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**Phylogeography and Population Dynamics of the Eastern Mediterranean Whiting (*Merlangius merlangus*) from the Black Sea, the Turkish Straits System, and the North Aegean Sea**

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1 **ABSTRACT**

2 In this study, the taxonomic resolution and phylogenetic relationship of two presumed  
3 subspecies of *Merlangius merlangus*, namely *M. m. euxinus* and *M. m. merlangus*, were  
4 investigated. In addition, the effect of the Turkish Straits System on the evolutionary  
5 history and genetic structure of *M. merlangus*, was explored. The mitochondrial  
6 cytochrome *c* oxidase subunit 1 and cytochrome *b* genes, and the nuclear recombination  
7 activating gene-1 were analysed. Our results indicate no clear distinction between the  
8 two presumed subspecies, which is attributed to the low resolution of recombination  
9 activating gene-1 and/or presence of potential gene flow between the two subspecies.  
10 The temporal pattern of divergence between the two presumed subspecies related to the  
11 Last Glacial Maximum (219 Kya), whereas the expansion of each main sampling  
12 location occurred after the flooding of the Black Sea by salt water from the  
13 Mediterranean (5 Kya), following a period of stability. Additionally, significant genetic  
14 differences are observed among the North Aegean samples and the collections from the  
15 Turkish Straits System and the Black Sea, along with some significant structure among  
16 sampling sites located in the Turkish Straits System and Black Sea. The lower genetic  
17 variability of the eastern Mediterranean *M. merlangus* when compared to Atlantic ones  
18 might be due to a potential population bottleneck before the last glacial period, a trend  
19 that is commonly found in these waters.

20 **Key words: Turkish Straits System, mitochondrial DNA, population structure,**  
21 **divergence, Gadoid.**

22

23

24

25 **1. INTRODUCTION**

26

27 Molecular phylogeographic studies have been used to resolve taxonomic relationships  
28 among species and assess evolutionary processes that can explain population diversity  
29 in time and space (Avice, 2000). In the marine environment where boundaries are not  
30 easily determined, molecular tools have proven to be extremely important in delineating  
31 species structure in fragmented geographical areas, and understanding dispersal patterns  
32 of organisms (Patarnello et al., 2007). Furthermore, analyses with these molecular tools  
33 can contribute to the conservation of individual species, and identification of marine  
34 protected areas (Avice et al., 1987). Previous studies of marine fishes have shown that  
35 biogeographical barriers and differences in environmental and oceanographic factors  
36 could affect population connectivity and cause strong genetic differences among stocks  
37 (Durand et al, 2013; Magoulas et al., 1996). Such knowledge is extremely important for  
38 the effective management of commercially important fish species, particularly in areas  
39 with pronounced geographical and oceanographic gradients, such as the Eastern  
40 Mediterranean.

41 The Turkish Straits System (TSS), consisting of the Bosphorus, the Sea of  
42 Marmara and the Dardanelles, plays a significant biogeographical role as the major  
43 connection between the Black Sea and the Mediterranean (Öztürk, 1998). The system  
44 serves as a barrier and/or a corridor for various marine species due to its distinguishing  
45 hydrographical characteristics (Öztürk and Öztürk, 1996). It acts as a physical barrier  
46 against dispersal of fish (Magoulas et al., 1996, 2006; Turan et al., 2009a, 2009b;  
47 Durand et al., 2013), fish larvae (Moraitou-Apostolopoulou, 1985), zooplanktonic  
48 species (Peijnenburg et al., 2004), and mammals (Viaud-Martinez et al., 2008). The

49 TSS also acts as a biological corridor for migratory species of fish (Turan et al., 2006)  
50 from the Black  
51 Sea and the Mediterranean Sea, and as feeding and spawning grounds for pelagic fish of  
52 Atlantic origin (Acara, 1957; Akşiray, 1987; Kocataş et al., 1993) during their migration  
53 from the Black Sea to the Sea of Marmara, or vice versa. Some examples include the  
54 bluefish (*Pomatomus saltatrix*) (Turan et al., 2006), the Mediterranean turbot  
55 (*Scophthalmus maximus*) (Suziki et al., 2004) and the flounder (*Platichthys flesus*  
56 *luscus*) (Borsa et al., 1997).

57 *Merlangius merlangus* (Linnaeus, 1758) (Gadidae) is a marine benthopelagic  
58 fish, found predominantly in the Northern hemisphere and in circumpolar temperate  
59 waters. It is presumed to be divided into two subspecies based on geographical  
60 distribution, and diagnostic morphological characters. The North Atlantic Ocean  
61 whiting, *Merlangius merlangus merlangus* (Linnaeus, 1758), that occurs along the  
62 European coasts from Iceland and south-western Barents Sea, to the western Baltic and  
63 the northern coasts of Portugal, exhibits a restricted distribution in the western  
64 Mediterranean. Conversely, *Merlangius merlangus euxinus* (Nordmann, 1840) inhabits  
65 the Black Sea, the adjoining areas of the Azov Sea, the Sea of Marmara, the Aegean,  
66 and the Adriatic Sea (Bailly, 2008; Parin et al., 2014). The two presumed subspecies are  
67 identified by the following diagnostic features: *M. m. euxinus* has a small barbel, and a  
68 pectoral fin reaching about 15.4-18.2% of the body length, while *M. m. merlangus* does  
69 not have a barbel and its pectoral fin is around 13.8-15.6 % of the body length  
70 (Whitehead et al., 1986).

71 Both presumed subspecies are of high commercial importance in their respective  
72 distributional ranges. In the Baltic Sea, *M. merlangus* is considered as Vulnerable due to  
73 a 30% decline of stocks over the last 12 years (HELCOM, 2013). In the Mediterranean

74 and Black Sea, however, *M. merlangus* has been reported as Least Concern (IUCN,  
75 2011). According to FAO Working Group on the Black Sea report (FAO, 2017),  
76 whiting has been overexploited in recent years (1994-2015). The total mean annual  
77 catch of whiting among Black Sea countries [(Bulgaria, Georgia, Romania, Russian and  
78 Ukraine (excluding Turkey)] was less than 0.6 thousand tons between 1996-2005  
79 (Shlyakhov and Charova, 2003). Turkey is the only country in the Black Sea with  
80 targeted trawling fisheries for whiting with mean annual catches dropping by  
81 approximately 40% between 1986-1995 and 1996-2005 (from 17.6 thousand tons to  
82 10.8 thousand tons, respectively), showing a general trend of decline in stocks. Illegal  
83 and unregulated fishing are the main threats that affect their abundance in the Black Sea  
84 (Özdemir et al., 2018).

85         Studies on the population structure of the whiting (*Merlangius merlangus*)  
86 revealed contrasting results. Nuclear markers, such as microsatellites, showed a small  
87 scale spatial structure in the North Atlantic (Rico et al., 1997; Charrier et al., 2007),  
88 whereas high gene flow was detected using the cytochrome *c* oxidase subunit I (COI)  
89 mitochondrial gene among sites in the North East Atlantic (Eiriksson and Arnason,  
90 2014). Additionally, Bektas and Belduz (2007) detected two main groups among *M. m.*  
91 *euxinus* populations in the Black Sea using Random Amplified Polymorphic DNA  
92 (RAPD) markers, whereas morphometric and meristic characters verified the presence  
93 of one stock in the area (İşmen, 1995, 2001). However, no taxonomic resolution of the  
94 two presumed subspecies of *Merlangius merlangus* has been previously investigated  
95 along the species' distribution. Here, we undertook multilocus analyses using both  
96 mitochondrial DNA (mtDNA) (COI and cytochrome *b*, *cyt-b*) and nuclear DNA  
97 (nuDNA) (Recombination Activating Gene-1, RAG1) markers. Intra- and inter-  
98 population analyses were applied to examine the taxonomic limits of the presumed

99 *Merlangius* subspecies. Additionally, phylogeographic analyses are presented to address  
100 intraspecific genetic structure, assess the stock status in the Northeastern Mediterranean  
101 Sea, and estimate the demographic history of *M. m. euxinus* populations.

102

## 103 **2. MATERIALS AND METHODS**

104

### 105 *2.1. Sampling sites*

106 Individuals of *Merlangius merlangus euxinus* were collected from eight stations, four in  
107 the Black Sea (Rize, Sinop, Inebolu and Zonguldak in Turkey), three in the TSS  
108 (Istanbul, Bandırma and Canakkale in Turkey), and one in the North Aegean Sea  
109 (Thermaikos Gulf in Greece). These areas (Black Sea, TSS and the North Aegean Sea)  
110 are described herein as the main sampling locations. Additionally, two *Merlangius*  
111 *merlangus merlangus* specimens were collected from one sampling station along the  
112 north coast of France (Ault) (Fig. 1, Supplementary Table 1). A total of 233 specimens  
113 were collected from otter trawl and handline fisheries between 2013-2015. Additionally,  
114 GenBank entries were included in the data set (Fig. 1, Supplementary Tables 1 and 2).  
115 The detailed descriptions (locations, references and regions) of all samples are provided  
116 in Fig. 1, and the Supplementary Tables 1, 2 and 3. Tissue was preserved in 80%  
117 ethanol at room temperature in the field, and at -20 °C in the laboratory, until further  
118 processing.

119

### 120 *2.2. DNA extraction, amplification and sequencing of genetic markers*

121 Genomic DNA was isolated from the muscle or caudal fin tissue with a PureLink®  
122 Genomic DNA Kit (Invitrogen, Carlsbad, USA), following the manufacturer's  
123 protocols. The mtDNA and nuDNA fragments were amplified via Polymerase Chain

124 Reaction (PCR) using the primers COI-Fish-F1 and COI-Fish-R1 (Eiriksson and  
125 Arnason, 2014; Kochzius et al., 2010) for COI, GluDg and Cb3h (Roques et al., 2006)  
126 for cyt-b, and newly designed primers for RAG1 (Supplementary Table 4).  
127 Amplifications were performed in a total volume of 25  $\mu$ L using different PCR profiles  
128 and amplification conditions per gene (Supplementary Appendix SI). All amplified  
129 products were purified and sequenced commercially (Macrogen Europe, Amsterdam,  
130 The Netherlands).

131

### 132 *2.3. Phylogenetic analyses*

133 All chromatograms were edited and aligned manually with Sequencher v.5.4.1  
134 (GeneCodes Corp.). The mtDNA PCR products were sequenced in the forward  
135 direction, as there were no indications of heterozygous sites. The resulting alignments  
136 were verified by eye. Additionally, stop codons were investigated for mtDNA genes to  
137 determine presence/absence of mitochondrial pseudogenes by using DAMBE v.6.4.1  
138 (Xia, 2017). Finally, potential amplification/sequencing errors were controlled by  
139 aligning all sequences to detect polymorphic sites and subsequently confirming the  
140 clarity of the peaks at these sites. The nuDNA products were sequenced bidirectionally  
141 to verify the presence/absence of heterozygous sites that were also confirmed in  
142 Sequencher.

143 The models of sequence evolution for the protein-coding mtDNA markers with  
144 partitions and the nuclear gene were selected using the Bayesian Information Criterion  
145 in PartitionFinder v.1.1.0 (Lanfear et al., 2012) (Supplementary Table 5). Phylogenetic  
146 analysis was performed in MrBayes v.3.2.2 (Ronquist et al., 2012) with two  
147 independent runs of  $5 \times 10^6$  generations and four parallel Markov chain Monte Carlo  
148 (MCMC) chains, discarding the initial 25% as burn-in. Maximum likelihood analyses



149 (ML) were performed in RAxML v.8.0 (Stamatakis et al., 2014) using 1,000 bootstrap  
150 replications. *Melanogrammus aeglefinus* [(DQ020497 (extracted from complete  
151 mitogenome) for COI, NC007396 for cyt-b, and AJ566336.2 for RAG1)], *Gadus*  
152 *morhua* (EU877731 for COI, NC002081 for cyt-b, and FJ215242, KP644390 for  
153 RAG1), and *Merluccius merluccius* (KX782819, KX782949 for COI, EU264016,  
154 EU492347 for cyt-b and JN230904 for RAG1) were used as outgroups.

#### 155 2.4. Population structure and demographic history analyses

156 Number of haplotypes, haplotype diversity ( $h$ ) (Nei and Tajima, 1981), nucleotide  
157 diversity ( $\pi$ ) (Nei, 1987), number of polymorphic sites, singleton and parsimony  
158 informative sites and the number of net nucleotide substitutions (percentage of pairwise  
159 sequence divergence) between subspecies were calculated in DnaSP v.5.10.1 (Librado  
160 and Rozas, 2009). Pairwise genetic distances between subspecies were also determined  
161 in MEGA v.7.0 (Kumar et al., 2016), based on the Kimura's two parameter model  
162 (Kimura, 1980).

163 Genetic differences among localities were estimated in Arlequin v.3.5.2.2  
164 (Excoffier and Lischer, 2010) using the genetic distance-based  $\Phi_{ST}$  and the pairwise  $F_{ST}$   
165 values using 10,000 randomizations. Bonferroni corrections (Rice, 1989) were used to  
166 adjust for the significance of the  $P$  values for multiple tests. A hierarchical analysis of  
167 molecular variance (AMOVA) to test for significance of differentiation between groups  
168 was performed in Arlequin. Data were grouped according to geographical locations,  
169 within the following hierarchy: i) TSS (Istanbul, Canakkale, Bandırma), ii) Black Sea  
170 (Rize, Sinop, Zonguldak, Inebolu) and iii) North Aegean Sea (Thermaikos Gulf,  
171 Greece). Analysis was performed only for *M. m. euxinus* due to sample size constraints.

172 Mismatch distribution of the number of pairwise differences (Slatkin and  
173 Hudson, 1991; Rogers and Harpending, 1992) between haplotypes, which can result

174 from demographic or spatial population expansion, were estimated with DnaSP.  
175 Additionally, Tajima's  $D$  (Tajima, 1989) and Fu's  $F_s$  (Fu, 1997) neutrality tests used to  
176 detect population growth, as well as the raggedness statistic " $rg$ " (Harpending et al.,  
177 1993; Harpending, 1994), which quantifies the smoothness of the fit of the observed  
178 mismatch distribution to one expected under a population expansion model, were  
179 performed in DnaSP. Significance of neutrality tests and  $rg$  values were tested with  
180 1,000 coalescence simulations. Past population demography of three different regions  
181 (TSS, Black Sea and North Aegean Sea) of *M. m. euxinus* was reconstructed using  
182 Extended Bayesian Skyline Plots (EBSP) (Heled and Drummond, 2008; Ho and  
183 Shapiro, 2011), as implemented in BEAST v.2.4.8 (Bouchkaert et al., 2014). A strict  
184 molecular clock was set using the multi-locus dataset (three genes: COI, cyt-b, RAG1),  
185 the previously selected substitution models (Supplementary Table 5), and time was  
186 scaled using the estimated rate for each marker (see Results section 3.5 Divergence time  
187 estimates). The Bayesian Markov Chain Monte Carlo (MCMC) was set for  $1 \times 10^8$   
188 generations, discarding the initial 10% of samples as burn-in. Convergence of runs was  
189 evaluated on TRACER v.1.6. (Rambaut et al., 2014). Visualization of the EBSP plots  
190 was done within RStudio v.1.1.463 in R v.3.5.1. (R Core Team, 2018). Finally,  
191 haplotype networks for each gene were constructed using the median joining method  
192 (Bandelt et al., 1999) as implemented in PopART v.1.7 (Leigh and Bryant, 2015).

193

#### 194 *2.5. Divergence time estimation*

195 The evolutionary divergence time of phylogenetic separation of the two potential *M.*  
196 *merlangus* subspecies was estimated in BEAST. *Melanogrammus aeglefinus* and *Gadus*  
197 *morhua* were used as outgroups. No calibration was used, and the best substitution  
198 model for each gene was applied (Supplementary Table 5). The coalescent constant

199 population size tree prior was applied to tackle divergence among closely related  
200 lineages (Ho et al., 2005), and an uncorrelated lognormal relaxed clock was used, as  
201 multiple loci can be incorporated into the analysis and also deal with different rates  
202 among loci. In order to account for the variation of substitution rates among the three  
203 genes (Chiriki-Adeeb and Chiriki, 2016), the rate for all markers was estimated. The  
204 analysis was executed twice for  $2 \times 10^8$  generations, sampling every 5,000 generations  
205 and discarding the initial 20% as burn-in. Convergence was confirmed in TRACER  
206 v.1.6, and the effective sample sizes (ESS) for all model parameters were assessed  
207 (values higher than 200) indicating adequate sampling intensity for all parameters.

208

### 209 **3. RESULTS**

210

#### 211 *3.1. Summary statistics*

212 Sequences of *M. merlangus* were obtained for COI (481 bp), cyt-b (611 bp) and RAG1  
213 (346 bp) regions, for 164, 129 and 173 individuals, respectively (Supplementary Table  
214 6). No stop codons or ambiguous sites were observed in any of the COI and the cyt-b  
215 sequences. *Merlangius merlangus euxinus* exhibited eight haplotypes and seven  
216 polymorphic sites in the COI marker, with haplotype and nucleotide diversities of  
217 0.1450 and 0.0003, respectively (Supplementary Table 6). Conversely, diversity indices  
218 were higher for the cyt-b (22 haplotypes; 19 polymorphic sites,  $h = 0.8730$ ,  $\pi = 0.0029$ )  
219 and the nuclear marker (22 haplotypes; 14 polymorphic sites;  $h = 0.4940$ ,  $\pi = 0.0025$ )  
220 (Supplementary Table 6). Interestingly, higher genetic diversity was observed along the  
221 Turkish coastline TSS (Istanbul) and Black Sea when compared to the North Aegean  
222 Sea (Thermaikos Gulf) for the mtDNA markers [COI (Black Sea:  $h = 0.2070$ ,  $\pi =$   
223  $0.0005$ ; TSS: Istanbul:  $h = 0.1950$ ,  $\pi = 0.0004$ , Thermaikos Gulf:  $h = 0.1080$ ,  $\pi =$

224 0.0002); cyt-b (Black Sea:  $h = 0.7970$ ,  $\pi = 0.0023$ ; TSS:  $h = 0.8800$ ,  $\pi = 0.0034$ ,  
225 Thermaikos Gulf:  $h = 0.7460$ ,  $\pi = 0.0020$ ], a trend that was generally reversed for the  
226 nuclear marker (Black Sea:  $h = 0.4300$ ,  $\pi = 0.0022$ ; TSS:  $h = 0.5050$ ,  $\pi = 0.0028$ ;  
227 Thermaikos Gulf:  $h = 0.5660$ ,  $\pi = 0.0019$ ) (Supplementary Table 6). The *Merlangius*  
228 *merlangus merlangus* samples exhibited higher values in diversity indices compared to  
229 the *M. m. euxinus* (Supplementary Table 6), however, the results were not taken into  
230 account due to their small size ( $n \leq 5$ ).

231 A total of 11 polymorphic sites were reported for the COI gene (2.2% of 481bp,  
232 164 sequences), of which seven positions (1.4%) were parsimony informative, and one  
233 genomic position (position 316) being fixed between the two potential subspecies  
234 (Supplementary Table 7). For this marker, the net pairwise sequence divergence  
235 between the presumed *M. m. euxinus* and *M. m. merlangus* groups was 0.331%.  
236 Additionally, 20 polymorphic sites (3.2% of 611 bp, 129 sequences) were detected in  
237 the cyt-b gene, of which nine positions (1.6%) were parsimony informative. One  
238 genomic position (position 611) was diagnostic between the two subspecies  
239 (Supplementary Table 8). For cyt-b, the net pairwise sequence divergence between the  
240 *M. m. euxinus* and *M. m. merlangus* groups was 0.337%. Thus, the genetic distance  
241 values for both markers between the two subspecies are very similar (approx. 0.3%).

242

### 243 3.2. Phylogenetic relationships

244 Both Bayesian and ML topologies of the concatenated dataset resolved one major clade  
245 for *M. merlangus*. Within *Merlangius*, there is a shallow lineage of *M. m. merlangus*  
246 with a low posterior probability and bootstrap support (0.53 and 55, respectively) that  
247 contained samples from the Black Sea, the TSS, North Aegean and France  
248 (Supplementary Figure 1). Additionally, two shallow geographical groups were also

249 revealed, one formed by the majority of the North Aegean Sea (Greece) specimens with  
250 posterior probability and bootstrap support of 0.60 and 35, respectively, and the second  
251 comprised of fish caught along the Turkish coast (Black Sea and TSS) with high  
252 posterior probability ( $> 0.70$ ) but low bootstrap values ( $< 70$ ). Similar trends were  
253 detected in the topologies of the individual markers, with the inclusion of additional  
254 samples from each sampling locality (Supplementary Figs. 2-4).

255

### 256 3.3. Population structure

257 Pairwise  $\Phi_{ST}$  values for the concatenated mtDNA dataset of *M. m. euxinus* subspecies,  
258 ranged from -0.045 to 0.364 (Table 1). Most pairwise comparisons involving the North  
259 Aegean Sea and the Turkish sampling localities (Istanbul, Canakkale, Bandırma, Rize,  
260 Sinop) showed significant differentiation following Bonferroni corrections (Table 1).  
261 The highest  $\Phi_{ST}$  value, which remained significant after Bonferroni corrections, was  
262 observed between Canakkale and Sinop ( $\Phi_{ST} = 0.364$ ). No significant differences were  
263 observed among localities within the main two Turkish sampling areas (TSS: Istanbul,  
264 Canakkale, Bandırma; Black Sea: Rize, Sinop, Zonguldak, Inebolu). Conversely,  
265 pairwise  $\Phi_{ST}$  values among three main regions (TSS, Black Sea, North Aegean Sea)  
266 were significant. Significant  $\Phi_{ST}$  values were observed between TSS and the Black Sea  
267 ( $\Phi_{ST} = 0.141$ ) and between TSS and the Aegean Sea ( $\Phi_{ST} = 0.171$ ) and between the  
268 Black Sea and the North Aegean Sea ( $\Phi_{ST} = 0.188$ ) (Supplementary Table 9).  
269 Hierarchical AMOVA (Table 2) showed significant variation among the three main  
270 groups ( $\Phi_{CT} = 0.1580$ , 15.80%). The highest percentage of genetic variation was  
271 detected within populations of these regions ( $\Phi_{ST} = 0.1833$ , 81.67%), however, no  
272 significant differences were detected among populations within groups ( $\Phi_{SC} = 0.0300$ ,  
273 2.53%) (Table 2).

274 Similar trends in  $F_{ST}$  values were detected for the nuclear RAG1 marker, ranging  
275 from -0.021 to 0.258 (Table 1). All but one (North Aegean Sea vs. Bandırma) pairwise  
276 comparisons between the North Aegean Sea and the Turkish sampling sites were  
277 significantly different. Moreover, no significant differences were observed among  
278 localities within the TSS (Istanbul, Çanakkale, Bandırma), a pattern that was not  
279 followed within the Black Sea sites (Rize, Sinop, Zonguldak, Inebolu), where the pair  
280 Rize and Inebolu exhibited the highest value ( $F_{ST} = 0.258$ ). Additionally, significant  $F_{ST}$   
281 values were also observed between TSS and North Aegean Sea ( $F_{ST} = 0.104$ ) and  
282 between Black Sea and North Aegean Sea ( $F_{ST} = 0.155$ ) (Supplementary Table 9). The  
283 AMOVA analysis detected no significant variation among groups ( $F_{CT} = 0.0419$ ,  
284 4.18%), whereas the variation among populations within groups and within populations  
285 was significant ( $F_{SC} = 0.0300$  and 2.51%;  $F_{ST} = 0.0866$  and 91.34 %, respectively)  
286 (Table 2).

287

### 288 3.4. Population history of *Merlangius merlangus*

289 A recent demographic population expansion was supported by the unimodal mismatch  
290 distributions (Supplementary Figs. 5, 6, and 7) of the three different genes from all *M.*  
291 *euxinus* main sampling localities. The population expansion model was also  
292 supported by the overall negative and significant  $F_S$  and  $D$  values, as well as the non-  
293 significant  $rg$  values (Supplementary Table 6).

294 No shared haplotypes were found among the presumed *M. m. euxinus* and *M. m.*  
295 *merlangus* individuals, whilst main haplotypes were separated by one mutational step in  
296 both mtDNA markers (Figs. 2a-b). A star-shaped haplotype network of the COI gene  
297 for the presumed *M. m. euxinus* subspecies showed that the most common haplotype  
298 (with a frequency of 79%) was found in eight different locations in the Black Sea, TSS

299 and the North Aegean Sea (Fig. 2a). A star-shaped haplotype pattern was not detected in  
300 the presumed *M. m. merlangus* (Fig. 2a). Interestingly, the haplotypes of the Black Sea  
301 specimens, which clustered with *M. m. merlangus* were separated by more mutational  
302 steps from the Black Sea *M. m. euxinus* than those from the Atlantic Ocean. A star-  
303 shaped network comprising haplotypes from the TSS, the Black Sea and the North  
304 Aegean Sea, was also observed in *M. m. euxinus* for cyt-b (Fig. 2b). Additionally, three  
305 haplotypes were the most common for *M. m. euxinus*, and were found in all sampling  
306 locations (Fig. 2b). For the RAG1 gene, the haplotype network revealed that the most  
307 common haplotype (70% of the individuals) was shared among all locations (Fig. 2c).  
308 As opposed to the mtDNA haplotype networks, the nuclear marker did not reveal any  
309 structure (Fig. 2c).

310         The EBSP analyses of the concatenated dataset indicated that the *M. m. euxinus*  
311 population size in the North Aegean Sea remained relatively constant until about 8 Kya,  
312 followed by a sharp population growth (Fig. 3a). Despite the initial trend of constant  
313 population size in the TSS and the Black Sea populations, a more recent and sharper  
314 onset of expansion (5-6 Kya) than the North Aegean was estimated (Figs. 3b-c).

315

### 316 3.5. Divergence time estimates

317 The divergence-time chronogram places the split between *Merlangius merlangus* and  
318 the two Gadoid species at an age of ~3.436 Ma (CI: 0.304-10.913 Ma). Within the  
319 *Merlangius merlangus* clade, the estimated divergence between the two presumed  
320 subspecies was about 219 Kya (CI: 25-687 Kya) (Fig. 4). The estimated mutation rates  
321 for the COI, cyt-b, and RAG1 were 5.083%, 3.444%, and 6.510% *per* million years,  
322 respectively.

323

## 324 **DISCUSSION**

### 325 *4.1. Phylogenetic analyses and genetic distances*

326 In this study, the phylogeography of *Merlangius merlangus* (*sensu lato*) was  
327 investigated with data from the Turkish coastal waters, the North Aegean Sea and the  
328 Atlantic region. Despite the large volume of studies describing the phylogeographical  
329 effect of the Strait of Gibraltar (Pillars of Hercules) on commercially important marine  
330 species (Rosel and Block, 1996; Ladoukakis et al., 2002; Bargelloni et al., 2003;  
331 Zardoya et al., 2004; Cimmaruta et al., 2005; Magoulas et al., 2006), little is known  
332 about the role of TSS and the underlying factors that could drive gene flow and  
333 diversification in the TSS and the seas adjacent to it.

334 Furthermore, there is a taxonomic uncertainty regarding the species under  
335 investigation, as two potential subspecies are often recognised in the literature:  
336 *Merlangius merlangus merlangus* and *Merlangius merlangus euxinus* (Özdamar et al.,  
337 1996; Bektas and Belduz, 2007; Nedreaas et al., 2014). The Black Sea whiting has been  
338 considered as a distinct subspecies (*M. m. euxinus* Nordmann, 1840), which is  
339 distinguished from *M. m. merlangus* by the presence of a barbel. However, the presence  
340 of individuals across the *M. m. merlangus* geographic distribution indicates that this  
341 character alone is not informative regarding the specific status of these presumed  
342 subspecies.

343 The available data from this study do not provide adequate arguments to verify  
344 or deny the subdivision of the *Merlangius merlangus*. For example, divergence time  
345 analyses using all three genes indicated two different subspecies lineages (Fig. 4). In  
346 addition, two geographically separated groups of *M. m. euxinus* from the North Aegean,  
347 and the TSS and the Black Sea, imply limited levels of gene flow between these water  
348 bodies. Furthermore, two geographically separated groups were also observed based on



349 the genetic structure analyses. On the other hand, topologies obtained from two  
350 approaches (Supplementary Figs. 1-4) revealed shallowly differentiated lineages of *M.*  
351 *m. euxinus* and *M. m. merlangus*. In addition, genetic distances and number of fixed  
352 polymorphisms were small: A total of seven and nine parsimony informative sites were  
353 observed for the COI and cyt-b genes, respectively, for both presumed subspecies,  
354 whilst only one absolute genomic position was fixed for differentiating between two  
355 subspecies based on each of the two mtDNA genes. Additionally, the genetic distance  
356 between the two presumed subspecies for the COI gene was about 0.3%, a value that is  
357 commonly found among conspecifics (Zhang and Hanner, 2011; Karahan et al., 2017).  
358 Furthermore, the relatively low nucleotide substitution values for both mitochondrial  
359 genes used in this study ( $D_a=0.00331$  and  $D_a=0.00337$  for COI and cyt-b genes,  
360 respectively) are consistent with the findings of distribution of genetic diversity within  
361 subspecies of other marine fish, e.g., *Mullus spp.* (Keskin and Can, 2009), and within  
362 other fish species, e.g. *Trachurus spp.* (Bektas and Belduz, 2008), and *Alosa spp.*  
363 (Turan et al., 2015a) from the same sampling locations, rather than between subspecies  
364 and species. Hence the signal from our data is not clear, in supporting or rejecting the  
365 hypothesis of two subspecies of *M. merlangus*. Further comparative molecular work  
366 (e.g. ddRAD) on the two subspecies is needed to test whether they represent different  
367 lineages or not.

368         The shallowly differentiated lineages of the two subspecies on the phylogenetic  
369 trees might have been influenced by different factors. Biologically speaking, gene flow  
370 between the two subspecies might be one of the reasons behind this lack of  
371 differentiation. Genetic homogeneity of the *Merlangius merlangus* populations, due to  
372 transportation and mixing of long-lived larvae and eggs by current system, was  
373 confirmed by the analyses of microsatellites in North Atlantic region (Rico et al., 1997;

374 Charrier et al., 2007) and the COI gene in the Northeast Atlantic (Eiriksson and  
375 Arnason, 2014). In addition, low numbers of specimens of *M. m. merlangus* that were  
376 sampled in our study (N=5) might also have contributed to the observed lack of  
377 differentiation. Finally, the RAG1 tree and haplotype network do not show indicate  
378 clear differentiation between the two presumed subspecies (e.g. there are no fixed  
379 differences between the two presumed subspecies), contributing to the lack of adequate  
380 differentiation.

381

382

#### 383 4.2. Molecular diversity, population expansion and evolutionary history

384 This study revealed that the overall COI haplotype and nucleotide diversities  
385 (Supplementary Table 6) of the presumed *M. m. euxinus* populations were lower than  
386 those reported for the Atlantic *M. m. merlangus* (Eiriksson and Arnason, 2014),  
387 particularly as sample sizes were similar ( $N_{\text{Atlantic}}= 139$ , vs.  $N_{\text{ThisStudy}}=159$ ). Low  
388 diversity levels in the area compared to those of the Atlantic were also observed in other  
389 fish species, such as the European sprat (*Sprattus sprattus*) and the thornback ray (*Raja*  
390 *clavata*) in the Mediterranean and the Black Sea (Chevolot et al., 2006; Debes et al.,  
391 2008; Limborg et al., 2012). Additionally, the low genetic variability of the eastern  
392 Mediterranean turbot (*Scophthalmus maximus*) compared to the western stock, was  
393 attributed to a population bottleneck (Suziki et al., 2004). Recent founder events,  
394 pollution and illegal fishing are also considered to be factors that negatively affect  
395 genetic diversities in marine species populations, such as Black Sea harbour porpoise  
396 (Viaud-Martínez et al., 2008). Similarly, the lower genetic variability of the eastern  
397 Mediterranean *M. merlangus* could be attributed to a potential population bottleneck

398 before the last glacial period and/or differences in population expansion patterns among  
399 the Atlantic and the Eastern Mediterranean/Black Sea stocks.

400 Past glaciation cycles and associated sea level changes seem to have affected the  
401 diversification and distribution of fish species in the Mediterranean (O'Regan et al.,  
402 2011). Here, divergence among the two presumed subspecies appears to predate the last  
403 glacial period, with estimated divergence time of 219 Kya. The mid-Saalian glaciation  
404 was characterised by interglacial episodes with subsequent fluctuation in sea level that  
405 could have potentially led to the diversification of the Eastern Mediterranean whiting  
406 (Waelbroeck et al., 2002). Moreover, the last glacial maximum (LGM) seems to have  
407 promoted lineage diversification and shaped geographical distribution within *M.*  
408 *merlangius euxinus* in the Eastern Mediterranean (Fig. 4). The Eastern Mediterranean  
409 basin has been previously considered to be a refuge during glaciations (Ekman, 1967;  
410 Patarnello et al., 2007). The post LGM sea level rise in rates of about 15 mm/year from  
411 16,000 to 12,500 and from 11,500 to 9,000 years ago (Lambeck et al., 2002) could have  
412 facilitated the dispersal and subsequent diversification of the species to the Aegean Sea.  
413 The proposed split (of approximately 219 Kya) might be consistent with a glacial or  
414 interglacial event, however, such lengthy periods of isolation should have had a stronger  
415 genetic impact on the evolution of the presumed subspecies.

416 The Eastern Mediterranean whiting has undergone a recent and sharp expansion  
417 in all three main sampling areas. The star-like haplotype networks, the significant  
418 negative  $F_s$  and  $D$  values, the unimodal mismatch distributions for all markers and the  
419 three EBSP plots corroborate such suggestion. Moreover, the EBSP results of the fully  
420 concatenated dataset revealed that the North Aegean Sea population was nearly constant  
421 until ~8 Kya. It also showed that the species underwent a recent expansion, which took  
422 place after the LGM and coincided with the opening of the Dardanelles (~8 Kya, Fig

423 3a). The timing of the expansion of both TSS and Black Sea *M. m. euxinus* populations  
424 significantly postdate the Last Glacial Maximum (~18 Kya) and the opening of the  
425 Dardanelles (~8 Kya) (Fig 3b-c). The time difference (~2000 years) between the  
426 expansion of the Black Sea and TSS (5-6 Kya) and the opening of the Dardanelles (~8  
427 Kya) might be attributed to the time required to create suitable habitat conditions for  
428 the settlement and expansion of the species, as suggested in previous studies (Rohnling  
429 et al., 2009; Fontaine et al., 2012). Before the opening of the Dardanelles, the Black Sea  
430 was considered to be a freshwater lake (Zaitsev and Mamaev, 1997), therefore low  
431 salinity waters would not have been a favourable environment for the survival of a  
432 marine species. The last opening of the Dardanelles and the flooding of the Black Sea  
433 basin by salt water from the Mediterranean Sea, might have allowed the subsequent  
434 geographic dispersal and establishment of *M. m. euxinus* into the Black Sea which  
435 occurred over the last 8 Kya (Ryan et al., 1997). Therefore, the Eastern Mediterranean  
436 whiting might have dispersed and expanded firstly into the North Aegean Sea, and  
437 subsequently into the TSS and Black Sea as observed in other fish species (Durand et  
438 al., 2013). Interestingly, the Eastern Mediterranean stocks showed a more recent  
439 expansion than the North East Atlantic whiting populations. The later have undergone  
440 an earlier sudden expansion, which took place approximately 70 Kya ago (Eiriksson and  
441 Arnason, 2014), in an area where high levels of gene flow were detected. This trend  
442 could also be attributed to water temperature and water level fluctuations, as well as  
443 interactions with other species (Eiriksson and Arnason, 2014).

444

#### 445 *4.3. Population differentiation*

446 Our results revealed a pattern of sub-structure and connectivity, with three  
447 geographically defined populations (Black Sea, TSS, North Aegean Sea), and relatively

448 low levels of gene flow among the sampling sites in the Black Sea and TSS. Significant  
449 genetic differentiation was observed among the main sampling locations of *M. m.*  
450 *euxinus* based on the concatenated mtDNA data, which was corroborated by the nuDNA  
451 marker results. Moreover, the high values of all molecular diversity indices, and the  
452 significant genetic structure between North Aegean and locations along the Turkish  
453 coast suggest that the TSS is likely to be a barrier to gene flow for *M. m. euxinus*.  
454 Despite the genetic isolation of the Black Sea and TSS from the North Aegean *M. m.*  
455 *euxinus*, low connectivity levels among Turkish sampling sites were identified in our  
456 results; a single lineage of *M. m. euxinus* was recovered (Supplementary Fig. 1) and  
457 non-significant pairwise genetic differences were also detected (Table 1). The  
458 Dardanelles Strait is extremely important to the water exchange of the Mediterranean  
459 and Black Seas systems (Kanarska and Maderich, 2008), creating differences in  
460 temperature, salinity and density between them (Sayın et al., 2011). Such physical  
461 barriers could instigate genetic substructuring despite the long pelagic stage of the  
462 species (Zheng et al., 2001). Previous studies have also highlighted that straits can act as  
463 barriers to dispersal for whiting. The Dogger Bank in the North Sea has been suggested  
464 to serve as a barrier that prevents mixing between southern and northern populations of  
465 whiting (Pilcher et al., 1989), a conclusion that has been confirmed by genetic studies  
466 (Rico et al., 1997; Charrier et al., 2007). Similarly, differences have been reported  
467 between the Eastern Mediterranean and Black Sea *Sarda sarda* populations (Roberti et  
468 al., 1993; Turan et al., 2015b), whilst two potential *Diplodus annularis* stocks were also  
469 reported based on the cyt-b gene analyses along the Turkish coasts (Bektas et al, 2016).

470 Interestingly, a few specimens from the Black Sea and the TSS clustered with *M.*  
471 *merlangus* samples from the Atlantic (Figs 2a-b, Supplementary Figs 2-3). As the  
472 total period of larval stages of *Merlangius merlangus* is longer than one month (Hislop,

473 1984; Fischer et al., 1987), the transoceanic and/or interoceanic dispersal from the  
474 Atlantic or the Mediterranean to the Black Sea is possible through ballast water  
475 discharge. Although *M. merlangus* individuals were not previously detected in ballast  
476 tanks, marine organisms such as blennies and gobies (Wonham et al., 2000), and fish  
477 eggs and larvae were detected (Carlton, 1985). Fish can survive up to 21 days in a range  
478 of vessels (Wonham et al., 2000), making specimen survival and transport a possibility  
479 for fish in general, and in this case for *M. merlangus merlangus* in particular.

480

#### 481 4.4. Conclusions

482 In this study, the presence of different lineages between the Eastern Mediterranean  
483 whiting (*M. m. euxinus*) to those of the Atlantic (*M. m. merlangus*) were reported,  
484 however, our markers were not informative enough to support the taxonomic separation  
485 of the two presumed subspecies, especially given that the phylogenetic trees of  
486 individual markers failed to separate them in reciprocal monophyly. Additionally, our  
487 results showed that *M. m. euxinus* specimens across the main sampling areas of the  
488 North Aegean Sea, TSS, and Black Sea should be treated as different stocks, as  
489 restricted gene flow is reported across the Turkish locations. However, additional  
490 sampling in different parts of the Eastern Mediterranean and the Atlantic is required to  
491 define the distributional ranges of each presumed subspecies, whereas analyses are  
492 imperative to unravel gene flow among sampling locations, and hence to manage  
493 whiting stocks in these waters responsibly. Moreover, separate conservation measures  
494 and monitoring regimes are necessary for the protection of current stocks of whiting (*M.*  
495 *m. euxinus*), as intense climate fluctuations and illegal and/or unregulated fishing could  
496 affect the species structure and dynamics.

497

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## TABLES

**TABLE 1.** Pairwise  $\Phi_{ST}$  values for the COI+cyt-b data, below diagonal and  $F_{ST}$  values for the RAG1 above diagonal of *Merlangius merlangus euxinus*. Values in italics and bold were significant before and after Bonferroni corrections, respectively.

		Istanbul	Çanakkale	Bandırma	Rize	Sinop	Zonguldak	İnebolu	Greece	
<i>M. m. euxinus</i>	TSS	Istanbul	-	0.041	0.031	<b>0.132</b>	-0.004	0.013	-0.015	<b>0.187</b>
		Çanakkale	0.022	-	0.011	0.013	-0.014	0.011	0.078	<i>0.129</i>
		Bandırma	-0.045	0.021	-	<i>0.125</i>	0.009	-0.017	0.033	0.057
	Black Sea	Rize	0.029	0.097	-0.028	-	<i>0.074</i>	<b>0.102</b>	<b>0.258</b>	<b>0.230</b>
		Sinop	<i>0.178</i>	<b>0.364</b>	<i>0.163</i>	0.082	-	-0.004	0.024	<b>0.183</b>
		Zonguldak	0.127	<i>0.295</i>	0.019	-0.013	0.109	-	-0.021	<i>0.119</i>
	North Aegean Sea	İnebolu	0.102	<i>0.294</i>	0.097	0.026	0.001	0.086	-	<b>0.253</b>
		Greece	<b>0.219</b>	<b>0.316</b>	<b>0.175</b>	<b>0.178</b>	<b>0.354</b>	<i>0.210</i>	<i>0.156</i>	-

**TABLE 2.** Hierarchical AMOVA results COI+cyt-b (top) and RAG 1 genes (bottom).

	Source of variation	Total variation	Percent of total	$\Phi_{CT}/F_{CT}$	$\Phi_{SC}/F_{SC}$	$\Phi_{ST}/F_{ST}$
<b>COI +cyt-b genes</b>	Among groups	0.1697	<b>15.80</b>	0.1580		
	(TSS, Black Sea, N. Aegean Sea)			(p=0.0049)		
	Among populations within groups	0.0272	<b>2.53</b>		0.0300	
					(p=0.2160)	
	Within populations	0.8773	<b>81.67</b>			0.1833
						(p= 0.0000)
	<b>Total</b>	1.0741				
<b>RAG 1</b>	Among groups	0.0190	<b>4.18</b>	0.04186		
	(TSS, Black Sea, N. Aegean Sea)			(p=0.1386)		
	Among populations within groups	0.0202	<b>4.46</b>		0.0466	
					(p =0.0106)	
	Within populations	0.4138	<b>91.34</b>			0.0866
						(p=0.0000)
	<b>Total</b>	0.4530				

## FIGURE CAPTIONS

**FIGURE 1.** Sampling locations of *Merlangius merlangus euxinus* [(this study (red diamond symbol), GenBank (red dotted circle symbol)], *M. m. merlangus* [(this study (yellow triangle), and GenBank (the yellow dotted circle symbol)]. The red dashed line represents the distribution of *M. m. euxinus* according to Bailly (2008) and Parin et al. (2014). Numbers on the figures indicate sampling locations. Localities information are found in Supplementary Table 1.

**FIGURE 2.** Haplotype networks of a) COI, b) cyt-b and c) RAG1 genes of *Merlangius merlangus*. Note: Yellow color indicates *Merlangius merlangus euxinus* and pink indicates *Merlangius merlangus merlangus*.

**FIGURE 3.** Extended Bayesian Skyline Plots for the concatenated COI, cyt-b, RAG1 genes of *Merlangius merlangus euxinus*, a) North Aegean Sea, b) TSS, and c) Black Sea populations reflecting changes in effective population size against time in thousands of years (Ya) before present. Central posterior density intervals are indicated as 95% CPD.

**FIGURE 4.** BEAST chronogram based on the concatenated COI, cyt-b, RAG1 genes of *Merlangius merlangus* without outgroup dating. Values above branches are the mean node height (age). Bars show 95% HPD. Values below branches are posterior probabilities. Legends indicate localities.









