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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ABSTRACT

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

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

1. INTRODUCTION

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

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

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

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

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

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

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

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

2. MATERIALS AND METHODS

2.1. Sampling sites

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

2.2. DNA extraction, amplification and sequencing of genetic markers

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

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

2.3. Phylogenetic analyses

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

The models of sequence evolution for the protein-coding mtDNA markers with partitions and the nuclear gene were selected using the Bayesian Information Criterion in PartitionFinder v.1.1.0 (Lanfear et al., 2012) (Supplementary Table 5). Phylogenetic analysis was performed in MrBayes v.3.2.2 (Ronquist et al., 2012) with two independent runs of $5x10^6$ generations and four parallel Markov chain Monte Carlo (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 (π**)** (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 Φ_{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

from demographic or spatial population expansion, were estimated with DnaSP. Additionally, Tajima's *D* (Tajima, 1989) and Fu's *F*s (Fu, 1997) neutrality tests used to detect population growth, as well as the raggedness statistic "*r*g" (Harpending et al., 1993; Harpending, 1994), which quantifies the smoothness of the fit of the observed mismatch distribution to one expected under a population expansion model, were performed in DnaSP. Significance of neutrality tests and *r*g values were tested with 1,000 coalescence simulations. Past population demography of three different regions (TSS, Black Sea and North Aegean Sea) of *M. m. euxinus* was reconstructed using Extended Bayesian Skyline Plots (EBSP) (Heled and Drummond, 2008; Ho and Shapiro, 2011), as implemented in BEAST v.2.4.8 (Bouchkaert et al., 2014). A strict molecular clock was set using the multi-locus dataset (three genes: COI, cyt-b, RAG1), the previously selected substitution models (Supplementary Table 5), and time was scaled using the estimated rate for each marker (see Results section 3.5 Divergence time estimates). The Bayesian Markov Chain Monte Carlo (MCMC) was set for $1x10^8$ generations, discarding the initial 10% of samples as burn-in. Convergence of runs was evaluated on TRACER v.1.6. (Rambaut et al., 2014). Visualization of the EBSP plots was done within RStudio v.1.1.463 in R v.3.5.1. (R Core Team, 2018). Finally, haplotype networks for each gene were constructed using the median joining method (Bandelt et al., 1999) as implemented in PopART v.1.7 (Leigh and Bryant, 2015).

2.5. Divergence time estimation

The evolutionary divergence time of phylogenetic separation of the two potential *M. merlangus* subspecies was estimated in BEAST. *Melanogrammus aeglefinus* and *Gadus morhua* were used as outgroups. No calibration was used, and the best substitution 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 $2x10^8$ 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 ambigious 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: $h = 0.1080$, $\pi =$

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

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

3.2. Phylogenetic relationships

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

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

3.3. Population structure

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

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

3.4. Population history of Merlangius merlangus

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

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

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

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

3.5. Divergence time estimates

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

DISCUSSION

4.1. Phylogenetic analyses and genetic distances

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

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

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

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

The shallowly differentiated lineages of the two subspecies on the phylogenetic trees might have been influenced by different factors. Biologically speaking, gene flow between the two subspecies might be one of the reasons behind this lack of differentiation. Genetic homogeneity of the *Merlangius merlangus* populations, due to transportation and mixing of long-lived larvae and eggs by current system, was 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 ($N_{Atlantic}$= 139, *vs.* $N_{ThisStudy}$=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 Mediterrenaen 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

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

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

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

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

4.3. Population differentiation

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

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

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

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

4.4. Conclusions

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

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TABLES

TABLE 1. Pairwise Φ_{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.

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

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

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

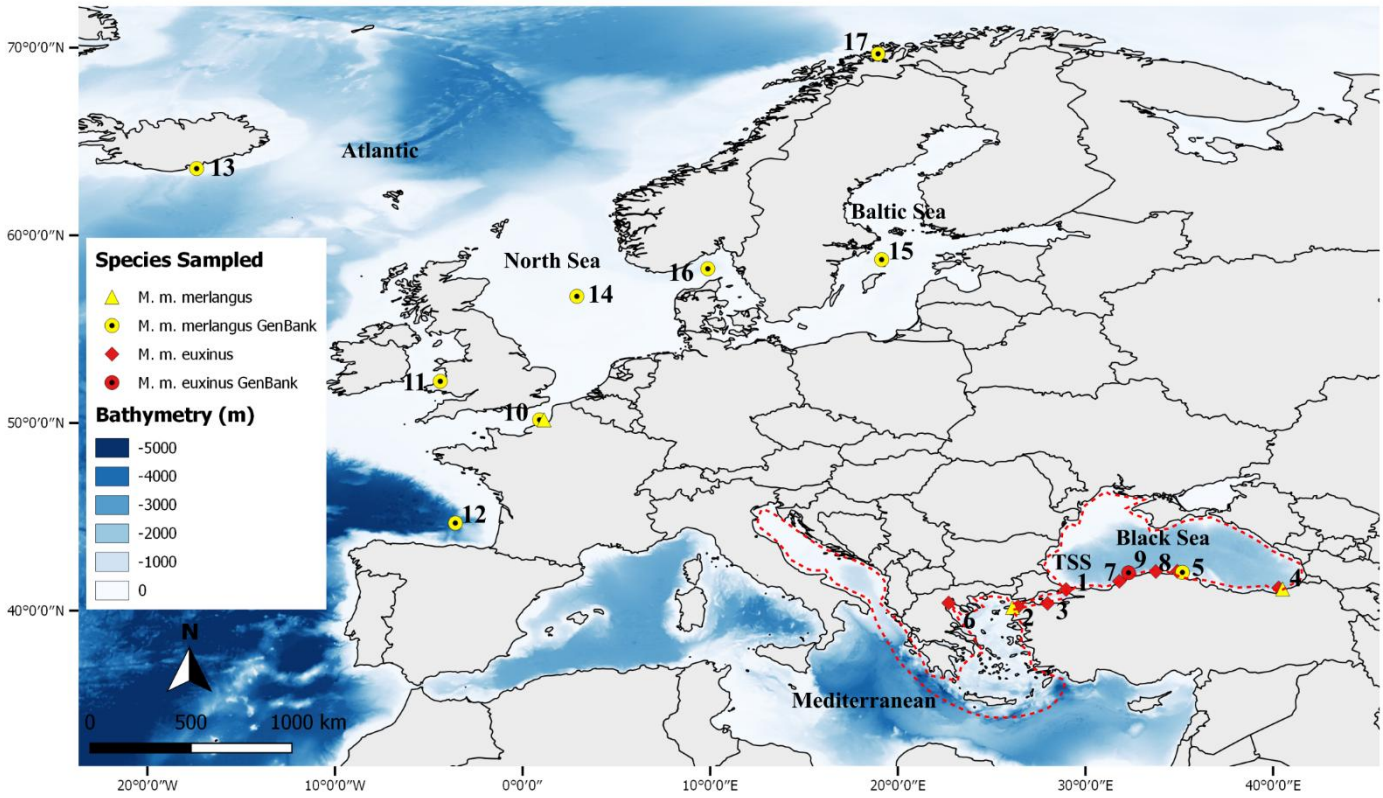
FIGURE CAPTIONS

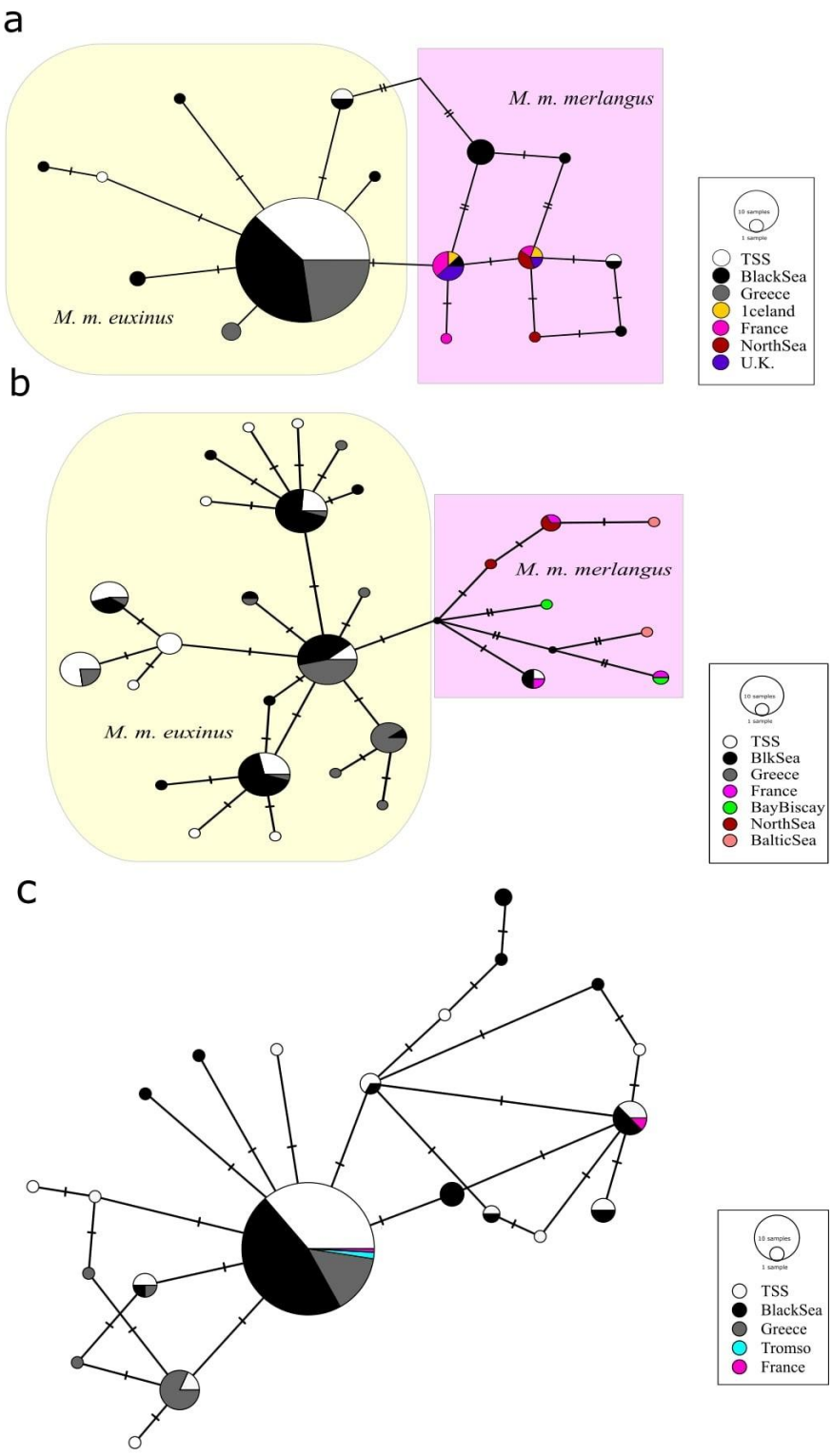
FIGURE 1. Sampling locations of *Merlangius merlangus euxinus* [(this study (red diamond symbol), GenBank (red dotted circle symbol)], *M. m. merlangus* [(this study (yellow triangle), and GenBank (the yellow dotted circle symbol)]. The red dashed line represents the distribution of *M. m. euxinus* according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

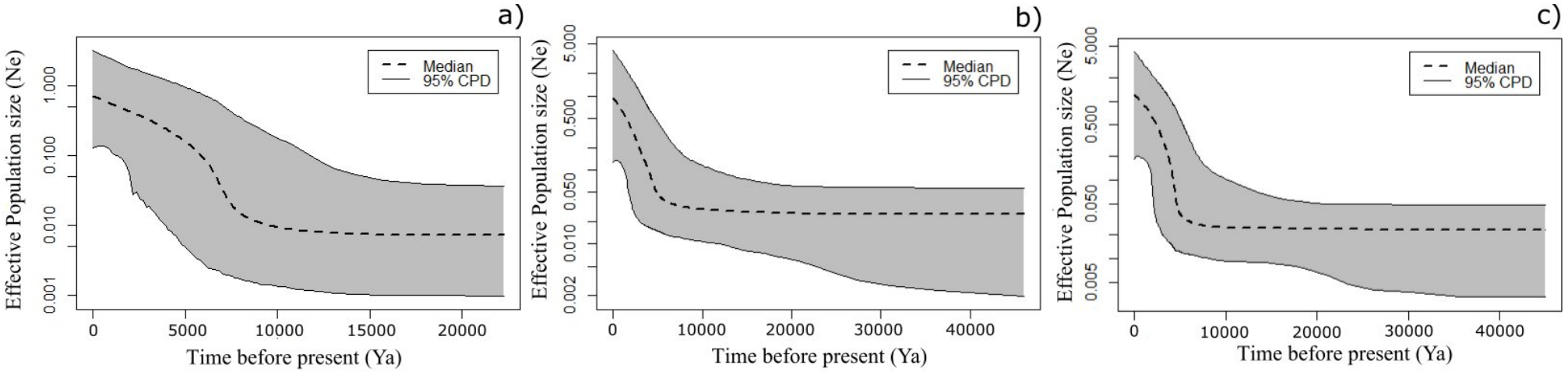
FIGURE 2. Haplotype networks of a) COI, b) cyt-b and c) RAG1 genes of *Merlangius merlangus.* Note: Yellow color indicates *Merlangius merlangus euxinus* and pink indicates *Merlangius merlangus merlangus*.

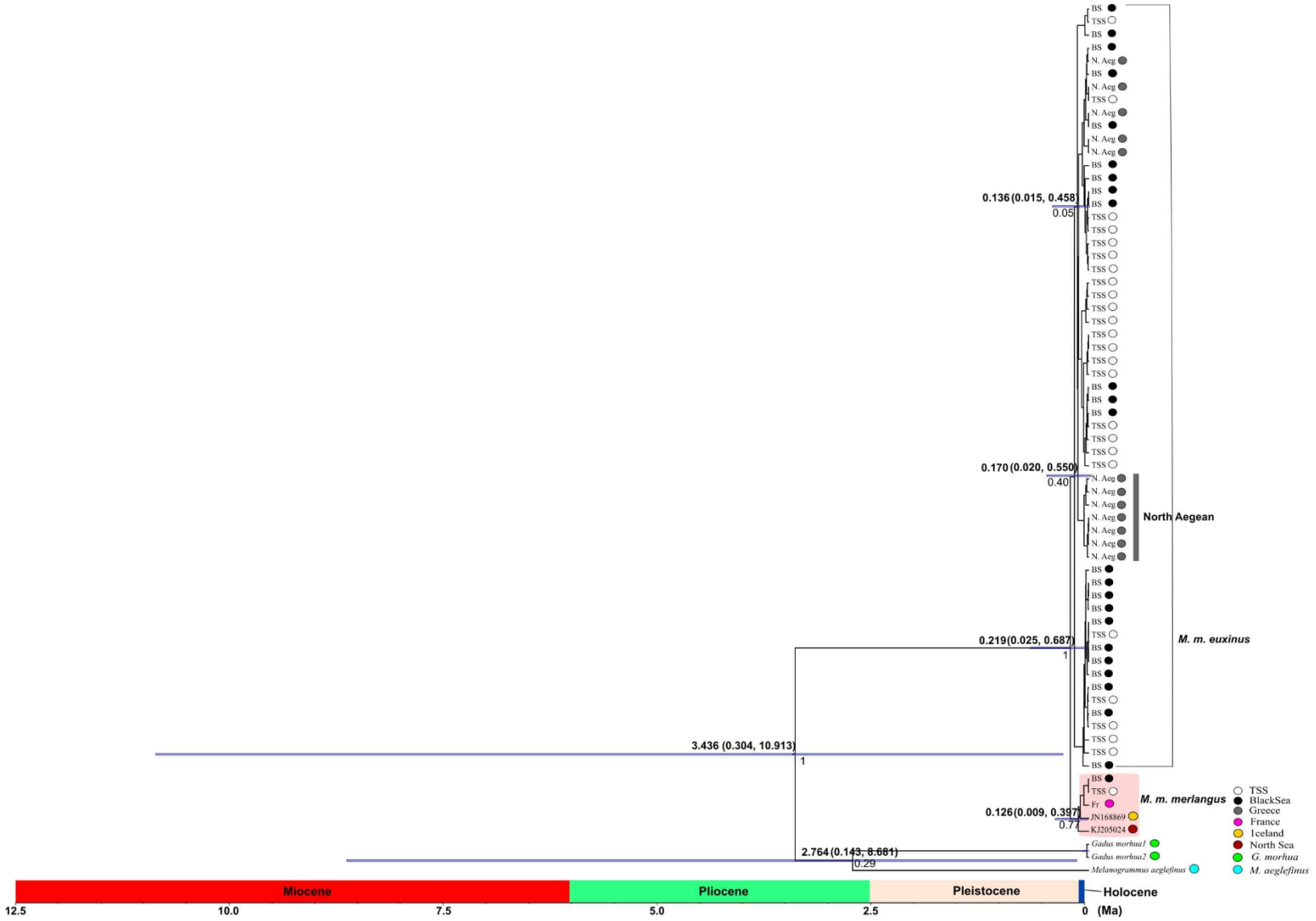
FIGURE 3. Extended Bayesian Skyline Plots for the concatenated COI, cyt-b, RAG1 genes of *Merlangius merlangus euxinus*, a) North Aegean Sea, b) TSS, and c) Black Sea populations reflecting changes in effective population size against time in thousands of years (Ya) before present. Central posterior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


70°0′0″N
60°0′0″N
50°0′0″N
40°0′0″N
20°0′0″W
10°0′0″W
0°0′0″
10°0′0″E
20°0′0″E
30°0′0″E
40°0′0″E
Species Sampled
M. m. merlangus
M. m. merlangus GenBank
M. m. euxinus
M. m. euxinus GenBank
Bathymetry (m)
-5000
-4000
-3000
-2000
-1000
0
N
0
500
1000 km
Atlantic
North Sea
Baltic Sea
Black Sea
Mediterranean
TSS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17


a
M. m. merlangus
M. m. euxinus
10 samples
1 sample
TSS
BlackSea
Greece
1celand
France
NorthSea
U.K.
b
M. m. merlangus
M. m. euxinus
10 samples
1 sample
TSS
BlkSea
Greece
France
BayBiscay
NorthSea
BalticSea
c
10 samples
1 sample
TSS
BlackSea
Greece
Tromso
France


a)
b)
c)
Median
95% CPD
Effective Population size (Ne)
Time before present (Ya)
0.001
0.010
0.100
1.000
0.002
0.050
0.500
5.000
0.005
0
5000
10000
15000
20000
30000
40000


0.136 (0.015, 0.458)
0.05
0.170 (0.020, 0.550)
0.40
North Aegean
M. m. euxinus
0.219 (0.025, 0.687)
1
3.436 (0.304, 10.913)
1
M. m. merlangus
0.126 (0.009, 0.397)
0.7
JN168869
KJ205024
2.764 (0.143, 8.681)
0.29
Gadus morhua1
Gadus morhua2
Melanogrammus aeglefinus
TSS
BlackSea
Greece
France
Iceland
North Sea
G. morhua
M. aeglefinus
Miocene
Pliocene
Pleistocene
Holocene
12.5
10.0
7.5
5.0
2.5
0
(Ma)