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Isolation and characterization of a satellite DNA family in *Achirus lineatus* (Teleostei: Pleuronectiformes: Achiridae)

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Abstract

Agarose gels stained with Ethidium bromide and Southern blot experiments of *Hind*III-digested genomic DNA of *Achirus lineatus* evidenced the presence of monomers and multimers of a DNA segment of about 200 bp, named here *Al-Hind*III sequence. No signals were observed in Southern blot experiments with genomic DNA of other flatfish species. The DNA sequencing of four recombinant clones showed that *Al-Hind*III sequences had 204 bp and were 63.72% AT-rich. FISH experiments using a *Al-Hind*III sequence as probe showed bright signals in the centromeric position of all chromosomes of *A. lineatus*.

Introduction

Flatfishes of the order Pleuronectiformes comprise a group of recognized economical and ecological importance. It presents approximately 570 species with representatives in freshwater, brackish water and saltwater (Nelson, 1994). Studies involving chromosome banding are still preliminary in the group. C-banding has been applied in 16 species (Kikuno, Ojima & Yamashita, 1986; Fan & Fox, 1991; Vitturi, Catalano & Colombera, 1993; Bouza, Sánchez & Martínez, 1994; Azevedo et al., in press a, b) and the number and location of nucleolus organizer regions (NORs) have been reported for 20 species (Kikuno, Ojima & Yamashita, 1986; Vitturi, Catalano & Colombera, 1993; Vitturi, Catalano & Schillaci, 1993; Pardo et al., 2001; Azevedo et al., in press a, b). Fishes of the family Achiridae are recognized by their wide karyotype diversification when compared with other flatfish families (Azevedo et al., in press a). The cytogenetic analysis of Achiridae species revealed other interesting characteristic that is the occurrence of C-band positive segments that are stained differently among the species of the family. Thus, *Catathyridium jenynsi*, *Gymnachirus nudus*, *Hypoclinemus mentalis* and *Trinectes paulistanus* exhibited C-band positive segments strongly stained, while *Achirus declivis* and *Trinectes* sp. did not present evident C-band segments (Azevedo et al., in press b).

Significant contribution to the knowledge of chromosome structure and evolution in fishes has been achieved with studies of satellite DNAs (Garrido-Ramos et al., 1995; Capana et al., 2002; Jesus et al., 2003; Vicente et al., 2003). Satellite DNAs are the main component of the constitutive heterochromatin (Miklos, 1985; Lohe, Hilliker & Roberts, 1993; Lohe & Hilliker, 1995) and represent a class of highly repetitive DNA molecules that are organized in tandem in the genome (Sumner, 1990; Epplen & Epplen-Haupt, 2002). The analysis of the highly repetitive DNA sequences is an important tool to understand the structure of heterochromatin. Moreover, satellite DNAs can be used to study phylogenetic

relationships among species by comparing their sequences (Garrido-Ramos et al., 1995, 1999; Canapa et al., 2002; among others). The study of chromosome markers of closely related species permits a better understanding of the relationship among these species since the satellite sequences usually have a very fast evolving pattern (Singer, 1982; Bachmann et al., 1993; Charlesworth, Sniegowski & Stephen, 1994; Pimpinelli et al., 1995). There is no information about satellite DNAs in flatfish. The available molecular cytogenetic data for flatfishes is restricted to the chromosome mapping of ribosomal DNA sequences (Pardo et al., 2001).

Detailed studies of flatfishes satellite DNA sequences may clarify important issues about genome organization and can also find application as a genetic marker to the fundamental and applied biology of such important fish group. In this way, we have investigated a satellite DNA family of *Achirus lineatus*, reaching a better understanding of the chromosome structure of this species and to develop further studies about the evolution of satellite DNA sequences in the order Pleuronectiformes.

Materials and methods

Specimens of *A. lineatus* were collected in the cities of Ubatuba and São Vicente, São Paulo State, Brazil. Specimens of *Gymnachirus nudus*, *Trinectes paulistanus* (collected in Ubatuba, São Paulo State, Brazil), and *A. declivis* (collected in Bragança, Pará State, Brazil) were used for comparative DNA analysis. The fishes were deposited in the fish collection of the Laboratório de Biologia e Genética de Peixes (LBP), UNESP, Botucatu, SP, Brazil.

Total DNA of a specimen of *Achirus lineatus* from Ubatuba was digested with the enzymes *Alu*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hinc*II, *Hind*III, *Hpa*I, *Msp*I, *Nru*I, *Pvu*II, *Rsa*I, *Sac*I, *Sca*I, and *Spe*I. The digestion with the enzyme *Hind*III displayed, in agarose gel, a band of about 200 base pairs (bp) that was isolated and inserted in the vector pUC18 *Hind*III-digested. The recombinant plasmids were used to transform DH5a *E. coli* competent cells (Subcloning Efficiency™ DH5α™ Competent Cells – Invitrogen). Plasmid DNAs were isolated using the phenol/chloroform method and sequenced with an ABI Prism 377 automated DNA sequencer using the BigDye Terminator kit (Applied Biosystems). Nucleic acid sequences were subjected to Blastn (Altschul et al., 1990) searches at the National Center for Biotechnology Information (NCBI), web site (http://www.ncbi.nlm.nih.gov/blast), and the sequence alignment was performed using Clustal W (Thompson, Higgins & Gibson, 1994) and by hands. The genetic distances among the obtained sequences were determined with the software MEGA 2.1 (Kumar et al., 2001).

For the Southern blotting analyses, genomic DNAs were partially and completely digested with *Hind*III, and transferred to nylon membranes (Southern, 1975). The membrane hybridization was conduced with the kit ECL-direct nucleic acid labeling and detection system (Amersham Bioscience) according to the manufacturer's recommendations.

Cytogenetic analyses were performed on chromosome preparations obtained from six females, 5 males and 10 unsexed specimens of *Achirus lineatus* from Ubatuba and São Vicente, São Paulo State, Brazil. Chromosome preparations were obtained from kidney tissues using the technique described by Foresti, Oliveira and Almeida-Toledo (1993). FISH experiments were performed according to the method described by Pinkel, Straume and Gray (1986). DNA probe was labeled by nick translation with biotin-14-dATP according to the instructions of the manufacturer's (Bionick labeling system – Invitrogen). Detection of hybridized probes was carried out with avidin–FITC conjugate (Sigma) followed by two rounds of signal-amplification with biotinilated anti-avidin (Sigma). Chromosomes were counterstained with Propidium Iodide (50 μg/ml) diluted in Antifade (Vector). C-banding was performed according to Sumner (1972). Metaphases were examined in a Zeiss Axiophot photomicroscopy and pictures were taken with a Kodak Gold Ultra 400 ASA film (FISH) and AGFA-HDP 13.

Results and discussion

The analysis of *Hind*III digested genomic DNA of *A. lineatus* showed clear bands in agarose gel, with the presence of monomers and multimers of a DNA segment of about 200 bp, named here *Al-Hind*III sequence. *Hind*III digestion analysis also evidenced a 200 bp band in the genome of

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Al-HindIII1 AAGCTTATTTCACGCTG-AAAACGTCAAAAACGCACATGTCCTGGGGAAATATGTTCTGCCTGGCATTT
Al-HindIII2 AAGCTTATTTCACGCTG-AAAACGTCAAAAACGCACATGTCCTGGGGAAATATGTTCTGCCTGGCATTT
Al-HindIII4 AAGCTTATTTTACGCTGCAAAACGTCAAAAACGCACATGTCCTGGGGAAATAGGTTTTGCTCTGCATTT
Al-HindIII6 AAGCTTATTTCACGCTGCAAAACGTCAAAAACGCACATGTCCTGGGGAAATATGTTCTGCCTGGCATTT

Al-HindIII1 TCAAAATCCAAGTGGTATATGCACAGAAACATCATAATATGATGGAATATGACCAAATAGACTGTTGCA
Al-HindIII2 TCAAAATCCAAGTGGTAAATGCACAGAAACATCATAATATGATGGAATATGACCAAATATACTGTTGCA
Al-HindIII4 TCAAAATCCAAGTGGTAAATGCACAGATATATCATAATACGATGGAATACAACCACATATAGTGTTGCT
Al-HindIII6 TCAAAATCCAAGTGGTAAATGCACAGAAACATCATAATATGATGGAATATGACCAAATATACTGTTGCA

Al-HindIII1 GTGAAGACA-TTTGTGGTTCAGTTTGCGTTTTCAGAGAATTACAGCAAAAGTGATGAAAACAAGCTT
Al-HindIII2 GTGAAGAAA-TTTGTGGTT-AGTTTGCGTTTTCAGAGAATTACAGCAAAAGTGATGAAAACAAGCTT
Al-HindIII4 GTGAAGAAAATTTGTGGTT-AGTTTGCGTTTTCAGAGAGTTACAGCAAAAGTGATGAAAACAAGCTT
Al-HindIII6 GTGAAGAAA-TTTGTGGTT-AGTTTGCGTTTTCAGAGAATTACAGCAAAAGTGATGAAAACAAGCTT
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Figure 1. DNA sequences of the cloned *Al-Hind*III fragment of *A. lineatus*. The sites of cleavage of *Hind*III endonuclease are underlined. Microrepeat sequences are in boxes and base differences are in boldface type.

A. declivis, but no bands were observed in *G. nudus* and *T. paulistanus*.

The DNA sequencing of four recombinant clones showed that the *Al-Hind*III sequences had 204 bp and were AT-rich (63.72%) (Figure 1) as usually found in satellite DNA sequences of freshwater and marine fishes (Ekker, Fritz & Westerfield, 1992; Garrido-Ramos et al., 1994; Koehler et al., 1997; Reed, Dorschner & Phillips, 1997; Fischer et al., 2000). A Blastn search conducted in the NCBI did not reveal similarity of *Al-Hind*III sequences and other sequences deposited in this data bank. The *Al-Hind*III sequence of *A. lineatus* showed the presence of a AATATG repeat (Figure 1).

Although the origin of satellite sequences is not resolved, the presence of a microrepeat inside the isolated *A. lineatus* satellite sequence reinforces the hypothesis that this satellite family was originated from smaller sequences that duplicated themselves and accumulated a series of mutations, given origin to a larger sequence that could be duplicated after some events of incorrect crossing-over, replication by rolling circle or slippage (Fanning, 1987; Walsh, 1987; Stephan, 1989; Charlesworth, Sniegowski & Stephen, 1994).

The *Al-Hind*III fragment isolated from *A. lineatus* only hybridized to membrane immobilized *Hind*III-digested DNA of this species. No hybridization signals were observed in digested DNA samples of other fish species (*A. declivis*, *G. nudus* and *T. paulistanus*) (Figure 2a). To check the genomic array of the isolated DNA segment, membrane hybridization using partially and completely *Hind*III-digested DNA of *A. lineatus* was performed, showing the presence of ladder-like pattern (Figure 2b) characteristic of satellite DNAs (Charlesworth, Sniegowski & Stephen, 1994).

The absence of any signal in the Southern blot experiments involving the genomic DNA of

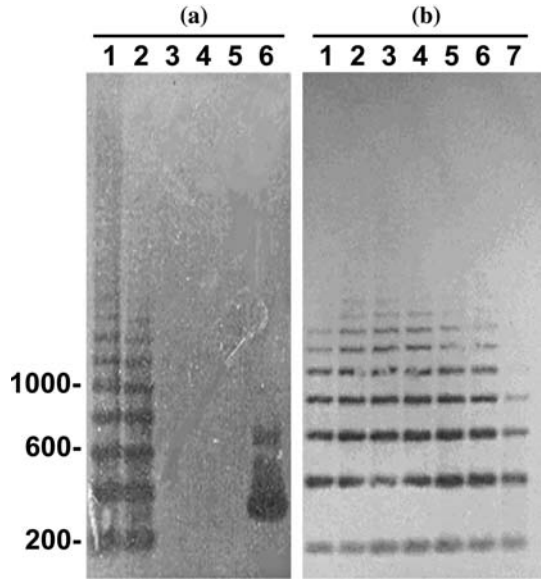

Figure 2. Genomic distribution and organization of *Al-Hind*III satellite family determined by Southern blot and membrane hybridization. (a) DNA samples of *A. lineatus* from Ubatuba (1) and São Vicente (2), *A. declivis* (3), *G. nudus* (4), *T. paulistanus* (5) and a plasmid fragment containing the *Al-Hind*III sequence (positive control) (6). (b) Partially to totally *Hind*III-digested genomic DNA of *A. lineatus* from 10 min to overnight (1–7). Molecular weight markers (bp) are shown on the left.

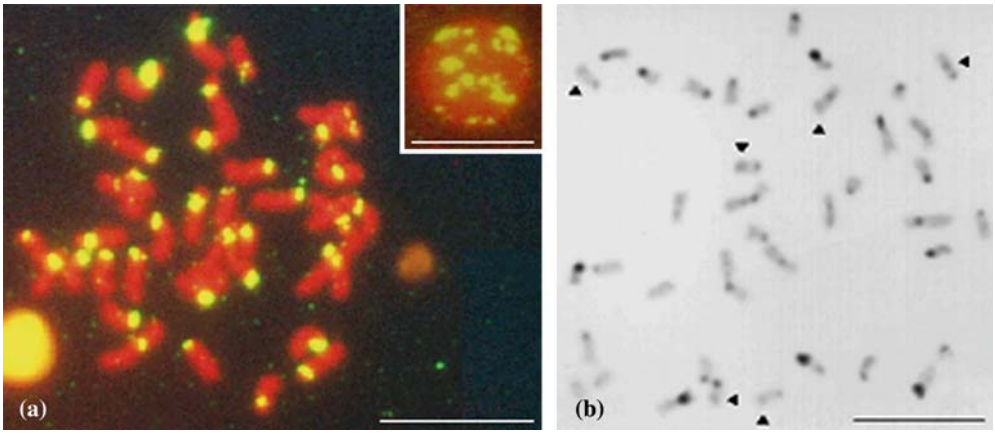

Figure 3. Metaphase chromosomes of *A. lineatus* showing the distribution of the *Al-Hind*III satellite family (a) and heterocrhomatin pattern distribution (b). In the inset in a, the organization of the *Al-Hind*III satellite family in the interphasic nucleus. The arrowheads indicate heterochromatin segments that do not hybridized to the *Al-Hind*III satellite. Bar = 10 μm.

A. declivis suggests that the band observed in the genomic DNA of this species after digestion by *Hind*III possibly corresponds to other satellite family in this species that has the same monomer size of the *Al-Hind*III satellite of *A. lineatus*. Further analysis of this other satellite sequence will be necessary to a better understanding of the relationships among these sequences. On the other hand, the presence of different satellite sequences in closely related species is not uncommon since a characteristic of these repetitive sequences is their rapid evolution (Singer, 1982; Charlesworth, Sniegowski & Stephen, 1994; Pimpinelli et al., 1995).

FISH experiments using *Al-Hind*III family as probe showed bright signals in the centromeric position of all chromosomes of *A. lineatus* (Figure 3a). The interphase nuclei usually exhibited the *Al-Hind*III DNA family organized in clusters, showing a tendency of the centromeres to stay associated (Figure 3a).

The presence of the *Al-Hind*III family in centromeric position of all chromosomes of *A. lineatus* may be associated to a specific function in this position or, alternatively, this satellite sequence may be associated to some centromeric sequence and are now evolving together. Although several studies have suggested some function to satellite sequences (Grady et al., 1992; Haaf et al., 1995; Dong et al., 1998; Li et al., 2002) any study demonstrated this putative function. Recent studies showed that some satellite sequences are transcribed (Bonaccorsi & Lohe, 1991) and, in some cases, a sex-specific transcription of satellite DNA was demonstrated (Rouleux-Bonnin, Bigot & Bigot, 2004). Further studies about transcription of *Al-Hind*III will be important to better understand the role of this sequence in the genome of in *A. lineatus*. The presence of a particular satellite sequence in the centromeres of all chromosomes of a species has been the most frequent situation found among the organisms so far studied (Hamilton, Honeyccut & Baker, 1990; Oliveira & Wright, 1998; Canapa et al., 2002; Viñas et al., 2004) showing the existence of a particular mechanism that promote a genomic turnover that is capable of homogenizing the satellite sequences. The observed tendency of the *Al-Hind*III family to be clustered in interphasic nuclei may be responsible for the homogenization of these sequences (Schweizer & Loidl, 1987).

A. lineatus showed C-band positive segments mainly distributed in the centromeric region of the chromosomes (Figure 3b), as usually found among fishes (Almeida-Toledo, 1998) and in other species of Achiridae (Azevedo et al. in press a, b). Besides the centromeric blocks, some smaller segments usually less stained were found in terminal and interstitial positions; these segments were not stained in FISH experiments (Figure 3a) showing that these additional C-band regions are composed by different satellite sequences.

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