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ORIGINAL PAPER

Cytogenetic analysis of three species of the genus *Haemulon* (Teleostei: Haemulinae) from Margarita Island, Venezuela

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Abstract This paper describes the karyotype analysis of *Haemulon aurolineatum*, *Haemulon bonariensis* and *Haemulon plumierii*, by Giemsa staining, C-banding, Ag-staining and fluorescent in situ hybridization (FISH), to locate the 18S and 5S rRNA genes. Diploid modal count in the three species was $2n = 48$ acrocentric elements. Except for pair 24, which exhibited an unmistakable secondary constriction in all three species, it was not possible to classify them as homologous to each other because differences in chromosome size were too slight between adjacent pairs within a size-graded series. Ag-NOR clusters were located in pair 24 in the three species with signal located on the secondary constriction of these chromosomes. C-banding demonstrated that the three species share the same distribution pattern of the constitutive heterochromatin with centromeric heterochromatic blocks in the 23 chromosome pairs and a pericentromeric block in pair 24 which is coincident with the NORs. FISH experiments showed that 18S rDNA sequences were located coincident with the Ag-NOR site in the three species; however, differences in both the number and chromosome distribution of 5S-rDNA cluster were detected among them. Our data suggest that chromosome evolution of *Haemulon* has been preserved from major changes in the karyotypic macrostructure, whereas microstructural changes have occurred.

Keywords C-banding · FISH · NOR · 5S rDNA · 18S rDNA · Perciformes · Haemulidae

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Introduction

The family Haemulidae (grunts) comprises coral-reef fishes found in abundance within the greater Caribbean area (Randall 1996; Lindeman 2002). In tropical regions, grunts are commercially (Appeldoorn and Lindeman 1985) and ecologically (Meyer and Schultz 1985) important. Haemulidae contains 17 genera and approximately 150 species (Nelson 1994) grouped into two different sub-families: Haemulinae and Plectorhynchinae, the latter not represented in the Americas (Cervigón 1993).

The genus *Haemulon* belongs to Haemulinae and comprises fishes of small size and lengthened body, laterally compressed, which in most cases exhibit fringes or lines of vivid colors in contrast with the general color of the bottom. Fourteen species have been recognized in Venezuela (Cervigón 1993). Despite their importance to coral-reef ecology, little is known about their life history and genetics.

Cytogenetic data on Haemulidae are scarce and less than 10% of the 150 recognized species have been karyotyped (Lima and Molina 2004). This paper describes the karyotype of *Haemulon aurolineatum*, *Haemulon bonariense*, and *Haemulon plumierii* by means of Giemsa staining, C-banding, silver staining

and Fluorescent in situ hybridization (FISH) to locate the ribosomal genes (18S rRNA and 5S rRNA).

Materials and methods

A sample consisting of 12 specimens (8 females and 4 males) of *H. aurolineatum*, 18 specimens (7 females and 11 males) of *H. bonariense* and six specimens (4 females and 2 males) of *H. plumierii* were analyzed. All specimens were collected around Margarita Island, Venezuela, and voucher specimens were deposited in the fish collection of the Escuela de Ciencias Aplicadas del Mar, Universidad de Oriente.

Chromosome preparation followed the technique described by Foresti et al. (1993). For the conventional karyotype, the preparations were stained for 20 min with 5% Giemsa in phosphate buffer pH 6.8. Detection of the Nucleolus Organizer Regions (NORs) was done following the silver ($AgNO_3$) staining method of Howell and Black (1980). C-bands were obtained according to the method described by Sumner (1972).

The 5S and 18S rDNA sites were identified using FISH, according to the method described by Pinkel et al. (1986). A segment of 1,800 base pairs of the 18S-rRNA gene of *Oreochromis niloticus* (Nile tilapia) cloned in pGEM-T plasmid was used as a probe to locate the sites for the 45S rDNA. PCR products containing 5S rDNA repeats from *H. aurolineatum* were used as probes for the chromosome mapping of 5S rDNA. The DNA was extracted from muscle (Sambrook and Russel 2001), and the 5S rDNA repeats generated by Polymerase Chain Reaction (PCR) with the primers 5SA (5′TAC GCC CGA TCT CGT CCG ATC3′) and 5SB (5′CAG GCT GGT ATG GCC GTA AGC3′), according to Martins and Galetti (1999).

The 18S rDNA and 5S rDNA sequences were labeled by nick translation with biotin-14-dATP, following the manufacturer's (Bionick^{TM} Labelling System-Gibco.BRL) instructions. The 18S rDNA and 5S rDNA probes were located in the chromosomes by Avidin-*N*-fluorescein Isothiocyanate (FITC) conjugate and the signal was enhanced by using biotinylated goat Antiavidin antibodies following a second round of Avidin-FITC detection. Chromosomes were counterstained with propidium iodide diluted in Antifade Vector (50 μg/ml).

Giemsa, Ag-stained and C-banded mitotic chromosomes were photographed using a digital camera and the images were digitally processed with Adobe Photoshop v. 7.0 software. The karyogram was constructed with chromosomes organized in order of decreasing size and the chromosomes classified according to Levan et al. (1964). Metaphases analyzed through FISH were examined with a Zeiss Axiophot photomicroscope and Kodak Gold Ultra 400 ASA film was used to take the pictures.

Results and discussion

The chromosome diploid modal count in the three species studied was $2n = 48$ acrocentric elements, obtained from 70.30%, 74.07%, and 76.10% of all the cells examined in *H. aurolineatum*, *H. bonariense* and *H. plumierii*, respectively. Although low, the frequency of cells with hypomodal and hypermodal chromosome numbers in the three species probably resulted from chromosome loss, overlap, miscounting, and additional chromosomes from another spread.

The representative karyotype for each species obtained by arranging the chromosomes in order of decreasing size is shown in Fig. 1. With the exception of chromosome pair 24, which presented a clear secondary constriction in all three species, it was not possible to accurately classify chromosomes as homologous pairs, since differences in chromosome size were too slight between adjacent pairs within a size-graded series. No differences were observed between male and female karyotypes. Species of Haemulidae karyotyped so far are showed in Table 1. All these species share $2n = 48$ acrocentric chromosomes with the exception of *H. sciurus* (Reagan et al. 1968), which has been reported as possessing a bimodal karyotype suggesting the possibility of a chromosome polymorphism with two cytotypes in the population ($2n = 46$ and $2n = 48$).

Although several authors (Ohno 1974; Garcia et al. 1987; Vitturi et al. 1991) suggest that the 48 uniarmed-chromosome type represents the ancestral complement in fish of diploid origin, Brum and Galetti (1997) theorized that this characteristic should not be assumed as a primitive feature, since most of the basal vertebrates like Agnatha, Condrichthyes, and Sarcopteygii possess higher diploid ($2n$) and fundamental numbers (FN) like Chondrostei, Gynglimodi, Halecomorpha, Osteoglossomorpha and Elopomorpha. Based on this observation, the basic chromosome number in Teleostei would be approximately $2n = 60$, with few metacentric chromosomes. Thus, the diploid number $2n = 48$ could have arisen, at the macrostructural level, through fusions and deletions from the ancestral karyotype and extended as a synapomorphic character in the Clupeomorpha and Euteleostei, which could have conserved this karyotype mainly in the marine species belonging to the Atherinomorpha and Percomorpha (Brum and Galetti 1997).

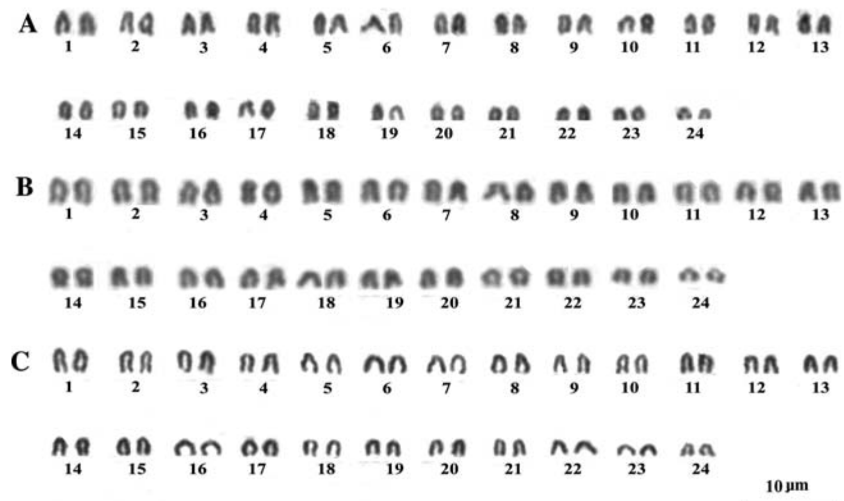

Fig. 1 Karyotypes of *Haemulon aurolineatum* (**A**), *H. bonariense* (**B**), and *H. plumierii* (**C**) after Giemsa staining

C-banding (Fig. 2A–C) revealed that the three species share the same distribution of constitutive heterochromatin with centromeric heterochromatic blocks in the 24 chromosome pairs with the exception of *H aurolineatum*, which presented a conspicuous heterochromatic block coincident with the NOR sites on the secondary constriction of the chromosome pair 24 (Fig. 2A). This distribution of heterochromatin restricted to centromere is also shown by *H. flavolineatum* from Margarita Island, Venezuela (Ron and Nirchio 2005). On the other hand, pericentromeric and telomeric heterochromatic blocks have been reported in samples of *H. parra*, *H. striatum* and *H. plumierii* from Brazil (Lima and Molina 2004), suggesting that constitutive heterochromatin distribution could be applied as cytogenetic markers for the discrimination of species or populations in the Haemulidae family.

Silver staining of the selected metaphase spreads revealed that the NOR clusters are located in pair 24 in all three species studied. Ag-NOR signals were located interstitially on the secondary constriction of these chromosomes (Fig. 2D–F). In the genus *Anisotremus* the NORs are located on chromosome pair 18 (Accioly and Molina 2004). Lima and Molina (2004) were unable to accurately identify the NOR-bearing chromosomes in *H. parra*, *H. plumierii* and *H. striatum* from the western Atlantic because of the minute variation in size among chromosomes. In *H. flavolineatum* from Venezuela the NORs are located on chromosome pair 24 (Ron and Nirchio 2005).

FISH with rDNA-probing is a useful technique to characterize some species. It allows researchers to know whether differences in Ag-NOR number are related only to the differential activities of these re-

Table 1 Diploid number (2*n*), chromosome arm number (FN), Karyotype formula, number and position of Nucleolus Organizer Regions (NORs) and constitutive heterochromatin distribution (C-Bands) in different Haemulidae species

Species	2*n*	FN	Karyotype formula	NORs	C-bands	References
Anisotremus moricandi	48	48	48a	1 pair (I)	–	Accioly and Molina (2004)
Anisotremus surinamensis	48	48	48a	1 pair (I)	–	Accioly and Molina (2004)
Anisotremus virginicus	48	48	48a	1 pair (I)	–	Accioly and Molina (2004)
Haemulon aurolineatum	48	48	48a	–	–	Duran et al. (1990)
Haemulon flavolineatum	48	48	48a	1 pair (I)	C	Ron and Nirchio (2005)
Haemulon parra	48	48	48a	1 pair (I)	P and T	Lima and Molina (2004)
Haemulon plumierii	48	48	48a	1 pair (I)	P and T	Lima and Molina (2004)
Haemulon sciurus	46	48	2 sm/st, 44a	–	–	Reagan et al. (1968)
Haemulon sciurus	48	48	48a	–	–	Reagan et al. (1968)
Haemulon striatum	48	48	48a	1 pair (I)	P and T	Lima and Molina (2004)
Orthopristis ruber	48	50	2 sm, 36st, 10a	–	C and T	Brum (1996)
Orthopristis ruber	48	48	48a	–	–	Brum (1996)
Pomadasys corvinaeformis	48	48	48a	1 pair (I)	C and P	Accioly and Molina (2004)

sm = submetacentric; st = subtelocentric; a = acrocentric; C = centromeric; T = telomeric; P = pericentromeric; I = interstitial

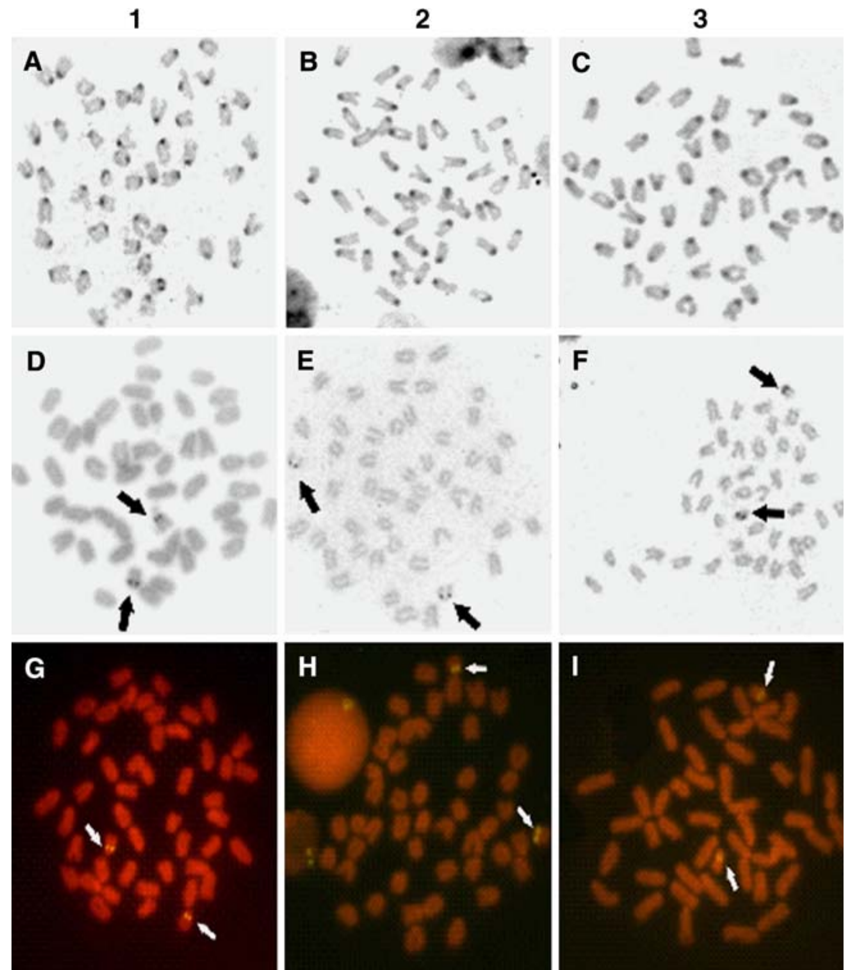

Fig. 2 Metaphase plates after C-banding (**A**–**C**), Ag-NOR (**D**–**F**); and FISH with 18S rDNA probe (**G**–**I**). Species are arranged in columns: *Haemulon aurolineatum* (1), *H. bonariense* (2), and *H. plumierii* (3). Arrows indicate localization of positive signals (see ''Results and discussion'')

gions or to different numbers of NORs in the genome of diverse species (Gornung et al. 1997; Nirchio et al. 2003, 2004; Gromicho et al. 2005). The FISH experiments performed here show that 18S rDNA signals coincide with signals seen in the Ag-NOR-bearing chromosomes (Fig. 2G–I), indicating that the three species here studied do not possess additional NOR sites and that all NOR-cistrons are active.

When 5S rDNA sequences were evidenced through FISH (Fig. 3a–c) it was observed that *H. aurolineatum* has two chromosome pairs marked by FISH. One pair presents two 5S rDNA clusters close to the centromeric

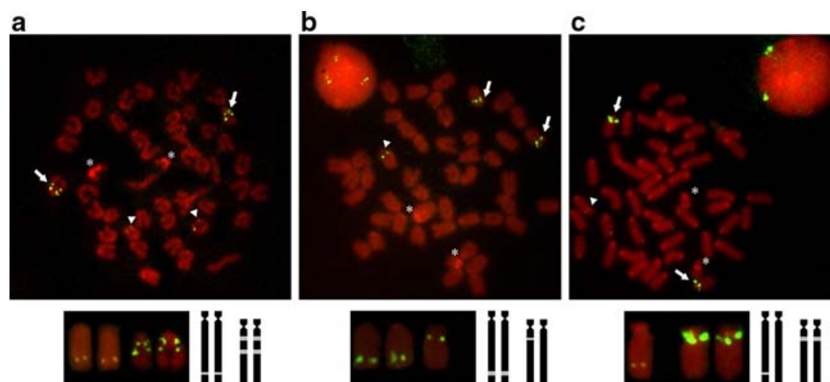

Fig. 3 Metaphase plates showing the 5S rDNA position. Arrows and arrowheads indicate location of strong and tenuous positive signals, respectively. Asterisks indicate NOR regions evidenced under the propidium iodide staining after FISH with the 5S rDNA probe. Details of 5S-bearing chromosomes are shown in the partial karyotype analysis and ideogram below each metaphase for *Haemulon aurolineatum* (**a**), *H. bonariensis* (**b**), and *H. plumierii* (**c**)

area and another pair has a single signal more tenuous than the double marks and is located next to the telomere of the long arm (Fig. 3a). On the other hand, *Haemulon bonariensis* presents conspicuous sites in the terminal position of the long arm of one chromosome pair and a faint site in a second chromosome (Fig. 3b). *H. plumierii* showed one chromosome pair with a strong signal close to the centromeric area and a second faint signal in the terminal position of a single chromosome (Fig. 3c). Sequential Ag-NOR staining of 5S rDNA/FISH probed showed that the 5S and 18S rDNAs are clustered in different chromosomes in the three Haemulidae analyzed (data not shown). Chromosomal sites of the NOR were also detected after the propidium iodide staining of the 5S rDNA/FISH probed metaphases (Fig. 3). The divergent locations of 18S and 5S rDNA loci seem to be the most common situation observed in fish (Martins and Wasko 2004), and by far the most frequent distribution pattern observed in vertebrates (Lucchini et al. 1993; Suzuki et al. 1996).

According to Sola et al. (1981), similar karyotypes are considered indicators of a relatively recent separation. Thus, the cytogenetic steadiness herein observed in the genus *Haemulon* and the substantial degree of similarity revealed by Cequea and Pérez (1971) through electrophoretic studies on haemoglobin and plasma protein in *H. steindachneri*, *H. bonariensis*, *H. flavolineatum*, *H. aurolineatum*, *H. chrysargyreum* and *H. plumierii* suggests the monophyletic origin of the genus *Haemulon* and indicates this group as one of recent diversification. Indeed, although the conserved diploid number, the presence and location of secondary constrictions on chromosome 24, the 18S rDNA cluster location and activity and the C-band pattern do not have a potential cytotaxonomic value for discriminating among any of the species of *Haemulon* herein studied, the number of chromosomes bearing the 5S-rDNA sequences and the distribution of 5S-rDNA clusters allowed the clear differentiation of *H. aurolineatum* from the other species we analyzed. Thus, although the chromosome macrostructure of *Haemulon* has been preserved from major changes, our data on 5S rDNA suggest that microstructural changes may have played an important role in the karyotype evolution of these fishes.

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