

## Karyotypic variation of *Glanidium ribeiroi* Haseman, 1911 (Siluriformes, Auchenipteridae) along the Iguazu river basin

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(With 3 figures)

### Abstract

The Iguazu river is a tributary of the left margin of the Paraná river, isolated from this basin about 22 million years ago with the appearance of the Iguazu Falls. The Iguazu river is characterized by high endemism due to two factors: its rugged topography and the old isolation caused by formation of the Iguazu Falls. This study analyzed cytogenetically a population of *Glanidium ribeiroi* collected in a region at the final stretch of this basin, by Giemsa staining, C-banding, impregnation by silver nitrate, and FISH with probes of 5S rDNA, 18S rDNA, telomeric sequence [TTAGGG]<sub>n</sub>, and [GATA]<sub>n</sub> repeats. The diploid number was equal to 58 chromosomes. The heterochromatin was present in the terminal region of almost all chromosomes. The Ag-NORs were simple and presented interstitially on the short arm of the submetacentric pair 14, which was confirmed by FISH with 18S rDNA probe. The 5S rDNA-FISH marked only the submetacentric pair 16 on the long arm in interstitial position. The FISH with [TTAGGG]<sub>n</sub> probe presented all telomeres labeled as expected, with an absence of Interstitial Telomeric Sequence (ITS). The repetitive [GATA]<sub>n</sub> sequence was dispersed throughout the genome, with preferential location in the terminal region of all chromosomes. The data obtained are discussed herein with other species of Auchenipteridae, and other previously analyzed populations of *G. ribeiroi* from the Iguazu river, verifying differences among these populations, which should be mainly related to the rugged topography of this basin.

**Keywords:** geographical barriers, ribosomal DNA, *Glanidium ribeiroi*, Iguazu river.

### Variação cariotípica de *Glanidium ribeiroi* Haseman, 1911 (Siluriformes, Auchenipteridae) ao longo da bacia do rio Iguazu

#### Resumo

O rio Iguazu é um afluente da margem esquerda do rio Paraná, que foi separado desta bacia a aproximadamente 22 milhões de anos com o surgimento das Cataratas do Iguazu. Esse rio é caracterizado por elevado endemismo, o que se deve a dois fatores: sua acidentada topografia e ao antigo isolamento proporcionado pela formação das cataratas. No presente trabalho foi analisado cromossomicamente uma população de *Glanidium ribeiroi* coletada em uma região que corresponde ao trecho final desse rio, através de coloração com Giemsa, bandamento-C, impregnação pelo nitrato de prata e FISH com sondas de rDNA 5S, rDNA 18S, sequência telomérica [TTAGGG]<sub>n</sub> e repetições [GATA]<sub>n</sub>. O número diploide encontrado foi igual a 58 cromossomos. A heterocromatina se mostrou dispersa na região terminal de quase todos os cromossomos. As Ag-RONs são simples e presentes no braço curto em posição intersticial do par submetacêntrico 14, o que foi confirmado pela FISH com rDNA 18S. O rDNA 5S marcou apenas o par submetacêntrico 16 no braço longo em posição intersticial. A hibridização com sonda [TTAGGG]<sub>n</sub> revelou todos os telômeros marcados conforme esperado e ausência de Sequência Telomérica Intersticial (ITS). As repetições [GATA]<sub>n</sub> se apresentaram dispersas no genoma da espécie, com preferencial localização na região terminal de todos os cromossomos. Os dados aqui obtidos são discutidos com os de outras espécies de Auchenipteridae, especialmente de *G. ribeiroi* anteriormente analisados do rio Iguazu. Diferenças populacionais são constatadas em decorrência do isolamento geográfico ocasionado pelas inúmeras cachoeiras existentes no curso do rio Iguazu.

**Palavras-chave:** barreiras geográficas, DNA ribossomal, *Glanidium ribeiroi*, rio Iguazu.



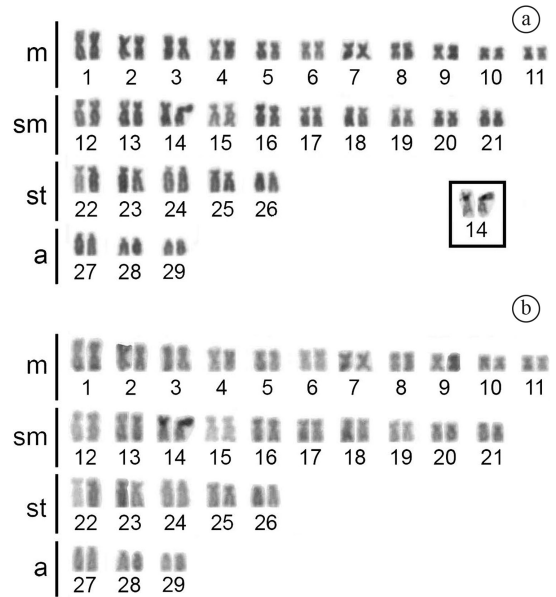
## 2. Material and Methods

Chromosomal analysis was performed in 43 specimens (20 males and 23 females) of *Glanidium ribeiroi* from the Iguazu river basin, municipality of Capanema – PR (25° 35' 19.6" S; 53° 54' 48.2" W) (see Figure 1). The specimens were deposited in the fish collection of the Museum of Zoology of the University of São Paulo, and were cataloged (MZUSP 109797). The preparation of cell suspensions of mitotic chromosomes was obtained from the anterior kidney cells (Foresti et al., 1993). The fundamental number (FN) was calculated considering metacentric (m), submetacentric (sm) and subtelocentric (st) chromosomes as having two arms, and acrocentric (a) chromosomes as having only one arm. The heterochromatic pattern was determined by Sumner (1972), with modifications (Lui et al., 2012a) in slides previously analyzed with Giemsa staining (sequential analysis). The Ag-NORs were identified using impregnation by silver nitrate (Howell and Black, 1980), and this preparation was performed on metaphases previously stained by Giemsa and C-banded (sequential analysis).

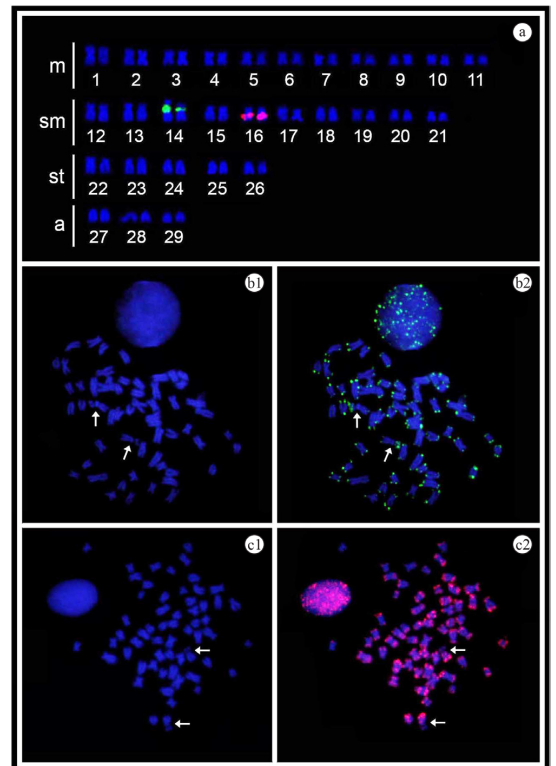
The fluorescence *in situ* hybridization (FISH) was performed according to Pinkel et al. (1986), using 18S rDNA (Hatanaka and Galetti Junior, 2004), 5S rDNA (Martins et al., 2000), [TTAGGG]<sub>n</sub> and [GATA]<sub>n</sub> probes, which were amplified without template DNA through PCR reaction, as described by Ijdo et al. (1991). The 18S rDNA probe was labeled with biotin-16-dUTP by nick translation according to the manufacturer's instruction (Biotin Nick Translation mix – Roche). The 5S rDNA probe was labeled with digoxigenin-11-dUTP by nick translation according to the manufacturer's instruction (Dig 11 Nick Translation mix – Roche). The [TTAGGG]<sub>n</sub> and [GATA]<sub>n</sub> sequences were labeled by Polymerase Chain Reaction (PCR), using biotin-16-dUTP and digoxigenin-11-dUTP respectively. All the hybridizations were performed with 77% stringency (200 ng from each probe, 50% deionized formamide, 10% dextran sulphate, 2xSSC; pH 7.0-7.2). The chromosomes were analyzed using an Olympus BX51 epifluorescence microscope. The software DP2-BSW (Olympus) was used for image capture.

## 3. Results

The diploid number found for *G. ribeiroi* was 58 chromosomes (22m + 20sm + 10st + 6a, NF = 110) (see Figure 2a). The heterochromatin was located in the terminal regions of several labeled pale chromosomes. In addition, a conspicuous labeled block corresponding to Ag-NORs, and one adjacent to this same site were observed (see Figure 2b). The impregnation by silver nitrate showed only the submetacentric pair 14 marked on the short arm in an interstitial position (see Figure 2, in box). The FISH with the 18S rDNA probe showed that only the submetacentric pair 14 was marked on the short arm in interstitial position. The 5S rDNA was observed in interstitial portion of the long arm of the metacentric pair 16 (see Figure 3a). The FISH with telomeric probe



**Figure 2.** Karyotype of *Glanidium ribeiroi* of the Iguazu river (final stretch of the basin, municipality of Capanema – PR) stained with Giemsa (a) and C-banded (b). Ag-NORs are presented in the box.



**Figure 3.** Karyotype of *Glanidium ribeiroi* of the Iguazu river hybridized with 18S rDNA (FITC, green probe) and 5S (Rhodamine, red probe). Metaphases of *Glanidium ribeiroi* hybridized with telomeric sequence [TTAGGG]<sub>n</sub> (b1 and b2) and [GATA]<sub>n</sub> repetitions (c1 and c2).

[TTAGGG]<sub>n</sub> showed all telomeres hybridized as expected, with no ITS (Interstitial Telomeric Sequence), and a more prominent hybridization signal in the terminal region of the short arm of pair 14, adjacent to the NOR (see Figure 3b1, 3b2). The hybridization with the repetitive [GATA]<sub>n</sub> sequence shows that this marker is dispersed throughout the genome, with a preferential location in the terminal region of the chromosomes; however, a lesser amount was also observed in the interstitial chromosomal regions (see Figure 3c1, 3c2).

#### 4. Discussion

The few chromosomal studies in Auchenipteridae suggest that the diploid number equal 58 chromosomes is a characteristic of this family (Ravedutti and Julio Junior, 2001). Except for the *Ageneiosus* species, the other genera with cytogenetic analyses (*Auchenipterus*, *Glanidium*, *Parauchenipterus* and *Tatia*) also show this conserved pattern of the diploid number 58. The sister-group of Auchenipteridae is the Doradidae family (Pinna, 1998), which presents a basal diploid number equal to 58 chromosomes (Eler et al., 2007; Milhomem et al., 2008), the same that is considered basal for Auchenipteridae (Lui et al., 2013a). This same diploid number was found for the population of *G. ribeiroi* in this paper, and for other populations of the same species previously studied (see Table 1) (Ravedutti and Julio Junior, 2001; Fenocchio et al., 2008). The fundamental number of the population this study was 110, but quite different from the other three populations due to their karyotypic formulas. Examples in which the diploid number is conserved while there are differences in the karyotype formula are very common in Neotropical fish, for example, in *H. malabaricus* Bloch, 1794 (Erythrinidae) (Blanco et al., 2010); *Pimelodus maculatus* Lacepède, 1803 (Pimelodidae) (Mazzuchelli et al., 2007) and *Hypostomus* spp. (Loricariidae) (Bueno et al., 2012). Rearrangements like translocations and/or pericentric inversions are normally considered responsible for these inter-population differences. This fact is reinforced by FISH with the [TTAGGG]<sub>n</sub> telomeric sequence in this

study, which did not show any ITS, a fact that confirms the proposition that non-Robertsonian rearrangements are more closely related to the karyotypic evolution of this species. The non-detection of ITS does not confirm the absence of Robertsonian rearrangements, because an event of fusion need not necessarily result in a chromosome that presents remnants of the telomeric sequence in the interstitial chromosome region (Slijepcevic, 1998). However, this is an aspect that reinforces what already may be suggested with regard to the conservation in the diploid number accompanied by variation in the karyotypic formula in *G. ribeiroi*.

Most species of Auchenipteridae, analyzed by C-banding of genera *Ageneiosus*, *Auchenipterus*, *Glanidium*, *Parauchenipterus* and *Tatia* (Ravedutti and Julio Junior, 2001; Fenocchio et al., 2008; Lui et al., 2009, 2010, 2013a, b; present paper), presented the heterochromatin as preferentially located in terminal regions with pale blocks, which seems to be a characteristic of the family. One prominent heterochromatic block was observed in the short arm of the pair where the NORs are located in the species of this study. The population of the Salto Caxias reservoir (Ravedutti and Julio Junior, 2001) also presented this heterochromatic block interspersed with NORs. The other two populations (Salto Segredo and Salto Osório reservoirs) (Fenocchio et al., 2008) did not present this site as positive for C-banding. Thus, differences in the pattern of C-banding related to the short arm of the NORs bearing pair can be detected among the populations.

In the population this paper, the impregnation by silver nitrate showed only a submetacentric pair marked on the short arm in an interstitial position (pair 14), as confirmed by FISH with 18S rDNA probe. In the other three populations of *G. ribeiroi*, a submetacentric pair bearing the NORs was observed; however, small variations in its location on the karyotype can be seen among the three. There are two interesting factors related to the pair carrier of the NOR, regarding the hybridization with the telomeric sequence [TTAGGG]<sub>n</sub> and the repetitive [GATA]<sub>n</sub> sequence. The highest hybridization signal with

**Table 1.** Cytogenetic data of different populations of *Glanidium ribeiroi* of the Iguazu river.

Locality	2n	FN	Karyotype formula	Ag-RONs/ 18S rDNA	5S rDNA	[TTAGGG] <sub>n</sub>	[GATA] <sub>n</sub>	Ref.
Reservoir of Segredo, PR, Brazil	58	106	22m+16sm+10st+10a	s, p, pair 13, sm	-	-	-	1
Reservoir of Salto Osório, PR, Brazil	58	106	22m+16sm+10st+10a	s, p, pair 13, sm	-	-	-	1
Reservoir of Salto Caxias, PR, Brazil	58	112	28m+16sm+10st+4a	s, p, pair 17, sm	-	-	-	2
Iguazu river, municipality of Capanema, PR, Brazil	58	110	22m+20sm+10st+6a	s, p, pair 14, sm	s, q, pair 16, sm	Telomeres marked, no ITS	Disperse coincident with terminal heterochromatin	3

PR = State of Paraná; 2n = diploid number; FN = fundamental number; s = single; p = short arm; q = long arm; sm = submetacentric. References: 1) Fenocchio et al. (2008); 2) Ravedutti and Julio Junior (2001); 3) Present study.

the [TTAGGG]<sub>n</sub> probe is in the terminal region of the short arm adjacent to the site of 18S rDNA. Amplification of the telomeres adjacent to NORs should have the function of increasing the protection and stability of these regions of ribosomal DNA, since these genes are essential for the cellular maintenance. Regarding the [GATA]<sub>n</sub> repetitions, the pair 14 showed the terminal region on the long arm (strong signal) and proximal to the centromere labeled; however, in the short arm, where the NORs are located in interstitial position, it was not possible to visualize the hybridization signal.

The repetitive element [GATA]<sub>n</sub> is conserved in different animal species, including humans (Subramanian et al., 2003; Srivastava et al., 2008). This sequence appears to be associated with the determining and evolution of sex chromosomes in groups of snakes (Jones and Singh, 1985); however, to date, there are not differentiated sex chromosomes described in Auchenipteridae. Nevertheless, [GATA]<sub>n</sub> is highly dispersed throughout the genome of *G. ribeiroi*, as shown in this paper. This same situation is found on the genome of another species of Auchenipteridae, *A. inermis* Linnaeus, 1766 (Lui et al., 2013a). The location of the [GATA]<sub>n</sub> sequence in *G. ribeiroi* corresponded to the pallid heterochromatin in the terminal region of almost all chromosomes, also occurring in a lesser amount in the interstitial regions (Figure 3c1, 3c2). According to Subramanian et al. (2003), this sequence might be related to various functions in the human genome, including gene regulation. In addition, it is concurrently found in the region of the Y chromosome where the genes of sex determination are, being suggested that this sequence can also be functionally related. Little can be said about the dispersed location in the genome of *G. ribeiroi*; however, the [GATA]<sub>n</sub> repeats can be a simple repetitive sequence that is part of the heterochromatin structure of this species, or, hypothetically, be related to the regulatory functions, as has already been established previously for humans.

The physical mapping of 5S rDNA for Auchenipteridae is rarer than 18S rDNA. *Parauchenipterus galeatus* Linnaeus, 1766 (Lui et al., 2010), *Tatia jaracatia* and *Tatia neivai* Ihering, 1930 (Lui et al., 2013b) present 5S rDNA in more than one pair, which is different to *A. inermis*, which showed only one pair marked (Lui et al., 2013a). Comparing the submetacentric pair 16 (bearing 5S rDNA) of *G. ribeiroi* with the other species of Auchenipteridae, it is possible to find one correspondent pair on the genome of *P. galeatus*, *T. jaracatia* and *T. neivai*, considering interstitial location on the long arm and submetacentric morphology of the bearing pair. However, this correspondence cannot be established with *A. inermis*, due to the different morphology of the 5S rDNA-bearing pair.

The isolation of freshwater fish in their watersheds approaches relationships among the natural histories of ichthyofauna with the basins in which they belong. The changes that occur in basins and their tributaries, as a result of natural events or anthropogenic actions, influence the entire ecosystem, thus giving distinctive characteristics to the natural history of populations that exist within it.

According to Castro (1999), most species of small fish have a relatively restricted ability to dislocate within large river basins. Thus, over the millions of years during the evolution of Neotropical fish, large numbers of geographical and geological changes occurred in water systems, generating numerous examples of vicariance, and creating isolated populations with extremely restricted or non-existent gene flow among them. In a species of Auchenipteridae with a wide distribution in South American watersheds, *P. galeatus*, these same types of differences were detected and were important to investigate the biogeographical relationships of populations relative to a region of transposition on the river. The inferences elaborated through chromosomal data (Lui et al., 2010) were corroborated by molecular analysis of mitochondrial DNA (Lui et al., 2012b). The chromosomal data of *G. ribeiroi* in this study, when compared with previous papers (see Table 1), presents a similar situation regarding *P. galeatus* populations; however, it is noteworthy to mention that with *G. ribeiroi*, all populations exist in the same river.

Studies of several fish species of the Iguazu river basin show a high genetic variability among natural populations of the same species (Agostinho et al., 1999), which reinforces the importance of chromosomal data of *G. ribeiroi*. Other species from the Iguazu river also have similar data that suggest fragmentation of populations, like *Astyanax altiparanae* Garutti & Britski, 2000, *Oligosarcus longirostris* Menezes & Géry, 1983, *Corydoras paleatus* Jenyns, 1842 and *Pimelodus ortomanni* Haseman, 1911, among others (Kantek et al., 2007), the reason being that, for the most part, this data were obtained after the construction of dams in the Iguazu river. Regarding *G. ribeiroi*, existing data was collected from most parts of the Iguazu river, comprising the majority of the course with dams (Suzuki, 1999; Cassemiro et al., 2005; Agostinho et al., 2007), with exception to some rivers of high Iguazu (Ingenito et al., 2004). Despite the fact that some barriers have ceased to exist in recent history due to the construction of a series of hydroelectric dams (after 1975), it is very likely that population fragmentations may be a consequence of the series of waterfalls that existed previously on the Iguazu river.

This reveals that each population of the ichthyofauna of the Iguazu river represents a diversified genetic heritage that must be conserved. For example, on the Segredo reservoir alone, more than fifteen species were considered at risk of extinction (Agostinho et al., 1999). Due to the recent growth urban around this basin, conservation programs and management of the ichthyofauna of the Iguazu river are of great importance, not only for the high endemism at interspecific level, but also by genetic heritage unique at intraspecific level, in that local extinctions may lead to loss of genetic variability.

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