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Research Article

5S rDNA characterization in twelve Sciaenidae fish species (Teleostei, Perciformes): Depicting gene diversity and molecular markers

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Abstract

In order to extend the genetic data on the Sciaenidae fish family, the present study had the purpose to characterize PCR-generated 5S rDNA repeats of twelve species of this group through PAGE (Polyacrylamide Gel Electrophoresis) analysis. The results showed the occurrence of at least two different 5S rDNA size classes in all the species. Moreover, 5S rDNA repeats of one of the studied species - *Isopisthus parvipinnis* - were cloned and subjected to nucleotide sequencing and Southern blot membrane hybridization analyses, which permitted to confirm the existence of two major 5S rDNA classes. Phylogenetic analysis based on the nucleotide sequences of different 5S rDNA repeats of *I. parvipinnis* lead to their separation into two major clusters. These results may reflect the high dynamism that rules the evolution rate of 5S rDNA repeats. The obtained data suggest that 5S rDNA can be useful in genetic analyses to identify species-specific markers and determine relationships among species of the Sciaenidae group.

Key words: Sciaenidae, fish, *Isopisthus parvipinnis*, 5S rDNA, molecular markers.

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Introduction

The 5S ribosomal multigene family (5S rDNA) of higher eukaryotes is comprised of tandemly repeated units of hundreds to thousands of copies that consist of a highly conserved coding sequence of 120 base pairs (bp) and a variable non-transcribed spacer sequence (NTS) (reviewed in Long and David, 1980). Studies on 5S rDNA organization could provide useful data for the understanding of genome organization and dynamics of repetitive sequences, and also provide genetic markers for the identification of species, subspecies, population, strain, or hybrids (*e.g.* Martins and Wasko, 2004). Although 5S rDNA repeats have been characterized in several vertebrate species, present data are mostly restricted to mammals and amphibians. To date, few analyses have been conducted on fishes, specially taking into account the great number of species of this group (*e.g.* Martins and Wasko, 2004).

The Sciaenidae (Perciformes) family contains approximately 70 genera and 270 fish species (Schwarzhans, 1993; Nelson, 1994) that are distributed in Indian, Pacific and Atlantic Oceans (Longhurst and Pauly, 1987; Sasaki, 1996). Although Sciaenidae is considered a monophyletic group, a great diversity of body shape and mouth position can be observed in several species, associated with different feeding patterns and life histories (Chao and Musick, 1977). Moreover, most species of the group represent important fishery resources with a high commercial value. However, despite the economic importance and the great species diversity, genetic studies are still scarce in this fish group. The available genetic data refer to molecular population genetic (Turner *et al.*, 1998; Lankford *et al.*, 1999; Gold *et al.*, 2001; Cordes and Graves, 2003; O'Malley *et al.*, 2003; Santos *et al.*, 2003) and phylogenetic analyses (Chao, 1978; Sasaki, 1989; Vinson *et al.*, 2004).

To improve the genetic data on Scianidae fish, the present study characterized the PCR-generated 5S rDNA repeat patterns of twelve species of the group. Moreover, 5S rDNA repeats of one of the species - *Isopisthus parvipinnis* - were cloned and subjected to nucleotide sequencing, Southern blot-membrane hybridization and phylogenetic analyses.

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Materials and Methods

Fish samples, DNA extraction and PCR

Samples of twelve different species of the Sciaenidae fish family (*Nebris microps, Paralonchurus brasiliensis, Stellifer stellifer, S. rastrifer, S. microps, Isopisthus parvipinnis, Cynoscion jamaicensis, C. virescens, Menticirrhus americanus, Micropogonias furnieri, Ctenosciaena gracilicirrhus,* and *Larimus breviceps*) from Ubatuba (São Paulo State, Brazil) were analyzed.

Genomic DNA was extracted from gill tissue, according to the method described by Wasko *et al.* (2003). A set of primers (Primer A 5'-TACGCCCGATCTCGTCCGAT C-3' and primer B 5'-CAGGCTGGTATGGCCGTAAG C-3'), corresponding to nucleotides 24-44 and 1-21, respectively, of the 5S coding region, were designed from the 5S rRNA sequence of rainbow trout (Komiya and Takemura, 1979) to amplify the 5S rRNA genes and their non-transcribed spacer regions (Martins and Galetti, 1999). PCR amplifications were carried out in a total volume of 50 μL, using 20-100 ng of genomic template DNA, 150 pmol of each primer, 1.25 mM of each dNTP, 1x PCR buffer containing 1.5 mM $MgCl_2$, and 1U of *Taq* DNA polymerase (GE Healthcare Life Sciences), using a PTC-200 Programmable Thermal Controller (MJ Research, INC). The optimum cycling times were as follows: 94 °C (5 min) denaturation, 35 cycles of 1 min at 95 °C, 30 s at 63 °C, and 1 min at 72 °C. A final 5 min extension was performed at 72 °C. The 5S rDNA-PCR products were visualized in 6% polyacrylamide gels by silver nitrate staining.

Cloning, nucleotide sequencing and sequence analyses

The PCR products of two DNA samples of *Isopisthus parvipinnis* were selected to be cloned and sequenced, and to be used in Southern blot-membrane hybridization and phylogenetic analyses, since this species presented the most common banding pattern among the analyzed Sciaenidae fish species, which was also observed in *Stellifer microps, Stellifer rastrifer, Cynoscion jamaicensis,* and *Cynoscion virescens*. The PCR products of *I. parvipinnis* were inserted into pGEM-T (Promega), which was used to transform competent cells of *E. coli* DH5α strain (Invitrogen), according to the manufacturer's instructions. Positive recombinant clones were recovered and stored in 75% glycerol at -80 °C for subsequent analysis. Several clones were sequenced on an ABI Prism 377 automatic DNA sequencer (Applied Biosystems) with DYEnamic ET Terminator Cycle Sequencing (GE Healthcare Life Sciences), following the manufacturer's instructions. Nucleic acid sequences were subjected to BLASTN (Altschul *et al.*, 1990) searches at the National Center for Biotechnology Information (NCBI), and the sequence alignment was performed using the computer program Clustal W (Thompson *et al.*, 1994) and by eye. Neighbor-Joining (NJ) phylogenetic analyses employing the Kimura-two-parameter genetic distance model (Kimura, 1980) were conducted using MEGA version 3.1 software (Kumar *et al.*, 2004). Bootstrap resampling using 1000 replicates (Felsenstein, 1985) was applied to assess support for individual nodes.

Southern blot hybridization

Genomic DNA samples of *Isopisthus parvipinnis* (8 μg) were completely and partially digested with *Hind* III. This restriction enzyme was chosen since it cuts once inside the 5S rRNA gene of most teleost fish species (Martins and Wasko, 2004). The digestion products were subjected to 1% agarose gel electrophoresis and Southern-transferred to a Hybond-N nylon membrane (Southern, 1975). The hybridization of the filter-immobilized DNA was performed using as probes clones containing repeat units of *I. parvipinnis* 5S rDNA. Probe labeling, hybridization and detection steps were performed with the kit ECL-Direct nucleic Acid Labeling and Detection System (GE Healthcare Life Sciences) following the manufacturer's instructions.

Results and Discussion

PCR amplification of 5S rDNA repeats of twelve Sciaenidae species generated distinct polyacrylamide gel electrophoresis banding patterns (Figure 1). The different sizes of the amplified fragments may reflect the intense dynamism that rules the evolution of the 5S rDNA tandem arrays in the fish genome. As the 5S rDNA repeats consist of a 120 bp conserved coding sequence, the observed differences among several Sciaenidae species is related to the NTS regions that can be extremely variable due to inser-

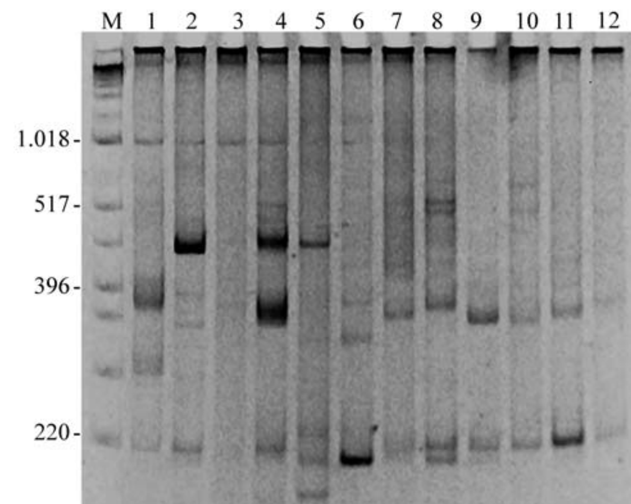

Figure 1 - 5S rDNA PCR products of Sciaenidae species visualized on 6% polyacrylamide gel: (1) *Larimus breviceps*, (2) *Nebris microps*, (3) *Paralonchurus brasiliensis*, (4) *Stellifer stellifer*, (5) *Menticirrhus americanus*, (6) *Micropogonias furnieri*, (7) *S. microps*, (8) *Ctenosciaena gracilicirrhus*, (9) *S. rastrifer*, (10) *Isopisthus parvipinnis*, (11) *Cynoscion jamaicensis*, and (12) *C. virescens.* M1 and M2 represent molecular mass markers of 1 kb Plus and 10 bp, respectively. The corresponding molecular masses are indicated on the left of the figure.

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Ip5S49d TACGCCCGAT CTCGTCCGAT CTCGGAAGCT AAGCAGGGTC AGGCCTGGTT AGTACTTGGA CGGGTGACCG CCTGGGAATA CCAGGTGCTG
Ip5S49f .......... .......... .......... .G........ .......... .......... .......... .......... .......T..
Ip5S49h .......... .......... .......... .....T.... .......... .......... .......... .......G.. ..........
Ip5S49i .......... .......... .......... .......... .......... .......... .......... .......... ..........
Ip5S49j .......... .......... .......... .......... .......... .......... .......... .......... ..........
Ip5S49o .......... .......... .......... .......... .......... .......... .......... .......... ..........
Ip5S55m .......... .......... .......... .....A.... .......... .......... T...A..... .......... ..........
Ip5S55D .......... ......G... .......... .......... G...C..... .......... T...A..... .......... ..........
Ip5S55H .......... .......... .......... .......... G......... .......... T...A..... .......... ..........

Ip5S49d TAAGCTTTTT CACAAAACCT C-CATCGGCT CCTTCAACGG CCTCGTTGTT G-ATGGATGA CGTACA---C ATAGGCGCCG TTTCTCACCC
Ip5S49f .......... .......T.. .-........ ........A. .......... .-........ ......---. .......... ..........
Ip5S49h .......... .......... .-........ .......TA. ...T...... .-........ ......---. .......... ..........
Ip5S49i .......... .......... .-...G.... ........A. .......... .-........ ......---. .......... ..........
Ip5S49j C......... .......... .-........ ........A. ....T..... .-....A... ......---. .......... ..........
Ip5S49o .......... .......... .-........ ........A. .......... .-........ ......---. .......... ..........
Ip5S55m .......... .....G.G-- ---..A.TG. ....A..T.. ..A....... .-....C... .AC..T---. ...TAG.G.A ...G....A.
Ip5S55D .......... ...TCCT.T. TG..GA..AC G..G.TGTT. .T..T.G.C. .C.CA.GCAG ..CT..GAA- .C.CTA..A. C..GC..G..
Ip5S55H .......... ...TCCT.T. TG..GA..AC G..G.TGTT. .T..T.G.C. .C.CA.GGAG ..CT..GAA- .C.C-A..A. C..GC..G..

Ip5S49d ACCAAGA--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49f .......--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49h .......--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49i .......--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49j C......--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49o .......--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S55m ......C--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S55D GA..C.CTGA AATGTCTAAG ATATTTATTG AACT-GCCTG ACACTTATTT GCCTCAGACG CTTGACTCCG GTCTCTAGCT GGTCATGAGG
Ip5S55H GA..C.CTGA AA------AG ATATTTATTG AACTTGCCTG ACACTTATTT GCCTCAGACG CTTGACTCCG GTCTCTAGCT GGTCATGAGG

Ip5S49d ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49f ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49h ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49i ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49j ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S49o ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S55m ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ip5S55D CCTGTGAAAT GAGTGCCACG GACAGCGGTG GAAGTGCCAG GATGTCTCTC CGGATGAAAA CGTCCACAAA C-GGATCGGC TACGGTGAGG
Ip5S55H CCTGTGAAAT GAGTGCCACG GACAGCGGTG GAAGTGCCAG GATGTCTCTC CGGATGAAAA CGTCCACAAA C-GGATCG-C TACGGTGAG-

Ip5S49d ---------- ---------- ---------- ---------- ------GGCT TACGGCCATA CCAGCCTGA [205]
Ip5S49f ---------- ---------- ---------- ---------- ------.... .......... ......... [205]
Ip5S49h ---------- ---------- ---------- ---------- ------.... .......... ......... [205]
Ip5S49i ---------- ---------- ---------- ---------- ------.... .......... ......... [205]
Ip5S49j ---------- ---------- ---------- ---------- ------.... .......... ......... [205]
Ip5S49o ---------- ---------- ---------- ---------- ------.... .......... ......... [204]
Ip5S55m ---------- ---------- ---------- ---------- ------.... .......... ......... [201]
Ip5S55D CGCGGCCATG TATAAATAAG CGTAATTTGC CGGCTGTCAC CTCGTG.... .......... ......... [426]
Ip5S55H CGCGGCCATG TATAAAT-AG CGTATTTTGC --GCTGTC-C CTCGTG.... .......... ......... [413]
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Figure 2 - Alignment of 5S rDNA nucleotide sequences of *Isopisthus parvipinnis*. The 5S rRNA gene coding sequence is in bold face and the primer regions are underlined. Dots indicate sequence identity, hyphens represent indels.

tions/deletions, minirepeats, and pseudogenes (Nelson and Honda, 1985; Leah *et al.*, 1990; Sajdak *et al.*, 1998).

On the other hand, it was possible to identify similar 5S rDNA amplification patterns for some species of the same genus, such as *Stellifer microps* and *S. rastrifer,* and *Cynoscion jamaicensis* and *C. virescens* (Figure 1). Moreover, *Isopisthus parvipinnis* also presented a PCR amplification pattern that resembles the pattern evidenced for the four former species (Figure 1). It was also possible to note that the amplified fragments obtained for *Stellifer stellifer* were very similar to the ones obtained for *Nebris microps* (Figure 1). Similar sized 5S-PCR products may reflect a higher genetic similarity among some Sciaenidae species, as already evidenced for other fish (*e.g.* Wasko *et al.*, 2001). However, we could not discard the possibility that similar DNA fragment sizes may also present large nucleotide differences. Although mitochondrial DNA sequence analysis has shown a distant relationship between *Cynoscion* and *Stellifer* (Vinson *et al.*, 2004), previously published morphometric data (Aguirre and Shervette, 2005) are in accordance with the present results that suggest a closer relationship between these two genera.

Despite the greater resemblance in the 5S rDNA PCR products of *Steliffer microps*, *S. rastrifer, Cynoscion jamaicensis*, *C. virescens*, and *Isopisthus parvipinnis*, all the other studied Sciaenidae species presented unique 5S rDNA amplification banding patterns, leading to the identification of species-specific DNA markers. 5S rDNA PCR

amplified products clearly discriminate other fish species, such as the Atlantic salmon (*Salmo salar*), the brown trout (*Salmo truta*), and their hybrids (Pendás *et al.*, 1995). Several species of the genus *Brycon* (Wasko *et al.*, 2001), and *Solea solea* and *Reinhardtius hippoglossoides* (Céspedes *et al.*, 1999) also show 5S rDNA PCR species-specific patterns. PCR amplification of 5S rDNA repeats thus represents a potential and simple methodology that can be applied to identify several fish species.

PAGE analysis of the 5S rDNA PCR products also showed at least two amplified fragments for all the Sciaenidae species. Each fragment could correspond to a distinct 5S rDNA class, as already detected for other fish species through PCR electrophoresis and nucleotide sequencing analyses (Martins and Galetti, 2001; Martins and Wasko, 2004). Although the occurrence of two distinct 5S rDNA classes represents the most common feature in fish (Martins and Wasko, 2004), some species may also present more than two 5S rDNA types, as shown for some of the analyzed Sciaenidae species with three or more clearly distinct PCR generated fragments (Figure 1).

In order to characterize the 5S rDNA nucleotide sequence and genomic organization for one of the analyzed species and also to verify the occurrence of two distinct classes of this repetitive ribosomal DNA in Sciaenidae fish, the two amplified fragments of *Isopisthus parvipinnis* - with around 200 and 400 base pairs - were cloned, sequenced, and subjected to Southern blot-membrane hybridization and phylogenetic analyses. The nucleotide sequencing analysis of these two fragments supports the existence of two different 5S rDNA size classes, named 5S rDNA type I (201-205 bp) and 5S rDNA type II (413-426 bp) (Figure 2). Both classes showed a conserved 120 bp 5S rRNA gene sequence and a variable NTS ranging between 80 bp (5S rDNA type I) to 280 bp (5S rDNA type II). These variations were characterized by insertions/deletions, and base substitutions. These 5S rDNA classes were separated in the phylogenetic analysis in 100% of the generated trees (Figure 3). Yet, one single sequence of 5S rDNA type I was quite different and could be considered a subclass since it appeared separated in 98% of the generated trees (Figure 3). Although the occurrence of two 5S rDNA repeat classes represents the most common feature in fishes (Martins and Galetti 2001; Martins and Wasko 2004), the presence of variant subclasses also seems to be frequent in this vertebrate group. The presence of two major repeated 5S rDNA classes in *I. parvipinnis* was confirmed by Southern blot hybridization which revealed the presence of tandem repeats of 200 and 400 bp, in agreement with the PCR products and the nucleotide sequencing data (Figure 4).

We take these results as reflecting the high dynamism that governs the evolution rate of the 5S rDNA repeats in the genome of *Isopisthus parvipinnis* and also in other Sciaenidae species. Moreover, the present data indicate that 5S rDNA can be useful for general and applied genetic analyses of different sciaenids, in order to identify species-specific markers and determine species relationships.

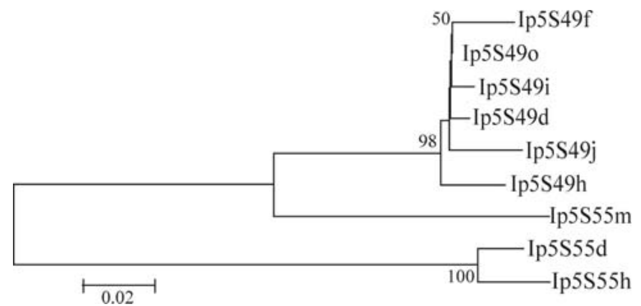

Figure 3 - Neighbor-joining three based on the 5S rDNA nucleotide sequences of *Isopisthus parvipinnis*. The numbers at each node indicate the percentage recovery (> 50%) of the particular node (1000 bootstrap replicates).

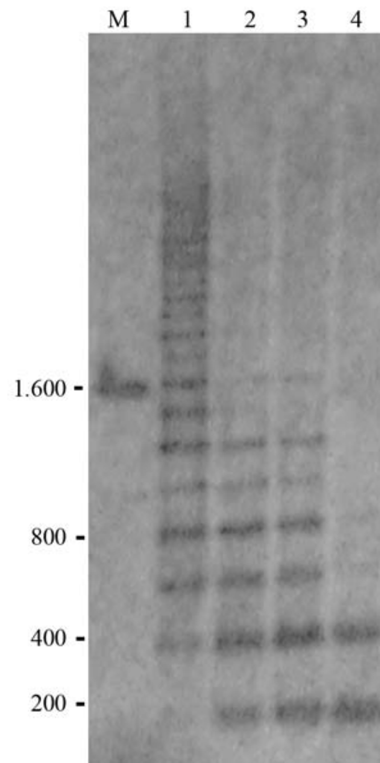

Figure 4 - Southern blot hybridization results of 5S rDNA sequences to genomic DNA of *Isopisthus parvipinnis* digested with *Hind*III endonuclease. 1, 2 and 3 are partially digested genomic DNA samples (10, 30 and 60 min digestion, respectively), and 4 is a totally digested DNA sample. M, molecular mass marker (bp).

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Internet Resources

National Center for Biotechnology Information (NCBI) (http:www.ncbi.nlm.nih.gov/blast).

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