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Identification of juveniles of grey mullet species (Teleostei: Perciformes) from Kuriat Islands (Tunisia) and evidence of gene flow between Atlantic and Mediterranean *Liza aurata*

Monia TRABELSI[1], Didier AURELLE[2], Nawzet BOURIGA[1,4], Jean-Pierre QUIGNARD[3], Jean-Paul CASANOVA[4] and Eric FAURE[4]*

(1) *Unité de Biologie marine, Faculté des Sciences, Campus Universitaire, 2092 Manar II, Tunis, Tunisie*

(2) *UMR 6540 DIMAR, Station Marine d'Endoume, Rue de la batterie des Lions, 13007 Marseille, France*

(3) *Laboratoire d'Ichtyologie, Université Montpellier II, Montpellier, France*

(4) *LATP, CNRS-UMR 6632, Evolution biologique et modélisation, case 5, Université de Provence, Place Victor Hugo, 13331 Marseille cedex 3, France*

**Corresponding author: Tel: 00 (33) 491 10 61 77, Fax: 00 (33) 491 10 62 25, E-mail: Eric.Faure@univ-provence.fr*

Abstract: Unidentified juveniles of a grey mullet species from the Kuriat Islands (Tunisia) were compared with Mediterranean and Atlantic candidate species (*Mugil* spp. or *Liza* spp.) using a mitochondrial gene (*cytochrome b*). These analyses have shown that juveniles are *L. aurata* individuals; phylogenetic analyses supported this grouping with very high bootstrap values and also shown evidence of gene flow between Atlantic and Mediterranean populations. Moreover, phylogenetic analyses were congruent with the analyses of the number of pyloric caeca. As mugilid juveniles for aquaculture are still obtained from wild stocks, these data provided a valuable baseline for further investigations on identification of these fish. Moreover, using the polymerase chain reaction, sufficient DNA for phylogenetic analyses can be amplified from very small portions of caudal fins and these samples can be collected without sacrificing individuals, which is one important requirement for the study of species that young fry is used for stocking lagoons and lakes. In addition, the Kuriat Islands Coasts could constitute a nursery for *L. aurata* which are of economical interest in Tunisia.

Résumé : *Identification de juvéniles de mulet (Teleostei : Perciformes) pêchés sur les côtes des îles Kuriat (Tunisie) et évidence de flux géniques entre les populations atlantiques et méditerranéennes de* Liza aurata. Des gènes mitochondriaux (*cytochrome b*) provenant de diverses espèces de Mugilidae de Méditerranée et de l'Atlantique (*Mugil* spp. or *Liza* spp.) ont été utilisés pour positionner phylogénétiquement des juvéniles de mulet pêchés sur les côtes des îles Kuriat (Tunisie) et dont l'espèce n'était pas connue. Les analyses phylogénétiques ont montré qu'il s'agissait de juvéniles de *L. aurata* et aussi l'existence de flux géniques entre les populations atlantiques et méditerranéennes. L'analyse du nombre de caeca pyloriques a confirmé cette identification. Comme en aquaculture, les juvéniles de mugilidés sont encore prélevés dans les stocks sauvages, ces analyses pourraient fournir une base pour de futurs travaux d'identification de ces poissons. De plus, l'utilisation de la réaction de polymérisation en chaîne permet d'obtenir suffisamment d'ADN pour les analyses phylogénétiques à partir de très petites portions de nageoires caudales qui ne nécessitent pas de sacrifier les individus, ce qui est une condition importante pour l'étude d'espèces dont les alevins sont utilisés pour empoissonner les lagunes et des lacs.

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D'autre part, les côtes des îles Kuriat pourraient constituer une nurserie pour *L. aurata*, espèce présentant un intérêt économique en Tunisie.

Keywords: Mugilidae ● Grey mullets ● *Liza aurata* ● Mitochondrial DNA ● *Cytochrome b* ● Pyloric caeca

Introduction

Grey mullet species are members of the Mugilidae family, order Perciformes; they are cosmopolitan fish inhabiting all tropical and temperate seas (Thomson, 1997; Nelson, 2006). They are found inshore, and enter lagoons, estuaries and even rivers (Thomson, 1997). Eight species belonging to four genera inhabit the Mediterranean Sea (flathead grey mullet, *Mugil cephalus* Linnaeus 1758; thicklip mullet, *Chelon labrosus* Cuvier 1758; boxlip mullet, *Oedalechilus labeo* Cuvier 1829; thinlip grey mullet, *Liza ramada* Thomson 1986; golden grey mullet, *Liza aurata* Risso 1810; leaping mullet, *Liza saliens* Risso 1810; *Liza abu* Heckel 1843; *Liza carinata* Valenciennes in Cuvier & Valenciennes, 1836), but only the first six species are commonly found in this sea (Nelson, 2006). Moreover, *L. carinata* is a lessepsian species and has recently invaded the Eastern Mediterranean from the Red Sea through the Suez Canal (Ben-Tuvia, 1975).

The grey mullets play an important role in fisheries and aquaculture of many regions of the world, especially in cultural practices based on natural food webs (Chaoui et al., 2006; Imsiridou et al., 2007 and references therein). The phylogeny of Mugilids appears particularly obscure at both the intra- and inter-specific levels; it is extremely difficult to distinguish some species, especially the juvenile stages, because their morphological and physiological characters do not frequently exhibit significant differences (Caldara et al., 1996; Thomson, 1997). Grey mullets' fry identification is based on the pigmentation along the body flanks (Serventi et al., 1996), the melanophore patterns on the ventral side of the head (Minos et al., 2002), and also morphometric traits (Katselis et al., 2006). Moreover, three recent studies have shown that molecular tools can determinate the systematic status of adult (Heras et al., 2006) or juvenile Mugilids (Papa et al., 2003; Imsiridou et al., 2007).

As we have recently fished unidentified juvenile mullets on the coasts of the Kuriat Islands (Tunisia) that could constitute a nursery for mugilids which are of economical interest in Tunisia (Ben Khemis et al., 2006), their identification was important. For this purpose, we have used mitochondrial DNA (mtDNA) methods in which the samples can be collected with a partially invasive tissue sampling without sacrificing individuals. Moreover, a morphological control has been made.

Materials and Methods

Biological Samples

Eighteen DNA sequences were obtained from specimens collected by our team in the Western Mediterranean Sea; i.e., 12 specimens of unidentified juvenile mullets within a school of fish have been caught around the Kuriat Islands coasts (Tunisia) (35°48'N, 11°02'E) (Fig. 1); 2 specimens of *Liza saliens* and 4 specimens of *Mugil cephalus* caught off Sete (Gulf of Lion, France, 43°24'N, 03°42'E). The French fishes were identified by one of us (JPQ); they were stored in 70% ethyl alcohol at ambient temperature and at - 20°C as soon as possible. As for the mullets from Kuriat Islands, the number of pyloric caeca has been counted under a light microscope. The juveniles from the Kuriat Islands measured from 2.8 to 3.6 cm.

DNA extraction and PCR reaction

Total DNA was extracted from approximately 0.15 cm^2 of caudal fin using a previously described method (Trabelsi et

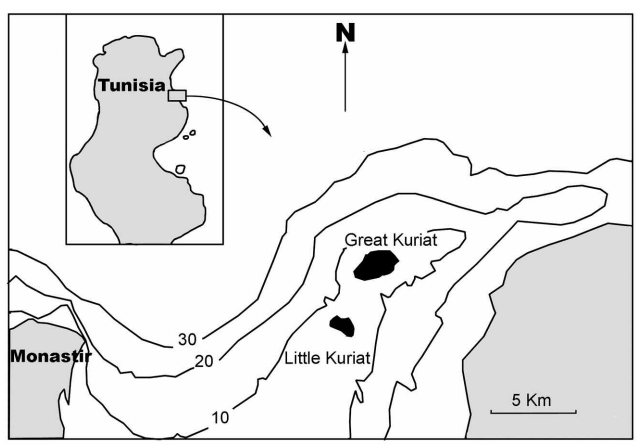

Figure 1. *Liza aurata.* Map of the Kuriat Islands.
Figure 1. *Liza aurata.* Carte des îles Kuriat.

al., 2002). A section of approximately 400 bp of mtDNA genome from the *cytochrome b (cyt b)* gene was amplified using published specific primers New-For 5'-AGCCTAC-GAAAACCCACCC-3' and 34-Rev 5'-AAACTGCAGCC-CCTCAGAATGATATTTGTCCTCA-3'. Polymerase chain reaction (PCR) components per 50 µl reaction were as follows 50 ng template DNA, 0.2 µM of each primer, 2.0 U. HiTaq *Taq* polymerase, dNTPs 0.2 mM, 5 µl of the reaction buffer provided by the *Taq* manufacturer (Bioprobe, France). The cycling parameters were as follows 92°C for 2 min., 5 times (92°C for 15 sec., 48°C for 45 sec., and 72°C for 1.5 min), 30 times (92°C for 15 sec., 52°C for 45 sec., and 72°C for 1.5 min), and 72°C for 8 min. Using the single-stranded DNA as a template, the nucleotide sequence was determined with an automated DNA sequencer (Macrogen, Seoul, South-Korea). These sequences will be deposited in GenBank.

Sequence analyses

The characteristics of the *cyt b* mtDNA sequences used for phylogenetic analyses are in Table 1. These sequences have been aligned with the BioEdit software (Hall, 1999). The alignment length was 376 bp, with four shorter haplotypes: *Chelon labrosus* Z70772 (286 bp), *Liza ramada* Z70779 (277 bp), *Liza saliens* Z70774 (285 pb) and *Oedalechilus labeo* Z70777 (286 bp). Three different approaches have been used. Maximum Parsimony (MP) analysis was performed in PHYLIP version 3.6 alpha 3 (Felsenstein, 2002) accessed at http://bioinfo.hku.hk/services/menuserv .html. Maximum likelihood (ML) has been made using the algorithm of Guindon & Gascuel (2003) implemented in the Phyml software (Guindon & Gascuel, 2003). The model of molecular evolution used for reconstruction was chosen with the Modeltest software (Posada & Crandall, 1998) using the FindModel website at Los Alamos National Laboratory (http://hcv.lanl.gov/content/hcv-db/findmodel /findmodel.html). This allowed us to choose among 28 nucleotide models with the Akaike Information Criteria (Akaike, 1974). Neighbor-joining (NJ) analysis was carried out using PAUP* 4.0 (Swofford, 2003) with a maximum likelihood distance correction set with the parameters obtained from MODELTEST (Posada & Crandall, 1998). For both ML and NJ analyses, the chosen model was a General Time Reversible (GTR; Tavaré, 1986) with Gamma distribution of rate variation among sites. The parameter of the Gamma distribution as well as the base frequencies were estimated by the software. Robustness of nodes was estimated by running a bootstrap test with 100 replicates for MP tree and 1000 for NJ and ML trees.

Results

Molecular phylogeny using partial cytochrome b gene

For the analyses of a portion of the *cyt b* gene, we have sequenced 18 individuals of Mugilidae, and extracted from GenBank 16 other Mugilidae sequences belonging to the six species most commonly found in Mediterranean Sea. Figure 2 presents the ML tree with bootstrap results. The trees using three methods (ML, NJ, and MP) give similar topologies for nodes which are statistically supported, and bootstraps values are high for all the species nodes (≥ 88%). The numbers of variable and informative sites are respectively 201/83.

The molecular analyses using the three methods (MP, ML and NJ) show that the fish from Tunisia were *Liza aurata*. This grouping was supported by high bootstrap values (BP) (≥ 86%). The phylogenetic tree is divided into two groups. The first group contains all the *Mugil cephalus* sequences, while the second contains those of the genera *Liza, Chelon* and *Oedalechilus*. Nevertheless, this second group is not well supported (BP ≥ 81%). Intra-specific nodes are never statistically supported, but when two or more strictly identical sequences are present (for example, in *M. cephalus* or in *L. aurata* analyses). Concerning, *Liza, Chelon* and *Oedalechilus* sequences, none inter-specific branching is statistically supported and the monophyly of the genus *Liza* has never been supported. In addition, although no statistically supported, the *L. aurata* sequences are divided into two groups which both contain sequences with Atlantic and Mediterranean (including the Kuriat Islands in one case) origins.

Pyloric caeca

In order to confirm the species identification using phylogenetic analyses, the counting of pyloric caeca has been made. The number of pyloric caeca set from twelve fish was seven for all of them and they decreased in size backwards. These characteristics suggest that they were *Liza aurata* which has 7 to 8 pyloric caeca decreasing in size (Louisy, 2002).

Discussion

Although not the topic of this study, we have already underlined the uncertainty of the phylogenetic relationships between grey mullets due to the conservative morphology displayed by all the species; thus, very few key characters are useful to establish unambiguously these relationships (Caldara et al., 1996 and references therein). Moreover, in all the published molecular studies but one, an allozyme analysis of Papasotiropoulos et al. (2001), the monophyly

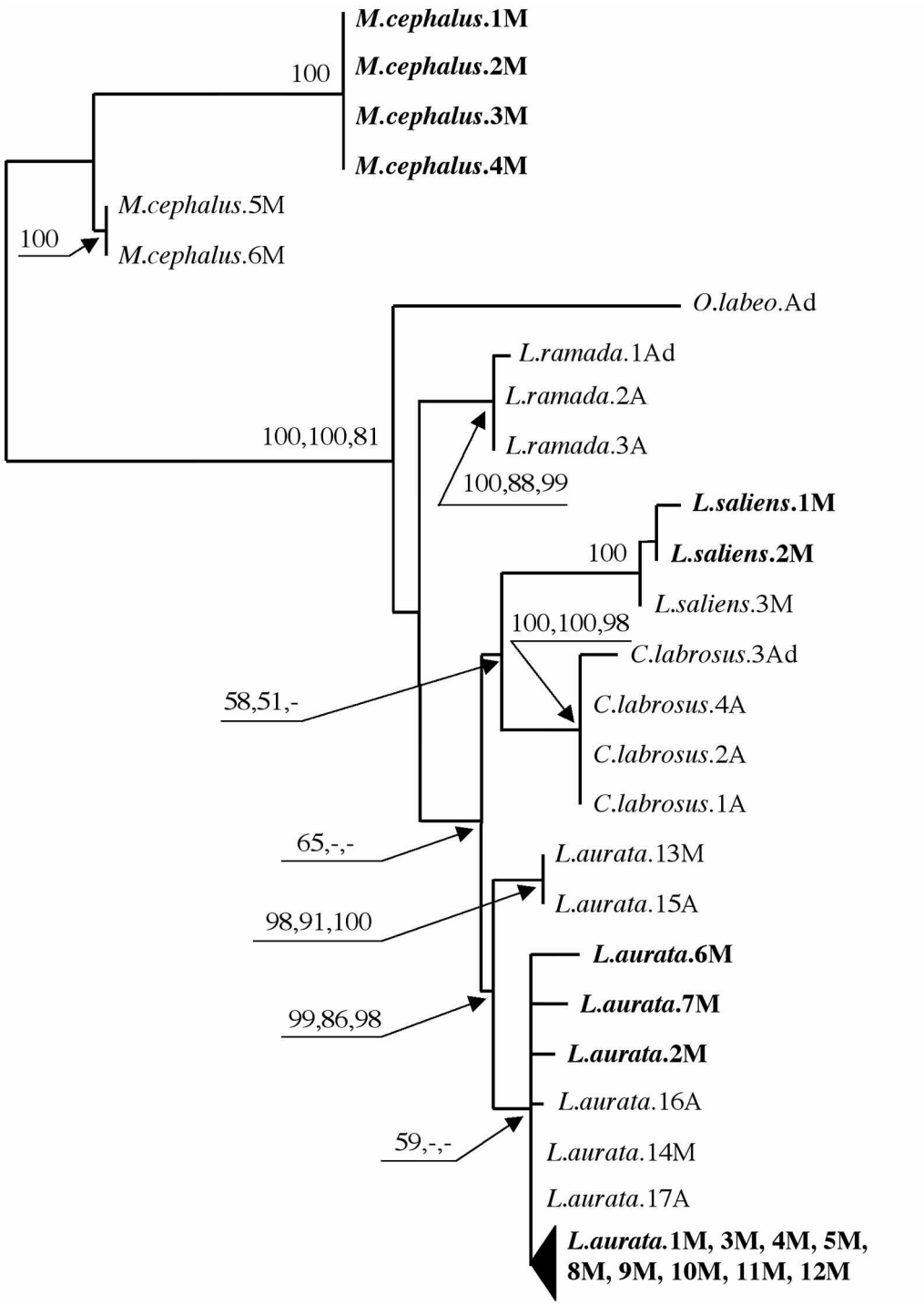

Figure 2. *Liza aurata.* Unrooted phylogenetic tree using the maximum likelihood method. Bootstrap analyses carried out with 100 or 1000 iterations using portions of the *cyt b* gene among 36 sequences of Mediterranean and Atlantic Mugilid species. Bootstrap values (BP) are given for each node only if they excess 50 in percentage, ML (left BP), NJ (middle BP) and MP (right BP). When the number 100 is indicated alone, it signifies that the three BP are 100. Sequence names from fish sequenced for this article are in bold letters.

Figure 2. *Liza aurata.* Arbre phylogénétique non enraciné obtenu en utilisant la méthode du maximum de vraisemblance. Cent ou mille itérations ont été effectuées lors des analyses de bootstrap qui portent sur des portions du gène *cyt b* provenant de 36 séquences de mugélidés méditerranéens et atlantiques. Les valeurs de bootstrap (BP) sont données pour chaque nœud, si la valeur est supérieure à 50 en pourcentage, ML (BP à gauche), NJ (BP au milieu) et MP (BP à droite). Lorsqu'un seul nombre 100 est noté, cela signifie que les trois valeurs de boostrap sont égales à 100. Les noms des poissons séquencés pour cet article sont notés en gras.

Table 1. Data concerning the various sequences uses for phylogenetic analyses (*, shorter sequence). Samples sequenced expressly for this study are in bold letters. Abbreviations: A, Atlantic Ocean; Ad, Adriatic Sea; M, Mediterranean Sea.

Tableau 1. Données concernant les diverses séquences utilisées lors des analyses phylogénétiques (*, séquence dont la taille est plus courte). Les noms des échantillons séquencés pour cet article sont indiqués en caractères gras. Abréviations: A, Océan Atlantique; Ad, Mer Adriatique; M, Mer Méditerranée.

Species	Corresponding name(s) in the phylogenetic tree and accession number	Site of collection	References
Chelon labrosus Cuvier 1758 thicklip grey mullet	*C.labrosus*.1A (EF427544)	A: Cantabric Sea, Spain	www.fishtrace.org
	C.labrosus.2A (EF427545)	A: Cantabric Sea, Spain	www.fishtrace.org
	C.labrosus.3Ad (Z70772)*	Ad: Italy, Lagoon of Venice	Caldara et al., 1996
	C.labrosus.4A (DQ197935)	A: Spain, Canary Islands, Gran Canaria	www.pescabase.org
Liza aurata Risso 1810 golden grey mullet	***L.aurata*.1M (EU122431)**, sequence identical to ***L.aurata*.3M, *L.aurata*.4M, *L.aurata*.5M, *L.aurata*.8M, *L.aurata*.9M, *L.aurata*.10M, *L.aurata*.11M** and ***L.aurata*.12M**	M: Tunisia, Kuriat Islands	present study
	***L.aurata*.2M (EU122432)**	M: Tunisia, Kuriat Islands	present study
	***L.aurata*.6M (EU122433)**	M: Tunisia, Kuriat Islands	present study
	***L.aurata*.7M (EU122434)**	M: Tunisia, Kuriat Islands	present study
	L.aurata.13M (EF439541)	M: Western part	www.fishtrace.org
	L.aurata.14M (EF439540)	M: Western part	www.fishtrace.org
	L.aurata.15A (EF427572)	A: Cantabric Sea, Spain	www.fishtrace.org
	L.aurata.16A (EU224056)	A: France, Pertuis d'Antioche	www.fishtrace.org
	L.aurata.17A (EU224057)	A: France, Pertuis d'Antioche	www.fishtrace.org
Liza ramada Thomson 1986 thinlip grey mullet	*L.ramada*.1Ad (Z70779*)	Ad: Italy, Lagoon of Venice	Caldara et al., 1996
	L.ramada.2A (EU224058)	A: France, Pertuis d'Antioche	www.fishtrace.org
	L.ramada.3A (EU224059)	A: France, Pertuis d'Antioche	www.fishtrace.org
Liza saliens Risso 1810 leaping mullet	***L.saliens*.1M (EU122428)**	M: Lion Gulf, France	present study
	***L.saliens*.2M (EU122428)**	M: Lion Gulf, France	present study
	L.saliens.3Ad (Z70774)*	Ad: Italy, Lagoon of Venice	Caldara et al., 1996
Mugil cephalus Linnaeus 1758 flathead grey mullet	***M.cephalus*.1M (EU122430)**, sequence identical to ***M.cephalus*.2M, *M.cephalus*.3M** and ***M.cephalus*.4M**	M: Lion Gulf, France	present study
	M.cephalus.5A (EU036449)	M: North Aegean Sea, Greece, Kavala	direct submission
	M.cephalus.6A (EU036450)	M: North Aegean Sea, Greece, Kavala	direct submission
Oedalechilus labeo Cuvier 1829	*O.labeo*.Ad (Z70777)*	Ad: Italy, Lecce	Caldara et al., 1996

of the genus *Liza* has never been supported. In addition, other molecular analyses have provided controversial results (Caldara et al., 1996; Rossi et al., 2004; Turan et al., 2005; Semina et al., 2007). Our present analyses provide evidence for the long-suspected paraphyly of the genus *Liza* and show the difficulty to analyse the relationships between *C. labrosus, L. aurata, L. ramada, L. saliens* and *O. labeo*. Moreover, the difficulty in discriminating *Chelon, Oedalechilus* and *Liza* was already revealed by investigations based on chromosome analysis (Nirchio et al., 2007 and references therein). In addition, on the basis of morphological data, the paraphyly of *Liza* with respect to *Chelon* has already been suggested (Senou et al., 1996).

Surprisingly, in spite that all the *L. aurata* from Kuriat Islands have been caught in the same school, their partial *cyt b* sequences exhibit a relatively great level of nucleotides differences; for example, 7 (~ 19%) and 6 (~ 16%) out to 376 amino acid residues are different between

respectively, *L. aurata*.2M and the nine sequences which are identical (*L. aurata*.1M, *L. aurata*.3M, *L. aurata*.4M, *L. aurata*.5M, *L. aurata*.8M, *L. aurata*.9M, *L. aurata*.10M, *L. aurata.*11M and *L. aurata*.12M) versus *L. aurata*.7M. Moreover, the nine identical sequences are also strictly similar to the corresponding sequences from a Mediterranean fish (*L. aurata*.14 M) and an Atlantic fish (South Brittany coasts, *L. aurata*.17A), with only three differences out to 1141 nt between these two last sequences. Similarly, on the 376 nt used for the phylogenetic analyses, another sequence from an Atlantic fish (Cantabric Sea, *L. aurata*.15A) is similar to those of an Mediterranean fish (*L. aurata*.13M) and only one difference has been found on the 1141 nt sequences found in GenBank. This suggests recent gene flow and no genetic isolation between populations of *L. aurata* on each side of the Strait of Gibraltar. This is a new example showing that the Straits of Gibraltar acts are not a barrier to gene flow as it has been already shown for several other marine species (reviewed in Patarnello et al., 2007). Moreover, the Mediterranean and Atlantic haplotypes have been in sympatric *L. aurata* individuals, as it has been previously observed for Algerian dusky grouper (*Epinephelus marginatus,* Lowe 1834) (Gilles et al., 2000) and several other fish, this is probably due to the Atlantic current flowing southeastward from the Spanish coast to the coast of North Africa (Maurin, 1968).

Even more than adults, it is extremely difficult to distinguish the species from one another at the larval and fingerling stages. This represents a major issue in aquaculture practice, because, artificial breeding of grey mullet's fry is not a common process and all the cultural practices are mainly based on fishing of wild fry. Hence, in order to improve the cultivation process, a more basic knowledge of their is needed. Molecular analyses can successfully determine the systematic status of adult (Heras et al., 2006) or juvenile mugilids (Papa et al., 2003; Imsiridou et al., 2007). Recently, different methods have been used, including allozyme and amplified fragment length polymorphisms (AFLP) (Papa et al., 2003), and sequencing of three mitochondrial genes (Heras et al., 2006) and nuclear *5S rDNA* genes (Imsiridou et al., 2007). Here, we propose a relatively easy mtDNA method to identify the mullets from Kuriat Islands as a population of *L. aurata*. Moreover, fin rays sampling is easily performed with basic dissecting tools and does not involve sacrificing the fish, indeed, analysis of regenerative capabilities in many teleosts has shown that they have the ability to regenerate their fins after injury (Géraudie & Singer, 1992). More generally, molecular tools can be useful to identify larvae or juvenile from other fish groups. Nevertheless the efficiency of such barcoding approach always relies on the differences between intra- and inter-specific divergences at the molecular level (Hajibabaei et al., 2007).

The Kuriat Islands lie at 18 km off the coast of Monastir and consist of two small islands: Little Kuriat which is *ca.* 0.7 km^2 and the larger Great Kuriat which is *ca.* 2.7 km^2 in area. Kuriat Islands houses a high diversity of fish species; mullets occur in abundance, and at least three species are found there. In the future, localization of spawning and nursery areas will be intensively studied, since juvenile mugilids (from 3 to 7 cm) are caught along the Tunisian Coasts in order to stock with fish in natural lakes and in lake dams (El Cafsi et al., 2003). Moreover, the Kuriat Islands could constitute a very interesting area with non-polluted fish. Indeed, in numerous Tunisian regions, contamination of *Mugil* species by various pollutants have been mentioned (Masmoudi et al., 2007 and references therein). In the future, the incidence of skeletal fish deformities of Kuriat Islands, which can be considered as indices of environmental disturbances (including pollution) (Ayed et al., 2007) will be analysed.

In conclusion, identification of juvenile mugilids is of major interest for aquaculture purpose, as captive breeding of these species is still very uncommon, and molecular tools could help to solve this problem. In this study, we have use a relatively rapid method for fry discrimination, on the basis of PCR amplication of species-specific fragments of a mtDNA gene. This approach could be applied to some other mugilid species and could facilitate aquaculture units to identify accurately the species of fry mullets supplied by fishermen. Among the exploited fishes, the golden grey mullet (*Liza aurata*) is particularly well appreciated by Tunisian consumers. The Kuriat Islands seems to be an important nursery area. In the future, we will collect all over-the-year monitoring data about the species composition of juvenile mugilids in these islands. Indeed, understanding of the recruitment dynamics of juveniles in wild populations is important for rational management of grey mullet stocks. Moreover, a more extensive genetic survey of representatives of mugilid species, including species of *Liza, Chelon* and *Oedalechilus*, is needed to shed more light on their phylogeny. In addition, we will make analyses of biological traits and genetic variables of numerous *L. aurata* populations to understand the Atlantic-Mediterranean phylogeographical patterns of this species.

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