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Mitochondrial DNA Sequence and Body Size Variations in Turkish Sardine (*Sardina pilchardus*) Stocks

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Abstract: Sardine (*Sardina pilchardus*) is one of the most important species among Turkish fisheries and is broadly distributed along its coastal waters. In the present study, mitochondrial DNA sequences from the *cytochrome b* (*cytb*) gene were examined to assess the genetic diversity of sardines inhabiting Turkish coastal waters. A fragment of sardine *cytb* DNA from each sample collected from 8 representative regions along the coastal zones was amplified by PCR analysis and subsequently sequenced. The results of sequence analysis determined the existence of variations in 16 single nucleotide sites within the 452 bp fragment of the *cytb* gene examined in the present study. Phylogenetic trees and pairwise analyses demonstrated a very small divergence (0.002%-0.44%) between the populations, suggesting the lack of population subdivisions. Furthermore, the results of this study revealed a pattern of high nucleotide homology among the adjacent populations, and a small number of nucleotide changes among disjunct populations, leading us to conclude that there is a genetic admixture among the populations inhabiting the coastal waters of Turkey, especially in those geographically close to each other. The results of this study suggest that sardine populations of coastal Turkey are part of a larger, self-recruiting population whose boundaries extend beyond the investigated area.

Key Words: Sardine, *Sardina pilchardus*, mitochondrial DNA, *cytb*, genetic divergence

Mitokondrial DNA Analiz Yöntemiyle Ülkemiz Denizlerindeki Sardalya Balığı (*Sardina pilchardus*) Populasyon Yapılarının İncelenmesi

Özet: Türkiye'nin kıyı sularında geniş bir yayılım gösteren sardalya (*Sardina pilchardus*), ülkenin en önemli balıkçılık ürünlerinden birini oluşturmaktadır. Bu çalışmada Türkiye kıyı sularında yaşayan sardalyalarda genetik farklılıkların belirlenmesi amacıyla, mitokondrial DNA'nın *cytochrome b* (*cytb*) gen sekansı ve vücut büyüklükleri ile ilişkisi incelenmiştir. Sekiz temsili bölgeden toplanan sardalyaların *cytb* DNA fragmenti örnekleri PCR analizi ile amplifiye edildikten sonra sekans analizleri yapılmıştır. Sekans analiz sonuçları, *cytb*'nin incelenen 452 nükleotidlik bölümünde 16 tek nükleotid değişimi, olduğunu göstermektedir. Yapılan filogenetik ağaç ve çiftli analizler, populasyonlar arası ayrılımın çok küçük bir yüzde (0,002-0,44) olduğunu göstererek populasyon içinde alt bölünmelerin bulunmadığını ortaya koymaktadır. Ayrıca analizler komşu populasyonlar arası nükleotid homolojisinin yüksek olduğunu ve komşu olmayan populasyonlar arasında da az sayıda nükleotid farklılığı bulunduğunu göstermektedir. Dolayısıyla bu sonuçlar Türkiye kıyı sularını habitat edinmiş sardalya populasyonları arasında bir genetik karışımın olduğunu ortaya çıkarmaktadır. Ayrıca sonuçlar, sardalya populasyonlarının çalışma sırasında incelenenden daha geniş bir alanı içine alan daha büyük bir populasyonun parçası olduğunu işaret etmektedir.

Anahtar Sözcükler: Sardalya, *Sardina pilchardus*, mitokondrial DNA, *cytb*, genetik sapma

Introduction

Sardine is an important fish species of great economic importance to Turkey, as well as to many other countries (Finney et al., 2002; Finney et al., 2002; Williams, 2003). Worldwide production of sardine is 940,700 tons/year (FAO, 1999), whereas in Turkey it is approximately 8,700 tons/year (DIE, 2004). Most of the yield in Turkey comes from the Aegean Sea (5,000 tons/year) and the Sea of Marmara (2,700 tons/year), while only a small portion (856 tons/year) is harvested

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from the Mediterranean Sea (DIE, 2004). Although official records show that about 154 tons/year of sardine were harvested from the entire Black Sea (DIE, 2004), it has been reported that big schools of sardine have not been caught there. In general, only small quantities of sardine have been harvested from the Black Sea, along with big schools of some other fish species, such as *Spratus spratus phalericus*.

In addition to the differences in the amount of sardine harvested from the different coastal zones, there is a distinct size difference among the sardines of Turkey. The sardines harvested from northern coasts, such as İstanbul and Bandırma, are much smaller than those harvested from southern coasts, such as Mersin and Adana. This might simply be due to the effect of temperature on the growth rate (lower water temperature of the northern coastal regions vs. warmer Mediterranean waters); however, the genetic background of different populations might also have some effect on size differences. Therefore, it is necessary to study whether there is genetic heterogeneity among the populations inhabiting the coastal zones of Turkey.

For the management and sustainability of fish populations, studying genetic diversity is critical. Nevertheless, studies on the geographic distribution and genetic population structure of many commercial fish species stocks have not been completed (Atarhouch et al., 2005). Data concerning sardine populations inhabiting Turkish coastal waters are currently very limited and fragmented (Cihangir, 1996; Turker, 1998; Kara and Ozekinci, 2002). To the best of our knowledge the genetic diversity of Turkey's sardine populations has yet to be investigated using modern DNA technology. Because lack of genetic variability causes a decline in the survival fitness of local populations (Hutchings, 2000; Jackson et al., 2001; Knutsen et al., 2003), lack of data on genetic diversity raises concerns, especially in those cases in which there is overexploitation of local stocks, which in turn may cause a genetic bottleneck and consequent depletions. The depletion of the Safi sardine stocks of Morocco in the 1970s, which is likely to have been caused by the genetic homogeneity of this population (Atarhouch et al., 2005), demonstrates the importance of studying the genetic variability of local stocks.

Analysis of mitochondrial DNA (mtDNA) has proved to be a powerful tool for addressing issues of genetic diversity among a great number of organisms (Hewitt, 1996; Avise, 2000; Saccone et al., 2000). Because of mtDNA's very important molecular features, such as compact organization, primarily maternal inheritance, and absence of recombination, it has provided a unique tool for such studies (Stabile et al., 1996; Saconne et al., 2000). Furthermore, a large number of studies have shown it to be, in general, a good phylogenetic marker for vertebrate phylogenetic analysis (Samonte et al., 2000; Sebastio et al., 2001). The cytochrome b (*cytb*) gene has been specifically chosen by many investigators because of its sufficient point mutation rate, its usefulness in discriminating closely related fish species, and in determining the degree of intraspecific variability in pelagic fish species for population identification (Reilly and Ward, 1999; Jerome et al., 2003; Lecomte et al., 2004). Analysis of differences in mtDNA sequences has led to the identification of Mediterranean and Eastern Atlantic populations of the sardinella, *Sardinella aurita* (Chikhi et al., 1997).

The aim of the present study was to determine whether there is genetic diversity among the sardine populations inhabiting the coastal zones surrounding Turkey. The sardines of these populations significantly vary ($P < 0.0001$) in body size according to coastal zone. We devised a molecular mtDNA analysis technique to assess the genetic diversity among the populations of sardine inhabiting Turkish coastal waters.

Materials and Methods

Fish Samples

The sardine samples used in the present study were collected between March and August 2002. In all, 10 different geographic locations were initially chosen to represent the coastal regions of Turkey for sample collection; however, due to the limited presence of sardine in the Black Sea, an adequate number of sardine samples could not be obtained from eastern and central regions of the Black Sea during the course of the present study. Therefore, this study was carried out using samples harvested from 8 different locations in the western Black Sea, the Sea of Marmara, Aegean Sea, and Mediterranean Sea (Figure 1). Samples were obtained from commercial vessels immediately after the catch, stored in dry ice, and shipped to our laboratory. Upon arrival at the laboratory the species of fish were confirmed (Astray, 1987), and then total weight and fork

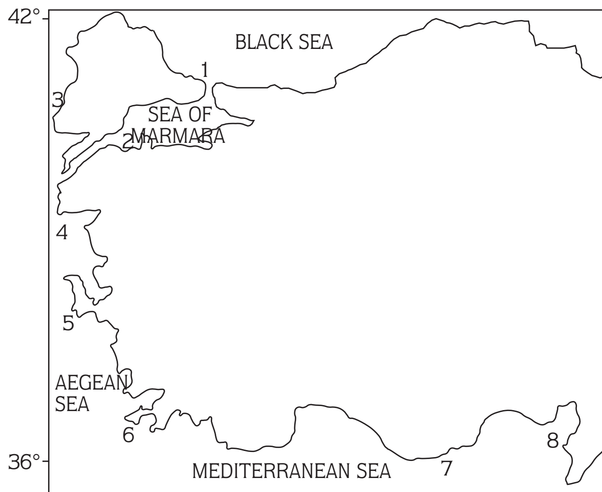

Figure 1. *Sardina pilchardus* sampling locations in the coastal zones of Turkey (1: İstanbul; 2: Bandırma; 3: Çanakkale; 4: Ayvalık; 5: İzmir; 6: Muğla; 7: Mersin; 8: Adana).

length of each fish were measured and recorded separately for each region (Table 1). Measurement of metric parameters was performed on 30 fish samples for each region; in total, 240 fish. Metric measurement showed that body size of sardines from different populations varied significantly (P < 0.0001). Following measurement of metric parameters the fish were then stored at –20 °C for further use in DNA analysis.

Table 1. *Sardina plichardus* size variation in the coastal zones of Turkey.

Region	Total length (cm)*	STDEV*	Total weight (g)*	STDEV*
İstanbul	13.28	0.44	26.67	1.92
Bandırma	12.68	0.29	22.21	1.24
Çanakkale	11.49	0.29	14.41	1.51
Ayvalık	14.57	0.89	31.06	6.98
İzmir	12.68	0.23	22.31	2.03
Muğla	13.30	0.42	25.83	2.61
Mersin	16.93	0.56	65.56	6.48
Adana	15.66	0.89	57.20	8.89

*Values represent the average of 30 samples from each region.

Statistical Analysis

Results shown in Table 1 are the mean ± SE of 30 fish samples from each region. Data analyses were performed by one way ANOVA, followed by Fisher's protected least significant difference test, using StatView 4 (Abacus Concepts, Berkley, CA, USA). Differences were considered statistically significant at P < 0.05.

DNA Extraction

DNA samples were prepared by extracting total DNA from frozen tissues (Sambrook et al., 1989). Extracted DNA was precipitated with ethanol in the presence of sodium acetate and then resuspended in either dH_2O for immediate use or in TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)) (Sambrook et al., 1989) for storage.

PCR Amplification and Sequencing

In order to design the primer set for the PCR amplification of the *Sardina pilchardus cytb* gene, a complete CDS sequence from GeneBank (accession number: AF472582) was used. A 1002-bp region of sardine *cytb* was amplified using an upstream primer (5'-ATCGCCAACGACGCACTAGTCGAC-3') and a downstream primer (5'-ACGCAGTTCTTGTTCTGA-3'). PCR conditions were as follows: Initial denaturation: 2 min at 94 °C; denaturation: 35 cycles at 94 °C for 30 s; annealing: 48 °C for 30 s; extension: 72 °C for 1 min; final extension: 72 °C for 10 min. All PCR reactions were performed with a hot-start using Pfu Polymerase (Fermentas) with high fidelity. The PCR reactions were carried out in a final volume of 50 µl, that contained 50 mM of KCl, 10 mM of Tris-HCl (pH 8.3), 2.5 mM of $MgCl_2$, 200 µM of each dNTP, 2 units of Pfu polymerase, and 0.2 µM of forward and 0.2 µM of reverse primers. PCR amplification for each region was repeated at least 3 times. The linear PCR product DNA samples were then sequenced by an automatic DNA sequencer (ABI Prism 310 DNA sequencer, Genewiz, NJ, USA) using the 5' AGCCATGCACTACACCTC 3' primer, which is located 152 bp downstream of the PCR fragments. DNA samples from 5 different fish samples from each region were sequenced. A representative sequence of the resultant *cytb* gene sequences located between 209 bp and 661 bp for each geographic region was deposited in the GeneBank (MA, USA). The GeneBank accession numbers are listed in Table 2.

Phylogenetic Analysis

Sequences of the 452 bp *cytb* region were aligned (Figure 2) using the BioEdit v.5.0.9 program. Alignment was then manually checked and corrected. Phylogenetic trees were then constructed based on neighbor-joining (Saitou and Nei, 1987) and maximum likelihood

Table 2. Accession numbers of the representative *cytb* sequences deposited in the GeneBank database.

Region	GeneBank accession #
İstanbul	AY946032
Bandırma	AY946029
Çanakkale	AY946033
Ayvalık	AY946030
İzmir	AY946031
Muğla	AY946028
Mersin	AY946026
Adana	AY946027

(Felsenstein, 1981) analyses, using the PHYLIP (Felsenstein, 1995) program. *Sardinops melanosticus cytb* sequences located between 14611 and 15063 bp (GeneBank accession number: NC-002616) were used as an outgroup in the construction of the phylogenetic tree. Pairwise sequence divergence among populations, and the number of transitions and transversions were calculated using the DNADIST program in PHYLIP, according to Kimura's 2-parameter model (Kimura, 1980). Pairwise nucleotide divergence was analyzed using both the rate of transition/rate of transversion, and percent divergence.

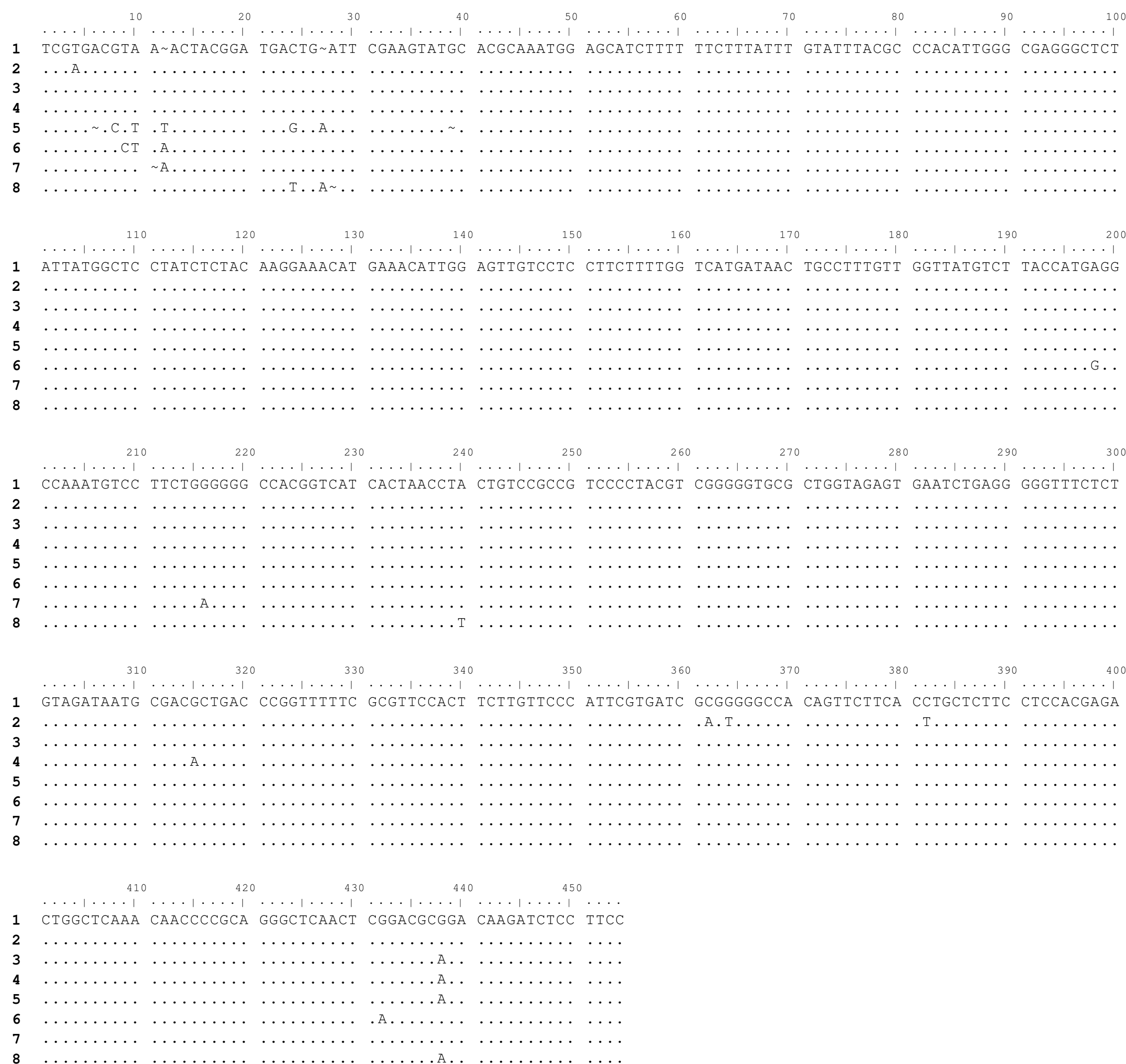

Figure 2. Sequence alignments of the *Sardina pilchardus cytb* gene in the 452-bp region (numbers are as in Figure 1).

Results

Size Variation among *Sardina pilchardus* Populations

Size differences were readily noticeable between the samples obtained from different coastal zones (Table 1). The length of samples from the Black Sea, Sea of Marmara, and Aegean Sea was relatively shorter (11.5-14.6 cm) than that of samples collected from the Mediterranean Sea (15.7-17 cm). When the length of the samples were compared, the differences were statistically significant ($P < 0.0001$); however, in the case of body weight, differences were more remarkable (also statistically significant; $P < 0.0001$). Weight differences became more obvious when samples collected from the northern regions (Aegean Sea and Sea of Marmara), which had much lower body weight (14.4-31.1 g), were compared to those harvested from the Mediterranean Sea, which had much higher body weight (57.2-65.6 g). Statistical analyses of body length and body weight data demonstrated that the size differences between all regions were statistically significant.

Nucleotide Variation in the *cytb* Gene

The 452 bp region of the *cytb* gene was used to perform sequence analysis. For each region, sequence samples obtained from 5 separate individuals were aligned and compared; however, no differences were detected between individuals from the same region (data not shown). Comparison of the 452 bp sequences between fish samples from different localities revealed the presence of nucleotide variations in 16 different sites. Most of the nucleotide variations in each site were unique for an individual group (Figure 2); however, variations were shared by more than 1 group for 5 different nucleotide changes; in particular, nucleotide site 438 (Figure 2) had 2 variations, each of which was harbored by 4 different groups. The extent of variation varied among the groups with some groups having as many as 5-7 variations and others having only 1. The nucleotide variations were mostly on the third base of the codons; therefore, they did not cause any change in the protein sequence, with the exception of 2 regions, İzmir and Muğla (data not shown), where there were only 3 amino acid changes in the first 4 amino acid sequences. When the nucleotide sequences of the groups investigated in the present study were compared with an outgroup sequence of sardines inhabiting the western part of the Mediterranean Sea (GeneBank accession number: AF472582), there were only a handful of nucleotide changes, and the protein sequences were identical (data not shown).

Sequence-Based Phylogeny

The phylogenetic trees were constructed (Figure 3A and Figure 3B) using neighbor-joining and maximum likelihood methods. Although the trees constructed using the 2 different methods were not identical, essentially very similar topology was obtained, which revealed very little divergence in the *cytb* region investigated in this study. When *S. melanosticus* was included as an outgroup in the construction of the phylogenetic tree (Figure 3C), the outgroup branch did reveal a big difference from the

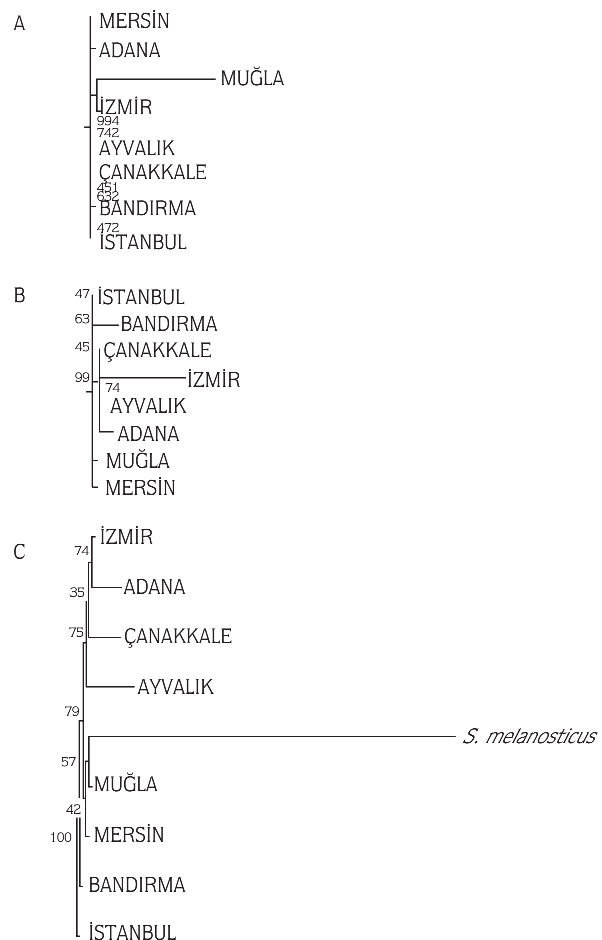

Figure 3. Phylogenetic trees illustrating the genetic relationships between sardine stocks (A: neighbor-joining tree based on Saitou and Nei (1987); B: maximum likelihood tree based on Felsenstein (1981); C: neighbor-joining tree in the presence of an outgroup, based on Saitou and Nei (1987). Numbers above are the branches that represent the bootstrap values of 100 replicates.

samples included in this study; however, it was interesting to determine that the location of the outgroup branch was closer to an Aegean (Muğla) stock. The data obtained from pairwise nucleotide divergence analysis, varying between 0.2% and 1.5%, also further confirmed the result obtained from phylogenetic tree analysis. The branch lengths were extremely short, ranging from 0.000 to 0.008 (data not shown), and showed that the populations were closely related to each other. The rate of transition was 2-fold greater than that of transversion (Table 3), another important factor affecting the divergence rate among populations. It has been reported that, in general, transitions cause less divergence than transversions (Hewitt, 1996; Saccone et al., 2000). The pairwise nucleotide similarity values, ranging from 97.3% to 99.7% (Table 3), also proved the lack of diversity among the populations investigated in this study.

Discussion

Sardine fisheries and snapper fisheries of the Moroccan coasts experienced severe stock declines due to overfishing (Belveze and Erzini, 1983; Hauser et al., 2002) in recent decades. Sardine stocks, in particular, suffered from significant population reductions. Although overall stocks were able to recover after these collapses and currently no serious signs of collapse of sardines are predicted (Beverton, 1990) for Moroccan sardine populations, Atarhouch et al. (2005) demonstrated that the historical collapse of Moroccan Safi sardine stocks in the 1970s is likely to have been caused by the genetic homogeneity of this population. Knowledge of genetic diversity and population distribution is vital for the effective management and sustainability of any regional fishery (Reilly and Ward, 1999). Investigations to determine diversity at the molecular level have not been commonly performed; however, recently reported investigations have demonstrated the importance and the usefulness of the mtDNA sequence analysis technique in determining the genetic diversity of regional fish species. The presence of 2 anchovy populations in the Mediterranean Sea was successfully confirmed by molecular phylogenetic DNA analysis (Borsa, 2002; Borsa et al., 2004). Furthermore, the signature of the early genetic bottleneck in a population of Moroccan Sardines (*Sardina pilchardus*) was also determined using mtDNA analysis (Atarhouch et al., 2005).

Metric data obtained from the present study revealed that there was a remarkable difference in the size of sardines, especially in the weight of those collected from the Mediterranean Sea, as compared to samples collected from the other sampling locations. Statistical analyses showed that the differences among all the groups were statistically significant. This phenomenon was previously observed (Cihangir, 1996; FAO, 1999); however, it has not yet been explained if this was a reflection of isolated populations occupying limited areas within the coastal zones of the country.

The phylogenetic trees constructed using neighbor-joining and maximum likelihood methods determined that the sardine stocks investigated in the present study were not from different populations. In 2 of the trees (Figure

Table 3. Pairwise nucleotide divergence of the *cytb* gene (452 bp fragments) of sardines inhabiting the coastal waters of Turkey.*

Region	İstanbul	Bandırma	Çanakkale	Ayvalık	İzmir	Muğla	Mersin	Adana
İstanbul		0.002/0.007	0.002/0.000	0.004/0.000	0.009/0.022	0.259/0.438	0.0.002/0.000	0.004/0.002
Bandırma	0.009		0.004/0.007	0.007/0.007	0.011/0.029	0.263/0.440	0.0044/0.007	0.007/0.009
Çanakkale	0.002	0.011		0.002/0.000	0.007/0.022	0.259/0.438	0.004/0.00	0.002/0.002
Ayvalık	0.004	0.013	0.002		0.009/0.022	0.259/0.438	0.007/0.000	0.004/0.002
İzmir	0.009	0.018	0.067	0.009		0.254/0.429	0.011/0.022	0.007/0.024
Muğla	0.009	0.018	0.011	0.013	0.015		0.261/0.438	0.259/0.436
Mersin	0.002	0.011	0.004	0.007	0.013	0.011		0.007/0.002
Adana	0.007	0.015	0.004	0.007	0.009	0.015	0.009	

*Above diagonal: rate of transition/rate of transversion. Below diagonal: percentage of divergence.

3A and Figure 3B), İstanbul, Bandırma, and Çanakkale sardines were clustered in close proximity. The main difference between these 2 trees was the branch lengths for İzmir and Muğla; however, when the actual branch lengths, ranging from 0.000 to 0.008, were taken into consideration, it was clear that this was not a significant difference. It was interesting to determine that the branch length of the outgroup (*S. melanosticus*) (Figure 3C) was located close to an Aegean Sea stock (Muğla); however, the branch length of this outgroup was much longer (0.012). This finding provides additional data demonstrating the close similarity among the sardines of the region. Moreover, divergence between 0.2% and 1.5% further proves how closely they are related; therefore, we report that based on the results obtained from molecular analyses, there is a lack of isolated populations and heterogeneity among the sardines inhabiting the coasts of Turkey. Despite the high disproportion in size between sample collection locations observed in the present study, phylogenetic analysis did not reveal any intrapopulation genetic diversity. Although previous studies involved the use of different methodologies and different fragments of DNA, this phenomenon was also observed for Aegean Sea (Spanakis et al., 1989), Spanish Mediterranean Coast (Ramon and Castro, 1997), and Adriatic and Ionian stocks of sardine (Tinti et al., 2002). Tinti et al. (2002) reported that there is gene flow between Adriatic-Ionian and Spanish sardines. Furthermore, they concluded that these sardine stocks might be part of a large population in this region. Low-level nucleotide diversity of mtDNA among the *Sardinop*s inhabiting zones of the Indian-Pacific Ocean was reported by Grant and Bowen (1998). They suggested that the populations may be subject to periodic extinction and recolonization due to climatic-associated dynamics, which explain the low level of nucleotide diversity. Genetic differentiation between sardine populations of Pasajes (Bay of Biscay, Atlantic Ocean), and those of the Mediterranean Sea and the Moroccan Coast was reported by Atarhouch et al. (2005); however, they also presented evidence of an early genetic bottleneck in the population of Moroccan sardine. Furthermore, they reported the existence of gene flow between populations of the Mediterranean Sea and those of the Moroccan Atlantic Coast.

Results from the present study agree with earlier studies that concluded species such as sardine, with high rates of dispersal, exhibit a low level of genetic diversification in population structure, whereas species with little capability for dispersal have a significant degree of interpopulation genetic diversity (Stabile et al., 1996). A number of earlier studies have shown that biogeography, evolution, and long-term climate change are among the primary influences that cause genetic diversity and morphological variation within a species (Hewitt, 1996; Grant and Bowen, 1998; Avise, 2000). The degree of gene flow can act as a strong force in the maintenance or homogenization of genetic differences among adjacent and disjunct populations. The results of our study reveal a lack of haplotypic diversity, which in turn suggests homogenization of genetic variation among the sardines inhabiting the coastal waters of Turkey; however, it needs to be acknowledged that the results of the present study are based on the analysis of *cytb* sequences. It has been reported that certain nucleotide sites in the control region of mtDNA are targets of more frequent mutations than those of *cytb* (Brown, 1983; Harrison, 1989); therefore, the control region of mtDNA may also need to be investigated in future studies. Samonte et al. (2000) were able to demonstrate 2 haplotypes of *Sardinella tawilis* using control region sequences, which were not detected with *cytb* sequence analysis.

Sardine is the third most economically important fish species in Turkey, with a yield of 493,500 tons/year (DIE, 2004), making its economic importance undeniable. In addition to its economic value, it is undoubtedly one of the most crucial species to the biodiversity of the marine aquatic system of the region. Turkey is an important genetic diversity location (Williams, 1997). Both the economic and ecological significance of this species demonstrate how vital knowledge its genetic diversity is for the effective management and sustainability of the region's fisheries. The present study, as well as those previously published (Spanakis et al., 1989; Tinti et al., 2002; Atarchous et al., 2005), suggests that there is a lack of genetic heterogeneity among the local stocks of sardine studied, suggesting that there may be a large sardine population whose boundaries are wider than the limited area studied by different investigators. The determination of the limits of this large sardine population will be invaluable to the management and sustainability of sardine in the region.

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