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Genetic identification and phylogenetic inferences in different Mugilidae species using 5S rDNA markers

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Abstract

A DNA methodology was developed to discriminate fry of six Mugilidae species found in the Mediterranean, namely *Mugil cephalus, Mugil so-iuy, Chelon labrosus, Liza aurata, Liza ramada* and *Liza saliens*. Polymerase chain reaction (PCR) amplification of the 5S rDNA gene was used for the identification of the above six species. PCR products of two species showed different patterns on EtBr-stained agarose gels; *M. so-iuy* gave a pattern of three bands, while *L. saliens* gave a pattern of one band. *Mugil cephalus, C. labrosus, L. aurata* and *L. ramada* gave a pattern of two bands. Subsequent sequencing analyses revealed unique haplotypes for each of the remaining four species. This is a genetic technique that could be applied in hatcheries, for identification of fry mullet's species. Furthermore, 5S rDNA sequences of each of five of the studied species (i.e. *M. cephalus, C. labrosus, L. aurata, L. ramada* and *L. saliens*), as well as a sequence of *Oedalechilus labeo* that belongs to the Mugilidae family, were aligned and used for the examination of the phylogenetic relationships among them. Phylogenetic trees produced in our study are in general agreement with those presented in the literature. This is the first study to use a nuclear marker to elucidate the phylogeny of Mugilidae species.

Keywords: species identification, Mugilidae, 5S rDNA, phylogeny, sequencing

Introduction

The Mugilidae family comprises 17 genera and more than 60 species (Nelson 1994), with a worldwide distribution. They inhabit marine, estuarine and freshwater environments at all latitudes. Eight species of the Mugilidae family inhabit the Mediterranean Sea: *Mugil cephalus* (Linnaeus, 1758) *Mugil so-iuy* (Basilewsky, 1855), *Chelon labrosus* (Risso, 1826), *Oedalechilus labeo* (Cuvier, 1829), *Liza aurata* (Risso, 1810), *Liza ramada* (Risso, 1826), *Liza saliens* (Risso, 1810) and *Liza carinata* (Ehrenberg, 1836).

The grey mullets play an important role in fisheries and aquaculture of many regions in the world (Nash & Shehadeh 1980). In 2006, 42 738 tonnes of grey mullets (about 60% of their total production) were produced from aquaculture in marine, brackish and inland waters of countries bordering Mediterranean and Black Sea (FAO 2006). In Greece, grey mullets have been traditionally cultured mainly on natural food webs. As artificial breeding of grey mullet's fry is not such a common process, all the cultural practices are based mainly on fishing of wild fry. Normally, aquaculturists do not know the species of grey mullets fry supplied by the fishermen, which they subsequently use for stocking aquaculture ponds and inland waters. However, species identity is very important information, as each species has a different growth rate and salinity tolerance. Grey mullets' fry identification is based on the pigmentation along the body flanks (Zismann 1981; Cambrony 1984; Serventi, Harrison, Torricelli & Gandolfi 1996), the melanophore patterns on the ventral side of the head (Zismann 1981; Reay & Cornell 1988; Minos, Katselis, Ondrias & Harrison 2002) and also the morphological pattern based on morphometric traits (Katselis, Hotos, Minos & Vidalis 2006).

Nevertheless, the very conservative morphology displayed by all grey mullets makes the systematic taxonomy and the phylogenetic relationships among Mugilidae species very difficult (Caldara, Bargelloni,

Ostellari, Penzo, Colombo & Patarnello 1996; Papasotiropoulos, Klossa – Killia, Killias & Alahiotis 2001). Particularly at the larval and fingerling stages, it is extremely difficult to distinguish the species from one another, because the morphological and physiological characters do not show significant differences. This represents, among other problems, a major issue in aquaculture practice (Thomson 1981). Hence, in order to improve the cultivation process, there is a need for more basic knowledge on the genetics of Mugilidae species.

Most of the genetic methodologies for determination of species identity are based on the polymerase chain reaction (PCR) amplification of a mitochondrial DNA region, followed either by direct sequencing analysis or by restriction fragment polymorphism (RFLP) analysis (Cespedes, Garcia, Carrera, Gonzalez, Fernandez, Asensio, Hernandez & Martin 2000; Karaiskou, Triantafyllidis & Triantaphyllidis 2003). However, in higher eukaryotes the 5S rDNA repeats consist of 120 bp highly conserved coding sequences, which are separated from each other by a non-transcribed spacer (NTS) that shows an accentuated length variation. This unit is tandemly repeated, it is located to different chromosome pairs in fish and normally it is species specific (Pendas, Moran, Freije & Garcia-Vazquez 1994; Martins & Galetti 2001; Martins, Wasko, Oliveira, Porto – Foresti, Parise-Maltempi, Wright & Foresti 2002).

Furthermore, data suggest that 5S rDNA sequences are valuable molecular markers to access the evolutionary history among closely related species, i.e. among species of the genus *Leporinus* (Ferreira, Oliveira, Venere, Galleti & Martins 2007). The NTS of the 5S rRNA gene has been proven to be a very dynamic region of the genome because it is free to mutate. The variant forms that arise are neutral to natural selection and can be fixed or lost, causing differences among related species (Cronn, Zhao, Paterson & Wendel 1996). As all previous phylogenetic investigations of grey mullets based on allozymic data (Autem & Bonhomme 1980; Rossi, Capula, Crosetti, Campton & Sola 1998; Papasotiropoulos *et al.* 2001), mtDNA sequence data (Caldara *et al.* 1996), RFLPs – mtDNA data (Papasotiropoulos, Klossa – Killia, Killias & Alahiotis 2002) and allozyme/mtDNA sequence data (Rossi, Ungaro, De Innocentiis, Crosetti & Sola 2004) provided controversial results, the 5S rDNA haplotypes for five Mugilidae species were analysed in order to shed more light on their phylogeny.

The aim of our work was to develop a methodology for fry discrimination of six Mugilidae species, on the basis of PCR amplification of species-specific fragments of the 5S rDNA gene. This genetic approach could facilitate aquaculture units to identify accurately the species of fry mullets supplied by fishermen. Furthermore, the different 5S rDNA sequences of Mugilidae species were used for phylogenetic analysis, in order to elucidate unclear relationships among species of this family.

Materials and methods

Fish samples

Fry individuals (total length range 20–35 mm) of each of the five most common Mugilidae species (*M. cephalus, C. labrosus, L. aurata, L. ramada* and *L. saliens*) were collected from the coast of Nea Moudania, Northern Aegean Sea, Greece. For the newly introduced species in the Mediterranean sea, *M. so-iuy,* adult individuals were collected from Northern Aegean Sea. 180 individuals were collected and studied in total, for the six species. The species *O. labeo* and *L. carinata* do not inhabit the studied area, and for this reason they have not been included in the present investigation. The fishing period was 8 months (March–October 2006) in order to collect samples of each species. All specimens were morphologically identified (Cambrony 1984; Minos *et al.* 2002) and then immediately stored at − 20 °C until DNA preparation.

DNA extraction

Total DNA was extracted according to the CTAB method described by Hillis, Moritz and Mable (1996). The 5S rDNA gene was amplified using the primers described in Pendas *et al.* (1994) for the amplification of one unit of any tandemly arranged 5S rDNA (including both coding and NTS sequences) in salmon, rainbow trout and brown trout.

Polymerase chain reaction amplification

In total, 180 individuals – 30 per each species – were analysed using the PCR technique. Double-stranded DNA was amplified in a total reaction volume of 25 μL containing 1.5 units of Taq polymerase (PROMEGA, Madison, WI, USA), 5 μL of 10 × reaction buffer, 2 mM $MgSO_4$, 0.5 mM of each dNTP, 0.8 pmoles μL^{-1} of each primer (Pendas *et al.* 1994) and approximately 50–100 ng of DNA. Polymerase chain reaction amplification conditions were as follows: one preliminary denaturation step at 95 °C (4 min), followed by strand denaturation at 95 °C (20 s), annealing at 65 °C (50 s)

and primer extension at 72 °C (30 s) repeated for 30 cycles and a final extension at 72 °C (7 min).

Electrophoresis of 3 μL of the PCR product was performed in 1 × TBE buffer for 1 h at 150 V, in 1.5% agarose gel containing 0.5 μg mL^{-1} ethidium bromide. The size of the PCR products was checked against a 100 bp DNA ladder. After the end of the electrophoresis, the resulting DNA fragments were visualized by UV transillumination and photographed.

Cloning and DNA sequencing

Polymerase chain reaction bands of all species were extracted and purified using an agarose gel extraction kit (Invisorb Spin DNA Extraction Kit from Invitek GmbH, Berlin, Germany) according to the manufacturer's specifications. The purified products were cloned into the TOPO TA plasmid (Invitrogen, Carlsbad, CA, USA) and used to transform competent cells of *Escherichia coli* strain DH5a (GibcoBRL, Gaithersburg, MD, USA). We applied the cloning procedure for two main reasons: (a) to read the whole sequence of the 5S rDNA gene and (b) to elucidate the nucleotide sequence of the 440 bp band. A total of 18 clones (two individuals per species) were sequenced by automated DNA sequencing on the 3730 × l DNA Analyzer from Applied Biosystems (Foster City, CA, USA), using both the M13(− 20) forward and reverse primers.

Phylogenetic analysis

The nucleotide sequences of all species were aligned using the CLUSTAL X software (Thompson, Gibson, Plewniak, Jeanmougin & Higgins 1997) and the BIOEDIT software (Hall 1999), set to default parameters and corrected by eye.

Using Kimura's two-parameter method (Kimura 1980), a pairwise distance matrix was generated, representing the degree of genetic distances among species. We inferred the phylogenetic relationships among the investigated taxa by maximum parsimony (MP) reconstruction, using the MEGA v 3.1 programme (Kumar, Tamura & Nei 1993). Maximum parsimony was performed by an exhaustive search of the most parsimonious tree(s); transitions and transversions were equally weighted. The robustness of MP trees was assessed using bootstrap analysis (Felsenstein 1992), with 1000 replications. The 5S rDNA sequence of species *Oedalechilus labeo* from GenBank (accession number: AM 260689) was used as an outgroup.

Results

Polymerase chain reaction analysis

In total, 180 individuals – 30 per each species – were analysed using the PCR technique. The 5S rDNA gene was amplified successfully in all six species, and the size of the amplified fragments was estimated in agarose gel. As shown in Fig. 1, two out of six species revealed species-specific patterns. *Mugil so-iuy* gave a pattern of three bands: a band of approximately 280 bp, a band of 600 bp and one of 620 bp. *Liza saliens* gave a pattern of one band, whose size was approximately 220 bp. The other four species (*M. cephalus, C. labrosus, L. aurata* and *L. ramada*) gave a pattern of two bands, whose sizes were around 220 and 440 bp. No intraspecific polymorphism was detected, as all the individuals of each species revealed the same PCR pattern.

Sequencing analysis

The amplification products of all species were purified, cloned and subsequently sequenced, apart from *M. so-iuy,* as this species revealed a completely distinct pattern. A total of 18 clones (two individuals per species), which correspond to the small (220 bp) and the large (440 bp) PCR fragments of each species, were sequenced by automated DNA sequencing. The results showed that the 220 bp PCR product of *M. cephalus, C. labrosus, L. aurata* and *L. ramada* corresponds to one unit of the 5S rDNA gene and that the 440 bp fragment of these species consists of two tandemly arranged units of this amplicon (both 5S rDNA gene and NTS) (Fig. 2). Similarly, the 220 bp fragment from *L. saliens* was confirmed to be a single unit of this amplicon. A similar structure of the 5S rDNA repeats was revealed and for other fish species like *Solea solea* (Cespedes, Garcia, Carrera, Gonzalez, Fernandez, Hernandez & Martin 1999), *Brycon cephalus* (Wasko, Martins, Wright & Galetti 2001) and *Brycon* sp. (Wasko *et al.* 2001). The size of the NTS was 100 bp in all five species.

The nucleotide sequences of the five species were aligned, and the polymorphic sites are indicated in Fig. 3. In total, 43 polymorphic sites were found, compared with *L. ramada* as a reference sequence. Three of them were found in the coding sequence and the rest in the NTS. The sequences were deposited in GenBank under the accession numbers DQ780572-6. The 220 bp bands (after extraction from agarose and without cloning) of three more individuals per

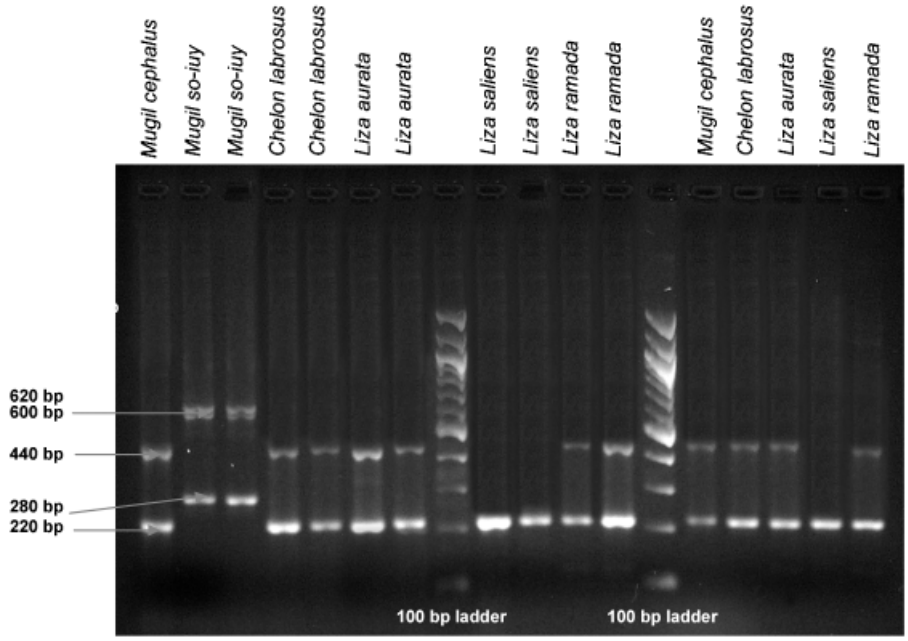

Figure 1 Electrophoretic analysis on 1.5% agarose gel of the 5S rDNA gene polymerase chain reaction (PCR) products, obtained from the six Mugilidae species (*M. cephalus, M. so-iuy, C. labrosus, L. aurata, L. saliens* and *L. ramada* – two different individuals per species). The size of the PCR products was checked against a 100 bp DNA ladder.

species were directly sequenced by automated DNA sequencing using both 5S forward and reverse primers, in order to verify the nucleotide differences among the five species analysed. The polymorphic sites remained the same in each species, inside both the NTS and the coding region, as all five sequences of each species were exactly the same. Low or zero intraspecific diversity for the same five Mugilidae species was also reported in Papasotiropoulos *et al.* (2002), after the PCR-RFLP analysis of three mtDNA segments. The five haplotypes of each species were used for phylogenetic analysis.

The BIOEDIT program (Hall 1999) was used to search all the possible restriction sites present in the 220 bp band of the 5S rDNA gene, in sequences of the four species (*M. cephalus, C. labrosus, L. aurata* and *L. ramada*) that show the same PCR pattern, in order to find restriction endonoucleases that would allow species discrimination. Unfortunately, no restriction enzymes were found on the basis of the predicted specific patterns that they would produce, which would enable species identification.

The whole PCR products (220 and 440 bp bands) of one more individual per species were directly sequenced (without extraction from agarose and subsequent cloning) by automated DNA sequencing using both 5S forward and reverse primers, in order to elucidate whether we could read the results and speed up the technique. Hopefully, the nucleotide sequence of the 220 bp band was very well analysed and for this reason we could adopt the PCR – direct sequencing analysis of the 5S rDNA gene, for discrimination of fry mullet's species.

Phylogenetic analysis

The pairwise distance matrix among species is given in Table 1. The nucleotide pairwise distances range from 0.005 (between *L. ramada* and *L. aurata*) to 0.209 (between *M. cephalus* and *C. labrosus*). The genetic distances among the species of the genus *Liza* (0.005–0.033) are very low. *Chelon labrosus* seemed to be quite distant from genus *Liza* (genetic distances: 0.037–0.042), while *M. cephalus* was the most distant from both *Liza* (0.179–0.191) and *Chelon* (0.209) genera. It is obvious that the differences are larger among non-congeneric species than among species of the same genus (Rossi *et al.* 1998; Papasotiropoulos *et al.* 2001).

The phylogenetic tree produced by the MP method reveals three main clusters (Fig. 4). *Mugil cephalus* seems to be the most genetically distinct species in a separate branch, with the highest (100%) bootstrap value. *Liza ramada* and *L. aurata* are clustered together with strong support in the bootstrap values

L. ramada

70

TACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGGGTCGGGCCTGGTTAGTACTTGGATGGGAGACCG

140

CCTGGGAATACCAGGTGCTGTAAGCTTTTGATTTCCGTCAGTAGTAATAGGAACATTCGCATCATAATGA

210

TGGACACAATGATGATTTCCATCATCTATTAATACTCTTTCTGTGACTACAGCAAAA**GCTTACGGCCATA**

280

CCAGCCTGAACACGCCCGATCTCGTCTGATCTCGGAAGCTAAGCAGGGTCGGGCCTGGTTAGTACTTGGA

350

TGGGAGACCGCCTGGGAATACCAGGTGCTGTAAGCTTTTGATTTTCATCACGAGTAATAGGAACATTCAC

420

ATCATAGTGATGGACACAATGATGATTTCCATCATCTATTAATACTCTTTCTGTGACTACAGCAAAA**GCT**

440

TACGGCCATACCAGCCTGAA

M. cephalus

70

TACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCACGGTCGGGCCTGGTTAGTACTTGGATCGGAGACCG

140

CCTGGGAATACTAGGTGCTGTAAGCCTTTCATTTTCATCAGGAGACATGGTAATATTTACATCATCAAAA

210

TACATGCAATGATGATTTTGAGCACTTATATATACTGCGTTTGTGACAGTTACCATT**GCTTACGGCCATA**

280

CCAGCCTGATTACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGTGTAGGGCCTGGTTAGTATTTGGA

350

TGGGAGACCGCCTGGGAATACCAGGTGCTGTAACCTTTTCATTCTCATCAGGAAACATGGTAACATTTAC

420

ATCATCAAAATACATGCAATGATCATTTTGACCACTTATATATACTGCTTTAGTGACATTTGACATT**GCT**

440

TACGGCCATACCAGCCTGAA

Figure 2 Dimeric 5S rDNA tandem arrays of *L. ramada* and *M. cephalus* species. The coding sequence of the 5S rDNA is in boldface type. The primers used to amplify the 5S rDNA are underlined.

(90%). The last clade includes *L. saliens* and *C. labrosus*, with lower bootstrap values (73%). The species *O. labeo* is always the outgroup.

Discussion

Species identification

Numerous analytical methodologies have been developed for fish species identification, mainly based on protein analysis (Andrews 1998; Mackie, Pryde, Gonzales-Sotelo, Medina, Pérez-Martin, Quinteiro, Rey-Mendez & Rehbein 1999). Among many nuclear markers, the 5S rDNA gene is of special interest in species identification because of its noteworthy structure that makes it a species–specific marker in higher eukaryotes (Pendas *et al.* 1994). The fact that the organization of 5S rDNA presents no intraspecific polymorphism, and on the other hand high interspecific variability, makes it a very good candidate for comparison of closely related species. This gene has been extensively used for species discrimination either with a simple PCR amplification (Cespedes *et al.* 1999; Rodriguez, Garcia, Gonzalez, Asensio, Fernandez, Lobo,

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                                                                                    70
L.ramada      TACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGGGTCGGGCCTGGTTAGTACTTGGATGGGAGACCG
L.aurata      ......................................................................
L.saliens     ......................................................................
C.labrosus    ..............................C.......................................
M. cephalus   ...................................C..................................

                                                                                   140
L. ramada     CCTGGGAATACCAGGTGCTGTAAGCTTTTGATTTTCGTCAGTAGTAATAGGAACATTCGCATCATAATGA
L.aurata      ....................................A.................................
L.saliens     ....................................A...........T........G..........A.
C.labrosus    ....................................A..........A................C...A.
M. cephalus   .........................C...C......A....G..AC..G.T......TA......C.AA.

                                                                                   210
L. ramada     TGGACACAATGATGATTTCCATCATCTATTAATACTCTTTCTGTGACTACAGCAAAAGCTTACGGCCATA
L.aurata      ......................................................................
L.saliens     ...................A.....................A......G.....................
C.labrosus    ........G................................A...G..G.....................
M. cephalus   .AC.TG............TG.C..CT...AT.....GCG.T......AGTTA.C.TT.............

                                                                                   220
L. ramada     CCAGCCTGAA
L.aurata      ..........
L.saliens     ..........
C.labrosus    ..........
M. cephalus   ..........
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Figure 3 Aligned nucleotide sequences of the 5S rDNA gene, for the five Mugilidae species. The coding sequence of the 5S rDNA is in boldface type. Dots indicate base identities.

Table 1 Pairwise distance matrix calculated for the five Mugilidae species

OTUs	1	2	3	4	5
1	–	[0.004]	[0.012]	[0.014]	[0.032]
2	0.005	–	[0.011]	[0.014]	[0.031]
3	0.033	0.028	–	[0.013]	[0.030]
4	0.042	0.037	0.037	–	[0.034]
5	0.191	0.185	0.179	0.209	–

In the lower-left matrix are reported the distances, in the upper-right matrix are reported the standard errors. OTUs labels are as follows: 1, *Liza ramada*; 2, *Liza aurata* 3, *Liza saliens*; 4, *Chelon labrosus*; 5, *Mugil cephalus*.

Hernandez & Martin 2001; Karaiskou *et al.* 2003) or with a PCR-RFLPs analysis (Aranishi 2005a, b). In our study, we used the PCR amplification of the 5S rDNA gene followed by direct sequencing analysis, in order to identify fry individuals of the six Mugilidae species.

Previous genetic identification of grey mullet's species has been achieved by mitochondrial DNA analysis, using species–specific D-loop amplification primers (Murgia, Tola, Archer, Vallegra & Hirano 2001). Later on, Klossa – Killia, Papasotiropoulos, Killias and Alahiotis (2002) used PCR-RFLP analysis of the 16S rRNA mtDNA segment in order to discriminate *M. cephalus* ovaries, from the fish roe of other four Mugilidae species. Both studies aimed to identify the origin of Mugilidae ovaries used for commercial processed products, for food quality control purposes. Also, allozyme analysis and glucose-6-phosphate isomerase-A (GPI-A) allelic differences were used for

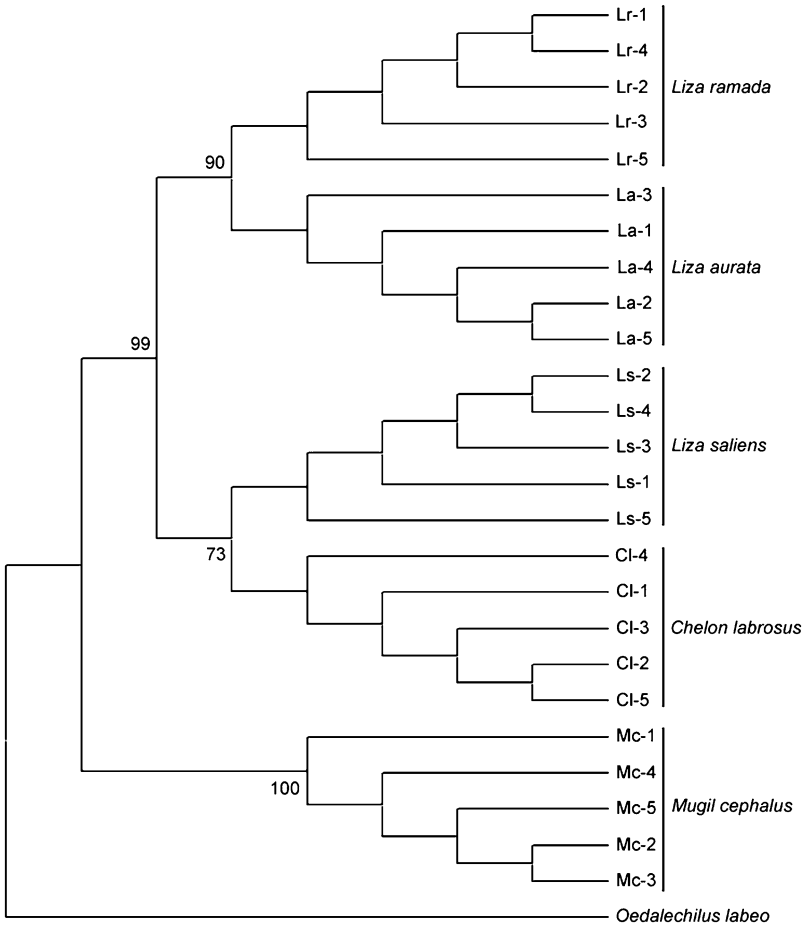

Figure 4 Tree topology obtained from Maximum parsimony analyses of 5S rDNA sequencing data, for five Mugilidae species. The numbers above the branches correspond to the percentage (%) bootstrap values (after 1000 replicates). The 5S rDNA sequence of *Oedalechilus labeo* was used as an outgroup.

discrimination of resident and migratory grey mullet (*M. cephalus*) stocks (Huang, Weng & Lee 2001). At the end, several allozyme loci (Papasotiropoulos *et al.* 2001) and different restriction patterns of mtDNA segments (Papasotiropoulos *et al.* 2002) can be used as diagnostic markers for distinguishing different Mugilidae species.

Mugil so-iuy and *L. saliens* are the two species (of the studied ones) that can be discriminated with a simple PCR reaction, as they reveal a unique pattern. *Mugil cephalus* seems to be the most divergent species compared with the rest four and reveals a distinct DNA haplotype. This species is the most important for the aquaculture units as it has a higher growth rate on natural food webs and on aquaculture units. *Mugil cephalus* ovaries extracted from the ripe females also have also high marketable price. Each of the rest three species *L. ramada*, *L. aurata* and *C. labrosus* reveals its own haplotype, which is unique, species specific and with nucleotide substitutions in different sites. Hence a PCR reaction of the 5S rDNA gene, followed by direct sequencing analysis, could lead to accurate discrimination of the species of fry mullets filling the hatcheries.

Phylogenetic inferences

Phylogenetic relationships among Mugilidae species have been investigated through biochemical and molecular markers, but all the previous studies provided some conflicting results, probably due to the different genetic systems analysed. All of the above studies are in agreement only with one major point: *Mugil cephalus* belongs to a monophyletic group and seems to be the most genetically distinct species. The results of the present study corroborate the previous conclusion as *M. cephalus* is grouped separately in the MP tree with the highest (100%) bootstrap value (Fig. 4), and values of its genetic distance among the rest species (0.179–0.209) are the highest observed in our study (Table 1). The above-mentioned hypothesis

is in agreement with chromosome (Cataudella, Civitelli & Capanna 1974; Delgado, Molina, Lobillo, Alonso & Camacho 1992) and hemoglovine (Rizzotti 1993) studies, but the high genetic differentiation observed between *M. 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 (Caldara *et al.* 1996; Rossi *et al.* 1998).

Our phylogenetic reconstruction is also in agreement with previous studies, clustering together *L. ramada* and *L. aurata*. Hence, the *L. ramada* – *L. aurata* cluster identified in our study with high (90%) bootstap values, was also revealed from allozymic data (Papasotiropoulos *et al.* 2001; Rossi *et al.* 2004) as well as from RFLP-mtDNA data (Papasotiropoulos *et al.* 2002). This is the first DNA study that reveals this clade as it was not recognized by previous mtDNA sequencing data (Caldara *et al.* 1996; Rossi *et al.* 2004). We should state here that *L. ramada* and *L. aurata* species share only one nucleotide substitution in the whole gene and they present the lowest genetic distance (0.005) among all the species (Table 1), facts that denote that these are the closest taxa in the Mugilidae family.

The MP tree of our study reveals that *L. saliens* and *C. labrosus* are clustered together, which is also identified by Caldara *et al.* (1996) based on mitochondrial 12S rDNA and cytochrome b sequences, and by Papasotiropoulos *et al.* (2002) based on the RFLP analysis of three PCR amplified mtDNA segments. From the DNA studies (including the present one), only Rossi *et al.* (2004) could not recognize this clade, based on sequences of mitochondrial 16S rDNA gene. Our results corroborate the very close similarity between *Chelon* and species of *Liza*, and show once more the non-monophyletic origin of the genus *Liza*. The close resemblance of the two genera is revealed also from the range of genetic distance between them (0.037–0.042) (Table 1). Similar difficulty in discriminating *Chelon* from *Liza* has been also revealed from chromosome studies (Cataudella *et al.* 1974) and morphological data (Schultz 1946; Harrison & Howes 1991). A more extensive genetic study of representatives of the two genera is needed to contribute to the systematic debate on whether the two genera should be synonymized, or whether they represent distinct clades.

Conclusively, our study proposes a possible genetic approach for discrimination of fry mullet's species. From the phylogenetic point of view, it reveals the high genetic divergence of *M. cephalus*, the non-monophyly of the genus *Liza* exclusive of *Chelon* and the close resemblance of *L. ramada* and *L. aurata*, based on sequences of nuclear DNA. The 5S rDNA sequencing analysis of more individuals of all Mugilidae species could finally clarify the systematic relationships among taxa.

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