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Molecular characterization and phylogeography of Mediterranean picarels (*Spicara flexuosa*, *S. maena* and *S. smaris*) along the coasts of Turkey and the Eastern Mediterranean

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ABSTRACT

In this study, molecular taxonomy associated with *Spicara flexuosa*, *Spicara maena*, and *Spicara smaris* was examined with a geographical focus along the coasts of the Turkey and the Mediterranean. In addition, the effects of the Turkish Straits System on the evolutionary history and phylogeography of *Spicara flexuosa* were investigated. The mitochondrial cytochrome c oxidase I, cytochrome b, and the nuclear Interphotoreceptor retinoid-binding protein genes were used for these purposes. Our results indicated that the distinction of the three taxa under *Spicara* is possible with the two different mitochondrial DNA markers. Nuclear DNA analyses indicated that reproductive isolation of the *S. maena* and *S. flexuosa* was complete. Demographic analyses support the sudden population growth of *S. flexuosa* after a potential bottleneck. The result of the dating analyses suggested that the three *Spicara* species were differentiated during the early or middle Pliocene. An absence of genetic structure between *Spicara flexuosa* subpopulations from Turkey indicated the connectivity, within sampling locations, suggesting that the Turkish Straits System is a corridor for gene flow for this species. As picarel populations have experienced rapid decline in their distribution areas in recent years, separate population studies are necessary to help make informed conservation and management decisions for *S. maena* and *S. flexuosa*.

Key words: Eastern Mediterranean; molecular taxonomy; phylogeography; sister species; Turkish Straits Systems.

1. INTRODUCTION

Accurate species identification is the main step in monitoring biodiversity, which is critical for taxonomic identification and conservation efforts (Tillett et al., 2012). In the marine environment, fish species identification based on only external morphological characters can be hard due to morphological similarities among sister species (Chiba et al., 2009; Teletchea, 2009). Such identification problems may have negative impact on fisheries management (Vecchione et al., 2000) and important implications for the conservation of exploited populations (Garcia-Vazquez et al., 2012). In this regard, molecular data can be useful in resolving phylogenetic and taxonomic uncertainties between morphologically similar sister fish species (Chiba et al., 2009; Li et al., 2020), and help to alleviate the aforementioned problems.

One of the unresolved questions in fish phylogeny and taxonomy concerns the family Sparidae, consisting of 39 genera, 166 valid species and 260 synonyms (Nelson et al., 2016; Parenti, 2019; Fricke et al., 2020) including the genera, *Spicara* and *Centracanthus*. Orrell et al. (2002) and Orrell and Carpenter (2004) showed that currently defined sub-families of Sparidae were not monophyletic and *Spicara* was a member of Sparidae. In addition, the monophyly of the family Sparidae is not supported (Chiba et al., 2009), primarily because of the uncertainty of the position of its sister family Centracanthidae, which includes the genera *Spicara* and *Centracanthus*. The disagreement in the taxonomy is also reflected in the online marine fish databases as well; according to the World Register of Marine Species (WoRMS), the Integrated Taxonomic Information System (ITIS), and the Marine Species Identification Portal, *Spicara* spp. are currently placed under the Centracanthidae. However, FishBase,

49 Catalog of Fishes (Fricke et al., 2020) and Encyclopedia of Life (EOL) (<https://eol.org>)
 50 place the genus *Spicara* within the Sparidae.

51 The taxonomic uncertainty within the Sparidae family is largely influenced by
 52 their biogeographical and evolutionary history. Previous biogeographical analyses
 53 among the Sparidae family members showed that the common ancestor of Sparid clades
 54 has undergone adaptive radiation in order to take advantage of various environmental
 55 conditions in the Central Tethys Sea during the Early Paleocene (Orrell and Carpenter,
 56 2004; Chiba et al., 2009). At that time, diversification of the clades occurred
 57 simultaneously, and as a result many species evolved and were distributed worldwide
 58 in a short time period, as observed for other fish species, such as some coral reef fish
 59 (Rocha et al., 2007; 2008) and cichlids (Sugawara et al., 2002). This feature is reflected
 60 in the northeastern Atlantic and the Mediterranean distribution of the Sparidae family
 61 members, as 24 fish species of this family were reported along these coasts (Bauchot
 62 and Hureau, 1986). Adaptive radiation and evolutionary plasticity of many
 63 morphological characters could be the main underlying reason behind the
 64 morphological similarities and difficulties in the identification of Sparid species (Chiba
 65 et al., 2009).

66 Difficulties in morphological discrimination also exist within the genus *Spicara*,
 67 represented by *Spicara smaris* L. 1758, *Spicara maena* L. 1758, and *Spicara flexuosa*
 68 (Rafinesque 1810) in the Eastern Atlantic, the Mediterranean, and the Black Sea.
 69 Certain species-specific characteristics within the *Spicara* genus, such as protogynous
 70 hermaphroditism (Salekhova, 1979; Karakulak et al., 2006; Saygılı et al., 2016) and
 71 secondary sex characteristics (e.g. size, body color and marking) (Pollard and Pichot,
 72 1971; Arculeo et al., 1996), contribute to their confused taxonomy. Consequently, some
 73 studies (Quero et al., 2003; Eschmeyer, 2010; Froese and Pauly, 2020) suggest that *S.*

maena and *S. flexuosa* should be considered as synonyms whereas others (Tortonose, 1986; Fischer et al., 1987; Costa, 1991; Louisy, 2002; Golani et al., 2006; Vasileva, 2007; Bilecenoğlu et al., 2014) support the distinction of the two species. More recent studies based on both genetic (Chiba et al., 2009; Imsiridou et al., 2011; Georgiadis et al., 2014; Bektaş et al., 2018) and morphological data (Minos et al., 2013) also support the latter conclusion. The discrepancy is reflected in various fish-related taxonomic databases as well. The Catalog of Fishes (Fricke et al., 2020), and ITIS (<http://www.itis.gov>) accept *S. flexuosa* as a valid species, while FishBase (Froese and Pauly, 2020), WoRMS, EOL and the Marine Species Identification Portal consider *S. flexuosa* as an unaccepted/unresolved and synonym of *S. maena*.

Regarding the biogeographical distribution of the three *Spicara* species, *Spicara maena* is of Atlanto-Mediterranean origin and inhabits temperate to warm waters, and is largely distributed in the Mediterranean, including the Adriatic Sea. It is also found from Portugal to Morocco in the Atlantic, and with a limited distribution in the Black Sea (Froese and Pauly, 2017). *Spicara flexuosa* is observed in the eastern Atlantic, Mediterranean, and the Black Sea (Salekhova, 1979; Tortonose, 1986; Golani et al., 2006). Finally, *Spicara smaris* is recorded in the Mediterranean, the Eastern Atlantic coasts, and with a restricted distribution in the Black Sea (Tortonose, 1986).

Taxonomic descriptions based on the main morphological characters of the three *Spicara* species are given below.

Family: Centracanthidae Gill, 1893

Genus: *Spicara* Rafinesque, 1810

Spicara flexuosa (Rafinesque, 1810)

Common name: Picarel.

Diagnosis: Protrusible mouth, not more than 73 lateral line scales; head length equals or greater than body depth. Color: Clear yellowish or grey-brownish. There is a brownish spot at the base of the pectoral fin. Color varies with age, sex and season. Habitat: On sand or muddy bottoms, down to about 130 m. Demersal schooling fish, eggs and larvae are planktonic. Size max: 20 cm. (Tortonese, 1986; Golani et al., 2006).

Spicara maena (Linnaeus, 1758)

Common name: Blotched picarel.

Diagnosis: Protractile premaxilla; head length shorter than body depth, Color: Bluish grey above, sides silvery and there is also brownish dark spot at the base of the pectoral fin which is larger than *S. flexuosa*. Color varies with age, sex and season. Habitat: Posidonia beds, rocks and mud down to about 100 m. Demersal spawner species. Size max: 25 cm. (Tortonese, 1986; Golani et al., 2006).

Spicara smaris (Linnaeus, 1758)

Common name: Picarel, Zerrow.

Diagnosis: Protractile premaxilla. Head length equals and longer than body depth, 75 or more lateral line scales; black spot on the body side above pectoral fin. Color: Grey above, silvery below. Color varies with age, sex and season. Habitat: Posidonia beds and muddy bottoms at about 15-100 m. Size max: 15 cm. (Tortonese, 1986; Golani et al., 2006).

The main aim of this study was to use molecular tools based on two mitochondrial genes, cytochrome c oxidase I (COI) and cytochrome *b* (cyt-*b*), and one nuclear gene, the Interphotoreceptor retinoid-binding protein (IRBP), in order to elucidate the taxonomic and phylogenetic status of *Spicara flexuosa*, *S. maena*, and *S. smaris* along the coasts of Turkey and the Eastern Mediterranean. Furthermore, the possible effects of the Turkish Straits System (TSS) on the evolutionary history and

phylogeography of *Spicara* spp. were investigated. The TSS forms a transition zone between the Mediterranean and the Black Sea and serves as a biogeographic boundary/corridor that controls fauna and flora characteristics of the region (Öztürk and Öztürk, 1996; Magoulas et al., 1996; 2006). Finally, the results of this study were also used to determine the relative presence of *S. maena* or *S. flexuosa* along the Turkish coastal waters, as their identification based on morphology is contentious.

2. MATERIAL AND METHODS

2.1. Sample collection

We sampled 279 specimens of *Spicara flexuosa* and *Spicara maena* representing a broad geographic coverage of their distribution along the Turkish coast line and Greece (Figure 1, Supporting Information, Table S1). Individuals were collected from ten stations by fishermen using trawlers, hand line fishing and purse seine nets between 2013-2015; two from the Black Sea (Sinop, Rize, Turkey), three from the TSS (Istanbul, Armutlu, Canakkale, Turkey), two from the Central/South Aegean Sea (Izmir, Mugla, Turkey), one from the North Aegean Sea, (Kavala, Greece), and two from the Levantine Sea (Mersin, Antalya, Turkey). As there are no explicit field guides for the identification or discrimination of *S. maena* and *S. flexuosa*, information on differences based on the diagnostic morphological characters of the two species (Tortonose, 1986; Lythgoe and Lythgoe, 1992; Golani et al., 2006), along with the experience of the field collector were used to sample and identify 30 specimens of *S. maena* from Greece. These morphological differences included *S. maena* having: i) rather deep body than *S. flexuosa*, ii) head length being less than its body depth (*vice versa* for *S. flexuosa*), iii) head elevated posteriorly and profile concave above the head. A black mark which is larger than the one found in *S. flexuosa*, occurs on the side of

the pectoral fin (Figures: 2a-d). In addition, the information that *S. maena* is encountered more often over *Posidonia oceanica* beds in the Aegean Sea, was used when sampling for the species with purse seine nets. The Turkish samples were initially collected as *S. flexuosa/maena*, without initial taxonomic assignment at the species level. All specimens (from Turkey and Greece) were genetically analyzed, and their species identities were determined (see subsequent analyses). GenBank entries were also included in the dataset (Figure 1, Supporting Information, Tables S1, 2, and 3). The collected tissue was preserved in 80% ethanol at room temperature and at -20 °C in the laboratory, until further processing.

2.2. DNA extraction and amplification

Total genomic DNA was isolated from the muscle or caudal fin tissue with a PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, USA), following the manufacturer's protocols and stored at -20°C. Two mitochondrial (COI, cyt-b) and one nuclear (IRBP) genes were amplified (for primers and PCR conditions used see Supporting Information, Table S4, Supporting Information, Appendix SI). All amplified products were purified and sequenced commercially (Macrogen Europe, Amsterdam, The Netherlands). The nuclear DNA products were sequenced bidirectionally to verify the presence/absence of heterozygous sites. New *Spicara flexuosa* and *S. maena* sequences have been deposited to GenBank under the following accession numbers: XX111111-XX111111 for COI, YY222222-YY222222 for cyt-b, and ZZ333333-ZZ333333 for IRBP genes, respectively.

2.3. Statistical analyses

2.3.1. Summary statistics

All chromatograms were edited and aligned manually with Sequencher v.5.4.1 (GeneCodes Corp.). Potential amplification/sequencing errors were controlled by

aligning all sequences to detect polymorphic sites and subsequently confirming the clarity of the peaks at these sites. After trimming all ambiguous bases on both sides, 454, 355 and 385 bp fragments were used for further analyses of COI, cyt-b, and IRBP genes, respectively. Stop codons were investigated for mitochondrial (mtDNA) genes to determine presence/absence of mitochondrial pseudogenes by using DAMBEv.6.4.1 (Xia, 2017). In the nuclear DNA data analysis, phased haplotypes were estimated for IRBP gene using Bayesian methods in PHASE (Stephens and Donnelly, 2003). Recombination was examined using a PHI Test (Bruen et al., 2006) in SPLITSTREE 4 (Huson and Bryant, 2006). No significant signals of recombination were detected, and the full non-recombining sequences were used in the subsequent analyses.

For species assignments of each individual and gene, two approaches were applied: i) sequence similarity-based identification, and ii) phylogenetic network based identification. First, a similarity test was used between our sequences and the reference sequences in the genetic databases in BOLD and GenBank using similarity scores in BLASTn (Altschul et al., 1990) option in NCBI web-based site (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=tblastn). Second, a phylogenetic network construction was used to assign unidentified sequences to species based on clustering to estimate the relationship between the reference sequences and the query sequence.

To describe genetic diversity of all samples, number of haplotypes, haplotype (h) (Nei and Tajima, 1981) and nucleotide (π) (Nei, 1987) diversities, number of polymorphic, singleton and parsimony informative sites, total number of mutations, and the average number of net nucleotide substitutions (percentage of pairwise sequence

divergence) between species were calculated in DnaSP v.5.10.1 (Librado and Rozas, 2009).

2.3.2. Haplotype networks and phylogenetic analyses

Genealogical relationships among haplotypes of *Spicara* specimens were constructed per gene using the median joining method (Bandelt et al., 1999) in PopART v.1.7 (Leigh and Bryant, 2015).

Phylogenetic relationships between haplotypes were determined by neighbour-joining, maximum likelihood, and maximum parsimony methods using the software MEGA v.7.0 (Kumar et al., 2016). Nucleotide substitution models (COI: GTR; cyt-b: GTR+I; IRBP: GTR+G+I) to be used were determined using MODELTEST v.3.7 (Posada and Crandall, 1998), with the AIC (Akaike, 1974) and the Akaike's information criterion (AIC), (Hurvich and Tsai, 1989) and Bayesian Information Criterion (BIC) (Schwarz, 1978) statistics. The General Time Reversible model (GTR) that best fitted the data, was used for building the maximum likelihood trees, for the different species and gene combinations, as it is a biologically realistic model for protein coding sequences (Shapiro et al., 2006). The robustness of the trees was tested with 1,000 replicates. Neighbour-joining trees (maximum composite likelihood distances) (Tamura et al., 2004) were constructed in MEGA with 1,000 bootstrap replicates. Phylogenetic relationships were also determined with Bayesian analysis using BEAST v.2.4.8. (Bouckaert et al., 2014) under the same models above. Two independent runs of Markov Chain Monte Carlo (MCMC) for 1×10^8 generations with sampling every 1,000 generations were performed, whereas convergence was confirmed in Tracer v.1.6 (Rambaut et al., 2014). *Spondylisoma cantharus* sequences (GenBank Accession numbers JQ624002, EU036508 and EU638155 for COI, cyt-b and IRBP genes, respectively) were included in all phylogenetic analyses as an

outgroup. Due to the lack of publicly available *Spicara smaris* IRBP sequences, the phylogenetic relationships between haplotypes were determined: i) for *Spicara flexuosa*, *S. maena* and *S. smaris* using the concatenated COI and cyt-b dataset, ii) for all three *Spicara* species using the COI and cyt-b genes individually, and iii) only for *S. flexuosa* and *S. maena* for the IRBP gene.

2.3.3. Divergence times estimates

The evolutionary divergence time of the *Spicara maena*, *S. flexuosa* and *S. smaris* were estimated in BEAST using the concatenated mtDNA dataset. Sequence data from *Spondylisoma cantharus* (GenBank Accession numbers were provided in 2.3.2) and *Centracanthus cirrus* (GenBank Accession numbers, KY176422 and MF149916) for COI and cyt-b genes, respectively, were used as outgroups. No calibration was used due to the lack of knowledge of published fossil date results. The Yule speciation process, strict clock and a molecular clock rate of 0.6% *per* million years for the COI gene for marine teleosts (Bermingham et al., 1997), and 2% *per* million years for the cyt-b gene were used based on other Sparids (Bargelloni et al., 2003). The analysis was executed for 2.5×10^8 generations, sampling every 5,000 generations, and discarding the initial 10% as burn-in. Convergence was confirmed in TRACER v.1.6, and the effective sample sizes (ESS) for all model parameters were assessed, by confirming that the ESS values were higher than 200 that indicate adequate sampling intensity for all parameters.

2.4. Genetic structure of *Spicara flexuosa*

2.4.1. AMOVA, Φ_{st} and F_{st}

A hierarchical AMOVA (Analysis of Molecular Variance) was performed in Arlequin v.3.5.2.2 (Excoffier and Lischer, 2010) to test for significance between groups of

sample collections based on the concatenated mtDNA and nuclear (nuDNA) markers. Individuals were grouped according to geographical location: i) TSS (Istanbul, Canakkale, Armutlu), ii) the Aegean Sea (Izmir, Mugla), iii) the Black Sea (Rize, Sinop), and iv) the Levantine (Antalya, Mersin). Estimations of the pairwise Φ_{ST} and F_{ST} for the concatenated mtDNA and nuDNA data sets, respectively, between sampling locations were conducted in Arlequin (with 10,000 permutations). Bonferroni corrections (Rice, 1989) were also used to adjust for the significance of the P values for multiple tests.

2.4.2. Demographic history analyses

Mismatch distribution of the number of pairwise differences (Slatkin and Hudson, 1991; Rogers and Harpending, 1992) and the Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) neutrality tests per species were estimated with DnaSP. Past population demography of *S. flexuosa* and *S. maena* was also reconstructed using Extended Bayesian Skyline Plots (EBSP) (Heled and Drummond, 2008; Ho and Shapiro, 2011), as implemented in BEAST using the multi-locus mtDNA dataset (COI, cyt-b). The BEAST input file was produced in BEAUTi v.2.4.8 (Bouckaert et al., 2014). The best model of nucleotide substitution was selected for each locus and time was scaled based on the molecular clock rates (COI and cyt-b rates were provided in 2.3.3). A strict molecular clock was set as prior and the Bayesian MCMC was run for 1.5×10^8 generations for both *S. flexuosa* and for *S. maena*, discarding the initial 10% of samples as burn-in. Convergence of runs was evaluated on TRACER. Visualization of the EBSP plots was made with the RStudio v.1.1.463 using R v.3.5.1 (R Development Core Team, 2018).

3. RESULTS

3.1. Summary statistics

The COI gene was successfully amplified from 198 out of 279 *Spicara* individuals (175 from Turkey, 23 from Greece). The sequences of the remaining 81 individuals were either of low quality, or failed to amplify. Moreover, 57 *Spicara* specimens (one *S. flexuosa*; 24 *S. maena*, and 32 sequences of *S. smaris*) were retrieved from GenBank and the Barcode of Life Database (BOLD) (Supporting Information, Table S2) and used for further analyses. All COI sequences were perfectly matched to the reference libraries [(including Cannas et al., retrieved from GenBank, 2010, GenBank accession numbers, HM038500-HM038506)] ranging from 99.78% to 100%, based on the BLASTn tool. A 454 bp fragment was used for all analyses, and a total of 18 haplotypes were found within 255 sequences.

Overall, 175 samples were identified as *Spicara flexuosa* and 23 as *Spicara maena* from the COI haplotype network (Figure 3a, for more details species identification see previous sections 2.1. and 2.3.1., and the following section 3.1.1.). Additionally, *S. flexuosa* had the lowest mean haplotype and nucleotide diversities ($h=0.0340$, $\pi=0.0001$). Conversely, the mean haplotype and nucleotide diversities of *S. maena* were higher than those reported for the *S. flexuosa* ($h=0.6050$, $\pi=0.0015$, Supporting Information, Table S5). Among the *S. flexuosa* sampling locations, the Black Sea exhibited the highest haplotype ($h=0.0650$) and nucleotide diversities values ($\pi=0.0001$) (Supporting Information, Table S5). Moreover, a total of 72 (15.8%) nucleotide sites were variable, of which 61 positions (13.4%) were parsimony informative (Supporting Information, Table S6; 27 for the *S. maena* and *S. flexuosa* group; 18 for the *S. flexuosa* and *S. smaris* group; 16 for the *S. maena* and *S. smaris* group). The number of mutations between the three *Spicara* species ranged from 43

(between *S. flexuosa* and *S. maena*) to 56 (between *S. maena* and *S. smarís*) and pairwise sequence divergences ranged from 8.11% (between *S. flexuosa* and *S. maena*) to 9.31% (between *S. maena* and *S. smarís*) (Supporting Information, Table S7).

The cyt-b gene was amplified from 157 individuals of *Spicara* spp. (139 from Turkey, 18 from Greece). The remaining 122 individuals had either low quality sequences or were not amplified, and thus were eliminated. After trimming of both ends the ambiguous sequences, a 355 bp fragment was used for subsequent analyses. Moreover, five *S. maena*, two *S. flexuosa* and four *S. smarís* sequences were added from GenBank (Supporting Information, Table S3). All cyt-b sequences were matched to the reference libraries [(including FishTrace, 2007, GenBank accession numbers (EU036502-EU036507)], with similarities ranging from 99.72% to 100%, based on the BLASTn tool. A total of 10 haplotypes were found among 168 individuals.

Among all samples, 139 fish were identified as *S. flexuosa* and 18 as *S. maena* based on the cyt-b haplotype network (Figure 3b, for species identification used to see aforementioned information). *Spicara flexuosa* exhibited the highest haplotype and nucleotide diversities ($h=0.0710$, $\pi=0.0002$). Specifically, Mersin ($h=0.3890$; $\pi=0.0011$) and Izmir ($h=0.2860$; $\pi=0.0008$) had the highest haplotype and nucleotide diversities. Region-wise, the Levantine showed the highest haplotype and nucleotide diversities ($h=0.1910$; $\pi=0.0005$). (Supporting Information, Table S8). On the other hand, *Spicara maena* showed the lowest haplotype and nucleotide diversities ($h=0$, $\pi=0$) (Supporting Information, Table S8). A total of 51 (14.3%) nucleotide sites were variable, of which 48 (13.5%) positions were parsimony informative. A total of 17 positions were parsimony informative for *S. flexuosa* and *S. maena*, 14 for *S. smarís* and *S. flexuosa*, and 17 for *S. maena* and *S. smarís* (Supporting Information, Table S9). The total number of mutations ranged from 35 (between *S. flexuosa* and *S. maena*) to

37 (between *S. flexuosa* and *S. smarís*), and the pairwise sequence divergences ranged from 9.01% (between *S. flexuosa* and *S. maena*) to 9.35% (between *S. maena* and *S. smarís*) (Supporting Information, Table S7). Total number of mutations and pairwise sequence divergence indicated similar levels of differentiation among the three *Spicara* species. In addition, stop codons were not observed for the COI and cyt-b genes.

Finally, a 380 bp fragment of the IRBP gene from 195 individuals of *Spicara* spp. (174 from Turkey, 21 from Greece) was used for further analyses. The target gene from the remaining 84 individuals either failed to amplify or resulted in low quality sequences, thus eliminated from further analyses. After the addition of one *S. maena* sample from France (Lautredou et al., 2013), (JX627893), a total of 38 haplotypes were detected (Figure 3c). *Spicara flexuosa* (n=174) had higher mean haplotype and nucleotide diversities ($h=0.5030$; $\pi=0.0016$) around the Turkish coastal waters than *S. maena* (n= 21) from Greece (North Aegean Sea) ($h=0.1380$; $\pi=0.0003$). Amongst the Turkish samples, the Black Sea sampling localities had the highest haplotype and nucleotide diversities ($h=0.6550$; $\pi=0.0023$), with Sinop showing the highest values ($h=0.7150$; $\pi=0.0026$) (Supporting Information, Table S10). Singleton variable and parsimony informative sites of *S. flexuosa* were also higher than those of *S. maena*. A total of 17 nucleotide sites were variable, of which eight positions (2.1%) were parsimony informative. Unlike mtDNA, only one position (243) was parsimony informative for identifying *S. maena* and *S. flexuosa* (Supporting Information, Table S11). The total number of mutations and pairwise sequence divergence between *S. flexuosa* and *S. maena* were 26 and 0.0036, respectively (Supporting Information, Table S7).

3.1.1. Haplotype networks and phylogenetic analyses

Three haplogroups, corresponding to *S. flexuosa*, *S. maena* and *S. smarís* were observed in the COI haplotype network (Figure 3a). Samples from Turkey shared the same haplotype (Hap 9), and were grouped with *S. flexuosa* from Sardinia (Cannas et al., retrieved from GenBank, 2010; GenBank and BOLD numbers are found in Supporting Information Table S2), whereas samples from Greece (Hap 10 and Hap 11), clustered with *S. maena* samples from Sardinia (Cannas et al., retrieved from GenBank, 2010) and Malta (KJ709919). In addition, 32 *S. smarís* samples retrieved from GenBank, (GenBank numbers are found in Supporting Information Table S2) (Hap 1, Hap 2, Hap 3, Hap 4, Hap 5, Hap 6, Hap 7 and Hap 8) were found in the *S. smarís* haplogroup. BLASTn results and our haplotype network descriptions showed that some of the sequences deposited in GenBank and BOLD originated from *Spicara* genus, were probably misidentified. The results indicated that one sample from Italy, 13 from Israel, and six from Portugal (reported as *S. maena* in GenBank) (Hap 9, Hap 17), (their GenBank and BOLD numbers were displayed on the Supporting Information Table S2) should have been identified as *S. flexuosa*.

Similarly, three *Spicara* haplogroups were identified on the *cyt-b* gene haplotype network, according to the reference sequences (EU036502-EU036507) from the N. Aegean Sea collected for the FishTrace project (FishTrace, 2007; Zanzi and Martinsohn, 2017). Samples collected from the Turkish coastal waters (Hap 1) were clustered with *S. flexuosa* from Greece (GenBank, EU036502, EU036503) whereas samples collected as *S. maena* from Greece (Hap 6) were clustered with reference sequences of *S. maena* from Greece (GenBank, EU036504, EU036505) (Figure 3b). Moreover, two *S. flexuosa* samples from Italy (previously identified as *S. maena* on GenBank, AF240737; KT883671), (Hap 1) and 139 samples from Turkey were found in the *S. flexuosa* haplogroup. One sample from Spain, previously classified as *S. maena*

(GenBank, EF439598) (Hap 8) was placed in the *S. maena* haplogroup. Finally, four *S. smarís* samples from Greece (EU036506; EU036507), Italy (KT883672) and Spain (EF439600) (Hap 3, Hap 4, and Hap 5) retrieved from GenBank, were in the *S. smarís* haplogroup. BLASTn analyses corroborated our haplotype networks results.

Additionally, 35 haplotypes were determined as *S. flexuosa* and three as *S. maena* in the IRBP haplotype network. All Greek samples (Hap 36-38) were grouped with previously identified *S. maena* specimens from GenBank (France; Lautredou et al., 2013). In contrast to the mtDNA haplotype networks, which exhibited high number of mutations between *S. maena* and *S. flexuosa* (35 for COI and 30 for cyt-b), single base changes were seen between the same species (Figure 3c) on the IRBP haplotype network.

The concatenated COI (454 bp) and cyt-b (355 bp) data consisted of an alignment of 809 bases and a total of 10 haplotypes were obtained from 119 *Spicara* specimens (104 from Turkey and 15 from Greece), and 10 sampling locations. The phylogenetic relationships revealed that *S. flexuosa*, *S. maena*, and *S. smarís* haplogroups were clearly differentiated on the concatenated mtDNA (COI and cyt-b genes) topologies (Figure 4, Supporting Information, Figures S1a-c), whilst they exhibited moderate to high bootstrap (≥ 70) and posterior probabilities (≥ 0.70) values. Additionally, *S. flexuosa* and *S. smarís* were closely related when compared to *S. maena*. The same patterns were also observed for trees constructed using individual COI gene (Supporting Information, Figures S2a, 3a, 4a, and 5a). On the other hand, *S. flexuosa* and *S. maena* clades were reciprocally monophyletic with *S. smarís* being basal in all individual cyt-b trees constructed using various phylogenetic approaches (Supporting Information, Figures S2b, 3b, 4b, and 5b). *Spicara flexuosa* and *S. maena*

were also generally reciprocally monophyletic in the IRBP trees (Supporting Information, Figures S6a-d).

3.1.2. Divergence Times Estimates

The BEAST chronogram indicated a split between *Centracanthus cirrus* and *Spondyllosoma cantharus* around 9.85 Mya (CI: 8.99-10.76). The estimated divergence time between *Spondyllosoma cantharus* and the three *Spicara* species was estimated at 6.05 Mya (CI: 5.30-6.79) (Figure 5). Within the three *Spicara* species, *S. maena* was separated from *S. flexuosa* and *S. smaris* approximately 4.30 Mya (CI: 3.50-5.08), and the split between *S. smaris* and *S. flexuosa* was estimated approximately 3.58 Mya (CI: 2.82-4.35).

3.2. Genetic structure of *Spicara flexuosa*

3.2.1 AMOVA, Φ_{ST} and F_{ST}

Pairwise Φ_{ST} values for the concatenated mtDNA dataset of *Spicara flexuosa* ranged from -0.19 to 0.01. Lack of genetic structure was observed among the Turkish sampling areas (Table 1). AMOVA results also indicated that variation was the highest within sampling locations (102.14%, Φ_{ST} = -0.0213), and the lowest values were observed among locations within groups (-4.74%, Φ_{SC} = -0.0486) and among the four different groups (the TSS, the Black Sea, the Aegean Sea, and the Levantine, 2.60%, Φ_{CT} = 0.0260) (Table 2).

Pairwise F_{ST} values ranged from 0.00 (Rize-Canakkale; Sinop-Canakkale; Sinop-Izmir and Sinop-Rize) to 0.12 (Istanbul-Armutlu; Armutlu-Mugla). Significant P values were observed between Armutlu and four other locations: Istanbul (F_{ST} = 0.12), Mugla (F_{ST} = 0.12), Mersin (F_{ST} = 0.11) and Antalya (F_{ST} = 0.07). Significant values

were also observed between Rize and Mugla ($F_{ST}=0.06$) and Antalya and Istanbul ($F_{ST}=0.05$). (Table 1). AMOVA results showed that variation values were low and non-significant among the four different groups in Turkish coasts (1.31%, $F_{CT}=0.0131$), whereas they were significant among and within sampling locations (2.36%, $F_{SC}=0.0239$; 96.33%, $F_{ST}=0.0367$), respectively (Table 2).

3.2.2. Demographic analyses of *S. flexuosa* and *S. maena*

Neutrality tests showed that *S. flexuosa* populations had unimodal mismatch distributions for each gene (Supporting Information, Figures S7a, 7c, 7e for COI, cyt-b, and IRBP genes, respectively). Therefore, *S. flexuosa* experienced recent demographic expansion, which was corroborated by the negative and significant F_S and D values (Table 3). Unimodal mismatch distributions were also observed in *S. maena* in all markers, suggesting recent demographic expansion (Supporting Information, Figures S7b, 7d, and 7f for COI, cyt-b, and IRBP genes, respectively). Similarly, neutrality test values (F_S and D) were all negative, supporting the population expansion model (Table 3).

The EBS plot of the concatenated dataset showed that the *S. flexuosa* population sizes remained relatively constant until about 5 Kya, followed by a sharp population growth (Figure 6a). Conversely, the *S. maena* revealed constant population sizes until about 15 Kya, followed by a subtler population growth (Figure 6b).

4. DISCUSSION

4.1. Genetic differentiation of *Spicara maena*, *Spicara flexuosa* and *Spicara smaris*

In this study, the distinction of the three taxa under the genus *Spicara* was undertaken through sequence analysis of two different mtDNA genes. The phylogenetic analyses

using data from these two genes, different tree reconstruction algorithms, and haplotype networks, show three units with different evolutionary histories, corresponding to *Spicara maena*, *Spicara flexuosa* and *Spicara smaris*. For example, the phylogenetic trees based on COI gene, and the concatenated COI and cyt-b data set indicated that *S. smaris* and *S. flexuosa* were the most closely related to each other. On the other hand, trees based on the cyt-b gene showed that *S. flexuosa* and *S. maena* were the two most closely related species among the three. Such contradictory results have been described in the literature as well. Chiba et al. (2009) found *S. flexuosa* and *S. smaris* lineages to be reciprocally monophyletic on their cyt-b phylogenetic trees. However, Bektaş et al. (2018) showed that *S. flexuosa* and *S. maena* were the two most closely related species on their cyt-b trees.

Based on the genetic analyses (COI gene), 175 samples collected from Turkey were *S. flexuosa*. In contrast, all 23 samples collected from Greece were found to be *S. maena*, in accordance with their morphological identification during collection. In several former studies, *S. flexuosa* was reported to be present in the Turkish coastal waters (Tortonose, 1986; Salekhova, 1979; Fischer et al., 1987; Nelson, 1994; Ragonese et al., 2004; Golani et al., 2006; Turan et al., 2007; Turan, 2011; Bektaş et al., 2018). Interestingly, other studies along the Turkish coast, consider *S. flexuosa* and *S. maena* to be conspecific (Karakulak et al., 2006; Soykan et al., 2010; Keskin et al., 2011; Saygılı et al., 2016; Froese and Pauly, 2017). The result of this study indicates that the distinction of the three taxa under *Spicara* species is possible, as shown in previous studies (Chiba et al., 2009; Imsiridou et al., 2011; Georgiadis et al., 2014; Bektaş et al., 2018).

According to the genetic analyses (sequence similarity-based identification and phylogenetic network-based identification) carried out in this study, it appears that

some sequences previously submitted to GenBank and BOLD have been incorrectly classified. Considering the *cyt-b* gene, specimens from Italy (Orrell et al., 2002; Sanciangco et al., 2016), which were previously identified as *S. maena*, actually belong to *S. flexuosa*. Focusing on the COI gene, a total of six sequences from Portugal (Atlantic) (Costa et al., 2012; Landi et al., 2014), one sequence from Italy, and 13 sequences from Israel (that were previously identified as *S. maena* in GenBank), should be classified as *S. flexuosa*. Landi et al. (2014) found two distinct lineages, previously identified as *Spicara maena*, based on barcoding (COI) sequences. Since *S. flexuosa* is considered as a synonym of *Spicara maena* in FishBase, Landi et al. (2014) indicated that one of the lineages of the *Spicara maena* could probably be *S. flexuosa*. Consequently, the main problem in studies on the *Spicara* genus in the Mediterranean region stems from the morphological similarity between *S. flexuosa* and *S. maena*. Systematic confusion due to variation in coloration, chromatic and morphological modifications during the reproductive period, and similarity between males of *S. flexuosa* and females of *S. maena* due to protogynous hermaphroditism (Pollard and Pichot, 1971; Arculeo et al., 1996; Vidalis and Tsimenidis, 1996; Imsiridou et al., 2011) could be some of the other possible reasons for morphological misidentification. To overcome this problem, Minos et al. (2013) evaluated similarity/dissimilarity of *S. maena* and *S. flexuosa* using multivariate analysis of morphometric characters. Their discriminant analysis showed that 83.2 % of the examined individuals could be classified correctly by this method. Minos et al. (2013) further argued that 13% of non-discrimination could be related to biological aspects, such as sexual dimorphism and protogynous hermaphroditism.

Estimated divergence times among the three species indicated that they began to diversify during the early or middle Pliocene (4.30-3.58 Mya), based on the analyses

carried out using concatenated mtDNA sequences, which dates the divergence after the Messinian Salinity Crisis that took place around 6 Mya (Por, 1989). In this perspective, we propose the following scenario regarding the diversification of *S. maena* and *S. flexuosa* throughout the Atlantic, the Mediterranean and the Black Sea:

According to this scenario, *S. flexuosa* is considered of Atlantic-Mediterranean origin, and the species might have expanded from Atlantic to the Mediterranean and Black Sea regions. An Atlantic–Mediterranean origin was suggested for several marine taxa, such as *Pagrus pagrus* and *Dentex dentex* (Bargelloni et al., 2003), *Sprattus sprattus* (Debes et al., 2008), *Sygnatus typhle* (Wilson and Veraguth, 2010), *Engraulis encrasicolus* (Magoulas et al., 2006), and *Mugil cephalus* (Durand et al., 2013). This scenario assumes that during the early or middle Pliocene (5.00-3.50 Mya) two important events took place: The isolation of the Mediterranean Sea from the Atlantic Ocean and the cessation of the connection between the Mediterranean and the Black Sea (Moraitou-Apostolopoulou and Kiortsis, 1985). It seems probable that the differentiation of the *Spicara* spp. species took place in the post Messinian period (in the early or middle Pliocene) in this closed Mediterranean region, with differentiation of the ancestral *S. maena* and *S. flexuosa* having taken place in the western and eastern Mediterranean basins, respectively. An eastern Atlantic origin of *S. maena* is also possible. During the Pleistocene, with each glacial and interglacial cycle, *S. flexuosa* could have gradually expanded into the Black Sea, but might not have been able to tolerate the low salinity and temperature conditions there, as suggested by Bektaş et al. (2018), and as it has been reported for other marine fish such as the whiting (*Merlangius merlangus*) (Şalcıoğlu et al., 2020) and anchovy (*Engraulis encrasicolus*) (Grant, 2005).

The expansion of *S. flexuosa* from the Mediterranean to the Black Sea might have been initiated approximately 5 Kya (the estimated time for the onset of population expansion based on the EBSP analysis) during the post glacial warming period after the end of the last glacial maximum (Ryan et al., 1997). During this period, there was outflow from Black Sea into Aegean Sea through the Bosphorus and Dardanelles (Kvasov, 1975). Hence the population expansion from the Mediterranean to the Black Sea might have occurred via the lower layer current after the opening of the Dardanelles, which occurred around 8 Kya (Ryan et al., 1997). Further and extensive sampling in the eastern Atlantic and the western Mediterranean, similar to that undertaken around Turkish coasts in this study, is necessary for a better understanding of the origins of *S. flexuosa* and *S. maena*.

4.2. Connectivity of the Turkish *Spicara flexuosa* populations between Eastern Mediterranean and Black Sea

The absence of genetic structure between subpopulations, low values of mtDNA molecular diversity indices and star shaped haplotype networks indicate the connectivity of the *S. flexuosa* subpopulations from Turkey, suggesting that the TSS is a biological corridor (Turan et al., 2016), rather than a barrier to gene flow, for this species. The absence of significant genetic structure was deduced from the generally low and insignificant pairwise Φ_{ST} values between populations of *S. flexuosa* along the Turkish coasts. This might have been affected by the life histories of the species (e.g. pelagic larval phase), and the TSS not being a hydrological barrier for the species, with these two effects not being mutually exclusive. The dispersal of pelagic larvae as a result of hydrological processes may enhance the gene flow between *S. flexuosa* from different seas, as was previously observed for other Sparid fish (Vigliola et al., 1998) and *Trachurus mediterraneus*, *Trachurus trachurus*, and *Trachurus picturatus*.

populations, based on the cyt-b gene analysis (Bektaş and Belduz, 2008), and Atlantic, Mediterranean, and Aegean *Trachurus* populations based on 16S rRNA (Karaïskou et al., 2003). Lack of genetic structure of other fish species has also been reported along the Turkish coasts (Debes et al., 2008; Wilson and Veraguth, 2010; Durand et al., 2013). In addition, Suziki et al. (2004) and Limborg et al. (2012) suggested that historical population bottleneck resulting from strong Pleistocene glaciations was the underlying reason for the low genetic structure, as well as for low level of genetic diversity in the eastern Mediterranean basin, and this could also be true for the Turkish *S. flexuosa* stock.

Considering overall molecular diversity of *S. flexuosa*, we see that the two lowest haplotype ($h=0.0620$, $h=0.0730$) and nucleotide diversities ($\pi=0.0001$, $\pi=0.0002$) were found in the COI and cyt-b genes, respectively. Geographically speaking, the highest haplotype and nucleotide diversities values were found in Mersin ($h=0.3890$, $\pi=0.0010$) based on the cyt-b gene. On the other hand, certain regions showed haplotype diversities that were zero, such as Black Sea region for cyt-b, and TSS for COI. Low levels of genetic variability were observed in other fish species, such as *Psetta maxima* in isolated eastern basins, including the Aegean, the Sea of Marmara, Black Sea and the Azov Sea (Suziki et al., 2004). In addition, Suziki et al. (2004) found two distinct lineages in the western Mediterranean and eastern Mediterranean for *P. maxima*, and suggested that the eastern Mediterranean lineage showed lower genetic variability than the western lineage, potentially due to a population bottleneck in the last glacial period that took place in the Black Sea as well as in the Eastern Mediterranean. Low levels of molecular diversities were also observed in the Eastern Mediterranean whiting (*Merlangius merlangus*) as compared to Atlantic (Şalcıoğlu et al., 2020) due to potential population bottleneck before the last glacial period and/or differences in population

expansion among the Atlantic and the Eastern Mediterranean/Black Sea stocks. The results of the current study are in agreement with those of Bektaş et al. (2018), who also detected low levels of molecular diversities values for *S. flexuosa* along the Black Sea coast.

In addition to historical factors, more recent events such as overfishing could have contributed to small (effective) population sizes. Turan et al. (2015) reported that high fluctuations of total catch and heavy fishing pressure were probably the main reasons for low levels of genetic variation of *Sarda sarda* among the Turkish coastal waters. In recent years, *S. flexuosa/maena* have been exposed to heavy fishing pressure and their annual production have drastically decreased from a total of 2.250 tons in 2001 to 218 tons in 2019 (TUIK, 2019). Thus, its populations might have been severely impacted, likely resulting in a decrease of the population numbers.

Star-shaped haplotype networks of the mtDNA genes of *S. flexuosa* recorded in the present study indicate that the species might have also undergone a population expansion in the past. The network also shows the connectivity of *S. flexuosa* sampling locations as has been previously observed for other protogynous hermaphrodite fish (e.g., *Pagellus bogavareo*, Bargelloni et al., 2003; Stockley et al., 2005), protandrous hermaphrodite fish (e.g., *Diplodus puntazzo*, (Bargelloni et al., 2005), and fresh to brackish water fish (e.g., *Coregonus clupeaformis*, Bernatchez et al., 1989). Neutrality test results (significantly negative F_S and Tajima D values), unimodal mismatch distribution and EBSF also support the scenario of sudden population growth (5 Kya) of *S. flexuosa* after a potential bottleneck in the eastern Mediterranean. Wilson and Veraguth (2010) also detected unimodal mismatch distributions among *Syngnathus typhle* (pipefish) from the Black Sea and the Sea of Marmara. In addition, a star-shaped haplotype network and a recent and sharp expansion of a single whiting lineage were

also reported from the Eastern Mediterranean, Black Sea, the TSS, and the North Aegean Sea (Şalcıoğlu et al., 2020). As opposed to these results, Debes et al. (2008) found ragged mismatch distribution among the European sprat populations between the Black Sea and the Bosphorus, not supporting a scenario of demographic expansion after having a small population size. Hence, along the Turkish coasts and TSS, the presence/absence of bottleneck and demographic expansion scenarios can be species-specific in fish, and for *S. flexuosa* we see support for their presence.

Nuclear DNA analysis results of *S. flexuosa* and *S. maena* revealed that the previous phylogenetic analyses were in accordance with the taxonomies of the two species as no haplotypes were shared between them. The genetic differentiation between these two species of *Spicara* confirms that reproductive isolation of the two species was complete. Moreover, the total number of mutations and the number of parsimony informative sites between the two *Spicara* species were not as high in the IRBP gene as those between the mtDNA genes, suggesting that the lack of the discrimination could be due to slower evolution rate of the former (Verma et al., 2004).

5. CONCLUSION AND RECOMMENDATIONS

In this study, molecular taxonomy, phylogeography of *Spicara* spp. around the Turkish and Eastern Mediterranean coasts were investigated. The three species of *Spicara* were clearly distinguished by two different mtDNA markers. Although not as clear-cut as in mtDNA, the nuDNA also showed the differentiation of *S. maena* and *S. flexuosa*. The result of the dating analyses suggested that the three *Spicara* species were differentiated during the early or middle Pliocene (4.30-3.58 Mya). Considering, *S. flexuosa*, low values of mtDNA molecular diversity indices, an absence of genetic structure between subpopulations, and star-shaped haplotype networks indicate the connectivity of the

examined *S. flexuosa* sampling locations from Turkey, suggesting that the TSS is a corridor, rather than a barrier to gene flow, for this species. Moreover, demographic analysis supports the scenario of sudden population growth of *S. flexuosa* after a potential bottleneck. Based on the EBSF analysis, *S. flexuosa* and *S. maena* expansions were relatively recent (ca. 5 Kya and 15 Kya, respectively) along the Eastern Mediterranean. *S. flexuosa* is currently considered as a synonym of *S. maena*, and therefore, data on its population statistics have not been reported in the literature. This is a significant problem, as it can result in inaccurate determination and assessment of current stock levels for both *S. maena* and *S. flexuosa*. As picarel populations have experienced rapid decline in the Black Sea, Aegean and Eastern Mediterranean in the last 10 years (Pollard et al., 2014), it is recommended that separate statistics and population studies should be used for conservation and management decisions for *S. maena* and *S. flexuosa*.

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Ethical Issue

The work raises no ethical issues. All fish examined were from commercial fishing activities and none of them were killed for the specific purpose of the study.

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1009 **TABLES**

1010 **Table 1.** Pairwise Φ_{ST} values of the concatenated data of COI + cyt-b genes (below
 1011 diagonal) and *Fst* values of IRBP genes (above diagonal) of *S. flexuosa*. Values
 1012 in italics and bold were significant before and after Bonferroni corrections,
 1013 respectively. TSS: Turkish Strait System.

			İstanbul	Çanakkale	Armutlu	İzmir	Muğla	Rize	Sinop	Antalya	Mersin
<i>Spicara flexuosa</i>	TSS	İstanbul	-	<i>0.03</i>	0.12	0.01	-0.00	<i>0.05</i>	<i>0.03</i>	0.05	0.02
		Çanakkale	-0.00	-	<i>0.03</i>	-0.00	<i>0.02</i>	0.00	0.00	<i>0.03</i>	<i>0.02</i>
		Armutlu	0.00	0.01	-	<i>0.04</i>	0.12	0.00	0.01	0.07	0.11
	Aegean Sea	İzmir	0.00	-0.13	0.00	-	0.01	0.01	0.00	<i>0.03</i>	<i>0.03</i>
		Muğla	0.00	-0.04	0.00	0.00	-	0.06	<i>0.03</i>	<i>0.05</i>	<i>0.03</i>
	Black Sea	Rize	0.01	0.00	0.03	-0.13	-0.04	-	-0.01	<i>0.03</i>	<i>0.04</i>
		Sinop	0.00	-0.19	0.00	0.00	0.00	-0.19	-	<i>0.02</i>	<i>0.03</i>
	Levantine	Antalya	-0.00	0.00	0.00	-0.13	-0.05	0.00	-0.19	-	0.01
		Mersin	0.00	-0.06	0.00	0.00	0.00	-0.05	0.00	-0.06	-

1014 **Table 2.** Hierarchical AMOVA results COI+cyt-b (top) and IRBP genes (bottom).
 1015 TSS: Turkish Strait System.

	Source of variation	Total variation	Percent of total	Φ_{CT}/F_{CT}	Φ_{SC}/F_{SC}	Φ_{ST}/F_{ST}
COI +cyt-b genes	Among groups (TSS, Black Sea, Aegean Sea and Levantine)	0.0007	2.60	0.0260 (p = 0.3142)		
	Among populations within groups	-0.0014	-4.74		-0.0486 (p = 0.7799)	
	Within populations	0.0304	102.14			-0.0213 (p = 0.6215)
	Total	0.0297				
IRBP gene	Among groups (TSS, Black Sea, Aegean Sea and Levantine)	0.0041	1.31	0.0131 (p = 0.1485)		
	Among populations within groups	0.0074	2.36		0.0239 (p = 0.0000)	
	Within populations	0.3038	96.33			0.0367 (p = 0.0000)
	Total	0.3154				

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Table 3. Neutrality test results for *S. flexuosa* /*S. maena* (including all GenBank individuals); N: Number of the samples; D: Tajima's *D*; *F_s*: Fu's *F_s*; (2 sequences per individual for IRBP gene). Significant values ($P < 0.05$) are indicated in bold. TSS: Turkish Strait System.

	Sampling sites	N	<i>D</i>	<i>F_s</i>
COI gene	Total <i>S. flexuosa</i>	196	-1.6251	-7.8670
	TSS	58	-	-
	Black Sea	31	-1.1447	-1.2385
	Aegean Sea	41	-1.1218	-1.4706
	Levantine	45	-1.1131	-1.5482
	Total <i>S. maena</i>	27	-0.7274	-1.4952
cyt-b gene	Total <i>S. flexuosa</i>	143	-1.6899	-7.6158
	TSS	62	-1.0804	-1.8164
	Black Sea	25	-	-
	Aegean Sea	22	-1.1624	-0.9567
	Levantine	30	-1.2555	-1.6685
	Total <i>S. maena</i>	21	-1.1635	-0.9188
IRBP gene	Total <i>S. flexuosa</i>	348	-2.2274	-56.5358
	TSS	120	-2.0581	-15.5390
	Black Sea	64	-2.0239	-13.5505
	Aegean Sea	76	-2.1256	-11.7787
	Levantine	88	-1.9022	-14.8931
	Total <i>S. maena</i>	43	-1.3023	-2.1189

FIGURE CAPTIONS

Figure 1. Sampling locations of three *Spicara* species in this study and GenBank, based on the COI, cyt-b and the IRBP genes, and the taxonomic evaluations from literature [(*Spicara smaris* L. 1758, *Spicara maena* L.1758, and *Spicara flexuosa* (Rafinesque 1810)]. GenBank samples are indicated by the GB suffix. Numbers on the figures indicate sampling locations. Localities information are found in Supporting Information Table S1.

Figure 2. a.) *Spicara maena* FishTrace specimen SpiMae-EM-04, b) *S. flexuosa*: FishTrace specimen SpiFle-EM-03, (Photos by Panos Leontarakis/Laurence Favre-Krey), c) *S. maena* collected from Greece, d) *S. flexuosa* collected from Turkish coastal waters.

Figure 3. Haplotype networks of a) COI, b) cyt-b and c) IRBP genes of *Spicara* spp. Note: Background of pink color indicates *S. flexuosa*, blue color indicates *S. maena*, and white color indicates *S. smaris*. The numbers correspond to number of mutations between haplotypes in A, B and C.

Figure 4. Bayesian topology of the concatenated mtDNA (COI and cyt-b) genes (total 809 bp) of *Spicara* spp. The numbers on the branches are the Bayesian posterior probabilities. TSS: Turkish Straits System. Hap1: *S. smaris* GenBank accession no: COI: KT883623, cyt-b: KT883672.

Figure 5. BEAST chronogram based on the concatenated mtDNA (COI and cyt-b) genes of *Spicara* spp. without outgroup dating. Values above branches are the mean node height (age). Bars show 95 % HPD. Values below branches are posterior probabilities.

1035 **Figure 6.** Extended Bayesian Skyline Plots for a) *S. flexuosa*, b) *S. maena*, reflecting
1036 changes effective population size against time in years (Ya) before present. CPD
1037 indicates central posterior density interval.

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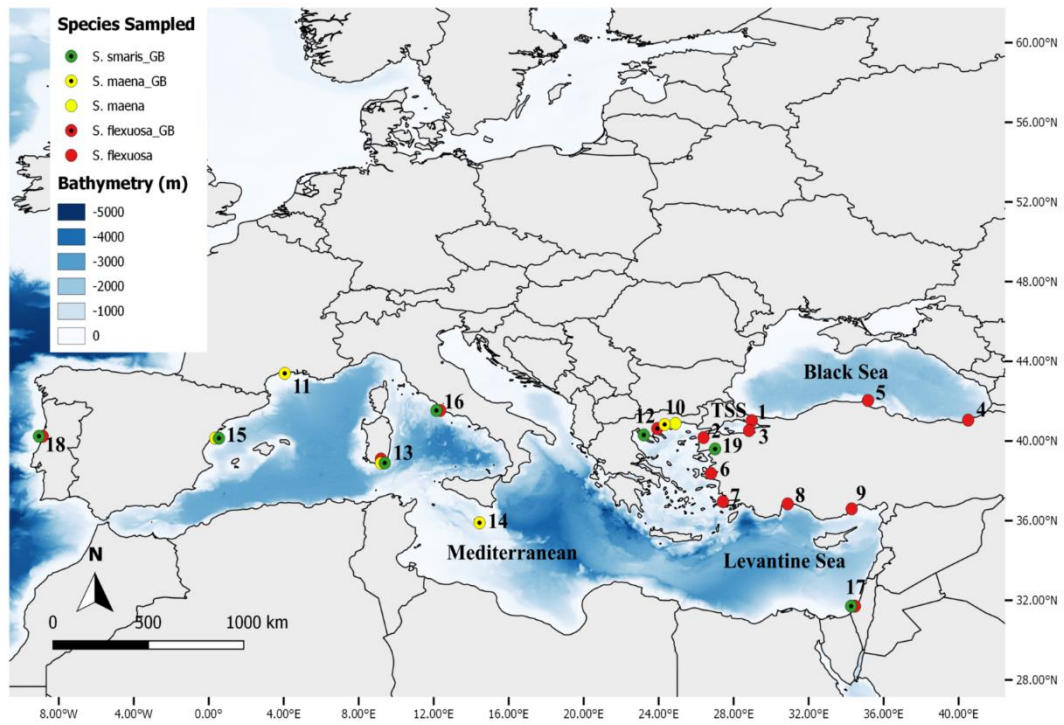
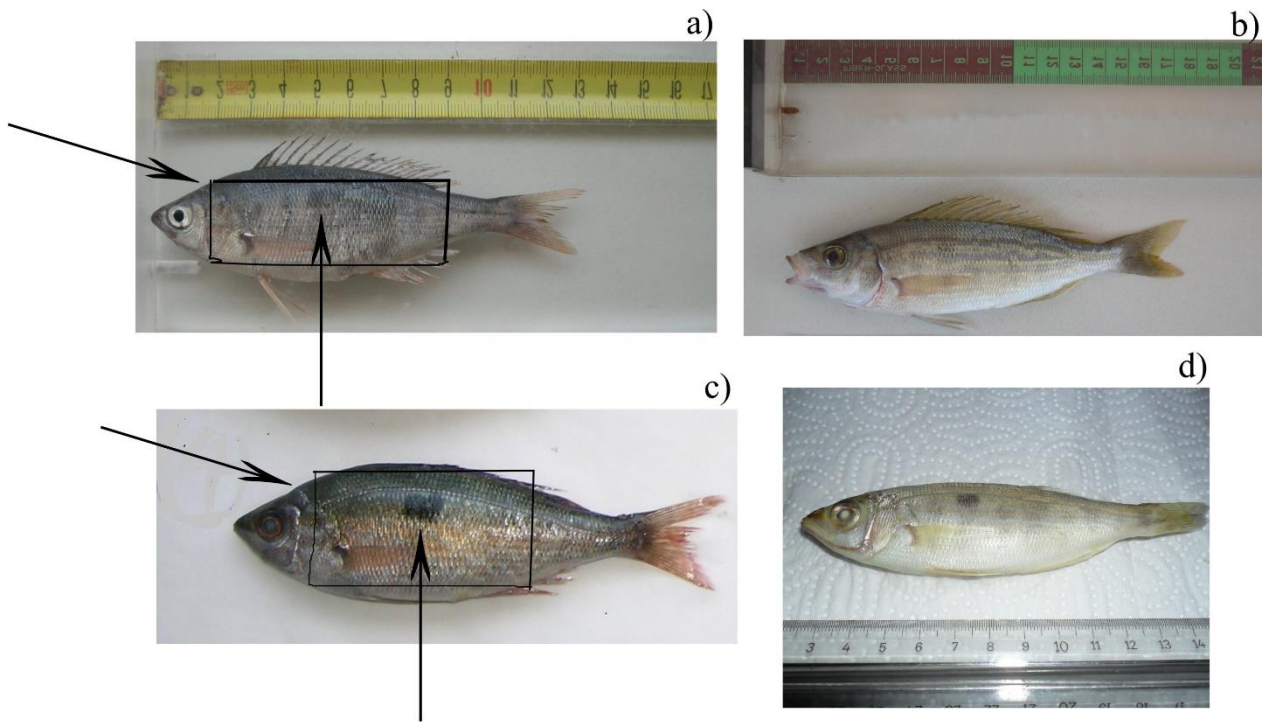


Figure 1.



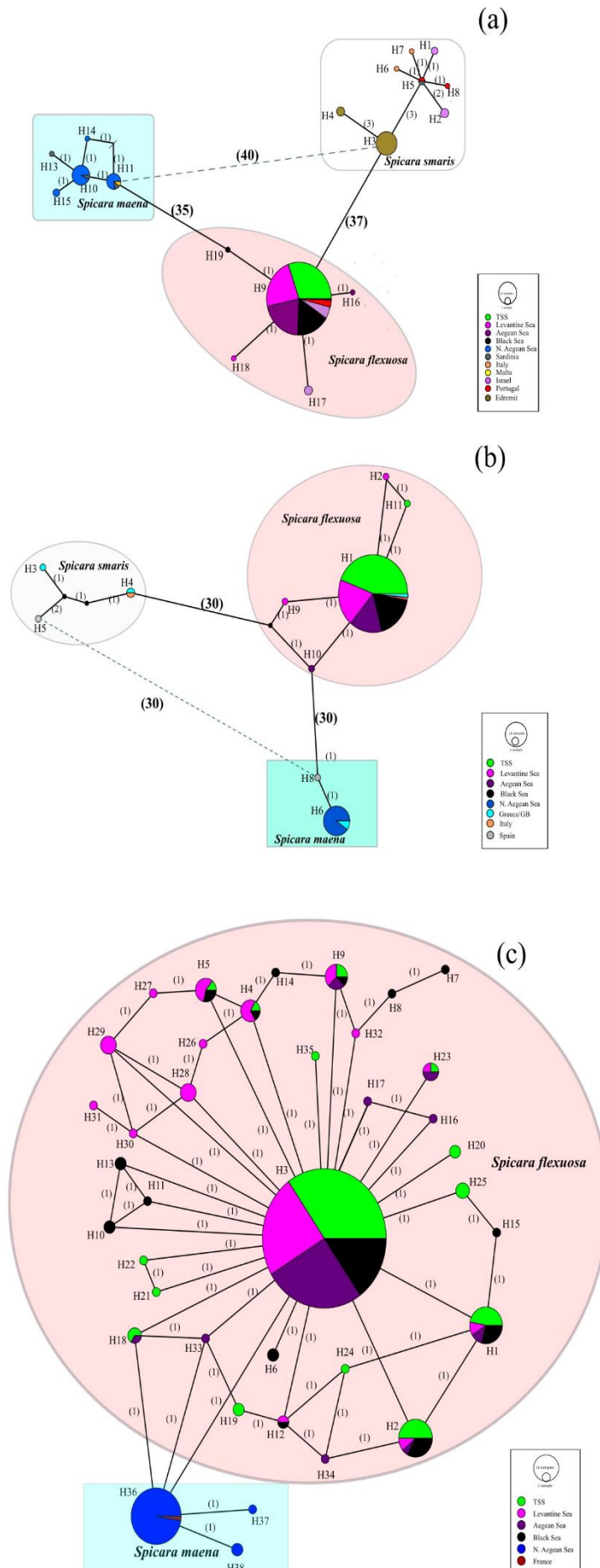
1057 Figure 2.

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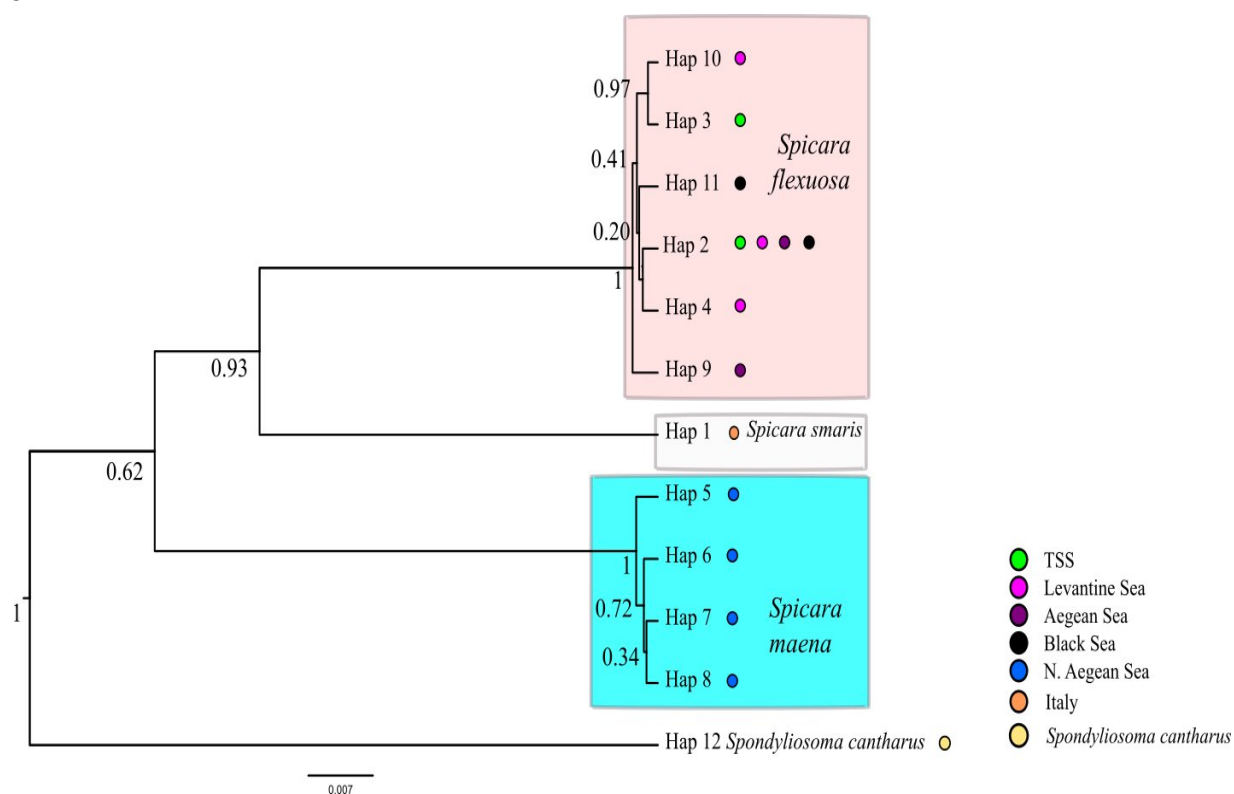


Figure 4.

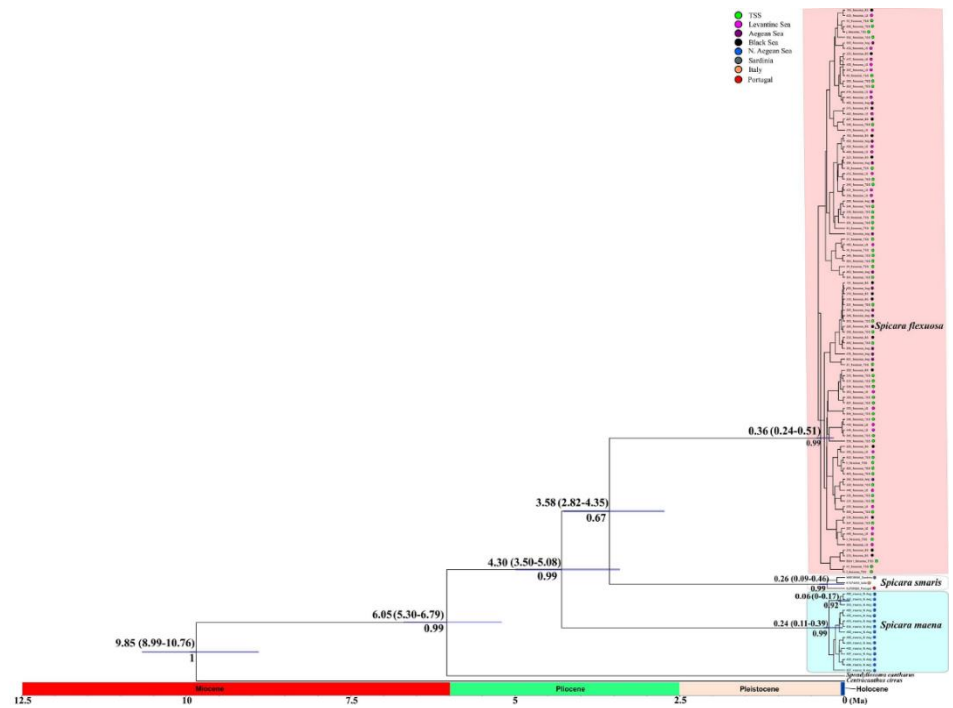


Figure 5.

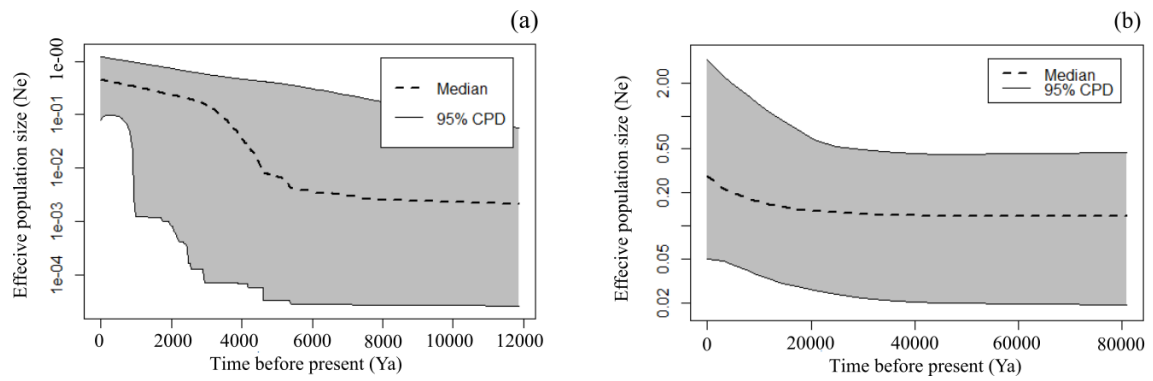


Figure 6.