# Molecular organization of 5S rDNA in fishes of the genus *Brycon*

**Adriane Pinto Wasko, Cesar Martins, Jonathan M. Wright, and Pedro Manoel Galetti, Jr.**

**Abstract**: There are few reports on the genomic organization of 5S rDNA in fish species. To characterize the 5S rDNA nucleotide sequence and chromosomal localization in the Neotropical fishes of the genus *Brycon*, 5S rDNA copies from seven species were generated by PCR. The nucleotide sequences of the coding region (5S rRNA gene) and the nontranscribed spacer (NTS) were determined, revealing that the 5S rRNA genes were highly conserved, while the NTSs were widely variable among the species analyzed. Moreover, two classes of NTS were detected in each species, characterized by base substitutions and insertions–deletions. Using fluorescence in situ hybridization (FISH), two 5S rDNA chromosome loci that could be related to the two 5S rDNA NTS classes were observed in at least one of the species studied. 5S rDNA sequencing and chromosomal localization permitted the characterization of *Brycon* spp. and suggest a higher similarity among some of them. The data obtained indicate that the 5S rDNA can be an useful genetic marker for species identification and evolutionary studies.

*Key words*: *Brycon*, FISH, nontranscribed spacer, nucleotide sequence, 5S rDNA.

**Résumé** : Il y a peu de travaux rapportés sur l'organisation génomique de l'ADNr 5S chez les poissons. Afin de caractériser la séquence nucléotidique et la localisation chromosomique de l'ADNr 5S chez des poissons néotropicaux du genre *Brycon*, des copies de l'ADNr 5S ont été obtenues par PCR chez sept espèces. La séquence nucléotidique de la région codante (gène d'ARNr 5S) et de l'espaceur non-transcrit (NTS) a été déterminée. Ces travaux ont révélé que les gènes d'ARNr 5S étaient très conservés tandis que les régions NTS étaient très variables parmi les espèces analysées. De plus, deux classes de NTS ont été détectées chez chacune des espèces et ces séquences se distinguaient en raison de substitutions nucléotidiques et d'insertions–délétions. Deux locus chromosomiques d'ADNr 5S ont été observés par hybridation in situ en fluorescence (FISH) chez au moins une des espèces étudiées, ce qui pourrait être lié à la présence de deux classes de NTS. Le séquençage et la localisation de l'ADNr 5S ont permis de caractériser les espèces du genre *Brycon* et suggèrent une plus grande similarité entre certaines espèces. Les données obtenues montrent que l'ADNr 5S peut être un marqueur génétique utile pour des fins d'identification des espèces et pour des études de l'évolution.

*Mots clés* : *Brycon*, FISH, espaceur non-transcrit, séquence nucléotidique, ADNr 5S.

[Traduit par la Rédaction]

## Introduction

Ribosomal DNA (rDNA) is organized in higher eukaryotes into two distinct gene classes composed of tandemly repeated units of hundreds to thousands of copies. The major class (45S rDNA) comprises the 18S, 5.8S, and 28S rRNA genes, and is related to the nucleolar organizer regions (NORs), and the minor class (5S rDNA) is represented by the 5S rRNA gene family. The 5S rDNA repeats consist of a highly conserved coding sequence of 120 bp and a nontranscribed flanking DNA (nontranscribed spacer; NTS) (reviewed in Long and David 1980). Variations in the NTS, related to insertions–deletions, minirepeats, and pseudogenes, are often species specific and have been successfully used in evolutionary studies (Nelson and Honda 1985; Leah et al. 1990; Sajdak et al. 1998). In addition, the chromosomal localization of the 5S rDNA has been reported for some animals, including fish (Pendás et al. 1994; Móran et al. 1996; Fujiwara et al. 1998; Murakami and Fujitani 1998; Sajdak et al. 1998; Martins et al. 2000), although few analyses have been performed in Neotropical species (Martins and Galetti 1999, 2000, 2001; Born and Bertollo 2000).

Fishes of the genus *Brycon* have been reported for all main hydrographic Brazilian systems and represent important fishery resources and (or) hatchery stocks in some areas. However, human activities have threatened or led to the extinction of some populations (Braga 1982), and conservation actions in the wild are badly needed. Moreover, little is

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**Table 1.** Description of *Brycon* spp. analyzed.

| Species | Collection site | Clones | Length (bp) | | | GenBank No. |
|---|---|---|---|---|---|---|
| | | | Gene | NTS | 5S repeat | |
| *B. lundii* | São Francisco River (Township of Três Marias, Minas Gerais State) | Bl 122 (1) | 120 | 123 | 243 | AF250513 |
| | | Bl 125 (1) | 120 | 122 | 242 | AF250514 |
| | | Bl 124 (1) | 120 | 123 | 243 | AF250515 |
| | | Bl 51 (1) | 120 | 123 | 243 | AF250516 |
| | | Bl 52 (2) | 120 | 108 | 228 | AF250511 |
| | | Bl 56 (2) | 120 | 108 | 228 | AF250512 |
| *B. orbignyanus* | Paraná River (Township of Porto Rico, Paraná State) | Bo 2 (1) | 120 | 120 | 240 | AF250519 |
| | | Bo 5 (1) | 120 | 121 | 241 | AF250520 |
| | | Bo 3 (2) | 120 | 106 | 226 | AF250517 |
| | | Bo 4 (2) | 120 | 106 | 226 | AF250518 |
| *B. microlepis* | Cuiabá River (Township of Cuiabá, Mato Grosso State) | Bm 12 (1) | 120 | 120 | 240 | AF250521 |
| | | Bm 18 (1) | 120 | 120 | 240 | AF250522 |
| | | Bm 4 (1) | 120 | 120 | 240 | AF250523 |
| | | Bm 16 (1) | 120 | 120 | 240 | AF250524 |
| | | Bm 15 (1) | 120 | 120 | 240 | AF250525 |
| | | Bm 13 (2) | 120 | 104 | 224 | AF250526 |
| | | Bm 14 (2) | 120 | 104 | 224 | AF250527 |
| *B. cephalus* | Amazonas River (Township of Manaus, Amazonas State) | Bc 13 (2) | 120 | 227 | 347 | AF250530 |
| | | Bc 12 (2) | 120 | 229 | 349 | AF250531 |
| | | Bc 15 (2) | 120 | 227 | 347 | AF250532 |
| | | Bc 14 (d) | 120 | 232 | 352 | AF250528 |
| | | Bc 33 (d) | 120 | 232 | 352 | AF250529 |
| *Brycon* sp. | Araguaia River (Township of Aragarças, Goiás State) | Bsp 3 (2) | 120 | 220 | 340 | AF250533 |
| | | Bsp 7 (2) | 120 | 221 | 341 | AF250534 |
| | | Bsp 11 (2) | 120 | 221 | 341 | AF250535 |
| | | Bsp 17 (d) | 120 | 222–223 | 342–343 | AF250536 |
| *B. brevicauda* | Araguaia River (Township of Aragarças, Goiás State) | Bb 6 (1) | 120 | 119 | 239 | AF250537 |
| | | Bb 12 (1) | 120 | 117 | 237 | AF250538 |
| | | Bb 5 (1) | 120 | 118 | 238 | AF250539 |
| | | Bb 1 (2) | 120 | 677 | 797 | AF250540 |
| | | Bb 13 (2) | 120 | 678 | 798 | AF250541 |
| *B. insignis* | Paraíba do Sul River (Township of Pindamonhangaba, São Paulo State) | Bi 2 (1) | 120 | 115 | 235 | AF250545 |
| | | Bi 8 (1) | 120 | 115 | 235 | AF250546 |
| | | Bi 13 (2) | 120 | 391 | 511 | AF250542 |
| | | Bi 14 (2) | 120 | 391 | 511 | AF250543 |
| | | Bi 15 (2) | 120 | 391 | 511 | AF250544 |

**Note:** Values in parentheses indicate whether the isolated clone was a 5S rDNA NTS-I (1), a 5S rDNA NTS-II (2), or a dimeric 5S rDNA (d) unit.



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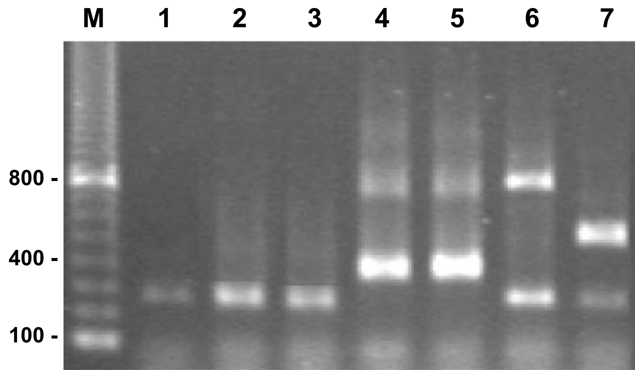


**Fig. 1.** PCR-generated 5S rDNA products obtained from *B. lundii* (lane 1), *B. orbignyanus* (2), *B. microlepis* (3), *B. cephalus* (4), *Brycon* sp. (5), *B. brevicauda* (6), and *B. insignis* (7). Lane M is a 100-bp molecular weight ladder.

known of the genetics of this fish group, which is considered to be a monophyletic unit with about 40 species (Howes 1982). As part of an ongoing effort to characterize species of the genus *Brycon* and to examine relationships among them, the 5S rDNA nucleotide sequence and chromosomal localization were analyzed in seven species of the group. To address this issue, PCR-generated 5S rDNA repeats were cloned, sequenced, and used as probes in chromosomal fluorescence in situ hybridization (FISH).

## Materials and methods

### PCR

Seven species of the genus *Brycon* from different Brazilian river systems were studied (Table 1). Genomic DNA was extracted from the liver according to the phenol–chloroform method detailed in Sambrook et al. (1989). A set of primers corresponding to nucleotides 24–44 (primer A, 5′-TACGCCCGATCTCGTCCGATC-3′) and nucleotides 1–21 (primer B, 5′-CAGGCTGGTATGGCCGTAAGC-3′) of the 5S coding region were designed from the 5S rDNA sequence of rainbow trout (Komiya and Takemura 1979), to amplify the 5S rRNA genes and their NTS regions (Pendás et al. 1994; Martins and Galetti 1999). PCR amplifications were carried out in a total volume of 50 μL containing 50 ng of genomic template DNA, 150 pmol of each primer, 1.25 mM of each dNTP, PCR buffer containing 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Pharmacia.Biotech), using a Perkin Elmer 2400 thermocycler. The optimum cycling times were as follows: 5 min at 94°C (denaturation); 35 cycles of 1 min at 95°C, 30 s at 63°C, and 1 min at 72°C (amplification); and a final 7 min at 72°C (extension). The 5S rDNA-PCR products were visualized by ethidium bromide staining of a 1.5% agarose gel.

### Cloning and sequencing

The PCR products were cloned in the plasmids pGEM-T or pGEM-T Easy (Promega) and used to transform competent cells of *Escherichia coli* strain DH5α (GibcoBRL). A total of 36 clones were sequenced on a LICOR 4200 automatic sequencer with a T7 Sequenase 7-deza-dGTP sequencing kit (Amersham); sequence alignment was performed using Clustal W (Thompson et al. 1994). 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).

**Table 2.** Percent sequence identity of 5S rRNA genes (above the diagonal) and NTS-I and NTS-II regions (below the diagonal) among the *Brycon* spp. analyzed.

| | | Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5S rDNA (NTS-I) | 1 | *B. lundii* | — | 0.97 | 0.96 | 0.95 | 0.94 | 0.99 | 0.97 | 0.94 | 0.92 | 0.93 | 0.94 | 0.94 |
| | 2 | *B. orbignyanus* | 0.95 | — | 0.99 | 0.98 | 0.94 | 0.96 | 1.00 | 0.97 | 0.94 | 0.95 | 0.96 | 0.96 |
| | 3 | *B. microlepis* | 0.82 | 0.86 | — | 0.97 | 0.94 | 0.95 | 0.99 | 0.96 | 0.94 | 0.94 | 0.95 | 0.95 |
| | 4 | *B. brevicauda* | 0.79 | 0.80 | 0.80 | — | 0.94 | 0.94 | 0.98 | 0.96 | 0.94 | 0.95 | 0.96 | 0.96 |
| | 5 | *B. insignis* | 0.88 | 0.89 | 0.83 | 0.81 | — | 0.94 | 0.94 | 0.93 | 0.93 | 0.94 | 0.94 | 0.94 |
| 5S rDNA (NTS-II) | 6 | *B. lundii* | 0.99 | 0.93 | 0.78 | 0.76 | 0.80 | — | 0.96 | 0.94 | 0.93 | 0.94 | 0.94 | 0.94 |
| | 7 | *B. orbignyanus* | 0.84 | 0.87 | 0.88 | 0.76 | 0.80 | 0.84 | — | 0.97 | 0.94 | 0.95 | 0.96 | 0.96 |
| | 8 | *B. microlepis* | 0.83 | 0.89 | 0.89 | 0.82 | 0.82 | 0.83 | 0.99 | — | 0.93 | 0.94 | 0.94 | 0.94 |
| | 9 | *B. cephalus* | 0.22 | 0.20 | 0.23 | 0.22 | 0.31 | 0.25 | 0.37 | 0.16 | — | 0.94 | 0.96 | 0.95 |
| | 10 | *Brycon* sp. | 0.22 | 0.13 | 0.23 | 0.22 | 0.16 | 0.25 | 0.22 | 0.16 | 0.93 | — | 0.95 | 0.95 |
| | 11 | *B. brevicauda* | 0.24 | 0.17 | 0.27 | 0.27 | 0.09 | 0.28 | 0.29 | 0.21 | 0.66 | 0.63 | — | 0.99 |
| | 12 | *B. insignis* | 0.29 | 0.24 | 0.24 | 0.23 | 0.29 | 0.33 | 0.27 | 0.58 | 0.55 | 0.57 | 0.30 | — |

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**Fig. 2.** Alignment of 5S rDNA consensus sequences of *B. lundii* (*a*), *B. orbignyanus* (*b*), *B. microlepis* (*c*), *B. cephalus* (*d*), *Brycon* sp. (*e*), *B. brevicauda* (*f*), and *B. insignis* (*g*). The coding sequence of the 5S rDNA is in boldface type and the primers used to amplify the 5S rDNA are underlined. Hyphens indicate gaps. Base substitutions are indicated in red letters. TATA-like elements are indicated in blue and a conserved 5′-NTS region is shown in green.

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(a)                                                                                                       93
NTS-I    TACGCCCGATCTCGTCCGATCTCGGAAACAAAGCAGGGTCTGGCCTGATTAGTACTTGGATGGGAGACTGCTTGGGAATACCAGGTGCCGTAA
NTS-II   TACGCCCGATCTCGTCCGATCTCGGAAACAAAGCAGGGTCTGGCCTGATTAGTACTTGGATGGGAGACTGCTTGGGAATACCAGGTGCTGTAA
                                                                                                        186
NTS-I    GCTTTTCCAACCCGGCAAACAATCGAAGGTTGAATTTGCTTTATATCTTTTTGCTTTATAGCTTACATTTGCTTCATTTTGTTTATTTTTTTT
NTS-II   GCTTTTCCAACCCGGCAAACAATCGAAGGTTGAATTTGCTTTATA--------------GCTTACATTTGCTTCATTTTGTTTATTTTTTTT
                                                       243
NTS-I    CATTTCCTTTATACTCTGATACTCAACGAAGCTTCTGCTTACGGCCATACCAGCCTG
NTS-II   CATTTCCTTTATACTCTGATACTCAACGAAGCTTCTGCTTACGGCCATACCAGCCTG

(b)                                                                                                       93
NTS-I    TACGCCCGATCTCGTCCGATCTCGGAAGCAAAGCAGGGTCGGGCCTGATTAGTACTTGGATGGGAGACTGCCTGGGAATACCAGGTGCCGTAA
NTS-II   TACGCCCGATCTCGTCCGATCTCGGAAGCAAAGCAGGGTCGGGCCTGATTAGTACTTGGATGGGAGACTGCCTGGGAATACCAGGTGCCGTAA
                                                                                                        186
NTS-I    GCTTTTCCAACCCTGCAAACAATCGAAGGTTGAATTTGCTTTATATCTTTTTGCTTTATAGCTTACATTTGCTTTATTTTTTATTTTTTTTCA
NTS-II   GCTTTTCCAACCCTGCAAACAATCGAAGGTTGCATTTGCTTTATA--------------GCTTACATTGGCTTAATTTTTTATTTTTTTTCA
                                                     241
NTS-I    TTTCCTTTATACTCTGCTTCTCAAAGAAGCTTCTGCTTACGGCCATACCAGCCTG
NTS-II   TTTGCTTTATACTCTGCTTCTCAAACTGTTTTTAGCTTACGGCCATACCAGCCTG

(c)                                                                                                       93
NTS-I    TACGCCCGATCTCGTCCGATCTCGGAAGCAAAGCAGGGTCGGGCCTGATTAGTACTTGGATGGGAGACTGGCTGGGAATACCAGGTGCCGTAA
NTS-II   TACGCCCGATCTCGTCCGATCTCGGAAGCCAAGCAGAGTCGGGCCTGATTAGTACTTGGATGGGAGACTGCCTAGGAATACCAGGTGCCGTAA
                                                                                                        186
NTS-I    GCTTTTCCAACCCTACAAACAATCGAAGGTTGAATTTGCTTTATATCTTTTTGCTTTATAGCTTAGATTTGCTTTATTATATATTTTTTTTAT
NTS-II   GCTTTTCCAACCCTGCAAACAATCGAAGGTTGCATTTGCTTTATA--------------GCTTACATTGGCTTTATTTTTTTTTTTTTTCAT
                                                    240
NTS-I    TTGCTTTTTACTCTACTTCTCAAAATATTTTTAGCTTACGGCCATACCAGCCTG
NTS-II   TTGCTTTATACTCTGCTTCTCAAA-TGTTTTTAGCTTACGGCCATACCAGCCTG

(d)                                                                                                       93
NTS-II   TACGCCCGATCTCGTCCGATCTCGGAAGCTAACCAAGGTCGGGCCTGGTTAGTACTTGGATGGGAAACTGCCTGGGAATACCAGGTGCTGTAA
                                                                                                        186
         GCTTTTTTCCAACCTGAAGCACTTACATGCACGTACATTCAGTTCAGTGTCTTTACTTTGTTTTACTCCATCTCATTTTATGTTTTCAGAAAT
                                                                                                        279
         GTACAAGAGATTACACTATAGTTTTATATACATCACTGTATTACATTTCATACAGCCTTTGGAGTCTTGAGAGTGAAAAAAATGTAGATACC
                                                                      349
         CTTTGAAAAGCTTTGCATTTATAAATAGGCTCTCTGGAGCAGTGCTCTAGCTTACGGCCATACCAGCCTG

(e)                                                                                                       93
NTS-II   TACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGGGTTGGGCCTGATTAGTACTTTGAAGGGAGACTGCCTGGGAATACCAGGTGCTGTAA
                                                                                                        186
         GCTTTTTTCCAACCTGAAGCACTTTCATGCACGTACATTCAGTTCAGTGTCTTTACTTTGTTTTACTCCATCTCATTTTATGTTTTCAGAAAT
                                                                                                        279
         GTACACGAGATTACACTATAGTTTTATATACATCACTGTATTACATATCATACAGCTTTGGAGCTGAGAGTGAAAATGTGAAAATGCAGACAC
                                                              341
         CTTTGAAACTTTGCATTATATAGCTATCTGGAGCATATCTCGCTTACGGCCATACCAGCCTG

(f)                                                                                                       93
NTS-I    TACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGGGTCGGGCCTGATTAGTACTTGGATGGGAGACTTCCTGGGAATACCAGGTGCCGTAA
NTS-II   TACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGGGTCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAA
                                                                                                        186
NTS-I    GCTTTTTCCAACCC------------TGCAAA-------CAATCGAAGGT-TGAATTTGC---------------------------------
NTS-II   GCTTTTTCCAACCTCACGCACGTACATGCACATTTTTTTCAGTTGAATCTCTTTACTTTGTTTTACTCCACCTAACTTTCTGTTTTCACAAAT
                                                                                                        279
NTS-I    ----------------------TTTATATC----------TT---TTT------GCCTTT---------------------------------
NTS-II   GTACAAGAGATGACACTAGAGTTTTATATACATCACTGTATTACATTTCACACAGCCTTTGGAGTCTTGAGAGTGAAAACATATGAGCGTCAG
                                                                                                        372
NTS-I    ---------------ACCT--------------------------------------------------------------------------
NTS-II   CTGCGCTTTAAACCCACCTGCCTGGCCTGCCATGTCATGTGAATGAATAAATTGTACTTAGGCTTAATTCCATTTAATTTCTCTGAAAACAAA
                                                                                                        465
NTS-I    ---------------------TCCTTTGCAT---------------------------------------------TATCAGTATTTT-----
NTS-II   ACACAGAAACATACAGTGCAATCATTTGTATGAAAGGAATGCTGAGAATAAAAATACCTGAAGCCTGCCAGTAGCTTGCCAGTACTTTTCACA
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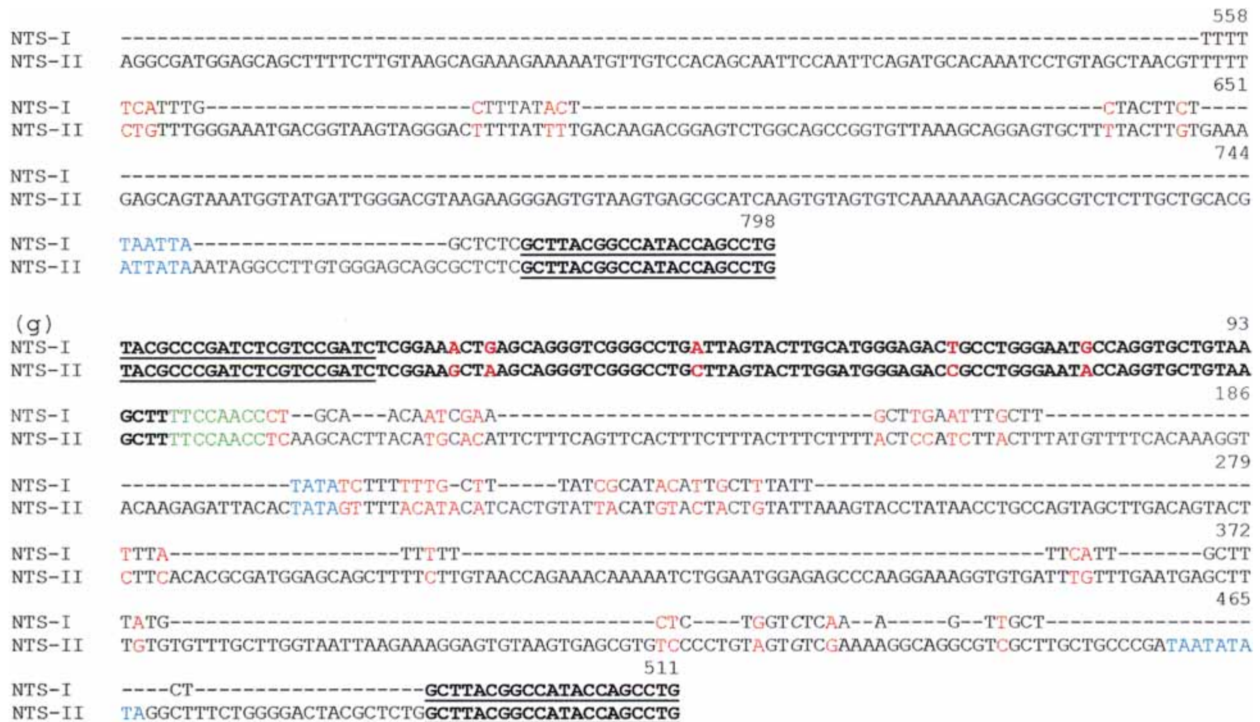
**Fig. 2** (*concluded*).



**Chromosomal localization**

Mitotic chromosomes were obtained from suspensions of anterior kidney cells using a conventional air-drying technique (Bertollo et al. 1978) and short-term solid tissue culture (Fenocchio et al. 1991). FISH was performed as detailed in Wasko and Galetti (2000), using a PCR-generated 5S rDNA of *B. lundii* as a probe (labeled by nick translation with biotin-dATP; Bionick$^{TM}$ Labeling System; GibcoBRL).

## Results and discussion

PCR amplification of 5S rDNA in seven *Brycon* spp. generated distinct agarose gel electrophoresis band patterns among some species: a band of approximately 250 bp for *B. lundii*, *B. orbignyanus*, and *B. microlepis*, two bands of about 350 and 700 bp for *B. cephalus* and *Brycon* sp., two bands of about 250 and 800 bp for *B. brevicauda*, and two bands of approximately 250 and 500 bp for *B. insignis* (Fig. 1). Sequencing analyses showed that *B. lundii*, *B. orbignyanus*, and *B. microlepis* presented two similarly sized 5S PCR products of 224–228 and 240–243 bp that were not distinguishable in agarose gel where they were seen as a single band of about 250 bp.

After cloning and sequencing the PCR fragments of each species, a highly conserved region that corresponded to the 5S rRNA gene was identified which had an average sequence identity of 95% among *Brycon* spp. (Table 2). Some of the few observed differences, related to base substitutions, may be the result of nucleotide misincorporation by Taq DNA polymerase, as the dNTP and $MgCl_2$ concentrations used in the amplification reactions could lead to a cumulative error frequency of about 0.25% after 30 PCR cycles (Saiki et al. 1988). Searches using the BLASTN program at NCBI (Altschul et al. 1990) detected a low divergence among the 5S rRNA gene sequences of *Brycon* spp. and many other vertebrates (average similarity of 88.8%), including the fishes *Salmo salar* (Pendás et al. 1994), *Oncorhynchus mykiss* (Móran et al. 1996), genus *Coregonus* (Sajdak et al. 1998), *Oreochromis niloticus* (Martins et al. 2000), and several species of *Leporinus* (Martins and Galetti 2001). The 5S rDNA NTSs, however, were highly variable and species specific, and no sequence correspondence was seen between the NTSs of *Brycon* spp. and those of other fishes, suggesting that this spacer region evolves rapidly. The NTS regions are presumed to be free to vary, since these variants are selectively neutral or nearly neutral and can become either fixed or lost, thereby causing differences. In contrast, most mutations in the 5S rRNA gene are presumed to be selectively neutral or nearly neutral only when they occur in subcritical proportion (Cronn et al. 1996).

Two distinct NTS classes, named NTS-I and NTS-II, were detected in *B. lundii*, *B. orbignyanus*, *B. microlepis*, *B. brevicauda*, and *B. insignis* that were characterized by insertions–deletions and base substitutions (Table 1; Fig. 2). In *B. cephalus* and *Brycon* sp., two different-sized fragments (approximately 350 and 700 bp) were visualized by agarose electrophoresis (Fig. 1) but they represented a monomeric and a dimeric 5S rDNA unit. Although only one NTS class was detected in *B. cephalus* and *Brycon* sp., it is possible that these two species also have two distinct NTS regions that are yet to be identified. The 5S rDNA monomers of *B. cephalus* and *Brycon* sp. could belong to the NTS-II class, as they have a very distinct size and several nucleotide differences. In contrast, NTS-I sequences were very similar among the species, with a mean identity of 84% (Table 2). Figure 2 presents the alignment of the consensus sequences of the two different 5S rDNA unit size classes of the species studied. Different clones of each species (Table 1) were used to establish the consensus sequences. The sequence

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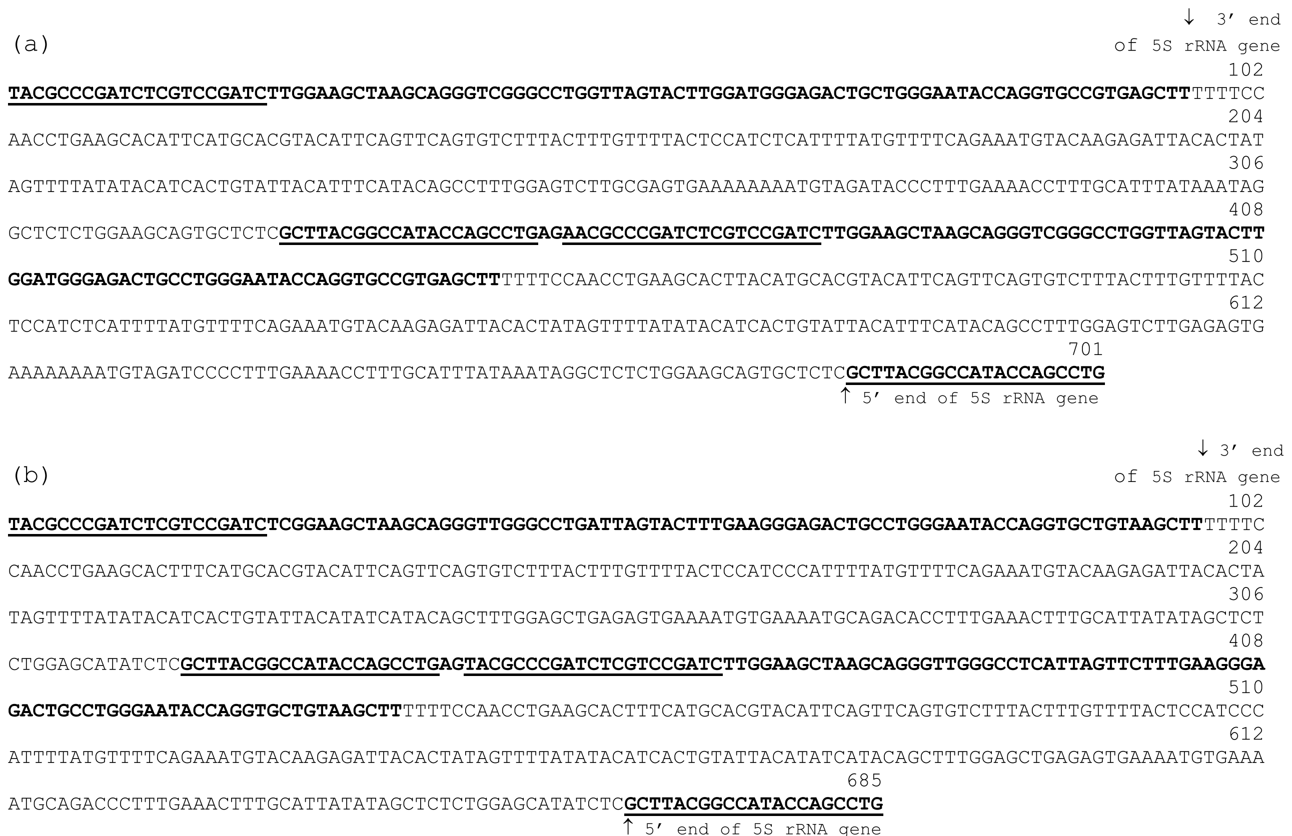
**Fig. 3.** Dimeric 5S rDNA tandem arrays from *B. cephalus* (*a*) and *Brycon* sp. (*b*). The coding sequence of the 5S rDNA is in boldface type and the primers used to amplify the 5S rDNA are underlined.




alignment of all clones is not shown, because there is a reduction of heterogeneity in each 5S rDNA size class within a genome. A high nucleotide identity was observed among all 5S rDNA NTS-I clones and among all 5S rDNA NTS-II clones of each species, as a consequence of some particular homogenization mechanism(s), such as gene conversion or unequal crossing over (Dover 1986).

5S rDNA variations have been well characterized in several plants (Gottlob-McHugh et al. 1990; Cronn et al. 1996; Danna et al. 1996) and some animals, such as mouse (Suzuki et al. 1994) and Atlantic salmon and brown trout (Pendás et al. 1994). These polymorphism have been useful as genetic markers for distinguishing closely related species, subspecies, lines, and hybrids (Pendás et al. 1995) and for evolutionary analysis (Suzuki et al. 1994; Joffe et al. 1995; Udovicic et al. 1995; Cronn et al. 1996; Crisp et al. 1999; Baker et al. 2000). Variations in the 5S rDNA of the species analyzed were mainly observed in the NTSs, especially in those of the NTS-II class. Table 2 indicates the percent identity among the NTS-II sequences of the species studied. *Brycon lundii*, *B. microlepis*, and *B. orbignyanus* presented very similar NTS-II sequences, with a mean identity of 88%, which could suggest a greater resemblance among these species. *Brycon cephalus* and *Brycon* sp. also seem to be more related to each other, since they presented very similar NTS-II sequences (identity of 93%). The NTS-II of *Brycon insignis* presented a mean sequence identity of 43% with the NTS-IIs of other species studied. The most distinct NTS-II was observed in *B. brevicauda*, which had a mean identity of 39% with those of the other species.

Despite the heterogeneity observed for the NTSs among *Brycon* spp., a relatively conserved sequence in the NTSs of approximately 120 bp was found to be common to all the species. This sequence is represented by the NTS-I, which is very similar among all the species analyzed (Fig. 2; Table 2) and could be the ancestral 5S rDNA NTS of the *Brycon* group. This same conserved sequence of around 120 bp also appears within the NTS-II sequence, although it is discontinuous, owing to several internal insertions–deletions that have occurred in each species (Fig. 2). This has led to a mean identity of only 46% among NTS-II sequences. In *B. lundii*, *B. orbignyanus*, and *B. microlepis*, the conserved sequence inside the NTS-II shows a deletion of 15 bp, when compared with the NTS-I sequence (Figs. 2*a*–2*c*). The data observed may indicate a common origin for both NTS classes and also that the NTS-IIs are evolving faster in each species.

A conserved NTS 5′-end region (TTCCAACC) is another common feature of the two NTS classes (Fig. 2), suggesting a regulatory role, such as the control of transcription by RNA polymerase III (Nederby-Nielsen et al. 1993; Pendás et al. 1994; Suzuki et al. 1996). Moreover, as in the NTSs of other fish species (Felgenhauser et al. 1990; Pendás et al. 1994; Sajdak et al. 1998; Martins et al. 2000, 2001), a TATA-like sequence was observed upstream of the 5S rRNA gene in *Brycon*, at the –19 to –27 position (Fig. 2). Other TATA-like elements were also detected, although these re-

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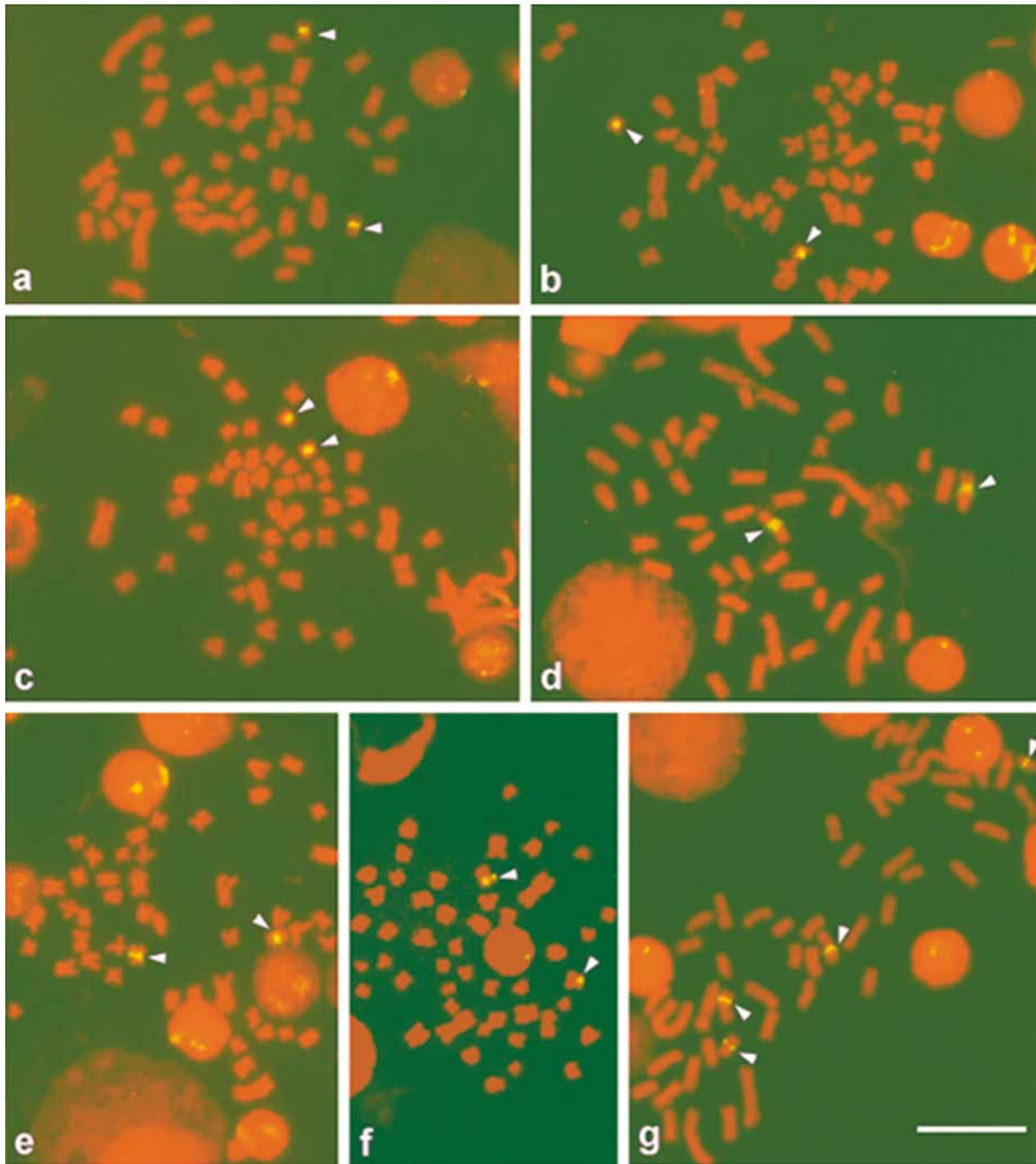
**Fig. 4.** 5S rDNA FISH of metaphase chromosome spreads of *B. lundii* (*a*), *B. microlepis* (*b*), *B. orbignyanus* (*c*), *B. cephalus* (*d*), *Brycon* sp. (*e*), *B. brevicauda* (*f*), and *B. insignis* (*g*). Arrows indicate the 5S rDNA loci. Scale bar = 3 µm.




gions have a particular location in *B. lundii*, *B.orbignyanus*, and *B. microlepis* and a similar location in *B. cephalus*, *Brycon* sp., and *B. brevicauda* (Fig. 2).

Sequencing of 5S rDNA dimers of *B. cephalus* and *Brycon* sp. (Fig. 3) determined the complete 5S rRNA gene nucleotide sequence of these two species. Within the limits of our analyses, we concluded that the repeated copies of the 5S gene within each species were nearly identical (similarity of 99.14% in *B. cephalus* and 99.12% in *Brycon* sp.), with only a few differences that were mainly due to some base substitutions. As with 18S–28S rDNA and other tandemly repeated multigene families, the several repeats within the 5S arrays seem to retain a high degree of identity, owing to homogenizing forces, which are referred to as concerted evolution (Cronn et al. 1996).

Previous cytogenetic studies have shown that the diploid genome of different species of the genus *Brycon* consists of 50 chromosomes, and the karyotype formula of the seven species analyzed is already well established (Almeida-Toledo et al. 1996; Margarido and Galetti 1996). FISH revealed 5S clusters located on two chromosomal sites that appear to be shared by the species analyzed. The 5S rDNA-bearing chromosomes in the karyotype of each species were identified by the chromosome pairing of several FISH meta-

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phases (data not shown). The first cluster was detected in the pericentromeric region of the long arm of a small submetacentric chromosome pair, and was observed in *B. lundii* (pair 23), *B. microlepis* (pair 22), and *B. orbignyanus* (pair 23) (Figs. 4*a*–4*c*). The second cluster, localized in the pericentromeric region of the long arm of two medium-sized submetacentric chromosomes corresponding to pair 15, was detected in *B. cephalus* and *Brycon* sp. (Figs. 4*d* and 4*e*). In *B. brevicauda*, the 5S rDNA was visualized interstitially on the short arm of two medium-sized submetacentric chromosomes that seem to correspond to chromosome pair 15 of *B. cephalus* and *Brycon* sp. *Brycon brevicauda* was the only species that presented 5S clusters on the short arm of a chromosome, and we suggest that this is the result of a pericentric inversion (Fig. 4*f*). In *B. insignis*, the 5S rDNA was mapped to the pericentromeric region of the long arms of two chromosome pairs: a small submetacentric pair (pair 21) and a medium-sized submetacentric pair (pair 15) (Fig. 4*g*).

Although four hybridization signals were only seen on the chromosomes of *B. insignis*, the possibility that minor clusters of a few 5S gene copies at other loci may have gone undetected using the FISH protocol cannot be eliminated, since the small size of these loci could impede their detection. An additional small fluorescent signal could be observed on a third chromosome in a few metaphases of *B. lundii*, *B. orbignyanus*, *B. microlepis*, *B. cephalus*, *Brycon* sp., and *B. brevicauda* (data not shown), which could indicate the occurrence of another 5S cluster in these species. Multiple 5S rDNA loci have been observed in amphibians (Vitelli et al. 1982; Schmid et al. 1987; Lucchini et al. 1993) and several fish species (Móran et al. 1996; Fujiwara et al. 1998; Murakami and Fujitani 1998; Martins and Galetti 1999; Martins et al. 2000).

Two main families of 5S rRNA genes that are expressed differently in oocytes and somatic cells have been described in amphibians (reviewed by Krämer 1985) and fishes (Komiya et al. 1986). Although these 5S rDNAs present a great sequence similarity in the coding region, their NTSs are very distinct. In *Xenopus laevis*, for example, the oocyte unit is about 750 bp and includes the 120-bp gene, a NTS, and a pseudogene (Carrol and Brown 1976; Jacq et al. 1977), while the somatic unit has approximately 880 bp and does not contain pseudogenes (Peterson et al. 1980). The two NTS classes (NTS-I and NTS-II) observed in *Brycon* spp. could be related to this dual system, which appears to represent paralogous copies of the 5S unit that may have evolved in separate regions of the genome and could represent the two chromosomal loci observed in at least one of the species analyzed, *B. insignis*. Two 5S rDNA arrays with distinct chromosome locations were also identified in *Leporinus* spp. (Martins and Galetti 2001).

No detectable polymorphism, in either the size or intensity of the fluorescent signals, was observed between homologous chromosomes. Variations in the size of the hybridization signal were detected between the two 5S rDNA-bearing chromosome pairs of *B. insignis* (Fig. 4*g*), characterizing a major and a minor locus. As an additional fluorescent signal could be observed on a third chromosome in a few metaphases of the other species analyzed, it is possible that all species also have two 5S rDNA loci. In *B. lundii*, *B. microlepis*, and *B. orbignyanus*, the major locus was detected by FISH in a small submetacentric chromosome pair, with a postulated minor locus on a medium-sized submetacentric pair. This pattern seems to be reversed for *B. cephalus*, *Brycon* sp., *B. brevicauda*, and *B. insignis*, with the major locus located in a medium-sized submetacentric chromosome pair and a postulated minor locus in a small submetacentric pair. The 5S rDNA size polymorphism between chromosome pairs can be due to differences in the number of tandemly repeat ribosomal units (Warburton et al. 1976; Martins and Galetti 1999). Physical 5S rDNA chromosome mapping—*B. lundii*, *B. orbignyanus*, and *B. microlepis* present conspicuous 5S clusters on a small submetacentric pair and *B. cephalus* and *Brycon* sp. present evident 5S clusters on a medium-sized submetacentric pair—supports the presumptive relationships, based on NTS data, among these species.

Of note, all major 5S rDNA chromosome loci were coincident with heterochromatic regions that have been characterized previously by C-banding (Almeida-Toledo et al. 1996; Margarido and Galetti 1996). Similar association was also observed in salmonids (Fujiwara et al. 1998). While the major *Brycon* 5S rDNA loci were coincident with dark positive C-bands, the minor ones were negative or weak for C-banding. The presence of heterochromatin associated with 5S rDNA may facilitate an accumulation of these loci by unequal exchange (Warburton and Henderson 1979; Jhanwar et al. 1981).

In *Brycon* spp., the 5S sites were not syntenic to the NORs. Silver nitrate staining (Margarido and Galetti 1996) and FISH with an 18S rDNA probe (Wasko and Galetti 2000) showed two NORs in the end of the long arm of a unique large submetacentric pair in all seven species analyzed. Such disjunct chromosome locations for a NOR and 5S rDNA loci have already been reported for *Anguilla anguilla* (Martínez et al. 1996), *Coregonus artedti* and *C. zenithicus* (Sajdak et al. 1998), genus *Leporinus* (Martins and Galetti 1999), genus *Schizodon* (Martins and Galetti 2000), *Hoplias malabaricus* (Born and Bertollo 2000), and *Oreochromis niloticus* (Martins et al. 2000) and is, by far, the most frequent condition observed in vertebrates (Lucchini et al. 1993; Drouin and Moniz de Sá 1995; Suzuki et al. 1996).

Not only have 5S rDNA nucleotide sequencing and chromosomal localization permitted the characterization of seven *Brycon* spp. and inferred a higher similarity among some of them, but two distinct 5S rDNA NTS classes have also been detected. The data obtained indicate that the 5S rDNA can serve as a suitable genetic marker for evolutionary studies and for the genetic identification of related species in the genus *Brycon*.

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