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The systematic status of the Mediterranean Spicara species (Centracanthidae) inferred from mitochondrial 16S rDNA sequence and morphological data

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Abstract

The mitochondrial 16S ribosomal DNA together with morphological data were used to elucidate monophyly of the family Centracanthidae and interrelationships of *Spicara* and *Centracanthus* genera, including four species, *Spicara maena, Spicara flexuosa, Spicara smaris* and *Centracanthus cirrus*. Examination of the gene revealed a moderate amount of thymine and abundance of adenine. The 16S rDNA dataset contained 92 variable and 69 parsimony informative sites with a mean nucleotide diversity of 0.099. Haplotype diversity was found to be 0.71. No genetic differences were observed between *S. maena* and *S. smaris*, and the genetic divergence between *S. flexuosa* and both *S. maena* and *S. smaris* was found to be 0.005. The intergeneric divergence was found to be very high (0.237) between *S. alta* and *C. cirrus*. For the other *Spicara* species, intergeneric divergence ranged from 0.170 between *C. cirrus* and both *S. maena* and *S. smaris* to 0.176 between *C. cirrus* and *S. flexuosa*. Minumum evolution, neighbor joining and parsimony trees revealed same tree topologies, and the monophyly of the genus *Spicara* was not supported. *S. maena* and *S. smaris* clustered together and showed close relationship and *S. flexuosa* was nodded with this group. Therefore *S. maena* was found to be more closely related to *S. smaris* rather than *S. flexuosa*. On the other hand, *S. alta* highly divergently clustered outside of this group and branched with *C. cirrus*.

Multivariate analysis of morphological data was congruent with the genetic data and revealed similar pattern of relationship among Centracanthidae species.

Key Words: Centracanthidae, *Spicara***,** *Centracanthus*, Systematics, mtDNA, Sequence, Morphology

Introduction

The family Centracanthidae is one of the most common families of fish distributed throughout the Mediterranean Sea and represented with two genera including four species (*Spicara maena, Spicara flexuosa, Spicara smaris, Centracanthus cirrus*) (Fischer *et al.* 1987; Haemstra 1990; Nelson 1994; Turan *et al*. 2008). *Spicara maena* (Linnaeus 1758) occurs in the Mediterranean, Black Sea, in the Atlantic from Portugal to Morocco and the Canaries, and usually found on Posidonia beds, rocks, and mud down to about 100 m (Tortonese 1986). *S. maena* used to be recognized as subspecies *S. m. maena* (Fischer *et al.* 1987; Aksiray 1987). *Spicara flexuosa* Rafinesque, 1810 occurs in the Mediterranean, Black Sea and off Portugal, inhabit on muddy bottoms, down to about 130 m (Tortonese 1986; Fischer *et al*. 1987; Nelson 1994). *S. flexuosa* also used to be recognized as subspecies of *S. maena* and named *S. m. flexuosa* (Fischer *et al.* 1987; Aksiray 1987). *Spicara smaris* (Linnaeus 1758) is a very common Mediterranean demersal fish species and found in the entire Mediterranean, the Black Sea, the southern Sea of Azov, and Atlantic coasts from Portugal to Morocco, and inhabits on Posidonia beds and muddy bottoms. *Spicara alta* (Osório 1917) is a commercial fish species and found eastern central Atlantic: Dakar, Senegal to southern Angola (Tortonese 1986). *Centracanthus cirrus* Rafinesque, 1810 occurs in the Mediterranean, Aegean Sea, in the Atlantic from Portugal to Morocco and off Maderia, found over rocky or gravelly bottoms to depth of 200 m (Tortonese 1986; Haemstra 1990).

Several publications have given detailed information about the biology, distribution and the identification characteristics of the species of the family Centracanthidae (Fischer *et al.* 1987; Aksiray 1987; Ismen 1995; Vidalis *et al.* 1997; Dulcic *et al*. 2000). However the phylogenetic

relationship of these species is still ambiguous. Because the classification of the family Centracanthidae have mainly been based on morphological characters, which have conflicted and not provided conclusive answers to the phylogenetic questions. Genetic relationships and amount of genetic divergence between these species are still lacking enough evidence to support present species even family status (Pollard and Pichot 1971; Orrell *et al*. 2002; Orrell and Carpenter 2004).

Mitochondrial DNA analysis is a very useful tool for molecular systematics because of its special features (Meyer *et al.* 1990; Normark *et al.* 1991; Meyer 1992). The pattern of maternally inheritance and rapid rate of evolutionary change of mtDNA compared to nuclear DNA makes it suitable tool to accomplish genetic studies among taxa of several fish groups at multiple taxonomic levels (Kocher and Stepien 1997; Zardoya and Doadrio 1999; Durand *et al.* 2002). The mitochondrial 16S rDNA gene has proven a valuable evolutionary marker for fishes because it has produced robust phylogenies at various taxonomic levels (Brown *et al.* 1982; Karaiskou *et al.* 2003; Perez *et al.* 2005; Turan *et al*. 2008).

In the present study the pattern of phylogenetic relationships of two genera, including four species, *Spicara maena, Spicara flexuosa, Spicara smaris, Centracanthus cirrus,* living in the Mediterranean Sea, were investigated together with mtDNA sequence and morphological data. *Spicara alta* obtained from GenBank database was also used only in the molecular analyses.

Materials and Methods

Samples

Individual fish from three species (*Spicara maena, Spicara flexuosa, Spicara smaris)* were collected from Iskenderun Bay in North-eastern Mediterranean Sea, and *Centracanthus cirrus* was sampled from Izmir in the Aegean Sea. The number and location of the samples used in the sequence and morphological analyses are given in Table 1. The samples were placed individually in plastic bags, and kept frozen at -20°C until

transportation. In the laboratory the muscle tissues were dissected and stored at -20°C until the molecular analyses are carried out and that the specimens were kept frozen until they were examined for morphology.

Table 1. Sampling details of Centracanthidae species and Gen Bank accession no for 16SrRNA segment. n-S and n-M sample size sequenced and used in morphological analysis respectively.

Species	Sampling location	Latitude	n-S	n-M	GenBank Accession No.
S. flexuosa	N. Mediterranean Sea -Iskenderun Bay	36° 05'N 36° 65'E	2	43	JF795028 JF795029
S. maena	N. Mediterranean Sea - Iskenderun Bay	36° 05'N 36° 65'E	2	30	JF795030 JF795031
S. smaris	N. Mediterranean Sea - Iskenderun Bay	36° 05'N 36° 65'E	3	47	JF795032 JF795033 JF795034
C. cirrus	Aegean Sea-Izmir Bay	26° 85'N 38° 35'E	3	20	JF795025 JF795026 JF795027

Morphology

Meristic and morphometric characters commonly used to distinguish Centracanthidae species were used for morphological analysis (Tortonese 1986; Fischer *et al*. 1987; Aksiray 1987). Numbers of unbranched and branched rays in first dorsal fin (DFR), ventral fin (VFR), anal fin (AFR), pectoral fin (PFR), gill rakers (GR) and scales in lateral line (LS) under a binocular microscope were recorded. Vertebrate numbers (VN)

were counted after taking X-ray films of fish. Additionally eye diameter (ED), head width (HW), body depth (BD), body width (BW), pectoral fin length (PFL), distance between snout and eye (N-E), eye and operculum (E-OP), mount and beginning of pectoral fin (pre-pectoral length; PPL) were measured for morphometric data.

There were significant correlation ($P<0.05$) between standard length and morphological data. Therefore it was necessary to remove size effect from the data. Thus principal component analysis (PCA) was used to remove size effect from the shape measures (Somers 1986). This method extracts first component as allometric size factor, allowing the subsequent components to be interpreted as summarizing shape variation independent of size and random variation among the sampled individuals. Hierarchical cluster analysis using the squared Euclidean distances was performed, and the neighbor-joining method was used to estimate phenotypic relationships between species. Morphological analyses were performed using SPSS*v*13, SYSTAT *v*11 and PHYLIP program packages (Felsenstein 1993).

Sequences

Total DNA was isolated from a piece of muscle tissue using DNA extraction Kit (AGOWA, Germany). The PCR reactions were performed with the following cycle parameters: initial denaturation at 94^{o}C for 4 min, followed by 35 cycles of 94^{o}C/30s strand denaturation, 52 ^{o}C/20s annealing and 72 ^{o}C/1 min 30 sec primer extension, and a final 7 min elongation at 72 ^{o}C. The following reagents were used in each PCR reaction: 1.5 µl 10 x polymerase buffer, 0.5 µl dNTP (10 mM), 0.3 µl Teg DNA polymerase (3 U/ µl) equivalent to Taq DNA polymerase, 0.05 µl 16Fi140 primer (100 µM) (5´-CG(CT)AAGGGAA(ACT)GCTGAAA-3´), 0.05 µl 16Fi1524 primer (100 µM) (5´-CCGGTCTGAACTCAGATCACGTAG-3´), 3-5 µl DNA from AGOWA purification, and water for a total reaction volume of 15 µl. Amplified DNA was purificated with Exo/Sap enzymes according to supplier's protocol (Cleveland, Ohio, USA). Finally, all the samples were sequenced in the forward (16Fiseq1463:

5′-TGCACCATTAGGATGTCCRGATCCAAC-3′) and reverse (16sarL: 5′-CGCCTGTTTAACAAAAACAT-3′) directions with an automated sequencer (Model ABI3730, Applied Biosystems).

The initial alignments of partial 16S rDNA 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 pairwise nucleotide variation and to determine nucleotide composition for each taxon using MEGA 3.1 (Kumar *et al*. 2004). The computer program ModelTest (Posada and Crandall 1998) was used to determine the best-fit model of DNA evolution. The HKY (Hasegawa *et al.* 1985) model was determined to be the appropriate model for our dataset. The molecular phylogenetic tree was constructed using the three distinct phylogenetic approaches: a distance-based method using neighbor joining (NJ), (Saitou and Nei 1985) a cladistic approach using the maximum parsimony (MP) criterion, and minimum evolution (ME). The reliability of the inferred phylogenies was evaluated using the bootstrap method (Felsenstein 1985) with 1000 replicates. One species from Sparidae family, morphologically similar to the family Centracanthidae were included in the molecular phylogenetic trees and rooted as out group species from published sequences in GenBank under accession number (*Boops boops*, AF247426). Moreover, 16S rDNA sequence of *Spicara* species obtained from GenBank database (*Spicara alta*, Accession No: AF247435; *Spicara maena*, Accession No: AF247434) was also included in the molecular analyses.

Results

Morphology

Range of observed meristic characters of four sampled Centracanthidae species were in the range of their description given by Tortonese (1986), Fischer *et al*. (1987) and Turan *et al*. (2007) (Table 2).

Table 2. Formula of observed meristic characters of Centracanthidae species. Roman letters show spine rays. DFR, dorsal fin rays; VFR, ventral fin rays; AFR, anal fin rays; PFR, pectoral fin rays; GR, gill rakers; LS, scales in lateral line; VN, vertebra numbers. Sample size is given in Table 1.

Species	DFR	VFR	AFR	PFR	GR	LS	VN
S. flexuosa	XI 11-12	I 5	III 10	15-17	23-30	23-24	67-76
S. maena	XI 11-12	I 5	III 9-10	15-16	26-30	23-24	69-74
S. smaris	XI 10-12	I 5	III 9-10	15-16	24-31	23-24	68-83
C. cirrus	XIII 10-12	I 5	III 10	16-18	26-29	26-27	86-92

In principal component analysis, 33% of the total variation was presented in first principal component which presents allometric size factor and excluded from the analyses. The subsequent components represented 77% of the variation which were used in Hierarchical cluster analysis. VFR was not considered in the analysis because these variables were constant among groups. Squared Euclidiean distances of morphologic data revealed that morphological divergence was very high between the two genera (Table 3). The highest morphologic divergence was observed between *C. cirrus* and *S. flexuosa*, and the lowest was detected between *S. smaris* and *S. maena*.

Table 3. Pairwise genetic distance (below diagonal) and the squared Euclidean distances (above diagonal) between the species.

Species	*S. flexuosa*	*S. maena*	*S. smaris*	*S. alta*	*C. cirrus*	*B. boops*
S. flexuosa	-	376	507		3612	
S. maena	0.005	-	202		3085	
S. smaris	0.005	0.000	-		2173	
S. alta	0.175	0.168	0.168	-		
C. cirrus	0.176	0.170	0.170	0.237	-	
B. boops	0.393	0.389	0.389	0.491	0.557	-

In the neighbor-joining analysis based on the squared Euclidean distances, *S. smaris* and *S. maena* are closely related in one node while *S. flexuosa* is in the neighbouring node. *C. cirrus* highly divergently clustered from the species of the genera *Spicara* (Figure 1). High bootstrapping values were detected for each node on the neighbor-joining tree.

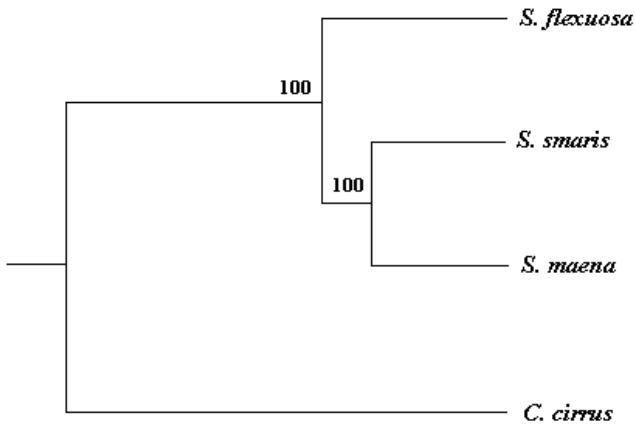

Figure 1. Neighbor-joining tree based on squared Euclidean distances of morphologic data. Numbers on nodes indicate the bootstrap values.

Examination of the contribution of each variable to the principal components showed high contributions from meristic (lateral scale numbers, vertebrate numbers, anal fin rays) and morphometric (distance between snout and eye, eye and operculum and pre-pectoral length) characters, indicating that these characters play important roles to species differentiation (Figure 2).

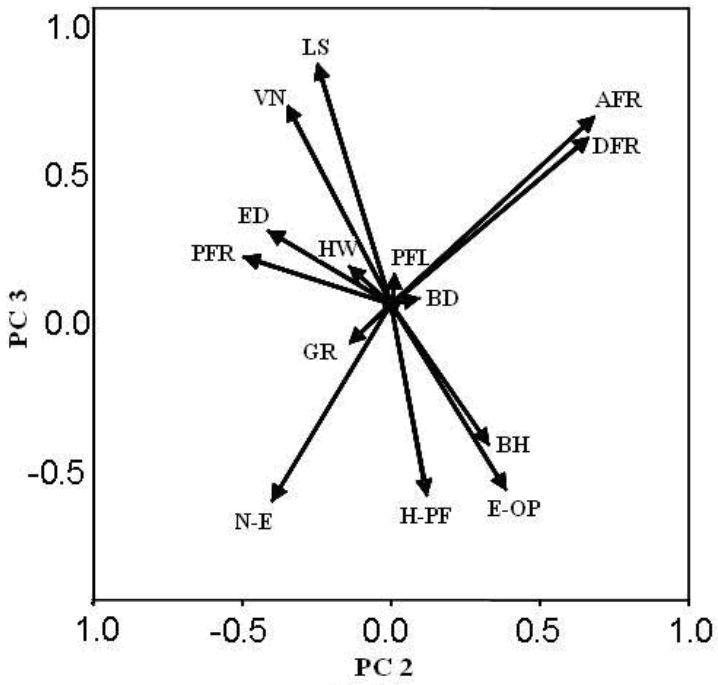

Figure 2. Contribution of meristic and morphometric variables to the principal components. Vectors indicate the loadings of the scores for each variable on principal components for species differentiation.

Sequences

After alignment, the partial 16S rDNA gene sequences consisted of 898 bp. Examination of the gene reveals a moderate amount of thymine (T; 21.5%) and abundance of adenine (A; 30.9%). The 16SrRNA dataset contained 92 variable and 69 parsimony informative sites, and the mean nucleotide diversity (*Pi*) was found to be 0.099. Haplotype diversity was found to be 0.71, and 4 different haplotypes were observed. Minimum spanning tree illustrating the phylogenetic relationships between 16S rDNA gene haplotypes are given in Figure 3.

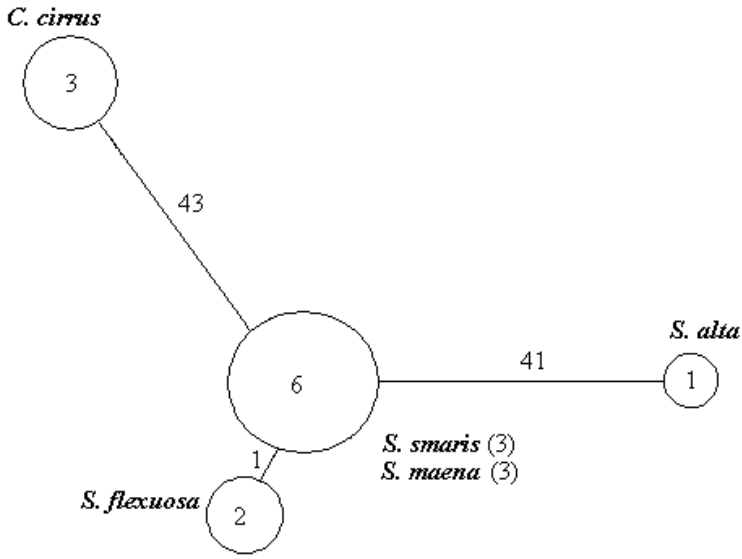

Figure 3. Minimum spanning tree that shows the relationships among the haplotypes. Each circle represents a different sequence. The size of the circle represents the sequence's relative frequency; connecting line is also labelled with the number of positional differences between the two sequences.

Pairwise genetic distances between the species were given in Table 3. For inter-generic comparisons, there was no genetic difference between *S. maena* and *S. smaris*, and the genetic divergence between *S. flexuosa* and both *S. maena* and *S. smaris* was found to be 0.005. High genetic divergences were observed between the two genera, *Spicara* and *Centracanthus*. Moreover, over high intergeneric divergence was found to be 0.237 between *S. alta* and *C. cirrus*. The resultant matrix of the genetic distance showed significant correlation (r=0.96, $P<0.001$) when compared with the Squared Euclidean distance of morphologic data.

The three different phylogenetic approaches resulted in same tree topologies and the clades are well supported (Figure 4). However in the molecular phylogenetic tree construction, the monophyly of the genus *Spicara* was not supported. *S. flexuosa* clustered with *S. maena* and *S.*

smaris, and showed close relationship. On the other hand, *S. alta* highly divergently clustered outside of this group and branched with *C. cirrus*.

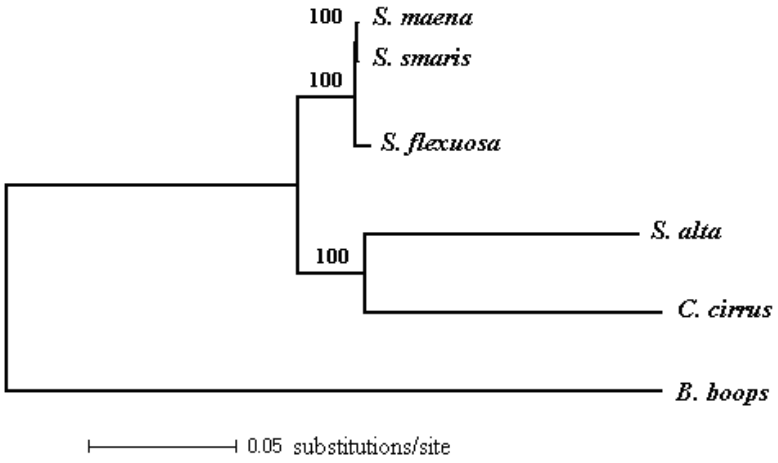

Figure 4. Neighbour joining tree for 16Sr DNA. Bootstrap values are show on the tree. *B. boops* was used as an outgroup.

Discussion

This study present first reported phylogenetic analysis based on mtDNA sequencing data, including five species and two genera of the family Centracanthidae. 16S rDNA gene sequence data used in this study was not congruent with the present monophyletic status of *Spicara* genus and support the present taxonomic status of *C. cirrus* within the family Centracanthidae. On the other hand, *S. alta* does not seems to be belong to the genus *Spicara*. Similar controversy was also reported by Orrell and Carpenter (2004) who investigated phylogenetic relationships of the species of the family Sparidae with inclusion of two *Spicara* species, *S. maena* and *S. alta*, based on 16S rDNA gene sequence data and concluded that *S. alta* should be placed in the family Sparidae. The present data also support the taxonomic revision of *S. alta* at least at the

genus level, allowing room for the plea to get more data to resolve the systematics of the family Centracanthidae.

The phylogenetic species concept considers a phylogenetic species as an irreducible cluster of organisms possessing at least one diagnostic character (Baum 1992). Therefore diagnosable taxa have species-specific genetic characters that discriminate between taxa (Harvey 1990; Baum 1992). No species-specific character was found between *S. maena* and *S. smaris* within the genus *Spicara*. The detected zero genetic differences between these lineages question the current taxonomic description of these taxa. Therefore the molecular phylogenetic topology and genetic differences between these lineages may suggest that the classically designated species *S. maena* and *S. smaris* belong to the same species. However the amount of morphologic divergence was enough to separate species within genus. *S. maena* was found to be more closely related to *S. smaris* rather than *S. flexuosa*, and the magnitude of the morphological divergence of *C. cirrus* was big enough to be considered as different genus. On the other hand, fishes demonstrate greater variance in morphological traits both within and between species than other vertebrates, and are more susceptible to environmentally-induced morphological variation (Dunham *et al*. 1979; Allendorf 1988; Wimberger 1992). The observed phenotypic differences in morphology between these taxa should not reflect environmentally induced phenotypic variation because the species sampled occur in the same habitat and in the same geographic region. Alternatively, there is increasing evidence that differentiation at the nuclear DNA level may not be shown in mitochondrial genes, (Ferguson *et al.* 1991; Ward and Grewe 1994; Turan *et al*. 1998) though there remain many cases to the converse (Ward *et al*. 1989; Reeb and Avise 1990; Hansen and Loeschcke 1996). Therefore applications of nuclear genes or/and more mtDNA segments can improve the indefinite taxonomic definition of these species.

The degree of molecular divergence between *S. flexuosa* and both *S. maena* and *S. smaris* was low, and does not provide substantial evidence to maintain different species. However, the percentage of sequence divergence based on 16S rDNA (0.5%) fell within the values reported for

some marine species (Doukakis *et al*. 1999; Tinti and Piccinetti 2000; Faria *et al*. 2006). For example; Tinti and Piccinetti (2000) investigated molecular systematics of the Solea species, and found that sequence divergences of 16S rDNA between species ranged from 0.72% to 11.09%. Moreover these species could have originated recently and they would not have had time to accumulate enough variation. On the other hand, the amount of genetic distance (17%) and topology found was enough to classify *C. cirrus* as intergeneric species. For example, interspecific mullid sequence divergences ranged from 7.15% to 21.68% in 16S rDNA.

In conclusion, the 16S rDNA data revealed that the inferred species-level topology of the genus *Spicara* is not congruent with the existing classic taxonomic classification of the genus *Spicara*. The species *S. maena* and *S. smaris* are found to be genetically contiguous; also *S. alta* seems to be not a member of the genus *Spicara*, and could belong to a new genera different from other species in Centracanthidae**.** Present analyses also support the existing taxonomic status of the genus *Centracanthus* in the family Centracanthidae. Additional molecular genetic analyses based on different parts of the mtDNA and nuclear genome could improve the findings presented here.

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