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Research Article

# Chromosome mapping of 5S rRNA genes differentiates Brazilian populations of *Leporellus vittatus* (Anostomidae, Characiformes)

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## Abstract

Among the anostomid fishes, the genus *Leporellus* is represented by only three species: *L. nattereri*, endemic of the Amazon River, *L. retropinnis*, endemic of the Piracicaba River, and *L. vittatus*, widely distributed in rivers from Peru, Colombia, Guianas, and different major hydrographic basins of Brazil. A cytogenetic study carried out on specimens of *Leporellus vittatus* from three major Brazilian hydrographic basins evidenced a karyotype of 54 metacentric and submetacentric chromosomes. C-banding analysis revealed the presence of large pericentromeric heterochromatic segments in all chromosomes and a telomeric block coincident with the NOR sites. Ag, $CMA_3$ or MM staining, and FISH with ribosomal probes located the 45S ribosomal genes on the terminal region of the long arm of the 12$^{th}$ chromosome pair of all populations. Nevertheless, in the specimens from the Paraná and São Francisco Basins the 5S rDNA clusters were interstitially located by FISH on the long arm of the 2$^{nd}$ chromosome pair, while in the specimens from the Tocantins-Araguaia Basin these sites were observed on the long arm of the 9$^{th}$ chromosome pair and on the short arm of the 17$^{th}$ chromosome pair. These data suggest that the species currently named *Leporellus vittatus* may comprise a complex of cryptic species.

*Key words:* karyotype, C-bands, 45S rDNA, 5S rDNA.

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## Introduction

The Anostomidae family comprises 12 genera of typically Neotropical fishes, occurring from Central America to South America (Garavello and Britski, 2003). According to the current taxonomic classification, based on morphological characters, the genus *Leporellus* is the smallest one of this family, represented by only three species: *L. nattereri*, endemic of the Amazon River (Northern Brazil), *L. retropinnis*, endemic of the Piracicaba River (Southeastern Brazil), and *L. vittatus*, widely distributed in rivers from Peru, Colombia, Guianas, and three major Brazilian hydrographic basins: Paraná, São Francisco and Tocantins-Araguaia (Fowler, 1950; Garavello and Britski, 2003).

Nevertheless, there is little agreement as to the limits of the genus. The wide geographical distribution of *L. vittatus* has raised questions about the cospecificity of local populations of this species. As the major drainage basins of Brazil began to develop during the Tertiary (Buerlen, 1970) and several teleost fish species of the families Cichlidae, Characidae, and Curimatidae have already been described as endemic of distinct hydrographic basins (Kullander, 1983; Menezes, 1988; Vari, 1988), it is possible that, due to the limited gene flow, some populations have genetically diverged, although the conservative morphology prevents the detection of such differentiation. Therefore, the species known as *Leporellus vittatus* may be actually representing a complex of species.

Classical population models of chromosome evolution have postulated that small and/or restricted populations may show a higher karyotypic diversity than migratory and/or large populations, which seem to retain more conservative karyotypes, at least at the macrostructure level (Lande, 1979).

Previous cytogenetic studies have determined the diploid number, the constitutive heterochromatin distribution pattern (C-bands), and the location of the nucleolus organizer regions (NORs) by silver-staining on specimens of just one population of *L. vittatus* from the Paraná Basin (Galetti *et al.*, 1991).

In view of these considerations, the aim of the present study was to investigate the possible geographical variation

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of *Leporellus vittatus* through cytogenetic comparisons. Samples collected in three major Brazilian hydrographic basins were cytogenetically analyzed and Giemsa karyotypes, C-banding patterns, and ribosomal DNA sites (rDNA) studied by silver staining, base-specific fluorochromes (chromomycin $A_3$ or mithramycin, and 4',6-diamidino-2-phenylindole) and fluorescence *in situ* hybridization were investigated in order to find potential chromosome markers.

## Material and Methods

### Sampling sites

Samples of *Leporellus vittatus* representing three distinct populations of major Brazilian hydrographic basins were collected. Seventeen specimens from the Mogi-Guaçu River, Pirassununga, São Paulo State (21° 9' S and 47° 4' W), Paraná Basin, Southeastern Brazil; four from the São Francisco River, Três Marias, Minas Gerais State (18° 2' S and 45° 2' W), São Francisco Basin, Southeastern Brazil; and three from the Araguaia River, Barra do Garças, Mato Grosso State (15° 9' S and 52° 3' W), Araguaia-Tocantins Basin, Central Brazil were analyzed (Figure 1).

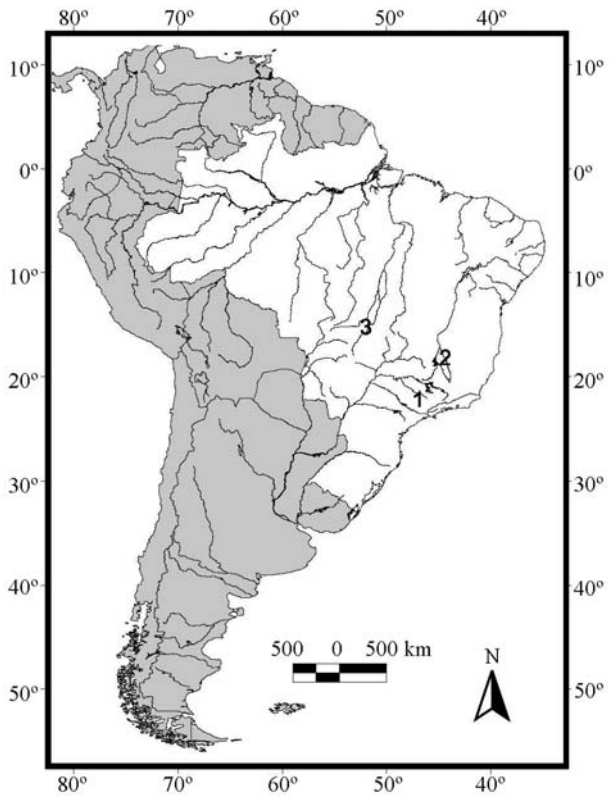


**Figure 1** - Collection sites of *Leporellus vittatus*: Mogi-Guaçu River, Pirassununga - Paraná Basin (1); São Francisco River, Três Marias - São Francisco Basin (2); Araguaia River, Barra do Garças - Tocantins-Araguaia Basin (3).

### Chromosome staining techniques

Mitotic chromosomes were obtained from kidney cellular suspensions through the air-drying technique (Bertollo *et al.*, 1978) or, alternatively, by short term solid tissue culture (Fenocchio *et al.*, 1991). Giemsa karyotypes were established for the three populations. The constitutive heterochromatin distribution pattern was investigated by barium hydroxide treatment (Sumner, 1972). The nucleolus organizer regions (Ag-NORs) were observed by colloidal silver-staining (Howell and Black, 1980). Fluorochrome staining with the GC-specific chromomycin $A_3$ ($CMA_3$) or mithramycin (MM), and the AT-specific 4',6-diamidino-2-phenylindole (DAPI) was carried as described by Schmid (1980) and Schweizer (1978), respectively.

### Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was performed basically according to Pinkel *et al.* (1986), using a cocktail of 18S and 28S cloned fragments of the rDNA of *Xenopus laevis* (Cortadas and Pavon, 1982), and a 5S rDNA probe of the fish *Leporinus elongatus*, obtained by PCR (polymerase chain reaction) as described in Martins and Galetti (1999). The probes were labeled with biotin-16-dUTP by nick translation. The metaphase chromosome slides were incubated with RNase (40 µg/mL) for 1.5 h at 37 °C in a moist chamber. The chromosomal DNA was denatured for 5 min at 70 °C in a solution of 70% formamide in 2 x SSC. After that, 40 µL of hybridization mixture (1 µg of denatured probe, 50% formamide, and 10% dextran sulphate in 2 x SSC) were applied to the slides under a glass coverslip. The hybridization was performed overnight at 37 °C in a moist chamber. The slides were then washed three times at 37 °C, once in a solution of 50% formamide in 2 x SSC, and twice in 2 x SSC, for 15 min each. The probes were detected by avidin-FITC conjugate. The signal was enhanced by biotinylated anti-avidin and avidin-FITC. Afterwards, the chromosomes were counterstained with 70 µL of propidium iodide (100 µg/mL) and the slides were mounted with 25 µL of the anti-fading Vectashield® Mounting Medium.

## Results

A total of 24 specimens were cytogenetically analyzed. Specimens from all populations had a modal diploid number of 54, composed of metacentric and submetacentric chromosomes (FN = 108) (Table 1 and Figure 2a).

The different applied staining techniques produced a pattern common to specimens of both sexes from all the populations. C-banding analysis revealed the presence of large pericentromeric heterochromatic segments in all chromosomes and a block at the terminal region of a middle-sized chromosome pair (Figure 2b).

Silver nitrate and chromomycin $A_3$ or mithramycin staining revealed the presence of two NOR-bearing chro-

**Table 1** - Diploid numbers found in *Leporellus vittatus*: populations from (♦) Mogi-Guaçu River, (■) São Francisco River and (●) Araguaia River.

| Specimens | Sex | Total number of cells | Diploid numbers | | | | |
|---|---|---|---|---|---|---|---|
| | | | 50 | 51 | 52 | 53 | 54 |
| 10093 (♦) | M | 5 | - | - | - | - | 5 |
| 10094 (♦) | F | 8 | - | - | - | - | 8 |
| 10095 (♦) | M | 3 | - | - | - | - | 3 |
| 10096 (♦) | M | 38 | - | - | 1 | - | 37 |
| 10097 (♦) | M | 24 | - | - | - | - | 24 |
| 10098 (♦) | M | 25 | - | - | 1 | - | 24 |
| 10102 (♦) | M | 8 | - | - | 1 | - | 7 |
| 10103 (♦) | F | 15 | - | - | 1 | - | 14 |
| 10104 (♦) | F | 17 | - | - | - | - | 17 |
| 10105 (♦) | F | 6 | - | - | 1 | - | 5 |
| 10106 (♦) | F | 7 | - | - | - | - | 7 |
| 10107 (♦) | F | 27 | - | - | 2 | - | 25 |
| 10108 (♦) | U | 10 | - | - | - | - | 10 |
| 10109 (♦) | U | 11 | - | 1 | - | - | 10 |
| 10112 (♦) | U | 16 | - | - | - | - | 16 |
| 10113 (♦) | U | 2 | - | - | - | - | 2 |
| 10125 (♦) | F | 9 | - | 1 | - | - | 8 |
| 5596 (■) | M | 8 | - | - | - | - | 8 |
| 11636 (■) | F | 10 | - | - | - | - | 10 |
| 13079 (■) | F | 41 | - | - | 1 | - | 40 |
| 13087 (■) | F | 132 | - | - | 2 | - | 130 |
| 331 (●) | F | 67 | 1 | - | 4 | 1 | 61 |
| 362 (●) | U | 2 | - | - | - | - | 2 |
| 363 (●) | U | 8 | - | 1 | - | - | 7 |

mosomes in this species (Table 2). The NOR sites were located near the telomere of the long arm of the medium-sized chromosome pair 12 and are apparently coincident with the conspicuous secondary constrictions, which appeared positive C-banded (Figure 3a and 3b). DAPI staining produced negative bands in these regions, while all the remaining chromosomes appear almost uniformly stained (Figure 3c).

The number of NORs and their unique location on chromosome pair 12 were further confirmed by FISH with

**Table 2** - Frequencies of ribosomal genes bearing chromosomes in specimens from the distinct populations of *Leporellus vittatus*: (♦) Mogi-Guaçu River, (■) São Francisco River and (●) Araguaia River.

| Specimens | Ag-NORs | | $CMA_3$ or MM | | FISH (45S rDNA) | | FISH (5S rDNA) | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 1 | 2 | 1 | 2 | 2 | 4 |
| 10094 (♦) | 0 | 84 | 1 | 13 | - | - | - | - |
| 10096 (♦) | 1 | 14 | 1 | 6 | - | - | - | - |
| 10097 (♦) | 1 | 29 | 2 | 75 | 7 | 156 | 141 | 0 |
| 10098 (♦) | 3 | 45 | 2 | 105 | 8 | 92 | 92 | 0 |
| 10103 (♦) | 0 | 4 | 2 | 8 | - | - | - | - |
| 10104 (♦) | 0 | 7 | 2 | 74 | 3 | 54 | 15 | 0 |
| 10105 (♦) | 0 | 10 | 0 | 6 | - | - | - | - |
| 10107 (♦) | 1 | 23 | 1 | 105 | 6 | 210 | 67 | 0 |
| 5596 (■) | 1 | 27 | 0 | 30 | - | - | - | - |
| 11636 (■) | 3 | 35 | 1 | 35 | 0 | 41 | 14 | 0 |
| 13079 (■) | 1 | 33 | 0 | 34 | - | - | 16 | 0 |
| 13087 (■) | 4 | 168 | 0 | 200 | 5 | 237 | 290 | 0 |
| 331 (●) | 2 | 75 | 3 | 174 | 5 | 211 | 0 | 288 |
| 362 (●) | 0 | 14 | 0 | 12 | - | - | 0 | 33 |
| 363 (●) | 0 | 8 | 0 | 5 | - | - | 0 | 8 |

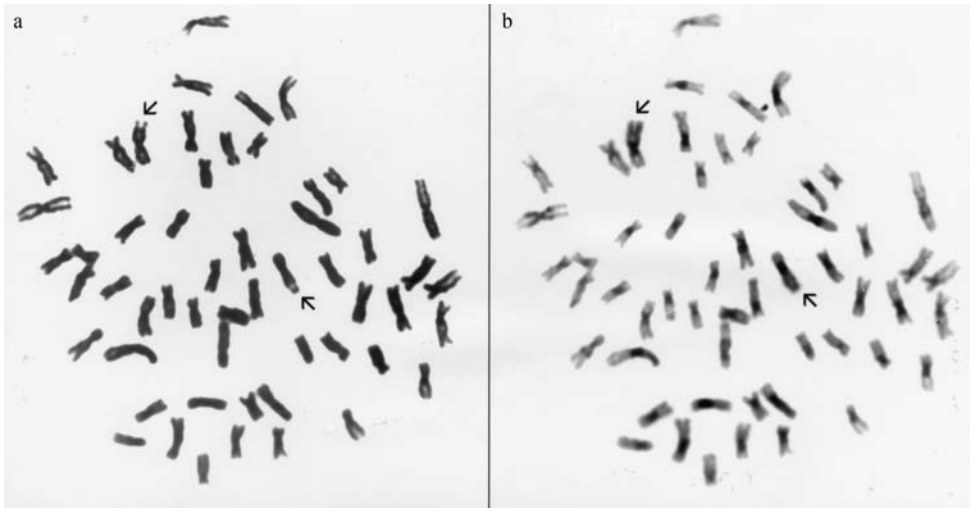


**Figure 2** - Metaphase plate of *Leporellus vittatus* after Giemsa staining (a) and sequential C-banding (b). Arrows indicate the NOR-bearing chromosomes.

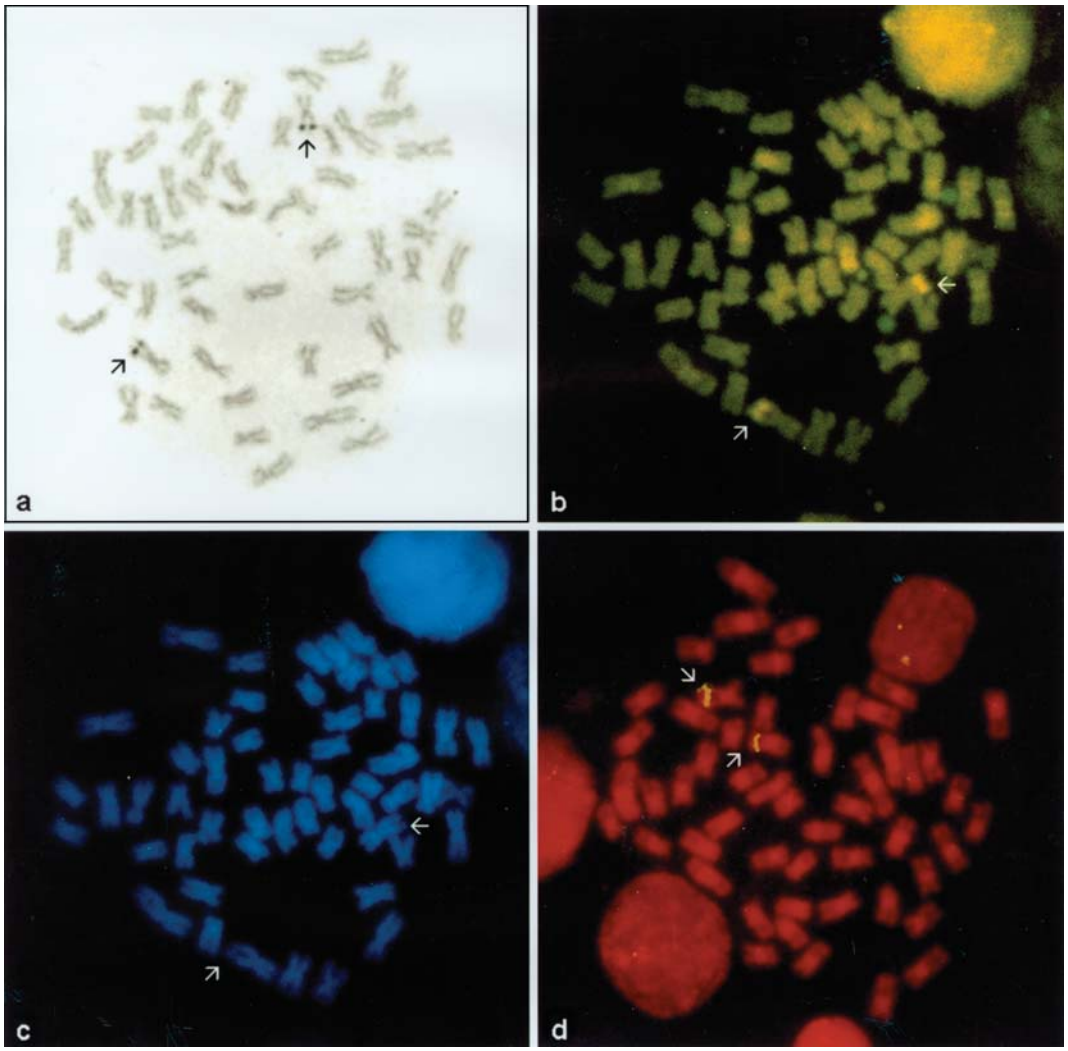


**Figure 3** - Metaphase plates of *Leporellus vittatus*: silver staining (a); sequential $CMA_3$ (b) and DAPI (c) staining; FISH with 45S ribosomal DNA probe (d). Arrows indicate the NOR-bearing chromosomes.

biotinylated 18S and 28S rDNA (45S rDNA) probes (Figure 3d).

The 5S ribosomal genes were detected by FISH on chromosome pairs distinct from the ones bearing the 45S rDNA clusters. In the specimens from the Paraná and São Francisco Basins the 5S rDNA clusters were interstitially located on the long arm of the 2nd chromosome pair (Figure 4a and b). The specimens from the Tocantins-Araguaia Basin showed two chromosome pairs bearing these sites. In this population the 5S rDNA sites were observed on the long arm of the 9th chromosome pair and on the short arm of the 17th chromosome pair (Figure 4c).

## Discussion

According to Buerlen (1970), the major drainage basins of Brazil began to develop during the Tertiary. Although the precise time of formation of each hydrographic system cannot be determined, several teleost fish species of the families Cichlidae, Characidae, and Curimatidae have been described as endemic of distinct hydrographic basins, reinforcing the supposed vicariant events (Kullander, 1983; Menezes, 1988; Vari, 1988).

The cytogenetic markers studied here included different banding techniques, fluorochrome staining, and in situ hybridization with ribosomal probes, allowing a careful investigation of the constitutive heterochromatin and, particularly, of the ribosomal sites, which are chromosomal regions often described as variable in fishes.

Although the three studied populations of *Leporellus vittatus* share the same karyotypic structure already described by Galetti *et al.* (1991) for the population from the Paraná Basin and no differences could be detected in the heterochromatin distribution pattern and in the number and location of the 45S rDNA sites, interpopulation differences were evidenced concerning the number and location of the 5S rDNA clusters.

The association between heterochromatin and NOR sites observed in all the three studied populations seems to

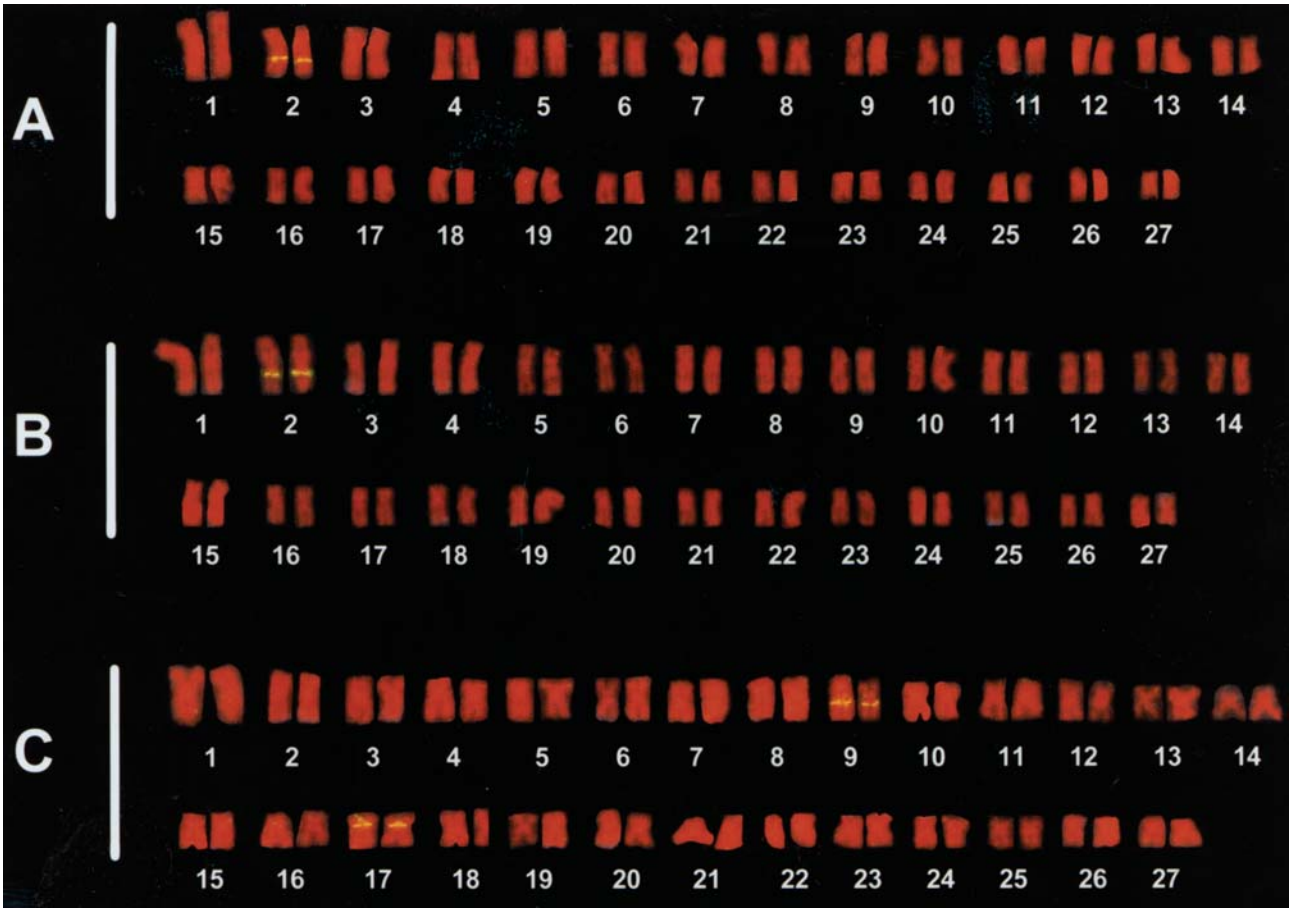


**Figure 4** - Karyotypes of *Leporellus vittatus*: FISH with 5S ribosomal probe in specimens from Mogi-Guaçu (a), São Francisco (b) and Araguaia (c) Rivers.

be a common feature in fishes and has also been described for many other fish species (Galetti *et al.*, 1991; Rossi *et al.*, 1996; Aguilar and Galetti, 1997; Martins and Galetti, 1997). This NOR-associated heterochromatin, positive stained with $CMA_3$, is thus GC-rich as originally reported by Amemiya and Gold (1986). DAPI staining, an AT-specific fluorochrome, confirmed the GC-rich nature of this heterochromatin, since negative bands coincident to the NOR sites were observed.

The lack of large chromosomal differences among the populations is not surprising since the karyotypic macrostructure of *L. vittatus* seems to be conserved among the Anostomidae (Galetti *et al.*, 1981; Martins and Galetti, 1997, 1998) and other related Characiformes families, such as Curimatidae (Venere and Galetti, 1989; Feldberg *et al.*, 1992), Parodontidae (Jesus and Moreira-Filho, 2000), and Prochilodontidae (Pauls and Bertollo, 1990), suggesting that the karyotype with 2n = 54, FN = 108, is ancient among the Characiformes.

Nevertheless, subtle changes in the chromosomal microstructure involving distinct rearrangements in the ribosomal regions seem to have occurred during the evolutionary diversification of the Anostomidae and may be strictly related to species differentiation. Intrapopulational chromosomal polymorphisms involving the number of 45S rDNA sites have already been reported in *Leporinus friderici* and *Leporinus trifasciatus* (Galetti *et al.*, 1991; Galetti *et al.*, 1995a,b), despite the conserved chromosome structure (2n = 54, FN = 108). In the present study we identified differences in the number and location of the 5S rDNA clusters among distinct populations of *Leporellus vittatus* from major Brazilian hydrographic basins.

In Neotropical characiform fishes, the 5S rDNA clusters are generally distributed in an interstitial position in two autosomal chromosome pairs and are usually not syntenic to the 45S rDNA sites, suggesting that this could be a common condition for the 5S rRNA gene organization in the genome of these fishes (Martins and Galetti, 1999, 2000, 2001; Born and Bertollo, 2000).

However, variations concerning the number and location of 5S rDNA sites have already been described for many other fish species. Multiple sites have been described for some salmonid species that show up to eight sites located on distinct autosomal chromosome pairs (Fujiwara *et al.*, 1998), and for *Astyanax scabripinnis*, a characid fish, that presents eight 5S rDNA sites located on four distinct

chromosome pairs (Ferro *et al.*, 2000). The 5S rDNA clusters have also been located on the X or Y sex chromosomes of some salmonid species (Moran *et al.*, 1996; Iturra *et al.*, 2001; Stein *et al.*, 2001), and on the Y-chromosome of the males of *Chionodraco hamatus*, an Antarctic fish (Mazzei *et al.*, 2004). Frequently, differences in the number and position of 5S rDNA sites have been reported as good chromosome markers to discriminate closely related fish species, such as in some families of the orders Mugiliformes and Perciformes (Gornung *et al.*, 2001; Molina and Galetti, 2002; Rossi *et al.*, 2005). Accordingly, the interpopulation differences observed in the present study concerning the number and location of the 5S rDNA clusters between Paraná/São Francisco and Tocantins-Araguaia populations of *L. vittatus* suggest that the species currently named *Leporellus vittatus* may comprise a complex of cryptic species.

The recent discovery that, during the construction of the Furnas hydroelectric power dam in the upper Paraná River Basin in the early 1960s, the Piumhi River drainage outflow was diverted into the headwaters of the São Francisco River Basin has raised questioning about the current São Francisco watershed ichthyofauna structure (Moreira-Filho and Buckup, 2005). As this transposition event allowed the entire fish fauna of the Piumhi River and associated swamps, lakes, and tributaries to intermingle with the fish fauna of the São Francisco Basin, it may have contributed to the chromosomal stability described by many authors for Paraná and São Francisco Neotropical fish populations, as also described in the present study. Therefore, further studies comparing the different populations of *L. vittatus* and other anostomid species through molecular approaches, such as DNA sequencing of mitochondrial genes, may probably give a more precise answer to the present question – Is *Leporellus vittatus* a complex of cryptic species?

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