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Highest Diploid Number Among Gymnotiformes: First Cytogenetic Insights into *Rhabdolichops* (Sternopygidae)

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Abstract

We report the first comparative cytogenetic analysis of two species from electrogenic fish of genus *Rhabdolichops* (Sternopygidae, Gymnotiformes): *Rhabdolichops troscheli* and *Rhabdolichops* cf *eastwardi*. *R. troscheli* has 2n = 54 (fundamental number [FN] = 66), whereas *R.* cf. *eastwardi* has 2n = 74 (FN = 78). C-banding revealed centromeric constitutive heterochromatin in both species. Ag-NORs mapped on pair 6 in *R. troscheli* and pair 30 in *R.* cf *eastwardi*. Fluorescense *in situ* hybridization with 18S rDNA probes confirmed the Ag-NOR staining results and revealed additional (presumably silent) ribosomal genes on pairs 12, 13, 21, 23, 26, and 27 in *R.* cf *eastwardi*. 5S rDNA was found on the centromeres of pair 7 in both species. Telomeric probes showed only distal locations. Dispersed signal patterns were obtained using probes for retrotransposons *Rex*1 and *Rex*3. Histone H1 and H3 genes were found together on pair 6 in *R.* cf *eastwardi*. The high diploid number found in *Rhabdolichops* suggests that chromosome fission may have contributed to its chromosomal evolution, phylogenetic relationship of the Sternopygidae suggests that this increase in diploid number could be a synapomorphic characteristic of genus *Rhabdolichops*. Although both species are phylogenetically close related, their karyotype structure has undergone divergent evolutionary directions. All in all, our results strongly suggest that *R.* cf *eastwardi* experencied recent intense genome reorganization.

Keywords: electric fish, chromosomes, biodiversity, Amazon rainforest, karyotypic diversity

Introduction

The electrogenic fish of the genus *Rhabdolichops* inhabit the lowlands of South America, and are found in the basins of the Amazon and Orinoco and the minor basins of the Guianas.[1] *Rhabdolichops*, *Archolaemus*, *Distocyclus*, *Eigenmannia*, *Japigny*, and *Sternopygus* together comprise the monophyletic family, Sternopygidae, whose members are widely distributed in Neotropical regions.[1,2] Ten species of *Rhabdolichops* are currently recognized; most of them feed on zooplankton, and they are commonly found in the benthic regions of deep rivers.[3–5]

Phylogenetic analyses revealed Sternopygidae to be a monophyletic group that comprises two clades with a sister taxon relationship.[2] The *Sternopygus* species form one clade, while the other clade, which is known as the Eigenmanninae subfamily, includes all remaining genera of this family. *Rhabdolichops* appears in a basal phylogenetic position among the Eigenmanninae genera, but the monophyly of these genera is called into question by the positioning of *Rhabdolichops lundbergi* and *Rhabdolichops nigricans*, which are more closely related to *Eigenmannia* than to the other *Rhabdolichops* species.[2]

Cytogenetics studies among Gymnotiformes are still in early stages. The species of genera *Gymnotus* and *Eigenmannia* have been the most widely studied to date (for review see Refs.[6,7]). The Gymnotiformes already studied show great karyotypic diversity, with diploid numbers ranging from

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2n = 22/24 in *Apteronotus albifrons*[8] to 2n = 54 in *Gymnotus inaequilabiatus*,[9] *Gymnotus carapo*,[9] *Gymnotus mamiraua*,[10] and *Gymnotus paraguensis*.[11]

In Sternopygidae, cytogenetic results have been published only for *Sternopygus macrurus*, which has a conserved 2n = 46 karyotype,[12,13] and several chromosomally diversified species of *Eigenmannia*, which reportedly have 2n = 28, 32, 32/31, and 38.[7,14,15] Sex-chromosome systems have been found in some species of *Eigenmannia*; they range from female heterogametic systems (ZZ/ZW) to single or multiple male heterogametic systems (XX/XY or $X_1X_1X_2X_2/X_1X_2Y$).[7,12,15–17]

The major ribosomal genes (the 18S or 45S rDNAs) and that encoding the 5S rDNA have been mapped in some *Eigenmannia* species, with the latter exhibiting multiple centromeric locations in most of the tested species.[14] Interstitial telomere sequences (ITSs) were found in a species of *Eigenmannia* with a low diploid number.[14] To date, no cytogenetic information has been published for *Archolaemus*, *Distocyclus*, *Japigny*, or *Rhabdolichops*.

In this study, we present the first conventional and molecular cytogenetic analysis of two species of *Rhabdolichops troscheli* and *Rhabdolichops* cf *eastwardi* from the eastern region of Amazon rainforest.

Materials and Methods

Samples

Four specimens of *R.* cf *eastwardi* (P-153/P-1540-1) and one *R. troscheli* (P-1903-4) were collected from two localities in the eastern Amazon rainforest (Fig. 1): one *R.* cf *eastwardi* and the *R. troscheli* in the Abaetetuba municipality (Caripetuba River: 01°37′23.49″S/048°55′33″W) and three *R.* cf *eastwardi* in the Barcarena Municipality (Arienga River: 1°36′28.40"S/48°48′16.70"W). Samples were collected with seine nets in agreement with Brazilian environmental protection legislation, under license 020/2005 (ICMBio Registration: 207419).

Specimens were kept alive with portable aeration and transported to the laboratory. They were then anesthetized with benzocaine hydrochloride solution and euthanized. Representative samples have been deposited in the Ichthyology Collections of the Centro de Estudos Avançados da Biodiversidade (CEABIO) of Universidade Federal do Pará (Belém, Brazil).

Chromosome preparations

Mitotic chromosomes were obtained from cephalic kidney cell suspensions.[18] Conventional karyotyping was performed

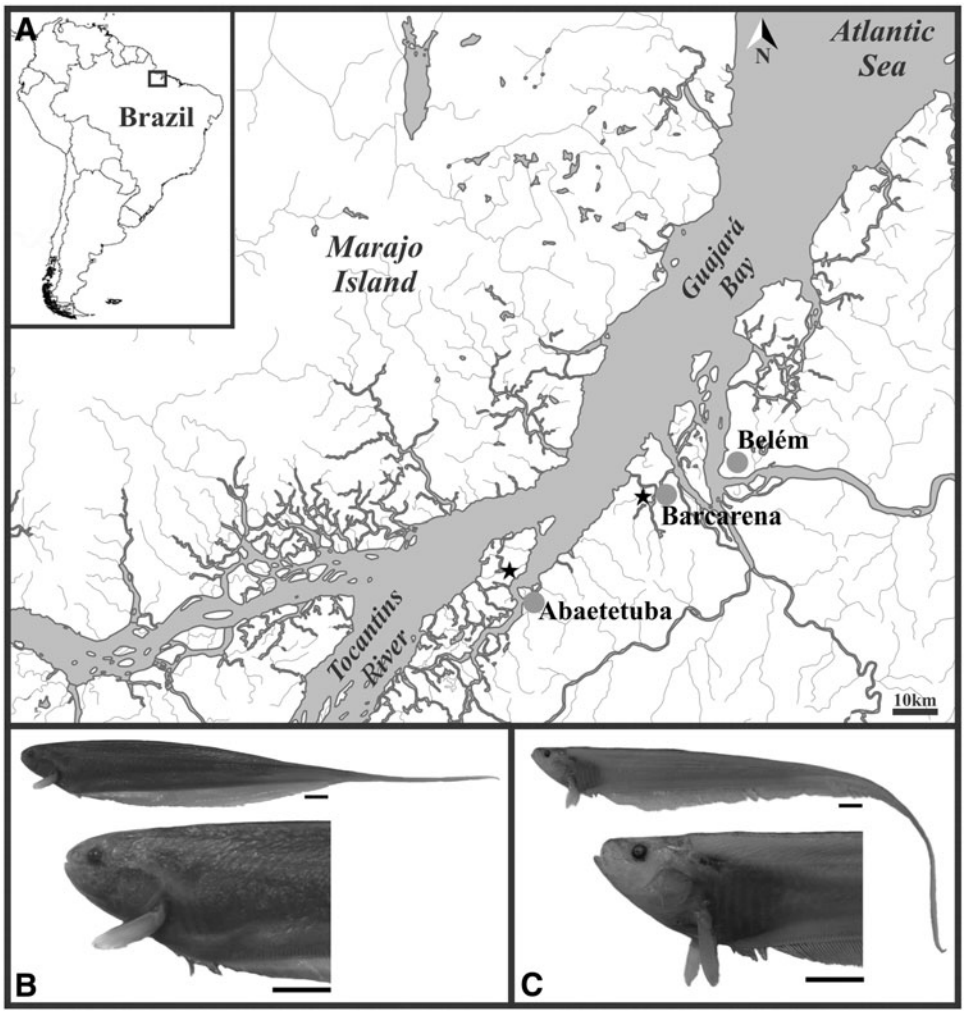

FIG. 1. Geographic locations (*stars*) of the sampling sites **(A)**, and specimens of *Rhabdolichops* cf *eastwardi* **(B)** and *Rhabdolichops troscheli* **(C)**. Bar **(B, C)** = 1 cm.

with Giemsa staining. Constitutive heterochromatin (CH) was characterized by C-banding.[19] Impregnation of NOR (nucleolar organizer region) with silver nitrate (Ag-NOR staining) was used to detect the nucleolar activity of major ribosomal genes.[20] DAPI/CMA_3 (4′,6-diamidino-2-phenylindole and Chromomycin A3) fluorochrome staining was used to identify AT- and GC-rich regions.[21]

For molecular characterizations of chromosomes, we performed fluorescense *in situ* hybridization (FISH)[22] with probes for 18S rDNA, 5S rDNA, telomeric sequences (TTAGGG)n, histone H1, histone H3, and the retrotransposable elements *Rex*1 and *Rex*3. The probes were labeled with digoxigenin-11-dUTP or biotin-16-dUTP and detected using avidin labeled with Cy3 (cyanine 3) or FITC (fluorescein isothiocyanate) or antidigoxigenin (labeled with FITC). Images were captured using a Nikon H550S fluorescent photomicroscope equipped with the Nis-Elements software. Karyotypes were built by digital handling with Adobe Photoshop CS5. Chromosomes were classified as described[23]: they were distributed according to their arm ratios as submetacentric or subtelocentric/acrocentric chromosomes and arranged by decreasing size in each group.

Probe preparation

Cell suspensions of *R.* cf *eastwardi* and *R. troscheli* were used for DNA purification with the DNAzol reagent (Invitrogen). The major ribosomal 18S rDNA genes were amplified[24] and labeled with digoxigenin-11-dUTP using the DIG-Nick Translation Mix (Roche). The coding region of the minor ribosomal 5S rDNA was amplified by polymerase chain reaction (PCR) using own design primers 5rF (5′-GCC ACA CCA CCC TGA ACA C-3′) and 5rR (5′-GCC TAC GAC ACC TGG TAT TC-3′). PCR was performed in a final volume of 25 μL containing 100 ng of genomic DNA, 1 × NH4-based polymerase reaction buffer, 200 μM of each dNTP (or for labeling PCR experiments, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 140 μM dTTP, and 60 μM biotin-16-dUTP), 0.4 μM of each primer, 1.5 mM of $MgCl_2$, and 2 U of BIOTAQ™ DNA Polymerase (BIOLINE).

The cycling conditions were as follows: 5 min at 95°C; 30 cycles of 1 min at 95°C, 30 s at 50°C, and 45 s at 72°C; and a final extension step at 72°C for 5 min. A probe for the general vertebrate telomeric sequence (TTAGGG)n was generated and PCR labeled[25] using primers $(TTAGGG)_5$ and $(CCCTAA)_5$. The coding domains of the reverse transcriptase genes of the retrotransposons, *Rex*1 and *Rex*3, were PCR amplified with primers RTX1-F1 (5′-TTC TCC AGT GCC TTC AAC ACC-3′) and RTX1-R3 (5′-TCC CTC AGC AGA AAG AGT CTG CTC-3′) for *Rex*1,[26] and RTX3-F3 (5′-CGG TGA YAA AGG GCA GCC CTG-3′) and RTX3-R3 (5′-TGG CAG ACN GGG GTG GTG GT-3′) for *Rex*3.[27]

Histone H1 was amplified by PCR using own design primers H1f (5′-AGA RGA GCG GCG TGT-3′) and H1r (5′-CYT CTT CRC CTT CYT KG-3′), and H3 were amplified with degenerate primers H3F1 (5′GGC NMG NAC NAA RCA RAC) and H3R1 (5′TGD ATR TCY TTN GGC ATD AT),[28] respectively. All PCR products were confirmed on 1% agarose gels and quantified using an Epoch™ Multi-Volume Spectrophotometer System (Bio Tek).

Results

Rhabdolichops troscheli

All the analyzed specimens exhibited karyotypes with a diploid number (2n) of 54 chromosomes, a karyotypic formula (KF) of 12 sm (submetacentrics) and 42 st/a (subtelocentrics/acrocentrics), and no sex chromosome system with a fundamental number (FN) = 66 (Fig. 2A). Ag-NOR staining was found pericentromerically on the short arm of pair 6 (Fig. 2A, square), which was consistent with the results of our *in situ* hybridization experiments with the 18S rDNA probes (Fig. 3A). The C-banding pattern corresponded to mainly centromeric CH (Fig. 2B). The DAPI/CMA_3 pattern indicated the presence of AT-rich CH (Fig. 3A) and CG-rich NORs (Fig. 3B). FISH with 5S rDNA probes showed a centromeric signal on pair 7 (Fig. 3A, square).

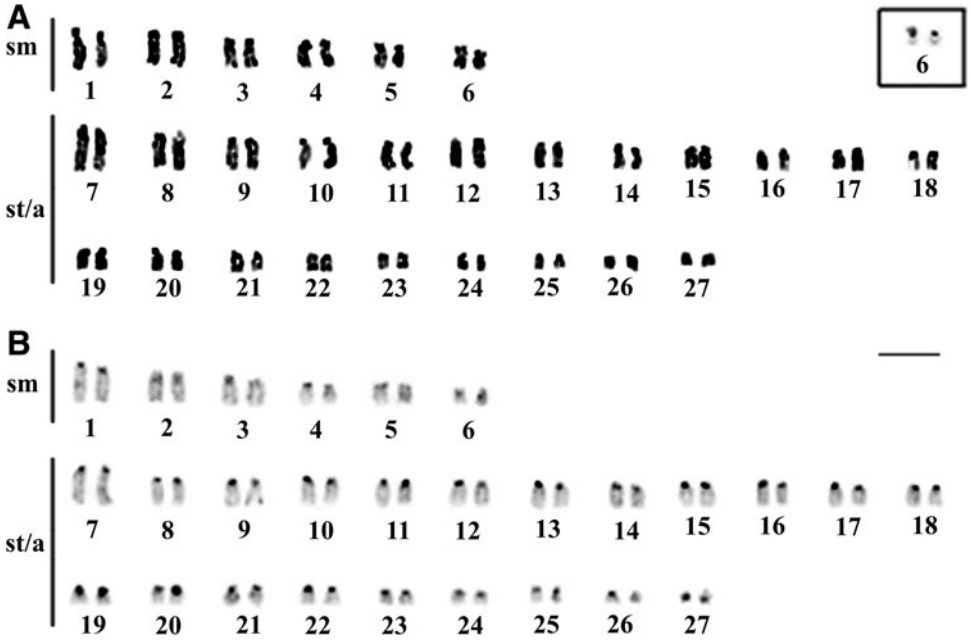

FIG. 2. *R. troscheli.* **(A)** Giemsa-stained karyotype and the Ag-NOR signal (*square*) on chromosome pair 6. **(B)** Karyotype showing the C-banding pattern. Bar = 5 μm.

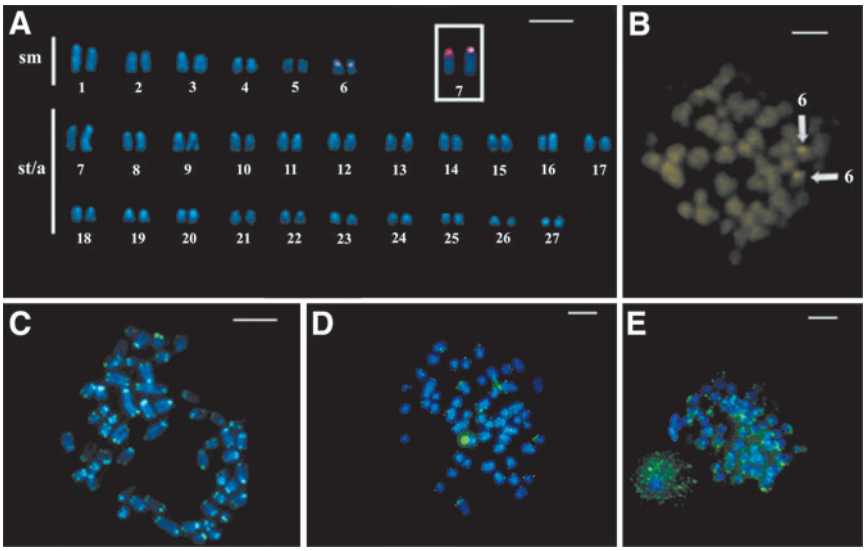

FIG. 3. *R. troscheli.* **(A)** DAPI-counterstained karyotypes showing FISH with 18S rDNA probes (*red* mark on pair 6) and 5S rDNA (*square* on pair 7). **(B)** CMA_3-stained metaphase chromosomes exhibiting pericentric positivity on the short arm of pair 6 (*white arrows*). **(C)** FISH with telomeric probes shows signals only at the chromosome tips. **(D)** FISH with *Rex*1 probe shows a scattered signal pattern. **(E)** FISH with *Rex*3 probe shows a scattered signal pattern. Bar = 5 μm. FISH, fluorescense *in situ* hybridization.

The probe (TTAGGG)n detected the terminal sites in all chromosome complements with different signal intensities, and no ITSs were detected (Fig. 3C). Finally, FISH with the *Rex1* and *Rex3* probes revealed the presence of these repetitive elements on some heterochromatic regions (Fig. 3D and E, respectively).

Rhabdolichops cf *eastwardi*

All specimens of this species exhibited karyotypes of 2n = 74 chromosomes, a KF of 4 sm and 70 st/a (FN = 78) (Fig. 4A), and no sex chromosome system. Ag-NOR positivity was detected distally on pair 30 (Fig. 4A, square). The C-banding pattern indicated that CH was present at almost all centromeric regions (Fig. 4B). Fluorochrome banding indicated that most of the centromeric CH corresponded to AT-rich (DAPI-positive) regions (Fig. 5A), although there were three distal CG-rich (CMA_3-positive) regions, which were observed on pairs 23, 24, and 30 (Fig. 5B). *In situ* hybridization experiments revealed that an 18S rDNA signal coincided with the distal Ag-NOR signal on pair 30 and also with the telomeres of pairs 11, 12, 13, 21, 23, 24, 26, and 27

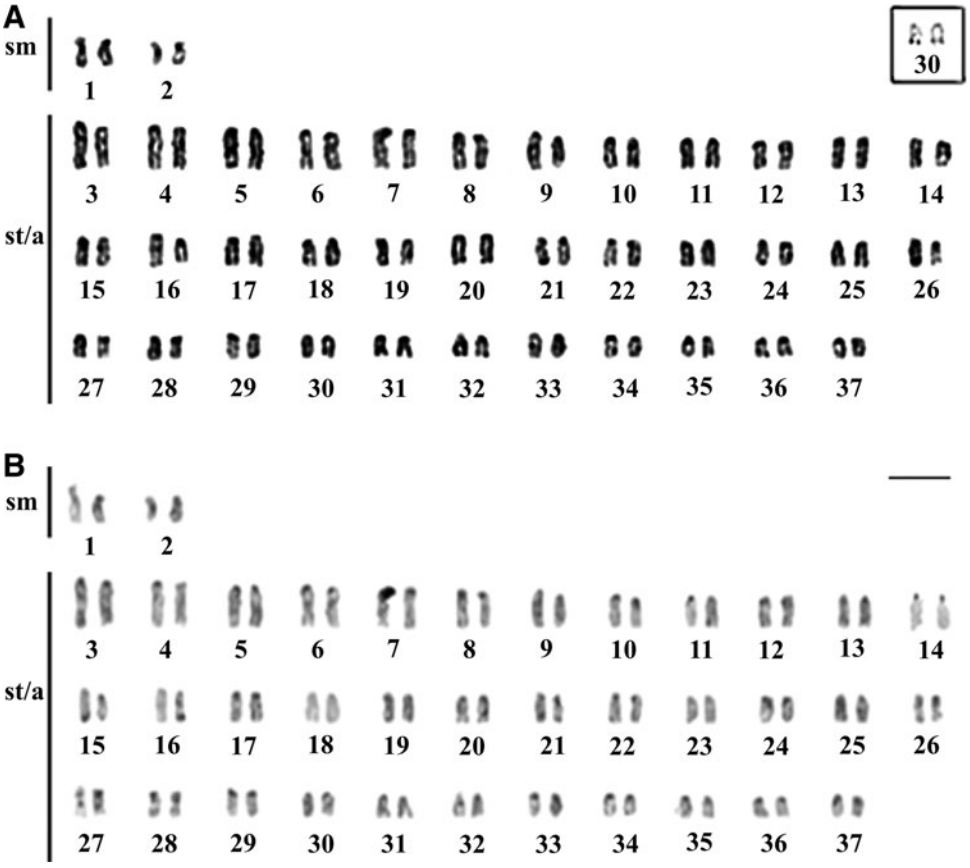

FIG. 4. Karyotype of *R.* cf *eastwardi*. **(A)** Giemsa-stained karyotype and the Ag-NOR staining (*square*) on chromosome pair 30. **(B)** C-banding pattern. Bar = 5 μm.

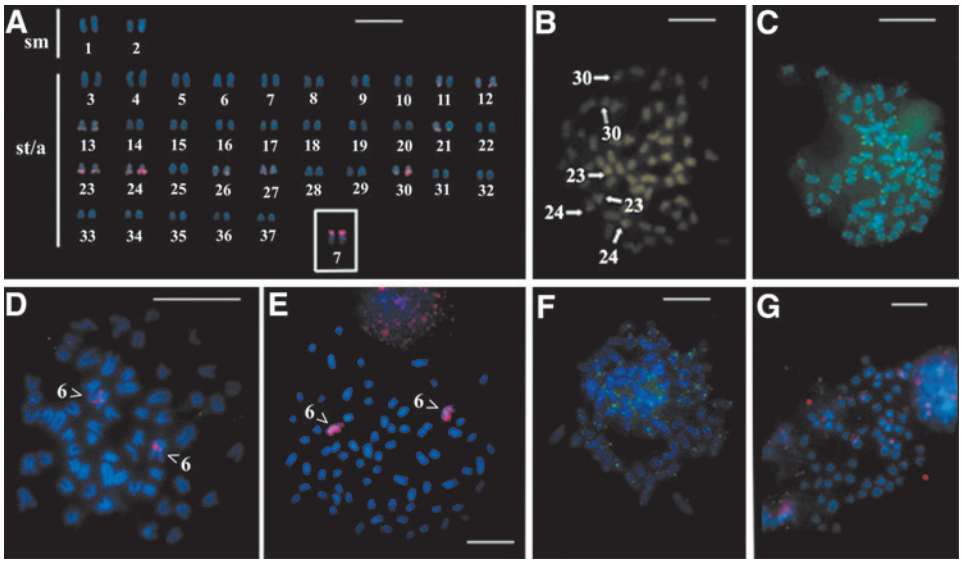

FIG. 5. *R.* cf *eastwardi.* **(A)** DAPI-counterstained karyotypes showing FISH with 18S rDNA probes (*red* marks on pairs 11–13, 21, 23–24, 26–27, and 30) and the 5S rDNA (*square* on pair 7). **(B)** CMA_3-stained metaphase chromosomes exhibiting positive bands on pairs 23–24, and 30 (*white arrows*). **(C)** FISH with telomeric probes shows signals only at the chromosome tips. **(D)** FISH with histone H1 probe. **(E)** FISH with histone H3 probe. **(F)** FISH with *Rex*1 probe shows a scattered signal pattern. **(G)** FISH with *Rex*3 probe shows a scattered signal pattern. Bar = 5 μm.

(Fig. 5A). FISH with 5S rDNA probe mapped this sequence in pericentromeric region of pair 7 (Fig. 5A, square).

The probe (TTAGGG)n detected the terminal sites in all chromosome complement with different signal intensities, and no ITS were detected (Fig. 5C). Probes for histones H1 and H3 revealed an interstitial signal on chromosome pair 6 (Fig. 5D and E, respectively). FISH with *Rex*1 and *Rex3* probes shows the presence of these repetitive elements on some heterochromatic regions (Fig. 5F and G, respectively).

Discussion

R. troscheli (2n = 54; FN = 66) shares the higher 2n already described for Gymnotiformes such as *G. inaequilabiatus*,[9] *G. carapo*,[9] *G. mamiraua*,[10] and *G. paraguensis.*[11] However, we herein report a new highest 2n for this family, which we found in *R.* cf *eastwardi* (2n = 74; FN = 78). This expands the karyotypic diversity known for this group of fish and suggests more complex karyotypic evolution for Gymnontiformes.

Among the Sternopygidae, the karyotypes of the diversified species of *Eigenmannia* (2n = 28–38; FN = 42–50) and a conserved *S. macrurus* (2n = 46; FN = 92) have relatively low diploid numbers compared with *R. troscheli* (2n = 54; FN = 66) and *R.* cf *eastwardi* (2n = 74; FN = 78). The former group, however, have higher FNs. This is due to the presence of more biarmed chromosomes, especially in *S. macrurus* (2n = 46, FN = 92), which has only biarmed chromosomes.[12,13] The karyotypic formulae of the studied *Rhabdolichops* species resemble those found in most species of *Eigenmannia*, especially, those with higher diploid numbers.[7,13,14] However, they are quite different from the published karyotypes of *Sternopygus* species, which only have biarmed chromosomes.[12,13]

The high diploid number found in *Rhabdolichops* suggests that chromosome fission may have contributed to the chromosomal evolution of this genus. Given the absence of interstitial telomeric signals in *Rhabdolichops*, we speculate that pericentric inversions and/or short arm additions could account for the increases in FN. Another possibility is that centromeric repositioning could be acting to promote the observed differences.[29] The phylogenetic relationship of the Sternopygidae[2] suggests that increases in diploid number could be a synapomorphic characteristic of genus *Rhabdolichops* among the Sternopygidae.

A high degree of AT-rich heterochromatin, such as observed in this study for *Rhabdolichops*, has been commonly observed among Sternopygidae; for example, it was previously described in *S. macrurus*[13] and for several *Eigenmannia* species.[7,16,18]

A single NOR is the most common situation among the Sternopygidae, as confirmed by physical mapping of these genes in species of *Eigenmannia.*[7,13,16,18] Consistent with this, we found only a single NOR in each of the studied species: on pair 6 of *R. troscheli* (Figs. 2 and 3) and on pair 30 of *R.* cf *eastwardi* (Figs. 4 and 5). The additional positive 18S rDNA clusters observed through FISH mapping in *R.* cf *eastwardi* are presumed not to reflect active NOR. Although a 5S rDNA probe reportedly hybridized to multiple locations among *Eigenmannia* species,[14] our 5S rDNA probe yielded only one signal in each of the tested species of *Rhabdolichops*.

The presence of major and minor ribosomal genes at multiple locations may reflect several kinds of chromosomal rearrangement, while the distal locations of the signals for 18S rDNA (at the telomeres) and 5S rDNA (near the centromeres of acrocentric chromosomes) may suggest that Rabl orientation-associated factors have been involved in the spread of these ribosomal genes. In *R.* cf *eastwardi*, only three of the nine 18S rDNA genes were found to be associated with CMA_3-positive CG-rich regions (chromosome pairs 23, 24, and 30) (Fig. 5A, B). This is a relevant result since it is commonly accepted that 18S rDNA genes are associated to GC-rich heterochromatin in fish. Our findings could indicate that these six 18S rDNA sites are so recent that heterochromatin has not yet had time to accumulate.

Also, Gromich *et al.*[30] found out by sequenced FISH/banding in fishes that ''not just some other regions besides NORs were stained with CMA3 and Ag, but also the majority of the 28S rDNA sites were not detected,'' meaning that it is always important to check the classical and molecular cytogenetics data.

In recent years, sequence data and physical mapping have shown that several retrotransposons can be used as important markers for karyotypic comparisons.[31,32] The *Rex*

retroelements are widespread among the genomes of different fish lineages, and they have been mapped by *in situ* hybridization in numerous fish.[33,34] The distribution patterns observed for the *Rex* retroelements have been found to vary across different groups: they are compartmentalized in pericentromeric heterochromatic regions among Cichlidae, Prochilodontidae, and Tetraodontidae[35–37]; they colocalize with ribosomal genes in some Cichlidae, Erythrinidae, and Loricariidae species[38–40]; they are associated with sexual heterochromatin in Channichthyidae[41] and Anostomidae[33]; and they are dispersed throughout the genome in many species of Erythrinidae, Artedidraconidae, Bathydraconidae, Bovichtidae, Nototheniidae, and Loricariidae.[32,39,41]

The previous pattern was observed herein for the two studied species of *Rhabdolichops* (Sternopygidae). This pattern compartmentalized in pericentromeric heterochromatic regions is consistent with the idea that these transposons move and insert themselves along the genome.

Finally, we herein report the first mapping of two histone (H1 and H3) genes in members of Sternopygidae. As in other fish species, these genes appear to be clustered,[42–46] and we observed their signals in the interstitial region of pair 6 for *R.* cf *eastwardi* (Fig. 5D, E), but were unable to physically map these genes in *R. troscheli*.

Our comparison between the karyotypes of *R. troscheli* and *R.* cf *eastwardi* show that, despite they can be found in the same geographic region and share some traits (a single active 18S rDNA site, a single 5S rDNA location, no ITSs, most of CH rich in AT pb and centromerically located, a heterochromatic pattern of Rex1 and Rex3 distribution), their karyotypes are quite different. *R. troscheli* has a more conserved karyotype when compared to other Gymnotiformes, while *R.* cf *eastwardi* shows a more divergent one, with higher diploid number, additional CG-rich distal CH, apparently new NOR sites. This is even more evident if we take into account that *R. troscheli* and *R.* cf *eastwardi* are phylogenetically close species.[47] All in all, our results strongly suggest that *R.* cf *eastwardi* experencied recent intense genome reorganization.

In the future, extending these observations to other *Rhabdolichops* species could help us identify the chromosomal rearrangements involved in the chromosomal evolution of this genus, potentially improving our understanding of the phylogenetic relationship among *Rhabdolichops* species.

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Disclosure Statement

No competing financial interests exist.

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