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Localization of ribosomal genes in Pleuronectiformes using Ag-, CMA_3-banding and *in situ* hybridization

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In this paper we present the analysis of nucleolar organizer regions (NORs) in five species of the order Pleuronectiformes (*Scophthalmus maximus*, *Scophthalmus rhombus*, *Platichthys flesus*, *Solea solea* and *Solea lascaris*), a group of fish poorly studied cytogenetically. In spite of the small chromosome sizes, which characterize the karyotypes of this group, we implement both classical (Ag- and CMA_3-staining) and molecular (*in situ* hybridization with a major rDNA probe) techniques for NOR location. NORs were localized in a single chromosome pair in all species studied and showed size variation within each species. Evidence of a NOR-site polymorphism was detected in *Platichthys flesus* and *Scophthalmus maximus*. NOR location was apparently the same within families (*S. rhombus* vs. *S maximus*, and *S. solea* vs. *S. lascaris*) but clearly differed among families, and supports the phylogenetic relationships proposed for the order Pleuronectiformes.

Keywords: *in situ* hybridization, NOR location, NOR polymorphism, phylogeny, Pleuronectiformes.

Introduction

Flatfishes constitute a group of fish not well known cytogenetically. Hinegardner (1968) demonstrated that some fish, in particular the Tetradontidae, have very small genomes. The cellular DNA contents of flatfish are among the lowest found in fish (being 20% of that of Mammals). In fact, they have very small chromosomes, and their chromosome number lies around the modal value described within Teleosts, $2n = 48$ acrocentric chromosomes, this karyotype being considered as the primitive one in this group (Ohno, 1970). The phylogeny of the order Pleuronectiformes is rather controversial and some authors (Vernau *et al*., 1994), comparing molecular and morphological data, have suggested that the family Soleidae could be the ancestral one within the order. In contrast, Le Grande (1975) proposed the Pleuronectidae as the ancestral family, using cytogenetic data. However, there still remain many unsolved questions in connection with chromosomal evolution in Pleuronectiformes (Sakamoto & Nishikawa, 1980; Patro & Prasad, 1981; Kikuno *et al*., 1986; Vitturi *et al*., 1993).

Out of the more than 500 species of the order Pleuronectiformes, only 37 (belonging to the families Pleuronectidae, Soleidae, Scophthalmidae, Bothidae and Cynoglossidae) have been subjected to karyotype analysis. In the majority of these studies only Giemsa staining and four types of banding analysis (C-, Q-, RE-banding and Ag-staining) have been reported (Kikuno *et al*., 1986; Fan & Fox, 1991; Vitturi *et al*., 1993; Bouza *et al*., 1994).

Nucleolar organizer regions are usually detected in fish by silver nitrate and GC-specific fluorochrome staining (Galetti *et al*., 1984). Silver treatment stains only active NORs (Hubbell, 1985), and therefore is more appropriate for the study of NOR activity than for NOR location. Molecular techniques offer new opportunities for fish cytogenetics. *In situ* hybridization (ISH) has proved to be useful for the localization of repeated gene families. *In situ* hybridization with rDNA probes directly detects the location of ribosomal RNA genes, regardless of their activation status.

In the present paper we describe the location of major ribosomal genes in several species of the order Pleuronectiformes, a group of fish of high interest for fish farming. Five species belonging to three different families were analysed using conventional cytogenetic techniques (Ag- and CMA_3-staining) and *in situ* hybridization with an 18S rDNA probe: Scophthalmidae: turbot (*Scophthalmus maximus*) and brill (*Scophthalmus*

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rhombus); Pleuronectidae: flounder (*Platichthys flesus*); and Soleidae: sole (*Solea solea*) and sand sole (*Solea lascaris*). Karyotype analysis is useful for studying phylogenetic relationships among related species, and also as a tool for genome mapping, a primary goal for genetic improvement in fish culture.

Materials and methods

Samples and cell cultures

Most individuals analysed were collected from natural populations, 10 specimens of each of the five species studied (*Scophthalmus maximus*, *Scophthalmus rhombus*, *Platichthys flesus*, *Solea solea*, and *Solea lascaris*) being taken from this source. In addition, 12 specimens of *S. maximus* from a fish farm were analysed.

Chromosome spreads were obtained from spontaneously dividing cells of kidney and spleen, as previously described by Bouza *et al*. (1994).

Ag-staining

Ag-staining was performed according to Howell & Black (1980). Around 20 metaphases were checked in each individual for analysing the Ag-NOR pattern.

CMA_3-staining

Slides were treated with 0.5 mg/mL CMA_3 for 2 h and counterstained with 0.1 mg/mL distamycin A (DA) for 15 min, as described by Schweizer (1979). Ten metaphases were checked in each individual. Photographs were made using Kodak plus X Pan 125 ASA film in an Olympus Vanox microscope with the standard blue excitation filter.

rDNA in situ *hybridization (ISH)*

The pB plasmid, which includes a long fragment of the human rDNA transcription unit (the promoter and most of the 18S gene; Wilson *et al*., 1978) was used as a probe to detect rDNA clusters in the individuals analysed. The probe was labelled by nick-translation with biotin 16-dUTP (Boehringer). Prior to hybridization, RNAse-pretreated slides were denatured for 2 min at 70°C with 70% formamide in 2 × SSC and dehydrated through a graded ethanol series at −20°C, and then air dried. After 10 min denaturation at 100°C, 30 μL of the hybridization mixture containing 50% formamide in 2 × SSC, 10% dextran sulphate, 100 ng/mL of herring sperm DNA and 60 ng of biotinylated probe were applied to each slide under a coverslip. Hybridization was performed overnight at 37°C in a humid chamber. Post-hybridization washes were carried out in 2 × SSC for 5 min, three times in 50% formamide with 0.05% of Tween 20 for 5 min, twice in 2 × SSC for 5 min (all these washes at 42°C) and twice in TNT (0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20) for 3 min at room temperature. Hybridization probes were detected according to the method of Kerstens *et al*. (1995), using the TSA-Indirect kit (NEN Life Science Products). Five to 10 metaphases were checked in each individual.

Results and discussion

Scophthalmus rhombus showed a diploid number of 44 chromosomes and 48 chromosome arms (NF), comprising two metacentric, one submetacentric and 19 acrocentric chromosome pairs (Fig. 1). Ag-staining, which reveals active nucleolar organizer regions (NORs) in the previous interphase (Howell & Black, 1980; Hubbell, 1985), localized active NORs in the short arms of a large submetacentric pair (Fig. 2d). CMA_3-staining, which reveals the presence of GC-rich heterochromatin, is usually associated with NOR regions in fish and Amphibia (Schmid, 1982; Phillips & Ihssen, 1985; Mayr *et al*., 1986). In *S. rhombus*, NOR regions were CMA_3 positive (Fig. 2e), and no other region showed positive staining with this technique. rDNA-ISH confirmed the presence of ribosomal genes in the short arms of the same Ag- and CMA_3 positive submetacentric pair (Fig. 2f). This karyotypic constitution appears very similar to that reported previously in *S. maximus* (Bouza *et al*., 1994), the location of rDNA cistrons in pair number 3 being confirmed after applying Ag- and CMA_3-staining, and rDNA-ISH (Fig. 2a–c).

A rather incomplete description of the karyotypes of *Solea solea* ($2n = 42$; Barker, 1972), and *S. lascaris* ($2n = 42$, NF = 50; Vasil'ev, 1978) has been previously reported only with Giemsa staining. The karyotypes of these species, as revealed in our work, showed $2n = 42$ and NF = 56–58, comprising 4 metacentric, 4–5 submetacentric and 12–13 acrocentric chromosome pairs. The variation in the number of chromosome arms and, accordingly, in the number of submetacentric and acrocentric chromosomes, was due to the length polymorphism of the NOR-bearing chromosome pair observed in both species. This pair ranged from acrocentric to submetacentric, and resulted in NFs of 56 (both acrocentric), 57 (acrocentric/submetacentric) or 58 (both submetacentric) chromosome arms, in different individuals. Our observations for *S. lascaris* clearly disagreed with the karyotype described for this species by Vasil'ev (1978), who only reported four biarmed chromosome pairs (1 M + 3 SM). This incompatibility could in part be due to the polymorphism observed in the NOR-bearing pair, but also to misclassification of

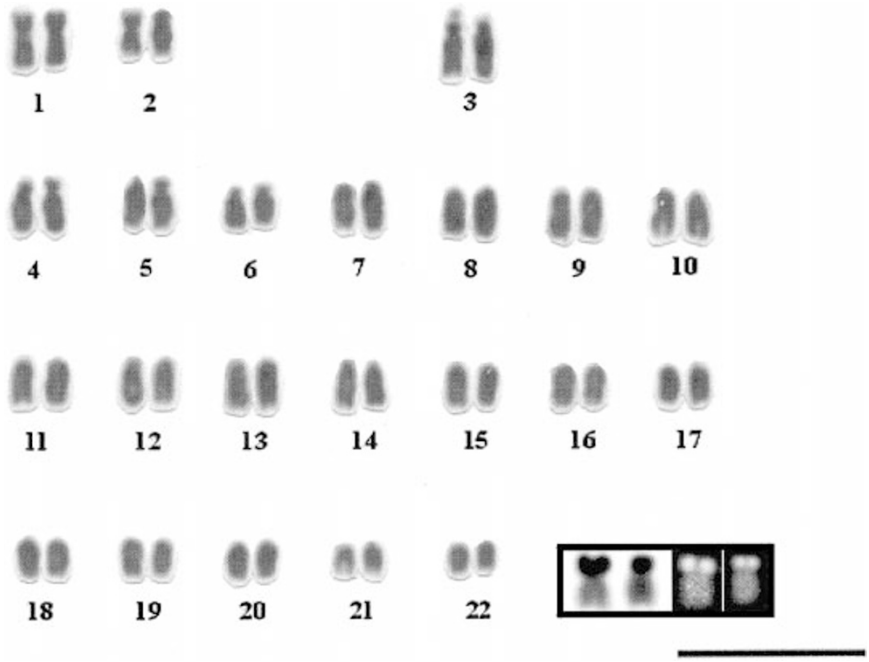

Fig. 1 Standard karyotype of *Scophthalmus rhombus* (brill) obtained with Giemsa staining. The NOR-bearing chromosomes are shown in the insets after Ag- and CMA_3-staining. Bars = 1 μm.

several biarmed chromosomes due to low technical resolution. After Ag- (Fig 2j, m), and CMA_3-staining (Fig 2k, n), and rDNA-ISH (Figs 2l, o), NOR regions appeared localized in the short arm of a medium submetacentric pair in both species. The karyotypes of these two species are very similar, with NORs apparently located in the same chromosome pair.

Platichthys flesus exhibited a karyotype of $2n = 48$, close to the ancestral one of Teleosts (Klinkhardt *et al*., 1995), with 24 pairs of acrocentric chromosomes and $NF = 48$, as found in most members of the family Pleuronectidae analysed (Fukuoka & Niiyama, 1970; Barker, 1972). NORs appeared localized in the short arms of a medium acrocentric pair after Ag- and CMA_3-staining, and rDNA-ISH (Fig. 2 g–i).

The most frequent localization of NORs in fish is telomeric (Gold, 1984). In Pleuronectiformes, Ag-staining has demonstrated a subcentromeric position in one chromosome pair in *Paralichthys olivaceus* (Kikuno *et al*., 1986) and a centromeric one in a large metacentric pair in *Bothus podas* (Vitturi *et al*., 1993). In the species of this order analysed in the present study, NORs showed locations more typical within fish (Hartley, 1987; Amemiya & Gold, 1988), comprising the whole short arm of a submetacentric pair in the genera *Scophthalmus* and *Solea*, and of an acrocentric pair in *P. flesus*.

Several authors have described NOR regions as highly polymorphic in fish. This variability has been in size and expression, as well as the number and position of NORs (Foresti *et al*., 1981; Phillips *et al*., 1988; Castro *et al*., 1996). In Pleuronectiformes, Vitturi *et al*. (1993) described the existence of NOR size variation in *B. podas*. By contrast, in *P. olivaceus* (Kikuno *et al*., 1986) no variation with regard to NOR size was detected. In the present study, a NOR size heteromorphism was detected with Ag-staining in the majority of the individuals of the five species analysed (Fig. 2). This variation was confirmed with CMA_3-staining and rDNA-ISH by using a human 18S rRNA probe (Fig. 2). Therefore, size differences observed between NOR-bearing chromosomes are mainly attributable to variation in the number of rRNA genes. Also, additional NORs were detected in *S. maximus* (one of 22 specimens analysed, data not shown) and *P. flesus* (one of 12 specimens; Fig. 2 g–i). This site polymorphism was confirmed with CMA_3-staining and rDNA-ISH (Fig 2 h, i). NOR-site polymorphisms have been widely described in fish (Pendás *et al*., 1993; Castro *et al*., 1996; Gornung *et al*., 1997). In most cases the new positions were confirmed to consist of rRNA genes after applying rDNA-ISH, as in our study.

In spite of the low incidence of the NOR-site variation observed in our work, its importance should not be underestimated, especially from an applied perspective to fish culture. The constant number of Ag-stained nucleoli in diploid organisms in the absence of such NOR polymorphisms makes possible a straightforward evaluation of triploidization success on the basis of Ag-staining (Phillips *et al*., 1986). The existence of a low frequency of polymorphism in the present study suggests a cautious application of this technique for monitoring

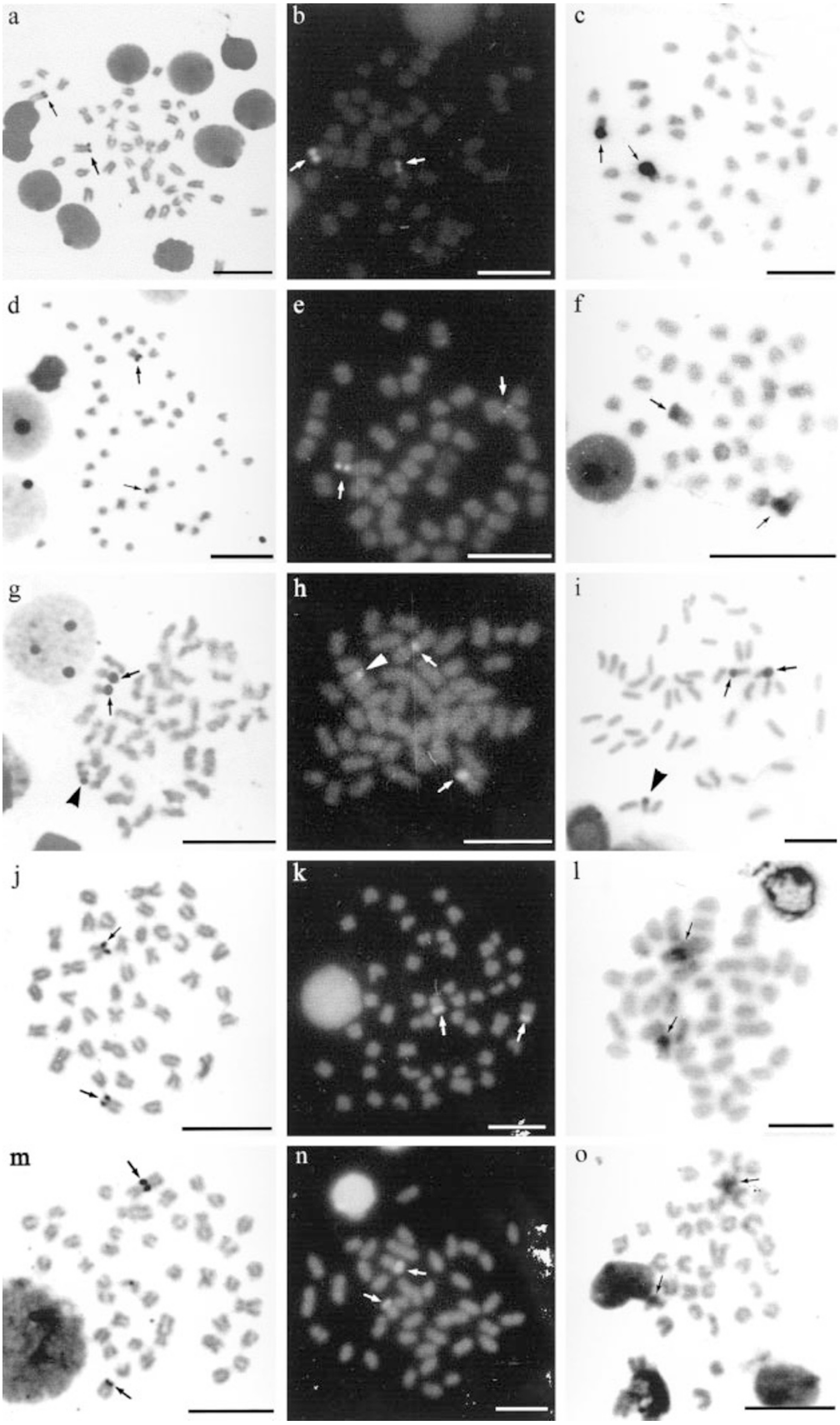
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Fig. 2 Metaphase spreads of *Scophthalmus maximus* (**a, b, c**), *Scophthalmus rhombus* (**d, e, f**), the NOR-site polymorphic individual of *Platichthys flesus* (**g, h, i**), *Solea solea* (**j**, **k**, **l**) and *Solea lascaris* (**m**, **n**, **o**) after Ag- (**a, d, g, j, m**), and CMA_3- (**b, e, h, k, n**) staining, and rDNA-ISH (**c, f**, **i, l, o**). Arrows indicate standard NOR regions in the five species. Arrowheads indicate the extra NOR in the polymorphic specimen of *P. flesus*. Bars = 1 μm.

triploidization studies, as shown in turbot (Piferrer *et al*., 2000).

Different patterns of NOR location have been observed in fish. Some taxa exhibit their ribosomic cistrons located in a single, either constant or variable, chromosome pair (Galetti *et al*., 1984), while others show a multichromosomal NOR distribution (Foresti *et al*., 1981; Phillips *et al*., 1988; Castro *et al*., 1996). Sometimes the location of NORs is highly conservative within taxa, with species of the same group showing a constant position. For example, in the genus *Brycon*, all the species investigated showed a common NOR-bearing chromosome pair (Margarido & Galetti, 1996). Other groups of fish presented different NOR locations among species, even when closely related. Galetti *et al*. (1984) described differences in NOR location in two morphologically cryptic species of the genus *Leporinus*. The situation in Pleuronectiformes is rather conservative with respect to NOR location. The five species analysed belonging to three families showed their ribosomal genes located in a single chromosome pair, apparently the same within each family, but with evident differences among families. More species from different families of this order should be analysed to get a more accurate picture of NOR location and to obtain the pattern of NOR evolution within flatfish.

Karyotypic analysis and, specifically, the number and location of NORs have been used as cytotaxonomic tools. One NOR-bearing pair is considered the plesiomorphic condition in fish and many other organisms (Hartley, 1987). The comparative location of NORs in the species analysed revealed close relationship within families and divergence among families, supporting their taxonomic status within the Pleuronectiformes. Le Grande (1975) using cytogenetic data suggested the family Pleuronectidae as the ancestral one within this order. The high karyotypic similarity observed in this study between *S. rhombus* and *S. maximus* ($2n = 44$; NF = 48; NOR location) gives evidence of more evolved karyotypes than the ancestral one of Teleosts ($2n = 48$), observed in *P. flesus*, which supports the hypothesis of Le Grande (1975). These two scophthalmid karyotypes could be explained by centric fusions (Bouza *et al*., 1994), also invoked to explain the centromeric location of NOR in the flatfish *B. podas* (Vitturi *et al*., 1993). On the other hand, the chromosomal constitution observed in the two soleid species analysed ($2n = 42$; NF = 56–58) cannot be explained in terms of Robertsonian changes, but require some other rearrangements, like pericentric inversions, also proposed by Le Grande (1975). Some authors have pointed out the high degree of karyotypic diversification (variation $2n$/NF) within Pleuronectiformes (Sakamoto & Nishikawa, 1980; Patro & Prasad, 1981), probably indicating a more complex karyotype evolution, which requires more detailed karyotypic studies in the future.

A controversy has arisen with regard to the taxonomic status of both species of the family Scophthalmidae, with some authors suggesting the inclusion of *S. maximus* in the genus *Psetta* (Nielsen, 1986). This study shows high karyotypic similarity between them, which represents additional support to isozyme data (Blanquer *et al*., 1992; Bouza *et al*., 1997) and the effective hybridization between both species (Purdom, 1976), in line with the proposal by Wheeler (1992) to maintain both species within the same genus.

Flatfish cytogenetics has received little attention to date, because of the intrinsic difficulties in analysing karyotypes with a large number of small chromosomes. Some members of this group, like turbot and sole, are important species for aquaculture. The localization of repeated gene families, such as ribosomal genes, should contribute to advances in physical mapping in this group of fish.

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