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Chromosomal evolution in Mugilidae: karyotype characterization of *Liza saliens* and comparative localization of major and minor ribosomal genes in the six Mediterranean mullets

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Abstract This study continues a comparative cytogenetic analysis of the fish family Mugilidae, reporting the karyotype characterization of the leaping mullet, *Liza saliens*, by C-banding, Ag- and fluorochrome-staining, and completing the fluorescence in situ hybridization (FISH)-mapping of the 18S and 5S rRNA genes (rDNA) to the chromosomes of the six Mediterranean mullets, namely *L. saliens*, *L. ramada, L. aurata*, *Mugil cephalus*, *Chelon labrosus* and *Oedalechilus labeo*. In all species, except *M. cephalus*, the 5S rDNA sites were localized on a medium-sized acrocentric chromosome pair, which was considered homeologous in all of them. In *L. saliens*, an additional 5S rDNA site was detected in a location close to the one shown by major ribosomal genes in *M. cephalus*, i.e. the subtelomeric region of chromosome pair 1. The 5S rDNA site in *M. cephalus* is located on the smallest chromosome pair of the complement, which, on the other hand, though on a different position, bears 18S rDNA in all the species of *Liza* and *Chelon* examined. The heterochromatin composition and the major and minor ribosomal gene locations suggest that the karyotype of *L. saliens* (subgenus *Protomugil*) can be considered intermediate between the karyotype of the more primitive *M. cephalus* and those of the other *Liza* (subgenus *Liza*) species and of the representatives of the more derived genera *Chelon* and *Oedalechilus*.

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Introduction

Six out of the 62–80 species included in the Mugilidae family (Nelson 1994; Thomson 1997) are present in the whole Mediterranean area. These species belong to four genera: *Mugil* Linnaeus, 1758, *Liza* Jordan and Swain, 1884, *Chelon* Artedi, 1793, and *Oedalechilus* Fowler, 1903, the systematics of which, though long debated, has recently been revised by Thomson (1997). In order to contribute to the cytotaxonomic knowledge of the family and to try to detect, in a "homogeneous" systematic context, a more general trend of karyotypic evolution in teleosts of the 48-uniarmed-chromosome type, we continue our cytogenetic survey (Rossi et al. 1996, 1997, 2000) of Mediterranean mullets to: (1) extend the cytogenetic characterization to *Liza saliens* Risso, 1810, for which only the Giemsa-stained karyotype description was available (Cataudella et al. 1974; Arefyev 1989); (2) complete the chromosomal localization of 18S rDNA by fluorescence in situ hybridization (FISH) in this species and in those mullets – *L. aurata* Risso, 1810 and *Chelon labrosus* Risso, 1827 – for which only the Ag-detection of nucleolar organizer regions (NORs) has been reported (Delgado et al. 1991); and (3) describe the chromosomal location of 5S rDNA in all six species of Mugilidae inhabiting the Mediterranean Sea, i.e. the three species mentioned above, plus *Mugil cephalus* Linnaeus, 1758, *L. ramada* Risso, 1826 and *Oedalechilus labeo* Cuvier, 1829, which have been previously cytogenetically studied (Rossi et al. 1996, 1997, 2000). Indeed, the number and the chromosomal locations of the major (18S, 28S, and 5.8S, clustered at the NORs) and minor (5S) rDNA loci are essentially species-specific and in fish constitute important karyotypic markers (Fujiwara et al. 1998), which may provide phylogenetic information. This is the first report on the chromosomal locations of 5S ribosomal genes in the family.

Materials and methods

Juveniles of *Mugil cephalus* (20 specimens), *Liza saliens* (8 specimens), *L. ramada* (8 specimens), *L. aurata* (6 specimens) and *Chelon labrosus* (7 specimens) were collected during autumn 1999 in Orbetello Lagoon (Tuscany, Italy). Metaphases were prepared using conventional air-drying techniques from pooled spleen, gill and cephalic kidney cells of specimens processed either immediately or after transfer to the laboratory. For *Oedalechilus labeo*, chromosomal preparations from juvenile specimens previously studied (Rossi et al. 2000), kept at –20°C, were used for FISH.

Silver-stained NORs (Ag-NORs) were obtained as described by Howell and Black (1980). Fluorescence staining with the GC-specific stain chromomycin A_3 (CMA_3) and the AT-specific stain 4′,6-diamidino-2-phenylindole (DAPI) was carried out according to Sola et al. (1992). For FISH, a whole pBR350 plasmid, with a 1.8 kb insert containing the *Xenopus laevis* 18S rDNA and a zebrafish 5S rDNA repeat (Genbank no. AF213516; Gornung et al. 2000), biotin-labeled by random priming (BRL), were used as probes. Standard procedures for hybridization of repetitive sequences (Lichter et al. 1992) were carried out, followed by high-stringency post-hybridization washes at 42°C. Signals were detected and amplified by a three-round application of Avidin-FITC/biotynilated anti-Avidin (Vector). At least 20 metaphases per individual were examined after FISH with either probe with a Zeiss Axiophot epifluorescence microscope, equipped with appropriate selective filters. Microphotographs were taken with a SenSys 1400 CCD camera (Photometrics), using the Iplab program (version 2.4, Windows). Digital images were processed using AdobePhotoshop 5.0 (Windows).

Results

Liza saliens

The karyotype (Fig. 1a) of the leaping mullet ($2n = 48$) is made of 46 acrocentric and 2 subtelocentric chromosomes. These latter are the smallest of the chromosome complement and show C- (Fig. 1b), Ag- (Fig. 1c–e) and CMA_3- (Fig. 1f) positive short arms. Ag-NOR-bearing chromosomes of different individuals of *L. saliens* may be quite heteromorphic in short-arm size (Fig. 1d). In one specimen an additional Ag-NOR (Fig. 1e) was observed in the paracentromeric region of a medium-sized acrocentric chromosome, classified as no. 9. C-banding revealed constitutive heterochromatin on the pericentromeric regions of all the chromosomes (not shown) in addition to the C- (Fig. 1b) and CMA_3-positive (Fig. 1f) and the DAPI-negative (Fig. 1g) short arms of chromosome pair 24. CMA_3 (Fig. 1f) and DAPI (Fig. 1g) otherwise produce a uniform staining pattern along chromosomes.

18S rDNA

Hybridization with the *Xenopus laevis* 18S rDNA probe confirmed that in *L. saliens* chromosome pair 24 is the main NOR-bearing pair (Fig. 2a). In three out of the eight specimens analyzed, additional small NOR-sites were detected in proximity to the centromeres of one or both homologues (Fig. 2a) of chromosome pair 9.

L. aurata (Fig. 2b) and *Chelon labrosus* (Fig. 2c) show hybridization signals on the short arms of chromosome pair 24.

5S rDNA

In *Mugil cephalus* (Fig. 3a), 5S ribosomal genes were localized exclusively in a paracentromeric position on the smallest chromosome pair (no. 24). In *L. saliens* (Fig. 3b), FISH signals for the 5S ribosomal genes were observed in subcentromeric position of one medium-sized

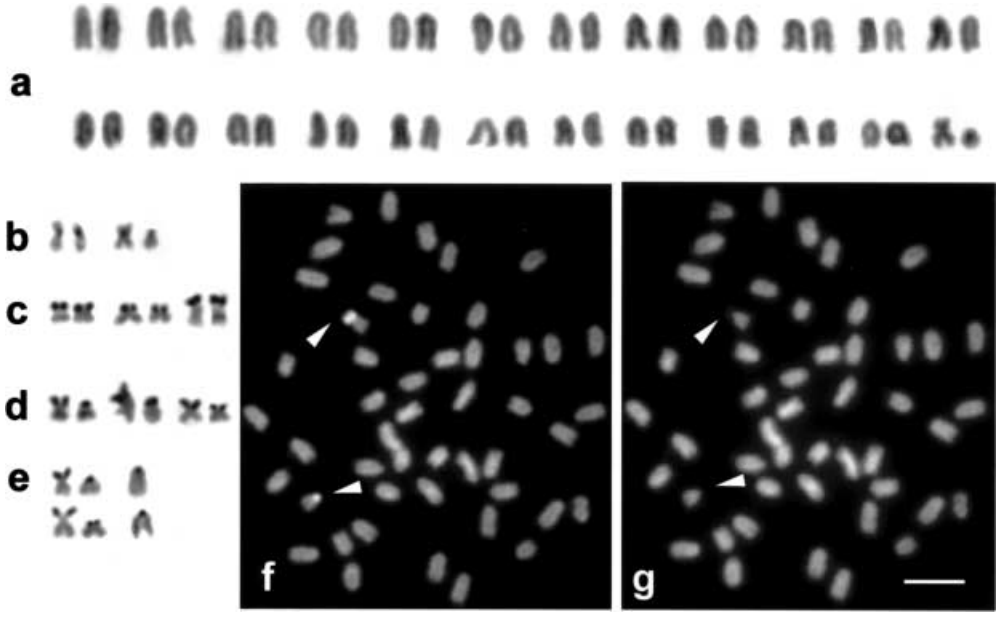

Fig. 1a–g *Liza saliens*. **a** Giemsa-stained karyotype; **b–e** selected examples of partial karyotypes showing: **b** C-banded chromosome pair 24, and **c** homomorphic, **d** heteromorphic and **e** additional Ag-NORs; **f** CMA_3- and **g** DAPI-stained metaphase plate. *Arrowheads* indicate the NOR-bearing chromosome pair 24 (bar = 5 μm)

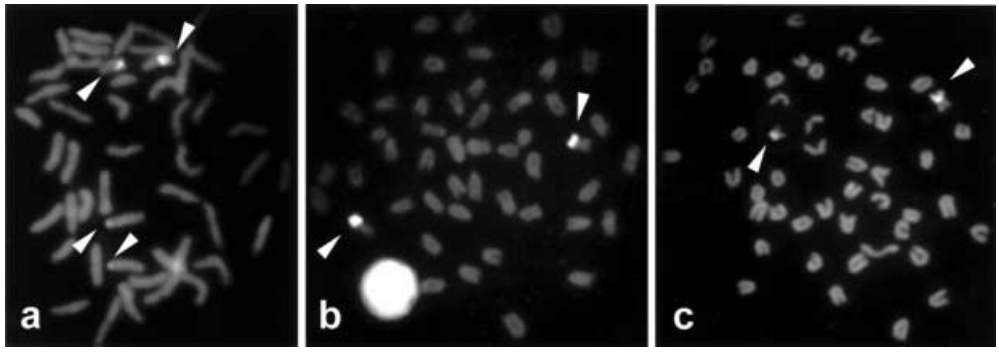

Fig. 2a–c Metaphases after FISH with 18S rDNA in: **a** *Liza saliens,* **b** *L. aurata* and **c** *Chelon labrosus*. *Arrowheads* indicate 18S rDNA clusters (=NORs)

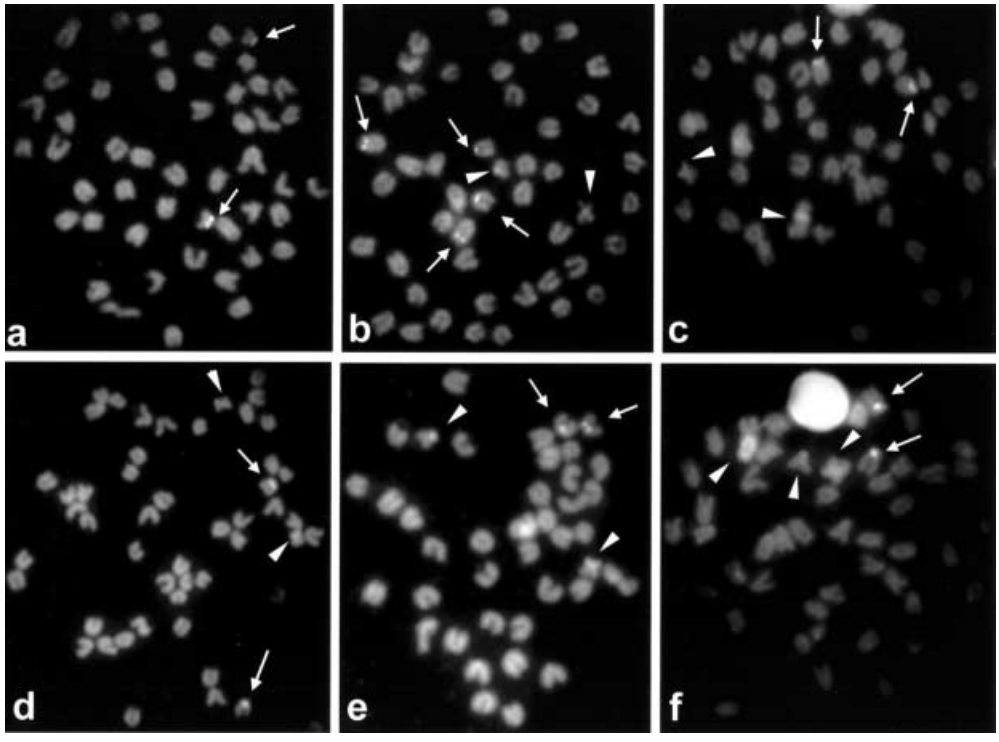

Fig. 3a–f Metaphases after FISH with 5S rDNA in: **a** *Mugil cephalus*, **b** *Liza saliens*, **c** *L. ramada*, **d** *L. aurata*, **e** *Chelon labrosus* and **f** *Oedalechilus labeo*. *Arrows* indicate 5S rDNA clusters, *arrowheads* indicate NOR-bearing chromosomes

chromosome pair, classified as no. 8, and in subtelomeric position of long arms of both chromosomes no. 1. In *L. ramada* (Fig. 3c), *L. aurata* (Fig. 3d) and *C. labrosus* (Fig. 3e), the 5S ribosomal genes were detected in a single-chromosome location, in a subcentromeric position of one medium-sized chromosome pair, considered homeologous to chromosome pair 8 of *L. saliens*.

Chromosome preparations from individuals of *Oedalechilus labeo* having different NOR-patterns (Rossi et al. 2000) were used for FISH with the 5S rDNA probe. In all the specimens, the 5S ribosomal genes were found to be restricted to a single site in subcentromeric position of chromosomes no. 8 (Fig. 3f). These are undoubtedly different from the NOR-bearing chromosomes, which are easily recognizable after denaturation for FISH, owing to enhanced propidium iodide fluorescence of NORs (Rab et al. 1995).

Discussion

Liza saliens

The karyotype we found in specimens of *L. saliens* from Orbetello Lagoon corresponds to the one previously described for specimens from Lesina Lagoon, Puglia, Italy (Cataudella et al. 1974) and from the Black Sea (Arefyev 1989). The chromosome number and formula conform to the homogeneous cytotaxonomic picture so far framed from approximately 15 species of Mugilidae (cf. Klinkhardt et al. 1995; Rossi et al. 1996, 1997, 2000), with the exception (unusual for fish) of the highly differentiated karyotype of *Mugil curema* ($2n=28$ chromosomes, 20 of which are metacentrics; LeGrande and Fitzsimons 1976).

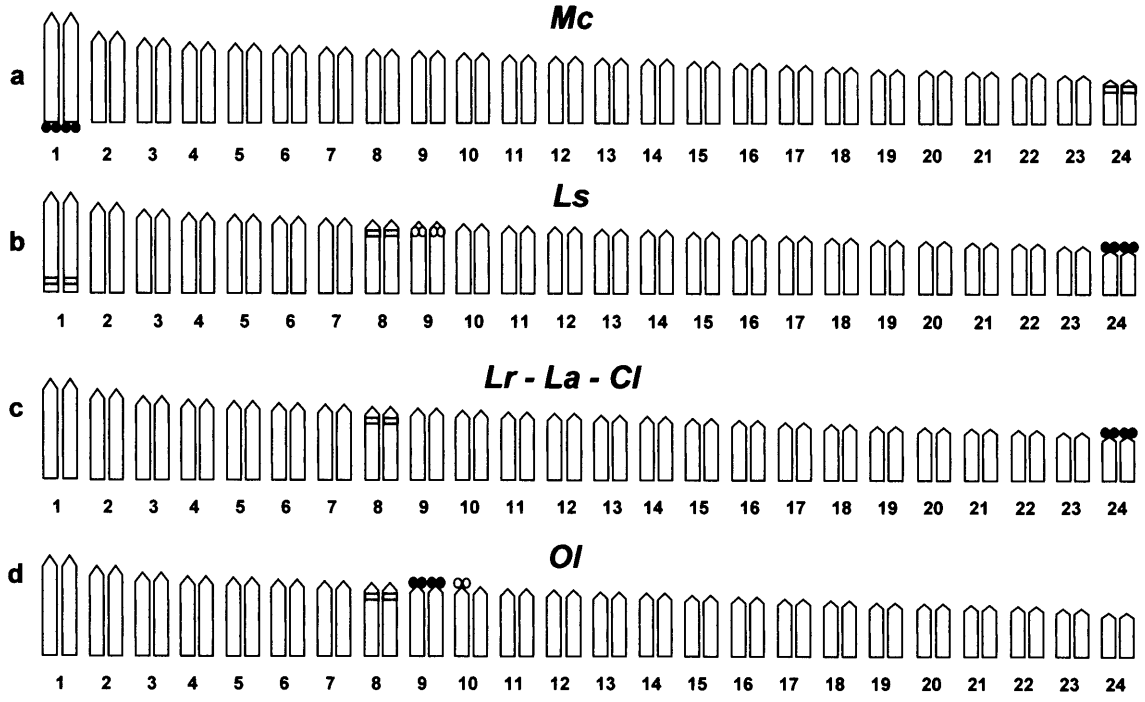

Fig. 4a–d Idiograms of: **a** *Mugil cephalus* (*Mc*), **b** *Liza saliens* (*Ls*), **c** *L. ramada* (*Lr*), *L. aurata* (*La*), *Chelon labrosus* (*Cl*) and **d** *Oedalechilus labeo* (*Ol*). *Solid circles* represent constant and *open circles* represent variable locations of 18S rDNA. *Grey bars* represent locations of 5S rDNA

In the leaping mullet, the distribution of constitutive heterochromatin is similar to that observed in other mullets (Jordão et al. 1992; Rossi et al. 1996, 1997, 2000). Fluorochrome staining indicates a homogeneous base composition of both eu- and heterochromatin, as reported in *M. cephalus* (Rossi et al. 1996) and *Oedalechilus labeo* (Rossi et al. 2000) while in *L. ramada* a GC-richness of centromeric heterochromatin was observed (Rossi et al. 1997). This result therefore does not support the previous hypothesis (Rossi et al. 1997) which stated that the accumulation of GC-rich DNA sequences observed in *L. ramada* might have played a role in the lineage split of the genus *Liza*. In fact, as discussed below, this might reflect the different systematic position of the two species in the genus, *L. saliens* being the type of the subgenus *Protomugil* Popov, 1930 and *L. ramada* belonging to the subgenus *Liza* Jordan and Swain, 1884 (Thomson 1997).

18S and 5S rDNA

To compare all data, presently or previously obtained (Rossi et al. 1996, 1997, 2000), idiograms were drawn (Fig. 4) reporting the chromosomal locations of 18S and 5S rDNA sites for each Mediterranean mullet species. A parsimonious criterion was pursued to minimize the arbitrariness in classification. Chromosomes of similar size sharing a similar location of either rDNA sequence were therefore considered to be homeologous.

Among the genera of Mugilidae presently investigated, *Mugil* and *Liza* are respectively considered as a more primitive and a more derived form from a common ancestor (Thomson 1997). The two other genera, *Chelon* and *Oedalechilus,* both containing just two species, display many features in common with *Liza* and are considered to be specialized offshoots of the latter (Trewavas and Ingham 1972; Thomson 1997). The high affinity among these four genera was also demonstrated by molecular data (Caldara et al. 1996). The present cytogenetic data generally fit into the evolutionary relationships proposed for the investigated genera and add new information on the intrageneric relationships among the *Liza* species.

In agreement with the phylogenetic position of the genus, the all-acrocentric 48 chromosome complement of *M. cephalus* is the one closest to the 48 uniarmed karyotype considered ancestral for all teleosts (Ohno 1974) and shows NORs located on the telomeric region of chromosome pair 1 (Fig. 4a), as observed also in *Mugil platanus* (Jordão et al. 1992). The other mullet species investigated here show a karyotype which differs from that of *M. cephalus* for the presence of short arms on a single chromosome pair (either no. 9 or no. 24). Present and previous data (Rossi et al. 1996, 1997, 2000) demonstrate that whichever the bibrachial chromosome, these short arms bear NORs (Fig. 4b–d). Considering that the size of chromosomes no. 1 in *M. cephalus* is larger than in other mullet species (Rossi et al. 1997) the derived karyotypes might therefore have originated from an ancestral karyotype similar to the one found in *M. cephalus* through a translocation of NORs from the terminal region of chromosomes no. 1 to the short arms of chromosomes no. 24 (*L. saliens*, *L. ramada*, *L. aurata*, *C. labrosus*) or chromosomes no. 9 (*O. labeo*). These

data therefore support a previous hypothesis, proposed for serranids (Sola et al. 1993), which stated that the shift from a 48 acrocentric karyotype to a karyotype composed of 46 acro- plus 2 subtelocentrics (or 2 submetacentrics – depending on the authors' classification) is related to the "appearance" of NORs over the short arms of the latter "new" chromosomes bearing them. No data on NOR-patterns are available for the further five species of *Mugil* and two species of *Liza* investigated so far (cf. Klinkhardt et al. 1995; Rossi et al. 1996, 1997, 2000).

Also with respect to the 5S rDNA location, *M. cephalus* (Fig. 4a) has distinctive cytogenetic features. In fact, 5S rRNA genes cluster in a subcentromeric position of chromosome pair 24, the same pair which bears NORs, though on its short arms, in the *Liza* (Fig. 4b,c) and *Chelon* (Fig. 4c) species. All the other mullet species (Fig. 4b–d) share a 5S rDNA location in a subcentromeric position of chromosome pair 8. The additional 5S rDNA location on chromosome pair 1 clearly distinguishes *L. saliens* (subgenus *Protomugil*) (Fig. 4b) from the other species of *Liza* (subgenus *Liza*) investigated here (Fig. 4c), as well as from *C. labrosus* (Fig. 4c) and *O. labeo* (Fig. 4d) and may indicate a higher affinity of *L. saliens* to *M. cephalus*. Indeed, *L. saliens* (Fig. 4b) shows alternative and reverse locations of major and minor rRNA genes on chromosome pairs 1 and 24 compared to *M. cephalus* (Fig. 4a); thus, an event of reciprocal translocation for these two sets of genes with respect to a common ancestral karyotype might be hypothesized. On the other hand, *L. saliens* (Fig. 4b) shares the main 18S rDNA location with the other *Liza* species (Fig. 4c) and with *C. labrosus* (Fig. 4c), and the additional NOR sites observed on a medium-sized pair of chromosomes might reveal its homeology to the NOR-bearing chromosome pair 9 of *O. labeo* (Fig. 4d). All these data suggest that *L. saliens* (subgenus *Protomugil*) has cytogenetic features which are intermediate between those of *M. cephalus* and those of the other *Liza* (subgenus *Liza*) species and of the representatives of the genera *Chelon* and *Oedalechilus*. In this context, the similar patterns of distribution of NORs and 5S rDNA sites in *L. ramada*, *L. aurata* and *C. labrosus* (Fig. 4c) underline a higher affinity of *Chelon* to the subgenus *Liza*.

In conclusion, present cytogenetic data suggest that *L. saliens*, and probably the whole subgenus *Protomugil*, should be considered as the most primitive in the *Liza–Chelon–Oedalechilus* series and that, owing to its NOR-pattern and the degree of the CMA_3-negative reaction of pericentromeric heterochromatin (Rossi et al. 2000), *Oedalechilus* might be a derived branch of *Protomugil*. The composition of the centromeric heterochromatin of other *Liza* species has to be investigated to verify whether this sub-branching exists between the two subgenera, *Protomugil* and *Liza*, within the genus.

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