hydro ecological characteristics [82]. These findings also support the hypothesis that the physicochemical characteristics (temperature, salinity, water column) [82,83] and oceanographic conditions (current speeds) [84] prevailing in the Amvrakikos Gulf may reduce gene flow between locations within and outside the Gulf. Further mtDNA or microsatellite analysis of sardine samples from more locations in the Ionian Sea could facilitate the discrimination of these populations, and give more data for a possible characterization of this stock as a PGI product.

The apparent lack of a spatial pattern to the genetic differentiation observed in the present study, especially the lack of a correlation between differentiation and geographical distance, indicates a mechanism of gene flow that is independent of distance. The observed structuring was unlikely to be caused by isolation due to geographical distance. Passive transport of egg or larvae seems the most likely explanation, affected by a combination of various processes, such as hydrodynamics, geographical structure, and environmental conditions. Furthermore, any differentiation at such a restricted geographical scale may be interpreted as an innate tendency for population sub-structuring and not just a consequence of external forces.

P. kerathurus

Multiple studies of genetic diversity in natural and hatchery populations of several penaeid species have been conducted, most of which have been of allozyme variation [35]. Many authors suggested population genetic structuring over large or short-geographic distances in several penaeid species [85-88] and this may affect the management of the species. Levels of worldwide genetic population structure vary widely in different penaeid species, so that genetic structure can be strong over

very short distances and weaker across large geographic scales [35,88-91]. Despite its economic and ecological importance, little is known about the genetic stock structure of *P. kerathurus.* Most of the studies focus mainly on allozyme data [38,92-95] but there are also few mtDNA studies [39,96,97].

Our results revealed a significant population differentiation in the total data set and a strong genetic structure among all the pairs of populations. The total F_{st} value found in the present study indicated that about 12% of the total gene diversity observed was due to population differentiation, and almost 88% was due to variation among individuals within populations. The mean F_{st} revealed from the present work has a higher/similar value compared with previous findings (Table 11SM).

Unique haplotypes were found in *COI* gene and *D-loop* region for individuals from Thracian Sea population. Additionally, as for *cytb* gene, the common H1 haplotype was found in just a single individual from Thracian Sea population, and all the other haplotypes were unique for this population. As a result, the highest F_{st} values were revealed for prawn population from Thracian Sea, while populations from Amvrakikos Gulf and Ionian Sea were less differentiated. The study of the genetic variability of nine Mediterranean and two Atlantic samples of caramote prawn with the mtDNA *COI* region revealed similar significant genetic structure between populations from Kavala (Thracian Sea) and Amvrakikos Gulf (with a higher value of F_{st} = 0.532) [39]. Generally, geographic patterns of genetic variation in invertebrates species suggest that isolation by distance occurs, but only over large geographic scales [40]. In the present study the distance between the Thracian Sea population and the Amvrakikos Gulf population was approximately 1100 km, whereas the estimated distance between the Thracian Sea population and the Ionian Sea population was 1200 km.

The genetic structure observed here for *P. kerathurus* populations reflects the two distinct biogeographical regions of Aegean and Ionian Sea. The Mediterranean has sometimes been referred as a "sea of seas" because of its division into different sub basins, each having its own distinct characteristics, which includes partially enclosed current systems and likely different ecological conditions. Also, the Aegean Sea has a complex archipelago, highly irregular coastline, and it is in fact a combination of semi-isolated deep basins [98]. Thus, distinct hydrographic and ecological conditions may be sufficient to reduce gene flow [31]. It is possible that the three localities sampled in the present study belong to distinct reproductive units (stocks) that have apparently been genetically isolated from one another. Natural selection through local adaptation, vicariance events, and/or current dispersal could explain this pattern of genetic differentiation.

Furthermore, this pattern of genetic structure may be explained by hydrographic incidents during the Pleistocene. Throughout this period the sea level has dropped and modified coast lines, splitting apart the eastern and western basins. During this period, climate fluctuations produced episodes of habitat fragmentation and promoted genetic discontinuities across geographical ranges. However, other contemporary factors that limit effective genetic dispersal, including oceanographic currents (in benthic species with long pelagic larval stages like *P. kerathurus*, water currents are assumed to play an important role in shaping the structure of genetic polymorphism) and larval behaviour may also be of critical importance [99,100]. However, the predominant mechanisms leading to population differentiation are not always clear [40].

In our case, this model of heterogeneity is probably linked to the particular biological cycle of *P. kerathurus*. This cycle strictly depends on low water salinity, thus limiting the geographical dispersion of the species to a few favourable places, where the larva-adult-larva cycle can be completed [101]. Hence, the distribution of the reproductive populations of this species can be very patchy. Genetic changes are thought to alter substantially the genetic architecture of such populations, allowing rapid accumulation of many genetic differences that can lead to reproductive isolation. The well-known genetic processes of mutation and selection may be the most powerful forces creating reproductive isolation [40].

The *P. kerathurus* population from Thracian Sea (Northern Aegean Sea) revealed a significant genetic differentiation and a higher value of F_{st}, compared with the other samples. Based on our finding, it could be suggested that this population constitute a different genetic stock and a possible PGI product. Future studies could reinforce the hypothesis of its characterization as a PGI product.

M. barbatus

A large number of genetic studies for species *M. barbatus* have been conducted implementing different methodologies such as allozymes [102-107], RAPDs [108], mitochondrial markers [109-112], microsatellite markers [113-119] as well as a combination of techniques [104,108,120].

In the present study three common haplotypes among all red mullet populations were found for both *COI* and *cytb* genes. This finding is an evidence of important gene flow between collecting sites. There was no clear discrimination of Amvrakikos Gulf population from Ionian Sea and Kalloni Bay populations in the NJ tree. Only population from Kalloni Bay was significantly differentiated from the Ionian Sea population ($P < 0.05$), while there was no significant differentiation among

the other pairs of populations.

Low levels of differentiation in marine organisms are most likely due to extensive gene flow [54,57] and exchange of pelagic forms is assumed to be the major mechanism uniting spatially discrete populations. Theoretically, gene flow of few individuals per generation would be sufficient to prevent the accumulation of significant genetic drift between locations. On the other hand, subtle population genetic differentiation does not necessarily imply that structuring does not exist, but that more powerful tools are required to detect it [57]. Marine organisms, even weakly differentiated on a small geographical scale, often show evidence of differentiation over larger distances, probably because the long distance acts as an isolation mechanism. Indeed, because marine species can often experience very high gene flow, the differentiation of populations can be detected on very large spatial and long-time scales [64].

Furthermore, *M. barbatus* is a demersal species, displaying a close relationship between habitat characteristics (depth, salinity, temperature, etc.) and life history. Larvae and juveniles are pelagic and when they move they follow currents at the surface. On the contrary, the adults inhabit deeper water (10-300 m) and they probably move over large areas covering long distances. Consequently, the distribution pattern of red mullet involves continuous admixture.

The genetic polymorphism of Greek *M. barbatus* populations has been studied with different techniques: allozymes [104,120], RAPDs [108,120] and mtDNA-RFLPs [111,120]. In both allozymes [104] and RARDs [108] analyses of Greek red mullet samples the populations from Ionian Sea formed a separate cluster in the UPGMA dendrogram and were differentiated from the Aegean Sea populations. On the contrary, samples of *M. barbatus* showed no genetic structuring with mtDNA-RFLPs analysis, and Corfu/Amvrakikos populations were clustered separately with no obvious differentiation [111]. A non-significant differentiation between the populations from Amvrakikos Gulf and Ionian Sea and zero differentiation between Amvrakikos Gulf and Kalloni Bay populations were revealed in the present work. The assumption of high gene flow has been discussed above. In addition, the only pelagic stage that the species goes through is the post larval stage, during which it exhibits intentional movement. Consequently, the distribution pattern of *M. barbatus* must be less random and more fixed, directed toward species biotopes.

In a previous study [108] the Aegean Sea samples seemed to diverge from the Ionian Sea samples and it was concluded that gene flow levels were insufficient to homogenise completely the red mullet populations. In the present study a significant genetic differentiation among Ionian Sea and Kalloni Bay populations (F_{st} = 0.04768, $P < 0.05$) was revealed. These results strengthen the hypothesis that there is a differentiation between the two basins (i.e., Ionian basin, Aegean basin). Bathymetric constraints may act as physical barriers, limiting migration of red mullet adults between Aegean Sea and Ionian Sea. Mass transportation of pelagic eggs and larvae between the two basins may also be limited by oceanographic conditions [108]. Both of these assumptions could support an explanation in our case. A similar significant differentiation between an Ionian Sea and an Aegean Sea population was revealed (F_{st} = 0.021, $P < 0.01$) from microsatellite data [119]. Additionally, red mullet populations from Ionian Sea were found to be significantly differentiated from other eastern and western Mediterranean Sea populations (F_{st} values ranged from 0.021 to 0.082) [115], or to have subtle but significant differences from Adriatic and western Mediterranean Sea populations [116]. The mean F_{st} found in the present study (0.03146) has a higher/similar/lower value compared with previous findings (Table 5).

F_{st}	Area	Type of analysis	Reference
0.03146	Aegean Sea, Ionian Sea	mtDNA sequencing	present study
0.003	Adriatic Sea, Mediterranean Sea	microsatellites	[116]
0.005	Adriatic Sea	microsatellites	[113]
0.007	Western Mediterranean Sea	microsatellites	[118]
0.009	Mediterranean Sea	allozymes	[105]
0.027	Adriatic Sea, Mediterranean Sea	microsatellites	[119]
0.043	Mediterranean Sea	allozymes	[104]
0.047	Gulf of Pagasitikos	allozymes	[120]
0.057	Gulf of Pagasitikos	RAPDs	[120]
0.5	Mediterranean Sea	mtDNA-RFLPs	[111]
0.5	Gulf of Pagasitikos	mtDNA-RFLPs	[120]

Table 5: F_{st} values among *Mullus barbatus* populations, estimated from previous studies.

For both *S. pilchardus* and *P. kerathurus*, the analysis of *D-loop* region has given similar results with the two other markers and with the complete nucleotide data set. Given that, the amplification failure of the control region for *M. barbatus* would not modify the overall analysis significantly.

Data from the present work as well as from previous studies [108,115,116,119] indicates a significant genetic heterogeneity of the Ionian Sea red mullet stock. These preliminary findings if combined with further genetic analyses from more sampling sites in the Ionian basin, could contribute to a future definition of this stock as a PGI product.

The significant population differentiation and the strong genetic structure among all pairs of *P.kerathurus* populations are probably connected with the particular biological cycle of the species: the nursery grounds are located in shallow waters near river estuaries areas and the benthic adults inhabit nearshore and offshore waters. These conditions limit the geographical dispersion of the species to a few favourable places. Mutations modify substantially the genetic architecture of the reproductive populations, allowing rapid accumulation of genetic differences that can lead to reproductive isolation. On the other hand, the weak genetic structure and low levels of genetic differentiation found for *S. pilchardus* and *M. barbatus* populations could be justified by their wide dispersal range. Specifically, *S. pilchardus* is a pelagic species with pelagic eggs and *M. barbatus* life cycle includes several pelagic phases involving eggs, larvae, post-larvae and juveniles. As a consequence, populations of the two species can widen their dispersal range to a scale of hundreds of kilometres, usually leading to weak genetic structure.

Conclusion

In this work the PCR and Sanger sequencing analysis of three mitochondrial segments were used to investigate the possibility of characterizing certain populations of three Greek marine species (*Sardina pilchardus, Penaeus kerathurus, Mullus barbatus*) as PGI products. The *P. kerathurus* population from Thracian Sea could be defined as a PGI product, as it reveals the highest significant genetic differentiation from the other samples. For *S. pilchardus* and *M. barbatus* the Ionian Sea populations showed a subtle but significant genetic structure. Nevertheless, these findings need to be strengthened by more genetic information of additional sampling sites in the Ionian and Aegean basins, for a future characterization of the Ionian stocks as PGI products. Generally, for the identification of individuals and populations along the food chain and their future determination as PGI/PDO products, microsatellite genotyping, single nucleotide polymorphisms or HRM analysis have been used. This study represents the first attempt to use the population structure of a marine organism for this purpose and the results are quite encouraging. Mitochondrial markers have been proven appropriate and useful for population differentiation analyses and further discrimination of labeled products.

Acknowledgments

This work was funded by the Research project "Identification of innovative PDO or PGI products of specific species of Amvrakikos Gulf, in the framework of exploitation and sustainable management of the Gulf fish stocks" by the Ministry of Economy, Competitiveness and Shipping (GR). The authors are indebted to Dr. Tata Despoina and Baka Vasilili MSc for her valuable help in correcting the manuscript.

Conflicts of Interest

All authors declare no conflicts of interest in this article.

References

1. Parrish RH, Serra R, Grant WS (1989) The Monotypic Sardines, *Sardina* and *Sardinops*: Their Taxonomy, Distribution, Stock Structure, and Zoogeography. Can J Fish Aquat Sci 46: 2019-2036.
2. Furnestin J (1943) Contribution à l'étude biologique de la Sardine atlantique (*Sardina pilchardus* WALBAUM). Rev Trav Off Peches Marit 13: 221-386.
3. Barkova NA, Chukhgalter OA, Scherbitch LV (2001) Problèmes structuraux des populations de sardines (*Sardina pilcardus*, Walbaum, 1792) habitant au large des côtes de l'Afrique du Nord-Ouest. In: Groupe de Travail de la FAO sur l' évaluation des petits pélagiques au large de l'Afrique Nord-Occidentale; 24-31 Mars 2001; Nouadhibou, Mauritanie (657): 120-133.
4. GFCM (2006) Report of the eighth session of the sub-committee on stock assessment (SCSA). Rome, Italy. GFCM:SAC9/2006/Inf.8.
5. FAO (2008) Report of the FAO working group on the assessment of small pelagic fish of Northwest Africa. Saly, Senegal. FAO Fish Rep.No. 882.
6. ICES (2009) Report of the working group on anchovy and sardine. ICES Document CM 2009/ACOM:13.
7. Hellenic Statistical Authority ELSTAT (2016). Press release: Marine fishing Report for year 2016. Piraeus, Athens, Greece.
8. Garcia S, Le Reste L (1981) Cycles vitaux, dynamique, exploitation et aménagement des stocks de crevettes penaeides côtières. FAO Doc Tech Pêches 203: 210.
9. Hureau J-C (1986) Mullidae. In: Whitehead PJP, Bauchot M-L, Hureau J-C, Nielsen J, Tortonese E (eds.). Fishes of the north-eastern Atlantic and the Mediterranean. Volume II, UNESCO, Paris, France. Pg no: 877-882.
10. Ben-Tuvia A (1990) Mullidae. In: Quero JC, Hureau JC, Karrer C, Post A, Saldanha L (eds.). Check-list of the fishes of the

eastern tropical Atlantic (CLOFETA). Volume II, JNICT, Lisbon, SEI, Paris and UNESCO. Pg no: 827-829.

11. Tserpes G, Fiorentino F, Levi D, Cau A, Murenu M, et al. (2002) Distribution of *Mullus barbatus* and *M. surmuletus* (Osteichthyes: Perciformes) in the Mediterranean continental shelf: implications for management. Sci Mar 66: 39-54.

12. Ganopoulos I, Bosmali I, Madesis P, Tsaftaris A (2012) Microsatellite genotyping with HRM (High Resolution Melting) analysis for identification of the PGI common bean variety Plake Megalosperma Prespon. Eur Food Res Technol 234: 501-508.

13. Ropars J, Lopez-Villavicencio M, Snirc A, Lacoste S, Giraud T (2017) Blue cheese-making has shaped the population genetic structure of the mould *Penicillium roqueforti*. PLoS One 12: 1-10.

14. Giannino ML, Buffoni JN, Massone E, Feligini M (2011) Internal transcribed spacer as a target to assess yeast biodiversity in Italian taleggio PDO cheese. J Food Sci 76: 511-514.

15. Palla M, Cristani C, Giovannetti M, Agnolucci M (2017) Identification and characterization of lactic acid bacteria and yeasts of PDO Tuscan bread sourdough by culture dependent and independent methods. Int J Food Microbiol 250: 19-26.

16. Allen AR, Taylor M, McKeown B, Curry AI, Lavery JF, et al. (2010) Compilation of a panel of informative single nucleotide polymorphisms for bovine identification in the Northern Irish cattle population. BMC Genetic 11: 5.

17. Ganopoulos I, Sakaridis I, Argiriou A, Madesis P, Tsaftaris A (2013) A novel closed-tube method based on high resolution melting (HRM) analysis for authenticity testing and quantitative detection in Greek PDO Feta cheese. Food Chem 141: 835-840.

18. Nicoloso L, Crepaldi P, Mazza R, Ajmone-Marsan P, Negrini R (2013) Recent advance in DNA-based traceability and authentication of livestock meat PDO and PGI products. Recent Pat Food Nutr Agric 5: 9-18.

19. Saccone C, De Giorgi C, Gissi C, Pesole G, Reyes A (1999) Evolutionary genomics in Metazoa: The mitochondrial DNA as a model system. Gene 238: 195-209.

20. Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of Birds through DNA Barcodes. PLOS Biology: 1657-1663.

21. Folmer O, Black MB, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotech 3: 294-299.

22. Montiel-Sosa JF, Ruiz-Pesini E, Montoya J, Roncalés P, López-Pérez MJ, Pérez-Martos A (2000) Direct and highly species-specific detection of pork meat and fat in meat products by PCR amplification of mitochondrial DNA. J Agr Food Chem 48: 2829-2832.

23. Simmons RB, Weller SJ (2001) Utility and evolution of cytochrome b in insects. Mol Phylogenet Evol 20: 196-210.

24. Lin WF, Hwang DF (2008) Application of species-specific PCR for the identification of dried bonito product (Katsuobushi). Food Chem 106: 390 396.

25. Bucklin A, Smolenack SB, Bentley AM, Wiebe PH (1997) Gene flow patterns of the euphausiid, *Meganyctiphanes norvegica*, in the NW Atlantic based on mtDNA sequences for cytochrome b and cytochrome oxidase I. J Plankton Res 19: 1763-1781.

26. Harrison RG (1989) Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. Trends Ecol Evol 4: 6-11.

27. Birky-Jr CW, Fuerst P, Maruyama T (1989) Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. Genetics 121: 613-627

28. Knutsen H, Jorde PE, André C, Stenseth N CHR (2003) Finescaled geographical population structuring in a highly mobile marine species: The Atlantic cod. Mol Ecol 12: 385-394.

29. Alarcon JA, Magoulas A, Georgakopoulos T, Zouros E, Alvarez MC (2004) Genetic comparison of wild and cultivated European populations of the Gilthead Sea bream (*Sparus aurata*). Aquaculture 230: 65-80.

30. Castilho R, Ciftci Y (2005) Genetic differentiation between close eastern Mediterranean *Dicentrarchus labrax* (L.) populations. J Fish Biol 67: 1746-1752.

31. Magoulas A, Castilho R, Caetano S, Marcato S, Patarnello T (2006) Mitochondrial DNA reveals a mosaic pattern of phylogeographical structure in Atlantic and Mediterranean populations of anchovy (*Engraulis encrasicolus*). Mol Phylogenet Evol 39: 734-746.

32. Rolland JL, Bonhomme F, Lagardère F, Hassan M, Guinand B (2007) Population structure of the common sole (*Soleasolea*) in the Northeastern Atlantic and the Mediterranean Sea: revisiting the divide with EPIC markers. Mar Biol 151: 327-341.

33. Turan C, Gurlek M, Erguden D, Yaglioglu D, Uyan A, et al. (2015) Population genetic analysis of Atlantic bonito *Sardasarda* (Bloch, 1793) using sequence analysis of mtDNA *D-loop* region. Fresen Environ Bull 24: 3148-3154.

34. Hellberg ME (1996) Dependence of gene flow on geographic distance in two solitary corals with different larval dispersal abilities. Evol 50: 1167-1175.

35. Benzie JAH (2000) Population genetic structure in penaeid prawns. Aquac Res 31: 95-119.

36. Nikula R, Väinölä R (2003) Phylogeography of *Cerastodermaglaucum* (Bivalvia: Cardiidae) across Europe: a major break in the Eastern Mediterranean. Mar Biol 143: 339-350.

37. Triantafyllidis A, Apostolidis AP, Katsares V, Kelly E, Mercer J, et al. (2005) Mitochondrial DNA variation in the European lobster (*Homarusgammarus*) throughout the range. Mar Biol 146: 223-235.

38. Zitari-Chatti R, Chatti N, Elouaer A, Said K (2007) Genetic

variation and population structure of the caramote prawn *Penaeus kerathurus* (Forskäl) from the eastern and western Mediterranean coasts in Tunisia. Aquac Res 39: 70-76.

39. Pellerito R, Arculeo M, Bonhomme F (2009) Recent expansion of Northeast Atlantic and Mediterranean populations of *Melicertus* (*Penaeus*) *kerathurus* (Crustacea: Decapoda). Fisheries Sci 75: 1089-1095.

40. Palumbi SR (1994) Genetic divergence, reproductive isolation, and marine speciation. Annu Rev Ecol Syst 25: 547-572.

41. Hillis DM, Moritz C, Mable BK (1996) Molecular Systematics. 2ndedn, Sinauer Associates Inc., Sunderland, Massachusetts, USA.

42. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple alignment aided by quality analysis tool. Nucleic Acids Res 25: 4876-4882.

43. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

44. Xia X (2017) DAMBE6: New tools for microbial genomics, phylogenetics, and molecular evolution. J Hered 108: 431-437.

45. Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870-1874.

46. Hall BG (2013) Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol 30: 1229-235.

47. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111-120.

48. Saitou N, Nei M (1987) The Neighbor-Joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.

49. Sneath PHA, Sokal RR (1973) Numerical Taxonomy. The principles and practice of numerical classification. WH Freeman Company, San Francisco, USA.

50. Rzhetsky A, Nei M (1993) Theoretical foundation of the minimum-evolution method of phylogenetic inference. Mol Biol Evol 10: 1073-1095.

51. Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under linux and windows. Mol Ecol Resour 10: 564-567.

52. Cheng YZ, Xu TJ, Jin XX, Tang D, Wei T, et al. (2012) Universal primers for amplification of the complete mitochondrial control region in marine fish species. Mol Biol 46: 727-730.

53. Nathan R (2001) The challenges of studying dispersal. Trends Ecol Evol 16: 481-483.

54. Avise JC (2000) Phylogeography - the history and formation of species. Harvard University Press, Cambridge, Massachusetts, USA.

55. Smith PJ, Jamieson A (1986) Stock discreteness in herrings: a conceptual revolution. Fish Res 4: 223-234.

56. Ovenden JR (1990) Mitochondrial DNA and marine stock assessment: a review. Aust J Mar Freshwat Res 41: 835-853.

57. Waples R (1998) Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. J Hered 89: 438-450.

58. Palumbi SR (2001) The ecology of marine protected areas. In: Bertness MD, Gaines SD, Hay ME (eds.). Marine community ecology. Sinauer Associates, Sunderland, Massachusetts, USA. Pg no: 509-530.

59. Palumbi SR (1995) Using genetics as an indirect estimator of larval dispersal. In: McEdward L (ed.). Ecology of marine invertebrate larvae. CRC Press, Taylor & Francis Group, USA. Pg no: 369-387.

60. Allendorf FW, Phelps SR (1981) Use of allelic frequencies to describe population structure. Can J Fish Aquat Sci 38: 1507-1514.

61. Gaffney PM (2000) Molecular tools for understanding population structure in Antarctic species. Antarct Sci 12: 288-296.

62. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791.

63. Ryman N (2002) Population genetic structure. NOAA Technical Memoranda, Northwest Fisheries Science Centre, Washington, USA.

64. Hilbish TJ (1996) Population genetics of marine species: the interaction of natural selection and historically differentiated populations. J Exp Mar Biol Ecol 200: 67-83.

65. Ward RD, Woodmark M, Skibinski DOF (1994) A comparison of genetic diversity levels in marine, freshwater and anadromous fishes. J Fish Biol 44: 213-232.

66. Árnason E, Pálson S, Arason A (1992) Gene flow and lack of population differentiation in Atlantic cod, *Gadus morhua* L., from Iceland, and comparison of cod from Norway and Newfoundland. J Fish Biol 40: 751-770.

67. Nei M (1987) Molecular Evolutionary Genetics. Columbia University Press, New York, USA.

68. Atarhouch T, Rüber L, Gonzalez EG, Albert EM, Rami M, et al. (2006) Signature of an early genetic bottleneck in a population of Moroccan sardines (*Sardina pilchardus*). Mol Phylogenet Evol 39: 373-383.

69. Spanakis E, Tsimenides N, Zouros E (1989) Genetic differences between populations of sardine, *Sardina pilchardus*, and anchovy, *Engraulis encrasicolus*, in the Aegean and Ionian seas. J Fish Biol 35: 417-437.

70. Ramon MM, Castro JA (1997) Genetic variation in natural stocks of *Sardina pilchardus* (sardines) from the western Mediterranean Sea. Heredity 78: 520-528.

71. Chlaida M, Kifani S, Lenfant P, Ouragh L (2006) First approach for the identification of sardine populations *Sardina pilchardus*

(Walbaum 1792) in the Moroccan Atlantic by allozymes. Mar Biol 149: 169-175.

72. Laurent V, Planes S (2007) Effective population size estimation on *Sardina pilchardus* in the Bay of Biscay using a temporal genetic approach. Biol J Linn Soc 90: 591-602.

73. Laurent V, Caneco B, Magoulas A, Planes S (2007) Isolation by distance and selection effects on genetic structure of sardines *Sardina pilchardus* Walbaum. J Fish Biol 71: 1-17.

74. Chlaida M, Laurent V, Kifani S, Benazzou T, Jaziri H, et al. (2009) Evidence of a genetic cline for *Sardina pilchardus* along the Northwest African coast. ICES J Mar Sci 66: 264-271.

75. Tinti F, Di Nunno C, Guarniero I, Talenti M, Tommasini S, et al. (2002) Mitochondrial DNA sequence variation suggests the lack of genetic heterogeneity in the Adriatic and Ionian stocks of *Sardina pilchardus*. Mar Biotechnol 4: 163-172.

76. Sarmaşik A, Çolakoğlu FA, Altun T (2008) Mitochondrial DNA sequence and body size variations in Turkish sardine (*Sardina pilchardus*) stocks. Turk J Zool 32: 229-237.

77. Fadhlaoui-Zid K, Oueslati S, Abdallah LB, Jarboui O (2010) Mitochondrial DNA sequence variation in Tunisia sardine (*Sardina pilchardus*) stocks. Rapp Commint Mer Médit 39: 508.

78. Baibai T, Oukhattar L, Quinteiro JV, Mesfioui A, Rey-Mendez M, et al. (2012) First global approach: morphological and biological variability in a genetically homogeneous population of the European pilchard, *Sardina pilchardus* (Walbaum, 1792) in the North Atlantic coast. Rev Fish Biol Fisher 22: 63-80.

79. Gonzalez EG, Zardoya R (2007) Relative role of life-history traits and historical factors in shaping genetic population structure of sardines (*Sardina pilchardus*). BMC Evol Biol 7: 197.

80. Kasapidis P, Silva A, Zampicinini G, Magoulas A (2012) Evidence for microsatellite hitchhiking selection in European sardine (*Sardina pilchardus*) and implications in inferring stock structure. Sci Mar 76: 123-132.

81. Atarhouch T, Rami M, Naciri M, Dakkak A (2007) Genetic population structure of sardine (*Sardina pilchardus*) off Morocco detected with intron polymorphism (EPIC PCR). Mar Biol 150: 521-528.

82. Ferentinos G, Papatheodorou G, Geraga M, Iatrou M, Fakiris E, et al. (2010) Fjord water circulation patterns and dysoxic/anoxic conditions in a Mediterranean semi-enclosed embayment in the Amvrakikos Gulf, Greece. Estuar Coast Shelf S 88: 473-481.

83. Panayotidis P, Pancucci A, Balopoulos E, Gotsis-Skretas O (1994) Plankton distribution patterns in a Mediterranean dilution basin: Amvrakikos Gulf (Ionian Sea, Greece). Mar Ecol 15: 93-104.

84. Voutsinou-Taliadouri F, Balopoulos ETH (1991) Geochemical and physical oceanographic aspects of the Amvrakikos Gulf (Ionian Sea, Greece). Toxicol Environ Chem 31: 177-185.

85. De la Rosa-Vélez J, Escobar-Fernández R, Correa F, Maqueda-Cornejo M, De la Torre-Cueto J (2000) Genetic structure of two commercial penaeids (*Penaeus californiensis* and *P. stylirostris*) from the Gulf of California, as revealed by allozyme variation. Fish B-NOAA 98: 674-683.

86. Sugama K, Haryanti, Benzie JAH, Ballment E (2002) Genetic variation and population structure of the giant tiger prawn, *Penaeus monodon*, in Indonesia. Aquaculture 205: 37-48.

87. Ramos-Paredes J, Grijalva-Chon JM (2003) Allozyme genetic analysis in hatchery strains and wild blue shrimp, *Penaeus* (*Litopenaeus*) *stylirostris* (Stimpson), from the Gulf of California. Aquac Res 34: 221-234.

88. Gusmão J, Lazoski C, Solé-Cava AM (2005) Population genetic structure of Brazilian shrimp species (*Farfantepenaeus sp*., *F. brasiliensis*, *F. paulensis* and *Litopenaeus schmitti*: Decapoda: Penaeidae). Genet Mol Biol 28: 165-171.

89. Ball AO, Chapman RW (2003) Population genetic analysis of white shrimp, *Litopenaeus setiferus*, using microsatellite genetic markers. Mol Ecol 12: 2319-2330.

90. McMillen-Jackson A, Bert TM (2003) Disparate patterns of population genetic structure and population history in two sympatric penaeid shrimp species (*Farfantepenaeus aztecus* and *Litopenaeus setiferus*) in the eastern United States. Mol Ecol 12: 2895-2905.

91. McMillen-Jackson A, Bert TM (2004) Genetic diversity in the mtDNA control region and population structure in the pink shrimp *Farfantepenaeus Duorarum*. J Crustacean Biol 24: 101-109.

92. Lester LJ (1979) Population genetics of penaeid shrimps from the Gulf of Mexico. J Hered 70: 175-180.

93. Ko G, Pasteur N, Bonhomme F, Liao D (1983) Variabilitè gènètique de quelques espéces de crevettes peneides d'intèrêt aquicole. Bases biologiques de l'aquaculture. Montpellier. Actes de Colloques 1: 175-180.

94. de Matthaeis E, Allegrucci G, Caccone A, Cesaroni D, Sbordoni V, et al. (1983) Genetic differentiation between *Penaeus kerathurus* and *P. japonicus* (Crustacea, Decapoda). Mar Ecol-Prog Ser 12: 191-197.

95. Mattoccia M, La Rosa G, De Matthaeis E, Cobolli-Sbordoni M, Sbordoni V (1987) Patterns of geneticvariability in Mediterranean populations of *Penaeus kerathurus* (Crustacea, Decapoda). In: Tiews K (ed). Selection, hybridization and genetic engineering in aquaculture. Heenemann-Verleg, Berlin, Germany. Pg no: 131-142.

96. Zitari-Chatti R, Chatti N, Fulgione D, Caiazza I, Aprea G, et al. (2009) Mitochondrial DNA variation in the caramote prawn *Penaeus* (*Melicertus*) *kerathurus* across a transition zone in the Mediterranean Sea. Genetica 136: 439-447.

97. Milli S, Ennouri R, Bouriga N, Missaoui H (2017) Phylogenetic variability of the caramote prawn *Penaeus* (*Melicertus*) *kerathurus* in the Tunisian waters. Sustainable Livestock Management 4: 61-68.

98. Olson DB, Kourafalou VH, Johns WE, Samuels G, Veneziani V (2007) Aegean surface circulation from a satellite-tracked

drifter array. J Phys Oceanogr 37: 1898-1917.

99. Palumbi SR, Grabowsky G, Duda T, Geyer L, Tachino N (1997) Speciation and population genetic structure in tropical Pacific sea urchins. Evolution 51: 1506-1517.

100. Riginos C, Nachman MW (2001) Population subdivision in marine environments: the contributions of biogeography, geographical distance and discontinuous habitat to genetic differentiation in a blennioid fish, *Axoclinusnigricaudus*. Mol Ecol 10: 1439-1453.

101. Lumare F, Scordella G (2001) Biological cycle, growth and reproduction of the Penaeid shrimp *Penaeus* (*Melicertus*) *keraturus* of the Adiatic coasts. Int Workshop Statodella Pesca e Dinamica di Popolazione del Gambero Mediterraneo *Penaeus* (*Melicertus*) *kerathurus* in Alcune Areedella Costa Adriatica, Lecce, Italy (in Italian).

102. Basaglia F, Callegarini C (1988) Biochemical characteristics of red mullet of the central Mediterranean. Comp Biochem Physiol 89: 731-736.

103. Cammarata M, Parrinello N, Arculeo M (1991) Biochemical taxonomic differentiation between *Mullus barbatus* and *Mullus surmuletus* (Pisces, Mullidae). Comp Biochem Physiol 99: 719-722.

104. Mamuris Z, Apostolidis AP, Triantaphyllidis C (1998) Genetic protein variation in red mullet (*Mullus barbatus*) and striped red mullet (*M. surmuletus*) populations from the Mediterranean Sea. Mar Biol 130: 353-360.

105. Arculeo M, Lo Brutto S, Cammarata M, Scalisi M, Parrinello N (1999) Genetic variability of the Mediterranean Sea red mullet, *Mullus barbatus* (Pisces, Mullidae). Russ J Genet 35: 292-296.

106. Turan C (2006) Phylogenetic relationships of Mediterranean Mullidae species (Perciformes) inferred from genetic and morphological data. Sci Mar 70: 311-318.

107. Ivanova PP, Dobrovolov IS, Tsekov AG (2014) Genetic differentiation between *Mullus barbatus* from the western part of the Black Sea and *Mullus surmuletus* (Pisces, Mullidae) from the Mediterranean Sea. Ecol Bal 6: 37-44.

108. Mamuris Z, Apostolidis AP, Theodorou AJ, Triantaphyllidis C (1998) Application of random amplified polymorphic DNA (RAPD) markers to evaluate intraspecific genetic variation in red mullet (*Mullus barbatus*). Mar Biol 132: 171-178.

109. Apostolidis AP, Mamuris Z, Triantaphyllidis C (2001) Phylogenetic relationships among four species of Mullidae (Perciformes) inferred from DNA sequences of mitochondrial cytochrome b and 16S rRNA genes. Biochem Syst Ecol 29: 901-909.

110. Apostolidis AP, Georgiadis A, Karaiskou N, Sandaltzopoulos R (2008) Reliable and rapid discrimination of congeneric species by mtDNA SNP analysis by multiplex PCR: application on three *Trachurus* and two *Mullus* fish species as model cases. Hydrobiologia 614: 401-404.

111. Mamuris Z, Stamatis C, Moutou KA, Apostolidis AP, Triantaphyllidis C (2001) RFLP analysis of mitochondrial DNA to evaluate genetic variation in striped red mullet (*Mullus surmuletus* L.) and red mullet (*Mullus barbatus* L.) populations. Mar Biotechnol 3: 264-274.

112. Keskin E, Can A (2009) Phylogenetic relationships among four species and sub-species of Mullidae (Actinopterygii, Perciformes) based on mitochondrial cytochrome b, 12 rRNA and cytochrome oxidase II genes. Biochem Syst Ecol 37: 653-661.

113. Garoia F, Guarniero I, Piccinetti C, Tinti F (2004) First microsatellite loci of red mullet (*Mullus barbatus*) and their application to genetic structure analysis of Adriatic shared stock. Mar Biotechnol 6: 446-452.

114. Galarza JA, Turner GF, Macpherson E, Carreras-Carbonell J, Rico C (2007) Cross-amplification of 10 new isolated polymorphic microsatellite loci for red mullet (*Mullus barbatus*) in striped red mullet (*Mullus surmuletus*). Mol Ecol Notes 7: 230-232.

115. Galarza JA, Turner GF, Macpherson E, Rico C (2009) Patterns of genetic differentiation between two co-occurring demersal species: the red mullet (*Mullus barbatus*) and the striped red mullet (*Mullus surmuletus*). Can J Fish Aquat Sci 66: 1478-1490.

116. Maggio T, Lo Brutto S, Garoia F, Tinti F, Arculeo M (2009) Microsatellite analysis of red mullet *Mullus barbatus* (Perciformes, Mullidae) reveals the isolation of the Adriatic Basin in the Mediterranean Sea. ICES J Mar Sci 66: 1883-1891.

117. Vogiatzi E, Hanel R, Dailianis T, Lagnel J, Hassan M, et al. (2012) Description of microsatellite markers in four mullids based on the development and cross-species amplification of 18 new markers in red mullet (*Mullus barbatus*). Biochem Syst Ecol 44: 279-285.

118. Félix-Hackradt FC, Hackradt CW, Pérez-Ruzafa Á, García-Charton JA (2013) Discordant patterns of genetic connectivity between two sympatric species, *Mullus barbatus* (Linnaeus, 1758) and *Mullus surmuletus* (Linnaeus, 1758), in south-western Mediterranean Sea. Mar Environ Res 92: 23-34.

119. Matić-Skoko S, Šegvić-Bubić T, Mandić I, Izquierdo-Gomez D, Arneri E, et al. (2018) Evidence of subtle genetic structure in the sympatric species *Mullus barbatus* and *Mullus surmuletus* (Linnaeus, 1758) in the Mediterranean Sea. Sci Rep 8: 1-14.

120. Apostolidis A, Moutou K, Stamatis C, Mamuris Z (2009) Genetic structure of three marine fishes from the Gulf of Pagasitikos (Greece) based on allozymes, RAPD, and mtDNA RFLP markers. Biologia 64: 1005-1010.

121. Ward RD, Zemlac TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia's fish species. Philos T Roy Soc B 360: 1847-1857.

122. Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, et al. (1989) Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 86: 6196-6200.

123. Kochzius M, Seidel C, Antoniou A, Botla SK, Campo D, et al. (2010) Identifying fishes through DNA barcodes and

microarrays. PLoS One 5: 12620.

124. Chu KH, Li CP, Tam YK, Lavery S (2003) Application of mitochondrial control region in population genetic studies of the shrimp Penaeus. Mol Ecol Notes 3: 120-122.

Species	*COI*	*cytb*	*D-loop*
Sardina pilchardus	FishF2- 5′TCGACTAATCATAAA GATATCGGCAC3′ FishR2- 5′ACTTCAGGGTGACCG AAGAATCAGAA3′ [121]	L14841- 5′AAAAAGCTTCCATCCAACAT CTCAGCATGATGAAA3′ H15149- 5′AAACTGCAGCCCCTCAGAATG ATATTTGTCCTCA3′ [122]	Marinefish-Dloop-Thr-F 5′AGCACCGGTCTTGTAA ACCG3′ Marinefish-Dloop-Phe-R 5′GGGCTCATCTTAACAT CTTCA3′ [52]
Penaeus kerathurus	LCO1490- 5′GGTCAACAAATCATA AAGATATTGG3′ HCO2198- 5′TAAACTTCAGGGTGA CCAAAAAATCA3′ [21]	L14841- 5′AAAAAGCTTCCATCCAACAT CTCAGCATGATGAAA3′ H15149- 5′AAACTGCAGCCCCTCAGAATG ATATTTGTCCTCA3′ [122]	12S- 5'AAGAACCAGCTAGGAT AAAACTTT3' PCR-1R 5'GATCAAAGAACATTCT TTAACTAC3' [124]
Mullus barbatus	FishF2- 5′TCGACTAATCATAAA GATATCGGCAC3′ FishR2- 5′ACTTCAGGGTGACCG AAGAATCAGAA3′ [121]	CytbF- 5′GGCTGATTCGGAATATGCAYG CNAAYGG3′ CytbR- 5′GGGAATGGATCGTAGAATTG CRTANGCRAA3′ [123]	Marinefish-Dloop-Thr-F 5′AGCACCGGTCTTGTAA ACCG3′ Marinefish-Dloop-Phe-R 5′GGGCTCATCTTAACAT CTTCA3′ [52]

Table 1SM: Primers for each mitochondrial segment per species, used in the present study.

Haplotype	Amvrakikos Gulf	Ionian Sea	Kalloni Bay
H1	7	3	10
H2		4	
H3		2	
H4		1	1
H5	1		1
H6	1		
H7	1		
H8	1		
H9	1		
H10	1		
H11	1		
H12	1		
H13	1		
H14	1		
H15	1		
H16	1		
H17		1	
H18		1	
H19		1	
H20		1	

H21		1	
H22		1	
H23		1	
H24		1	
H25		1	
H26		1	
H27			1
H28			1
H29			1
H30			1
H31			1
H32			1
H33			1
Total	19	20	19

Table 2SM: Distribution and frequencies of *S. pilchardus* mtDNA *COI* haplotypes.

Haplotype	Amvrakikos Gulf	Ionian Sea	Kalloni Bay
H1	6	7	10
H2	2		
H3	2		
H4	1		
H5	1		
H6	1		
H7	1		
H8	1		
H9	1		
H10			1
H11			1
H12			1
H13			1
H14			1
H15			1
H16		1	
H17		1	
H18		1	
H19		1	
H20		1	
H21		1	
H22		1	
H23		1	
H24		1	
H25		1	
Total	16	17	16

Table 3SM: Distribution of mtDNA *cytb* haplotypes and their frequencies in *Sardina pilchardus* populations.

Haplotype	Amvrakikos Gulf	Ionian Sea	Kalloni Bay
H1	2		
H2	1		1
H3	1		
H4	1		
H5	1		
H6	1		
H7	1		
H8	1		
H9	1		
H10	1		
H11	1		
H12	1		
H13	1		
H14	1		
H15	1		
H16		1	
H17		1	
H18		1	
H19		1	
H20		1	
H21		1	
H22		1	
H23		1	
H24		1	
H25		1	
H26		1	
H27		1	
H28		1	
H29		1	
H30		1	
H31		1	
H32		1	
H33		1	
H34		1	
H35		1	
H36			1
H37			1
H38			1
H39			1
H40			1
H41			1
H42			1
H43			1
H44			1
H45			1

H46			1
H47			1
H48			1
H49			1
H50			1
H51			1
H52			1
H53			1
Total	16	20	19

Table 4SM: Distribution of mtDNA *D-loop* haplotypes and their frequencies in *Sardina pilchardus* populations.

Haplotype	Amvrakikos Gulf	Ionian Sea	Thracian Sea
H1	6	8	
H2			10
H3	8		
H4	1		
H5	1		
H6	1		
H7	1		
H8	1		
H9	1		
H10		1	
H11		1	
H12		1	
H13		1	
H14		1	
H15		1	
H16		1	
H17		1	
H18		1	
H19		1	
H20		1	
H21		1	
H22			1
H23			1
H24			1
H25			1
H26			1
H27			1
H28			1
H29			1
H30			1
Total	20	20	19

Table 5SM: Distribution of mtDNA *COI* haplotypes and their frequencies in *Penaeus kerathurus* populations.

Haplotypes	Amvrakikos Gulf	Ionian Sea	Thracian Sea
H1	9	2	1
H2		3	
H3		2	
H4		2	
H5	2		
H6	1		
H7	1		
H8	1		
H9	1		
H10	1		
H11	1		
H12	1		
H13	1		
H14	1		
H15		1	
H16		1	
H17		1	
H18		1	
H19		1	
H20		1	
H21		1	
H22		1	
H23		1	
H24		1	
H25			1
H26			1
H27			1
H28			1
H29			1
H30			1
H31			1
H32			1
H33			1
H34			1
H35			1
H36			1
H37			1
H38			1
H39			1
H40			1
H41			1
Total	20	19	18

Table 6SM: Distribution of mtDNA *cytb* haplotypes and their frequencies in *Penaeus kerathurus* populations.

Haplotypes	Amvrakikos Gulf	Ionian Sea	Tracian Sea
H1	1		
H2	1		
H3	1		
H4	1		
H5	1		
H6	1		
H7	1		
H8	1		
H9	1		
H10	1		
H11	1		
H12	1		
H13	1		
H14	1		
H15	1		
H16	1		
H17	1		
H18	1		
H19		1	
H20		1	
H21		1	
H22		1	
H23		1	
H24		1	
H25		1	
H26		1	
H27		1	
H28		1	
H29		1	
H30		1	
H31		1	
H32		1	
H33		1	
H34		1	
H35		1	
H36		1	
H37		1	
H38			1
H39			1
H40			1
H41			1
H42			1
H43			1
H44			1

H45			1
H46			1
H47			1
H48			1
H49			1
H50			1
H51			1
Total	18	19	14

Table 7SM: Distribution of mtDNA *D-loop* haplotypes and their frequencies in *Penaeus kerathurus* populations.

Haplotype	Amvrakikos Gulf	Ionian Sea	Kalloni Bay
H1	4	7	3
H2	3	1	
H3	1	2	1
H4	1	1	1
H5	2		
H6	1		1
H7	1		
H8	1		
H9	1		
H10	1		
H11	1		
H12	1		
H13	1		
H14	1		
H15		1	
H16		1	
H17		1	
H18		1	
H19		1	
H20		1	
H21		1	
H22		1	
H23		1	
H24		1	
H25			1
H26			1
H27			1
H28			1
H29			1
H30			1
H31			1
H32			1
Total	20	21	14

Table 8SM: Distribution of mtDNA *COI* haplotypes and their frequencies in *Mullus barbatus* populations.

Haplotype	Amvrakikos Gulf	Ionian Sea	Kalloni Bay
H1	5	6	4
H2	8	3	3
H3	2	1	2
H4		4	
H5		1	1
H6	1	1	
H7	1		
H8			1
H9	1		
H10	1		
H11	1		
H12		1	
H13		1	
H14		1	
H15		1	
H16		1	
H17			1
H18			1
H19			1
Total	20	21	14

Table 9SM: Distribution of mtDNA *cytb* haplotypes and their frequencies in *Mullus barbatus* populations.

F_{st}	Area	Type of analysis	Reference
0.01232	Aegean Sea, Ionian Sea	mtDNA sequencing	Present study
0.005	Atlantic Ocean, Mediterranean Sea	microsatellites	[79]
0.008	Adriatic Sea, Ionian Sea	mtDNA sequencing	[75]
0.0093	Bay of Biscay	allozymes	[72]
0.017	Europe	microsatellites	[80]
0.026	Atlantic Ocean, Mediterranean Sea	mtDNA sequencing	[68]
0.034	Moroccan Atlantic coast	intron polymorphism	[81]
0.036	Moroccan Atlantic coast	microsatellites mtDNA sequencing	[78]
0.057	Atlantic Ocean, Mediterranean Sea	allozymes	[73]
0.074	Western Mediterranean Sea	allozymes	[70]
0.205	Moroccan Atlantic	allozymes	[74]
0.22	Moroccan Atlantic	allozymes	[71]

Table 10SM: F_{st} values among *Sardina pilchardus* populations, estimated from previous studies.

F_{st}	Area	Type of analysis	Reference
0.12096	Aegean Sea, Ionian Sea	mtDNA sequencing	Present study
0.040	Gulf of Mexico	allozymes	[92]
0.076	Tunisia	allozymes	[38]
0.194	Mediterranean Sea, Atlantic	mtDNA sequencing	[39]
0.2957	Siculo–Tunisian strait	mtDNA sequencing	[96]

Table 11SM: F_{st} values among *Penaeus kerathurus* populations, estimated from previous studies.