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Molecular phylogenetic relationships among mediterranean mugilidae species

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ABSTRACT

In order to explore phylogenetic relationships among six Mediterranean species of the Mugilidae family namely, *Mugil cephalus, Chelon labrosus, Liza aurata, Liza ramada, Liza saliens* and *Oedalechilus labeo*, polymorphism sequence of mitochondrial (16S rRNA, COI, CytB and 12S rRNA) and nuclear (5S DNA and Rhodopsin) gene were analysed. Phylogenetic trees built are in agreement with previous studies but the overall data set provide the finest picture of phylogenetic relationships among these species. *Mugil* appear as the most derivative genus among studied genera (*Liza, Chelon, Oedalechilus)* which is in agreement with morphometric specificity of *Mugil*. In contrast, our data disagree with a close relationship of *Oedalechilus* with *Chelon*. Last, our results using as well the mitochondrial as the nuclear markers corroborate previous studies that question the validity of *Chelon labrosus* and confirm that this species belong to the *Liza* genus. © 2009 Trade Science Inc. - INDIA

KEYWORDS

Mediterranean;
Mugilidae;
Phylogeny;
Mitochondrial markers;
Nuclear markers.

INTRODUCTION

Mugilidae is an important family distributed worldwide. They are euryhaline, they inhabit marine, estuarine, and freshwater environments. Various mugilid species are commercially important in fishery and aquaculture of many countries and are highly exploited throughout their distribution[11,12].

In the Mediterranean Sea, 6 species belonging to 4 genera have been described: *Mugil cephalus* Linneaeus, 1758; *Liza aurata* Risso, 1810; *Liza saliens* Risso, 1810; *Liza ramada* Risso, 1826; *Chelon labrosus* Risso, 1826; and *Oedalechilus labeo* Cuvier, 1829. Recently 2 other species can be locally observed: the Lessepsian invader, *Liza carinata* and *Mugil soiuy* which was introduced in the Black Sea. All native species can be observed in lagoons with exception of *Oedalechilus labeo* which exclusively inhabits marine environments and whose geographic distribution is limited to the Mediterranean Sea.

Mugilidae species show remarkable morphological uniformity that inevitably leads to misidentification and limit any accurate phylogenetic inference. To point out the poor phylogenetic utility of meristic and morphometric characters usually used in the Mugilidae systematic, genetic studies have been soon investigated

Mediterranean mugilids species using various genetic markers: cytogenetic[8,14,15,23,24,43-45], allozymes[1,6,36,46,57] and genes sequence polymorphism[9,25,37,38,46]. All these studies underlined the peculiar taxonomic status of *Mugil cephalus* which seems to be the most derivative species. They also stressed some conflicting results concerning the interspecific differentiation between *Liza* species and the phylogenetic position of *Chelon* genus. Some studies have questioned the monophyletic origin of the genus *Liza* as well as the validity of the *Chelon* genus[46]. The systematic classification of these two genera has been subject of a long-running debate. Schultz[49] did not recognize the genus *Liza* and included within *Chelon* all the species later reported as *Liza* by Thomson[55], who considered *Chelon* a valid genus with two nominal species: *C. labrosus* and *C. bispinosus* whereas all others species previously included in the *Chelon* genus where considered as belongin to *Liza*.

Most genetic approaches to the determination of species identity and phylogenetic relationships are based on amplification of a region of mitochondrial DNA by Polymerase Chain Reaction (PCR), followed either by direct sequence analysis of the amplified fragment, or restriction fragment length polymorphism analysis (e.g. Carrera *et al*. 1999). Most DNA analyses for fish species identification have been based on amplification of different mitochondrial DNA regions[13,42]. Mitochondrial genes are highly conserved among vertebrates, including fish[5], and the inheritance of mtDNA is usually maternal non-recombinational. MtDNA is a broadly used genetic tool, and one of its advantages is the high copy numbers of the mitochondrial genome compared with nuclear genome within a cell. Mitochondrial DNA markers have been successfully used to decipher evolutionary relationships at multiple taxonomic levels among different organisms[51]. Indeed, Caldara *et al.*[9] using the sequence polymorphisms of the cytochrome B suggested an apparent heterogeneous evolution rate among genus of the Mugilidae family. According to these authors the mitochondrial genome of the *Mugil* genus would present an evolution rate faster than the evolution rate observed in other Mugilidae family.

Apart from mtDNA, nuclear genes such as 5S ribosomal DNA (5S rDNA) are possibly suitable candidates for genetic discrimination of related species, because in higher eukaryotes, the 5S rDNA gene comprises a 120-bp highly conserved coding sequence and a variable non transcribed spacer (NTS). This unit is tandemly repeated, usually arranged head to tail, and is species-specific[3,40]. Furthermore, data suggest that 5S rDNA sequences are valuable molecular markers to access the evolutionary history among closely related species[19].

In this study, mitochondrial and nuclear DNA sequences were used to develop a robust phylogenetic hypothesis for the Mediterranean mugilidae species and more specifically on the existing debate regarding the phylogenetic relationships among the *Chelon* and *Liza* species. Furthermore, we would like to compare mitochondrial and nuclear data and to evaluate the effect of different data sets or different methodologies on the same problems. With the use of these markers analysis, we aimed to shed more light on the evolutionary history of the Mugilidae family.

MATERIALS AND METHODS

Biological Material

Two specimens from each of the five species of the Mugilidae family (*Liza aurata, Liza ramada, Liza saliens, Chelon labrosus* and *Mugil cephalus*) were collected from the lagoon of Hergla in Tunisia. These same samples were used in an allozymic studies[6]. Generally, *Oedalechilus labeo* is a marine species, for this reason they have been not collected from the lagoon. Even if this species was considered as rare in Tunisia, we successfully collected some specimens in Tunisian, precisely at Hammam el Ghzez coasts.

All specimens were identified according Farrugio's[17] keys and FAO criterias depicted in the FAO species identification sheets[21]. A small piece of fin was collected from each fish and preserved in 95% ethanol until the DNA extraction.

Genetic Analysis

Genomic DNA was extracted using conventional phenol–chloroform protocols[48] and examined for quantity and quality through agarose gel electrophoresis.

Several gene (cytochrome b (Cytob); 12S RNA; 5S RNA and Rhodpsin) of the six Mediterranean Mugilidae species were amplified and sequenced in other studies. The sequences were available in GenBank (TABLE 1). In this work, all these data were used and

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compiled with our sequences in the aim to analyze the phylogenetic relationships among the Mugilidae family using as well mitochondrial as nuclear markers and to analyze the evolutionary history of the Mugilidae family.

TABLE 1 : Accession number of the sequences of the four gene (CytB, 12S rRNA, 5S DNA and Rhod) of the six Mediterranean Mugilidae species from GenBank

	CytB	12S rRNA	5S DNA	Rhod
Size	1141 bp	570 bp	269 bp	460 bp
Liza aurata	EU224056	EF437077	DQ780572	EF439127
Liza ramada	EU224058	EF437079	DQ780576	EU224157
Liza saliens	***	EF437081	DQ780573	Y18670
Chelon labrosus	EF427544	EF437075	DQ780574	EF439095
Oedalechilus labeo	***	Z71995	AM706439	
Mugil cephalus	EU036449	EF437083	DQ780575	EU036557

Fragments of the mtDNA genes analysed (16S rRNA and COI) were amplified by PCR. PCR reactions were carried out in 50 µL volumes containing 1µl DNA template, 1µM of each primer, 1.2 mM MgCl2 (Promega, Madison, Wis., USA), 74 µM of each dNTP, and 0.13 µl Taq polymerase. For PCR amplifications of both mtDNA segments, two different sets of primers were used. For the 16S rRNA gene we used the universal primers 16SARL (5'-CGCCTGTTTATCAAAAACAT-3') and 16SBRH (5'-CCGGTCTGAACTCAGATCACGT-3') described by Palumbi *et al*.[35]. For the COI segment we used primers described by Ward *et al*.[59], FishF1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and FishR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'). PCR amplification conditions were as follows: preliminary denaturation at 92°C (5 min), strand denaturation at 92°C (30s), primer annealing at 50°C (30s) and primer extension at 72°C (45s) repeated for 35 cycles and final extension at 72°C (5 min). For the PCR products an enzymatic purification was used "Exosap" according to the supplier's protocol. PCR products were visualized on 1% agarose gels and the most intense products were selected for sequencing. Products were labelled using the BigDye Sequencing Kit (Promega) and sequenced bidirectionally using a capillary automated sequencer.

Phylogenetic Analysis

The nucleotide sequences of all species were aligned using Clustal W suite option of MEGA version 4[53]. All molecular analyses were performed using MEGA version 4[53]. The same type of analysis was applied to all sets of sequences. Transition/Transversion Ratio (R) was calculated using the same program, (R) is the ratio of the number of transitions to the number of transversions for a pair of sequences. Values of R (Transition/ Transversion Ratio) were estimated for the entire dataset, for the five genes separately and for the three mitochondrial genes (16S rRNA_COI_12S rRNA) combined, as well as for each codon position (1st, 2nd, 3rd). The number of variable nucleotide was estimated by the MEGA program, the variable sites contains at least two types of nucleotides, some variable sites can be singleton or parsimony-informative and the site that is not variable is referred to as a constant site.

Kimura's two parameter model[26] corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites Using Kimura's two-parameter method[26], a pairwise distance matrix was generated for each DNA segment as well as for the combined data set, representing the degree of genetic distances among species. We inferred the phylogenetic relationships among the investigated taxa by Neighbor-Joining (NJ) reconstruction, using the program MEGA version 4[53]. The robustness of NJ trees was assessed using bootstrap analysis, with 1000 replications.

For the construction of the phylogenetic trees, sequences of *Salarias fasciatus* (16S rRNA); *Atherinops affinis* (COI), *Abudefduf sordidus* (CytB and 12S rRNA)*; Oreochromis sp.*(with the combined data), *Leporinus octofasciatus* (5S RNA) and *Scorpaena porcus* (Rhodopsin) were used as outgroups to root the trees.

RESULTS

In total, four mitochondrial (cytochrome b (Cytob), 12S rRNA; 16S rRNA and COI) and two nuclear (5S RNA and Rhodopsin) sequences gene were analysed. As a result, a combined data set of 1531 nucleotide sites of three mt DNA genes (16S rRNA_COI_12S rRNA) was obtained. The number of variable sites ranged from 57 (10%) for 12S rRNA, 84 (10.95%) for 16s rRNA, 51 (11.08) for Rhodopsin, 298 (26.11%) for Cyt B; 167 (28.4%) for COI to 90 (33.45%) for the nuclear gene 5S RNA. Among the taxa examined,

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big size differences were revealed in the nuclear gene. From the combined data set, 314 sites out of 1531 varied among the different species. As deduced from the sequence analysis, the vast majority of nucleotide substitutions occurred between *M. cephalus* and the other species in all genes studied.

Using the Kimura's two-parameter method, a pairwise distance matrix was generated from the combined mitochondrial dataset (16S rRNA_COI_12S rRNA), Cyt B (TABLE 2) and the two nuclear genes (TABLE 3). The genetic distances among the species of the genus *Liza* are very low, *Chelon labrosus* seemed to be quite distant from genus *Liza* and *Oedalechilus labeo* showed the second highest genetic distances after *Mugil cephalus.* Much higher distances were displayed by *Mugil cephalus* compared with all the other Mugilidae taxa. It is obvious that the differences are larger among noncongeneric species than among species of the same genus. In 16S rRNA and CytB, the lowest divergence values were observed between *Liza aurata* and *Chelon labrosus*. But in 12S rRNA and COI the lowest values were observed between *Liza saliens* and *C. labrosus*, this same result was observed when the three mtDNA genes (16S rRNA_COI_12S rRNA) were combined. In contrast, in the nuclear markers, the lowest distances were observed between *L. aurata* and *C. labrosus*.

TABLE 2 : Kimura 2-parameter[26] distances calculated for mitochondrial sequences genes among the six Mediterranean Mugilidae species. Below diagonal: distances calculated for the combined mitochondrial data set (16S rRNA_COI_12S rRNA) segment. Above diagonal: distances calculated for Cytochrome B (Cyt B).

	L. aurata	*L. ramada*	*L. saliens*	*C. labrosus*	*O. labeo*	*M cephalus*
Liza aurata	*	0.093	-	0.090	-	0.244
Liza ramada	0.049	*	-	0.100	-	0.247
Liza saliens	0.044	0.055	*	-	-	-
Chelon labrosus	0.042	0.053	0.043	*	-	0.249
Oedalechilus labeo	0.099	0.104	0.105	0.102	*	-
Mugil cephalus	0.173	0.165	0.176	0.172	0.161	*

TABLE 3 : Kimura 2-parameter[26] distances calculated for nuclear gene segment among the six Mediterranean Mugilidae species. Below diagonal: distances calculated for (5S RNA). Above diagonal: distances calculated for Rhodopsin (Rhod).

	L. aurata	*L. ramada*	*L. saliens*	*C. labrosus*	*O. labeo*	*M. cephalus*
Liza aurata	*	0.009	0.007	0.007	-	0.040
Liza ramada	0.016	*	0.002	0.007	-	0.036
Liza saliens	0.021	0.027	*	0.004	-	0.033
Chelon labrosus	0.032	0.027	0.044	*	-	0.036
Oedalechilus labeo	0.164	0.158	0.158	0.164	*	-
Mugil cephalus	0.191	0.184	0.198	0.198	0.248	*

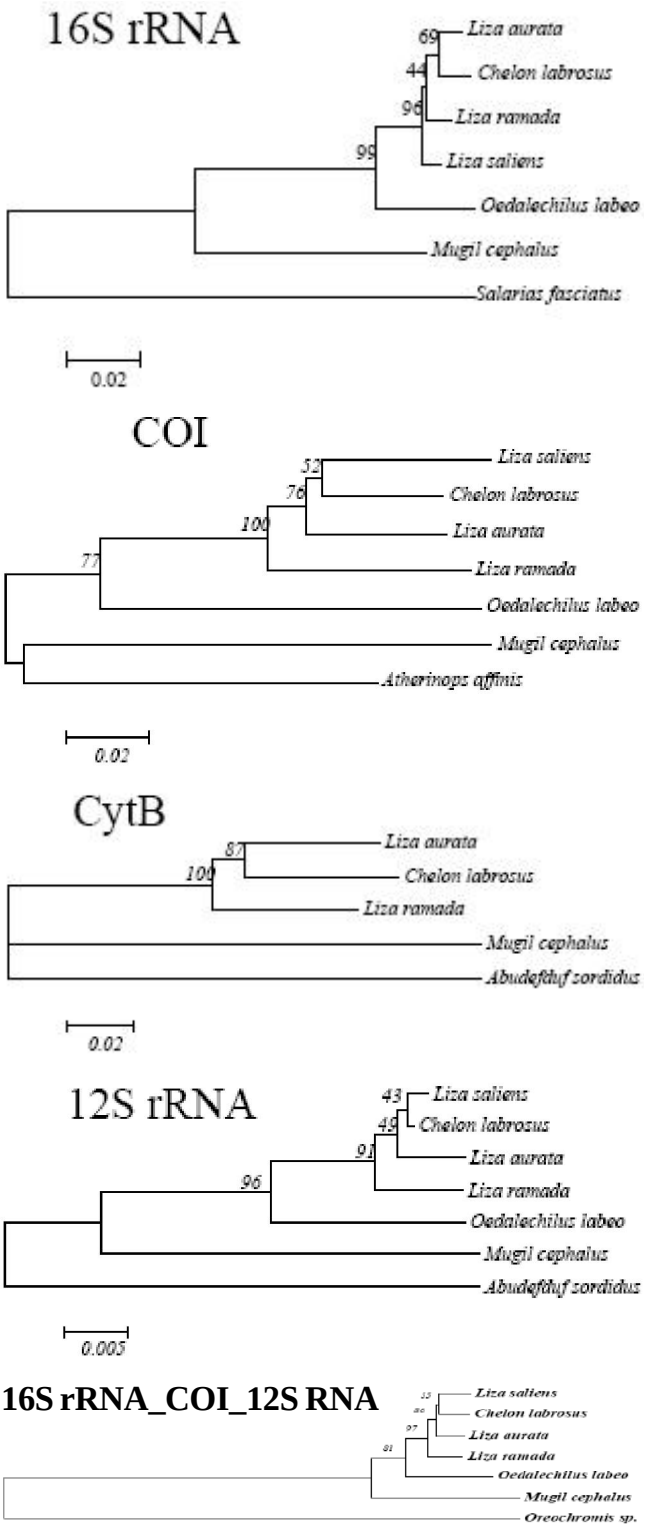

Figure 1 : Phylogenetic Neighbor-joining (NJ) trees obtained using mitochondrial sequences (16S rRNA, COI, Cyt B, 12S rRNA and the combined data set) of the six Mediterranean Mugilidae species. Numbers indicate the percentage of 1000 bootstrap replicates at each node in the majority rule consensus tree

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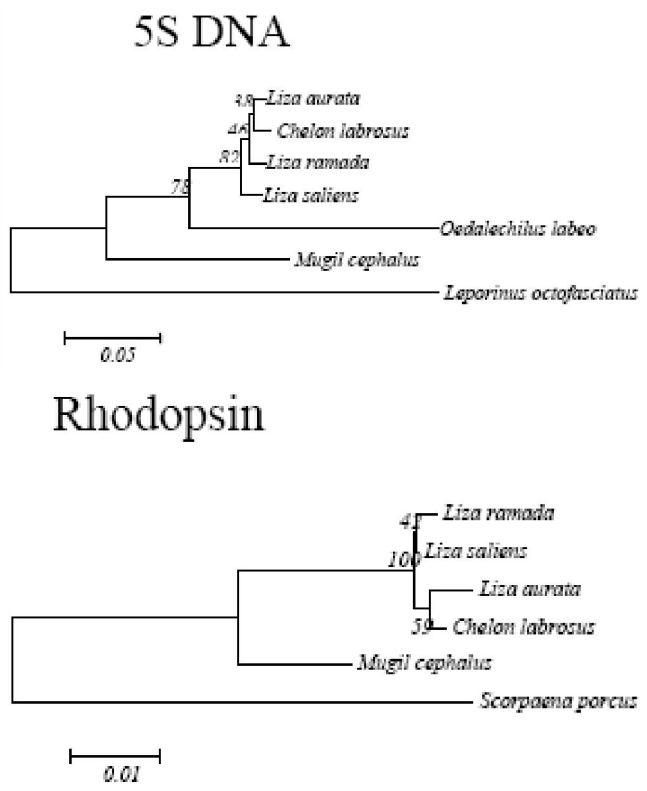

Figure 2 : Phylogenetic Neighbor-joining (NJ) trees obtained using nuclear sequences (5S DNA and Rhod) of the six Mediterranean Mugilidae species. Numbers indicate the percentage of 1000 bootstrap replicates at each node in the majority rule consensus tree.

Our results demonstrate a high degree of similarity between mtDNA derived and nuclear based phylogenetic reconstructions. Indeed, the phylogenetic trees obtained by the NJ method using mitochondrial (Figure 1) and nuclear genes (Figure 2) emphasizes the high divergence of *Mugil cephalus*. *Oedalechilus labeo* is the sister species of the other grey mullets. The three *Liza* species and *Chelon labrosus* were clustered together. The phylogenetic reconstruction obtained considering the nucleotide sequence of the three mitochondrial sequences combined suggested *Liza ramada* as the sister group to the *Liza- Chelon labrosus* lineage, whereas *C. labrosus* and *Liza saliens* resulted the closest taxa. The phylogenetic reconstruction obtained considering the nuclear nucleotide sequence showed the regrouping of *C. labrosus* and *L. aurata* together being the closest taxa. This clustering brings into question the monophyletic origin of the genus *Liza*.

DISCUSSION

The aim of this paper was to summarise all phylogenetic information available for Mediterranean Mugilidae. Due to the high morphometry conservation of species belonging to this family doubt about species identification lead researchers to question the systematic of these family around various localities in the Mediterranean area: France, Italy[9,46], Greece[25,36-38], Turkey[57], and Tunisia[6] harbouring same species. In this review, all available sequences were considered to provide the most accurate picture of phylogenetic relationships among Mediterranean Mugilidae family and definitely solve phylogenetic relationships among these genus and species.

The first observation when considering all results obtained in different areas, is the absence of cryptic species despites identification difficulties of mugilids species. All phylogenetic studies confirm the taxonomy and the existence in the Mediterranean sea of 6 natives species: *M. cephalus, O. labeo, L. ramada, L. saliens, L. aurata* and *C. labrosus*.

The levels of divergence estimated among the Mediterranean Mugilidae species using the mitochondrial and the nuclear are in general agreement with those reported by Billington & Hebert[5], as well as with those proposed by Gonzalez & Powers[22] for marine species. Moreover, the level of nucleotide divergence observed among the three *Liza* species is in congruence with that proposed by Avise *et al*.[2] and Moritz *et al*.[32] among congeneric species.

As to the phylogeny within the mugilidae family, The DNA sequence analysis of the entire data set strongly supports the position of *Mugil cephalus* as a separate lineage with a large genetic divergence from the other mugilidae considered in the present study. The highest degree of genetic divergence estimated among *M. cephalus* and all the other species was observed as well in the mitochondrial as in the nuclear markers, this could be the result of the faster substitution rate observed in this species, and it could be explained as a combined effect of nucleotide bias and saturation of signal[28,38] in molecular markers (both mitochondrial and nuclear genes). This is in agreement with all previous studies using allozymic and molecular markers[1,6,9,25,36-38,46,57]. This hypothesis is also supported by hemoglobins[47] and chromosome studies by Cataudella *et al*.[8]; Rossi

et al.[43] and Gornung *et al*.[23], who stated that the *Mugil cephalus* karyotype is considered closest to the karyotype described by Ohno[34] as ancestral for all teleosts. But the high genetic differentiation observed between *Mugil cephalus* and other Mugilidae species appears to sharply contrast with their high morphological similarity. This situation might be explained by the lack of parallel evolution between morphology and some portions of DNA[9,38,44], this has already been reported for other groups of fish[31,39,50] and might be explained by differences in the selective constraints operating on these two characters[9]. In the case of the grey mullets, their considerable morphological homogeneity may reflect a convergent adaptation of their body architecture[9].

Our phylogenetic reconstruction is also in agreement with previous studies[9,46] concerning the systematic position of *Oedalechilus labeo* that is considered as the second most divergent species after *M. cephalus*. Indeed, phylogenetic reconstructions from 5S rDNA sequences suggest an ancestral position of *O. labeo* compared with the genus *Liza*. But cytogenetic analysis[23,45] showed that *Oedalechilus* might be a derived branch of *Liza* (*Protomugil*) it present the subtelocentric chromosome pair for the 9th one whereas as *Liza* species and *C. labrosus* present this subtelocentric chromosome pair in 24. This cytogenetic and genetic congruence disagree with Thomson[55] assumption that consider the genus *Oedalechilus* in an evolutionary series as close to the genus *Chelon* both descendent of the genus *Liza*.

The relationships among the three species of *Liza* (*L. aurata, L. ramada* and *L. saliens*) and *Chelon labrosus* are poorly resolved, whereas the *Liza-Chelon* clade showed a reduced interspecific differentiation, which is always well supported by all the molecular markers used in this study as well the mitochondrial as the nuclear sequences gene, this is comparable to the results reported by previous molecular studies[9,25,37,38,46]. The difficulty in discriminating between *Chelon* and *Liza* was already revealed by cytogenetic analysis[8,23] which suggests a close relationship between the two genera. This similarity was not supported by allozyme data that showed an appreciable degree of genetic differentiation between *Liza* and *Chelon*[1,6,36,46,57]. All these data contribute to the long systematic debate carried out on the monophyletic origin on the genus *Liza*. Caldara *et al*.[9] did not reject the *Liza* monophyly because they showed that a certain degree of homoplasy might affect this phylogenetic analysis. Contrary to Rossi *et al*.[46] and Papasotiropoulos *et al*.[37, 38], Imsiridou *et al*.[25] and Gornung *et al*. who indicates that the Mediterranean Mugilidae species of *Liza* do not form a monophyletic group exclusive of *Chelon*, and thus, the monophyly of the whole genus should be reconsidered.

Our study is the first who considered mitochondrial and nuclear sequences gene in the same time to analyze the phylogenetic relationships among the Mediterranean Mullets, all our analyses place *Chelon labrosus* among the *Liza* species so suggesting the polyphyly of the *Liza* genus. This is will be true if the genus *Chelon* is really a valid genus as considered by Thomson[55]. The present molecular phylogenetic study based on the mitochondrial and nuclear analysis did not reject the monophyly for the *Liza* genus and have questioned the validity of the *Chelon* genus who provide important implications for the phylogenetic relationships in mugilidae. However, *Liza* is the genus which contain a highest number of species (more than 20) contrary to the *Chelon* genus which contains only two species in the world *Chelon labrosus* (Mediterranean, Eastern Atlantic coasts, North of Cape Verde) and *Chelon bispinosus* (Cape Verde Islands)[55]. In order to clarify definitively the validity of *Chelon* genus, therefore the mono or polyphyly of the *Liza* genus, a more extensive genetic survey of representatives of the two genera is needed.

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