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Evolutionary dynamics of rDNA genes on chromosomes of the *Eucinostomus* fishes: cytotaxonomic and karyoevolutive implications

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ABSTRACT. Several chromosomal features of Gerreidae fish have been found to be conserved. In this group, it is unclear whether the high degree of chromosomal stasis is maintained when analyzing more dynamic regions of chromosomes, such as rDNA sites that generally show a higher level of variability. Thus, cytogenetic analyses were performed on 3 Atlantic species of the genus *Eucinostomus* using conventional banding (C-banding, Ag-NOR), AT- and GC-specific fluorochromes, and fluorescence *in situ* hybridization mapping of telomeric sequences and 5S and 18S rDNA sites. The results showed that although the karyotypical macrostructure of these species is similar (2n = 48 chromosomes, simple Ag-NORs seemingly located on homeologous chromosomes and centromeric heterochromatin pattern), there are differences in the positions of rDNA subunits 5S

and 18S. Thus, the ribosomal sites have demonstrated to be effective cytotaxonomic markers in *Eucinostomus*, presenting a different evolutionary dynamics in relation to other chromosomal regions and allowing access to important evolutionary changes in this group.

Key words: Chromosome evolution; Gene synteny; Karyotype stasis; Mojarras

INTRODUCTION

The family Gerreidae comprises 44 species of small- to medium-sized fish living in shallow coastal estuarine waters, with wide distribution in tropical and subtropical latitudes (Nelson, 2006). In some areas they constitute typical groups of mangrove environments, where they represent an abundant and important component of the food chain in areas of high biological productivity (Ramírez-Luna et al., 2008). Some species have been considered potential candidates for intensive cultivation (Calado et al., 2013). In the Atlantic waters, this group of fish includes the genera *Diapterus*, *Eugerres*, *Eucinostomus*, *Gerres*, and *Ulaema* (Eschmeyer, 1998), with cytogenetic data available only for the first 3 of these genera.

Karyotypic studies are useful tools in taxonomy and identification of relationship patterns and phylogenies, particularly in groups with cryptic taxonomic characters in some life stages. Previous cytogenetic analyses of *Eucinostomus*, *Diapterus*, and *Eugerres* have demonstrated the potential of Ag-NOR sites for taxonomic discrimination (Ruiz-Carus and Uribe-Alcocer, 2004) and of the mapping of 18S and 5S rDNA sequences in the species identification in this family (Calado et al., 2013). The chromosomal mapping of ribosomal sequences, which are dynamic regions able to spread within the genome (Shishido et al., 2000), has received increasing attention for their usefulness in indirect identification of karyotype diversification in Perciformes, especially for species identification (Molina et al., 2012a,b) and/or fish stocks.

Thus, in this study, cytogenetic data obtained from 3 species of the genus *Eucinostomus*, silver mojarras *E. argenteus* (Baird and Girard, 1855), jenny mojarras *E. gula* (Quoy and Gaimard, 1824), and flagfin mojarras *E. melanopterus* (Bleeker, 1863), were determined using different classical and molecular cytogenetic methods, including dual-color fluorescence *in situ* hybridization (FISH) chromosomal mapping of 18S and 5S rDNA sites and telomeric $(TTAGGG)_n$ sequences. The data revealed the dynamics of the 18S and 5S ribosomal units in the genome of *Eucinostomus* species resulting from chromosomal rearrangements not detectable by macrostructural chromosome analysis. Thus, these rDNA sites are effective markers for increasing the understanding of evolutionary processes occurring in this group.

MATERIAL AND METHODS

Specimens and chromosome preparations

Twenty-seven individuals of 3 species of *Eucinostomus* (Gerreidae) were analyzed, including *E. argenteus* (N = 10, 6 males, 4 females), *E. gula* (N = 8, 5 males, 3 females), and *E. melanopterus* (N = 9, 4 males, 5 females), collected from the base levels of the Cunhaú River (6°18'26.97"S 35°01'55.45"W) in Canguaretama, and from the Açu River in Guamaré (5°05'42.33"S 36°16'31.66"W), in the State of Rio Grande do Norte, which is the northeast

region of Brazil.

Prior to chromosome preparations, the specimens were subjected to *in vivo* mitotic stimulation for 24 h, by intramuscular and intraperitoneal inoculation of complexes of bacterial and fungal antigens (Molina et al., 2010). The specimens were anesthetized with clove oil (eugenol) and euthanized for the removal of kidney tissue. Metaphase chromosomes were obtained from cell suspensions of fragments of the anterior kidney using an *in vitro* method of short duration (Gold et al., 1990). Cell suspensions were dripped onto slides coated with a film of distilled water heated to 60°C. Approximately 30 metaphases were analyzed in each specimen to determine the chromosome number and morphology. The best metaphases were photographed using an Olympus BX50 epifluorescent microscope with a digital camera Olympus DP73 (Tokyo, Japan).

Chromosome banding

The heterochromatic regions and NOR sites were identified using the methods described by Sumner (1972) and Howell and Black (1980), respectively. GC-rich chromosomal regions were evidenced by the chromomycin A_3 fluorochrome (CMA_3), using 4', 6-diamidino-2-phenylindole (DAPI) as counterstain (Schweizer and Loidl, 1987). Briefly, 3-day-old slides were stained with 0.1 mg/mL CMA_3 for 60 min and restained with 1 µg/mL DAPI for 30 min. Next, the slides were mounted in glycerol:McIlvaine buffer, pH 7.0 (1:1), and incubated for 3 days at 4°C before analysis with an epifluorescence microscope under appropriate filters.

FISH procedures

FISH using 5S rDNA, 18S rDNA, and telomeric was performed with minor modifications of the method recommended by Pinkel et al. (1986). The 5S and 18S rDNA probes, containing 200 and 1400 bp, respectively, were obtained by polymerase chain reaction (PCR) from the nuclear DNA of *Prochilodus lineatus* (Hatanaka and Galetti, 2004), using the primers A: 5'-TAC GCC CGA TCT CGT CCG ATC-3' and B: 5'-CAG GCT GGT ATG GCC GTA AGC-3' (Pendás et al., 1995), NS1: 5'-GTA GTC ATA TGC TTG TCT C-3', and NS8: 5'-TCC GCA GGT TCA CCT ACG GA-3' (White et al., 1990), respectively. The telomeric DNA sequence $(TTAGGG)_n$ was also used as a probe, previously generated by PCR (PCR-DIG Probe Synthesis Kit, Roche) using $(TTAGGG)_5$ and $(CCCTAA)_5$ as primers (Ijdo et al., 1991). The 5S and 18S rDNA sequences were detected by dual-color FISH. The 5S and 18S rDNA probes were labeled with biotin-14-dATP and digoxigenin-11-dUTP, respectively, by nick translation according to manufacturer recommendations (Roche, Mannheim, Germany). Chromosomes were treated with 20 mg/mL DNAse-free RNAse in 2X SSC at 37°C for 1 h with 0.005% pepsin in 10 mM HCl at 37°C for 10 min, fixed with 1% formaldehyde for 10 min, and then dehydrated in an alcohol series. Chromosomes were then denatured in 70% formamide/2X SSC at 72°C for 5 min. The hybridization solution consisted of 50% formamide, 2X SSC, 10% dextran sulfate, and 5 ng/µL denatured probe. After hybridization for 18 h at 37°C, 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 Tween 20 0.5%/4X SSC at room temperature. The hybridization signals of digoxigenin-labeled probes were detected using anti-digoxigenin-rhodamine conjugate (Roche); and anti-avidin fluorescein isothiocyanate conjugate (Sigma, St. Louis, MO, USA) was used for the biotinylated probes. The chromosomes were counterstained with 1.5 µg/mL Vectashield/DAPI (Vector Laboratories, Burlingame, CA, USA).

RESULTS

E. argenteus, *E. gula*, and *E. melanopterus* have 2n = 48 acrocentric chromosomes (FN = 48; Figure 1a, b, and c). The karyotypes are quite symmetrical with little size difference between the pairs.

Conspicuous heterochromatic blocks were observed in the pericentromeric regions of the chromosomes in the 3 species analyzed (Figure 1a, b, and c; right column). However, the heterochromatic segments more expanded in *E. argenteus* than in the other species (Figure 1b; right column). In *E. argenteus*, the pericentromeric heterochromatin was GC-positive both in ribosomal sites and most chromosome pairs (Figure 2a). Ag-NOR sites were identified in all 3 species only on chromosome pair 22, although in varying positions, since they were interstitially located in *E. argenteus* and *E. melanopterus* and in the terminal position in *E. gula* (Figure 2b, c, and d). A precise correlation was observed between the Ag-NORs and $CMA_3^{+}/DAPI^{-}$ sites in the 3 species (Figure 2b, c, and d). FISH with telomeric probes $(TTAGGG)_n$ showed markings exclusively in the terminal regions of almost all the chromosomes (Figure 2e, f, and g).

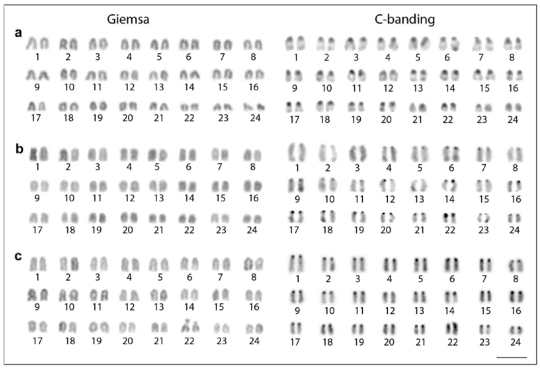

Figure 1. Karyotypes of *Eucinostomus argenteus* (**a**), *E. gula* (**b**), and *E. melanopterus* (**c**) with conventional staining and C-banding. Bar = 5 µm.

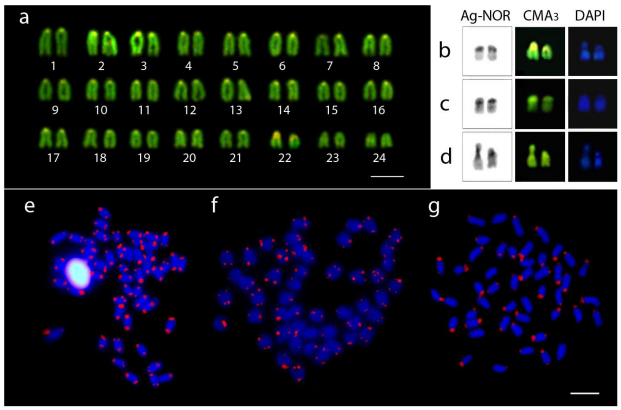

Figure 2. Fluorescent pattern of *Eucinostomus argenteus* chromosomes (**a**) stained with CMA_3 showing GC-rich pericentromeric heterochromatin on most chromosome pairs. Detail of the nucleolar organizer pair (pair 22) subjected to silver impregnation and staining with base-specific fluorochrome (**b**, **c**, **d**) and mapping of telomeric sequences $(TTAGGG)_n$ (**e**, **f**, **g**) in *E. argenteus* (**b**, **e**), *E. gula* (**c**, **f**), and *E. melanopterus* (**d**, **g**), respectively. Bars = 5 µm.

Mapping of 5S rDNA subunits showed single sites in the 3 species, but located on different chromosomes. In *E. argenteus*, these sites were located interstitially on pair 11. In *E. gula*, they were located in the terminal position of the short arms on pair 6, and in *E. melanopterus* they were located in interstitial position and colocalized with the 18S rDNA sites on the long arm of pair 22 (Figure 3a, b, and c).

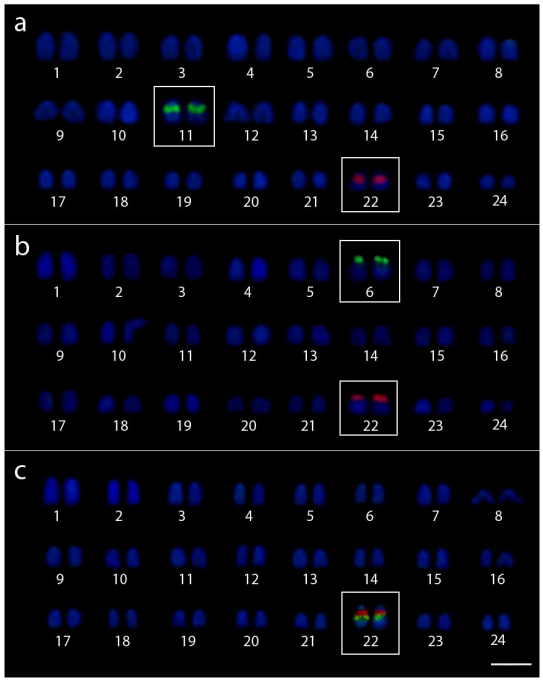

Figure 3. Location of 18S (red) and 5S (green) ribosomal genes using dual-color FISH mapping in *Eucinostomus argenteus* (**a**), *E. gula* (**b**), and *E. melanopterus* (**c**). Bar = 5 μm.

DISCUSSION

Eucinostomus species show a highly conserved karyotypic macrostructure (2n = 48, FN = 48). Substantial intrageneric similarity has also been observed among mojarras of the genera *Diapterus* and *Eugerres* (Calado et al., 2013). This karyotypic pattern, which is similar to the putative basal karyotype in Perciformes (Molina, 2007; Motta-Neto et al., 2011), is not only present in representatives of Gerreidae (Ruiz-Carus and Uribe-Alcocer, 2004; Calado et al., 2013), but also frequently observed among Perciformes (Arai, 2011). Together with macrostructural features, some chromosomal traits of *Eucinostomus* appear to be highly conserved, such as simple Ag-NOR sites ($C^+/GC^+/DAPI^-$) and pericentromeric heterochromatin regions.

Marine Perciformes exhibit a high karyotypic stability in some groups, with evidence of karyotypic stasis (Molina, 2007), where the cytogenetic differences fall short of phenotypic variation between closely related species (Motta-Neto et al., 2012). In contrast, the karyotypes of some Perciformes families, although subjected to a slower evolutionary rate regarding the fixation of structural rearrangements, have revealed some hypervariable chromosomal

regions, particularly those represented by ribosomal sites or positions adjacent to them (Affonso and Galetti Jr., 2005). Thus, in groups of fish with higher karyotypic stability, the mapping of the ribosomal has been particularly effective for identifying and quantifying chromosome dynamics (Motta-Neto et al., 2012), particularly among cogeneric species, as suggested by the *Eucinostomus* species analyzed in this study. In fact, analyses involving the mapping of 18S and 5S sequences and characterization of GC-rich regions in these species revealed diagnostic cytogenetic markers, enabling identification of unique chromosomal differences between them.

GC-positive extra-NOR heterochromatic segments in *E. argenteus*, which are not present in *E. gula* and *E. melanopterus*, represent a peculiar characteristic of this species. This condition, although unusual, has been reported in some fish species (Affonso and Galetti Jr., 2005; Molina et al., 2012a,b), suggesting that the high GC-rich content around the karyotype favors chromosomal rearrangements (Martinez et al., 2011). Aside from this characteristic derived from heterochromatin in *E. argenteus* (GC-rich), all species presented mainly centromeric heterochromatin. Such heterochromatin distribution pattern is considered to be conserved and widespread in Perciformes, particularly in marine groups (Molina, 2007).

Less conserved, the mapping with 5S and 18S ribosomal sequences revealed interspecific variation thus demonstrating that ribosomal sites may be effective cytotaxonomic markers or indicate the occurrence of chromosomal rearrangements, particularly among species that show low karyotypic diversity (Motta-Neto et al., 2012). Thus, although 18S rDNA sites are single and apparently located on homeologous chromosome pairs in the 3 species analyzed (pair 22), they have different positions on the chromosomes: at the interstitial position in *E. argenteus* and *E. melanopterus* and at the terminal position of the short arm in *E. gula*; these results suggest intrachromosomal rearrangements. The activity of NORs on a single chromosome pair may have provided a rare and casual interstitial colocalization of the 18S and 5S genes in *E. melanopterus.* In fish, 5S and 18S rDNA multigene families are most often located on different chromosome pairs, which appear to represent the most common and possibly basal condition for ribosomal gene arrangement (Martins and Galetti Jr., 1999). Thus, they may evolve independently and are subject to individual selective pressure (Amarasinghe and Carlson, 1998). Occasionally, in Perciformes, both the 5S and 18S rDNA sites may be diversified (Motta-Neto et al., 2012), standing out not only as efficient species markers but also as population markers (Lima-Filho et al., 2012), and may be involved in chromosome rearrangements (Jacobina et al., 2013).

In several fish species, the 5S rDNA sites are located in interstitial position on the chromosomes, as in salmonids (Fujiwara et al., 1998), anostomids (Martins and Galetti Jr., 2000), and parodontids (Vicente et al., 2001), among many others. In fact, this location is also the most common among *Eucinostomus* species. However, it remains unclear whether this condition is a plesiomorphic characteristic of this particular genus. Further studies should map these genes in a larger number of species.

Eventually, the 5S and 18S sites may exhibit synteny and become located on the same chromosome. Although this spatial conformation is unusual, it has been observed in some species of fish, including *Salmo salar* (L. 1758) (Morán et al., 1994), *Oreochromis niloticus* (L. 1758) (Pendás et al., 1994), *Triportheus nematurus* (Kner, 1858) (Diniz et al., 2008), and *Hoplias malabaricus* (Bloch, 1794) (Cioffi et al., 2009). The syntenic adjacent location detected in *E. melanopterus* indicates an autapomorphic condition for this species, since the dual-color

FISH mapping in other species of this and other Gerreidae genera reveal a non-syntenic position of ribosomal genes (Calado et al., 2013). Interestingly, in *E. argenteus* and *E. gula*, both ribosomal sites are located on separate chromosomes, exhibiting variations in intrachromosomal location (terminal, interstitial), whereas in *E. melanopterus* they are interstitially colocated on a single pair. These various physical arrangements of rDNA sites demonstrate their relative mobility in the genome, unlike other chromosomal regions such as the centromeric and telomeric sequences, which seem to be less dynamic in evolutionary terms.

Based on the position of the ribosomal sequences in the karyotypes of the 3 species of *Eucinostomus*, as with the dual-color FISH mapping data in species of *Diapterus* and *Eugerres*, 3 stages of chromosomal location of ribosomal sites of Gerreidae could be distinguished (Figure 4). The first pattern, observed in *E. argenteus*, is represented by both 5S and 18S sites in interstitial positions on different chromosome pairs. This pattern may correspond to the baseline condition of Gerreidae, given its greater frequency among species and a possible advantage that the interstitial location of ribosomal genes may protect against disruptive chromosomal rearrangements (Martins and Galetti Jr., 1999). Thus, the second pattern, present only in *E. gula* among the Gerreidae, corresponds to a derived condition, represented by both 5S and 18S sites in the terminal position on different chromosomes. Finally, the rare third pattern corresponds to an autapomorphy in *E. melanopterus*, represented by both 5S and 18S sites colocated on a single pair in the interstitial position.

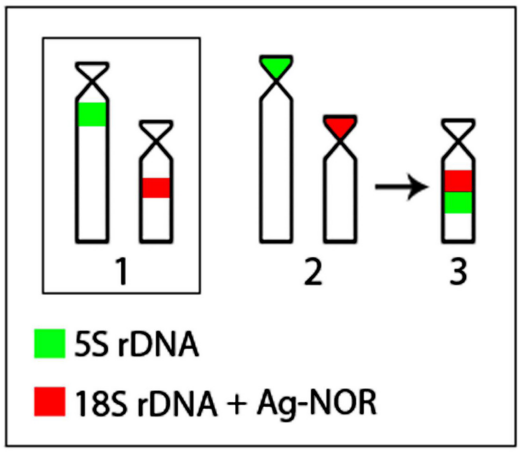

Figure 4. Idiogram representing the 3 stages of chromosomal location of ribosomal sites in *Eucinostomus* species. First pattern is represented by both 5S and 18S sites in interstitial position on different chromosome pairs; second, represented by both 5S and 18S sites in the terminal position on different chromosomes; and third, both 5S and 18S sites are colocated on a single pair in the interstitial position.

Telomeric probes have usually been employed in the identification of traces of recent evolutionary chromosomal rearrangements, as has been identified in Salmoniformes (Salvadori et al., 1995), Characiformes (Cioffi et al., 2010), and Perciformes (Gornung et al., 1998), among other groups. Mapping of repetitive sequences $(TTAGGG)_n$ in the karyotypes of the 3 species indicates an exclusive location in the terminal portions of all chromosomes of the complement. The failure to detect ectopic sites and the maintenance of the basal diploid number, associated with the dynamics presented by ribosomal sequences, suggests that the distribution

of ribosomal genes in interstitial or terminal positions may result from transposition or paracentric inversions; the latter process is rarely detected among fish (Porto-Foresti et al., 2004).

The macrokaryotypic conservatism observed in *Eucinostomus*, whose greatest detectable variation is related to the dynamics of ribosomal sites in the karyotype, finds support in previous evidence found in other genera of Gerreidae (Calado et al., 2013). In this karyoevolutionary context, with reduced rates of chromosomal diversification, cytogenetic analyses employing recent advances in molecular cytogenetic methods emerge as an interesting model for tracking the processes of diversification within the group.

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