Short Communication

Molecular Identification of Mediterranean three *Spicara* Species to Improve 16S rRNA Partial Region Sequences

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Abstract | In this study, the molecular identification of the three Mediterranean *Spicara* species was investigated by using the 16S rRNA gene for confirmation of the taxonomic status and also identify incorrectly classified sequences on the GenBank database. The results of the haplotype network and phylogenetic trees show three distinct *Spicara* haplotypes/haplogroups, corresponding to three different species. Additionally, the number of mutations and high values of sequence divergences were useful in differentiating the three different *Spicara*.

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Introduction

Spicara spp. (Picarels) are small to medium sized fish, and the genus is native to the eastern Atlantic and the western Indian Ocean (Tortonose, 1986; Froese and Pauly, 2022). *Spicara* genus is represented by three species (*Spicara flexuosa*, Rafinesque, 1810, *Spicara maena,* L, and *Spicara smaris*, L) in the Mediterranean and the Black Sea (Heemstra 1990; Şalcıoğlu *et al*., 2021).

Morphologic misidentification among the three Spicara species is possible, due to sexual dimorphism and protogynous hermaphroditism. Previous studies revealed that sex reversal which can be affected by protoygnous hermaphroditism (sex change from female to male) (Salekhova, 1979; Vidalis and Tsimenidis, 1996; Dulcic *et al*., 2000) were widely observed in picarels, which contributes to difficulties in their morphological identification. As a result of this, many studies (Tortonose, 1975; Heemstra, 1981, 1990; Quero *et al*., 2003; Eschmeyer, 2010) reported that *S. flexuosa* was considered as a synonym of *Spicara maena*. On the other hand, previous genetic studies (Imsiridou *et al*., 2011; Georgidias *et al*., 2014; Bektaş *et al*., 2018; Şalcıoğlu *et al*., 2021) indicated that *Spicara flexuosa* was separated from *S. maena.*

Mitochondrial DNA sequences especially partial 16S rRNA were previously used to resolve species delimitation among Sparids (Hanel and Sturmbauer, 2000). Previous phylogenetic study (Şalcıoğlu *et al*., 2021) showed that some of the sequences deposited in GenBank and BOLD originated from *Spicara* genus,

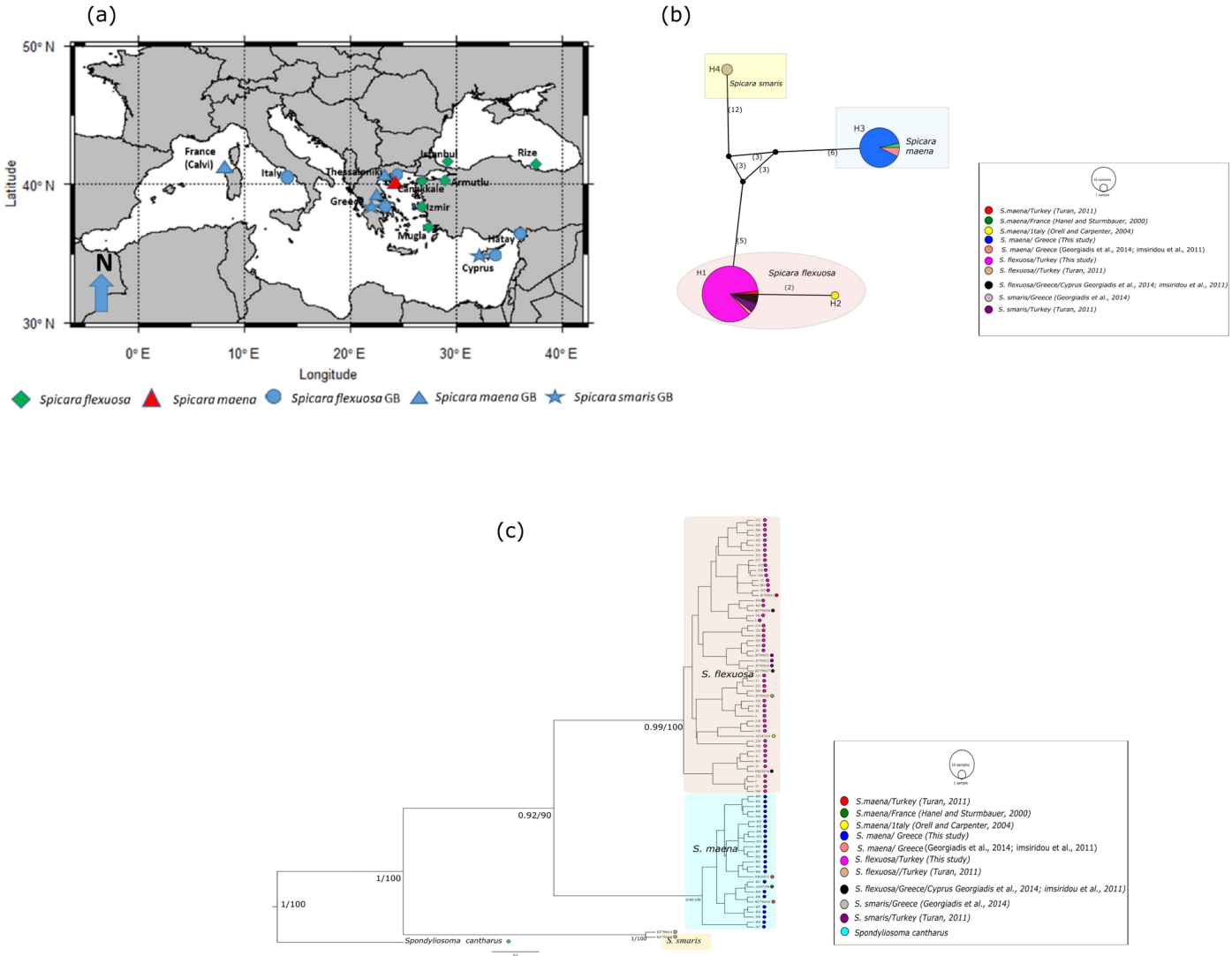

Figure 1: *(A) Map of sampling sites of three Spicara species in this study. Abbreviation is GB: GenBank. (B) The haplotype network based on 16S rRNA sequences of Spicara spp. generated for this study with Spicara spp. sequences available in GenBank. The numbers indicate the number of mutations between haplotypes. Small black circles between the three haplogroups represents missing haplotypes. (C) Phylogenetic trees of 16SrRNA gene of three Spicara species. The values above the branches are the BY posterior probabilities and ML bootstrap values, respectively.*

have been found misidentified based on the COI (Costa *et al.*, 2012; Landi *et al.*, 2014) and cyt-b (Orrell *et al.*, 2002; Sanciangco *et al.*, 2016) genes. Three *Spicara* species were previously evaluated 16S rRNA sequences in the literature (Imsiridou *et al.*, 2011; Georgiadis *et al.*, 2014; Bektaş *et al.*, 2018) however, none of the studies were actually represented incorrectly classified sequences. Therefore, the main aim of this study was to evaluate mtDNA (16S rRNA) gene to identify misidentified sequences and to improve 16S rRNA region sequences on the GenBank Database of the three *Spicara* species.

Materials and Methods

Sample collection

Total 70 individuals of *Spicara flexuosa* and *S. maena* individuals were sampled from Turkish coastal waters (that were represented in the three different Turkish seas) and Greece which were previously identified by two mtDNA and nuclear genes (Figure 1A). Specimens were collected by fishermen, who were using various fisheries equipments by using trawlers and handline fishing. Identification of specimens was based on the main morphological characters of the two species in the identification keys (Tortonose, 1986; Minos *et al.*, 2013). Additionally, experienced field collectors were used to identify *S. maena* based on morphology due to their comparative experience of two *Spicara* species in Greece. GenBank samples were also used in the data set (Figure 1A, Table 1). Fresh tissues of *S. flexuosa/maena* (muscle or caudal) were used and preserved in 80% ethanol in the field and at -20°C in the laboratory.

DNA analyses

Genomic DNA was isolated from the tissues of each specimen with a PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, USA) by using the manufacturer's protocol, and subsequently stored at -20 °C. New 16S rRNA gene primers (Forward: GTGTGCTGCATGGGAAAGAC; Reverse: CTGGTCCACATGGGGGTTTT) were designed by Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on a published sequence of *S. maena* from GenBank (JF795031) (Turan, 2011). The PCR reactions were performed in a total of 25 µl reaction volume, containing 2.5 µL of 10 X Taq Buffer with KCl (100 mM Tris–HCl, 500 mM KCl, pH 8.8), 2 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTPs (10 mM each), 0.5 µL of each primer (20 pM/µL), 0.25 µL of Taq polymerase (5U/µL), and 5 µL of DNA. Thermal cycle conditions for PCR amplification were as follows: 95 °C for 2 min for the initial denaturation step, followed by 35 cycles of denaturation 95 °C for 30 s, annealing at 56 °C for 40 s, extension 72°C for 1 min, and with a final extension step of 72 °C for 7 min (Turan, 2011). All amplified products were sequenced in the Macrogen Europe, Amsterdam, The Netherlands.

Statistical analyses

Alignment of all 16S rRNA gene sequences (including GenBank sequences) were edited manually with Sequencher v. 5.4.1. *(Genecodes Inc., Ann Arbor, MI).* A 469 bp fragment was used for further analyses of the 16S rRNA gene.

A sequence similarity based methods were used for species assignment of each individuals based on the reference sequences (Georgiadis *et al.* (2014), accession codes KF796614-KF796618, and Imsiridou *et al.* (2011) accession codes FJ625835-FJ625836) on the Basic Local Alignment Search Tool (BLAST).

Haplotype and nucleotide diversities, number of mutations, parsimony informative sites, and the number of net nucleotide substitutions between the three species were calculated in DnaSP v.5.10.1 (Librado and Rozas, 2009). Genetic distances between the three species were also determined in MEGA X (Kumar *et al.*, 2018) by using Kimura 2-parameter model (Kimura, 1980). Finally, the average nucleotide composition of the three species of *Spicara* was determined by MEGA X.

Haplotype network, phylogenetic analyses

Relationship among haplotypes of *Spicara* species was determined by haplotype network using the median joining method (Bandelt *et al.*, 1999) in PopART v.1.7 (Leigh and Bryant, 2015).

Phylogenetic relationships between the three species were determined by the Maximum Likelihood (ML) and Bayesian Inference (BY) using the software MEGA X and BEAST v.2.4.8 (Bouckaert *et al.*, 2014), respectively. HKY substitution model was used for building the ML and BY trees. *Spondyliosoma cantharus*, GenBank (AF2474031) was used as the outgroup sequence. The analysis was executed for 4.0×10^7 generations.

Results and Discussion

Statistical analyses

A total of four haplotypes were determined within the 84 sequences, including 14 *Spicara* specimens (four *Spicara flexuosa*; five *Spicara maena* and five sequences of *Spicara smaris*) were retrieved from GenBank. Blast tools analyses of the GenBank sequences are shown in Table 1. Furthermore, Blast tools analyses of the sequences from this study (haplotypes based on the collected samples) are also represented in Table 2. Percentage identity of those sequences ranged from 99.57 to 100 %.

Considering molecular diversities indices, *S. maena* had the lowest haplotype and nucleotide diversities (h=0; π =0). The mean haplotype and nucleotide diversities of *S. flexuosa* (h=0.0360; π =0.0001) were higher than those reported for the *S. meana*. The total number of mutations ranged from 16 (between *S. flexuosa* and *S. maena*) to 22 (between *S. maena* and *S. smaris*) as well as pairwise sequence divergences between the three *Spicara* species ranged from 3 (between *S. maena* and *S. flexuosa*) to 4.44 % (between *S. smaris* and *S. maena)*. Genetic distance values between the three *Spicara* species ranged from 0.031 to 0.046. Among the three species, a total of 28 nucleotide sites exhibited variable, of which 26 were parsimony informative. Of all parsimony informative sites, twelve positions were informative for *S. maena* and *S. flexuosa,* five positions for *S. smaris* and *S. maena* and six for *S. smaris* and *S. flexuosa* (Supplementary Table 1). The mean nucleotide frequencies of *S. flexuosa* was A=28.35, T=20.89, C=26.23, G=24.52; *S. maena* was A=28.57, T=22.17, C=24.95 G=24.31 and *S. smaris* was A=29.60, T=21.80, C=25.10 and G=23.9.

Table 1: *Blast tools analyses of the GenBank sequences used for 16S marker, alignment length, accession numbers, scientific names, total score, E-value, % Identity, mismatches and taxid number. Red bold fonts indicate misidentified sequences on GenBank.*

Species	Accession numbers	Location	Reference sequences' accession numbers	% identity	Alignment length	Total score	E value	Mismatches	Hit species	Taxid
S. maena	AJ247298	France	FJ625835.1; KF796618.1	100	481	889	0.0	0	*S. maena*	98823
S. maena	KF796618	Greece	FJ625835.1	100	566	1046	0.0	0	*S. maena*	98823
S. maena	FJ625835	Greece	KF796618.1	100	566	1046	0.0	0	*S. maena*	98823
S. maena	**JF795031**	**Turkey**	**KF796616.1; KF796617.1**	**100**	**648**	**1197**	**0.0**	**0**	***S. flexuosa***	**458843**
S. maena	**AF247434**	**Italy**	**KF796616.1; KF796617.1**	**99.48**	**577**	**1048**	**0.0**	**2**	***S. flexuosa***	**458843**
S. flexuosa	JF795029	Turkey	KF796616.1; KF796617.1	99.85	648	1192	0.0	1	*S. flexuosa*	458843
S. flexuosa	KF796616	Greece	FJ625836.1	100	566	1046	0.0	0	*S. flexuosa*	458843
S. flexuosa	KF796617	Cyprus	FJ625836.1	100	566	1046	0.0	0	*S. flexuosa*	458843
S. flexuosa	FJ625836	Greece	KF796616.1; KF796617.1	100	566	1046	0.0	0	*S. flexuosa*	458843
S. smaris	**JF795032**	**Turkey**	**KF796616.1; KF796617.1**	**100**	**648**	**1197**	**0.0**	**0**	***S. flexuosa***	**458843**
S. smaris	**JF795033**	**Turkey**	**KF796616.1; KF796617.1**	**100**	**648**	**1197**	**0.0**	**0**	***S. flexuosa***	**458843**
S. smaris	**JF795034**	**Turkey**	**KF796616.1; KF796617.1**	**100**	**648**	**1197**	**0.0**	**0**	***S. flexuosa***	**458843**
S. smaris	KF796614	Greece	KF796615.1	99.74	763	1399	0.0	2	*S. smaris*	119752
S. smaris	KF796615	Cyprus	KF796614.1	99.74	763	1399	0.0	2	*S. smaris*	119752

Table 2: *Blast tools analyses of this study sequences (Haplotypes) used for 16S marker, alignment length, reference sequences' accession numbers, scientific names, maximum score, total score, query cover, E- value, % Identity, mismatches and taxid number.*

Sequence names	Alignment length	Reference sequences' accession numbers	Scientific name	Max. score	Total score	Query cover	E value	% Identity	Mismatches	Taxid
Hap 1	469	KF796617.1; KF796616.1	*S. flexuosa*	867	867	100	0.0	100	0	458843
Hap 3	469	KF796618.1	*S. maena*	867	867	100	0.0	100	0	98823

Haplotype network, phylogenetic analyses

Overall, 46 specimens were determined as *Spicara flexuosa* and 24 as *Spicara maena* from the 16S network (Figure 1B). The results of the haplotype network show three distinct *Spicara* haplotypes/haplogroups, corresponding to three different species. According to classification of Imsiridou *et al.* (2011) and Georgiadis *et al.* (2014), those individuals collected from Turkey, which all have Hap 1, were clustered with reference sequences of *S. flexuosa* from Greece in GenBank. On the other hand, samples collected from Greece coast for this study, which all have Hap 3, were grouping together with reference sequences of *S. maena* in GenBank. Thus, the samples collected from Turkey and Greece corresponded to *S. flexuosa* and *S. maena*, respectively, based on the results for the 16S gene.

Phylogenetic relationships of the three *Spicara* species indicated that three *Spicara* species were also clearly differentiated on the 16 S rRNA topology (Figure 1C), with high posterior probabilities (≥ 0.70) and bootstrap (≥ 70) values. ML and BY analyses result almost identical. In addition, *S. flexuosa* and *S. maena* species were reciprocally monophyletic based on the 16S tree.

Overall results indicated that some sequences previously submitted to GenBank have been incorrectly classified. Focusing on the other GenBank entries, based on the 16S classification that is followed here, samples from Italy (Orell and Carpenter, 2004) as well as *S. maena* and *S. smaris* collected by Turan (2011) (GenBank accession numbers (JF795031-JF795034)) were probably misidentified. On the other hand, the classification of samples, whose sequences were retrieved from GenBank, was based on the studies of Imsiridou *et al.* (2011), Georgiadis *et al.* (2014) and Hanel and Sturmbauer (2000) as *S. maena* were confirmed as correct.

Considering the source studies for some of these sequences and their resolution for diagnosing the

three *Spicara* species, Imsiridou *et al.* (2011) found an interspecific sequence polymorphism between *S. maena* and *S. flexuosa*. The sequences submitted by Georgiadis *et al.* (2014) were previously used to diagnose three *Spicara* species by using real-time (melt-curve haplotype specific) PCR.

Misidentification problem of the three *Spicara* spp. was also observed in another study that used the 16S rRNA marker (Turan, 2011). The results of this study showed that samples assigned to the three different species by Turan (2011) are members of the same species, namely, *S. flexuosa*. Low levels of genetic divergence (0.5 %) between *S. maena* and *S. flexuosa* as well as no genetic divergence between *S. maena* and *S. smaris* were found by Turan (2011). However, the values reported in this study are higher (3.0- 4.4 %) than those recorded by Turan (2011). In the same study, morphological analyses of these *Spicara* spp. were also performed, and the result was congruent with the genetic results. Sexual and seasonal dimorphism, which is reinforced by sex inversion, morphological modifications during spawning seasons (Pollard and Pichot, 1971; Froese and Pauly, 2022; Minos *et al.*, 2013) might be some other reasons for morphological incorrect identification as observed by Turan (2011).

Genetic differentiation between *Spicara maena* and *S. flexuosa* was initially reported by Imsiridou *et al.* (2011). The results from the number of mutations (16) and genetic distance value (p=0.031) between *S. maena* and *S. flexuosa* were found nearly identical with Imsiridou *et al.* (2011), who found total 15 mutations and genetic distance values (p=0.027) between two *Spicara* species.

Recently, genetic differentiation of the three species of *Spicara* and intraspecific substructure of *S. flexuosa* were investigated by using 16S rRNA and cytochrome b genes (Bektaş *et al.*, 2018). The results from Bektaş *et al.* (2018) found that three *Spicara* species were clearly separated from each other based on the phylogenetic trees. The tree from this study also showed that *S. flexuosa* and *S. maena* are more closely related each other than *S. smaris* as observed in Bektaş *et al.* (2018). With regard to molecular diversity indices results as seen in Bektaş *et al.* (2018), three *Spicara* species had low levels of haplotype and nucleotide diversities for the cyt-b and 16S rRNA genes. Low levels of molecular diversities indices for COI (h=0.0340, π=0.0001) and cyt-b genes (h=0.0710, π=0.0002) and star-shaped haplotype network for *S. flexuosa* were also found in the previous study (Şalcıoğlu *et al.*, 2021), and this study. Moreover, relatively high interspecific sequence variations among the three *Spicara* species based on the 16S (2.90-4.44 %) and cyt-b genes (7.99-9.41 %) were reported by Bektaş *et al.* (2018), similar to 16S (3.0- 4.4 %) (this study) and cyt-b findings (9.01- 9.35 %) (Şalcıoğlu *et al.*, 2021).

Conclusions and Recommendations

The taxonomy of the genus *Spicara* has been problematic around the Mediterranean region. The result of the current phylogenetic analyses (ML and Bayesian) and the haplotype network using data of this gene indicated three different species namely, *S. maena, S. flexuosa, and S. smaris*. Overall results indicated that some of the sequences that belongs to studies by (Orell and Carpenter, 2004; Turan, 2011) previously submitted to GenBank have been incorrectly classified.

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Novelty Statement

The present study revealed the first focus on the study of the identification and confirmation of incorrectly classified sequences on the Genbank Database based on the 16S r RNA gene of Spicara species. The results of this study have been outperformed of the previous studies based on the phylogenetic analyses approach.

Data availability

New 16S rRNA sequences of each samples have been deposited to GenBank under the following accession numbers: OM935680-OM935682. Parsimony informative sites of each Spicara species are given in Supplementary Table 1.

Ethics approval

The work raises no ethical issues. All fish examined were from commercial fishing activities and none of them were killed for the specific purpose of the study.

Supplementary Material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.vsrr/2022.8.1.8.14

Conflict of ınterest

The author has declared no conflict of interest.

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