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Chromosome Studies of *Astyanax jacuhiensis* Cope, 1894 (Characidae) from the Tramandai River Basin, Brazil, Using *in situ* Hybridization with the 18S rDNA Probe, DAPI and CMA_3 Staining

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The genus *Astyanax* comprises 86 species of fish distributed in Brazilian river basins and is considered of the *Incertae sedis* group within the family Characidae. This study presents an analysis of 12 specimens of *Astyanax jacuhiensis* from the Tramandai River Basin, RS Brazil: 6 from the Maquiné River and 6 from the Quadros Lagoon. All specimens showed a diploid number equal to 50 chromosomes with different karyotypic formula between the two localities. The population from the Maquiné River showed 10m+26sm+6st+8a (FN=92). Fish from the Quadros Lagoon showed 12m+20sm+6st+12a (FN=88). AgNORs were evidenced in the short arm of one acrocentric chromosome pair in both populations, confirmed by FISH with the 18S rDNA probe. CMA_3 fluorochrome corresponded with the AgNOR sites, while DAPI staining was negative in these regions. C banding revealed that heterochromatin was weakly distributed, mainly in the pericentromeric and terminal regions in most chromosomes. Analyses of male gonadal tissue were conducted with the objective of characterizing the meiotic chromosome behavior in *A. jacuhiensis*. The following stages were evidenced: spermatogonial with 50 chromosomes, pachytene and metaphase I with 25 bivalents, and metaphase II with 25 chromosomes, thus confirming the diploid number of the species. Chromosomal abnormalities were not observed. This study shows preliminary data on *A. jacuhiensis* from the Tramandaí River Basin, contributing with more chromosomal information for this group of fish.

Key words: *Incertae sedis*, cytogenetics, heterochromatin, meiosis, NORs.

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The genus *Astyanax* Baird & Girard, 1854 comprises 86 species of fish distributed throughout the Brazilian river basins and is considered of the *Incertae sedis* group within the family Characidae (LIMA *et al*. 2003). In the genus *Astyanax*, a diploid number equal to 50 is usual, being found in *Astyanax altiparanae* GARUTTI & BRITSKI 2000 (HASHIMOTO *et al.* 2008; PERES *et al*. 2008; FERREIRA NETO *et al*. 2009; PACHECO *et al*. 2011), *A. scabripinnis* Jenyns 1842 (FERNANDES & MARTINS-SANTOS 2005; VICARI *et al*. 2008a, among others), *A. laticeps* COPE 1894 (ROSA *et al*. 2009), *A. bimaculatus* LINNAEUS 1758 (AFFONSO *et al*. 2007; PAMPONET *et al*. 2008), *A. bockmanni* CASTRO & VARI 2007 (KAVALCO *et al.* 2009), and *A. jacuhiensis* COPE 1894 (PACHECO *et al*. 2010). It can be considered a plesiomorphy and is very relevant for the separation of the species in more primitive groups, i.e., those that maintain the diploid number, and more derived groups, such as those that have different diploid numbers (DOMINGUES *et al*. 2007).

The genus *Astyanax* shows variation in the number and location of NORs. The multiple NOR system is the most commonly encountered, as observed in *A. scabripinnis* (FERNANDES & MARTINS-SANTOS 2005, VICARI *et al*. 2008a), *A. fasciatus* CUVIER 1819 (ALMEIDA-TOLEDO *et al.* 2002; PERES *et al*. 2009), and *A. giton* EIGENMANN 1908 (KAVALCO & MOREIRA-FILHO 2003). However, the simple NOR system has previously been documented for *A. altiparanae* (DOMINGUES *et al*. 2007; FERREIRA NETO *et al.* 2009; PACHECO *et al*. 2011), *A. lacustris* LUTKEN 1875 (PERES *et al*. 2008), and *A. jacuhiensis* (PACHECO *et al*. 2010).

The exact location of rDNA sites can be obtained through fluorescence *in situ* hybridization (FISH)

carried out with 18S rDNA, 28S rDNA or 45S rDNA, as has already been recorded for *A. scabripinnis* (PERES *et al.* 2008; VICARI *et al*. 2008a), *A. altiparanae* (DOMINGUES *et al*. 2007; FERREIRA NETO *et al.* 2009; PACHECO *et al*. 2011), *A. fasciatus* (ALMEIDA-TOLEDO *et al*. 2002; PAZZA *et al*. 2008a), *A. lacustris* (ALMEIDA-TOLEDO *et al*. 2002), and *A. jacuhiensis* (PACHECO *et al*. 2010).

In the genus *Astyanax*, the heterochromatin distribution is variable and species with a reduced amount of heterochromatin, such as *A. altiparanae* (DOMINGUES *et al*. 2007; FERREIRA NETO *et al*. 2009), *A. jacuhiensis* (PACHECO *et al.* 2010), and even species with large heterochromatic blocks, such as *Astyanax* sp. D (KANTEK *et al.* 2007; 2008), *A. scabripinnis* (MAISTRO *et al.* 2000; ABEL *et al*. 2006), *A. fasciatus* (ARTONI *et al.* 2006; PAZZA *et al*. 2008b) and *A. janeiroensis* EIGENMANN 1908 (VICARI *et al.* 2008b) have been described.

Due to the large variation observed in this genus, the aim of this study was to analyze cytogenetically *Astyanax jacuhiensis* from both the Maquiné River and the Quadros Lagoon, which belong to the Tramandaí River Basin, RS, thus contributing more chromosomal data for this group of fish.

Material and Methods

This analysis included 12 individuals of *Astyanax jacuhiensis* (Fig.1) collected in 2010, in the Tramandaí hydrographic basin, RS, in the municipality of Maquiné: 6 males from the Maquiné river (29°39'10.4''S e 50°12'31.8''W) and 2 females and 4 males collected in the Quadros Lagoon (29°46'21.2''S e 50°05'08''W).

Conventional staining

Mitotic chromosomes were obtained by direct preparation removing the anterior kidney, according to BERTOLLO *et al.* (1978) and meiotic chromosomes were obtained using spermatogonial cells using the technique developed by KLIGERMAN & BLOOM (1977), with modifications. The chromosomes were organized as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) for the preparation of a karyogram. Metacentric, submetacentric and subtelocentric chromosomes were considered biarmed and acrocentric uniarmed for determination of the fundamental number (FN) according to LEVAN *et al*. (1964).

Chromosome banding

Active nucleolar organizer regions (NORs) were detected by silver nitrate staining (HOWELL & BLACK 1980). The distribution of heterochromatin was analyzed by Giemsa and fluorochromes C-banding after treatments with formamide 50% for 2 min at 70°C and 2xSSC from 30 to 60 min at room temperature (FERNÁNDEZ *et al*. 2002). GC- and AT-rich bands were detected with chromomycin A_3 (CMA_3) and 4'-6-diamino-2-phenylindole (DAPI), respectively, according to SCHWEIZER (1980).

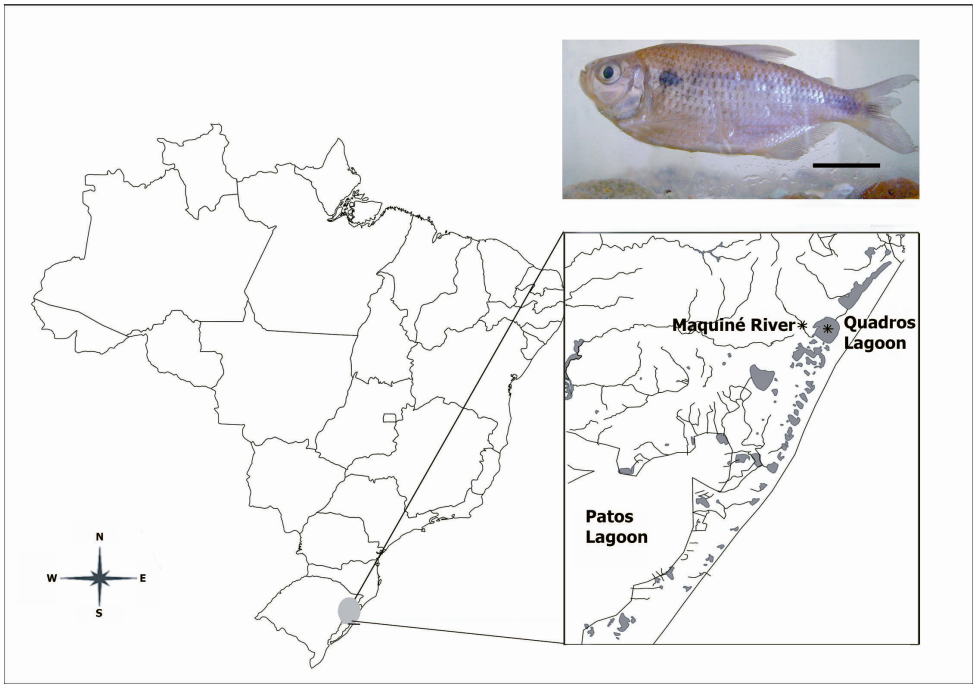

Fig. 1. Collection sites of *Astyanax jacuhiensis.* Map of Brazil showing the Rio Grande do Sul state in the selected area (left side); Tramandaí River Basin map (right) showing the Maquiné River and Quadros Lagoon. In detail the species *Astyanax jacuhiensis*. Bar 1cm.

Fluorescence in situ hybridization (FISH)

The *in situ* hybridization procedure was performed according to PINKEL *et al*. (1986), with modifications. The 18S rDNA probe of *Prochilodus argenteus* Agassiz, 1829 (HATANAKA & GALETTI Jr 2004) was labeled with biotin-14-dATP by nick. Slides were treated with 50 μl of hybridization mixture containing 100 ng of labeled probe (7,5 μl), 50% formamide (30 μl), dextran sulfate 50% (12 μl), 20 SSC (10,5 μl). The material was denatured at 80°C for 10 min, and hybridization was performed overnight at 37°C in a humidified chamber. Post-hybridization washes were carried out in 2 SSC for 5 min, in 1x PBS and 1x PBD (20xSSC, Triton 100, unfat milk and distilled water qsp 100, pH 7), all at 45°C. The probe was detected with 5 μl of FITC (1:100) and 45 μl of BSA (5%). In order to amplify the signal, we used 40 μl of solution of amplification (1 ml anti-avidin-biotin conjugate and 39 ml of 1x PBD). Slides were mounted with 25 μl of medium composed of 23 l of DABCO solution (1,4-diaza- bicyclo (2.2.2)-octane (2,3%), 20 mM Tris HCl, pH 8.0, (2%) and glycerol (90%), in distilled water), 1 l of MgCl2 50 mM and 1 μl of propidium iodide (50 μg/ml). All the images were acquired with a Leica DM 4500 B microscope equipped with a DFC 300FX camera and Leica IM50 4.0 software, and optimized for best constrast and brightness with iGrafx Image software.

Results and Discussion

The specimens of *Astyanax jacuhiensis* analyzed exhibited 2n=50 in both locations, however, with different karyotypic formulae. The population from the Maquiné River showed 10m+26sm+6st+8a (NF=92) (Fig. 2a), and that from the Quadros Lagoon presented 12m+20sm+6st+12a (NF=88) (Fig. 2b). This diploid number is usually found within this genus and has previously been reported for other species, such as *A. altiparanae* (DOMINGUES *et al*. 2007, PERES *et al*. 2008, FERREIRA NETO *et al*. 2009; PACHECO *et al*. 2011), *A. bimaculatus* (AFFONSO *et al*. 2007; PAMPONET *et al*. 2008), *A. fasciatus* (ARTONI *et al*. 2006), *A. laticeps* (ROSA *et al*. 2009) and *A. scabripinnis* (PERES *et al*. 2008; VICARI *et al*. 2008a).

The first karyotypic description of *Astyanax jacuhiensis* was given by PACHECO *et al*. (2010) who found 2n=50 with a karyotypic formula of 8m+30sm+4st+8a (NF=92) in specimens collected from the Guaíba Lake, RS. Despite the difference in the karyotypic constitution, the fundamental number is equal to that of the population from the Maquiné River. This means that these populations have the same number of chromosomes with 1 and 2 arms and the existing differences are ascribable to chromosomal rearrangements, such as pericentric inversions. The same can be said for the Quadros Lagoon population, which shows a greater variation in relation to the other two populations of *A. jacuhiensis* studied. However, the condensation of chromosomes can lead to errors in interpretation of chromosome classification.

Through silver nitrate impregnation, nucleolus organizing regions were observed on the short arm of only one pair of acrocentric chromosomes in both populations of *Astyanax jacuhiensis* (Fig. 2-box), occurring size polymorphism of AgNOR, since both homomorphic and heteromorphic NORs showing inter-and intra-individual variation were observed. FISH with 18S rDNA probe confirmed the simple NOR pattern but did not confirm the size heteromorphism between homologous chromosomes, thus meaning that the size difference observed after silver nitrate staining is not structural, but only phenotypic.

In *Astyanax jacuhiensis* from the Guaíba Lake, PACHECO *et al.* (2010) showed from 2 to 5 AgNOR chromosomes revealing, at first, a multiple system with size heteromorphism on the short arm of submetacentric pair 8, which is the most frequent Ag-NOR pair. However, FISH with 18S rDNA probe corroborated the presence of simple NOR patterns by marking only the eighth pair, also confirming the occurrence of size heteromorphism. The populations of the Maquiné River and Quadros Lagoon, however, presented NORs on a pair of acrocentric chromosomes. This variation in the NOR-bearing chromosomal type might also be attributable to the occurrence of chromosomal rearrangements, such as pericentric inversions. The simple NOR pattern is less often found in this genus, having been documented in populations of *A. altiparanae* (FERNANDES & MARTINS-SANTOS 2004; DOMINGUES *et al.* 2007; PERES *et al*. 2008) and *A. lacustris* (PERES *et al.* 2008).

In *Astyanax jacuhiensis* from the Maquiné River and Quadros Lagoon, the acrocentric pair corresponding to the NOR was CMA_3 positive and DAPI negative (Fig. 2), i.e. rich in GC base-pairs and low in AT base-pairs. In *A. jacuhiensis* from the Guaíba Lake, analyzed by PACHECO *et al.* (2010), besides CMA_3 positive signals corresponding to NORs, fluorescent markers were detected in some other chromosomes, which according to the authors, were probably related to GC-rich heterochromatic regions.

Heterochromatin in the two populations of *Astyanax jacuhiensis* analyzed proved to be weakly distributed in pericentromeric and terminal re-

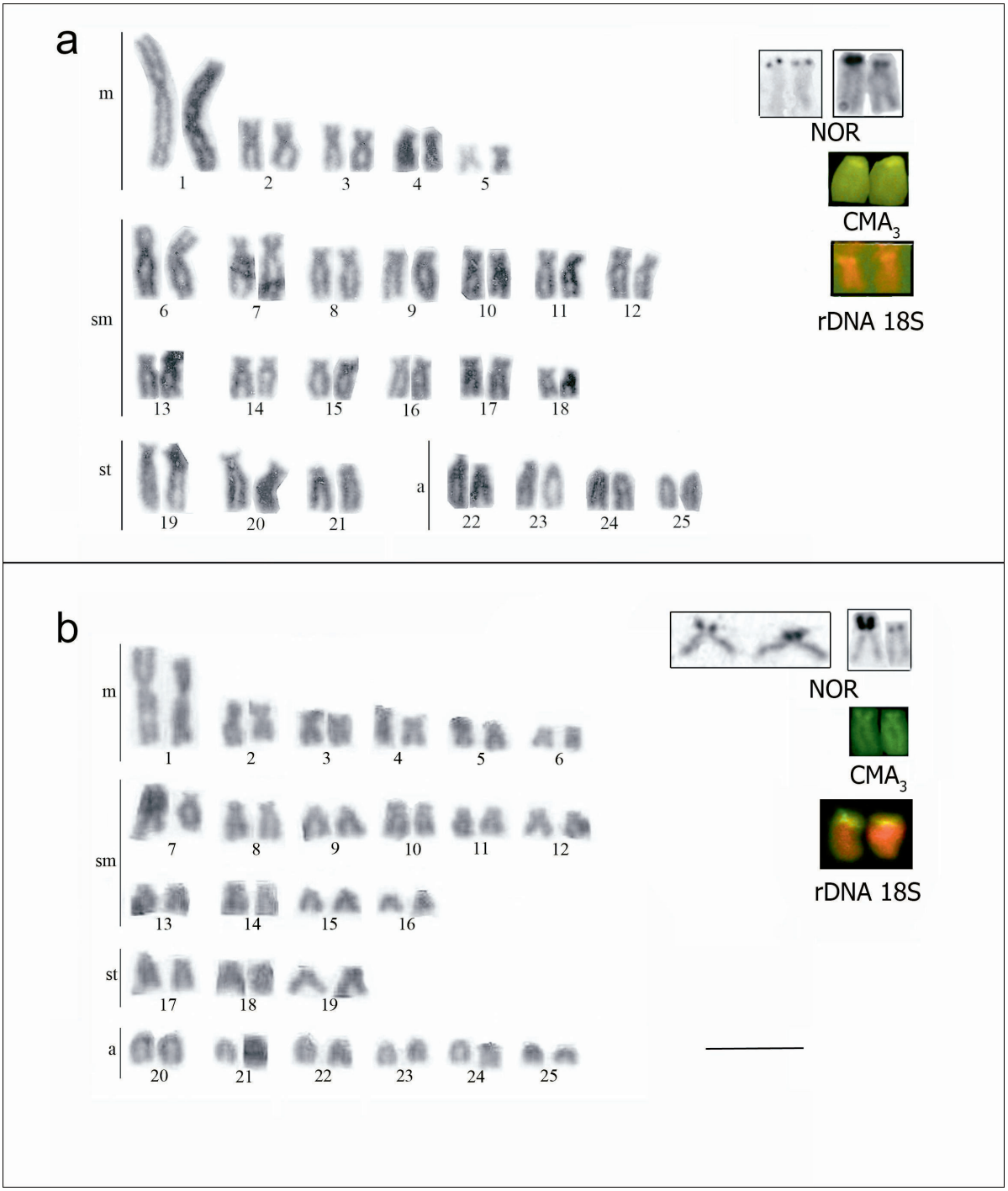

Fig. 2. Karyograms of *Astyanax jacuhiensis*: Maquiné River (a) and Quadros Lagoon (b) showing AgRONs, CMA_3^+ and rDNA 18S sites in boxes. Note size polymorphism of the AgNORs. Bar 5 μm.

gions in most chromosomes (Fig. 3A). Through sequential staining with CMA_3/DAPI after C-banding, both CMA_3^{+}/$DAPI^{+}$ signals were observed, meaning that these populations of *A. jacuhiensis* have two types of heterochromatin, one rich in GC and another in AT base-pairs (Fig. 3B-C). Figure 3B shows one chromosome with more evident CMA_3^{+} signals, which probably corresponds to the NOR-bearing chromosome, a result similar to that previously found by PACHECO *et al*. (2010).

Analyses in gonadal tissues were also performed on all males of *Astyanax jacuhiensis* of the Quadros Lagoon, to characterize the meiotic chromosome behavior of this species, and the following phases were observed: spermatogonial with 50 chromosomes, pachytene and metaphase I with 25 bivalents, and metaphase II with 25 chromosomes, thus confirming the diploid number, constituting the first meiotic data on this species (Fig. 4). It is worth mentioning that the meiotic behavior has not

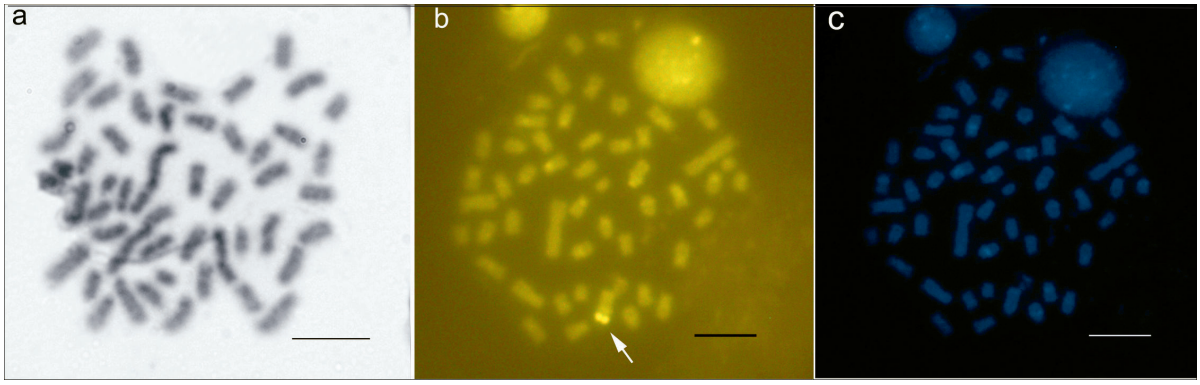

Fig. 3. Somatic metaphase of *Astyanax jacuhiensis* with C-banding: a) conventional Giemsa staining, b) sequential staining with CMA_3 and c) with DAPI. Note pericentromeric bands and some terminals CMA_3^+/$DAPI^+$. The arrow in (b) indicates, probably, the NOR-bearing chromosome with heterochromatin CMA_3^+. Bar 5 μm.

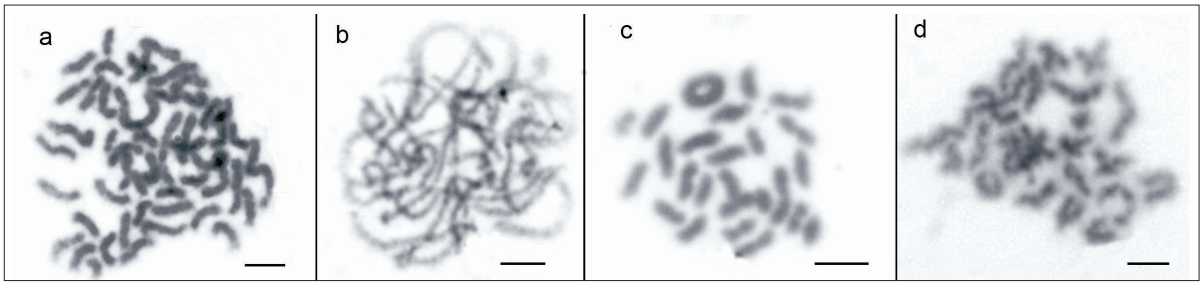

Fig. 4. Phases of meiosis in *Astyanax jacuhiensis* from Quadros Lagoon: a) Spermatogonial metaphase, with 50 chromosomes; b) Pachytene, with 25 bivalents; c) Metaphase I, with 25 bivalents and d) Metaphase II, with 25 chromosomes. Bar 5 μm.

changed, although pericentric inversions have occurred. The presence of 25 bivalents during metaphase I and 25 chromosomes in metaphase II shows that pairing during meiosis usually occurs. This means that the gametes are viable and shows that the species supports karyotypic variations resulting from chromosomal rearrangements, as these did not interfere with the meiotic behavior.

The data obtained herein are similar to those found by PACHECO *et al.* (2010) for *A. jacuhiensis* from the Guaíba Lake, RS. These authors observed the same diploid number in the two populations analyzed herein and the same fundamental number found in the population of the Maquiné River, evidencing karyotypic similarity between these populations. The maintenance of a simple NOR pattern was also observed for the three populations, a feature that distinguishes them from most species of the genus, which present multiple NORs. Small variations, such as different types of NOR-bearing chromosomes between them may indicate the occurrence of chromosomal rearrangements in the species, leading to a natural differentiation between populations, considering that although living close to each other, they dwell in distinct water basins.

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