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Brief report
Cytogenetic studies in *Sparus auratus* (Pisces, Perciformes): molecular organization of 5S rDNA and chromosomal mapping of 5S and 45S ribosomal genes and of telomeric repeats

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The gilthead seabream, *Sparus auratus*, lives in the coastal and brackish waters of the north-eastern Atlantic Ocean and the Mediterranean, where it is economically important both for fisheries and aquaculture. Through both classical (CATAUDELLA et al. 1980; VITTURI et al. 1992) and molecular techniques (GARRIDO-RAMOS et al. 1994, 1995, 1998; CUÑADO et al. 2000), several cytogenetic features of the species have been described, such as the karyotype and the chromosomal distribution of constitutive heterochromatin, of functional and structural nucleolar organizing regions (NORs), as well as some repetitive DNA sequences. By fluorescence in situ hybridization (FISH), GARRIDO-RAMOS et al. (1995) detected a polymorphism of NORs, due to a complete NOR deletion in one homologue of the only NOR-bearing chromosome pair, in a high number of individuals from a Spanish hatchery. The high frequency of this deletion was interpreted to be due to founder effects and/or to inbreeding in the hatchery, and raised interest in continuing surveys of stocks, together with natural populations, for such or other cytogenetic defects. The *Eco*RI-defined satellite DNA, isolated in the species (GARRIDO-RAMOS et al. 1994), has been found evenly distributed on the centromeres of all mitotic (GARRIDO-RAMOS et al. 1994) and pachytene (CUÑADO et al. 2000) chromosomes. CUÑADO et al. (2000) also localized the telomeric sequence repeat to the ends of each synaptonemal complex (SC) in the pachytene spermatocytes. Thus, with the exception of NORs, all the above-mentioned studies did not reveal any other distinctive chromosome marker in *S. auratus*.

This study was carried out on individuals from a natural population in order to localize another cytogenetic marker, the 5S rRNA genes, in the species. To this purpose, the gilthead 5S rDNA was cloned and its molecular organization and its chromosomal location were studied. This study represents the first report on the chromosomal distribution of these repeats in the family. In addition, FISH mapping of the major ribosomal genes and of telomeric repeats was carried out in order to verify the possible occurrence of NOR polymorphism in a natural population and to verify the exclusively terminal location of telomeric repeats in metaphase chromosomes.

MATERIAL AND METHODS

Twelve specimens of *Sparus auratus* were collected in Paola Lagoon (LT), along the central Tyrrhenian coast. Chromosome preparations were obtained using conventional air-drying techniques from pooled spleen, gill and cephalic kidney cells.

Genomic DNA was extracted from the white muscle by the conventional phenol/chloroform method. 5S rDNA was PCR-amplified using a pair of primers originally designed in *Salmo trutta* by PENDAS et al. (1994). PCR-products were resolved on a 2 % agarose gel, purified with a GFX-purification kit (Amersham), cloned into pGEM-T Easy vector (Promega), and sequenced in both strands, according to Promega recommendations. Nucleic acid sequences were subjected to BLASTN (ALTSCHUL et al. 1990) searches at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/blast).

For FISH, the purified 5S rDNA PCR-products or cloned genes and a whole pUC19 plasmid, with a 12kb insert containing the *Xenopus laevis* 45S rDNA, were used as probes after biotin-labeling by random priming (BRL). Telomeric probes were commercially synthesized as two complementary heptameres $(GGGTTA)_7/(TAACCC)_7$ end-labeled with biotin (M-Medical, Genenco). Standard procedures for the hybridization of repetitive sequences (LICHTER et al. 1992) were carried out. Post-hybridization washes were carried out either at RT or at 42–43°. Signals were detected and amplified by a three-round application of Avidin DCS-FITC/biotynilated anti-Avidin (Vector).

RESULTS AND DISCUSSION

The 5S rDNA was PCR-amplified, according to PENDAS et al. (1994), to enclose partial fragments of two consecutive coding regions and a complete non-transcribed spacer (NTS) between them. After electrophoresis, PCR products yielded prominent bands of about 200–220 bp and a few bands of larger size, approximately of 450 and 650 bp. DNA fragments were purified from each band and cloned. A total of 15 positive clones were sequenced. The alignment of the sequences revealed that the differences in length were due to the amplification of either monomeric or multimeric repeated units, the larger fragments likely to have resulted from non-saturation of the homologous priming sites (PENDAS et al. 1994).

The ten clones of larger size, containing more than one repeat, allowed us to obtain the complete coding sequence (Fig. 1a), including positions +22 and +23, usually missing due to the PCR primers design (PENDAS et al. 1994). In all the clones analyzed but one (pSA-5S-12-K1), the 120 bp 5S coding sequence is highly conserved (Fig. 1a), and the three elements (Box A, Box C and IE) of the internal control region (ICR), that functions as a promoter for the gene (HALLENBERG et al. 1994), are preserved. Clone pSA-5S-12-K1 (Fig. 1a) may be interpreted as a pseudogene, as it lacks a large part of Box A and shows numerous base substitutions. Based on the alignment of these clones, a consensus sequence was produced which, after searches using the BLASTN program (ALTSCHUL et al. 1990), shows an average sequence identity of 97 % with the 5S rDNA coding region of other fish species, including representatives from phylogenetically older orders, such as Cypriniformes, Characiformes, Salmoniformes, to more recent orders, such as Gadiformes, Perciformes and Tetraodontiformes.

Two types of NTSs, 77 and 87 bases long (Fig. 1b), were found which were characterized by a single ten-base deletion/insertion (at −67 to −59) and by three base substitutions in fixed positions at the beginning of the spacers. Their size is close to the minimum considered necessary for the maintenance

a

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                +1                                                                  Box A
consensus       GCTTACGGCC ATACCACTCT GAACACGCCC GATCTCGTCC GATCTCGGAA GCTAAGCAGG
pSA-5S-5K       __________ _______GC_ ___T_____ __________ __________ __________
pSA-5S-9K       __________ __________ __________ __________ __________ __________
pSA-5S-9F       __________ __________ __________ C_________ __________ __________
pSA-5S-10F1     __________ __________ __________ __________ __________ __________
pSA-5S-10F2     __________ __________ __________ __________ __________ __________
pSA-5S-12F1     __________ _______GC_ __________ __________ __________ __________
pSA-5S-12F2     __________ __________ __________ __________ __________ __________
pSA-5S-12K2     _________G_ __________ __________ __________ __________ __________
pSA-5S-13K      __________ __________ __________ __________ __________ __________
pSA-5S-12K1     __________ _______GC_ ________T_ _________AA _C_AAGCAGG ----------

                +70         IE                      Box C                          +120
consensus       GTCGGGCCTG GTTAGTACTT GGATGGGAGA CCGCCTGGGA ATACCAGGTG CTGTAAGCTT
pSA-5S-5K       __________ ____C_____ __________ __________ __________ __________
pSA-5S-9K       __________ __________ __________ __________ _C________ __________
pSA-5S-9F       __________ __________ __________ __________ _C________ __________
pSA-5S-10F1     __________ __________ __________ __________ __________ __________
pSA-5S-10F2     __________ __________ __________ __________ __________ __________
pSA-5S-12F1     __________ __________ __________ __________ __________ __________
pSA-5S-12F2     __________ ___G______ __________ __________ _________A_ __________
pSA-5S-12K2     __________ T_A_______ __________ _____A____ __________ __________
pSA-5S-13K      ____A_____ __________ __________ __________ __________ __________
pSA-5S-12K1     __________ __________ __________ __________ __________ __________
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b

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                -87
NTS87(pSA-5S-9F)   TTTAATTTCT TCCAGTGTCG ACCAGTGTCT TCTTGGAGAC ATAACTGTTG
NTS77(pSA-5S-13K)  ______G__G A________- ---------_ __________ __________

                                                                    -1
NTS87(pSA-5S-9F)   AACGGGTTTA AATAGTGTGT TTGTCTCCTG CACCTCG
NTS77(pSA-5S-13K)  __________ __________ __________ _______
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Fig. 1. 5S rDNA in *S. auratus*: alignment of (**a**) complete coding sequences obtained from large clones and the consensus sequence deduced from them, (**b**) NTS sequences from two clones, representing each length type. Dots denote identical bases; dashes, gaps inserted for alignment purpose. The primer homology regions are in boldface type. The ICR elements (Box A, IE and Box C) are underlined. The sequences of clones pSA-5S-9F and pSA-5S-13K are deposited in GenBank under accession numbers: AY 330701, AY 330702.

of the array (MARTINS and GALETTI 2001a). The regions immediately upstream of the transcription starting-point are invariable in all the clones. Both NTSs include presumed control/functional regions, such as an upstream TATA-box-like sequence (MARTINS and GALETTI 2001a) at −31 to −25, and T-blocks at their 5′ end, which may reinforce the terminator sequence of the coding region (SAJDAK et al. 1998).

Large clones, constituted by two or three consecutive repeats, contain variants of the coding sequence and both types of NTS. The NTSs are AT-rich (57.5 %), while the coding region is GC-rich (42.5 % AT).

The molecular organization of 5S rRNA genes in *S. auratus* is consistent with what has been observed in most eukaryotic organisms, including fish. Besides differences among repeats of different species, due to variation in lengths of non-transcribed spacers (compared to the highly conservative 120 bp coding sequence), much evidence is accumulating which indicates that more than one type of NTSs is normally found within the same species. This was interpreted (PENDAS et al. 1994; SAJDAK et al. 1998; WASKO et al. 2001) as reflecting the existence of a dual-gene system producing two types of 5S rRNA in fish, the oocyte- and the somatic-type, revealed by direct analysis of 5S rRNA in some species (*Tinca tinca*, DENIS and WEGNEZ 1977; *Misgurnus fossilis*, MASHKOVA et al. 1981). This dual-gene system is likely to be associated with the existence of two physically separated 5S rDNA arrays in several neotropical fish species (MARTINS and GALETTI 2001a,b). In the present study, two types of very similar NTSs have been found in *S. auratus*. Since very little is known about the regulatory role of NTSs it is difficult to hypothesize whether the observed variants of spacers can account for differences in the gene expression among different tissues.

After FISH with either 5S rDNA PCR amplification products (Fig. 2a) or with cloned genes (Fig. 2b), hybridization signals for 5S rDNA were found in an interstitial position of the smallest telocentric chromosome pair (number 24). According to MARTINS and GALETTI (2001a), this position seems to be a general feature for fish (approximately 60 species studied so far) and might be common in vertebrates

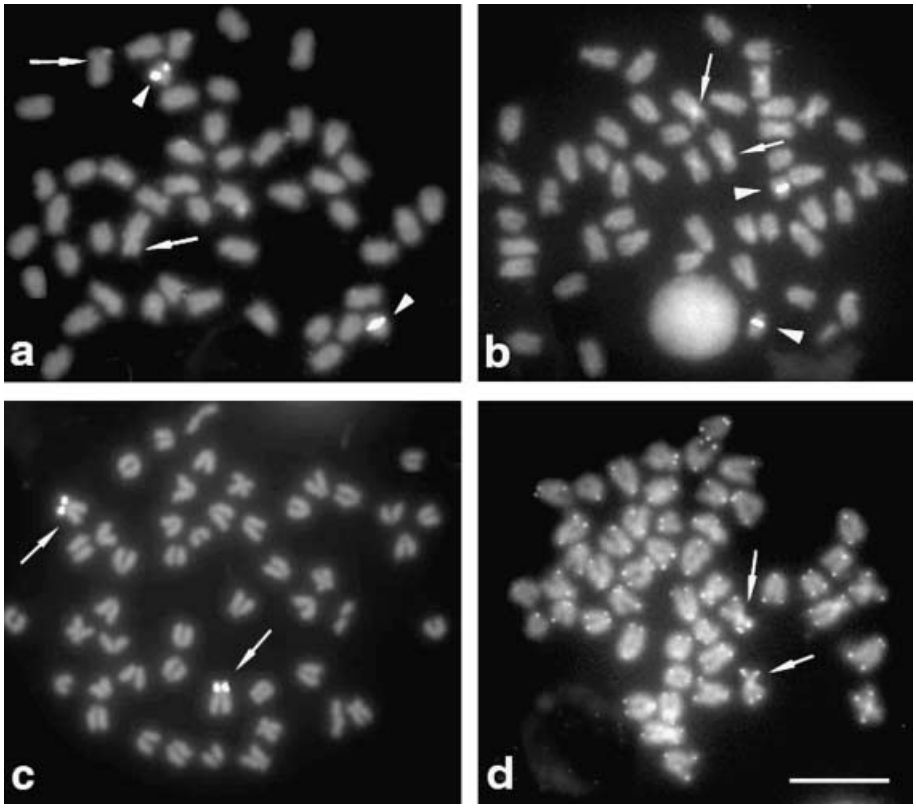

Fig. 2. Metaphases of *S. auratus* after FISH with: (**a**) PCR-amplified 5S rDNA, (**b**) 5S rDNA pSA-5S-12F clone, (**c**) 45S rDNA, and (**d**) telomeric probes. Arrows indicate NORs, arrowheads indicate 5S rDNA sites. In (**d**), hints of hybridization signals can be observed at the pericecentromeric regions of the biarmed chromosomes (low-stringency conditions). Scale bar = 1 μm.

in general. This organization may confer some advantage to the vertebrate genome. As far as the number of chromosomal sites for 5S rDNA array is concerned, including *S. auratus* only five Perciformes species, belonging to different families, have been analyzed so far, and three of them, *S. auratus* (present study), *Micropterus salmoides* (Centrarchidae, DEIANA et al. 2000) and *Epinephelus marginatus* (Serranidae, SOLA et al. 2000) show one single 5S rDNA-bearing chromosome pair, while *Oreochromis niloticus* (Cichlidae, MARTINS et al. 2000) and *Coris julis* (Labridae, MANDRIOLI et al. 2000) show two pairs. Considering that the former species, compared to the latter, show a karyotype which is closer to the 48 uni-armed-chromosome type considered ancestral for all Teleosts (OHNO 1974) and that the location of 5S rDNA on a single chromosome pair has been observed in representatives of all orders so far examined (MARTINS and GALETTI 2001a; GORNUNG et al. 2001; FONTANA et al. 2003), the number of one chromosomal site for 5S rDNA probably corresponds to the ancestral condition for this set of genes for fish.

The number and position of NORs were also verified by FISH with a 45S rDNA probe (Fig. 2c). Fluorescent signals were observed on one pair of chromosomes, specifically on the short arms of the largest biarmed chromosomes, the same location previously described in the species (VITTURI et al. 1992; GARRIDO-RAMOS et al. 1995). No unusual features of NORs were detected.

Thus, in *S. auratus*, the minor and major rDNA clusters are located on different chromosome pairs, which is so far the more common condition found in bony fish, including those species which have more than one chromosomal site for 5S rDNA, though evidence of co-localization of 5S and 18S rDNA has been reported for some species of Salmoniformes (PENDAS et al. 1994; FUJIWARA et al. 1998), Cypriniformes (INAFUKU et al. 2000) and Characiformes (ALMEIDA-TOLEDO et al. 2002).

Finally, the telomeric probe hybridized uniformly at the ends of all chromosomes-which is consistent with the observation of CUÑADO et al. (2000) on pachytene chromosomes. However, under low-stringency conditions, hints of hybridization signals can be observed at the pericentromeric regions of the biarmed chromosomes (Fig. 2d). These latter signals might be due to degenerated telomere-like sequences either representing remnants of true telomeres or of heterochromatic derivation. Indeed, telomere-like sequences were shown to be a component of satellite DNAs in several vertebrate species (PAGNOZZI et al. 2000), including bony fish (GARRIDO-RAMOS et al. 1998).

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