



## Genetic Differentiation of Three *Spicara* (Pisces: Centranchidae) Species, *S. maena*, *S. flexuosa* and *S. smaris*: and Intraspecific Substructure of *S. flexuosa* in Turkish Coastal Waters

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### Abstract

Interspecific and intraspecific differentiation of the picarel in Turkish coastal waters were examined using partial 16S rRNA (1021 bp) and whole cytochrome b gene (1141 bp) sequences of 244 samples from 7 picarel populations across their geographical distribution. Genetic distances and phylogenetic tree topologies revealed that three *Spicara* species were distinctly separated from each other and *S. flexuosa* and *S. maena* are genetically more closely related than *S. smaris*. Low-level genetic diversity and a star-like haplotype network can be attributed to the founder effect associated with demographic bottleneck in late Pleistocene Mediterranean basin. AMOVA of the genetic substructure for *S. flexuosa* does not support any grouping in the Turkish waters. The divergence time between the Mediterranean and Black Sea populations (0.015%, cyt b) of *S. flexuosa* estimated based on the mtDNA clock calibration of 2% sequence per million years suggests that the Mediterranean samples with reduced genetic diversity has colonized into the Black Sea through straits during the early Holocene (about 7500 kya). The current distribution and demographic pattern of *Spicara* species in the Eastern Mediterranean basin are shaped by climatic events during late Pleistocene and mid-Holocene (about 25-5 kya) and the biological and ecological characteristics specific to *Spicara* species.

**Keywords:** Centranchidae, *Spicara*, species discrimination, population structure, mtDNA.

### Introduction

*Spicara* is a centranchid genus inhabiting shallow, rocky, and muddy bottoms throughout the Mediterranean and the Black Sea, in eastern coastal water of the Atlantic including Portugal and Morocco, and around the Canary Island (Froese & Pauly, 2017). Moreover, *Spicara* genus is represented by three species [*Spicara maena* (Linnaeus, 1758); *Spicara flexuosa* (Rafinesque, 1810); *Spicara smaris* (Linnaeus, 1758)] in the Turkish coastal waters of the Mediterranean and Black Sea (Tortonese, 1986; Golani, Ozturk, & Basusta, 2006; Turan, 2011; Froese & Pauly, 2017). *S. maena* and *S. flexuosa* are morphologically quite similar and their distribution areas mostly overlap, causing identity problems in this region. Morphological similarity can be attributed to the color change resulting from sexual maturation and the coloration diversity associated with sexual dimorphism (Pollard & Pichot, 1971). However, metric and meristic characters of *Spicara* species have proved to be quite essential for identification purposes (Tortonese, 1986; Fischer, Bauchet, & Schneider, 1987; Rizkalla, 1996) despite the problems mentioned

above. In fact, Minos, Imsiridou, & Katselis (2013) showed that the identification of both species (*S. maena* and *S. flexuosa*) could easily be done depending on the external morphological characteristics.

The *Spicara* genus has been genetically examined previously as part of the ongoing efforts aimed at genealogy of the Sparid family based on partial 16S rRNA (Hanel & Sturmbauer, 2000; Orrell & Carpenter, 2004) and cytochrome b gene (Orrell, Carpenter, Musick, & Graves, 2002; Orrell & Carpenter, 2004; Chiba, Iwatsuki, Yoshino, & Hanzawa, 2009) sequences of mitochondrial DNA. Later, only a few genetic studies targeting the genus *Spicara* were directly conducted. At first, using mitochondrial 16S rRNA sequences, Imsiridou, Minos, Gakopoulou, Katsares, and Karidas (2011) have precisely separated *S. maena* from *S. flexuosa*, which had long been debated whether they were congeneric (Tortonese, 1975; Eschmeyer, 2013; Froese & Pauly, 2017) or not (Vasiliev, 1980; Papakonstantinou, 1988; Golani et al., 2006; Vasileva, 2007). Second, by using mitochondrial DNA partial 16S rRNA sequences Turan (2011) has



identifications of picarel were performed by examining some morphological features (Vidalis, Markakis, & Tsimenides, 1997; Karidas, Argiridis, & Minos, 2009; Minos et al., 2013) under expeditionary conditions.

### DNA Extraction, PCR Amplification and DNA Sequencing

Fin clips of 244 specimens were sampled and stored in 96% ethanol at  $-20^{\circ}\text{C}$  until DNA extraction. DNA was extracted from fin tissues of each picarel using Qiagen DNeasy Blood & Tissue Kit (Qiagen, USA) and purified. The obtained DNA were stored in  $-20^{\circ}\text{C}$  for PCR applications. The quality and quantity of isolated DNA were verified using NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). The PCR amplifications of full cytochrome b gene and partial 16S rDNA regions were performed using following primer pairs: SpcrCytbF: 5'-CAA GCC TTC GAA ARA CAC ACC C-3'; and SpcrCytbR: 5'-ACY TTR TTT TCT AWA GTT GCT GC-3'), Spcr16SF: 5'-GTA TGG GCG ACA GAA AAG G-3' and Spcr16SR: 5'-ACT GAC CTG GAT TAC TCC GG-3'). PCR reactions were performed in 50  $\mu\text{l}$  reaction volume containing 5 $\mu\text{l}$  10x PCR buffer, 1.5 mM  $\text{MgCl}_2$ , 0.5 mM dNTPs mix, 0.5 mM of each primer, 50 ng template DNA, and 1 U *Taq* DNA polymerase (Fermentas, Thermo Fisher Scientific Inc). The PCR amplifications were performed under following conditions using a gradient thermal cycler Biorad T100™ (Bio-Rad, Hercules, CA, USA): initial denaturation at  $94^{\circ}\text{C}$  for 5 min, denaturation at  $94^{\circ}\text{C}$  for 1 min, annealing (at  $59^{\circ}\text{C}$  for cyt b and  $50^{\circ}\text{C}$  for 16S rDNA) for 45 second, primer extension at  $72^{\circ}\text{C}$  for 1 min through 35 cycles and a final extension at  $72^{\circ}\text{C}$  for 7 min. The PCR products were run on 1.5% agarose gel stained with ethidium bromide (0.5  $\mu\text{g}/\text{mL}$ ), in TAE buffer, and were visualized under UV Quantum-Capt ST4 system (Vilber Lourmat, France). The concentrations of purified PCR products were estimated by NanoDrop 2000C UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). Double stranded PCR product were purified using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystem, Foster City, CA), and then were bidirectionally sequenced on an ABI Prism 3730x1 automatic DNA sequencer (Applied Biosystems, USA) using related PCR primers. The obtained sequences of haplotypes were deposited in GenBank under accession numbers (MF149882-MF149892 of 16S rRNA haplotypes; MF149896-MF149912 of cytochrome b haplotypes).

### Data Analysis

The obtained sequences were edited and aligned using software Bioedit (Hall, 1999), and genetic distances were calculated according to Kimura (1980) method. The molecular diversity indices such as

number of haplotypes (H), polymorphic sites, nucleotide diversity ( $\pi$ ) and haplotype diversity ( $H_d$ ) were computed for each species using the program DnaSP version 5.10.1 (Rozas, 2009). Intraspecific genealogy based on network inference of haplotypes were reconstructed by means of a median-joining (MJ) algorithm implemented in Network 4.6.1.3 software (Bandelt, Forster, & Röhl, 1999, www.fluxus-engineering.com). Detecting of the hierarchical population structure within *S. flexuosa* dispersed throughout Turkish waters was performed using hierarchical analysis of molecular variance AMOVA (Excoffier, Smouse & Quattro, 1992) implemented in Arlequin version 3.5 (Excoffier & Lischer, 2010) with 10,000 permutations. The phylogenetic analyses of *Spicara* haplotypes were performed through neighbour joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) analyses. MP analyses were conducted in software PAUP v. 4.0b10 (Swofford, 2003) using a heuristic search, with the tree-bisection and reconnection (TBR) branch swapping and 100 random addition replicates. On the other hand, ML analyses were performed by software PHYML version 3.0 (Guindon & Gascuel, 2003), with 100 bootstrap repetitions using the TIM2+G (for both markers) substitution models, as selected according to AICc in jModelTest 0.1.1 (Posada, 2008). The robustness of nodes was assessed with 100, 500 and 1000 replicates in ML, MP and NJ, respectively. In order to construct the phylogeny of genus *Spicara*, *Centrachanthus cirrus* (GenBank accession number: MF149893-MF149895 of 16S rRNA haplotypes; MF149913-MF149916 of cytochrome b haplotypes) and *Pagrus major* (GenBank accession number: AP002949) were used as outgroups because they are closely related to *Spicara* species.

## Results

### Genetic Diversity

The full mitochondrial DNA cytochrome b (1141 bp) and partial 16S rRNA (1021 bp) sequences were analyzed for 244 individuals of three *Spicara* species from seven geographic populations natively distributed in Turkish territorial waters (Figure 1; Table 1). The nucleotide frequencies for mitochondrial DNA cytochrome b were identified as 33.31% A, 22.16% T, 23.46% C, and 21.07% G. The overall transistion/transversion bias was  $R=2.612$ . A total of 17 mtDNA cyt b haplotypes (1141 base pairs) were defined; five from *S. maena* (N=74), eight from *S. flexuosa* (N=112) and four from *S. smaris* (N=58) (Table 1). In total, 149 variable sites were identified, 140 of which were polymorphic. On the other hand, the nucleotide frequencies identified for partial 16S rRNA were found as 24.32% A, 30.78% T, 29.28% C, and 15.63% G. The overall transistion/transversion bias was  $R=5.293$ . A total of 11 mtDNA 16S rRNA

haplotypes (1021 bp) were defined, four from *S. maena* (N=74), five from *S. flexuosa* (N=112) and two from *S. smarís* (N=58) (Table 1). The aligned partial sequences of 16S rRNA included 63 variable sites, including 54 parsimony informative.

### Population Structure

The obtained molecular findings pointed out that both mitochondrial cytochrome b and 16S rRNA sequences of the individuals of *S. maena*, *S. flexuosa* and *S. smarís* of *Spicara* genus in Turkish coastal waters showed low level of haplotype and nucleotide diversity [(Sm:  $Hd=0.1791$  and  $\pi=0.00016$ ;  $Hd=0.07997$  and  $\pi=0.00013$ ), (Sf:  $Hd=0.2768$  and  $\pi=0.00026$ ;  $Hd=0.1044$  and  $\pi=0.00029$ ) and (Ss:  $Hd=0.1654$  and  $\pi=0.00020$ ;  $Hd=0.09982$  and  $\pi=0.00020$ )], respectively (Table 2).

The mutational distance-based relationships between the mitochondrial cytochrome b and 16S rRNA haplotypes were utilized in order to elucidate the genetic structure of *Spicara* populations through a MJ (median joining) network analysis with few unique haplotypes linked by one or two mutations (Figure 2). The haplotype network revealed a star-like structure containing several unique haplotypes, linked to dominant haplotypes (Smc1:67, Sfc1:105, and Ssc1:53 for cyt b; Sms1:71, Sfs1:106, Sss1:55 for 16S rRNA) with one or two mutational steps in each *Spicara* species that clearly separated from each other by at least 28 mutational steps (Figure 2).

AMOVA revealed that most of the total molecular variance 84.69% can be attributed to regional differences among individuals within the population, while 13.93% was apportioned among the

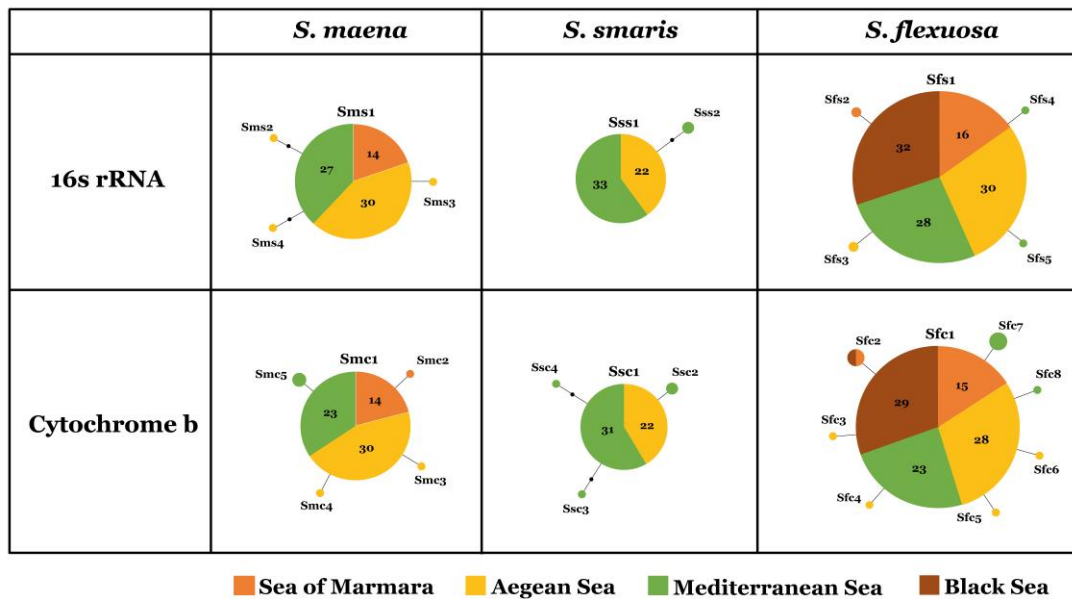
population within the group and 1.38% among groups for cytochrome b (Table 3). Genetic variations within *S. flexuosa* populations (fixation index within populations  $F_{SC} = 0.14121$ ,  $p < 0.001$ ) is higher than that among populations within groups (fixation index among populations within groups  $F_{ST} = 0.15306$ ,  $p < 0.001$ ) and among groups (fixation index between groups  $F_{CT} = 0.01380$ ,  $p < 0.001$ ) (Table 3). For 16S rRNA, AMOVA revealed that most of the total molecular variance 98.65% can be attributed to regional differences among individuals within the population, while 0.58% was apportioned among the population within the group and 0.77% among groups (Table 3). Genetic variations within *S. flexuosa* populations (fixation index within populations  $F_{SC} = 0.00583$ ,  $p < 0.001$ ) is higher than that among populations within groups (fixation index among populations within groups  $F_{ST} = 0.01351$ ,  $p < 0.001$ ) and among groups (fixation index between groups  $F_{CT} = 0.00733$ ,  $p < 0.001$ ) (Table 3). AMOVA and fixation indices did not suggest any hierarchical regional structuring among populations within group or among groups.

### Species Divergence and Phylogenetic Relationships

The phylogenetic trees obtained from different methods such as NJ, MP, and ML highlighted the existence of similar tree topologies with high reliability (71-100) (Figure 3, Figure 4) for both mtDNA markers. *Spicara* species in the coastal water surrounding Turkey were subdivided into two main clusters. The first cluster was composed of two main haplogroups, corresponding to *S. flexuosa* and *S. maena*, indicating that they are more closely related

**Table 2.** Main values of mtDNA variability in Turkish *Spicara* populations. Number of samples (N) and haplotypes numbers (H), haplotype diversity ( $Hd$ ), nucleotide diversity ( $\pi$ ) values

Species, Locality, Sea	16S rRNA				cytochrome b		
	N	H	$Hd$	$\pi$	H	$Hd$	$\pi$
<i>S. maena</i>							
Istanbul, Sea of Marmara	15	2	0.13333	0.00026	2	0.1333	0.00012
Canakkale, Sea of Marmara	16	1	0.00000	0.00000	1	0.0000	0.00000
Izmir, Aegean Sea	16	3	0.24167	0.00035	3	0.2416	0.00020
Antalya, Mediterranean Sea	15	1	0.00000	0.00000	2	0.4190	0.00037
Adana, Mediterranean Sea	12	1	0.00000	0.00000	1	0.0000	0.00000
	74	4	0.07997	0.00013	5	0.1791	0.00016
<i>S. smarís</i>							
Izmir, Aegean Sea	22	1	0.00000	0.00000	1	0.0000	0.00000
Antalya, Mediterranean Sea	19	2	0.28070	0.00089	3	0.3684	0.00040
Adana, Mediterranean Sea	17	1	0.00000	0.00000	2	0.1176	0.00021
	58	2	0.09982	0.00020	4	0.1654	0.00020
<i>S. flexuosa</i>							
Rize, Black Sea	20	1	0.00000	0.00000	2	0.2684	0.00024
Sinop, Black Sea	12	1	0.00000	0.00000	1	0.0000	0.00000
Istanbul, Sea of Marmara	18	2	0.20915	0.00020	2	0.2941	0.00026
Canakkale, Sea of Marmara	15	1	0.00000	0.00000	1	0.0000	0.00000
Izmir, Aegean Sea	17	2	0.22059	0.00022	5	0.4246	0.00041
Antalya, Mediterranean Sea	16	3	0.24167	0.00025	2	0.5000	0.00044
Adana, Mediterranean Sea	14	1	0.00000	0.00000	2	0.1428	0.00013
	112	5	0.10440	0.00029	8	0.2768	0.00026



**Figure 2.** Median-joining network for the mtDNA 16S rRNA and cytochrome b haplotypes of *Spicara* spp. from Turkish coastal waters. Mutation differences between haplotypes are indicated in the branches. Circle size is proportionate to the number of individuals carrying the same haplotype.

**Table 3.** Analysis of molecular variance (AMOVA) for cyt b and 16S rRNA sequences of *S. flexuosa* distributed throughout Turkish waters

Genes	Source of variation	d.f.	Variance components	Variance components	Percentage Variation
<i>cytochrome b</i>	Among groups	3	1.873	0.00244 Va	1.38
	Among populations within groups	3	1.576	0.02459 Vb	13.93
	Within populations	105	15.700	0.14952 Vc	84.69
	Total	111	19.149	0.17654	
	Fsc:0.14121, Fst: 0.15306, Fcr: 0.01380				
<i>16S rRNA</i>	Among groups	2	0.174	0.00165 Va	3.77
	Among populations within groups	4	0.135	-0.00059 Vb	-1.35
	Within populations	105	4.494	0.04280 Vc	97.58
	Total	111	4.802	0.04386	
	Fsc: -0.01404, Fst: 0.02415, Fcr: 0.03766				

than *S. smarlis*. Second cluster included only *S. smarlis* haplotypes (Figure 3, 4).

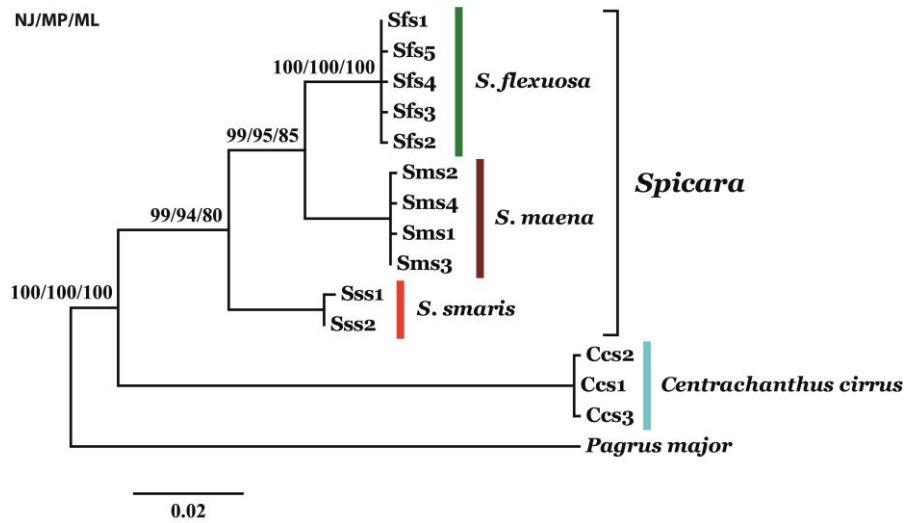
The pairwise sequence divergence among three *Spicara* species ranged from 7.99 to 9.41% for cytochrome *b* gene and 2.90 to 4.44% in 16S rRNA (Table 4). *S. flexuosa* and *S. maena* appear more closely related compared to *S. smarlis*. Assuming that a 2% cytochrome *b* divergence rate per million years (Grant & Bowen, 1998; Bargelloni, Alarcon, Alvarez, Penzo, Magoulas, Reis, & Patarnello, 2003), the differences measured among the samples of *S. flexuosa* from both Mediterranean and Black Sea (0.015%, Table 4) illustrated the precise isolation since the early Holocene (7500 years ago).

**Discussion**

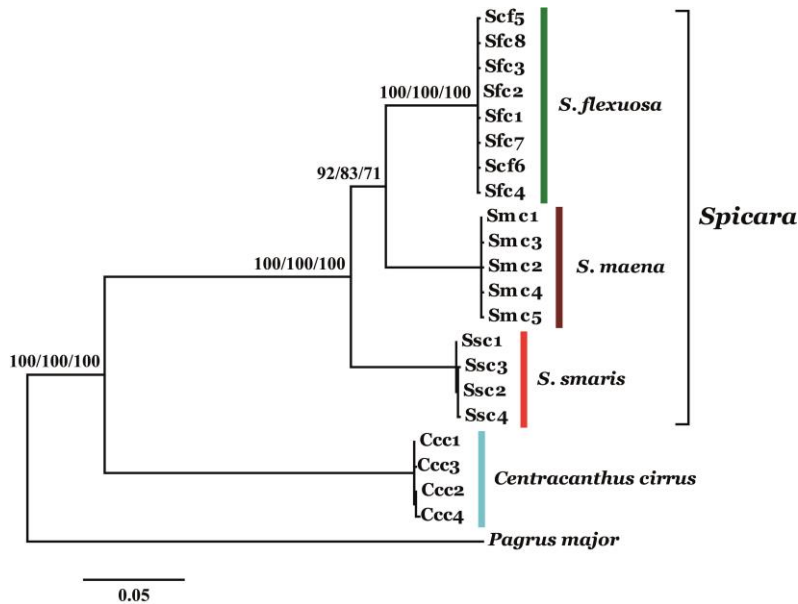
**Geographical Distribution of *Spicara* Species**

The boundaries of current distribution areas of

picarel species through field studies conducted during the present study were estimated. In addition, as in most marine species (Leppakoski, 1991), we speculated that the salinity level of the seawaters determines the limits of the geographical distribution of *Spicara* species. The Black Sea basin is partially isolated and thus differs from the Mediterranean in terms of ecological barriers such as the salinity factor. The salinity level of the Black Sea surface waters (16.5-18.5 psu, Oguz, Özsoy, Latif, Sur, & Ünlüata, 1990; Altıok, Sur, & Yuce, 2012) is quite lower than Mediterranean Sea (36.5-39 psu) (Topper & Meijer, 2015). For this reason, it is speculated that from south to north, the geographical distribution of picarel is limited with Dardanelles strait for *S. smarlis* and Bosphorus strait for *S. maena*. Because *S. flexuosa* are able to tolerate higher salinity levels (%17-39) (Leonart, 2008), compared to both *S. maena* and *S. smarlis*, we have only observed the existence of *S. flexuosa* in present-day Black Sea, which is



**Figure 3.** Maximum likelihood tree of the partial 16S rRNA gene sequences of three picarel species. The neighbor joining, maximum parsimony and maximum likelihood analysis resulted in similar and congruent trees. Bootstrap (only 50 % or higher) values are shown above nodes on trees.



**Figure 4.** Maximum likelihood tree of cytochrome b gene sequences of three picarel species. The neighbor joining, maximum parsimony and maximum likelihood analysis resulted in similar and congruent trees. Bootstrap (only 50 % or higher) values are shown above nodes on trees.

characterized as brackish.

### Species Divergence and Phylogenetic Relationships

The genetic distances (7.99-9.41%, cyt b and 2.90-4.44%, 16S rRNA) among the *Spicara* species in Turkish coastal waters revealed a precise genetical divergence when compared to the findings [%6.21'lik (16S+Cytb) (Orrell & Carpenter, 2004) and 10.5-22% (cyt b) (Junhong, 2007)] previously reported for some other Sparid taxa. In addition, upon the analysis of partial 16S rRNA sequences, a much higher interspecific variation (2.90-4.44%) level was

determined when compared to that of reported (0-0.5%) by Turan (2011), who previously investigated the systematic status of the Mediterranean *Spicara* species with the same marker. On the other hand, although Turan (2011) previously reported no genetic distance among *S. maena* and *S. smaris*, the existence of a 4.44% genetic distance based on the mitochondrial DNA 16S rRNA gene sequences was determined with this study. Similarly, the phylogenetic tree topologies obtained from the mitochondrial cyt b and 16S rRNA gene sequence analyses (Figure 3, 4) also supported a closer genetic relationship between *S. flexuosa* and *S. maena* than to

**Table 4.** Genetic distance (percentage) calculated with the kimura 2 parameter (K2P) model based on mitochondrial sequences of cyt b and 16S rRNA for *Spicara* spp.

Spp	Cytochrome b				16S rRNA			
	<i>Sm</i>	<i>Sf</i>	<i>Ss</i>		<i>Sm</i>	<i>Sf</i>	<i>Ss</i>	
<i>Sm</i>	0.16				0.13			
<i>Sf</i>	7.99	0.21			2.90	0.10		
<i>Ss</i>	9.30	9.41	0.20		4.44	4.35	0.20	
		BS	MS	MEDS		BS	MS	MEDS
<i>Sf</i>	BS	0.011			BS	0.000		
	MS	0.010	0.006		MS	0.006	0.004	
	MEDS	0.015	0.013	0.013	MEDS	0.008	0.007	0.002

Note: The lower diagonal shows the interspecies and interpopulation divergences for *Spicara* spp. and *S. flexuosa*, respectively. The intraspecific and intrapopulation distances shaded.

*S. smaridis*, confirming the results reported by previous genetic (Georgiadis et al., 2014) and morphological studies (Minos et al., 2013) on three different *Spicara* species. It is thus hypothesized that although being supplied from almost the same locations in Turkey's coastal waters, the current discrepancies related to the reports on the interspecific relationships in *Spicara* genus is most likely due to the inaccurate morphological descriptions from previous studies.

#### Population Structure of *S. flexuosa*

The potential distribution of planktonic larvae and eggs, and high gene flow mostly caused by the lack of geographical barriers among populations (McCusker & Bentzen, 2010) contributes to low genetic differentiation in marine fish (Wang, Meng, Liu, Zhang, & Lin, 2011). Contrary to these phenomena, there are three events favorable to the genetic differentiation for *Spicara* populations.

First, the picarel have a relatively low distribution potential in the early and adult stages of life cycles. Second is the presence of geographical barriers among the Turkish picarel populations (Mediterranean-Marmara-Black Sea) such as the Dardanelles and the Istanbul Straits. The distribution potential of picarel is low due to the presence of demersal eggs and the semi-pelagic forms of adults (Francour, Pollard, Bizsel, Kara, Yokes, & Goren, 2011). Thirdly, picarel individuals are protogynous hermaphrodite (Zei, 1941, 1949; Tortonese, 1975). For this reason, this reproduction pattern may be casual reason of the low level diversity of mtDNA (Richardson & Gold, 1997) in *S. flexuosa*, which are distributed throughout the coastal waters of Turkey.

Therefore, contrary to what is observed in some other marine fish such as anchovy (Grant, 2005) and sprat (Debes, Zachos, & Hanel, 2008), it is expected that there will be a remarkable genetic differentiation among the populations because picarel tend to live as dependent on a particular region in early and adult stages of life. The expectations related to the low genetic similarity is more understandable for the Aegean and Mediterranean populations in the absence

of significant geographical barriers. On the other hand, high proportion of genetic differentiation is expected for the Mediterranean, Aegean, Marmara and Black Sea populations, which are currently dealing with the both geographical (Bosphorus and Dardanelles straits) and some ecological obstacles (temperature and salinity differences). Despite these biotic and abiotic barriers that cause population differentiation through low gene flow, it is difficult to explain the low genetic differences [0.010-0.015% for cyt b; 0.006-0.008% for 16S rRNA (Table 4)] among groups (especially between the Mediterranean and the Black Sea, which are separated by a geographical barriers) of *Spicara* species by the ongoing gene flow. Moreover, as similar to the previously reported findings for anchovy (Grant, 2005) and horse mackerel (Bektas & Belduz, 2008) in the Mediterranean Sea and Black Sea, the low haplotype and nucleotide diversity (Table 2) and star-like network topologies (Figure 2) identified for *Spicara* species may be the main indicator of a founder effect associated with demographic bottleneck in Mediterranean basin during the late Pleistocene. AMOVA results showed that there was no significant difference in hierarchical levels among groups (Mediterranean Sea, Sea of Marmara and the Black Sea) (Table 3), separated by geographical obstacles such as Dardanelles and Bosphorus straits. In particular, the identified this genetic uniformity among the *S. flexuosa* populations distributing throughout the Turkish coastal waters point out to the phylogeographic history of the *Spicara* genus in the region unlike the ongoing gene flow.

#### Phylogeographic History of *S. flexuosa* in Turkish Coastal Waters

The geographical and climatic factors occurred during the Pleistocene glaciers (1.8 mya-11.5 kya) are the main factors responsible for the present genetic structure of existing marine life (Hewitt, 2000). Phylogenetic studies on aquatic organisms provide information on biotic responses to palaeohydrological dynamics associated with climatic oscillations (Faria,

Weiss, & Alexandrino, 2012). The Mediterranean ichthyofauna is characterized by the formation of habitats that are unsuitable for many marine organisms, developed during the Pleistocene glacial events (1,8 mya-present), such as sea-level fluctuations and the Messinian salinity crisis (Wilson, 2006). In this sense, changes in the Mediterranean Sea level related to the Pleistocene glaciation resulting from global climate changes have been more effective on the hydrological budgets of semi-enclosed or marginal sea basins such as the Black Sea (Ryan, Major, Lericolais, & Goldstein, 2003; Lericolais, Bulois, Gillet, & Guichard, 2009).

To support this hypothesis, the dropping of the global sea level during the last glacial period of Pleistocene (25-11.7 kya, Würm period), has led to the breakthrough of saline flow from the Mediterranean into the Black Sea through the straits (Popescu, Lericolais, Panin, Normand, Dinu, & Le Drezen, 2004; Lambeck & Purcell 2005, 2007; Lericolais, Bulois, & Gillet, 2006; Panin & Popescu, 2007). Afterwards, the ancient Black Sea (new euxinian) has become a low saline lake (salinity <5 psu) isolated from the Mediterranean Sea due to the contribution of large rivers for thousands of years (Neveeskaya, 1963, 1965; Grigor'ev, Isagulova, & Fedorov, 1984; Mudie, Rochon, Aksu, & Gillespie, 2002; Lericolais, Popescu, Guichard, Popescu, & Manolukakis, 2007). These new environmental conditions, which destroyed the halophile Mediterranean fauna in the Black Sea, were probably not suitable for *S. flexuosa* that are eligible to tolerate to high salinity (17-39%) (Leonart, 2008), similar to other marine life forms such as horse mackerel (*Trachurus* spp., Bektas & Belduz, 2008) and anchovy (*E. engrasicolus*, Grant, 2005) except for several estuarine forms such as Pipefish (Wilson & Veragut, 2010) and sprat (Debes et al., 2008).

After the Würm glacial period (between 7-5.3 kya), the significant rise in Mediterranean Sea level caused by the interchange of marine waters between Atlantic and Mediterranean Sea led to the establishment of marine connection between the Black Sea and the Mediterranean (Ryan et al., 2003; Ryan & Pitman, 2000; Lericolais et al., 2007). The Neouxinian lake (present-day Black Sea) has thus been colonized by the Mediterranean immigrants due to the gradual increase in salinity (Ryan et al., 1997; Ballard, Coleman, & Rosenberg, 2000; Reid & Orlova, 2002; Cristescu, Hebert, & Onciu, 2003; Boissin et al., 2016) following this reconnection of the Black Sea to Mediterranean Sea. The separation time calculated based on the genetic distances between the Mediterranean and Black Sea populations of *S. flexuosa*, coincided with the latest gradual recolonization process of the Black Sea by marine organisms that took place since about 7 kya (Ryan et al., 1997). For the last 10,000 years, marine organisms have invaded the Black Sea, and it is clear that this immigration still continues. Moreover, a slight

increase in the salinity of the Black Sea has been observed in recent years, resulting in the invasion of several marine species, a process known as "mediterraneanization" (Gomoiu, 1981; Cvetkov & Marinov, 1986) of the Black Sea fauna.

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