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-RESEARCH ARTICLE-

Phylogenetic Relationships of Turbot Species (Scophthalmidae) Inferred from the Mitochondrial COIII Gene and Morphological Characters

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Abstract

In this study, the validity, distribution and structure of three turbot species, *Scophthalmus maeoticus*, *S. maximus*, *S. rhombus*, belong to Scophthalmidae family in Turkish, Bulgarian and Russian coastal waters were determined with mtDNA sequencing of Cytochrome c oxidase subunit III (COIII). The sequencing of the COIII region revealed 8 bp variable and 6 bp parsimony informative sites between all turbot species. The overall genetic and haplotype diversities among all turbot species were found to be 0.004109 and 0.7655, respectively. Genetic distance analysis showed that the highest nucleotide differences was observed between *S. maximus* and *S. rhombus* species with a value of 0.09620 and, the lowest value (0.02482) was observed between *S. maximus* and *S. maeoticus* species. Neighbor Joining and Maximum Parsimony phylogenetic approaches resulted in the similar tree topologies that *S. maximus* and *S. maeoticus* were found as sister group, whereas *S. rhombus* was more divergent from this group. The mtDNA COIII gene is a useful genetic marker for species specific identification of the genus Scophthalmus due to its inter-specific heterogeneity producing a species-specific pattern. In morphological analyses, *S. rhombus* was most differentiated from *S. maximus* and *S. maeoticus*. The genetic data was supported by the detected morphometric variations among the turbot species.

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Introduction

The Scophthalmidae are a family of sinistral flatfish found in the North Atlantic Ocean, Baltic Sea, Mediterranean Sea, Black Sea and Marmara Sea are important species in the world due to their economic and ecological value (Chanet, 2003). Fishes of this family are known commonly as turbots which may exceed 1 m of length and 20 kg of weight (Froese & Pauly, 2018). Colors of these species are variable and they can adapt at colors of their environment. Scophthalmid fishes are benthic marine species, living on sandy and muddy bottoms, and younger individuals tend to live in shallower areas. These species generally feed on demersal and pelagic fish species, crustaceans and molluscs; the feeding ratio decreases during the spawning period and becomes more intensive in autumn and winter (Slastenenko, 1956; Karapetkova, 1980; Ivanov & Beverton, 1985).

In the world, the Scophthalmidae family is represented by 9 species (*Scophthalmus maximus, S. maeoticus, S. rhombus, S, aquosus, Lepidorhombus whiffiagonis, L. boscii, Zeugopterus punctatus, Z. regius, Phrynorhombus norvegicus*) within 4 genera, *Scophthalmus*, *Lepidorhombus*, *Zeugopterus*, *Phrynorhombus* (Nelson, 1994). Scophthalmidae family is represented with three species (*Scophthalmus maximus* (Linnaeus, 1758), *S. maeoticus* (Pallas, 1814), *S. rhombus* (Linnaeus, 1758) in the Black and Marmara Seas (Nelson, 1994; Turan, 2007). *Scophthalmus maximus, S. maeoticus* and *S. rhombus* are closely related congeneric species (Pardo et al., 2005; Turan, 2007; Azevedo et al., 2008) which show a similar distributional range (Blanquer et al., 1992; Pardo et al., 2001) and generally considered and excepted that there is one species, *Scophthalmus maximus*, exist in the Black Sea and Marmara Sea (Muus & Dahlström, 1978; Suziki et al., 2004). Unfortunately, the argument on the existence of the number of the species in these waters are still ongoing issue and need clarification.

In the Black and Marmara Seas there is a dilemma on the existence of the number of turbot species (Evseenko, 2003; Voronina, 2010; Froese & Pauly, 2018). The turbot, *Scophthalmus maximus* (Linnaeus, 1758), formerly recognized as *Psetta maxima* (Bailly & Chanet, 2010), distributed in the Northeast Atlantic, throughout the Mediterranean and along the European coasts to Arctic Circle as well as most of the Baltic Sea (Prado et al. 2018). The Black Sea brill, *Scophthalmus maeoticus* (Pallas, 1814), distributed in the Black Sea, was considered as a synonym of *S. maximus* by Bailly & Chanet (2010). Eschmeyer (2011) spelled a specific epithet as *maeotica* and accepted as *S. maeoticus*. The brill, *Scophthalmus rhombus* (Linnaeus, 1758), distributed in the Eastern Atlantic, Norway to Morocco and reported from Iceland (Jonsson, 1992), throughout the Mediterranean and Black Sea. Turan et al. (2016) reported that *S. rhombus* populations were seriously declined and rarely found in the Black Sea and Marmara Sea due to overfishing, habitat degradation and pollution and should be taken into consideration to be Near Threatened (NT) in the IUCN Red List of Threatened Species.

Molecular genetic studies on mtDNA have demonstrated useful in order to determine hypotheses about the phylogeny and phylogeography of marine species (Meyer, 1993; Avise, 1994; Turan et al. 2015a). The pattern of maternal inheritance and rapid rate of evolutionary change of mtDNA compared to nuclear DNA make it a suitable tool for genetic studies among taxa of several fish groups at multiple taxonomic levels (Kocher & Stepien, 1997; Zardoya et al., 1999; Durand et al., 2002). Sequence analysis of mtDNA regions may be a quick tool to reveal phylogenetic relationships of marine species (Avise, 1994; Tabata & Taniguchi, 2000; Turan et al. 2008). Since different regions of mtDNA evolve at different rates, specific mtDNA regions have been targeted for inter and intra specific variation (Hauser et al. 2001; Mohindra et al. 2007; Turan et al. 2015b).

In the present study, the validity, distribution and phylogenetic relationships of three turbot species, *Scophthalmus maeoticus*, *S. maximus*, *S. rhombus*, belong to Scophthalmidae family in the Black and Marmara Seas comprising Turkish, Bulgarian and Russian coasts were investigated with mitochondrial Cytochrome c oxidase subunit III (COIII) gene sequence data.

Materials and Methods

Sampling

S. maximus specimens were collected from three locations (at fishing ports) throughout the species' distributional range, comprising Trabzon and Düzce and Marmara Sea from Turkish Coasts, Varna Coast from Bulgaria and Sevastopol Coast from Russia. *S. maeoticus* specimens were collected from Trabzon, Düzce and Marmara Sea from Turkish Coasts. *S. rhombus* specimens were only found and collected from only the Marmara Sea (Fig. 1). All the specimens were sampled in winter season of 2013-2014. All samples were put in plastic bags individually and frozen at -20 °C until they were transported to the laboratory.

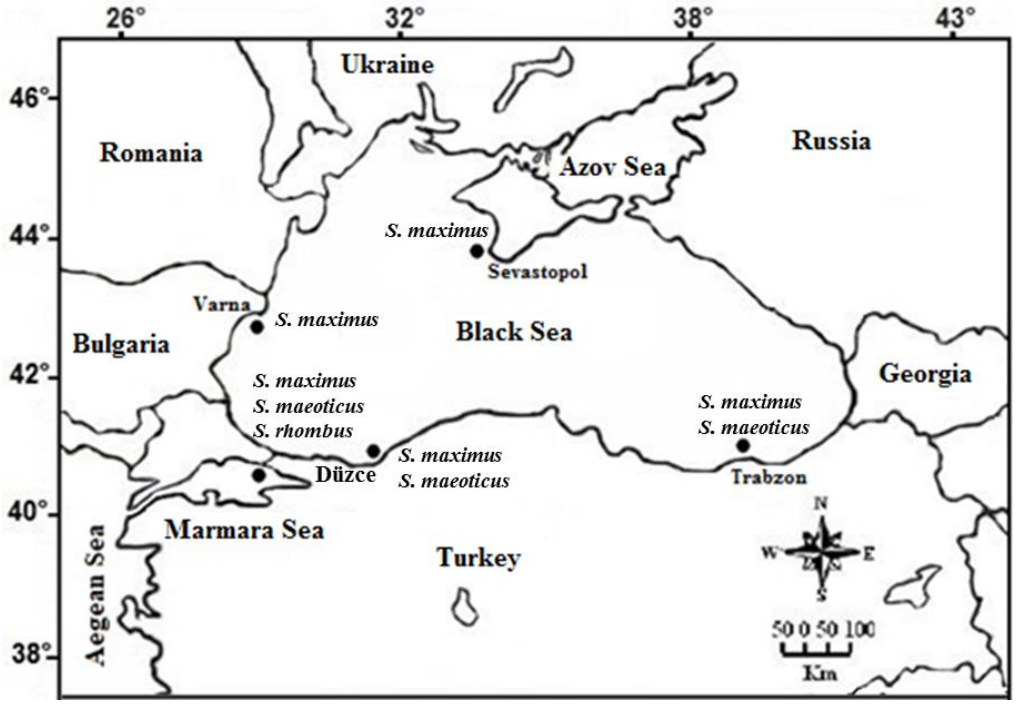

Figure 1. Sampling locations of turbot species analysed.

Morphology

Meristic characters commonly used to describe Scopthalmidae species such as dorsal fin, caudal fin, anal fin, ventral fin, pectoral fin, back pectoral fin, gill rakers number, vertebra numbers were examined. Moreover, morphological characters that differentiate the species were also used as illustrated in Figure 2.

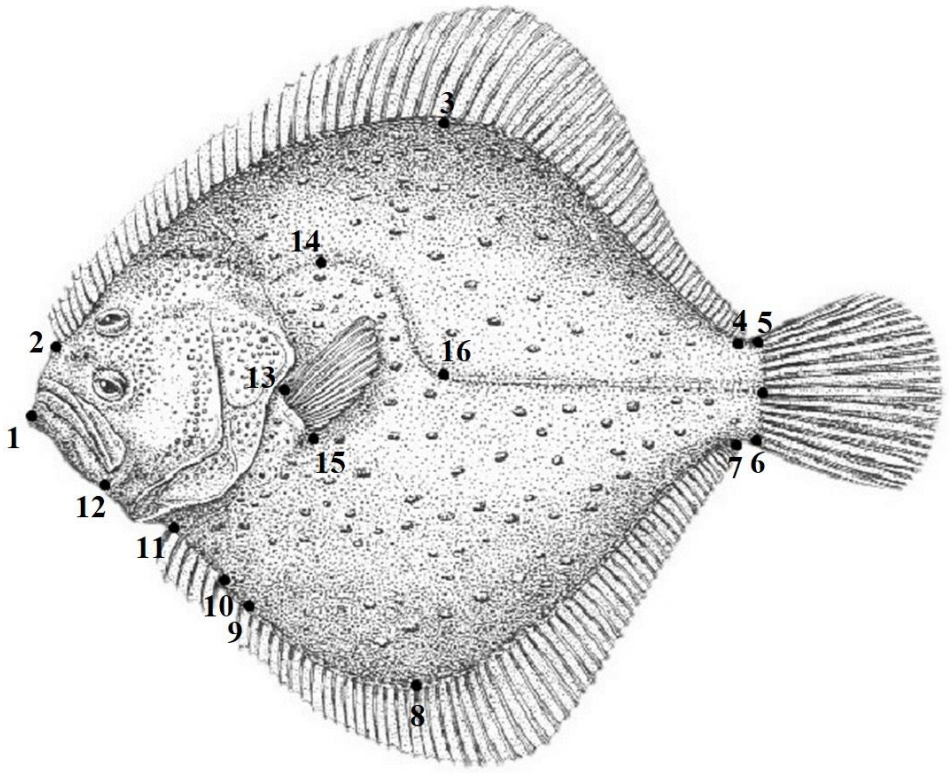

Figure 2. Morphometric distances between the numbers were taken as morphometric characters. Fish drawings is from Froese & Pauly (2018)

Most of the variability in morphological characters is due to size differences (Junquera & Perez-Gandaras, 1993, Turan, 1999). Therefore, shape analysis should be free from the effect of size to avoid misinterpretation of the results (Strauss, 1985). Therefore allometric size correction method (Elliott et al., 1995) was used to remove the effect of size variation. Principal component analysis (PCA) were used for detecting morphological characters which from differences among populations. Discriminant function analysis (DFA) were used for distinguishing morphological differences between populations (Turan, 1999). Univariate analysis of variance (ANOVA) was used to compare the variation between size-adjusted characters. SPSSv23 and SYSTATv13 were used for all statistical analysis.

Genetic analysis

All tissue samples were stored at -20 °C and 95 % ethanol till the analysis. Total DNA from all fish was extracted from the muscle using the standard phenol: chloroform: isoamyl alcohol procedure

(Sambrook et al., 1989). PCR amplification of the mitochondrial COIII gene was carried out using the universal primers (Valles-Jiménez, 2005):

COIII-F: 5'- AGC CCA TGA CCT TTA ACA GG -3'

COIII-R: 5'- GAC TAC ATC AAC AAA ATG TCA GTA TCA -3'

Polymerase chain reactions were conducted by using a reaction volume of 50 µl containing 5 units of *Taq* polymerase (Thermo scientific), 2 mM of each primer, 10 mM dNTPs (Thermo scientific), 25 mM $MgCl_2$ (Thermo scientific), 10 mM Tris-HCl pH 8.8, 50 mM KCI and 1 µl template DNA (~10–25 ng). The amplification was performed with pre-denaturation at 95°C for 1 min followed by 5 cycles of denaturation at 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s for 30 cycles and followed by a final extension for 7 min at 72 °C. After PCR amplification, 4 µl sample of each PCR product was controlled in 1.5% agarose gels. Quantitation of the PCR product was completed using spectrophotometer. The DNA sequencing was attempted to determine the order of the nucleotides of a gene. The chain termination method by Sanger et al. (1977) was applied with Bigdye Cycle Sequencing Kit V3.1 and ABI 3130 XL genetic analyzer.

Sequence alignment and genetic analysis

The initial alignments of partial COIII sequences were performed with Clustal W program (Thompson et al. 1994) and final alignment was completed manually with BioEdit (Hall, 1999). MtDNA sequence data were analyzed to assess levels of genetic variation and to determine nucleotide composition for each taxon using MEGA 6 (Tamura et al. 2011). Modeltest (Posada & Crandall, 1998) was used to determine the best-fit model of DNA evolution. Molecular phylogenetic analyses and molecular phylogenetic trees were conducted using MEGA version 6 (Tamura et al. 2011). A distance-based method as neighbor joining (NJ) (Saitou & Nei, 1987) and a cladistic phylogenetic tree as maximum parsimony (MP) criterion were used. The robustness of the internal branches of trees was assessed by bootstrapping (Felsenstein, 1985) with 1000 replicates. One species from the Solidae family was included in the molecular phylogenetic trees and rooted as an outgroup species *Solea senegalensis* from published sequences in GenBank under accession number, AB270760.

Results

Genetic results

There were 8 variable and 557 conservative nucleotides of which 6 were parsimony informative over 565 bp sequences. The average nucleotide composition was 23.01% for A, 28.80% for T, 19.77% for G and 28.41% for C. The Jukes-Cantor (Jukes-Cantor, 1969) model was determined to be the best model for our dataset. The mean genetic diversity between and within species was calculated as 0.004109 and 0.000446, respectively. The sequence analysis of COIII revealed 6 different haplotypes which were not shared between any species (Table 1). Average haplotype diversity between populations was found to be 0.7655. The minimum spanning tree showing the phylogenetic relationships among the COIII haplotypes was given in Figure 3. Ancestral haplotype (Hap_6) was only found in *S. rhombus*. The distribution of the haplotypes in the tree generally reflects the species-specific distribution of the samples.

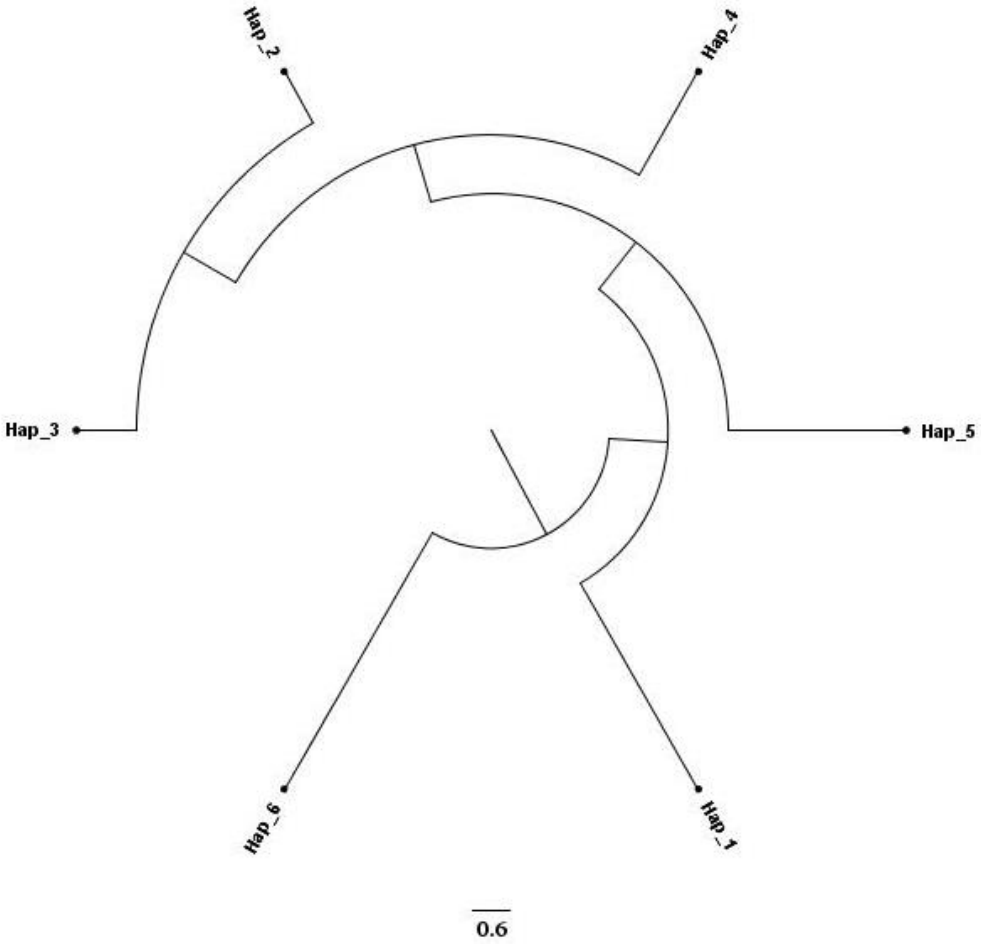

Figure 3. Minimum spanning tree that shows the relationships among the haplotypes.

The matrix of pairwise genetic distances within species and between species are presented in Table 2. Intra-specific genetic diversity was not observed within *S. rhombus* specimens while highest (0.000709) was found within *S. maeoticus* specimens. The lowest genetic distance (0.002482) was observed between *S. maximus* and *S. maeoticus* while the highest (0.009620) was observed between *S. rhombus* and *S. maximus*.

Table 1. The number of haplotype and its distribution among the species.

Haplotypes	***S. maeoticus***	***S. maximus***	***S. rhombus***	**Total**
Hap 1	8			8
Hap 2	1			1
Hap 3	1			1
Hap 4		8		8
Hap 5		2		2
Hap 6			10	10
Total	10	10	10	30

Table 2. The matrix of intra-specific genetic divergence given in bold (transversal diagonal) and pairwise genetic distances between species with statistical significance. ***, P<0.001.

	S.maeoticus	***S.maximus***	***S.rhombus***
S. maeoticus	**0.000709**		
S. maximus	0.002482***	**0.000630**	
S. rhombus	0.007471***	0.009620***	**0.000000**

Neighbor Joining and Maximum Parsimony phylogenetic approaches resulted in the similar tree topologies. In the both Neighbor Joining and Maximum Parsimony phylogenetic trees (Figure 4, 5), two phylogenetic nodes were detected; in the first node, *S. rhombus* was grouped separately. In the second node two branches were detected; *S. maximus* and *S. maeoticus* were grouped together.

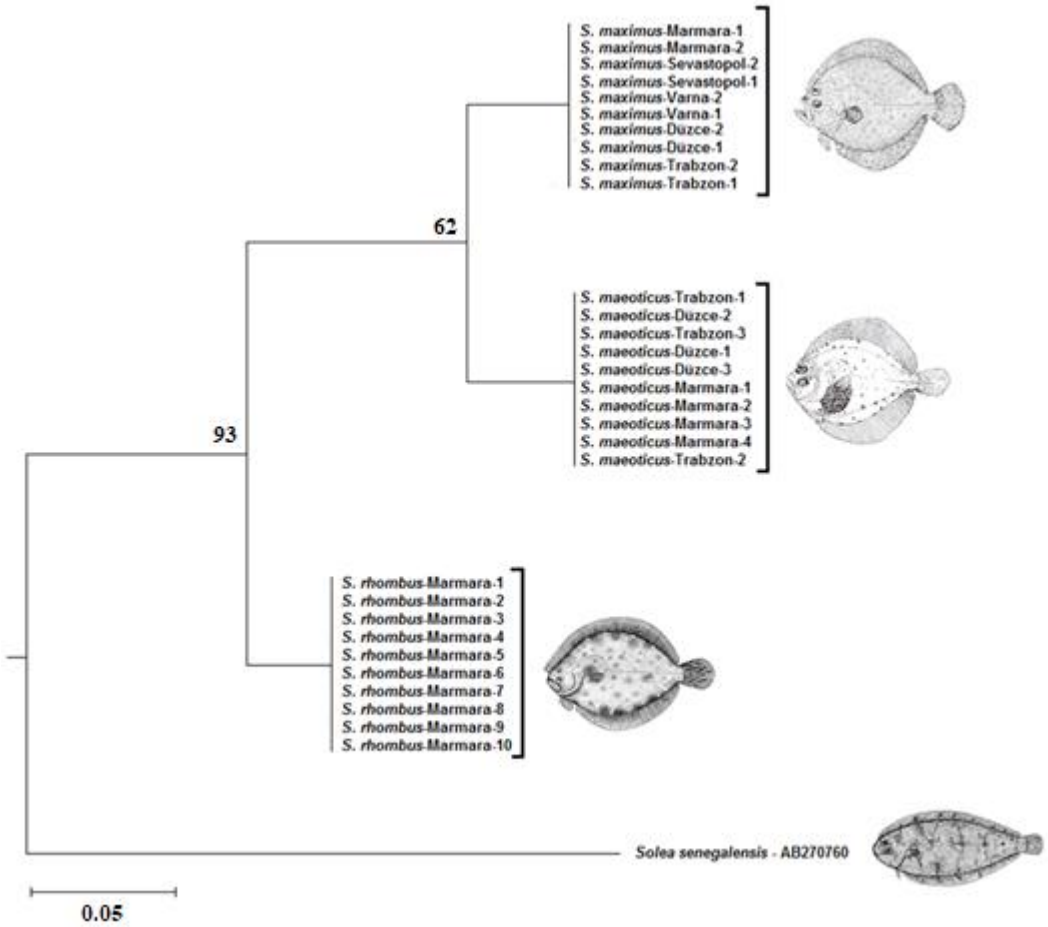

Figure 4. Neighbor joining phylogenetic tree based on COIII sequences. The tree was rooted using the outgroup species *S. senegalensis*. Numbers on nodes indicate the bootstrap values. Fish drawings: *S. maximus*, *S. rhombus* and *S. senegalensis* is from Froese & Pauly (2018) and *S. maeoticus* is from Zaharia (2002).

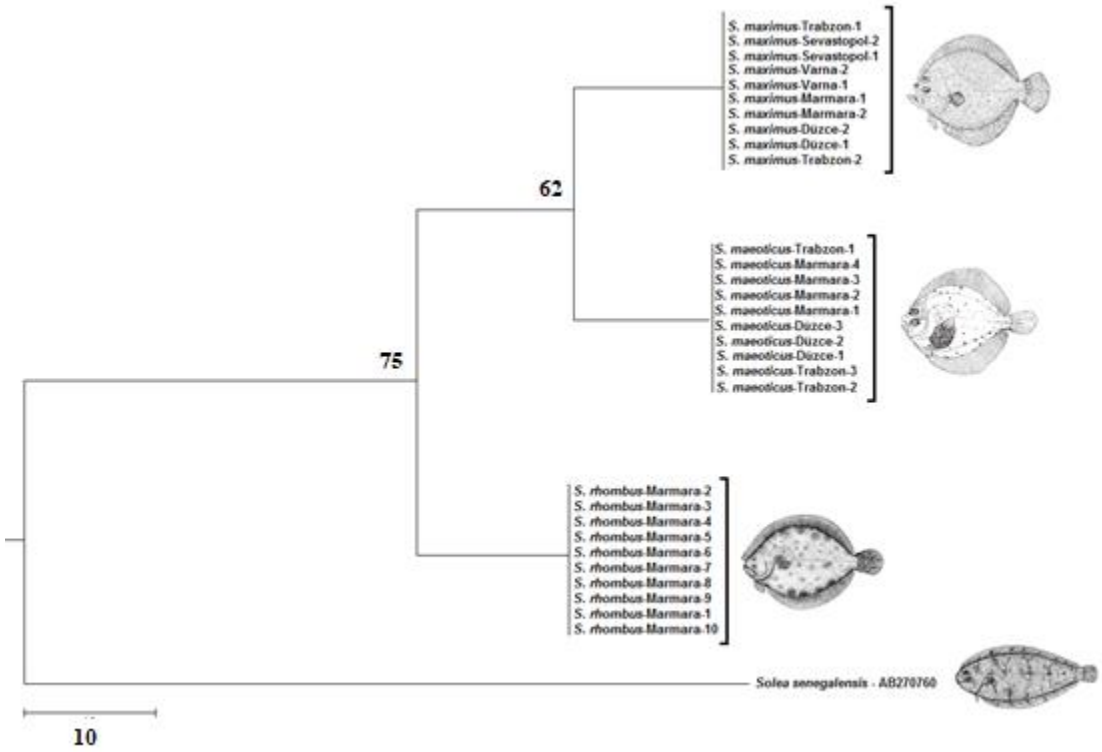

Figure 5. Maximum parsimony tree phylogenetic tree based on COIII sequences. The tree was rooted using the outgroup species *S. senegalensis*. Numbers on nodes indicate the bootstrap values. Fish drawings: *S. maximus, S. rhombus* and *S. senegalensis* is from Froese & Pauly (2018) and *S. maeoticus* is from Zaharia (2002).

Morphological results

The range of the meristic characters used to differentiate turbot species is given in Table 3. Dorsal fin ray number varied between 60 to 69 for *S. maximus*, 59 to 76 for S. *maeoticus* and 72 to 81 for *S. rhombus*. Anal fin ray number varied between 41 to 52 for *S. maximus*, 42 to 51 for S. *maeoticus* and 53 to 61 *S. rhombus*. Pectoral fin ray number varied between 9 to 14 for all species. Ventral fin ray number varied between 5 to 7 for all species. The gill raker number and vertebra number of all species were found different ranges from each other (Table 3).

Table 3. The range of the meristic characters observed in turbot species

Species	**N**	**DFR**	**PFR**	**AFR**	**VFR**	**BPFR**	**CFR**	**GRN**	**VN**
S. maximus	158	60-69	10-14	41-52	6-7	10-14	15-22	13-17	30-32
S. maeoticus	162	59-76	10-13	42-51	6	10-13	15-19	14-17	30-31
S. rhombus	25	72-81	10-12	53-61	6	10-12	12-18	15-18	36-38

DFR: Dorsal fin ray, PFR: Pectoral fin ray, AFR: Anal fin ray, VFR: Ventral fin ray, BPFR: Back pectoral fin ray, CFR: Caudal fin ray, GRN: Gill raker number VN: Vertebra number, N: number of individuals analyzed.

In PCA, 27 principal components (PCs), which contain the percentage of total variance of all variables, were produced, and 52.10 and 18.79 % of the total variation was presented in the first and second PCs. Morphometric characters generally highly contributed in differentiation, and meristic characters which are anal fin ray, back pectoral fin rays, vertebra numbers, dorsal fin rays were more efficient to differentiate turbot species (Table 4). In discriminant function analysis, the overall random assignment of individuals into their original group was 100%, showing a clear differentiation of species from each other.

Table 4. Contribution of each morphological character on the main principal components that played a role in discriminating each species.

	Component			
Variable	**1**	**2**	**3**	**4**
1_14	0.986	-0.152	0.015	-0.019
1_15	0.985	-0.154	0.015	-0.005
3_8	0.985	-0.144	-0.020	-0.015
7_9	0.985	-0.161	0.025	-0.009
8_14	0.984	-0.148	-0.014	-0.013
1_13	0.984	-0.155	0.017	-0.003
2_4	0.984	-0.164	0.027	-0.009
2_14	0.983	-0.157	0.017	-0.018
3_14	0.983	-0.150	-0.006	-0.007
1_12	0.981	-0.158	-0.010	0.003
5_6	0.980	-0.156	-0.019	-0.013
10_11	0.980	-0.142	-0.008	-0.013
Linea L.	0.970	-0.151	0.037	-0.011
1_2	0.970	-0.132	-0.010	-0.026
BVFR	0.412	0.740	-0.417	0.006
D_O	0.277	0.726	0.495	0.057
VN	-0.383	-0.717	0.404	-0.062
PFL	0.209	0.712	0.255	-0.045
DFR	-0.360	-0.699	0.354	-0.073
ID	0.227	0.683	0.378	0.087
HL	0.404	0.670	0.495	0.018
AFR	-0.457	-0.630	0.462	-0.080
PFR	0.189	0.486	-0.364	-0.006
ED	0.203	0.469	0.219	-0.276
M_E	0.114	0.566	0.623	-0.135

GRN	0.123	-0.486	0.536	0.096
VFR	0.080	-0.095	0.104	0.791
CFR	0.048	0.132	0.071	0.577

In phylogenetic tree showed morphological relationship among turbot species, *S. maximus* and *S. maeoticus* were branched together in the same nod. On the other hand, *S. rhombus* was found to be separated from other two turbot species (Figure 6).

Figure 6. Systematic relationship of *Scophthalmus maximus*, *S. maeoticus* and *S. rhombus* based on morphometric and meristic characters.

Discussion

The phylogenetic relationships of three commercially important turbot species belongs to Scophthalmidae family (*Scophthalmus maximus*, *S. maeoticus* and *S. rhombus*) from Turkish and Bulgarian coasts were investigated based on mtDNA COIII gene sequencing data in the present study. The mean intra-specific genetic diversity was found to be 0.000446 and the mean inter-specific genetic diversity was found to be 0.004109. The mean genetic divergence was low (0.00456). Feng et al. (2005) analyzed phylogenetic relationships of 10 flatfish species (*Psetta maxima*, *Platichthys flesus*, *Solea solea*, *Solea senegalensis*, *Microchirus variegatus*, *Monochirus hispidus*, *Synaptura kleini*, *Paralichthys olivaceus*, *Pseudorhombus cinnamomeus* and *Kareius bicoloratus*) belongs to the Pleuronectiformes order on the basis of 16S rRNA sequencing data and found genetic divergence values between these species ranged from 0.0141 to 0.2639. Karan & Turan (2019) reported significant genetic divergence (F_{ST}, 0.001593) based on COIII region of mitochondrial DNA between populations of *S. maeoticus* collected from the Black Sea and Marmara Sea. Feng et al. (2005) also reported that the turbot *Psetta maxima* and the thickback sole *Microchirus variegatus* has the genetically closer relationship (0.0395). Additionally, Feng et al. (2005) found genetic divergence of *P. maxima* from the other flatfish species, The European flounder *Platichthys flesus*, the common sole *Solea solea*, the whiskered sole *Monochirus hispidus*, the Klein's sole *Synaptura kleinii* and the Senegalese sole *Solea senegalensis*, to be 0.1492, 0.1798, 0.1969, 0.1734 and 0.1785, respectively. When we compare the mean genetic divergence finding with other flatfish species reported by Feng et al. (2005), genetic divergence is lower at turbots in the present study. It was thought that these turbot species may be distributed in close locations to each other in Turkish marine waters and may happen limited gene flow between them. On the other hand, similar biological and ecological features of the species together with their low mean genetic divergence may support the production of natural F_1 hybrids from inter-specific mating events in

the areas where they are sympatric. However, owing to the maternal inheritance of mtDNA in vertebrates, mitochondrial markers can reveal only the maternal genome sequence. These markers cannot elucidate inter-specific hybridization occurrences that may happen between species with covering reproductive, biological, and ecological features (Turan, 2008).

The genetic differentiation of *S. rhombus* was found to be relatively higher than *S. maximus* and *S. maeoticus* that was also found statistically significant ($P < 0.001$). Moreover, our results indicated that the relationship between *S. maeoticus* and *S. maximus* was closer than that both between *S. rhombus* and *S. maeoticus*, and *S. rhombus* and *S. maximus*, and confirmed the presence of three turbot species in Turkish marine waters. Pardo et al. (2005), investigated phylogenetic relationships of a total of 30 species belongs to 7 flatfish family including Scopthalmidae, Pleuroenectidae, Paralichthyidae, Cynoglossidae, Soleidae, Bothidae and Achiridae from the North East Atlantic, Southwest Atlantic and Indian Ocean inferred from 16S rRNA gene and found the close relationship between *S. maximus* and *S. rhombus*. Azevedo et al. (2008) studied phylogenetic analysis of 19 species belongs to the families of the Pleuronectiformes order (Achiridae, Bothidae, Cynoglossidae, Paralichthyidae, Pleuronectidae, Scophthalmidae and Soleidae) from the South America coasts based on 12S and 16S rRNA combined sequence data and found the close relationship between *S. rhombus* and *S. maximus*, as well. As a result, our findings about the relationship between *S. rhombus* and *S. maximus* were not supported by above mentioned studies. This case may be due to the fact that above mentioned studies were conducted by different gene, such as 16S rRNA, in different seas far away from Turkish marine waters and disadvantages of 16S rRNA in discriminating closely related species (Seyhan & Turan 2016).

There was congruent between genetic and morphological analyses that *S. rhombus* was genetically and morphologically differentiated from *S. maximus* and *S. maeoticus*. Phylogenetic studies of molecular genetic variation are increasingly being used to test evolutionary hypotheses about morphological, behavioral, and life-history evolution. In particular, the reconstruction of the evolutionary history of ecological interactions is most robust when phylogenies are based on characters independent of phenotypic characters involved in, or affected by, the history of these interaction (Brooks & McLennan, 2012; Hansen, 2014). Although suitable morphological characters may be found that resolve relationships and provide an independent template for testing evolutionary hypotheses about other phenotypic characters, mitochondrial genes provide a wealth of variation almost certainly independent of changes in ecologically relevant characters. This variation may be particularly useful in generating phylogenies in groups of taxa in which most variation in morphological characters is associated with apparent adaptation (such as in groups that have undergone recent adaptive radiation), or where excessive homoplasy in such morphological characters is apparent (Brown, 1994).

In conclusion, as a result of phylogenetic analysis for turbot species, the presence of three different turbot species is determined as *S. maximus, S. maeoticus* and *S. rhombus* in the Black Sea and the Marmara Sea. *S. rhombus* is genetically found as the most different species. In this study, it has been proven that *S. maeoticus*, which has not been reported from the Marmara Sea and Black Sea coasts of Turkey so far, is a distinct species from *S. maximus*. Consequently, the Scophthalmidae family has been began to be represented by six species with the inclusion of *S. maeoticus* in fish fauna of Turkey through this comprehensive study. The present study also suggest that COIII could be adopted as rational approach for resolving unambiguous identification of scophthalmid species in Turkish marine waters with applications in its management and

conservation. Moreover, further analyses on a larger data set and additional molecular markers, such as nuclear genes, could improve the findings and address for more explanation.

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