Comparative cytogenetic analysis of marine needlefishes (Beloniformes) from southern Brazil

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Abstract Cytogenetic studies have assisted in the taxonomic classification of organisms, especially those involving species with highly similar morphologic characteristics, or so-called cryptic species. *Strongylura marina* and *Strongylura timucu* collected from Paranaguá Bay, Paraná Coast in Southern Brazil are considered cryptic species, and the identification of interspecific variations based on the number and/or morphology of its chromosomes may serve as differentiating cytotaxonomic markers. Chromosomes of the two species were subjected to different banding and staining methods (C-, Ag-, and DAPI-CMA₃), as well as chromosomal mapping of major rDNA (45S), revealed with an 18S probe by fluorescence in situ hybridization (FISH). The pattern of

distribution of constitutive heterochromatin showed distinct features involving the pericentromeric and telomeric bands in both species. In *S. marina*, chromosome 1 represents the main species-specific marker, appearing almost entirely heterochromatic. In both species, the 45S rDNA is located at terminal region of the short arm of the chromosome 6, as detected by silver nitrate staining and FISH. Despite the apparent conserved diploid number of 48 chromosomes, data on the karyotype microstructure characterize the cytogenetic profile of the genus and may allow the establishment of cytotaxonomic and evolutionary inferences for these fishes.

Keywords Ag-NORs · C-band · Karyotype · FISH · Marine teleosts

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Introduction

The needlefish (Belonidae) is an ecologically diverse yet morphologically homogeneous family. It occurs mainly in tropical and subtropical regions, river basins of Central and South America, Australia, and Asia, as well as in the Pacific, Indian and Atlantic oceans (Collette 2003). It is among the main piscivorous families in estuaries, characterized by an elongated body, dorsal and anal fins positioned posteriorly, and both upper and lower jaws extended into long beaks filled with sharp teeth (Goulding and Carvalho 1984; Liao 2002). According to Collette et al. (1984), the Belonidae family consists of 48 species and subspecies distributed in 10 genera (*Ablennes*, *Belone*, *Belonion*, *Petalichthys*, *Platybelone*, *Potamorrhaphis*, *Pseudotyloturus*, *Strongylura*, *Tylosurus*, and *Xenentodon*). In terms of evolution, the genera *Strongylura*, *Tylosurus*, and *Ablennes* are considered as most recent.

Within the order Beloniformes, karyotypes have been described mainly for species of the family Adrianichthyidae (ricefish and medakas) (Arai 2011). Studies carried out in the Belonidae family reported karyotypes with two diploid numbers, 48 or 50 chromosomes, varying from all acrocentric chromosomes to diverse karyotypic formulas (Srivastava and Kaura 1964; Rish 1973; Rishi and Singh 1982), including the presence of the supernumerary chromosomes in *Strongylura microps* (Pastori et al. 1998).

Regarding the heterochromatic component of the genome, extensive studies on the organization, nature, and physical mapping of repetitive DNA has resulted in a better understanding of karyotype evolution of the species, including the diversification of sex chromosomes and the origin of B chromosomes (Vicari et al. 2010). In some cases, the maintenance of these repetitive elements in certain representatives of a clade has been used in generating phylogenetic inferences between species (Araújo et al. 2010; Sczepanski et al. 2010). Despite its role in chromosomal rearrangements in various fish species, constitutive heterochromatin generally has a uniform distribution, amount, and composition (John et al. 1985). It is believed that the heterochromatic content of the genome is not random and probably plays a role in genome evolution.

This study conducted a comparative analysis of the chromosomal distribution pattern of constitutive

heterochromatin and mapping of nucleolar organizer regions (NORs) in *Strongylura marina* and *Strongylura timucu*, in order to detect the existence of karyotypic differentiation between them and thus provide useful information in establishing the biodiversity of estuaries.

Materials and methods

Cytogenetic analyses were carried out in 14 specimens of *S. marina* (9 males and 5 females) and 17 *S. timucu* (11 males and 6 females) from Paranaguá Bay (25°26'45"S/48°23'11"W) State of Paraná, Brazil. Voucher specimens were deposited in the Capão da Imbuia National History Museum, in Curitiba, Paraná, Brazil. The procedures used in the current study were in compliance with the Ethics Committee on Animal Experimentation of the Universidade Federal do Paraná. The mitotic chromosomes were obtained from the anterior kidney using short time culture and air-drying conventional preparation (Fenocchio et al. 1991).

The Nucleolar Organizer Regions were stained with silver nitrate solution (AgNO_3) (Howell and Black 1980). The constitutive heterochromatin was performed according to Sumner (1972). Double staining using 4'-6-diamin-2-phenylindole (DAPI) and chromomycin A3 (CMA_3) was based on procedures described by Schweizer (1976). The chromosomal location of 18S ribosomal genes was performed by fluorescence in situ hybridization (FISH) (Pinkel et al. 1986) using 18S rDNA probes from *Prochilodus argenteus* (Hatanaka and Galetti 2004). A stringency of 77 % was maintained in all hybridization mixtures (1 μg from each probe, 50 % deionized formamide, 10 % dextran sulphate, $2\times$ SSC; pH 7.0; for 18 h). The probes were stained with biotin-14-dATP via nick translation (BioNickTM Labeling System, Invitrogen, Carlsbad, CA, USA) and the signal detection was accomplished using streptavidin-FITC conjugates.

The metaphases were examined on a Zeiss Axiophot epifluorescence microscope and the chromosome images were captured using the software Case Data Manager Expo 4.0 (Applied Spectral Imaging). For karyotyping, the chromosomes were identified according to their arm ratio, as proposed by Levan et al. (1964), and divided into metacentric (m), submetacentric (sm) and acrocentric (a).

Fig. 1 C-banded karyotypes: **a** *S. marina*; **b** *S. timucu*. The NOR-bearing chromosome pair is shown after silver impregnation (boxes). Bar = 10 μ m

Results

The karyotypes of the species in this study were previously described by Cipriano et al. (2008) using conventional staining. Both species presented the usual diploid number of 48 chromosomes. No sex chromosome heteromorphism was observed.

The C-banding revealed heterochromatic blocks at pericentromeric region of most chromosomes and telomeric bands on some pairs in both species (Fig. 1). In *S. marina*, chromosome 1 (metacentric) appears almost entirely heterochromatic (Fig. 1a).

The silver nitrate staining showed the presence of a single pair of NORs. The two species showed staining in the same chromosome pair (pair 6); however, in *S. marina*, staining was located on the proximal position of acrocentric chromosomes (Fig. 1a—box), whereas in *S. timucu*, these were at the terminal region of the short arm of a pair of submetacentric chromosome (Fig. 1b—box). This pattern was also visualized when metaphases were subjected to FISH using an 18S rDNA probe (Fig. 2b, d, respectively). In both species,

Fig. 2 Metaphases of the *S. marina* (**a**, **b**) and *S. timucu* (**c**, **d**) after DAPI-CMA₃ staining and FISH with 18S rDNA probes, respectively. The arrows indicate the NOR bearing chromosomes. Bar = 10 μ m

these regions also showed positive signals after CMA₃ staining (Fig. 2a, c, respectively), indicating that this region was GC-rich. Assessment of several metaphases also indicated that this region often presented a size heteromorphism.

Discussion

The modal value for the diploid number of chromosomes in teleosts is 48 (Ohno et al. 1968; Naruse et al. 2004), in agreement with a whole-genome duplication of an ancestral bony vertebrate genome composed of 24 chromosomes (Jaillon et al. 2004). This diploid number is shared by approximately 60 % of marine Perciformes, and has been explained taking into account the occurrence of large populations and therefore intense gene flow due to extensive dispersion of eggs, larvae, or adults in the marine environment would thus reduce karyotype diversification (Molina and Galetti 2007).

Despite the same diploid number, a difference in the chromosomal formula was observed: *S. timucu* presented a karyotype composed of six pairs of bi-armed chromosomes, fundamental number (FN = 60),

whereas *S. marina* almost exclusively presented a karyotype composed of uni-armed chromosomes (FN = 52). These differences were indicative of gross chromosomal rearrangements, mainly pericentric inversions, which are the main mechanism of chromosomal evolution of Perciformes (Galetti et al. 2006).

Nucleolar organizer regions location by silver nitrate staining and FISH using an 18S rDNA probe identified the NOR-bearing pair between the species (pair 6), although these occurred in different positions. This finding reinforces the role of pericentric inversions in chromosome and karyotype evolution. Size heteromorphisms between homologous chromosomes are frequently observed in the genomes of various species. Rearrangements such as spontaneous deletions, duplications, or unequal recombination are important evolutionary processes that involve tandem repetitive sequences, which in turn generate chromosomal heteromorphisms (Dover 1986).

The NORs were found to be adjacent or embedded within heterochromatic regions. The association between constitutive heterochromatin and rDNA cistrons has been frequently reported in fish (Molina et al. 1998; Born and Bertollo 2000). Repetitive blocks of ribosomal RNA genes are packaged into heterochromatic structures; this compaction generally decreases the chances of recombination (Gottlieb and Esposito 1989).

Both species presented a large amount of constitutive heterochromatin (satellite DNA), an unusual pattern in marine teleosts. Since these two species are morphologically similar, C-banding proved to be an accurate marker for the differentiation, given that the metacentric pair 1 in *S. marina* appears almost heterochromatic (Fig. 1a). In Teleostei, chromosome-specific satellite DNA may be a useful cytogenetic marker (Saito et al. 2007), and sometimes used to in resolving cytotaxonomic issues in species complexes such as that involving the *Astyanax scabripinnis* species group (Vicari et al. 2008; Kante et al. 2009). Among belonids, the heterochromatin can be considered an important component in the karyotype diversification of the group. Pastori et al. (1998) have also observed a remarkable pattern in other species, including entirely heterochromatic chromosomes (NOR-bearing and B chromosome).

In eukaryotic genomes, satellite DNA is one of the components of heterochromatin, which is also enriched with other dispersed repeated elements,

including transposons (Mazzuchelli and Martins 2009). The accumulation of satellite DNA in certain chromosomal regions may play a role in modeling the karyotype structure of species, causing both functional (position-effect variegation) and structural changes (fissions, fusions, and inversions), thus contributing to karyotypic diversity in some marine groups, as observed in Tetraodontiformes (Noletto et al. 2012) and Perciformes (Affonso and Galetti 2005).

The species here analyzed revealed a karyotypic variability, which allowed be distinguished from each other by their FN values, C-banding pattern and NOR location, thus making them cytotaxonomic markers. In view of the limited information on the marine fishes, the data on microstructural karyotypic features improve our understanding of the karyotype evolution within the magnitude of marine species diversity.

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