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Research Article

Mapping of ribosomal genes and chromosomal markers in three species of the genus *Serrasalmus* (Characidae, Serrasalminae) from the Amazon basin

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Abstract

Karyotypic characteristics of three species of the genus *serrasalmus* (*S. altispinnis*, *S. gouldingi* and *S. Serrulatus*) from the middle and lower Negro River, Amazon Basin, were investigated using different staining techniques and Fluorescent *in situ* hybridization with 5S and 18S rDNA probes. The diploid number was invariably 2n = 60 and the fundamental number was FN = 110. Nevertheless, the karyotypes differed from each other in composition: 24m, 20sm, 6st, 10a in *S. altispinnis*; 22m, 22sm, 6st, 10a in *S. gouldingi* and 20m, 22sm, 8st, 10a in *S. serrulatus.* The karyotype of *S. altispinnis* differed from the one previously described in a population from the Pitinga River. C-positive constitutive heterochromatin was mainly pericentromeric in the karyotypes of all species. Nucleolar organizer regions were multiple and preferentially located terminally on the short arms of the subtelocentric/acrocentric chromosomes, as evidenced by both silver nitrate staining and fluorescent *in situ* hybridization with the 18S rDNA probe. The maximum number of NORs varied among species, as did the NOR-bearing chromosomes. FISH with the 5S rDNA probe produced an interstitial signal on the long arms of the pair 7 in all species, coincident with a C-positive heterochromatic band. While some chromosome features were shared by the three species, some were species-specific and thus useful for cytotaxonomy.

Key words: chromosome banding, cytotaxonomy of piranhas, FISH, karyotype, ribosomal DNA.

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Introduction

The subfamily Serrasalminae includes 15 genera and 80 species restricted to the Neotropical region and known as pacus and piranhas. All these genera occur in the Amazon Basin, six of them are also present in the Paraná-Paraguay Basin and three in the São Francisco Basin (Jégu, 2003).

The genus *Serrasalmus* is one of the most diversified Serrasalminae and has a wide distribution throughout South America. It includes highly specialized voracious fishes displaying a typical laterally compressed and deep body, with a series of middle-ventral abdominal spines (Géry, 1972; Machado-Allison and Fink, 1996). Besides the 24 recognized species of *Serrasalmus*, four species still require a detailed characterization and formal recognition (Jégu, 2003).

Ten *Serrasalmus* species have already been karyotyped and 2n = 60 was hypothesized as the basal diploid number (Cestari and Galetti, 1992a; Nakayama *et al.*, 2002). The high intraspecific chromosome diversity of the genus is documented by the 17 distinct karyotypes that have already been reported (Nakayama *et al.*, 2002). For instance, four distinct karyotypes were found in *S. maculata* (junior synonyme - *S. spilopleura* Jégu and Santos, 2001) in the confluence of the Negro and Solimões Rivers, Amazon Basin (Nakayama *et al.*, 2000; Centofante *et al.*, 2002), while three karyomorphs were reported in the Paraná Basin population (Cestari and Galetti, 1992b). While *S. rhombeus* presented a karyotype with 2n = 60; a criptic species presented 2n = 58 and a karyotype probably derived from the 2n = 60 by chromosome fusions (Nakayama *et al.*, 2001), and a third new karyomorph was also reported (Teixeira *et al.*, 2006).

In this work, the karyotypes of three species of *serrasalmus* (*S. alispinnis*, *S. gouldingi* and *S. Serrulatus*) were investigated after C-banding, silver nitrate staining of the nucleolus organizer regions (Ag-NORs) and fluorescent *in situ* hybridization (FISH) with the 5S and 18S rDNA probes.

Material and Methods

Twenty-three specimens of *Serrasalmus altispinnis,* 18 specimens of *S. gouldingi* and 26 specimens of *S. serrulatus* from the middle and lower Negro River, Ama-

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zon Basin, state of Amazonas, Brazil, were analyzed (Figure 1; Table 1). The specimens were classified by Dr. Jansen Zuanon, from the Instituto Nacional de Pesquisas da Amazônia (INPA), where voucher specimens were deposited (*Serrasalmus altispinnis*: INPA 28887, 28890; *S. gouldingi*: INPA 28888, 28889; *S. serrulatus*: INPA 28884, 28885, 28886).

The chromosome preparations were obtained from kidney cells, according to the *in vivo* procedure described by Bertollo *et al.* (1978) and after mitotic stimulation with biological yeast, as described by Oliveira *et al.* (1988).

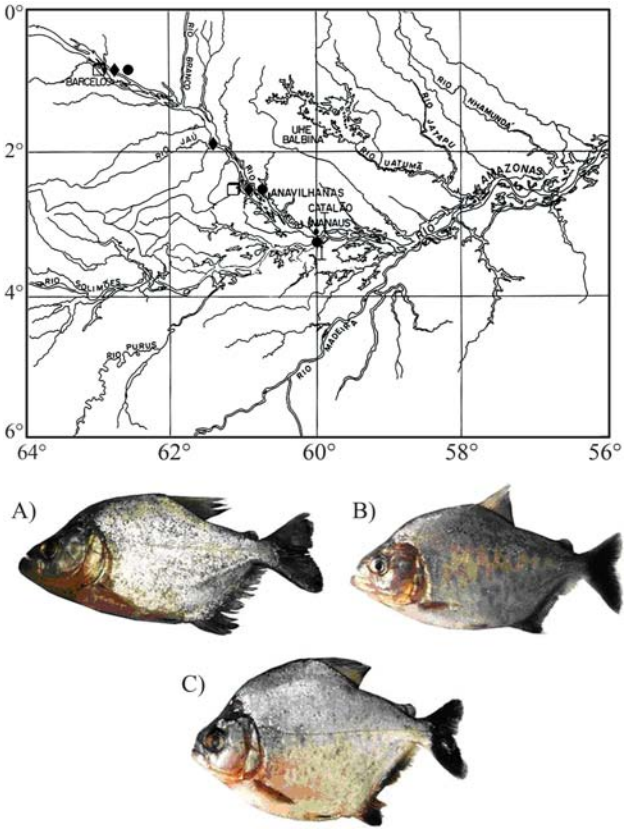

Figure 1 - Map of the collection sites and *Serrasalmus* species analyzed: a) ❒ *S. altispinnis* (total length =16.0 cm); b) ◆ *S. gouldingi* (total length = 14.0 cm), and c) ● *S. serrulatus* (total length = 15.5 cm).

Table 1 - Specimens of *Serrasalmus* from the Amazon Basin analyzed.

Species	Collection site	Males	Females	Total
Serrasalmus altispinnis	Anavilhanas	8	4	12
	Barcelos	8	3	11
Serrasalmus gouldingi	Barcelos	6	10	16
	Jaú	-	1	1
	Anavilhanas	1	-	1
Serrasalmus serrulatus	Anavilhanas	3	4	7
	Barcelos	5	7	12
	Catalão	4	3	7
Total		35	32	67

About 30 metaphases per individual were analyzed. The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) and arranged by decreasing size. The fundamental number (FN), or number of chromosome arms was determined considering that metacentric (m), submetacentric (sm) and subtelocentric (st) chromosomes are biarmed and that acrocentric (a) chromosomes are uniarmed. C-positive constitutive heterochromatin was evidenced by C-banding (Sumner, 1972) and the active nucleolar organizer regions were identified after silver nitrate staining (Ag-NORs), according to Howell and Black (1980).

Fluorescent *in situ* hybridization (FISH) was performed to locate 18S and 5S DNA sites on the chromosomes, according to Pinkel *et al.* (1986). The 18S rDNA probe was obtained by PCR from the DNA of the fish *Prochilodus argenteus* (Hatanaka and Galetti, 2004), using the primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (5'- TCCGCAGGTTCACCTACGGA-3'), according to White *et al.* (1990). The 5S rDNA probe was obtained from the fish *Leporinus obtusidens* (Martins and Galetti, 1999), using the primers A (5'-TACGCCCGATC TCGTCCGATC-3') and B (5'- CAGGCTGGTATGGCC GTAAGC-3'), according to Pendás *et al.* (1994). The probes were labeled by nick translation (BioNick Labeling System - Invitrogen) following the manufacturer's specifications. Denaturation was for 5 min in formamide 70%/2xSSC at 70 °C. Post-hybridization washes were performed in high stringency conditions (formamide 50%/2xSSC at 42 °C). The chromosomes were counterstained with propidium iodide (50 µg/mL and 200 µL of antifading solution) and analyzed in an epifluorescence microscope Olympus BX50. The images were documented using the software CoolSNAP-pro (Media Cybernetics).

Results

The three species presented karyotypes with 2n = 60 and FN = 110. However, some differences regarding to karyotypic formula were observed (24m, 20sm, 6st, 10a for *S. altispinnis*; 22m, 22sm, 6st, 10a for *S. gouldingi* and 20m, 22sm, 8st, 10a for *S. serrulatus*). No heteromorphic sex chromosomes were identified when comparing male and female karyotypes of the three species (Table 2; Figure 2).

After C-banding, constitutive heterochromatin was located mainly at the pericentromeric chromosome regions of the three species. Some chromosome pairs had conspicuous pericentromeric heterochromatic blocks, on the short or on the long arms. This was the case of pairs 3, 4, 9, 14, 16 and 27 in *S. altispinnis*, of pairs 26, 27and 29 in *S. gouldingi*, and of pair 22 in *S. serrulatus*. Some remarkably dark regions seen on the short arms of one homologue of pairs 14 and 18 of *S. serrulatus* were actually technical

Table 2 - Karyotypic features of the *Serrasalmus* species (2n = 60; FN = 110).

Species	Karyotype formulas	Ag-NORs	18S	5S
Serrasalmus altispinnis	24m+20sm+6st+10a	6-9a, p, t	9a, p, t	2m, q, i
S. gouldingi	22m+22sm+6st+10a	5-8a, p, t	6a, p, t + 2st, p, t	2m, q, i
S. serrulatus	20m+22sm+8st+10a	4-12st-a, p, t	8a, p, t + 2st, p, t +1m, p, t +1m, p, t	2m, q, i

m = metacentric; sm = submetacentric; st = subtelocentric; a = acrocentric; p = short arm; q = long arm, t = terminal region; i = interstitial region; Ag-NOR = silver stained nucleolar organizer regions; 18S = maximum number of 18S rDNA sites; 5S = maximum number of 5S rDNA sites.

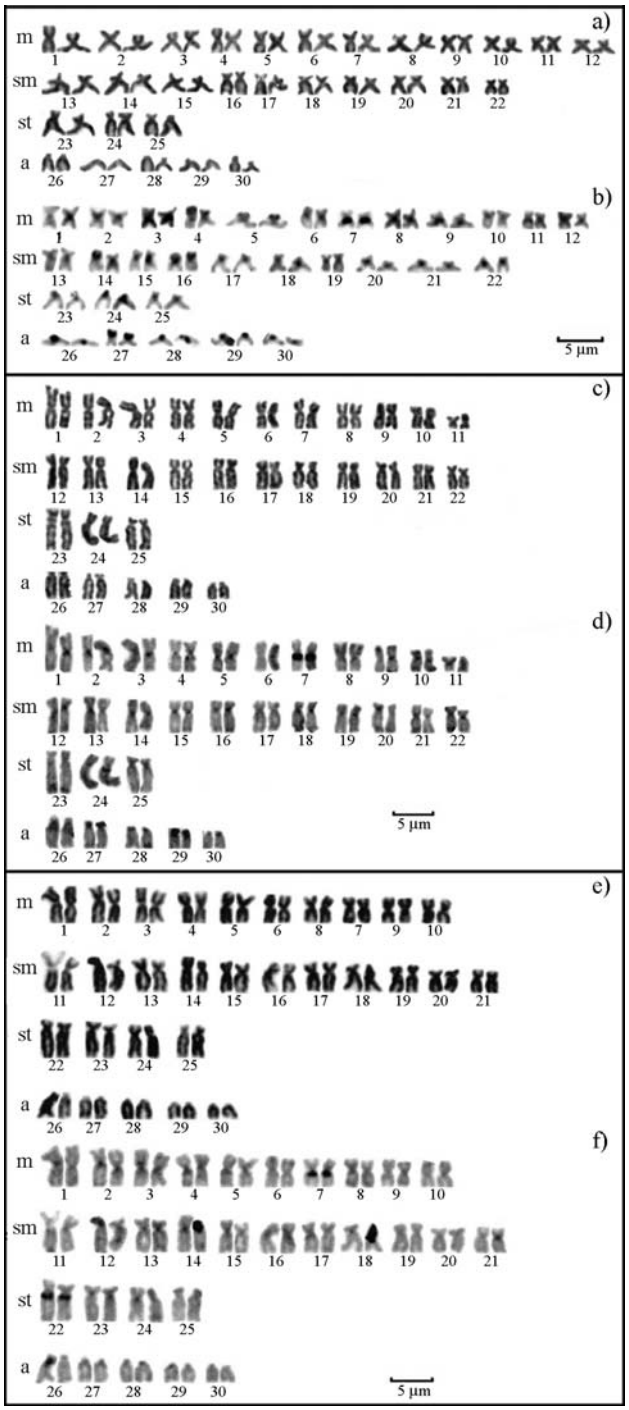

Figure 2 - Karyotype of *Serrasalmus altispinnis*, *S. gouldingi* and *S. serrulatus* after: Giemsa-staining (a, c, e) and C-banding (b, d, f).

artifacts (Figure 2 b, d, f). The metacentric pair 7 presented a remarkable C-band on its long arm close to the centromere in the three species (Figures 2 b, d, f and 5 a, c, e).

The multiple Ag-NORs were located at the terminal regions of the short arms of acrocentric/subtelocentric chromosomes. They varied inter- and intraindividually, as well as inter- and intraspecifically and their maximum numbers were nine in *S. altispinnis*, eight in *S. goldingi* and 12 in *S. serrulatus* (Table 2; Figure 4 a, c, e). Variations were also observed in the 18S rDNA sites. In *S. altispinnis*, the 18S rDNA signals were detected on the same acrocentric chromosomes that showed Ag-NORs. In *S. gouldingi* and *S.*

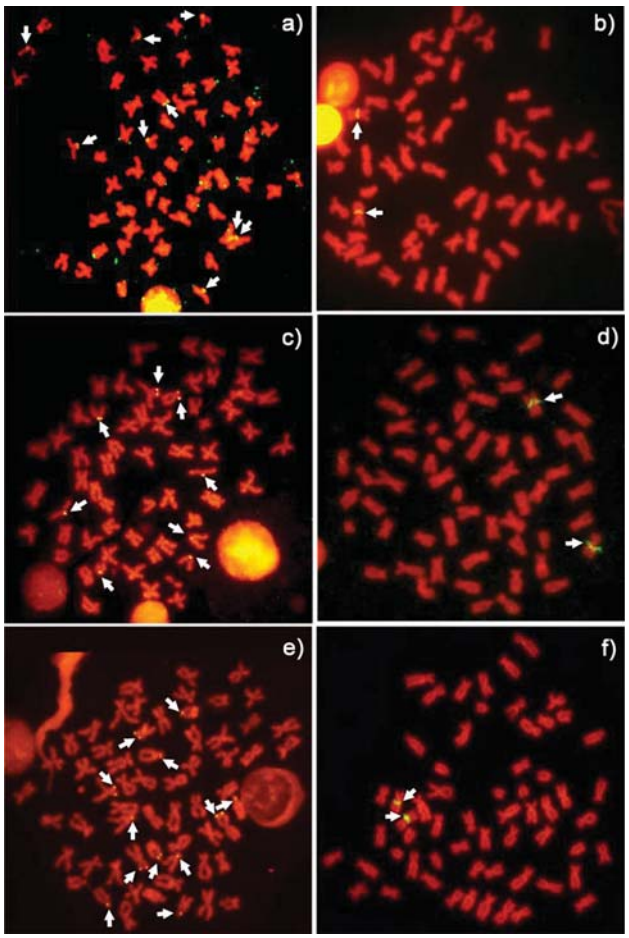

Figure 3 - Metaphases of *Serrasalmus altispinnis* (a, b), *S. gouldingi* (c, d) and *S. serrulatus* (e, f), showing 18S rDNA (a, c, e) and 5S rDNA (b, d, f) positive sites after FISH.

serrulatus the 18S rDNA sites were found on subtelocentric and metacentric chromosomes, respectively, which were not evidenced as Ag-NORs-bearing chromosomes (Table 2; Figure 3 a, c, e; Figure 4 b, d, f). The 5S rDNA sites were present on the long arms of the metacentric pair 7, close to the centromeric region in the three species (Table 2; Figure 3 b, d, f; Figure 5 b, d, f).

Discussion

Most of the ten *Serrasalmus* species previously analyzed showed 2n = 60 (Nakayama *et al.*, 2002); except for *S. hollandi* with 2n = 64 (Muramoto *et al.*, 1968) and a karyotypic variation of *S.* cf. *rhombeus* with 2n = 58 (Nakayama *et al.*, 2001).

Our work presents new chromosomal data for the *Serrasalmus* genus. Indeed, there are no reported data for *S. goulding* and *S. serrulatus* and a new karyotypic form was described herein for *S. altispinnis*. The three species showed 2n = 60 and FN = 110, but they can be differentiated by their karyotypic formulas, which indicated that distinctive non-Robertsonian rearrangements have occurred during the evolution of each species. Nakayama *et al.* (2002) detected a karyotype composed of 20m, 28sm, 2st, 10a and FN = 110 in *S. altispinnis* from the Pitinga River (Uatumã Basin), which differs from the pattern presently described in the specimens from the middle Negro River (22m, 22sm, 6st, 10a and FN = 110). Therefore, this species shows chromosome variation in its geographical range. The fundamental number is the same (FN = 110) in all specimens, thus pericentric inversions, involving at least six chromosomes in one of the karyotypic forms must be the cause of this variation. The fixation of such chromosome rearrangements might have been facilitated by the lack of gene flow between both populations. Actually, there is no current connection between the Uatumã and the Negro Rivers, which were probably separated from each other by Late Tertiary or Early Quaternary (Nogueira and Sarges, 2001). Further associations of these chromosome data with morphologic and genetic features may help to elucidate whether these two karyotypic forms of *S. altispinnis* represent cryptic species or populations under different evolutionary paths.

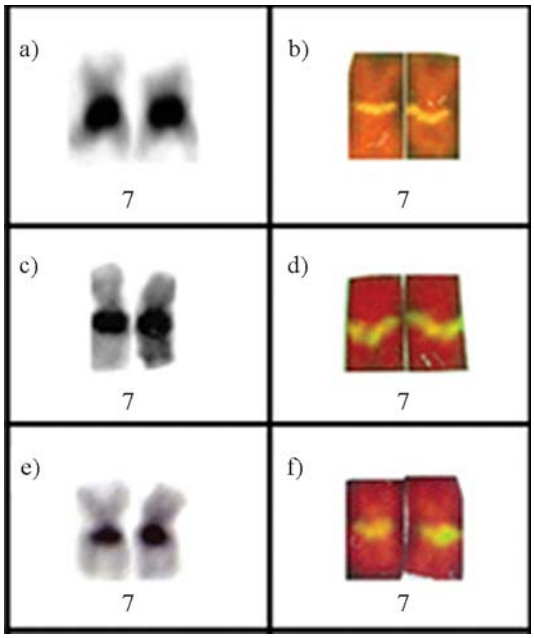

Figure 5 - Pair 7 of *Serrasalmus altispinnis* (a, b), *S. gouldingi* (c, d) and *S. serrulatus* (e, f) showing the co-localization of the C-positive heterochromatic region (a, c, e) and the 5S rDNA positive site (b, d, f).

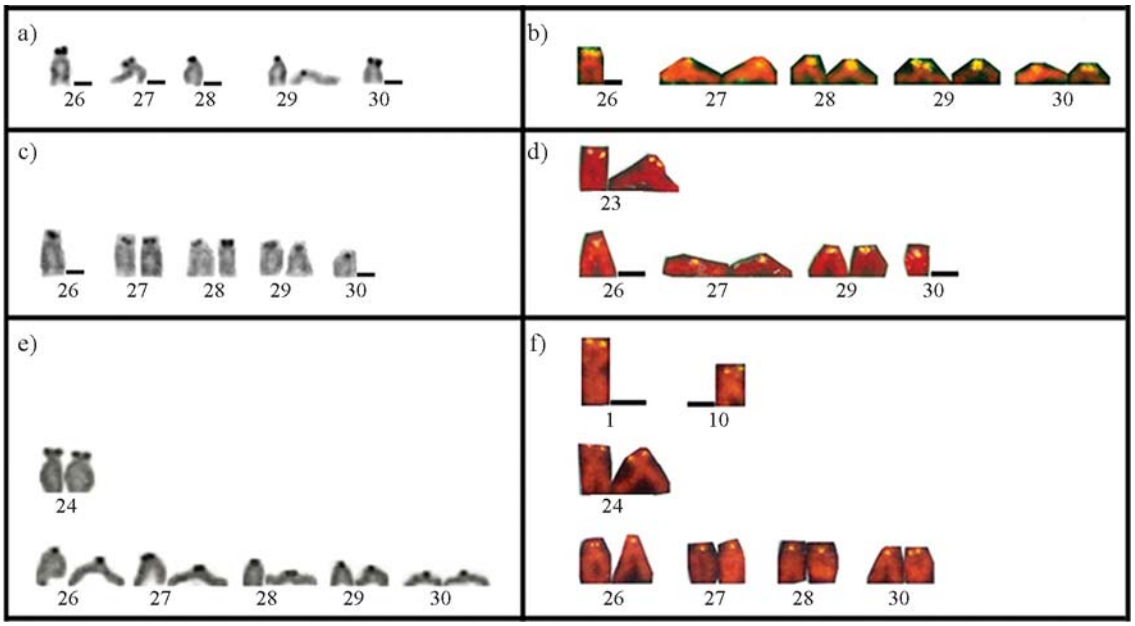

Figure 4 - Partial karyotypes of *Serrasalmus altispinnis* (a, b), *S. gouldingi* (c, d) and *S. serrulatus* (e, f) with Ag-NORs (a, c, e) and 18S rDNA sites (b, d, f).

C-banding was mainly located at the centromeric chromosome regions in the three species, although some bands were more conspicuous than others. A decreasing amount of heterochromatin seems to characterize the karyotypes of *S. altispinnis*, *S. gouldingi* and S. *serrulatus*, respectively. Furthermore, some conspicuous and easily identified species-specific C-bands were also observed, such as those located on long arms of the metacentric pairs 3 and 9 of *S. altispinnis*, on the long arms of the acrocentric pair 26 of *S. gouldingi* and on the long arms of the subtelocentric pair 22 of *S. serrulatus*. It is noteworthy that the three species had a positive C-band on the long arms of the metacentric pair 7, which agrees with the pattern observed in other *Serrasalmus* species (Nakayama *et al.*, 2002; Centofante *et al.*, 2002).

All species of Serrasalminae analyzed so far showed several rDNA sites, with inter- and intraindividual variations, ranging from 4 to 12 Ag-NORs. In species of *Serrasalmus*, the Ag-NORs were always located on the short arms of the acro-subtelocentric chromosomes (Galetti *et al.*, 1985; Cestari and Galetti, 1992a, b; Martins-Santos *et al.*, 1994; Nakayama *et al.*, 2001, 2002; Centofante *et al.*, 2002), coinciding with the pattern observed in the three species analyzed herein. Nonetheless, the maximum number of Ag-NORs differed among species (nine in *S. altispinnis,* eight in *S. gouldingi* and 12 in *S. serrulatus*). The maximum number of 18S rDNA sites and of Ag-NORs was the same in each species, but their localizations were not always coincident by FISH and by Ag-NOR, with the exception of *S. altispinnis*. *S. gouldingi* and *S. serrulatus* presented some subtelocentric and metacentric chromosomes with 18S rDNA sites, respectively, but not with Ag-NORs. Inversely, some Ag-NORs-bearing chromosomes presented no 18S rDNA signals.

The variation in the Ag-NORs could be explained by the differential activity among the distinct ribosomal sites within the species genome, since the silver nitrate staining can only detect NORs that were active in the preceding interphase (Miller *et al.*, 1976). Actually, the inactivity of some ribosomal sites is a common feature observed in fish with multiple NORs, like *Hoplias malabaricus* (Born and Bertollo; 2000); *Astyanax scabripinnis*, *A. parahybae, A. intermedius* and *A. giton* (Kavalco and Moreira-Filho, 2003), or with single NORs, such as some Cichlidae and Curimatidae (Feldberg and Bertollo, 1985; Feldberg *et al.*, 1992;). On the other hand, the lack of FISH signals in some Ag-NOR sites may be due to the small size of these regions. It is probable that the hybridization of the 18S rDNA was hindered or barely perceptible because there were only a few copies of the gene in these sites. Therefore, the real number of NORs in the karyotype of *S. gouldingi* and *S. serrulatus* should be higher than that detected by both silver nitrate staining and by FISH. However, NORs transposition among different chromosomes with the consequent variation in their location cannot be excluded.

The 5S rDNA is located in a single chromosome pair in many organisms. However, in amphibians (Lucchini *et al.*, 1993) and fishes (Martins and Galetti, 1999; 2000), the 5S rDNA cistrons can be present in several chromosomes. Besides, the 45S and 5S rDNA loci may be syntenic (Morán *et al.*, 1996) or located in distinct chromosome pairs (Martins and Galetti, 1999; Born and Bertollo, 2000), the last condition being commonly observed among fishes.The three *Serrasalmus* species showed a single 5S rDNA site, located on the same region of the medium-sized metacentric pair 7, coinciding with a C-positive heterochromatic band. Thus, the chromosome location of the 5S rDNA site and the heterochromatin band are features shared by the *Serrasalmus* species, representing additional important chromosome markers for this fish.

The evolutionary relationships among piranha species are still unclear and several studies have focused on this matter. Molecular and morphological data have been used in order to test the monophyletism of this group (Freeman *et al.*, 2007). Similarly, chromosome data have revealed useful cytotaxonomic features and provided data on the relationships of these species. Therefore, some relatively conserved characteristics such as diploid and fundamental numbers, and some chromosome markers, support the relationship between *S. altispinnis*., *S. gouldingi* and *S. serrulatus* and other *Serrasalmus* species. On the other hand, distinctive features, such as the karyotype formulas, species-specific heterochromatic bands, and the number and location of the 18S rDNA represent helpful tools for the cytotaxonomy of these species, allowing the detection of possible cryptic species, as suggested for *S. altispinnis.*

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