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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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1 **ABSTRACT**

2

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

20

21

22

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

25 1. INTRODUCTION

26

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

37 One of the unresolved questions in fish phylogeny and taxonomy concerns the
38 family Sparidae, consisting of 39 genera, 166 valid species and 260 synonyms (Nelson
39 et al., 2016; Parenti, 2019; Fricke et al., 2020) including the genera, *Spicara* and
40 *Centracanthus*. Orrell et al. (2002) and Orrell and Carpenter (2004) showed that
41 currently defined sub-families of Sparidae were not monophyletic and *Spicara* was a
42 member of Sparidae. In addition, the monophyly of the family Sparidae is not supported
43 (Chiba et al., 2009), primarily because of the uncertainty of the position of its sister
44 family Centracanthidae, which includes the genera *Spicara* and *Centracanthus*. The
45 disagreement in the taxonomy is also reflected in the online marine fish databases as
46 well; according to the World Register of Marine Species (WoRMS), the Integrated
47 Taxonomic Information System (ITIS), and the Marine Species Identification Portal,
48 *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.*

74 *maena* and *S. flexuosa* should be considered as synonyms whereas others (Tortonose,
75 1986; Fischer et al., 1987; Costa, 1991; Louisy, 2002; Golani et al., 2006; Vasileva,
76 2007; Bilecenoğlu et al., 2014) support the distinction of the two species. More recent
77 studies based on both genetic (Chiba et al., 2009; Imsiridou et al., 2011; Georgiadis et
78 al., 2014; Bektaş et al., 2018) and morphological data (Minos et al., 2013) also support
79 the latter conclusion. The discrepancy is reflected in various fish-related taxonomic
80 databases as well. The Catalog of Fishes (Fricke et al., 2020), and ITIS
81 (<http://www.itis.gov>) accept *S. flexuosa* as a valid species, while FishBase (Froese and
82 Pauly, 2020), WoRMS, EOL and the Marine Species Identification Portal consider *S.*
83 *flexuosa* as an unaccepted/unresolved and synonym of *S. maena*.

84 Regarding the biogeographical distribution of the three *Spicara* species, *Spicara*
85 *maena* is of Atlanto-Mediterranean origin and inhabits temperate to warm waters, and
86 is largely distributed in the Mediterranean, including the Adriatic Sea. It is also found
87 from Portugal to Morocco in the Atlantic, and with a limited distribution in the Black
88 Sea (Froese and Pauly, 2017). *Spicara flexuosa* is observed in the eastern Atlantic,
89 Mediterranean, and the Black Sea (Salekhova, 1979; Tortonose, 1986; Golani et al.,
90 2006). Finally, *Spicara smaris* is recorded in the Mediterranean, the Eastern Atlantic
91 coasts, and with a restricted distribution in the Black Sea (Tortonose, 1986).

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

94 Family: Centracanthidae Gill, 1893

95 Genus: *Spicara* Rafinesque, 1810

96 *Spicara flexuosa* (Rafinesque, 1810)

97 Common name: Picarel.

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

103 *Spicara maena* (Linnaeus, 1758)

104 Common name: Blotched picarel.

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

110 *Spicara smaris* (Linnaeus, 1758)

111 Common name: Picarel, Zerro.

112 Diagnosis: Protractile premaxilla. Head length equals and longer than body
113 depth, 75 or more lateral line scales; black spot on the body side above pectoral fin.
114 Color: Grey above, silvery below. Color varies with age, sex and season. Habitat:
115 Posidonia beds and muddy bottoms at about 15-100 m. Size max: 15 cm. (Tortonese,
116 1986; Golani et al., 2006).

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

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

129

130 **2. MATERIAL AND METHODS**

131 **2.1. Sample collection**

132 We sampled 279 specimens of *Spicara flexuosa* and *Spicara maena* representing a
133 broad geographic coverage of their distribution along the Turkish coast line and Greece
134 (Figure 1, Supporting Information, Table S1). Individuals were collected from ten
135 stations by fishermen using trawlers, hand line fishing and purse seine nets between
136 2013-2015; two from the Black Sea (Sinop, Rize, Turkey), three from the TSS
137 (Istanbul, Armutlu, Canakkale, Turkey), two from the Central/South Aegean Sea
138 (Izmir, Mugla, Turkey), one from the North Aegean Sea, (Kavala, Greece), and two
139 from the Levantine Sea (Mersin, Antalya, Turkey). As there are no explicit field guides
140 for the identification or discrimination of *S. maena* and *S. flexuosa*, information on
141 differences based on the diagnostic morphological characters of the two species
142 (Tortonose, 1986; Lythgoe and Lythgoe, 1992; Golani et al., 2006), along with the
143 experience of the field collector were used to sample and identify 30 specimens of *S.*
144 *maena* from Greece. These morphological differences included *S. maena* having: i)
145 rather deep body than *S. flexuosa*, ii) head length being less than its body depth (*vice*
146 *versa* for *S. flexuosa*), iii) head elevated posteriorly and profile concave above the head.
147 A black mark which is larger than the one found in *S. flexuosa*, occurs on the side of

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

157 **2.2. DNA extraction and amplification**

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

169 **2.3. Statistical analyses**

170 **2.3.1. Summary statistics**

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

173 aligning all sequences to detect polymorphic sites and subsequently confirming the
174 clarity of the peaks at these sites. After trimming all ambiguous bases on both sides,
175 454, 355 and 385 bp fragments were used for further analyses of COI, cyt-b, and IRBP
176 genes, respectively. Stop codons were investigated for mitochondrial (mtDNA) genes
177 to determine presence/absence of mitochondrial pseudogenes by using DAMBEv.6.4.1
178 (Xia, 2017). In the nuclear DNA data analysis, phased haplotypes were estimated for
179 IRBP gene using Bayesian methods in PHASE (Stephens and Donnelly, 2003).
180 Recombination was examined using a PHI Test (Bruen et al., 2006) in SPLITSTREE 4
181 (Huson and Bryant, 2006). No significant signals of recombination were detected, and
182 the full non-recombining sequences were used in the subsequent analyses.

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

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

197 divergence) between species were calculated in DnaSP v.5.10.1 (Librado and Rozas,
198 2009).

199

200 **2.3.2. Haplotype networks and phylogenetic analyses**

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

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

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

228

229 **2.3.3. Divergence times estimates**

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

244 **2.4. Genetic structure of *Spicara flexuosa***

245 **2.4.1. AMOVA, Φ_{st} and F_{st}**

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

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

256

257 **2.4.2. Demographic history analyses**

258 Mismatch distribution of the number of pairwise differences (Slatkin and Hudson,
259 1991; Rogers and Harpending, 1992) and the Tajima's D (Tajima, 1989) and Fu's F_s
260 (Fu, 1997) neutrality tests per species were estimated with DnaSP. Past population
261 demography of *S. flexuosa* and *S. maena* was also reconstructed using Extended
262 Bayesian Skyline Plots (EBSP) (Heled and Drummond, 2008; Ho and Shapiro, 2011),
263 as implemented in BEAST using the multi-locus mtDNA dataset (COI, cyt-b). The
264 BEAST input file was produced in BEAUTi v.2.4.8 (Bouckaert et al., 2014). The best
265 model of nucleotide substitution was selected for each locus and time was scaled based
266 on the molecular clock rates (COI and cyt-b rates were provided in 2.3.3). A strict
267 molecular clock was set as prior and the Bayesian MCMC was run for 1.5×10^8
268 generations for both *S. flexuosa* and for *S. maena*, discarding the initial 10% of samples
269 as burn-in. Convergence of runs was evaluated on TRACER. Visualization of the EBSP
270 plots was made with the RStudio v.1.1.463 using R v.3.5.1 (R Development Core Team,
271 2018).

272

273 **3. RESULTS**

274 **3.1. Summary statistics**

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

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

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

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

310 Among all samples, 139 fish were identified as *S. flexuosa* and 18 as *S. maena*
311 based on the *cyt-b* haplotype network (Figure 3b, for species identification used to see
312 aforementioned information). *Spicara flexuosa* exhibited the highest haplotype and
313 nucleotide diversities ($h=0.0710$, $\pi=0.0002$). Specifically, Mersin ($h=0.3890$;
314 $\pi=0.0011$) and Izmir ($h=0.2860$; $\pi=0.0008$) had the highest haplotype and nucleotide
315 diversities. Region-wise, the Levantine showed the highest haplotype and nucleotide
316 diversities ($h=0.1910$; $\pi=0.0005$). (Supporting Information, Table S8). On the other
317 hand, *Spicara maena* showed the lowest haplotype and nucleotide diversities ($h=0$,
318 $\pi=0$) (Supporting Information, Table S8). A total of 51 (14.3%) nucleotide sites were
319 variable, of which 48 (13.5%) positions were parsimony informative. A total of 17
320 positions were parsimony informative for *S. flexuosa* and *S. maena*, 14 for *S. smarís*
321 and *S. flexuosa*, and 17 for *S. maena* and *S. smarís* (Supporting Information, Table S9).
322 The total number of mutations ranged from 35 (between *S. flexuosa* and *S. maena*) to

323 37 (between *S. flexuosa* and *S. smaris*), and the pairwise sequence divergences ranged
324 from 9.01% (between *S. flexuosa* and *S. maena*) to 9.35% (between *S. maena* and *S.*
325 *smaris*) (Supporting Information, Table S7). Total number of mutations and pairwise
326 sequence divergence indicated similar levels of differentiation among the three *Spicara*
327 species. In addition, stop codons were not observed for the COI and cyt-b genes.

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

346 3.1.1. Haplotype networks and phylogenetic analyses

347 Three haplogroups, corresponding to *S. flexuosa*, *S. maena* and *S. smarís* were observed
348 in the COI haplotype network (Figure 3a). Samples from Turkey shared the same
349 haplotype (Hap 9), and were grouped with *S. flexuosa* from Sardinia (Cannas et al.,
350 retrieved from GenBank, 2010; GenBank and BOLD numbers are found in Supporting
351 Information Table S2), whereas samples from Greece (Hap 10 and Hap 11), clustered
352 with *S. maena* samples from Sardinia (Cannas et al., retrieved from GenBank, 2010)
353 and Malta (KJ709919). In addition, 32 *S. smarís* samples retrieved from GenBank,
354 (GenBank numbers are found in Supporting Information Table S2) (Hap 1, Hap 2, Hap
355 3, Hap 4, Hap 5, Hap 6, Hap 7 and Hap 8) were found in the *S. smarís* haplogroup.
356 BLASTn results and our haplotype network descriptions showed that some of the
357 sequences deposited in GenBank and BOLD originated from *Spicara* genus, were
358 probably misidentified. The results indicated that one sample from Italy, 13 from Israel,
359 and six from Portugal (reported as *S. maena* in GenBank) (Hap 9, Hap 17), (their
360 GenBank and BOLD numbers were displayed on the Supporting Information Table S2)
361 should have been identified as *S. flexuosa*.

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

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

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

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

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

398

399 **3.1.2. Divergence Times Estimates**

400 The BEAST chronogram indicated a split between *Centracanthus cirrus* and
401 *SpondylIOSoma cantharus* around 9.85 Mya (CI: 8.99-10.76). The estimated divergence
402 time between *SpondylIOSoma cantharus* and the three *Spicara* species was estimated at
403 6.05 Mya (CI: 5.30-6.79) (Figure 5). Within the three *Spicara* species, *S. maena* was
404 separated from *S. flexuosa* and *S. smarIS* approximately 4.30 Mya (CI: 3.50-5.08), and
405 the split between *S. smarIS* and *S. flexuosa* was estimated approximately 3.58 Mya (CI:
406 2.82-4.35).

407

408 **3.2. Genetic structure of *Spicara flexuosa***

409 **3.2.1 AMOVA, Φ_{ST} and F_{ST}**

410 Pairwise Φ_{ST} values for the concatenated mtDNA dataset of *Spicara flexuosa* ranged
411 from -0.19 to 0.01. Lack of genetic structure was observed among the Turkish sampling
412 areas (Table 1). AMOVA results also indicated that variation was the highest within
413 sampling locations (102.14%, $\Phi_{ST} = -0.0213$), and the lowest values were observed
414 among locations within groups (-4.74%, $\Phi_{SC} = -0.0486$) and among the four different
415 groups (the TSS, the Black Sea, the Aegean Sea, and the Levantine, 2.60%, Φ_{CT}
416 = 0.0260) (Table 2).

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

421 were also observed between Rize and Mugla ($F_{ST}=0.06$) and Antalya and Istanbul
422 ($F_{ST}=0.05$). (Table 1). AMOVA results showed that variation values were low and non-
423 significant among the four different groups in Turkish coasts (1.31%, $F_{CT}=0.0131$),
424 whereas they were significant among and within sampling locations (2.36%,
425 $F_{SC}=0.0239$; 96.33%, $F_{ST}=0.0367$), respectively (Table 2).

426

427 **3.2.2. Demographic analyses of *S. flexuosa* and *S. maena***

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

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

441

442 **4. DISCUSSION**

443 **4.1. Genetic differentiation of *Spicara maena*, *Spicara flexuosa* and *Spicara smaris***

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

446 using data from these two genes, different tree reconstruction algorithms, and haplotype
447 networks, show three units with different evolutionary histories, corresponding to
448 *Spicara maena*, *Spicara flexuosa* and *Spicara smaris*. For example, the phylogenetic
449 trees based on COI gene, and the concatenated COI and cyt-b data set indicated that *S.*
450 *smaris* and *S. flexuosa* were the most closely related to each other. On the other hand,
451 trees based on the cyt-b gene showed that *S. flexuosa* and *S. maena* were the two most
452 closely related species among the three. Such contradictory results have been described
453 in the literature as well. Chiba et al. (2009) found *S. flexuosa* and *S. smaris* lineages to
454 be reciprocally monophyletic on their cyt-b phylogenetic trees. However, Bektaş et al.
455 (2018) showed that *S. flexuosa* and *S. maena* were the two most closely related species
456 on their cyt-b trees.

457 Based on the genetic analyses (COI gene), 175 samples collected from Turkey
458 were *S. flexuosa*. In contrast, all 23 samples collected from Greece were found to be *S.*
459 *maena*, in accordance with their morphological identification during collection. In
460 several former studies, *S. flexuosa* was reported to be present in the Turkish coastal
461 waters (Tortonose, 1986; Salekhova, 1979; Fischer et al., 1987; Nelson, 1994;
462 Ragonese et al., 2004; Golani et al., 2006; Turan et al., 2007; Turan, 2011; Bektaş et
463 al., 2018). Interestingly, other studies along the Turkish coast, consider *S. flexuosa* and
464 *S. maena* to be conspecific (Karakulak et al., 2006; Soykan et al., 2010; Keskin et al.,
465 2011; Saygılı et al., 2016; Froese and Pauly, 2017). The result of this study indicates
466 that the distinction of the three taxa under *Spicara* species is possible, as shown in
467 previous studies (Chiba et al., 2009; Imsiridou et al., 2011; Georgiadis et al., 2014;
468 Bektaş et al., 2018).

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

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

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

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

500 According to this scenario, *S. flexuosa* is considered of Atlantic-Mediterranean
501 origin, and the species might have expanded from Atlantic to the Mediterranean and
502 Black Sea regions. An Atlantic–Mediterranean origin was suggested for several marine
503 taxa, such as *Pagrus pagrus* and *Dentex dentex* (Bargelloni et al., 2003), *Sprattus*
504 *sprattus* (Debes et al., 2008), *Sygnatus typhle* (Wilson and Veraguth, 2010), *Engraulis*
505 *encrasicolus* (Magoulas et al., 2006), and *Mugil cephalus* (Durand et al., 2013). This
506 scenario assumes that during the early or middle Pliocene (5.00-3.50 Mya) two
507 important events took place: The isolation of the Mediterranean Sea from the Atlantic
508 Ocean and the cessation of the connection between the Mediterranean and the Black
509 Sea (Moraitou-Apostolopoulou and Kiortsis, 1985). It seems probable that the
510 differentiation of the *Spicara* spp. species took place in the post Messinian period (in
511 the early or middle Pliocene) in this closed Mediterranean region, with differentiation
512 of the ancestral *S. maena* and *S. flexuosa* having taken place in the western and eastern
513 Mediterranean basins, respectively. An eastern Atlantic origin of *S. maena* is also
514 possible. During the Pleistocene, with each glacial and interglacial cycle, *S. flexuosa*
515 could have gradually expanded into the Black Sea, but might not have been able to
516 tolerate the low salinity and temperature conditions there, as suggested by Bektaş et al.
517 (2018), and as it has been reported for other marine fish such as the whiting (*Merlangius*
518 *merlangus*) (Şalcıoğlu et al., 2020) and anchovy (*Engraulis encrasicolus*) (Grant,
519 2005).

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

531 **4.2. Connectivity of the Turkish *Spicara flexuosa* populations between Eastern** 532 **Mediterranean and Black Sea**

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

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

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

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

574 In addition to historical factors, more recent events such as overfishing could
575 have contributed to small (effective) population sizes. Turan et al. (2015) reported that
576 high fluctuations of total catch and heavy fishing pressure were probably the main
577 reasons for low levels of genetic variation of *Sarda sarda* among the Turkish coastal
578 waters. In recent years, *S. flexuosa/maena* have been exposed to heavy fishing pressure
579 and their annual production have drastically decreased from a total of 2.250 tons in
580 2001 to 218 tons in 2019 (TUIK, 2019). Thus, its populations might have been severely
581 impacted, likely resulting in a decrease of the population numbers.

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

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

602 Nuclear DNA analysis results of *S. flexuosa* and *S. maena* revealed that the
603 previous phylogenetic analyses were in accordance with the taxonomies of the two
604 species as no haplotypes were shared between them. The genetic differentiation
605 between these two species of *Spicara* confirms that reproductive isolation of the two
606 species was complete. Moreover, the total number of mutations and the number of
607 parsimony informative sites between the two *Spicara* species were not as high in the
608 IRBP gene as those between the mtDNA genes, suggesting that the lack of the
609 discrimination could be due to slower evolution rate of the former (Verma et al., 2004).
610

611 **5. CONCLUSION AND RECOMMENDATIONS**

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

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

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642 **Ethical Issue**

643 The work raises no ethical issues. All fish examined were from commercial fishing
644 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>	F_S
	Total <i>S. flexuosa</i>	196	-1.6251	-7.8670
	TSS	58	-	-
COI gene	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
	Total <i>S. flexuosa</i>	143	-1.6899	-7.6158
	TSS	62	-1.0804	-1.8164
cyt-b gene	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
	Total <i>S. flexuosa</i>	348	-2.2274	-56.5358
	TSS	120	-2.0581	-15.5390
IRBP gene	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

1017 **FIGURE CAPTIONS**

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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.

1019 **Figure 2.** a.) *Spicara maena* FishTrace specimen SpiMae-EM-04, b) *S. flexuosa*:
1020 FishTrace specimen SpiFle-EM-03, (Photos by Panos Leontarakis/Laurence Favre-
1021 Krey), c) *S. maena* collected from Greece, d) *S. flexuosa* collected from Turkish coastal
1022 waters.

1023 **Figure 3.** Haplotype networks of a) COI, b) cyt-b and c) IRBP genes of *Spicara* spp.
1024 Note: Background of pink color indicates *S. flexuosa*, blue color indicates *S. maena*,
1025 and white color indicates *S. smaris*. The numbers correspond to number of mutations
1026 between haplotypes in A, B and C.

1027 **Figure 4.** Bayesian topology of the concatenated mtDNA (COI and cyt-b) genes (total
1028 809 bp) of *Spicara* spp. The numbers on the branches are the Bayesian posterior
1029 probabilities. TSS: Turkish Straits System. Hap1: *S. smaris* GenBank accession no:
1030 COI: KT883623, cyt-b: KT883672.

1031 **Figure 5.** BEAST chronogram based on the concatenated mtDNA (COI and cyt-b)
1032 genes of *Spicara* spp. without outgroup dating. Values above branches are the mean
1033 node height (age). Bars show 95 % HPD. Values below branches are posterior
1034 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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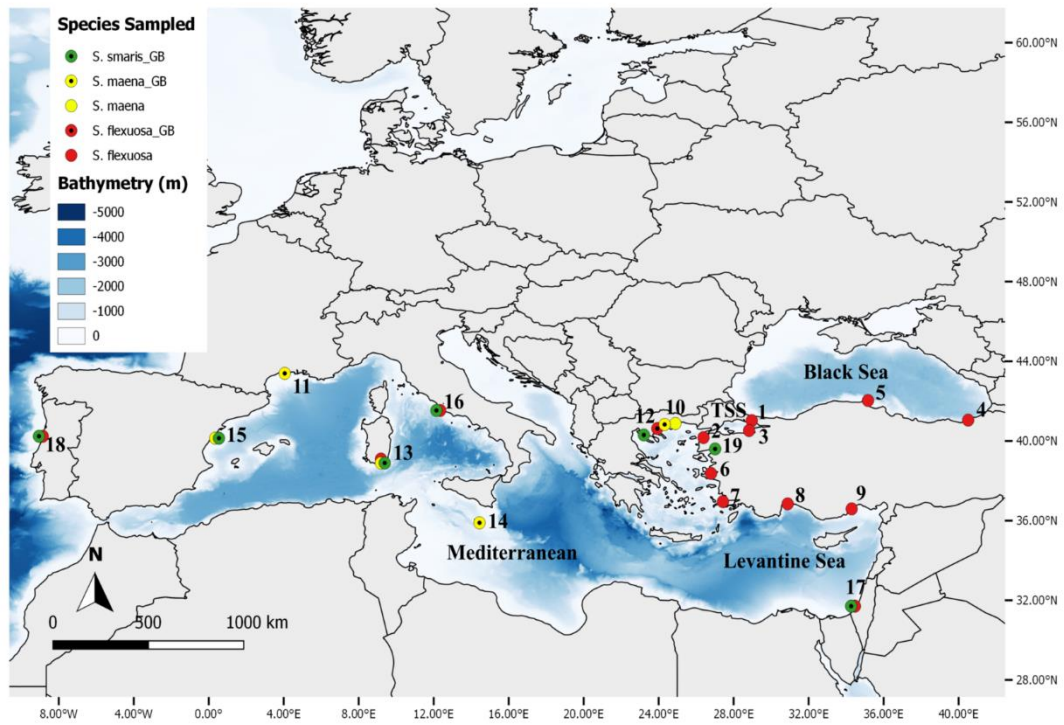
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1044 Figure 1.

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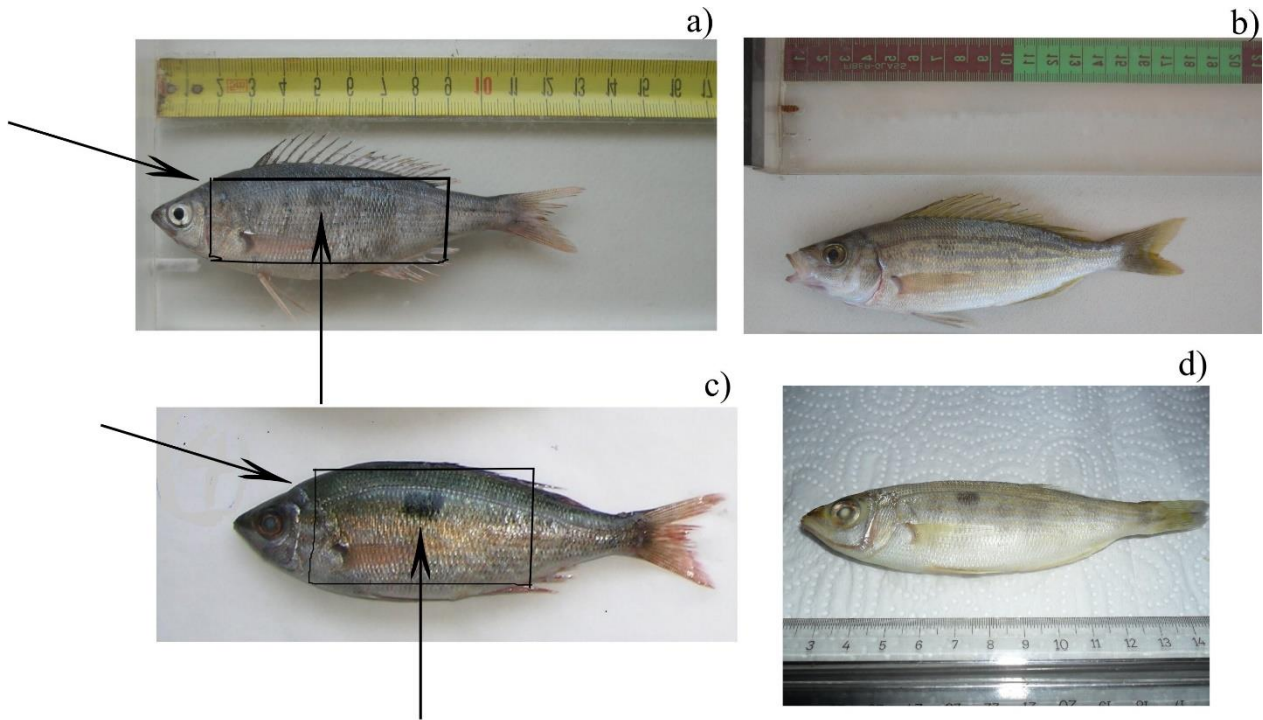
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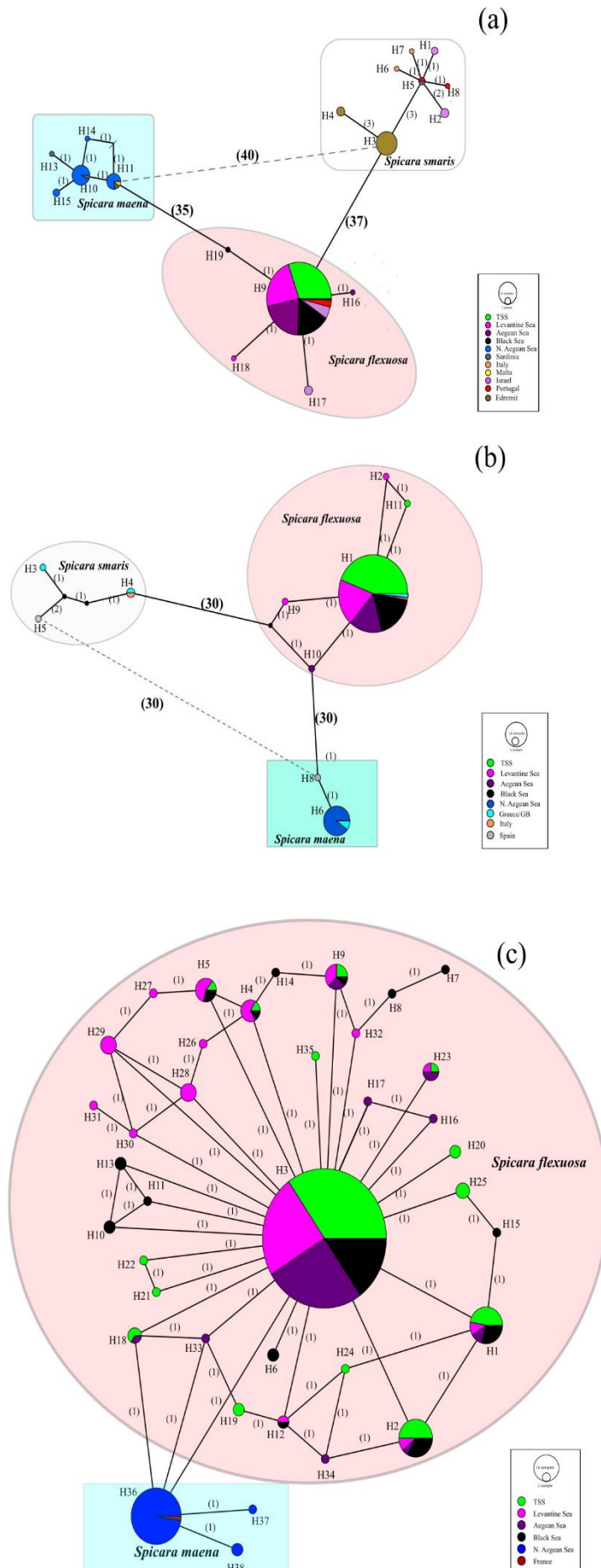
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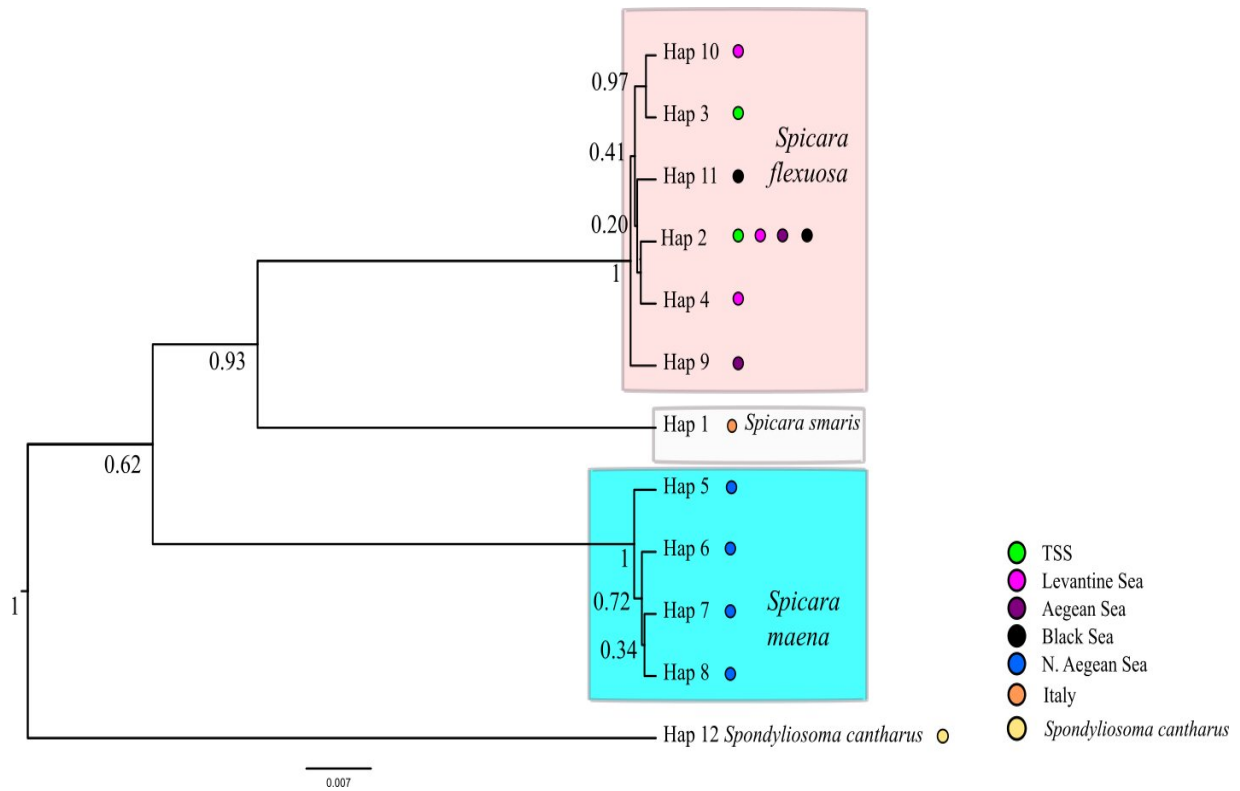
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Figure 3.



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1080 Figure 4.

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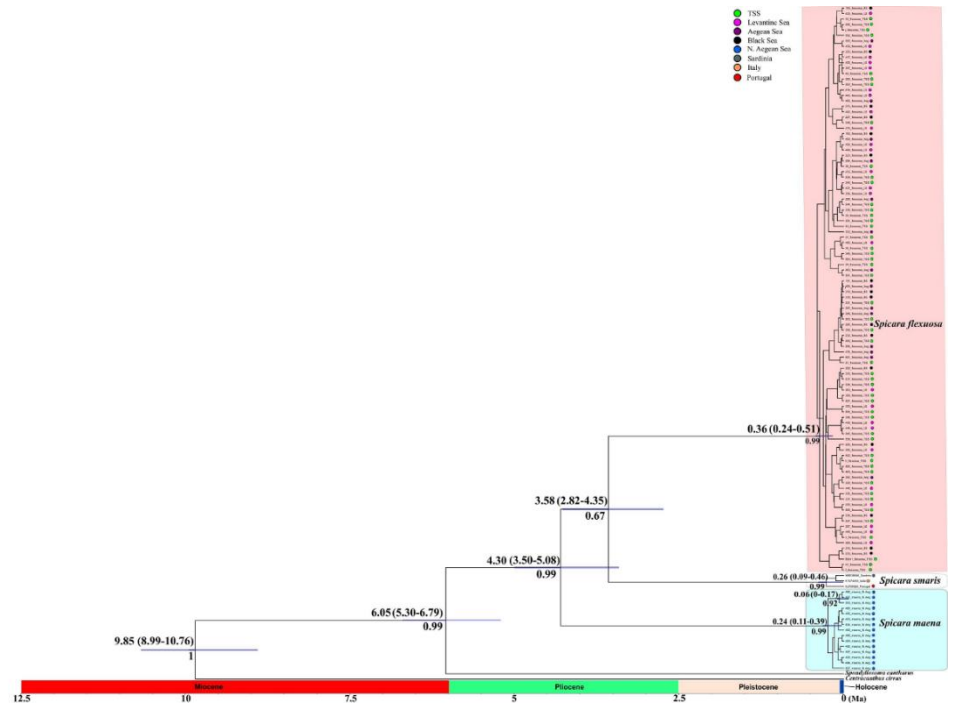
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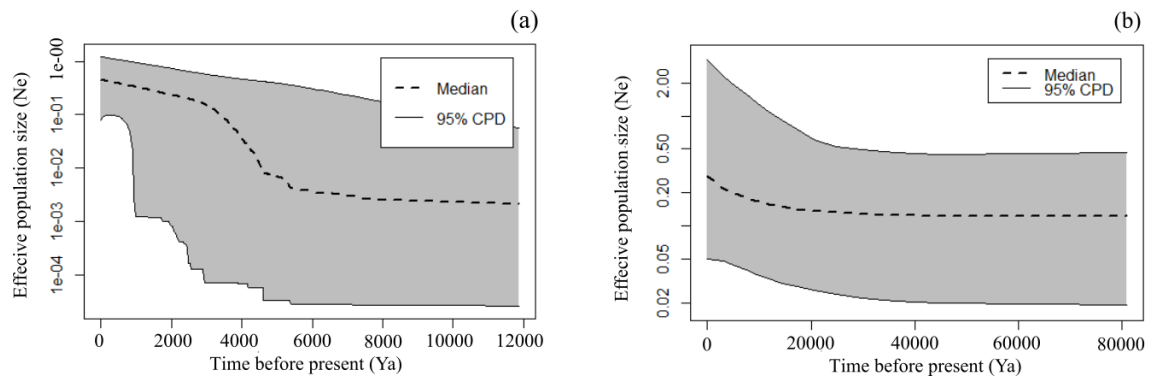
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1121 Figure 6.

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