

# POPULATION GENETIC ANALYSIS OF ATLANTIC BONITO *Sarda sarda* (BLOCH, 1793) USING SEQUENCE ANALYSIS OF MTDNA D-LOOP REGION

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## ABSTRACT

In this study mitochondrial DNA D-loop gene sequencing was used to investigate genetic structure of 11 Atlantic bonito *Sarda sarda* populations from the Black Sea, Marmara, Aegean, Mediterranean Seas and Adriatic Sea. The total sequence length, variable sites and parsimony informative sites were 868 bp, 12 bp and 7 bp from 222 individuals, respectively. The nucleotide frequencies were 32.55% A, 31.32% T, 14.44% C, and 21.68% G. The total number of haplotypes was 19, and the highest number of different haplotypes was observed in the northeastern Mediterranean (the Iskenderun Bay) sample, and the lowest was observed in the Bulgarian sample. Low genetic diversity was observed within populations, and the mean genetic diversity within populations and the mean genetic divergence between populations were 0.0009 and 0.0013, respectively. In the statistical analysis, *S. sarda* was divided into three genetically different populations ( $P < 0.001$ ); the Black and Marmara Sea populations comprise one genetic unit, and the Aegean and Mediterranean coast of Turkey populations constitute the genetically different second unit. The Adriatic Sea population from Croatian coast was also genetically different from these two units. The neighbor joining tree revealed three main phylogenetic nodes; in the first node, the Black Sea, Bosphorus and Marmara Sea samples were grouped close together. In the second main node, the Aegean and northeastern Mediterranean Seas samples were clustered close to each other, and the Adriatic Sea sample was far from these samples, but closer to the Aegean and northeastern Mediterranean samples than the Black Sea and Marmara Sea samples.

**KEYWORDS:** Atlantic bonito, *Sarda sarda*, Population Genetics, mtDNA, Sequencing

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## 1. INTRODUCTION

The Atlantic bonito *Sarda sarda* (Bloch 1793) is a commercially valuable small tuna-like species, which occurs along the tropical and temperate coasts of the Atlantic Ocean, the Mediterranean Sea and the Black Sea and inhabit pelagic waters limited by the continental shelf [1, 2]. Three discrete spawning grounds are assumed to exist for the bonito in the Mediterranean Sea. The Black Sea and Marmara Sea are one spawning grounds in at the eastern Mediterranean, and the area between Gibraltar, Balearic Islands and Algeria is the second spawning ground at the western Mediterranean [2], and the third is in the northern Balearic Sea [3]. Rey et al. [2] reported that there is no exchange of individuals between the east and west spawning populations, on the bases of a tag-recapture data.

There have been a number of genetic studies which also report limited gene flow between eastern and western Mediterranean populations of *Sarda sarda*. Roberti et al. [4] used mtDNA Cytochrome b gene and found a small but significant difference in haplotype frequency between a sample from the Sea of Marmara and two Mediterranean samples, one from the Aegean Sea and the other from the Ionian Sea, which in turn were not different from each other. Roberti et al. [4] reported that a barrier prevents gene flow between the Sea of Marmara as spawning area and Aegean Sea as feeding area. Pujolar et al. [5] found a small but significant differentiation between the Aegean Sea and two western samples, namely the Ionian Sea and the Ligurian Sea using allozyme electrophoresis and argued that the barrier to gene flow between east and west is located in the region that separates the Ionian and the Aegean seas. Therefore, allozyme data would appear concordant with the interpretation of Rey et al. [2] about an eastern and western subdivision, which is in contrast not agreed with the mitochondrial data given by Roberti et al. [4], but agreed with Vinas et al. [6]. Vinas et al. [6] used mtDNA control region sequences of *Sarda sarda* on the Balearic, Ligurian, Ionian and

Aegean Seas, and reported the lack of differentiation between the Balearic Sea and the Ionian Sea, and significant differentiation of the Balearic and the Ligurian from the Aegean Sea, and the Ionian Sea sample was not different from any other samples. The genetic data on mtDNA available only comprise the Aegean Sea from the northeastern part of the Mediterranean Sea, however there is no genetic study comprising all the marine waters of Turkey. Therefore, the level of genetic diversity and population structure of *Sarda sarda* to date remain equivocal. To draw stronger inferences on genetic diversity and population structure, it is important to obtain larger number of samples from across the natural range of the species.

The assessment of population dynamics and levels of gen flow among populations is essential for management of marine stocks [7, 8]. The mitochondrial DNA (mtDNA) has been widely used as a marker for population studies because of its compact size, fast evolutionary rate, and exclusive maternal mode of inheritance [9, 10]. The mitochondrial DNA of vertebrates has a small and closed circular structure, which contains 37 enzymes encoding genes and a control region that regulates the replication of H strand and transcription of all mitochondrial genes [11]. The mtDNA control region is also known as displacement loop (D-loop) region, which evolve much faster than the average because of the reduced functional constraints [12].

In the present study, mitochondrial DNA D-loop sequences were utilized to examine populations of Atlantic bonito *Sarda sarda* caught from the Black Sea, Marmara

Sea, Aegean Sea, northeastern Mediterranean Sea and Adriatic Sea for determining population structuring in order to develop policies for the management and conservation of its genetic resources.

## 2. MATERIAL AND METHODS

*S. sarda* samples were collected by commercial fishing vessels from eleven fishing ports, comprising 5 location in the Black Sea (Bulgarian Coast (BS1), Igneada (BS2), Duzce (BS3), Samsun (BS4), Trabzon (BS5)), 1 location from the Bosphorus (BP), 1 location from the Marmara Sea (Bandırma (MS)), 1 location from the Aegean Sea (Izmir Bay (AS)), 2 locations from the northeastern Mediterranean Sea (Antalya Bay (NMS1) and Iskenderun Bay (NMS2)), and 1 location from the Adriatic Sea (Croatian Coast (ADS)) (Fig. 1). All samples were put in plastic bags individually and frozen at  $-20^{\circ}\text{C}$  till they arrived to the laboratory. Dissected muscle from fish was preserved in 98% ethanol. Age of the samples was recorded by identifying and counting annuli of otoliths proposed by Rey et al. [13]. Sex was determined macroscopically from the gonads whenever possible. The muscle tissue was minced and then digested with proteinase K. Total genomic DNA was isolated using the standard phenol-chloroform extraction method [14]. The complete mtDNA D-Loop sequences were amplified *via* PCR reactions, which was performed in a total volume of 50  $\mu\text{l}$  containing 0,4  $\mu\text{M}$  of each primer, 0,2 mM of dNTP and 1.25U of *Taq* DNA polymerase in a PCR buffer that included

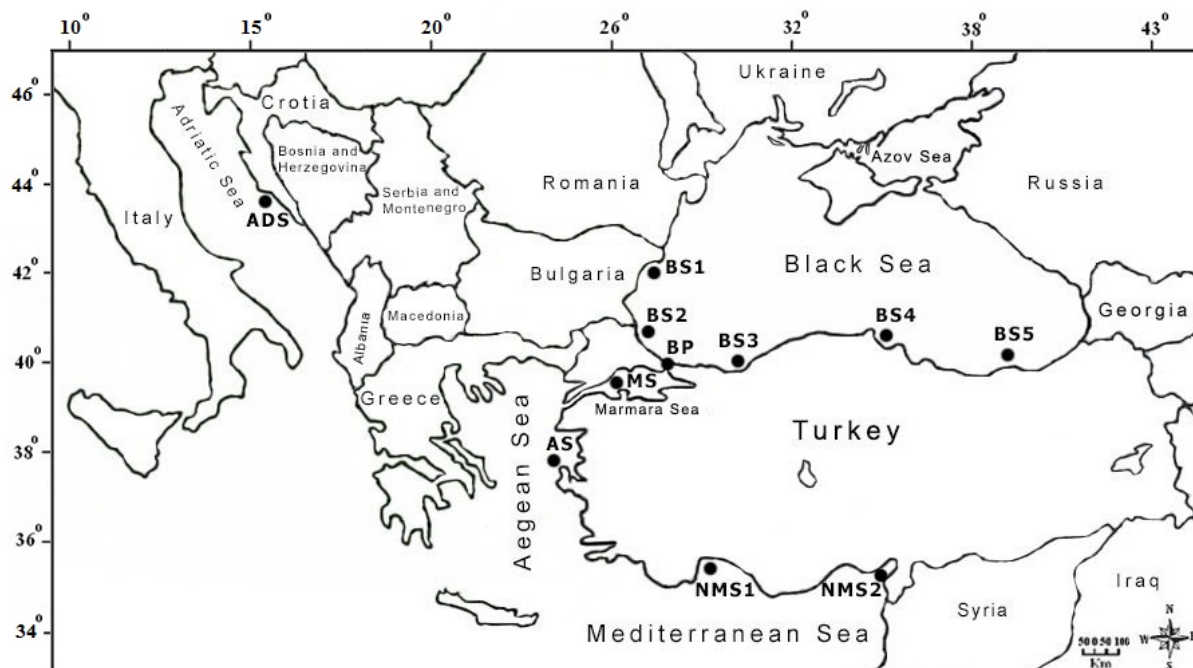


FIGURE 1 - Sampling locations of *S. sarda*. The abbreviation of the samples as: BS1, the Black Sea Bulgarian Coast (Varna); BS2, the Black Sea Igneada; the Black Sea Duzce (BS3); the Black Sea Samsun (BS4); the Black Sea Trabzon (BS5); Istanbul Bosphorus (BP); MS, Marmara Sea Bandırma; AS, the Aegean Sea Izmir; NMS1, the northeastern Mediterranean Sea Antalya Bay; NMS2, the northeastern Mediterranean Sea Iskenderun Bay; ADS, Adriatic Sea Croatian Coast.

20mM of Tris–HCl (pH 8.0), 1,5mM of MgCl<sub>2</sub>, 15 mM of KCl and 1–2 µl template DNA. Denaturation step at 94°C for 30 s, 50 °C for 30 s, and 72 °C for 45 s for 30 cycles and followed by a final extension for 7 min at 72 °C. The set of primers used for PCR amplification described by Vinas et al. [15]. D-Loop-F: 5'-TAC CCC AAA CTC CCA AAG CTA-3'; D-Loop-R: 5'-GCG GAG GCT TGC ATG TGTA-3'. Visualization of amplified D-Loop gene was performed on agarose gel. Quantitation of the PCR product was completed using spectrophotometer. The DNA sequencing was attempted to determine the order of the nucleotides of a gene. The chain termination method by Sanger et al. [16] was applied with Bigdye Cycle Sequencing Kit V3.1 and ABI 3130 XL genetic analyzer. The initial alignments of partial D-Loop sequences were performed with Clustal W program [17] and final alignment was completed manually with BioEdit [18]. After sequence alignment, the best model for sequence divergences were calculated using Mega v5, and the molecular phylogenetic tree was also constructed using Mega v5 [19]. Neighbor joining (NJ) phylogenetic tree was used to reveal genetic relationships of populations [20]. The statistical robustness in the nodes of the resulting tree was determined by 1000 bootstrap replicates [21].

### 3. RESULTS AND DISCUSSION

There were 12 variable and 856 invariable conservative nucleotides of which 7 were parsimony informative over 868 bp sequences. Examination of the gene fragment reveals a lack of guanine (G; 14.4%) and abundance of adenine (A; 32.6%). The average nucleotide composition was 31.3 and 21.7 % for thymine and cytosine, respectively. Tamura 3-parameter model (T92+G) was chosen as a best method for intra and interspecific variations.

The overall nucleotide diversity ( $\pi$ ) was 0.00131. The nucleotide diversities within the populations ranged from 0.0006 within both Igneada (BS2) and Bulgarian (BS1) populations to 0.0015 within the northeastern Mediterranean (the Iskenderun Bay) population (Table 2). Estimated pairwise genetic distances ( $\theta$ ) based on Tamura 3-parameter model between populations ranged from 0.0006 between both Bulgarian (BS1) and Igneada (BS2) and Bulgaria and Duzce (BS3) populations to 0.0027 between the Adriatic (ADS) and the northeastern Mediterranean (the Iskenderun Bay) populations with an average of 0.00231 (Table 2). Significant degree of population structure was observed in the overall analysis with all locations included and no groupings assigned ( $F_{ST}=0.3262$ ,  $P<0.001$ ). The Black Sea samples including the Bosphorus sample were significantly not different from each other, there were also no significant differences between the Black Sea and Marmara Sea samples (Table 2). Moreover, the Mediterranean and Aegean Sea samples were also not significantly different from each other, but significantly different from the Marmara and Black Sea samples (Table 2).

The overall haplotype diversity ( $h$ ) including all populations was 0.74. The number of unique haplotypes was found to be 19 out of 222 sequences based on nucleotide variability. Trabzon, Bandırma and Adriatic populations possessed some private haplotypes (Table 1). The dominant haplotype 1 accounted for 45% (100/222) of *S. sarda* specimens and appeared in each sampled population. Moreover, the population-specific haplotypes occurred at moderate frequencies (13%). The phylogenetic relationships among the identified haplotypes were constructed, and the result revealed a star-like shape, characterized by a remarkable number of unique haplotypes, which were mostly related to a central and most-abundant haplotype (hap1) (Fig. 2).

TABLE 1 - Distribution of mtDNA D-loop haplotypes and their frequencies in *S. sarda* samples.

Haplotype	f	Populations										
		NMS2	NMS1	AS	MS	BP	BS2	BS3	BS4	BS5	BS1	ADS
Hap 1	100	7	9	12	6	10	12	12	11	8	12	1
Hap 2	1	1										
Hap 3	22	4	11	6	1							
Hap 4	1	1										
Hap 5	10	5	1		3	1						
Hap 6	1	1										
Hap 7	1	1										
Hap 8	3		1	2								
Hap 9	47				6	8	8	4	4	9	8	
Hap 10	2				1	1						
Hap 11	1				1							
Hap 12	7						2	5				
Hap 13	2							1	1			
Hap 14	1								1			
Hap 15	4								4			
Hap 16	1											1
Hap 17	8											8
Hap 18	9											9
Hap 19	1											1
<b>Total</b>	<b>222</b>	<b>20</b>	<b>22</b>	<b>20</b>	<b>18</b>	<b>20</b>	<b>20</b>	<b>18</b>	<b>21</b>	<b>23</b>	<b>20</b>	<b>20</b>

TABLE 2 - Pairwise genetic distance ( $F_{ST}$ ) between populations of *S. sarda* (below diagonal), and genetic diversity ( $\pi$ ) within samples (transversal diagonal as given in bold). \*, indicate significance level at  $P < 0.001$  after the bonferonni correction.

	NMS2	NMS1	AS	MS	BP	BS2	BS3	BS4	BS5	BS1	ADS
NMS2	<b>0.0015</b>										
NMS1	0.0430	<b>0.0008</b>									
AS	0.0019	0.00	<b>0.0008</b>								
MS	0.1454*	0.2915*	0.2198*	<b>0.0013</b>							
BP	0.2421*	0.3998*	0.3180*	0.00	<b>0.0008</b>						
BS2	0.2696*	0.4201*	0.3335*	0.0275	0.00	<b>0.0006</b>					
BS3	0.1940*	0.3437*	0.2369*	0.0843	0.0585	0.0281	<b>0.0007</b>				
BS4	0.2261*	0.3532*	0.2650*	0.1273	0.0585	0.1027	0.00	<b>0.0009</b>			
BS5	0.2840*	0.3968*	0.3276*	0.0567	0.0235	0.0261	0.0894	0.1215	<b>0.0011</b>		
BS1	0.2696*	0.4206*	0.3335*	0.0275	0.00	0.00	0.0281	0.1027	0.0261	<b>0.0006</b>	
ADS	0.5469*	0.6495*	0.6240*	0.5838*	0.6295*	0.6295*	0.6201*	0.6003*	0.6026*	0.6542*	<b>0.0009</b>

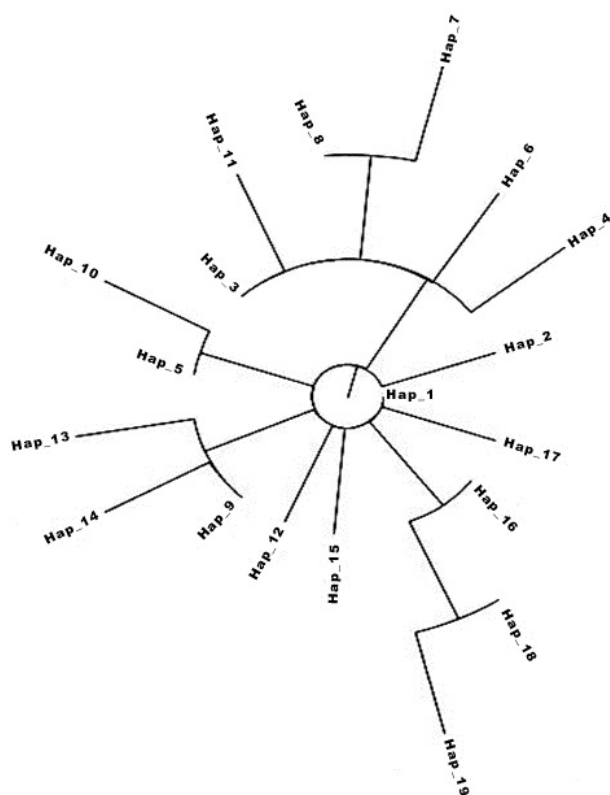


FIGURE 2 - Phylogenetic trees of the mtDNA D-loop haplotypes of *S. sarda* reconstructed with neighbor joining method.

The distribution of the haplotype in the tree generally reflects the geographic separation of the samples.

In the neighbor joining tree (Fig. 3), three main phylogenetic nodes were detected; in the first node, the Black Sea (BS1, BS2, BS3, BS4, BS5), Bosphorus (BP) and Marmara Sea samples were grouped close together. In the second main node; the Aegean Sea and Northeastern Mediterranean Sea samples were clustered close to each other. On the other hand, the Adriatic Sea samples showed very distinctive relationship.

Multi dimensional scaling analysis (MDS) of the  $F_{ST}$  values between the geographic samples showed that there are three genetically separated grouping; one include Black

Sea and Marmara Sea samples (BS1, BS2, BS3, BS4, BS5, BP, MS), and the second include Aegean and Mediterranean Samples (AS, NMS1, NMS2), and the third one the Adriatic sample (Fig. 4).

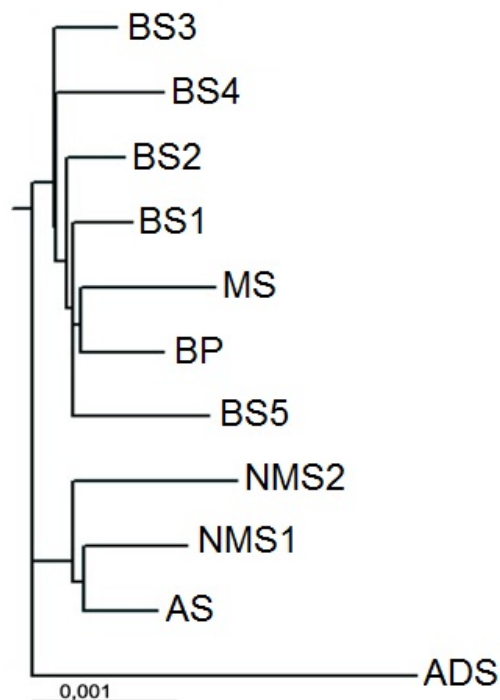


FIGURE 3 - Mitochondrial DNA D-loop neighbour joining tree of *S. sarda* samples.

The results of the present study support restricted gene flow at the margins of the geographical distribution. A three major genetic break was observed in *S. sarda*; the Black and Marmara Sea populations comprise one genetic unit, and the Aegean and Mediterranean coast of Turkey populations constitute the genetically different second unit, and the Adriatic Sea population was genetically different from these two units.

The Dardanel strait system seems to be a geographic barrier to limit gen flow between the Black Sea and Aegean Sea populations, and cause genetic differentiation of

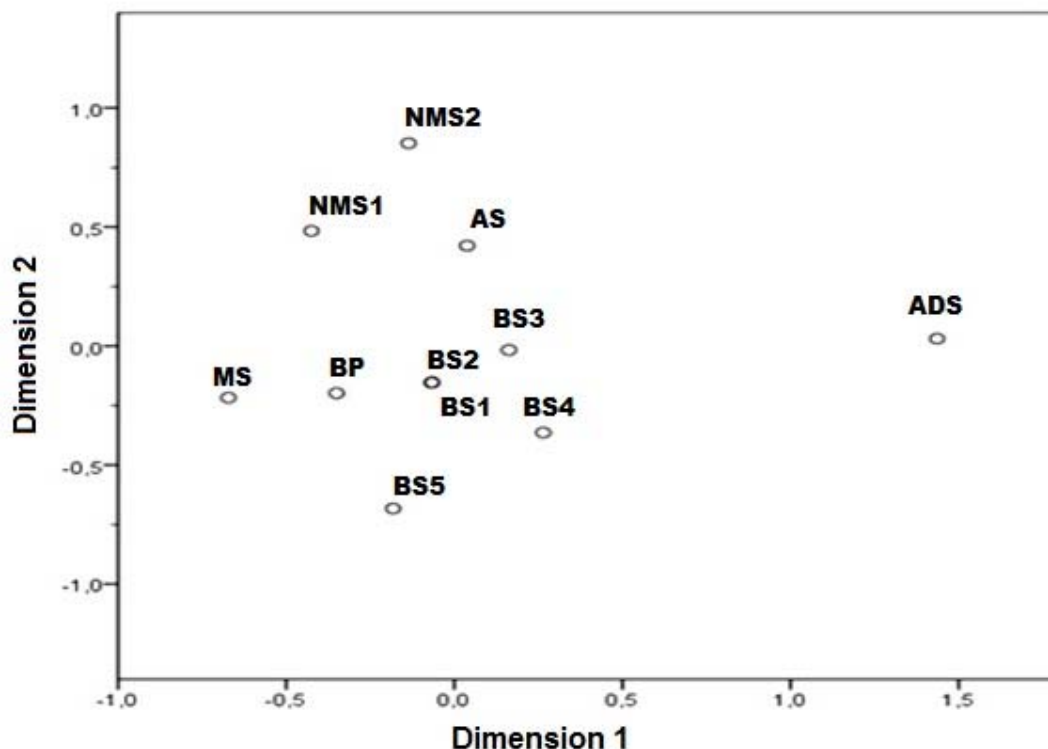


FIGURE 4 - Multi dimensional scaling plot of pairwise  $F_{ST}$  between the populations of *S. sarda*. Abbreviations of the samples are given in the Figure 1.

*S. sarda* populations. Moreover the wide geographic interval between the Adriatic and the other *S. sarda* populations seems to be limiting intermingling and rising the detected genetic differentiation. The NJ tree (Fig. 3) and MDS (Fig. 4) analyses also support the genetic differentiation of the geographically isolated three groups.

The Dardanel strait system has been reported to be acting as a geographic barrier to prevent gen flow for other marine species such as Anchovy (*Engraulis encrasicolus*), mullets (*Liza aurata*, *Liza saliens*, *Mugil cephalus*). Moreover, the detected genetic disconnectivity of the Aegean Sea and Marmara Sea populations was generally concordant with the results of the genetic population structure analyses by Vinas et al. [6], Roberti et al. [4] and Pujolar et al. [5].

Vinas et al. [15] reported the overall nucleotide and haplotype diversity to be 0.031 and from the control region of *S. sarda* populations from the Balearic, Ligurian, Ionian and Aegean Seas. Vinas et al. [22] investigated phylogenetic relationship of four *Sarda* species (*Sarda sarda*, *Sarda orientalis*, *Sarda australis* and *Sarda chilensis*) using D-Loop sequence analysis and reported the nucleotide diversity to be 0.071 for Mediterranean and 0.061 for Atlantic populations. In the present study the overall nucleotide diversity was found to be 0.0013 which is lower than the studies by Vinas et al. [15] and Vinas et al. [22]. The lower nucleotide diversity in the present study can be explained that there might be a past bottleneck effect on *S. sarda* in Turkish marine waters. Since there was high fluctuation of total catch of *S. sarda* in

Turkish marine waters that the catches of *S. sarda* in the adjacent seas of Turkey were 10.000 t in 2004, and in the following year (2005), the catches was raised to 70.000 t, and declined to 30.000 t and 10.000 t in 2006 and 2007, respectively [23].

Vinas et al. [6] reported the total haplotype diversity to be 0.993, and the number of haplotypes was 128 extracted from 198 individuals. In the present study the haplotype diversity was 0.74 and the number of different haplotypes was 19 extracted from 222 individuals. In the present study, only the Aegean Sea sampling location was geographically same with Vinas et al. [6]. The number of different haplotypes was 28 in the Aegean Sea sample in Vinas et al. [6], and in the present study only 3 unique haplotypes were found in the Aegean Sea sample (Table 1). Moreover, the number of unique haplotypes was 2 from Northwestern Black sea samples (Igneada and Bulgaria). The low haplotype diversity may indicate past bottleneck effect on *S. sarda* as discussed above. Since Vinas et al. [6] collected the *S. sarda* samples from the Aegean Sea in 1993, and the high fluctuation of total catch of *S. sarda* in Turkish marine waters was happened in 2004-2006.

The marine species usually demonstrate low genetic differentiation due to lack of major geographical barriers to dispersal and gene flow [24-27]. Marine species with high dispersal and large population size such as pelagic fish often result in low or no genetic structuring across large geographic scales. Genetic divergence between populations of

*S. sarda* found to be reasonably high in the present study and in the previous study [15] in comparisons to other marine species. Mean nucleotide divergence was found to be 0.00231 in the present study. Mean nucleotide divergence among populations of marine species were 0.00055 for striped red mullet *M. surmuletus* [28], 0.00027 for the long-tailed hake, *Macruronus magellanicus* [29], 0.000038 for sardine *Sardina pilchardus* [30], 0.803 for red drum *Sciaenops ocellatus* [31], 0.076 for spiny chromis damselfish *Acanthochromis polyacanthus* [32], 0.0037 for black sea bass *Centropristis striata* [33], 0.0059 for orange roughy *Hoplostethus atlanticus* and 0.00076 for hoki *Macruronus novaezelandiae* [34].

The detected significant genetic divergence indicates that restricted levels of gene flow are occurring between stocks of *T. mediterraneus*, relevant to geographical distance.

The phylogenetic relationships among the identified haplotypes revealed a remarkable number of unique haplotypes, indicate restricted gene flow between the geographically isolated groups. Complementarily, the most-abundant haplotype (hap1) was shared only once with the geographically most isolated Adriatic Sea population. Vinas et al. [6] also support the high haplotype differences between the geographically separated populations of *S. sarda* from the northeastern and northwestern Mediterranean. The phylogenetic trees (Fig. 3 and Fig. 4) also support the geographic separation is limiting factor to gene flow. Moreover the phylogenetic trees and haplotype distributions may also indicate that the *S. sarda* originated from the western side and spread towards eastern side of the Mediterranean.

#### 4. CONCLUSION

The present study support restricted gene flow at the margins of the geographical distribution and indicate that *S. sarda* in the Black and Marmara Sea populations comprise one genetically discontinuous unit, and the Aegean and Mediterranean coast of Turkey populations constitute the genetically different second unit, and the geographically highly separated Adriatic Sea population was genetically different from these two units. The management implications of populations depend on whether marked variation persists over time. Consistent differentiation of these populations may indicate its temporal and spatial integrity and thus would also require its consideration as a separate population for management purposes. Moreover, the utilization of nuclear genes with different genetic markers such as microsatellites would extend the reliability of these findings.

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