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Mapping 18S ribosomal genes in fish of the genus *Brycon* (Characidae) by fluorescence *in situ* hybridization (FISH)

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Abstract

The present study provides data on the nucleolar organizer regions (NORs) of seven *Brycon* species based on mapping of the 18S rRNA genes by fluorescence *in situ* hybridization (FISH). Fluorescent signals were observed on the telomere of the long arm of two large submetacentric chromosomes, thus confirming the number and location of NORs previously revealed by other classical cytogenetic techniques. Although there were no inter- or intra-individual variations in the number and location of the 18S loci, NOR size polymorphism was observed between homologous chromosomes. The clustering and conservation of NORs in a single chromosome pair indicates a high level of NOR stability among species of the genus *Brycon*.

INTRODUCTION

The nucleolar genes for ribosomal RNA (rDNA) are organized as multiple copies of a repeated unit that consists of a transcribed zone with coding regions for the 18S, 5.8S and 28S RNA genes separated by internal and external transcribed spacers and surrounded by non-transcribed spacer sequences (for review, see Long and David, 1980).

Nucleolar organizer regions (NORs) have been studied in many fishes using silver nitrate (Ag-NOR), mithramycin (MM) or chromomycin A_3 (CMA_3) staining (Amemiya and Gold, 1986; Phillips *et al*., 1989; Galetti Jr. and Rasch, 1993; Sola *et al*., 1997). Silver staining is more suitable for studying NOR expression because it detects only transcriptionally active NORs (Goodpasture and Bloom, 1975; Hofgatner *et al*., 1979; Howell and Black, 1980). GC-specific fluorochromes (MM, CMA_3) stain both active and inactive NORs in fish and amphibians (Mayr *et al*., 1986; Schmid and Guttenbach, 1988; Phillips and Hartley, 1988), probably as a consequence of the higher GC content of rDNA (Schmid and Guttenbach, 1988), but they can also detect different chromosome regions.

The location of NORs has been confirmed by isotopic and non-isotopic *in situ* hybridization of rRNA or rDNA probes in fixed chromosomes of several vertebrates, including amphibians, humans, chimpanzees (for review, see Long and David, 1980) and, more recently, fishes (Pendás *et al*., 1993a,b; Castro *et al*., 1996; Viñas *et al*., 1996; Abuín *et al*., 1996; Gornung *et al*., 1997; Martins and Galetti Jr., 1998). Both Ag and CMA_3 staining have been used in several species of the *Brycon* genus (Margarido and Galetti Jr., 1996; Almeida-Toledo *et al*., 1996).

In the present study, fluorescence *in situ* hybridization (FISH) was used to determine the chromosome location of the 18S rDNA genes of seven species of the genus *Brycon*, and to screen for additional minor rDNA units not detected by conventional techniques.

MATERIAL AND METHODS

Samples and mitotic chromosomal preparations

Seven species of the genus *Brycon* from different regions in Brazil were studied (Table I). Mitotic chromosomes were obtained from a suspension of anterior kidney cells using direct preparations (Bertollo *et al*., 1978) and short-term solid tissue culture (Fenocchio *et al*., 1991).

Fluorescence *in situ* hybridization

The rDNA probe was produced by a polymerase chain reaction (PCR) using genomic DNA from *Brycon lundii* (extracted from liver) and primers for the 18S genes (Hizume, 1994). The probe was isolated from agarose gels

Table I - Species of the genus *Brycon* and collection sites.

Species	Collection sites	N	
		F	M
B. lundii	São Francisco River (Três Marias, MG)	6	8
B. orbignyanus	Paraná River (Porto Rico, PR)	1	4
B. microlepis	Cuiabá River (Cuiabá, MT)	10	5
B. brevicauda	Araguaia River (Aragarças, GO)	6	5
Brycon sp.	Araguaia River (Aragarças, GO)	1	-
B. cephalus	Amazonas River (Manaus, AM)	3	2
B. insignis	Paraíba do Sul River (Pindamonhangaba, SP)	2	3

N = Analyzed individuals, F = females, M = males.

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using a commercial kit (Sephaglas™ BandPrep Kit, Pharmacia Biotech) and labeled with biotin-14-dATP by nick translation according to the kit manufacturer's instructions (Bionick™ Labeling System, Gibco). Prior to hybridization, the metaphase chromosome slides were pretreated with RNase (40 µg/ml in 2x SSC) and incubated for one hour in a moist chamber at 37°C followed by dehydration in an ethanol series. The slides were subsequently denatured in 70% formamide/2x SSC at 70°C for 5 min and then dehydrated in an ethanol series at -20°C. Forty microliters of the hybridization mixture, containing 1 µg of denatured probe, 50% formamide, 50% dextran sulfate and 2x SSC, was applied to each slide under a glass coverslip. After overnight incubation at 37°C in a moist chamber, the slides were washed in 50% formamide/2x SSC at 37°C (15 min), in 2x SSC (twice, 15 min each) and in 4x SSC (10 min). Avidin-fluorescein isothiocyanate (FITC) conjugate (Sigma) was added to the slides which were then incubated at 37°C in a moist chamber for one hour. The slides were subsequently immersed three times (5 min each) in blocking buffer ($NaHCO_3$, sodium citrate, Triton 20, nonfat dry milk) at 42°C. Biotinylated anti-avidin antibody was applied to the slides followed by incubating at 37°C in a moist chamber for 30 min and washing

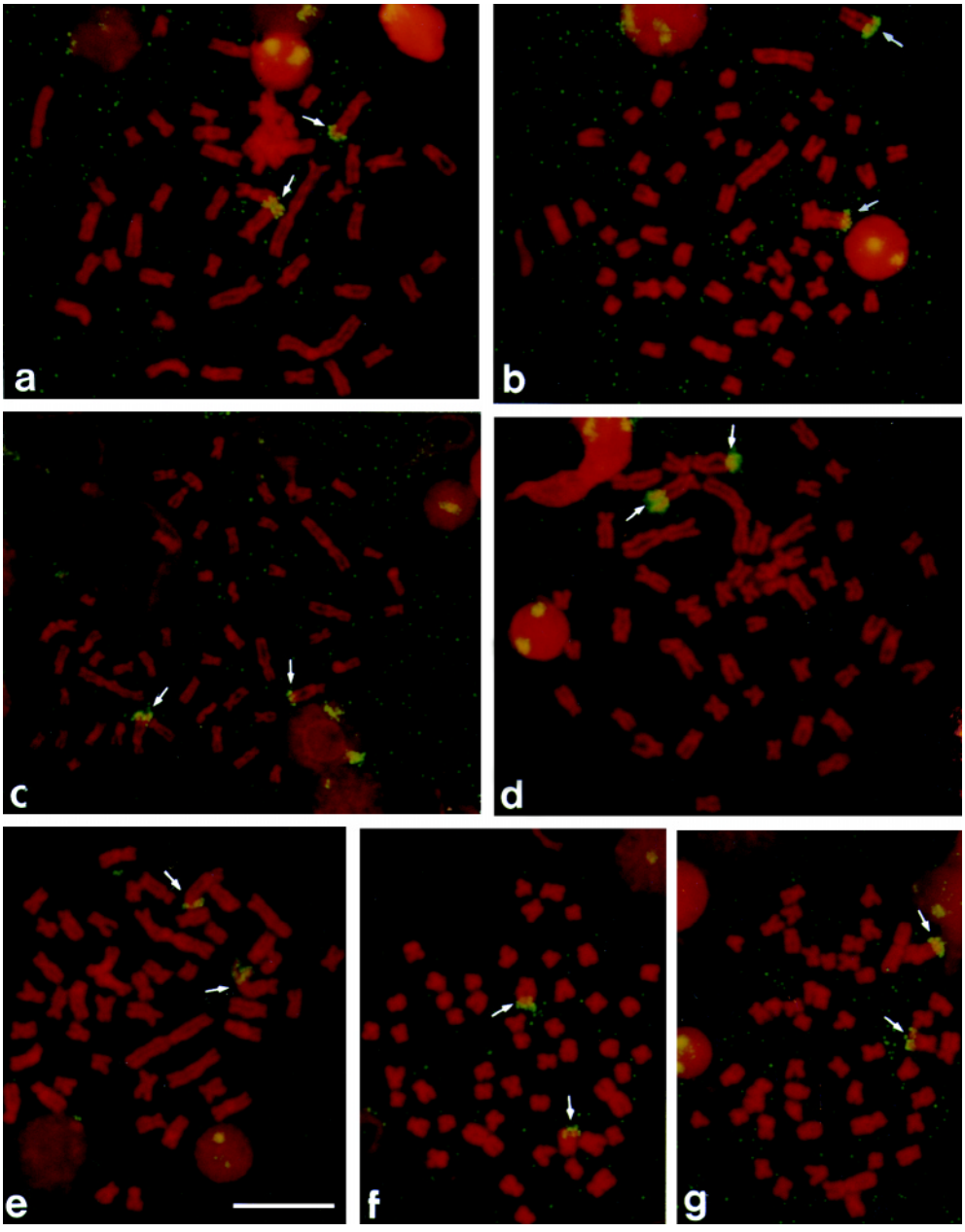

Figure 1 - 18S rDNA-FISH in *Brycon*. (a) *B. brevicauda*, (b) *B. cephalus*, (c) *B. microlepis*, (d) *B. orbignyanus*, (e) *B. lundii*, (f) *Brycon* sp., (g) *B. insignis*. Arrows indicate rDNA sites. Bar = 3 µm.

in the same buffer. The signal was enhanced by biotinylated anti-avidin and avidin-fluorescein. The slides were washed twice (3 min each) in 2% Triton 20/4x SSC after which they were mounted with fluorescence antifade solution (Vectashield antifade-Vector). The DNA counterstain propidium iodide (50 µg/ml) was included in the antifade solution. Metaphases were examined with an Olympus BX50 epifluorescence microscope. Photographs were taken using 400 ASA Kodak Gold Ultra film for color slides.

RESULTS AND DISCUSSION

The chromosomal organization of the seven species analyzed has been described in previous reports (Vascon *et al*., 1984; Bigoni *et al*., 1993; Margarido and Galetti Jr., 1996; Almeida-Toledo *et al*., 1996). A karyotype of 2n = 50, consisting of metacentric, submetacentric and subtelocentric chromosomes, is characteristic for the Bryconinae group.

Fluorescence *in situ* hybridization with a specific 18S rDNA probe resulted in a large, bright fluorescent signal spread over the terminal region of the long arm of a large submetacentric pair, and corresponded to the NOR-bearing chromosomes in all of the species (Figure 1). No additional fluorescent signals were detected and one or two nucleoli were observed in interphase cells.

The detection of NORs in a single chromosome pair agreed with previous data obtained by silver nitrate and GC-specific fluorochrome staining (Margarido and Galetti Jr., 1996; Almeida-Toledo *et al*., 1996) and confirmed the existence of a single locus for this gene family in the seven species of the genus *Brycon*. The maximum number of nucleoli observed in interphase cells also highlighted the absence of other 18S rDNA sites.

Although FISH revealed no inter- or intraindividual variations in the number and location of the 18S loci, heteromorphism in the size of the fluorescent signal was often detected between homologous chromosomes (see especially Figure 1d,e).

Variation in NOR size between homologous chromosomes is common in many vertebrates, including fishes (Foresti *et al*., 1981), in which NORs have been detected by Giemsa, C-banding, silver nitrate and mithramycin staining (Mestriner *et al*., 1995) and by rDNA-FISH (e.g. Wiberg, 1983; Sola *et al*., 1984; Viñas *et al*., 1996). Regional chromosomal duplications, produced either by DNA polymerase slippage or unequal crossing over (Warburton and Henderson, 1979; Jhanwar *et al*., 1981), could lead to the observed structural polymorphism. Moreover, genetic redundancy of rDNA regions and the tendency for association in nucleolar organization could facilitate non-reciprocal translocations between NORs (Goodpasture and Bloom, 1975; Ruiz, 1982; Schmid *et al*., 1987).

C-banding patterns (Margarido and Galetti Jr., 1996) for the species of the genus *Brycon* studied here indicate that the terminal region of the long arms of the NOR-bearing chromosomes consists mainly of constitutive heterochromatin, a common feature among other fish species (Moreira-Filho *et al*., 1984; Galetti Jr. *et al*., 1991; Pendás *et al*., 1993a,b; Martínez *et al*., 1996). The rDNA appears to be interspersed or adjacent to heterochromatin segments in these fishes (Pendás *et al*., 1993b; Galetti Jr. *et al*., 1995), and size polymorphism may be facilitated by this heterochromatin in the species of the genus *Brycon*.

The presence of NORs clustered and conserved in a single chromosome pair is a common character of all studied *Brycon* species and may be a primitive feature in the subfamily Bryconinae. This finding indicates a remarkable stability of the NOR phenotype pattern among these fishes.

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RESUMO

O presente estudo fornece dados sobre as regiões organizadoras de nucléolos (NORs) de sete espécies do gênero *Brycon*, obtidos através da localização dos genes RNAr 18S por hibridação *in situ* fluorescente (FISH). Sinais fluorescentes foram observados no telômero do braço longo de dois cromossomos submetacêntricos grandes e confirmaram o número e a localização das regiões organizadoras de nucléolos anteriormente detectadas através de outras técnicas citogenéticas clássicas. Embora não tenham sido detectadas variações inter ou intra-individuais no número e localização dos loci 18S, um polimorfismo de tamanho na região organizadora de nucléolo foi observado entre os dois homólogos. O alto nível de estabilidade das NORs observado no gênero *Brycon* é caracterizado pela presença destes sítios agrupados e conservados em um único par de cromossomos nestes peixes.

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