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Genetic Identification and Taxonomic Relationship of Mediterranean Mugilid Species Based on Mitochondrial 16S rDNA Sequence Data

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Abstract: This study examines eight mugilid species: *Mugil cephalus*, *Chelon labrosus*, *Oedalachelis labeo*, *Liza abu*, *Liza aurata*, *Liza saliens* and *Liza ramada* from the Mediterranean Sea and *Mugil soiuy* Black Sea on the basis 16S rDNA gene of mitochondrial DNA. The 16S rDNA dataset contained 121 variable and parsimony informative sites and the mean nucleotide diversity (Pi) was found to be 0.05. Haplotype diversity was found to be 0.88 and 7 different haplotypes were observed. Species specific haplotypes were detected and only *C. labrosus* and *L. ramada* shared the same Haplotype (H_3). Sequencing analysis revealed that *M. cephalus* was clearly separated from the other species. For inter-generic comparisons, there was no genetic difference between *C. labrosus* and *L. ramada* and *C. labrosus* and *O. labeo* should be considered within the genus *Liza*. Moreover *M. soiuy* and *L. abu* should be considered under the genus *Liza,* or new genus name should be given for these two species.

Key words: Phylogenetic relationship, mugilidae, mugilid species, mitochondrial DNA, 16S rDNA, sequence

INTRODUCTION

Mugilid species are distributed worldwide and inhabit marine, estuarine and freshwater environments in all tropical and temperate regions. Various Mugilid species play an important role in the fisheries and aquaculture of many regions of the world (Nash and Shehadeh, 1980), especially in aquacultural practices based on natural food webs.

The family Mugilidae includes 17 genera and >75 species (Nelson, 2006) in the world. Mugilid species are commonly found along the Mediterranean and Black Seas and represented with four genera and nine species. *Mugil cephalus, Liza aurata*, *Liza ramada*, *Liza saliens*, *Chelon labrosus* and *Oedalechilus labeo* have Atlanto Mediterranean distribution. *L. carinata* has recently spread to the South-Eastern Mediterranean from the Red Sea through the Suez Canal (Thomson, 1997). *Mugil soiuy* was initially introduced into the Azof Sea and now has been reported in the Black Sea and more recently in the Aegean Sea (Kaya *et al*., 1998). *Liza abu* is a freshwater species and found in the Orontes River, Turkey connected to Mediterranean Sea (Turan *et al*., 2004).

Due to the very conservative morphology displayed by mugilids many investigations based on various morphological characters did not elucidate their system atic problem (Schultz, 1946; Trewavas and Ingham, 1972; Stiassny, 1993), while the use of the pharyngobranchial organ as a key character to address the identification and taxonomy of mugilids also provided poor results (Harrison and Howes, 1991). Thomson (1997) revised Mugilidae family and reported 14 genera and a total of 64 valid species, most of these species are representatives of *Liza* and *Mugil* genera. Despite the fact that the Mugilid species have been revised many times, genetic identification and the taxonomic status of some species and genera within the family is still confused (Rossi *et al*., 1998; Turan *et al*., 2005).

Nowadays, mitochondrial DNA analysis is a very useful tool for molecular genetic studies because of its special features (Meyer *et al*., 1990; Billington and Hebert, 1991; Normark *et al*., 1991; Meyer, 1992). In most species mitochondrial DNA (mtDNA) is highly variable and is therefore a good marker for detecting possible genetic differentiation. Therefore, mtDNA variation has been widely investigated among several fish species including mugilidae (Papasotiropoulos *et al*., 2002; Caldara *et al*., 1996; Turan, 2008; Turan *et al*., 2009).

Although, the systematic relationships among these species have been investigated with the use of

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nonmorphological characters, such as biochemical and cytogenetic markers (Delgado *et al*., 1992; Rossi *et al*., 1996, 1997, 2000; Gornung *et al*., 2001, 2004; Nirchio *et al*., 2003), allozyme electrophoresis (Autem and Bonhomme, 1980; Papasotiropoulos *et al*., 2001; Rossi *et al*., 2004; Turan *et al*., 2005) and mtDNA analyse (Caldara *et al*., 1996; Murgia *et al*., 2002; Papasotiropoulos *et al*., 2002, 2007; Rossi *et al*., 2004; Fraga *et al*., 2007) they did not result in a clear taxonomic perspective (Cataudella *et al*., 1974; Delgado *et al*., 1992; Crosetti *et al*., 1993; Rossi *et al*., 1997, 2000).

In this study, taxonomic description and status of 8 mugilid species (*Mugil cephalus*, *M. soiuy*, *Liza ramada*, *L. aurata*, *L. abu*, *L. saliens*, *Chelon labrosus*, *Oedalechilus labeo*), living in the Mediterranean Sea, were investigated with mitochondrial 16S rDNA gene sequence data.

MATERIALS AND METHODS

Samples from eight mugilid species (*Mugil cephalus*, *Liza ramada*, *Liza aurata*, *Liza abu*, *Liza saliens*, *Chelon labrosus*, *Oedalechilus labeo*) were collected from Iskenderun Bay in North-eastern Mediterranean Sea and only one species *Mugil soiuy* was sampled from Trabzon in the Black Sea.

The number and location of the samples used in the sequence analysis are given in Table 1. The samples were placed on ice and kept frozen at -40°C in the laboratory. In the laboratory fin clips and muscle tissue were collected and preserved in 95% ethanol for DNA extraction.

Total genomic DNA was extracted from a peace of fin tissue (approximately 2 mm^2) using AGOWA mag Midi DNA isolation Kits (AGOWA, Berlin, Germany). The amplification of the mitochondrial 16S rDNA gene was performed using PCR with a profile of 94°C for 4 min, followed by 35 cycles of 94°C/30 sec strand denaturation, 52°C/20 sec annealing and 72°C/1 min 30 sec primer extension and a final 7 min elongation at 72°C.

The 16S rDNA amplification conditions were: 1.5 μL 10x polymerase buffer, 0.5 μL dNTP (10 mM), 0.3 μL Teg DNA polymerase (3 U μL^{-1}) equivalent to Taq DNA polymerase, 0.05 μL 16Fil 40 primer (100 μM) (5'-CG(CT)AAGGGAA(ACT)GCTGAAA-3'), 0.05 μL 16Fi1 524 primer (100 μM) (5'-CCGGTCTGAACTCAGATCACGT AG-3'), 3-5 μL DNA from AGOWA purification and water for a total reaction volume of 15 μL.

Amplified DNA was purified with Exo/Sap enzymes (Cleveland, Ohio, USA) following the manufacturer's instructions.

Finally, all the samples were sequenced in both directions using 16Fiseq1463 (5'-TGCACCAT TAGGATGTCCRGATCC AAC-3') and 16sarL (5'-CGCCTG TTTAACAAAAACAT-3') primers. The sequencing products were loaded onto an ABI3730 (Applied Biosystems) automated sequencer.

Sequences were aligned and ambiguous bases resolved by eye using Sequencer v.4.5 (Gene Codes Corp) and final alignment was done manually with BioEdit (Hall, 1999). MtDNA sequence data were analysed to assess levels of pairwise nucleotide variation and to determine nucleotide composition for each taxon using MEGA 3.1 (Kumar *et al*., 2004).

The molecular phylogenetic tree was constructed using the two distinct phylogenetic approaches: a distance-based method using Neighbor Joining (NJ) (Saitou and Nei, 1987) and a cladistic approach using the Maximum Parsimony (MP). Bootstrap resampling was applied to assess the relative stability of NJ trees produced with different substitution models.

Nucleotide diversity and DNA total divergence (Dxy; were estimated using MEGA v.4 (Tamura *et al*., 2007). In all models, phylogenetic trees were rooted using out group species *Carangoides armatus*, which belongs to the family Carangidae and its sequence is published in GenBank under accession number NC_004405. The sequences have been deposited in the GenBank with accession numbers given in Table 1.

Table 1: Sampling coordinates and GenBank accession numbers for the 16S rDNA segment sequenced in this study

Species	Sampling location	n-S	GenBank
M. soiuy	Black Sea (Trabzon)	2	GU449119
			GU449120
O. labeo	Northeastern Mediterranean Sea	2	GU449121
	(Iskenderun Bay)		GU449122
C. labrosus	Northeastern Mediterranean Sea	2	GU449123
	(Iskenderun Bay)		GU449124
L. aurata	Northeastern Mediterranean Sea	3	GU449125
	(Iskenderun Bay)		GU449126
			GU449127
L. abu	Northeastern Mediterranean Sea	2	GU449128
	(Iskenderun Bay)		GU449129
L. ramada	Northeastern Mediterranean Sea	3	GU449130
	(Iskenderun Bay)		GU449131
			GU449132
M. cephalus	Northeastern Mediterranean Sea	2	GU449133
	(Iskenderun Bay)		GU449134
L. saliens	Northeastern Mediterranean Sea	3	GU449135
	(Iskenderun Bay)		GU449136
			GU449137

n-S: Number of specimens used in the sequencing

RESULTS AND DISCUSSION

After alignment, the partial 16S rDNA gene sequences consisted of 788 bp. Examination of the gene reveals a moderate amount of guanine (G; 21.4%) and abundance of adenine (A; 31%). The 16S rDNA dataset contained 121 variable and parsimony informative sites and the mean nucleotide diversity (*Pi*) was found to be 0.05. Haplotype diversity was found to be 0.88 and 7 different haplotypes were observed. Variable nucleotide positions and frequencies of haplotypes are given in Fig. 1. Species specific haplotypes were detected and only *C. labrosus* and *L. ramada* shared the same Haplotype (H_3) (Table 2).

Pairwise genetic distances between the species were given in (Table 3). For inter-generic comparisons, there was no genetic difference between *C. labrosus* and *L. ramada* and highest genetic divergences were observed between *M. cephalus* and *O. labeo*.

The three different phylogenetic approaches resulted in same tree topologies and the clades are well supported (Fig. 2). *C. labrosus* and *L. ramada* clustered together on the same branch and were sister group to *L. saliens*, *O. labeo* clustered with *L. aurata* and *M. soiuy* branched with *L. abu*. On the other hand, *M. cephalus* highly divergently clustered outside of this group.

The present study based on 16S rDNA gene sequencing data of four genera and eight species of the family Mugilidae revealed different systematic classification from the current classification and question existence of *Chelon* and *Oedalechilus* genera. Mugilid species are generally morphologically uniform; the genus *Oedalechilus* by the different pattern of the jaw structure, that features a very broad upper lip, with the maxillare extending vertically backwards under the preorbitale. In other Mediterranean mugilid species (*L. abu*, *L. saliens*, *L. ramada*, *L. aurata* and *M. cephalus*), the upper lip is not broad and the mouth is rather horizontal, with the same extending maxillare. However, *M. cephalus* shows at least some features that make it diagnosable compared to *Liza*, *Chelon* and *Oadalechilus* and all the genetic and molecular studies (Caldara *et al*., 1996; Cataudella *et al*., 1974; Autem and Bonhomme, 1980; Rossi *et al*., 1998; Papasotiropoulos *et al*., 2001, 2002; Turan *et al*., 2005; Fraga *et al*., 2007; Semina *et al*., 2007) reported that *M. cephalus* shows the highest degree of genetic divergence among the other three genera. The present study is not congruent with the present monophyletic status of *Liza* and *Mugil* genera. There was no enough genetic differentiation of *Chelon* and *Oadalechilus* genera from *Liza* genus and *C. labrosus* and *O. labeo* seems to be belongs to the genus *Liza*.

The close relationship between *L. saliens C. labrosus* and *O. labeo* suggest that the separation of *Chelon, Oadelechilus* and *Liza* in three separate genera might be unnatural, making the monophyletic origin of the genus *Liza* questionable. In the previous studies, similar controversy was also reported. Papasotiropoulos *et al*. (2002) investigated the phylogenetic relationship of five

Table 2: Distribution and frequency of 16S rDNA haplotypes of mugilid species

Species	H_1	H_2	H_3	H_4	H_5	H_6	H_7
M. soiuy	2	-	-	-	-	-	-
O. labeo	-	2	-	-	-	-	-
C. labrosus	-	-	2	-	-	-	-
L. aurata	-	-	-	3	-	-	-
L. abu	-	-	-	-	2	-	-
L. ramada	-	-	3	-	-	-	-
M. cephalus	-	-	-	-	-	2	-
L. saliens	-	-	-	-	-	-	3

H: Haplotypes

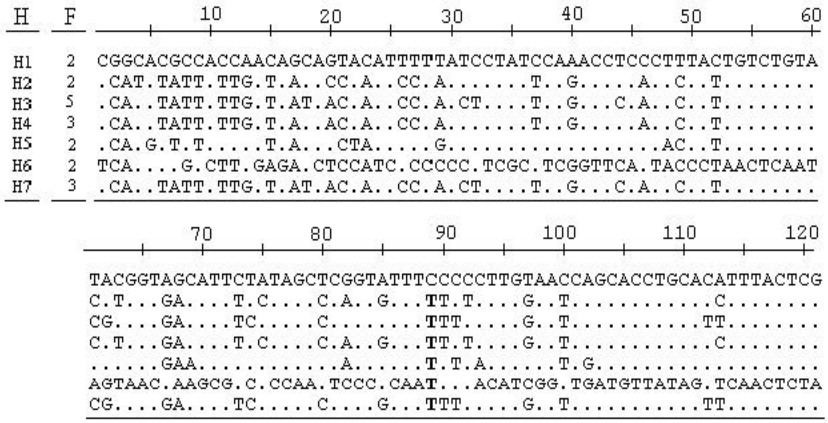

Fig. 1: Variable nucleotide positions and frequencies of 16S rDNA haplotypes in Mugilid species. For all haplotypes variable nucleotides are indicated, while identity is shown by dashes

Table 3: Total genetic distance between the species

Species	1	2	3	4	5	6	7	8	9
C. armatus	-	-	-	-	-	-	-	-	-
M. soiuy	0.1610	-	-	-	-	-	-	-	-
O. labeo	0.1671	0.0459	-	-	-	-	-	-	-
C. labrosus	0.1580	0.0486	0.0181	-	-	-	-	-	-
L. aurata	0.1640	0.0446	0.0026	0.0155	-	-	-	-	-
L. abu	0.1610	0.0260	0.0379	0.0379	0.0366	-	-	-	-
L. ramada	0.1580	0.0486	0.0181	0.0000	0.0155	0.0379	-	-	-
M. cephalus	0.1900	0.1299	0.1357	0.1284	0.1343	0.1212	0.1284	-	-
L. saliens	0.1595	0.0499	0.0168	0.0013	0.0142	0.0392	0.0013	0.1299	-

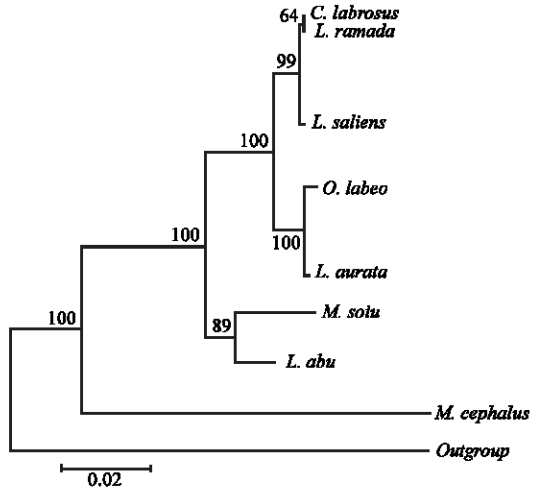

Fig. 2: Neighbour joining phylogenetic tree for 16S rDNA. Bootstrap values are shown on the tree. *C. armatus* was used as an outgroup

mugilid species by means of mtDNA PCR-RFLP and found that *L. saliens* and *C. labrosus* were the most closely relates species. Turan *et al.* (2005) investigated phylogenetic relationships of the nine species of the family Mugilidae based on allozyme data and suggested that *C. labrosus* and *O. labeo* should be placed in the genus *Liza*. Rossi *et al.* (2004) and Papasotiropoulos *et al.* (2007) investigated phylogenetic relationships of six mugilids (*M. cephalus*, *C. labrosus*, *O. labeo*, *L. aurata*, *L. ramada* and *L. saliens*) and five mugilid species (*M. cephalus*, *C. labrosus*, *L. aurata*, *L. ramada* and *L. saliens*) and found low level of sequence divergence between *Chelon labrosus* and *Liza aurata* using allozyme data and three segments of mtDNA. Investigations based on nuclear and mtDNA data provide good support for the hypothesis that *C. labrosus* and *O. labeo* should be placed in the genus *Liza*. The existence of such disorder in phylogenetic studies of Mugilidae in the study is not uncommon (Cataudella *et al.*, 1974; Menezes *et al.*, 1992; Caldara *et al.*, 1996; Papasotiropoulos *et al.*, 2002).

The present study also revealed close relationship between *M. soiuy* and *L. abu*. The molecular phylogenetic position of *M. soiuy* and *L. abu* was considered in this study first time. *L. abu* is a freshwater species and *M. soiuy* is in fact a freshwater fish originating from Amu Darya River Basin in Far East Asia (Berg, 1965). This species was later introduced to the Sabolat (Hacibey) Lagoon, 60 km from Odessa in the Northeastern Black Sea *M. soiuy* found a suitable environment on the eastern Black Sea coast of Turkey, after leaving the Sea of Azov and following the Northeastern coast of the Black Sea. In time, the species migrated to the west, reaching the Sea of Marmara and Aegean Sea via the Istanbul Bogazi (Kaya *et al.*, 1998). The present study gives new hypothesis that *M. soiuy* and *L. abu* should be considered in the genus *Liza*, or new genus name should be given for these two species.

16S rDNA gene sequencing data revealed a high level of genetic variability among the species examined. Only *C. labrosus* and *L. ramada* shared the same Haplotype (H_3) and the taxonomic tree support structuring of haplotypes by species designation. Any species-specific haplotypes could serve as an strict diagnostic marker for taxonomic and aquaculture practice purposes, which is especially, important at the larval and fingerling stages since the morphological and physiological characters do not show significant differences. The identification of several fish species by use of molecular markers proves a reliable tool facilitating discrimination of morphologically similar species. For example, McDowell and Graves (2002) used different mitochondrial and nuclear markers and concluded that the mitochondrial ND 4 and the nuclear locus unambiguously identified marlins, spearfishes, sailfishes and swordfishes; xiphiid billfishes that represent an important commercial and recreational fisheries resource.

CONCLUSION

The two genera *Chelon* and *Oedalechilus* should be considered together within the genus *Liza*. The present data also first time report the taxonomic revision the species of *M. soiuy* and *L. abu*, which should be considered together under the same genus.

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