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Physical mapping of 18S and 5S genes in pelagic species of the genera *Caranx* and *Carangoides* (Carangidae)

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ABSTRACT. In Carangidae, *Caranx* is taxonomically controversial because of slight morphological differences among species, as well as because of its relationship with the genus *Carangoides*. Cytogenetic data has contributed to taxonomic and phylogenetic classification for some groups of fish. In this study, we examined the chromosomes of *Caranx latus*, *Caranx lugubris*, and *Carangoides bartholomaei* using classical methods, including conventional staining, C-banding, silver staining for nuclear organizer regions, base-specific fluorochrome, and 18S and 5S ribosomal sequence mapping using *in situ* hybridization. These 3 species showed chromosome numbers of 2n = 48, simple nuclear organizer regions (pair 1), and mainly centromeric heterochomatin. However, *C. latus* (NF = 50) and *C. bartholomaei* (NF = 50) showed a structurally conserved karyotype compared with *C. lugubris* (NF = 54), with a larger number of 2-armed chromosomes. The richness of GC-positive heterochromatic segments and sites in 5S rDNA in specific locations compared to the other 2 species reinforce

the higher evolutionary dynamism in *C. lugubris*. Cytogenetic aspects shared between *C. latus* and *C. bartholomaei* confirm the remarkable phylogenetic proximity between these genera.

Key words: Chromosome markers; Cytotaxonomy; Karyotype evolution

INTRODUCTION

The Carangidae family represents one of the most morphologically diverse groups within the order Perciformes (Nelson, 2006), which includes economically and ecologically important species such as travellies, moonfishes, and pompanos. The approximately 140 species of this family are divided into 4 tribes of 32 genera, some of which have relatively undefined generic boundaries (Smith-Vaniz, 1984; Gushiken, 1988). In fact, the taxonomic relationships within the family are unclear for some groups, and nomenclature changes are typical of this family (Laroche et al., 1984; Gunn, 1990; Honebrink, 2000). Taxonomic analysis of the *Caranx* genus has shown that some species with a wide geographic distribution and cryptic taxonomic features constitute species complex (Smith-Vaniz and Carpenter, 2007). Interspecific hybrids have been reported for this genus (Murakami et al., 2007), suggesting genetic conservation after species diversification. In addition, overlapping intraspecific features are observed, and *Caranx* shows morphological similarities that make it difficult to distinguish from other *Carangoides* members. Such similarities led various authors to classify these species into the same genus (Smith-Vaniz, 1984; Reed et al., 2002). Known as neritic predators, species in the genera *Caranx* and *Carangoides* play an important ecological role in both tropical and subtropical environments, constituting a significant economic resource throughout the tropics (Meyer et al., 2001).

Cytogenetic studies constitute auxiliary tools for identifying existing biodiversity and aiding in taxonomy and phylogeny studies (Dias and Giuliano-Caetano, 2002). Cytogenetic data are important for identifying evolutionary significant units (Moritz et al., 1996) and have been employed in recent surveys for diverse groups of Carangidae (Accioly et al., 2012; Jacobina et al., 2012a,b, 2013). The genetic and ecological peculiarities of the family make this group of marine fish an important model for evolutionary studies. However, despite the evolutionary, ecological, and economic features of *Caranx*, chromosomal aspects of the genus are not well understood. In this study, we present cytogenetic data of the Atlantic species *Caranx latus* and *Caranx lugubris* as well as *Carangoides bartholomaei*, which were analyzed using standard cytogenetic techniques, including base-specific fluorochrome CMA_3/DAPI mapping of ribosomal DNA sequences rDNA 18S, and by dual-color fluorescence *in situ* hybridization (FISH).

MATERIAL AND METHODS

Cytogenetic analysis was conducted using insular samples of *C. lugubris* (N = 9) collected from the St. Peter and St. Paul Archipelago and *C. latus* (N = 6) and *C. bartholomaei* (N = 5) caught in Rocas Atoll's, sites located in the Atlantic Ocean across the northeastern coast of Brazil. Before chromosome preparation, specimens were subjected to *in vivo* mitotic stimulation for 24 h by intramuscular and intraperitoneal inoculation of complexes of bacterial and fungal antigens according to the method proposed by Molina et al. (2010). The specimens

were anesthetized using clove oil (eugenol) and sacrificed for kidney tissue removal. Metaphase chromosomes were obtained from cell suspensions of the anterior kidney fragments using a short *in vitro* method (Gold et al., 1990). Cell suspensions were dripped onto slides coated with a film of distilled water heated to 60°C, from which the best metaphase samples were photographed under an Olympus BX50 epifluorescence microscope (Olympus; Tokyo, Japan) with a magnification of 1000X using an Olympus DP73 digital image capture system.

Chromosome banding

Heterochromatic regions and ribosomal sites were identified using the methods of Sumner (1972) and Howell and Black (1980), respectively. In addition, chromosomes were subjected to double CMA_3/DAPI staining, using 4',6-diamidino-2-phenylindole (DAPI) for counterstaining (Barros e Silva and Guerra, 2010). Briefly, slides that had been aged for 3 days were stained with 0.1 mg/mL CMA_3 for 60 min and stained with 1 µg/mL DAPI for 30 min. Next, the slides were mounted in glycerol: McIlvaine buffer, pH 7.0, (1:1) and left to age for 3 days before analysis using an epifluorescence microscope under the appropriate filters.

FISH

FISH was performed using an 18S rDNA probe obtained from the nuclear DNA of *Prochilodus argenteus* Spix and Agassiz, 1829 (Hatanaka and Galetti, 2004), the 5S rDNA probe isolated from the genomic DNA of *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti, 1999), and a probe for the general vertebrate telomeric sequence $(TTAGGG)_n$ (Ijdo et al., 1991). Probes were labeled by polymerase chain reaction (PCR) using biotin-16-dUTP (Roche Applied Science; Upper Bavaria, Germany) for 18S rDNA or digoxygenin-11-dUTP (Roche Applied Science) for 5S rDNA and $(TTAGGG)_n$ probes. PCR labeling using primers was performed for rDNA clones using 20 ng template DNA, 1X *Taq* Reaction Buffer (200 mM Tris, pH 8.4, 500 mM KCl), 40 mM dATP, dGTP, and dCTP, 28 µM dTTP, 12 mM biotin-16-dUTP or digoxygenin-11-dUTP, 1 µM primers, 2 mM $MgCl_2$, and 2 U *Taq* DNA polymerase (Invitrogen; Carlsbad, CA, USA) under the following conditions: 5 min at 94°C, 35 cycles: 1 min at 90°C, 1 min 30 s at 52°C, and 1 min 30 s at 72°C, and final extension at 72°C for 5 min. The reaction for labeling the telomeric probe included 1X *Taq* Reaction Buffer, 40 µM dATP, dCTP, and dGTP, 28 µM dTTP, 12 µM digoxygenin-11-dUTP, 0.2 µM primers $(TTAGGG)_5$, 0.2 µM primer $(CCCTAA)_5$, 2 µM $MgCl_2$, and 2 U *Taq* DNA polymerase under the following conditions: the first amplification was performed with low stringency for 4 min at 94°C, 12 cycles of 1 min at 94°C, 45 s at 52°C, and 1 min 30 s at 72°C, followed by 35 high-stringency cycles: 1 min at 94°C, 1 min 30 s at 60°C, and 1 min 30 s at 72°C.

The overall hybridization procedure has been described by Pinkel et al. (1986) under high stringency conditions (2.5 ng/µL each probe, 50% formamide, 10% dextran sulfate, 2X SSC, pH 7.0-7.2) at 37°C overnight. After hybridization, the slides were washed in 15% formamide/0.2X SSC at 42°C for 20 min, 0.1X SSC at 60°C for 15 min, and 4X SSC/0.05% Tween at room temperature for 10 min, the latter consisting of two 5-min washes. Signal detection was performed using streptavidin-Alexa Fluor 488 (Molecular Probes; Eugene, OR, USA) for 18S rDNA and anti-rhodamine (Roche Applied Science) for 5S rDNA and $(TTAGGG)_n$ probes. The 5S and 18S genes were detected using dual-color FISH.

RESULTS

The 3 species analyzed contained 2n = 48 chromosomes with chromosome type variations. Macrostructural karyotype patterns of the species revealed a first submetacentric chromosome pair of similar size among the species. While *C. bartholomaei* and *C. latus* showed similar karyotypes composed of 46 acrocentric and 2 submetacentric pairs (NF = 50), *C. lugubris* had 42 acrocentric, 2 submetacentric, and 4 metacentric chromosomes (NF = 54) (Figure 1). In these species, the heterochromatic regions were distributed in pericentromeric positions in most chromosomes as well as in portions of some telomeric pairs. In *C. lugubris*, in addition to the ribosomal sites, heterochromatic regions were shown to be GC-rich (Figure 2).

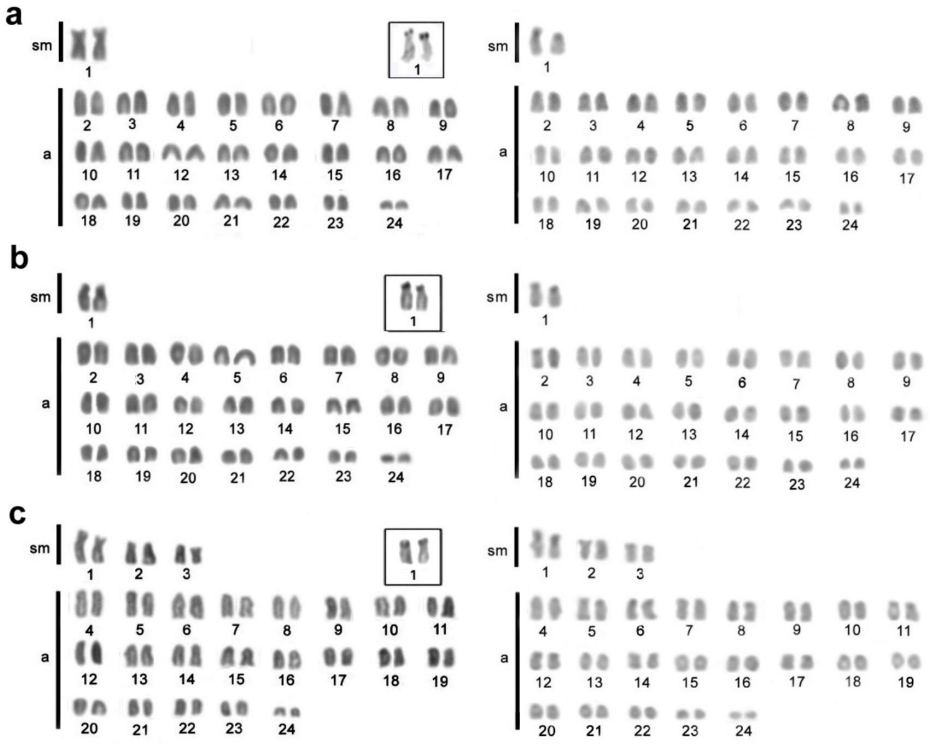

Figure 1. Karyotypes of *Carangoides bartholomaei* (**a**), *Caranx latus* (**b**), and *Caranx lugubris* (**c**) by Giemsa staining. The left panel shows the patterns of heterochromatin distribution for each species, respectively. The nucleolar organizer pairs are highlighted (pair 1). Bar = 5 μm.

The pair 1, submetacentric, of similar size between species, showed secondary constrictions (C +/Ag-NOR+/18S rDNA/GC+) in the subterminal region of the short arm. The 5S rDNA sites are located in non-syntenic conditions with the 18S genes. *In situ* mapping with 5S rDNA probes indicated the presence of these genes in a terminal position on the short arm of the pair 15 in *C. bartholomei* and *C. latus* and in a terminal position on the long arm of the pair 9 in *C. lugubris* (Figure 2).

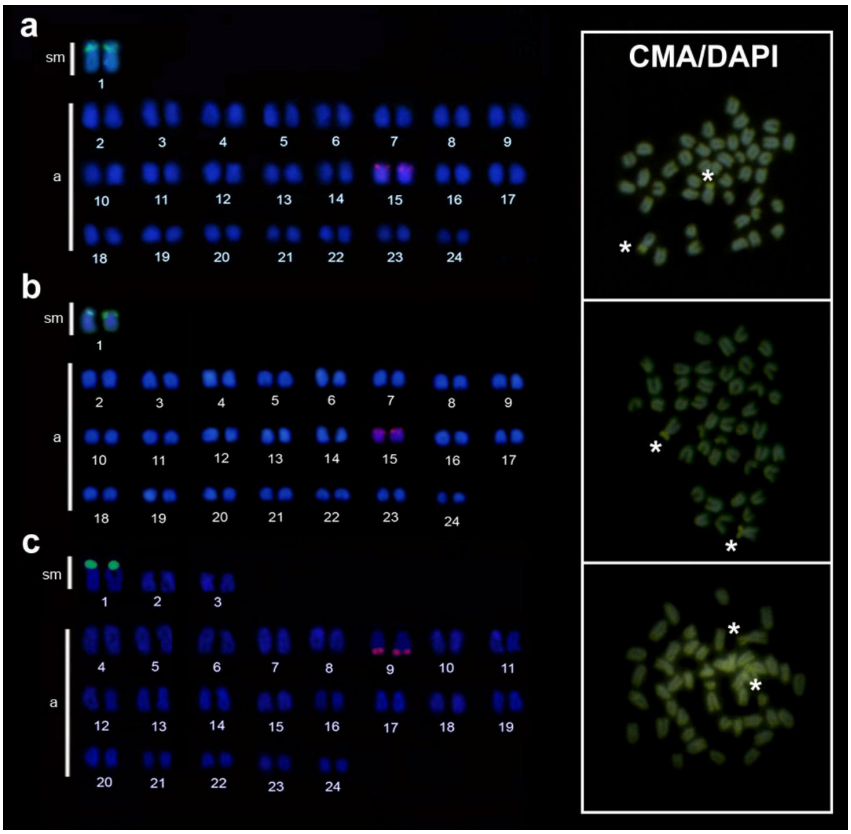

Figure 2. Chromosome mapping by dual-color FISH with 18S (green) and 5S (red) rDNA probes in *Carangoides bartholomaei* (**a**), *Caranx latus* (**b**), and *Caranx lugubris* (**c**). In the same row, results of sequential CMA_3/DAPI staining are shown. Asterisks indicate the GC-rich regions of the rDNA sites. Bar = 5 µm.

DISCUSSION

The carangids *C. bartholomaei*, *C. latus*, and *C. lugubris*, similarly to many other Perciformes species, have a karyotype of 48 chromosomes; this characteristic is considered to be basal and phylogenetically conserved within the order (Galetti et al., 2000; Molina and Galetti, 2007). In addition to conservation regarding the number of chromosomes, *C. bartholomaei* and *C. latus* share a similar karyotype (NF = 50).

Among species in this family, approximately one-third display the basal karyotype to Perciformes (2n = 48 acrocentric). With a higher number of acrocentric chromosomes than *C. lugubris* (NF = 54), *C. bartholomaei* and *C. latus* possess higher numbers of basal chromosomal characteristics. In fact, the presence of a single pair of 2-armed chromosomes in the karyotypes of 3 other species of *Caranx* (Chai et al., 2009) indicates that this is typical. The occurrence of this unique and conserved 2-armed pair among species supports the hypothesis that *C. lugubris*, with higher numbers of 2-arm chromosomes (22a and 4m), presents a more derived pattern than typically observed.

Pericentric inversions are one of the primary mechanisms responsible for karyotypic diversification in carangids (Caputo et al., 1996; Sola et al., 1997; Rodrigues et al., 2007; Chai et al., 2009). In some genera, such as *Trachinotus* (NF = 52-58) and *Trachurus* (NF = 50-66), pericentric inversions have played an important role in the karyotype diversification (Murofushi and Yoshida, 1979; Caputo et al., 1996; Rodrigues et al., 2007, Jacobina et al., 2012a; Accioly et al., 2012). Moreover, other sporadic mechanisms may have contributed to the chromosomal evolution of this group, particularly Robertsonian translocations, which are found in *Trachurus*, *Seriola*, and *Selene* (Vitturi et al., 1986; Jacobina et al., 2012b).

The phylogenetic relationship between *Caranx* and *Carangoides* is supported not only by the common karyotypes among *C. bartholomaei* and *C. latus*, but also by the presence of 18S rDNA sites, which appear at equilocal positions on the short arm of the first chromosome pair in all 3 species. This condition, as well as reduced heterochromatin and localized pericentromeric positions, indicates conservation of chromosome structure (karyotypic stasis, *sensu* Molina and Galetti, 2007), which is widely disseminated within the Perciformes. The occurrence of reduced heterochromatization processes, such as those observed in *Caranx* and *Carangoides*, are thought to play a restrictive role in the karyotypic differentiation of species (Galetti et al., 2000; Molina and Galetti, 2004, 2007).

The presence of ribosomal sites in 2-armed chromosomes, possibly from mechanisms of pericentric inversion and Robertsonian translocation, suggests that these sites have diversified functions. In this respect, the frequent occurrence of equilocal heterochromatin in the nuclear organizer regions (CG+) reinforces the hypothesis that repetitive DNA favors the occurrence and retention of chromosomal rearrangements (Redi et al., 1990; Fujiwara et al., 1998; Jacobina et al., 2012a,b; Accioly et al., 2012). In fact, *C. lugubris* that are GC-rich in most chromosomes show the most cytogenetic differences among the 3 species. High GC+ content dispersed throughout all chromosomes, although uncommon, has been identified in some marine families such as Tetraodontidae and Grammatidae (Martinez et al., 2011; Molina et al., 2012).

In groups of fish with conserved chromosomal characteristics, the mapping of 18 and 5S sequences using dual-color FISH, as used for *C. bartholomei*, *C. latus*, and *C. lugubris* allows analysis of karyotype evolution, rearrangements involved in diversification, and discrimination of species or populations (Accioly et al., 2012; Jacobina et al., 2013). The presence of 18S rDNA sites on the same homeolog pair (pair 1) and 5S rDNA sites located at the same position (short arm) of pair 15 in *C. bartholomei* and *C. latus* reinforce the conservation and phylogenetic proximity among the species in these 2 genera (*Carangoides* and *Caranx*), which are considered by some authors to belong to the same genus (Smith-Vaniz, 1984; Reed et al., 2002).

In marine Perciformes, greater diversification of 5S ribosomal sequences compared to 18S rDNA sites has been observed, making them efficient chromosomal markers for evolutionary studies and species discrimination (Motta-Neto et al., 2011). With a higher level of chromosomal diversification, *C. lugubris* shows 5S rDNA sites exclusively on the chromosome pair 9 in the terminal portion of the long arm, supporting a more derived condition compared to other species.

The cytogenetic patterns of the species *C. bartholomei*, *C. latus*, and *C. lugubris* support the phylogenetic proximity of *C. bartholomaei* to species of the genus *Caranx*, widening the issues to the real systematic positioning among these genera and highlighting the derived condition of *C. lugubris* compared to other species of this genus.

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