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Molecular and otolith shape analyses of *Scorpaena* spp. in the Turkish seas

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Abstract: This study aims to investigate the discrimination and the phylogenetic relationship of five *Scorpaena* species (*S. elongata, S. maderensis, S. notata, S. porcus,* and *S. scrofa*), sampled from eight locations in the four seas (Mediterranean Sea, Aegean Sea, Black Sea, and Sea of Marmara) in Turkey, using both molecular and otolith shape analyses. Totally, 1360 samples were examined for otolith shape analyses, and 51 of them were also used in the molecular analysis (COI sequences, 652 bp). Eighteen haplotypes were determined in the 5 species of the genus *Scorpaena*; 3 haplotypes for *S. elongata, S. maderensis* and *S. scrofa*, 4 haplotypes for *S. notata,* and 5 haplotypes for *S. porcus*. The highest interspecific genetic distance was observed between *S. maderensis* and *S. notata* (0.1569), and the lowest interspecific genetic distance (0.0527) was observed between *S. elongata* and *S. scrofa*. The lowest interspecies genetic distance was found to be considerably higher than the highest intraspecies genetic distances for these five *Scorpaena* species. The barcode range in the study alone provided a high rate of barcoding success in identifying species belonging to the genus *Scorpaena*. A Principal Component Analysis (PCA) and Canonical Discriminant Analysis (CDA) were conducted for interspecific discrimination of the five *Scorpaena* species. The CDA produced an overall classification success rate of 96.3% with the highest rate for *S. scrofa* (98.8%), followed by *S. maderensis* (97.5%), *S. notata* 96.6%), *S. elongata* (94.6%), and with the lowest rate for *S. porcus* (94.4%). The results indicated that molecular analysis was generally compatible with otolith shape analysis and confirmed that otoliths have phylogenetic signals for *Scorpaena* species. This is the first study in which five *Scorpaena* species sampled from eight locations in four seas have been distinguished using both otolith shape and molecular analyses.

Key words: *Scorpaena*, taxonomic classification, mtDNA-COI, elliptic Fourier coefficients, otolitholog

1. Introduction

Fishes are one of the most populous groups of vertebrate animals, inhabit nearly all aquatic habitats, and perform various biological functions in ecosystems (Nelson et al., 2016). However, the fish are affected by many environmental and ecological factors (water temperature, competitors, predators, abiotic and biotic components). Such events can cause morphological and genetic differentiation of fish species (Bruno et al., 2013; Gebrekiros 2016). In addition, some changes in fish biodiversity may occur in the relevant habitat as these factors cause habitat fragmentation and changes in connectivity between populations. Thus, it is possible to define the fish species in detail in all respects, to control possible species losses and reveal new species. Since fish have a wide distribution in the marine environment, they are likely to be exposed to genetic differentiation. For example, the high dispersal potential and absence of physical barriers may provide the species with an opportunity for allopatric speciation (Faria et al., 2021). However, the main difficulty in classifying and distinguishing marine species is the poor understanding of genetic differentiation patterns (Gandra et al., 2021).

Molecular markers, specifically from mitochondrial DNA (mtDNA) sequences are useful tools for distinguishing many species (Kocher et al., 1989). The widespread use of mtDNA in species discrimination is due to its maternal inheritance, which ultimately indicates that it provides information on evolutionary lineages (Ramírez-Pérez et al., 2010). In this field, especially 16SrRNA, Cytochrome b, and subunit I of the oxidase cytochrome (COI) genes are used for the distinction and identification of fish species (Lakra et al., 2009). Since its use in the field, the use of COI as a useful marker for the discrimination and identification of species under a familiar concept such as the DNA barcode has gained great interest (Hebert et al., 2003; Turan et al., 2017). Species identification by DNA barcodes can be reliable only if a significant difference between the average interspecific and average intraspecific genetic distance can be detected (Hebert et al., 2003). Barcoding gap is a threshold value defined between the lowest interspecific genetic distance and the highest intraspecific

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genetic distance. The barcoding range can be calculated over these threshold values. Barcoding success can be mentioned if the interspecific variation is considerably larger than the intraspecific variation (Hebert et al., 2003; Bingpeng et al., 2018). However, it has been reported that data overlap and DNA barcoding may be less effective if the intraspecific variation is greater than the interspecific variation (Meyer and Paulay, 2005). Besides being a robust evolutionary marker, COI has been shown to have a higher mutation rate in many fish species (Ward et al., 2005; Zang et al., 2019). Therefore, the mitochondrial COI gene is often preferred in studies where interspecies comparisons of fish species are made (Hebert et al., 2003; Ward et al., 2008; Roesma et al., 2020). Another useful and statistically robust tool is otolith shape analysis for the discrimination of fish species. Otoliths are bony structures in the inner ear of fish and play an important role in the hearing and balance of fish (Popper and Lu, 2000). In addition, otolith shape is species-specific for fish species. Otoliths have been preferred in many studies for the identification and differentiation of fish species due to the high degree of interspecific variation (Campana, 2004; Tuset et al., 2016). Several otolith shape methods such as wavelets, contour, and Fourier analysis were used for the identification of many fish species in the literature (Parisi-Baradad et al., 2005; Capoccioni et al., 2011; Tuset et al., 2016). Several studies have indicated that otolith shape can be used as an alternative tool for the discrimination of fish species, genus, and populations (Campana and Casselman, 1993; Bostanci et al., 2015; Bostanci and Yedier, 2018; Yedier 2021).

Scorpaenidae is a large family of dangerous marine fish that include lionfish, scorpionfish, and stonefish. Although members of the Scorpaenidae spread in tropical, temperate, and cold marine waters, the highest species diversity was recorded in the tropical waters of the Indian Pacific Ocean (Hureau and Litvinenko, 1986; Froese and Pauly, 2021). Many fish species in the Scorpaenidae are quite difficult to define morphologically because especially small individuals are very similar, and the characters used to describe species are not easy to use (Arculeo and Lo Brutto, 2014). The family comprises 25 genera and 230 bony fish species all over the world. The genus *Scorpaena* is represented by 64 species in the world (Froese and Pauly, 2021). Further, since the *Scorpaena* species can show coloration according to habitats, their camouflage characteristics are quite high (Hureau and Litvinenko, 1986; Froese and Pauly, 2021). For this reason, there are occasionally many problems in identifying and distinguishing the species belonging to the genus *Scorpaena*. Currently, six valid species are recognized in genus *Scorpaena* from the Turkish coasts; Cadenat's rockfish, *S. loppei*, Slender rockfish, *S. elongata*, and Madeira rockfish, *S. maderensis* from the Aegean Sea and the Mediterranean Sea, Small red scorpionfish, *S. notata,* and Black scorpionfish, *S. porcus* in the Black Sea, Aegean Sea, Sea of Marmara and the Mediterranean Sea, and Red scorpionfish, *S. scrofa* in the Aegean Sea, Sea of Marmara and the Mediterranean Sea (Bilecenoğlu et al., 2014; Froese and Pauly, 2021). A small number of *S. loppei* specimens was reported among fish species belonging to the genus *Scorpaena* in studies conducted from the Turkish waters (Keskin and Eryılmaz, 2009; Filiz et al., 2010).

Many studies have been focused on the biology and diagnostic features of the *Scorpaena* species (Hureau and Litvinenko, 1986; Morato et al., 2001; Yedier and Bostanci, 2021a). Although there are several studies based on morphology and karyology with the taxonomic status of this species (La Mesa, 2005; Turan et al., 2009), the amount of genetic difference and phylogenetic relationships between these species still needs to be investigated. As in many parts of the world, systematic knowledge of these species is quite limited in Turkish marine waters. Therefore, detailed knowledge of the *Scorpaena* systematic is important not only for evolutionary and taxonomic purposes but also for species delimitation in population and stock assessment studies.

Several studies that focused on the systematic and phylogeny of the species in the Scorpaenidae family have been conducted in limited areas. Their results frequently have emphasized the complexity of clarifying the position of the species and genera in this family (Kai et al., 2003; Shinohara and Imamura, 2007). Further, they reported a discrepancy between cytogenetic/genetic and morphological results, indicating the species belonging to this genus should be taxonomically reevaluated in detail. Thus, it was suggested that *Scorpaena* species can be evaluated in detail using different methods together (Arculeo and Lo Brutto 2014; Trojette et al., 2014; Yedier and Bostanci, 2021b) and using molecular and otolith biometry methods together can contribute to the differentiation of *Scorpaena* species and reveal the phylogenetic relationships between them. Under the light of previous literature, the present study aims to investigate the discrimination and the phylogenetic relationship of five *Scorpaena* species (*S. elongata, S. maderensis*, *S. notata*, *S. porcus*, and *S. scrofa*), sampled from four different seas (Mediterranean Sea, Aegean Sea, Black Sea, and Sea of Marmara) using both molecular and otolith shape analyzes.

2. Materials and methods

2.1. Sample collection

Fish samples of *S. elongata, S. maderensis, S. notata, S. porcus,* and *S. scrofa* were obtained using trawl and nets of different mesh sizes during the 2019-2020 fishing season from eight sites (Antalya, Balıkesir, Çanakkale,

Hatay, İzmir, Marmara Ereğlisi, Ordu and Şile) in the four different seas (Aegean Sea, Black Sea, Mediterranean Sea, and Sea of Marmara) in Turkey (Figure. 1). The total length (TL) of the samples was measured to the nearest 0.1 cm and then fish samples were sexed. Caudal fins of fish samples were stored in 96% ethanol for molecular analysis, and sagittal otoliths were stored dry in 96-well plates at room temperature for otolith shape analysis.

2.2. Molecular analysis

The present study used cytochrome c oxidase subunit I (COI) gene, which is widely employed in phylogenetic studies because it is highly conserved, for molecular identification of the *Scorpaena* species. Molecular analyzes were performed on individuals sampled from eight sites from the four seas. Overall, 51 individuals belonging to five *Scorpaena* taxa were collected from eight sites. Total DNA was extracted from the caudal fin tissues (approximately 2 mm^2) using a commercial DNA extraction kit protocol (Invitrogen PureLink Genomic DNA Purification Kit). The DNA extractions were verified using agarose gel electrophoresis (0.8%-Tris-Borate-EDTA), including the use of 0.5 mg/L of ethidium bromide for staining purposes and examination under UV light. DNA barcoding region, a 652-bp-long fragment of the mitochondrial COI gene, was amplified using universal fish barcoding primer pair Fish-F2 (5′-TCGACTAATCATAAAGATATCGGGAC-3′) and Fish-R2 (5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′) (Ward et al., 2005). PCR reactions contained 10 µL of template DNA (25–50 ng/µL), 5 µL each of forward and reverse primers (10 pM of each primer), 25 µL of PCR Master Mix (2x) (Thermo Fisher Scientific), and 5 µL of ddH_2O for 50 µL reaction mixture. Thermal cycler parameters were set to 95 °C for 2 min for an initial denaturation step, followed by 35 cycles at 95 °C for 30 s, annealing at 54 °C for the 30s, 72 °C for 60 s and finally 72 °C for 10 min (Keskin and Atar, 2013). PCR products were checked for optimal fragment size using agarose gel (1%) electrophoresis. Purification of PCR products was conducted using a commercial PCR purification kit protocol (Invitrogen PureLink PCR Purification Kit). When the success of the amplification was confirmed, the DNA sequence of the samples was determined using a Genetic Analyzer (Applied Biosystems) by Macrogen (MacroGen Inc., Netherlands).

2.3. Otolith shape analysis

A data set was created to include both left and right otoliths belonging to male and female individuals. A total of 160 otoliths (80 left and 80 right) belonging to 80 fish samples (40 males and 40 females) were selected for each *Scorpaena* species from each site. Each sagittal otolith was examined under a stereo-microscope fitted. Images of the otoliths were taken using a camera connected to the stereo-microscope. Overall, 2720 sagittal otoliths such as *S. elongata* (480), *S. maderensis* (480), *S. notata* (640), *S. porcus* (640), and *S. scrofa* (480) were analyzed for five *Scorpaena* species were collected from eight sites. The otolith width (OW, mm), otolith length (OL, mm), otolith perimeter (OP, mm), and otolith area (OA, mm^2) were measured using the Leica Application Suite (Version 3.7.0) program. These measurements were used to calculate six shape indices: aspect ratio (AR), ellipticity (EL), circularity

Figure 1. The geographical location of the sampling sites along the coastline of Turkey.

(C), form factor (FF), rectangularity (R), and roundness (RD) (Tuset et al., 2003; Ponton, 2006). These shape indices were calculated for the left and right sagittal otoliths of both females and males of the *Scorpaena* species. The contour shape from each otolith image was determined based on Elliptic Fourier Descriptors with the SHAPE software (Ver. 1.3) (Iwata and Ukai, 2002). Each harmonic was composed of four coefficients, and they were called the Fourier Coefficients (A, B, C, and D). Fourier power (FP) was calculated to determine the sufficient and necessary number of harmonics for the otolith shape (Crampton, 1995). Fourier power (FP) was calculated using $FP=(A_n^2+B_n^2+C_n^2+D_n^2)/2$ formula. As the first 20 harmonics reached 99.99% of the cumulated power for both left and right otoliths. Nevertheless, the coefficients derived from the 1st harmonic were not considered, since the outline reconstructed with these coefficients is a simple ellipse resulting in maximum FP. These coefficients would then mask the information derived from the other harmonics (Crampton, 1995). Therefore, 19 harmonics, and, thus, 76 Fourier coefficients were used to analyze otolith data from each *Scorpaena* species.

2.4. Data analyses

All sequences were assembled using MEGA X and consensus sequences were aligned using Muscle implemented in MEGA X (Kumar et al., 2018). Haplotypes were detected using DnaSP v6 software (Rozas et al., 2017). The genetic distance among observed clusters was calculated using the Kimura-two-parameter (K2P) distance model in MEGA X for the COI gene (Kumar et al., 2018). Neighbour-Joining (NJ) method was employed to evaluate phylogenetic relationships among *Scorpaena* species of COI data based on the Kimura-2 parameter genetic distance using MEGA X with bootstrap tests of 1000 replicates to verify the robustness of the tree, and to estimate the interspecific genetic distances (Kumar et al., 2018). Using the Kimura-2 parameters, the DNA barcode gap, which is the minimum distance of each species to the nearest neighbor versus the maximum intraspecific distance (Hebert et al., 2003), was calculated for *Scorpaena* species. *Dendrochirus brachypterus* (Cuvier, 1829) was used as an outgroup for all the phylogenetic analyses (JN312280). Neighbour-Joining (NJ) method was employed to evaluate phylogenetic relationships among *Scorpaena* species.

A standardized model (Elliott et al., 1995) was used to remove the total length effect on the otolith measurement. The six shape indices were calculated over these standardized measurement values. The differences between female-male and left-right otoliths data were examined by t-test and paired-t test, respectively. One-way analysis of variance (ANOVA) was used to explore the differences in otolith measurements and shape indices between species. A principal component analysis (PCA) was used on the first 20 harmonics of the Fourier series and otolith shape indices with measurements to reduce the number of variables. The comparison of Fourier coefficients and otolith shape indices with measurements in five species was conducted using canonical discriminant analysis (CDA). A dendrogram was constructed by hierarchical cluster analysis (UPGMA), based on the Euclidian distance values to assess the degree of similarity between *Scorpaena* species. Wilk lambda (λ) was used to evaluate the interspecific discrimination performance of the CDA. Total variance, interspecific variance, and their percentage of agreement between real and predicted group membership were calculated for each *Scorpaena* species. All tests were conducted using the Past (V.2.17c) and SPSS (V.21.0).

3. Results

A total of 1360 samples were examined in the study and all of them were used for the otolith shape analyses, while 51 of them were used in molecular analyses.

3.1. Molecular identification

COI sequences (652 bp) were obtained for 51 individuals of *S. elongata, S. maderensis, S. notata, S. porcus,* and *S. scrofa* in the eight locations from the four seas. There were 18 haplotypes in the five species of the genus *Scorpaena*; 3 haplotypes for *S. elongata, S. maderensis* and *S. scrofa,* 4 haplotypes for *S. notata* and 5 haplotypes for *S. porcus* (Table 1). The sequences obtained in the present study were deposited in GenBank (Accession numbers: MZ673813 – MZ673815 for *S. elongata*, MZ673813 – MZ673815 for *S. maderensis,* MZ673828 – MZ673831 for *S. notata,* MZ673817 – MZ673821 for *S. porcus,* MZ673822 – MZ673824 for *S. scrofa*) (Table 1). The intraspecific distances were ranged from 0 to 0.00307 for *S. elongata*, from 0 to 0.02341 for *S. maderensis*, from 0 to 0.01084 for *S. notata*, from 0 to 0.00618 for *S. porcus*, from 0 to 0.00617 for *S. scrofa* (Table 1). The interspecific distance was ranged from 0.0527 to 0.1569 in the five *Scorpaena* species (Table 2). The highest interspecific genetic distance was between *S. notata* and *S. maderensis* (0.1569), and the lowest genetic distance was between *S. elongata* and *S. scrofa* (0.0527) (Table 2). The genetic distance was also determined between the *Scorpaena* species and *D. brachypterus* (outgroup). High levels of genetic divergences were detected between the two genera. The greatest distance was found to be 0.2685 between *S. maderensis* and *D. brachypterus* (Table 2). Phylogenetic relationship based on the COI gene was revealed by the NJ tree among the five *Scorpaena* species (Figure. 2). The NJ phylogenetic tree showed that two main lineages: one including *S. porcus* and *S. notata*, and another comprising the remaining *Scorpaena* species. *S. scrofa* was the

Table 1. Average genetic distances (Kimura 2-parameters) among *Scorpaena* species and their haplotypes with GenBank accession numbers.

Species	Haplotypes	GenBank Accession Numbers	*S. elongata*			*S. maderensis*			*S. notata*				*S. porcus*					*S. scrofa*		
			Hap1	Hap2	Hap3	Hap1	Hap2	Hap3	Hap1	Hap2	Hap3	Hap4	Hap1	Hap2	Hap3	Hap4	Hap5	Hap1	Hap2	Hap3
S. elongata	Hap1	MZ673813																		
	Hap2	MZ673814	0.00154																	
	Hap3	MZ673815	0.00307	0.00153																
S. maderensis	Hap1	MZ673825	0.08437	0.08264	0.08263															
	Hap2	MZ673826	0.10000	0.09824	0.09822	0.01707														
	Hap3	MZ673827	0.10000	0.09824	0.09822	0.02341	0.00617													
S. notata	Hap1	MZ673828	0.13151	0.13341	0.13521	0.14936	0.15672	0.16060												
	Hap2	MZ673829	0.13161	0.13352	0.13532	0.14737	0.15473	0.15859	0.00772											
	Hap3	MZ673830	0.13544	0.13736	0.13917	0.15314	0.16053	0.16444	0.00772	0.00617										
	Hap4	MZ673831	0.13532	0.13724	0.13905	0.15322	0.16060	0.16452	0.00308	0.01084	0.00462									
S. porcus	Hap1	MZ673817	0.12510	0.12702	0.12879	0.14795	0.15544	0.15740	0.05743	0.06244	0.05908	0.05743								
	Hap2	MZ673818	0.12894	0.13086	0.13265	0.14795	0.15544	0.15740	0.05743	0.06244	0.05908	0.05743	0.00308							
	Hap3	MZ673819	0.12484	0.12674	0.12852	0.14429	0.15174	0.15369	0.05910	0.06413	0.06075	0.05910	0.00462	0.00616						
	Hap4	MZ673820	0.12702	0.12894	0.13072	0.14602	0.15349	0.15544	0.05910	0.06413	0.06075	0.05910	0.00462	0.00154	0.00462					
	Hap5	MZ673821	0.12307	0.12497	0.12674	0.14419	0.15164	0.15359	0.05745	0.06247	0.05910	0.05745	0.00307	0.00618	0.00154	0.00462				
S. scrofa	Hap1	MZ673822	0.05461	0.05630	0.05624	0.11089	0.12528	0.12159	0.13681	0.12744	0.13500	0.14064	0.13250	0.13638	0.13415	0.13444	0.13236			
	Hap2	MZ673823	0.05123	0.05291	0.05454	0.11089	0.12528	0.12159	0.13301	0.12370	0.13121	0.13681	0.12866	0.13250	0.13031	0.13058	0.12852	0.00308		
	Hap3	MZ673824	0.04793	0.04961	0.05123	0.10914	0.12349	0.11980	0.13482	0.12736	0.13491	0.13863	0.13044	0.13429	0.13209	0.13236	0.13031	0.00617	0.00307	
Dendrochirus brachypterus		JN312280	0.23622	0.23622	0.23826	0.26389	0.27313	0.26837	0.22827	0.22186	0.22625	0.22827	0.21263	0.21701	0.21442	0.21482	0.21244	0.21660	0.21224	0.21461

Table 2. Interspecific genetic distance (Kimura 2-parameters) and standard error values of five *Scorpaena* species with the outgroup.

	S. elongata	*S. maderensis*	*S. notata*	*S. porcus*	*S. scrofa*	*D. brachypterus*
S. elongata		0.0123	0.0161	0.0157	0.0093	0.0228
S. maderensis	0.0936		0.0164	0.0163	0.0141	0.0239
S. notata	0.1353	0.1569		0.0094	0.0155	0.0217
S. porcus	0.1277	0.1517	0.0598		0.0158	0.0211
S. scrofa	0.0527	0.1187	0.1334	0.1320		0.0204
D. brachypterus	0.2369	0.2685	0.2262	0.2143	0.2145	

Lower left diagonal: Genetic distance; Upper right diagonal: standard error values.

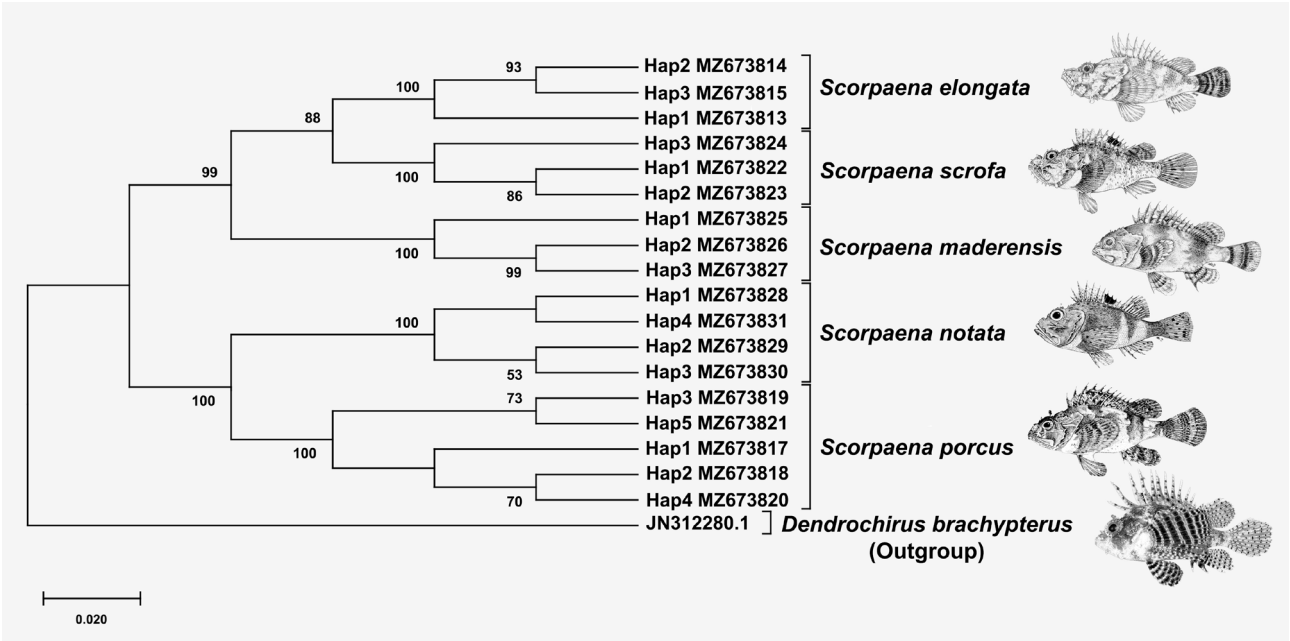

Figure 2. Phylogenetic tree based on Neighbour Joining method analysis of COI gene for five *Scorpaena* species and their haplotypes. Only bootstrap values greater than 50 were shown (1000 replicates). *D. brachypterus* was used as an outgroup.

sister group to *S. elongata*. Also, *S. maderensis* was the sister lineage of this group (Figure. 2). Besides, aligned sequences of the *Scorpaena* species in the study were compared with existing data from the barcode of life data (BOLD) and GenBank databases. The dataset for COI included 18 individuals sequenced by this study and 65 individuals from BOLD and GenBank (COI sequences, 652 bp) belonging to 27 *Scorpaena* species (Figure. 3). NJ tree was constructed under the assumption of the Kimura 2 parameter model to evaluate the phylogenetic relationships among *Scorpaena* species (Figure. 3).

Barcode gap and threshold values were calculated according to Kimura 2 parameters, and no overlap was found between intraspecific and interspecific genetic distances in the five *Scorpaena* species (Table 3). According to these results, the highest intraspecific and lowest interspecific threshold values of *S. elongata* were determined as 0.003074 and 0.052731, respectively (Table 3). Besides, the highest intraspecific and lowest interspecific threshold values of *S. maderensis* were determined as 0.023413 and 0.052731, respectively (Table 3). Moreover, the highest intraspecific and lowest interspecific threshold values of *S. notata* were determined as 0.010840 and 0.052731, respectively (Table 3). Furthermore, the highest intraspecific and lowest interspecific threshold values of *S. porcus* were determined as 0.006180 and 0.052731, respectively (Table 3). Besides, the highest intraspecific and lowest interspecific threshold values of *S. scrofa* were determined as 0.006170 and 0.052731, respectively (Table 3).

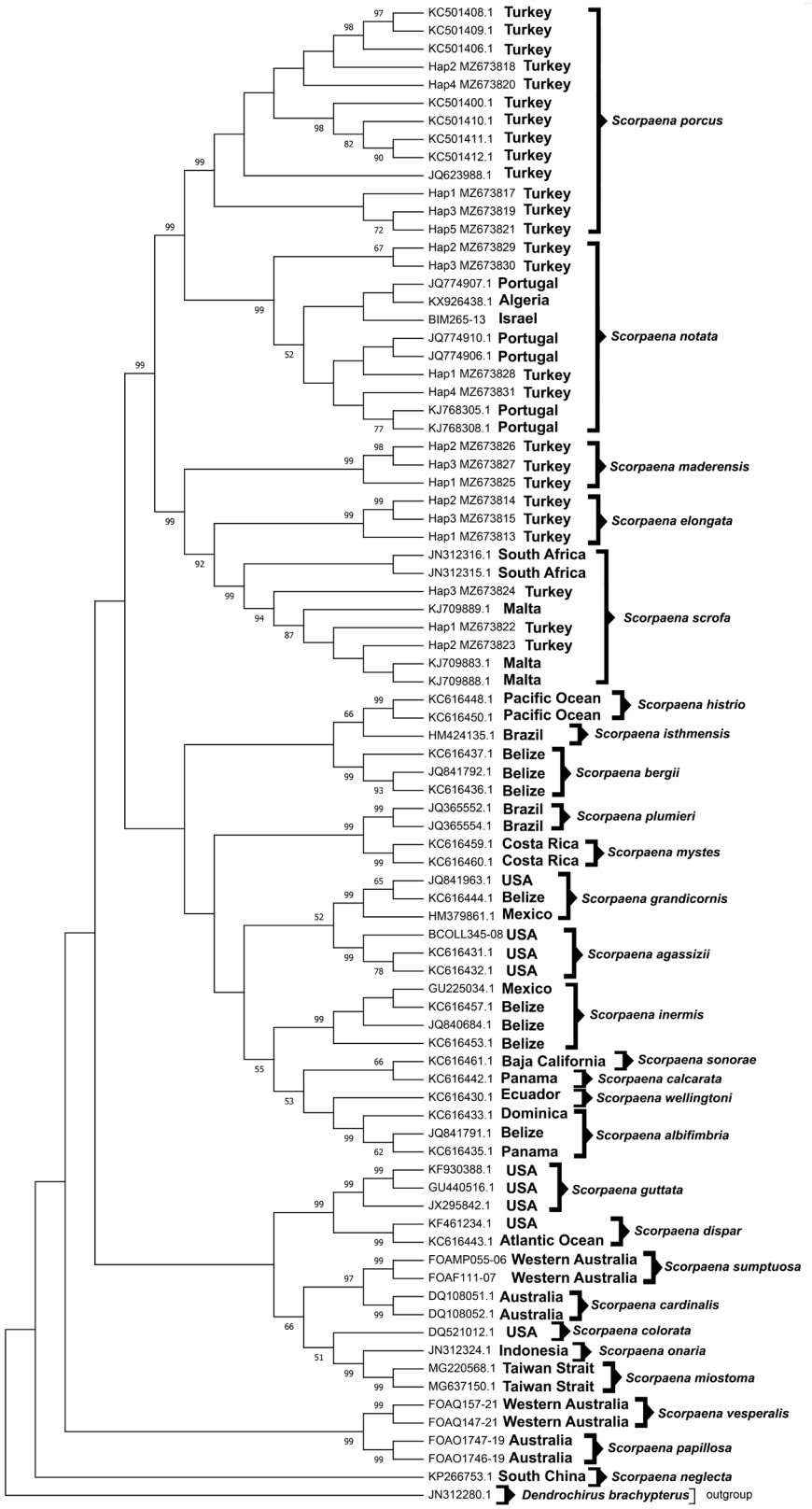

Figure 3. Phylogenetic tree based on Neighbour Joining method analysis of COI sequences of *Scorpaena* species obtained in the present study and found in GenBank and BOLD Systems. Only bootstrap values greater than 50 are shown. *D. brachypterus* was used as an outgroup.

3.2. Morphometric analysis

Descriptive statistics regarding the left and right otolith measurements and shape indices of the five *Scorpaena* species are present in Table 4. No significant differences were observed between the otolith morphometric measurements of females and males in the *Scorpaena* species (t-test, $p > 0.05$). Since the difference between right and left otoliths in most of the otolith measurements and shape indices for *Scorpaena* species was found statistically significant (Paired-t test, $p < 0.05$), the left and right otoliths of both sexes were pooled and used in the present study. One-way ANOVA results on the otolith measurements and shape indices showed that all the variables significantly differed among *Scorpaena* species ($p < 0.05$; Table 4).

Average shapes of the otoliths were reproduced by Principal Components Analysis (PCA) using the Fourier descriptors and otolith shape indices with the morphometric measurements. The first eleven principal components of the analysis were significant, and they were selected to construct the discriminant functions in the study. They were displayed detailed differences for each *Scorpaen*a species (Figure. 4).

UPGMA cluster analysis, using Euclidean distance, for otolith shape clearly distinguished the *Scorpaena* species. The dendrogram is based on Fourier coefficients and six otolith shape indices with four otolith morphometric variables (Figure. 5). Five *Scorpaena* species examined as a result of cluster analysis formed two groups. In the first group, the *S. porcus* lineage separated from the *S. notata*. In this second group, *S. maderensis* is also separated from *S. elongata* and *S. scrofa* (Figure. 5). The canonical discriminant analysis explains the interspecific variability among *Scorpaena* species based on the otolith shape indices with otolith measurements and otolith Fourier coefficients. The first four canonical discriminant functions (F1:49.1%, Wilks $\lambda=0.010$, $p < 0.001$; F2:40.3%; Wilks $\lambda=0.068$, $p <$

Table 3. Intraspecific and interspecific genetic distances (Kimura 2-parameters) and their descriptive statistical values used for the barcoding gap of the five *Scorpaena* species.

	Intraspecific					**Interspecific**
	S. elongata	***S. maderensis***	***S. notata***	***S. porcus***	***S. scrofa***	
Average	0.001537	0.011665	0.005734	0.003638	0.003079	0.116184
Standard Deviation	0.001255	0.010540	0.003540	0.001982	0.002520	0.036048
Standard Error	0.000627	0.005270	0.001340	0.000598	0.001260	0.011399
Minimum	0	0	0	0	0	0.052731
Maximum	0.003074	0.023413	0.010840	0.006180	0.006170	0.156985

Table 4. Descriptive statistics of left and right otolith measurements and shape indices (Mean±SD) with Tukey's HSD comparisons for the five *Scorpaena* species.

Species		**OA**	**OL**	**OP**	**OW**	**AR**	**EL**	**FF**	**RD**	**C**	**R**
S. elongata	Left Side	12.79±2.62^{b}	6.59±0.64^{c}	16.35±1.85^{c}	2.82±0.27^{c}	2.34±0.10^{c}	0.40±0.02^{d}	0.59±0.04^{a}	0.37±0.03^{a}	21.20±1.26^{d}	0.67±0.03^{a}
S. maderensis		8.73±0.72^{e}	5.72±0.14^{e}	14.27±0.54^{d}	2.25±0.10^{e}	2.55±0.11^{a}	0.44±0.02^{a}	0.54±0.05^{b}	0.34±0.02^{b}	23.47±2.26^{c}	0.68±0.05^{a}
S. notata		11.67±2.97^{c}	7.76±1.02^{b}	16.85±2.14^{b}	3.37±0.46^{b}	2.31±0.18^{c}	0.40±0.02^{d}	0.49±0.04^{d}	0.23±0.02^{d}	25.59±1.99^{a}	0.42±0.04^{c}
S. porcus		11.02±1.33^{d}	6.42±0.52^{d}	16.64±1.08^{bc}	2.51±0.22^{d}	2.56±0.16^{a}	0.42±0.02^{b}	0.49±0.03^{d}	0.33±0.03^{c}	25.52±1.50^{ab}	0.66±0.07^{b}
S. scrofa		23.71±2.79^{a}	9.36±0.67^{a}	24.33±2.02^{a}	3.88±0.29^{a}	2.42±0.16^{b}	0.41±0.03^{c}	0.51±0.05^{c}	0.34±0.03^{bc}	25.12±2.47^{b}	0.65±0.05^{b}
S. elongata	Right Side	12.71±2.60^{b}	6.58±0.64^{c}	16.32±1.86^{c}	2.81±0.27^{c}	2.34±0.10^{c}	0.40±0.02^{d}	0.59±0.04^{a}	0.37±0.03^{a}	21.28±1.29^{c}	0.67±0.03^{a}
S. maderensis		8.76±0.72^{e}	5.71±0.17^{e}	14.32±0.46^{d}	2.25±0.09^{e}	2.54±0.11^{a}	0.43±0.02^{a}	0.54±0.05^{b}	0.34±0.03^{b}	23.52±2.19^{b}	0.68±0.05^{a}
S. notata		11.70±2.98^{c}	7.76±1.02^{b}	16.88±2.19^{b}	3.37±0.47^{b}	2.31±0.17^{d}	0.40±0.02^{d}	0.50±0.04^{d}	0.23±0.02^{d}	25.50±2.06^{a}	0.43±0.04^{c}
S. porcus		11.03±1.34^{d}	6.41±0.55^{d}	16.62±1.12^{bc}	2.52±0.22^{d}	2.55±0.17^{a}	0.42±0.02^{b}	0.49±0.03^{d}	0.33±0.03^{c}	25.48±1.51^{a}	0.66±0.07^{b}
S. scrofa		23.67±2.79^{a}	9.36±0.69^{a}	24.28±2.00^{a}	3.86±0.30^{a}	2.43±0.16^{b}	0.42±0.03^{c}	0.51±0.05^{c}	0.34±0.03^{b}	25.07±2.44^{a}	0.65±0.04^{b}

OL: Otolith length; OW: Otolith width; OA: Otolith area; OP: Otolith perimeter; AR: Aspect ratio; EL: Ellipticity; FF: Form factor; RD: Roundness; C: Circularity; R: Rectangularity. Values in rows with different superscript lower case letters are significantly different ($p < 0.05$).

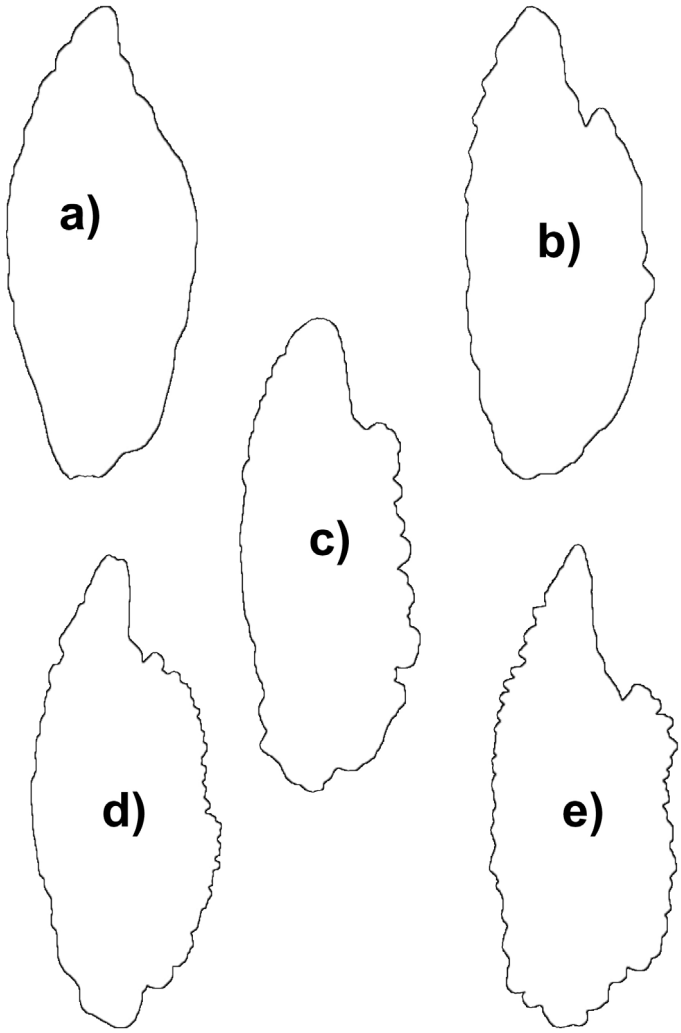

Figure 4. Average shapes of the otoliths in the five *Scorpaena* species, based on mean Fourier descriptors. a) *S. elongata*, b) *S. maderensis*, c) *S. porcus*, d) *S. notata*, e) *S. scrofa*.

0.001; F3:8.9%; Wilks λ=0.400, $p < 0.001$; F4:1.8%; Wilks λ=0.826, $p < 0.001$) were used in the analysis. The CDA produced an overall classification success rate of 96.3% with the highest rate for *S. scrofa* (98.8%), followed by *S. maderensis* (97.5%), *S. notata* (96.6%), *S. elongata* (94.6%), and with the lowest rate for *S. porcus* (94.4%) (Table 5).

4. Discussion

Fishes of the Scorpaenidae are important for global fisheries as well as in rocky-benthic-reef communities (Stewart and Hughes, 2010). The genus *Scorpaena* is distributed throughout the temperate and tropical seas of the world (Hureau and Litvinenko, 1986; Froese and Pauly, 2021). It is known that *Scorpaena* species are difficult to identify at the species level using visual observation alone, due to coloration similarities and overlapping morphological features in different habitats (Hureau and Livtinenko, 1986; Akalın et al., 2011; Froese and Pauly, 2021). For instance, *S. porcus* can be confused with other *Scorpaena* species in terms of both coloration and morphological characters. *S. porcus* was known as the black scorpionfish, but in a study conducted in the eastern English Channel, a sample of a red-colored fish was reported to be molecularly *S. porcus* species (Mahé et al., 2014). Besides, there were overlaps in dorsal fin rays and morphometric features of *Scorpaena* species (Akalın et al., 2011; Froese and Pauly, 2021), and these overlaps were more common in juvenile samples (Akalın et al., 2011). Insufficient work was done for the identification and discrimination of *Scorpaena* species on Turkish coasts. Hence, the current study was focused on the discrimination and identification of *Scorpaena* species in the different localities of Turkish coasts. The species

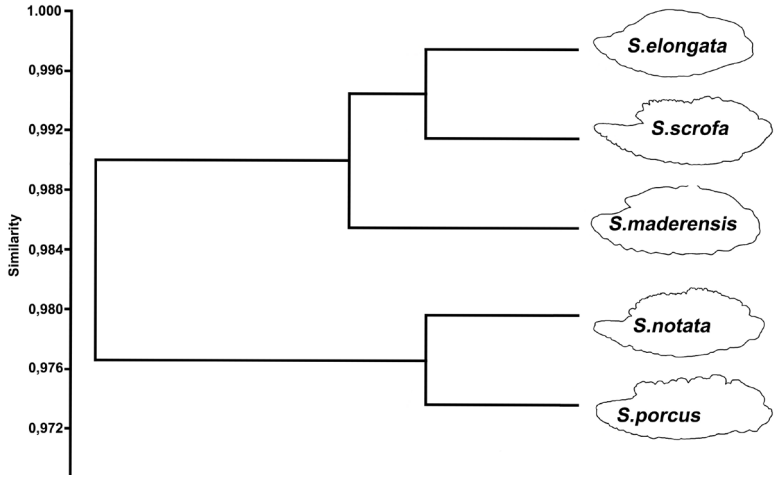

Figure 5. The dissimilarity of *Scorpaena* species based on the Euclidian distance, grouping by hierarchical cluster analysis (UPGMA).

Table 5. Cross-validated classification matrix of the canonical discriminant functions between *Scorpaena* species base on the otolith shape analysis.

Species	*S. elongata*	*S. maderensis*	*S. notata*	*S. porcus*	*S. scrofa*
S. elongata	**94.6(227)**	0	0	0	5.4(13)
S. maderensis	2.5(6)	**97.5(234)**	0	0	0
S. notata	0	0	**96.6(309)**	3.4(11)	0
S. porcus	0		5.6(18)	**94.4(302)**	0
S. scrofa	1.2(3)	0	0	0	**98.8(237)**
Overall classification success is **96.3%.**					

The correct classifications percentages and numbers are in bold; the number of individuals is given in parentheses.

identification and phylogenetic relationships based on traditional and molecular methods are mostly compatible, and they perform together to give more harmonious results (Ward et al., 2005). Therefore, in this study, molecular and otolith shape analyzes were used together for the discrimination and identification of five *Scorpaena* species from the Black Sea, Sea of Marmara, Mediterranean Sea, and the Aegean Sea. To the best of our knowledge, this is the first study to distinguish five *Scorpaena* species sampled from eight sites in four seas by both otolith shape and molecular analysis.

S. elongata, S. scrofa, S. notata, S. maderensis, and *S. porcus* species belonging to the Scorpaenidae family were investigated by 16S rDNA sequences from the Mediterranean Sea (Turan et al., 2009). However, there is no detailed comparison of these species on the Turkish coast of these four seas with respect to the COI gene region. The mitochondrial cytochrome oxidase subunit I (COI) is considered one of the most reliable genes for the discrimination and identification and characterization of many animals, such as fish (Hebert et al., 2003; Ward et al., 2005). There is a great deal of data in molecular databases on the COI gene regions of *Scorpaena* species such as *S. notata, S. porcus,* and *S. scrofa*. However, the COI sequences data of *S. maderensis* and *S. elongata* species are not currently available in GenBank nor BOLD database. This study provides evidence that the COI gene could be an effective tool to discriminate the five *Scorpaena* species distributed in four Turkish seas. In the present study, five *Scorpaena* species from Turkish coasts were found genetically distinct from each other based on COI a gene sequence.

Turan et al. (2009) investigated the systematic status of *S. elongata, S. maderensis, S. notata, S. porcus,* and *S. scrofa* by using mitochondrial 16S rDNA from İskenderun Bay in the Mediterranean Sea. According to the 16S rDNA molecular analysis of thirteen individuals sampled from the İskenderun Bay in the Mediterranean, it was reported that there were six haplotypes in total, two from the *S. elongata* species and one each from the *Scorpaena* species. Turan

et al. (2009) reported the lowest genetic divergence was detected between *S. notata* and *S. porcus*, while the highest genetic divergence was detected between *S. maderensis* and *S. notata*. According to the molecular analysis results of the COI gene regions of the five *Scorpaena* species evaluated in the present study, it was determined that the closest species were *S. elongata* and *S. scrofa*, and the most distant species were *S. maderensis* and *S. notata* (Table 2, Figure 2). However, according to mitochondrial 16S rDNA analysis, in a study conducted on *Scorpaena* samples from the Pula (Croatia), *S. porcus* and *S. scrofa* were phylogenetically close species, while the *S. notata* was the distant one (Saju et al., 2014). Arculeo and Brutto (2014) compared the mitochondrial 12S rRNA regions of *S. notata, S. porcus,* and *S. scrofa* from Mediterranean waters. In that study, the most distant species were *S. scrofa* and *S. porcus*, and the closest species were *S. notata* and *S. scrofa*, contrary to Saju et al.'s (2014) study. In the present study, it was concluded that among these three *Scorpaena* species (*S. notata*, *S. scrofa,* and *S. porcus*), the closest species are *S. notata* and *S. porcus*, while *S. scrofa* is quite different from these species. When the current study is compared with the two studies mentioned above, it is thought that these differences are caused by habitat and genetic region differences other as well as environmental-ecological factors.

A BOLD and GenBank evaluation revealed that the COI sequence of the *Scorpaena* species examined in the present study was quite similar to the previously published sequence (Figure. 3). As a result of the evaluation of the phylogenetic relationship of 83 individuals from 27 species belonging to the genus *Scorpaena* from different habitats in many countries, it was revealed that there were deep divergences between species and minimal differences within species (Figure. 3). In the Mediterranean and Aegean seas, several barcoding studies have been carried out on *S. scrofa* and *S. porcus* species among *Scorpaena* species (Keskin and Atar 2013; Landi et al., 2014). The mean genetic intraspecific distances of *S. elongata, S. maderensis, S. notata, S. porcus,* and *S. scrofa* species evaluated in the present study are 0.001537, 0.011665, 0.005734, 0.003638, and 0.003079, respectively. Keskin and Atar (2013) reported the mean intraspecific genetic distance from 0.040 to 0.062 for *S. scrofa* specimens. In previous DNA barcoding studies on fish species, the mean intraspecific genetic distance was calculated as ≤ 1% (Ward et al., 2005; Hubert et al., 2008; Nwani et al., 2011). For instance, Rasmussen et al. (2009) made molecular analyzes based on the COI gene region of six *Oncorhynchus* species such as *O. tshawytscha, O. nerka, O. keta, O. kisutch, O. gorbuscha*, and *O. mykiss* distributed in North America. The intraspecific genetic distance values for these six *Oncorhynchus* species were ranged from 0.04 to 0.40 (Rasmussen et al., 2009).

Many studies have reported that a good barcode should have a greater interspecific distance than intraspecific variation and exhibit a pattern commonly known as the barcode gap (Hebert et al., 2003; Meyer and Paulay 2005; Hajibabaei et al., 2007). Investigators have recommended that the genetic distance between species should be much greater than the intraspecific genetic distance, and species' limits are based on an average genetic distance of ≥2% among individuals in different species (Hebert et al., 2003; Ward, 2009). For the five studied *Scorpaena* species in the present study, the interspecific distances were greater than 0.02 and they were ranged from 0.052731 to 0.156985. Besides, for the five *Scorpaena* species studied in our study, the genetic distance between species was found to be considerably greater than the intraspecific genetic distance (Table 3). According to our data set, no intraspecific–interspecific distance overlap was detected, and a distinct barcoding gap was found between interspecific and intraspecific distances in each *Scorpaena* species. Similar results were observed by Keskin and Atar (2013), who studied the commercially important fish species in Turkey. Results of the current study indicate that the COI gene sequence can be effectively used to identify the five species of genus *Scorpaena* by DNA barcoding.

In the literature reviews, it was determined that otoliths are used intensely in studies on the age and growth characteristics of species belonging to the genus *Scorpaena* (La Mesa et al., 2010; Şahin et al., 2019). Many studies were conducted in the last two decades claim that otolith characteristics are genetically encoded for some fish species and therefore they were stated to have phylogenetic signals (Tuset et al., 2008; Teimori et al., 2014; Tuset et al., 2016). Otoliths are species-specific bony structures, and they are used to discriminate fish species and stocks in many studies (Tuset et al., 2008; Bostanci et al., 2015; Bostanci and Yedier, 2018; Yedier, 2021). Similarly, in our study, it was observed that otoliths, among five *Scorpaena* species, can be used in species discrimination, thanks to their unique characteristics. When the mean values of the otolith shape indices of these five *Scorpaena* species are evaluated comparatively, the aspect ratio value is the smallest in the *S. notata* and the largest in the *S. porcus*. The ellipticity value is the smallest in the *S. elongata* and the highest in the *S. maderensis*. The form factor value is the smallest in the *S. notata* and *S. porcus* and the highest in the *S. elongata*. The roundness value is the smallest in the *S. notata* and the highest in the *S. elongata*. The circularity value is in the smallest *S. elongata* and the highest in *S. notata*. The rectangularity value is the smallest in the *S. notata* and the highest in the *S. maderensis*. There is a limited number of studies on the use of otoliths in intraspecific discrimination of *Scorpaena* species. Intraspecific discrimination was made on both left and right otoliths values of *S. porcus* from Hammam

Fiber, Rafraf, and Djerba areas on the Tunisian coast. In that study, the otoliths can be used to discriminate *S. porcus* populations from the Tunisian coast (Trojette et al., 2014). Although some morphological and morphometric data of the otoliths of the five *Scorpaena* species examined within the scope of the study were given in the otolith atlas of Tuset et al.(2008), no study was found in the literature on the interspecific differentiation of *Scorpaena* species, which were evaluated in detail using otolith shape analysis. In the present study, the otolith shape analyses, including otolith shape indices with otolith morphometric measurements and Fourier coefficients, demonstrate the obvious interspecific variation of sagittal otolith shape and provide an efficient tool for the discrimination and identification of five *Scorpaena* species. Wilk lambda (λ) value varies between 0 and 1, and the better the discriminating power of the CDA is when it is close to 0 (Pothin et al., 2006). In the current study, the Wilk lambda (λ) values are determined as 0.010, 0.068, 0.400, and 0.826. These lambda values also support high accuracy in species discrimination. In the present study, the CDA performed on the otolith harmonics and shape indices produced the correct classification of the five species as high as 96.3%. Similarly, high accuracy was reported in many studies with different fish species such as *Sebastes* (Zhuang et al., 2015), *Alburnus* (Bostanci et al., 2015), *Sicyopterus* (Lord et al., 2012), and Caspian gobies (Bani et al., 2013). Such high accuracy indicates that there is clear discrimination between these five *Scorpaena* species based on otolith shape. It also provides robust evidence for the validation of these species. The data collected in the current study, including eight locations from the four different seas, show that interspecific variation of otolith shape can be used as a cost-effective method for the identification and discrimination of *Scorpaena* species. In conclusion, the reduction in fishery resources around the world has encouraged researchers to investigate both the structure and genetic diversity of many commercially important fish species and their populations (Gauldie, 1991; Sotelo et al., 1993; Mackie, 1996). Each fish species should be evaluated in detail to establish biological reference points for sustainable exploitation. For this, taxonomic identification of fish species should be as reliable as possible. It was determined that phylogenetic trees based on both molecular and otolith shapes show similar topologies. These results prove that an otolith shape is an effective tool for identifying and distinguishing fish species. Considering the results obtained in this study, molecular and otolith shape analyzes should be applied together, as in our study, in order to determine the similarities/differences between fish species and to improve the current taxonomic status of the relevant species in future studies.

Declaration of competing interest

The authors report that there is no conflict of interest.

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