Neotropical Ichthyology, 17(3): e190069, 2019
DOI: 10.1590/1982-0224-20190069

Journal homepage: www.scielo.br/ni
Published online: 30 September 2019 (ISSN 1982-0224)
Printed: 30 September 2019 (ISSN 1679-6225)

Original article

First chromosomal analysis of *Gymnorhamphichthys britskii*: the remarkable lowest diploid value within the family Rhamphichthyidae (Gymnotiformes)

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Gymnorhamphichthys britskii is a Neotropical electric fish of family Rhamphichthyidae described from the Paraná-Paraguay system. This study reports the first karyotypic description of *G. britskii* collected from the upper Paraná river basin, which presented 2n=38 chromosomes, karyotype composed of 14 metacentric, 8 submetacentric, 2 subtelocentric and 14 acrocentric chromosomes, and fundamental number as 62 for both sexes. Heteromorphic sex chromosomes were absent. A single pair of nucleolar organizing regions (NORs) was detected in the submetacentric chromosome pair number 9 by silver staining and confirmed by the 18S rDNA probe. The 5S rDNA was located in a single chromosome pair. Heterochromatic regions were clearly observed in the short arms of the NOR-bearing chromosome pair and in the telomeric positions of most acrocentric chromosomes. Besides the present data are valuable to help in understanding karyotypic evolution in Rhamphichthyidae, data from NORs confirmed the tendency of this family in presenting simple NORs sites, similar to the other Gymnotiformes clades. Yet, the presence of a large heterochromatic block in the NOR-bearing chromosome can be used as cytogenetic markers for *G. britskii*, and that centric fusions appear to be an important mechanism in the karyotype evolution and differentiation among Gymnotiformes species.

Keywords: Ag-NORs, C-banding, Fish cytogenetics, Karyotype evolution, Repetitive sequences.

Gymnorhamphichthys britskii é um peixe neotropical da família Rhamphichthyidae descrita no sistema Paraná-Paraguai. Este estudo relata a primeira descrição cariotípica de *G. britskii* coletado na bacia do alto rio Paraná, que apresentou 2n = 38 cromossomos, cariótipo composto por 14 metacêntricos, 8 submetacêntricos, 2 subtelocêntricos e 14 acrocêntricos, e número fundamental 62 para ambos sexos. Cromossomos sexuais heteromórficos estavam ausentes. Um único par de regiões organizadoras de nucléolos (RONs) foi detectado no par de cromossomos submetacêntricos número 9 por coloração com prata e confirmado pela sonda DNAr 18S. O DNAr 5S foi localizado em um único par cromossômico. Regiões heterocromáticas foram claramente observadas nos braços curtos do par de cromossomos que carrega a RON e nas posições teloméricas da maioria dos cromossomos acrocêntricos. Além dos dados presentes serem valiosos para auxiliar na compreensão da evolução cariotípica em Rhamphichthyidae, dados de RONs confirmaram a tendência desta família em apresentar sítios simples de RONs, semelhantes aos demais clados de Gymnotiformes. No entanto, a presença de um grande bloco heterocromático no cromossomo portador da RON, pode ser usado como marcador citogenético para *G. britskii* e as fusões cêntricas parecem ser um mecanismo importante na evolução e diferenciação cariotípica entre as espécies de Gymnotiformes.

Palavras-chave: Ag-RONs, Bandeamento C, Citogenética de peixes, Evolução cariotípica, Sequências repetitivas.

Introduction

The *Gymnorhamphichthys* Ellis, 1912 is a representant of the clade Rhamphichthyidae, a group of Neotropical electric fishes, widely distributed through the cis-Andean drainages of tropical South America, from Venezuela to northeast Argentina (Nijssen *et al*., 1976). The genus is composed of five valid species: *G. rondoni* (Miranda Ribeiro, 1920), *G. rosamariae* Schwassmann, 1989, *G. bogardusae* Lundberg, 2005, *G. hypostomus* Ellis, 1912, and *G. britskii* Carvalho, Ramos & Albert, 2011 (Fricke *et al*., 2019).

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Gymnorhamphichthys britskii is distributed from tributaries of the La Plata River system, and share with *G. hypostomus* a distinct color pattern of the dorsum composed of a few large, dark saddles, which is not present in any other species of Rhamphichthyidae (Carvalho *et al*., 2011). Previously, there were no records of *G. britskii* from the upper Paraná River before the construction of the Itaipu Dam. The distribution of *G. britskii* (referred to as *Gymnorhamphichthys* sp. by Graça, Pavanelli, 2007) in the upper Paraná River seems to be due to the construction of Itaipu Dam, which by elevating a portion of the lower Paraná River effectively eliminated the Sete Quedas falls as a barrier for dispersal (Langeani *et al*., 2007; Ota *et al*., 2018).

The families Hypopomidae and Rhamphichthyidae are closely related and constitute the superfamily Rhamphichthyoidea (Albert, 2001). No cytogenetic information is available for species of *Gymnorhamphichthys*. Among the other Rhamphichthyidae, cytogenetic information is available only for *Hypopygus lepturus* Hoedeman, 1962 (Almeida-Toledo, 1978), *Steatogenys duidae* (La Monte, 1929), *S. elegans* (Steindachner, 1880) (Cardoso *et al*., 2011), *Rhamphichthys hahni* (Meinken, 1937), *R. pantherinus* Castelnau, 1855 (cited as *R. marmoratus*) and *R. rostratus* (Linnaeus, 1766) (Mendes *et al*., 2012; Silva *et al*., 2013). Among the Hypopomidae, it is available for *Hypopomus artedi* (Kaup, 1856) (Almeida-Toledo, 1978), eight *Brychyhypopomus* species (Almeida-Toledo, 1978; Mendes *et al*., 2012; Cardoso *et al*., 2018) and *Microsternarchus bilineatus* Fernández-Yépez, 1968 (de Jesus *et al*., 2016; Batista *et al*., 2017).

There are still few cytogenetic studies regarding Rhamphichthyoidea, but they show that these species present great differences in relation to the number of chromosomes and karyotype macrostructure. In Rhamphichthyidae all species had 2n = 50 chromosomes but differed in their karyotypes (Almeida-Toledo, 1978; Cardoso *et al*., 2011; Mendes *et al*., 2012; Silva *et al*., 2013). Among the Hypopomidae, the diploid number ranged from 36 (*Brachyhypopomus brevirostris* (Steindachner, 1868)) to 48 (*M. bilineatus*) chromosomes (de Jesus *et al*., 2016; Batista *et al*., 2017; Cardoso *et al*., 2018).

The physical mapping of ribosomal DNAs, especially 5S and 18S rDNAs, has been frequently used by cytogenetic studies on Neotropical fish, including fishes of the order Gymnotiformes (Fernandes *et al*., 2017a,b) to assist the clarification of taxonomic, biogeographical and phylogenetic problems. In Rhamphichthyidae, cytogenetic studies about the distribution of ribosomal genes (18S rDNA) are restricted to karyotypes of *S. duidae*, *S. elegans* (Cardoso *et al*., 2011), *R. hahni*, *R. pantherinus* and *R. rostratus* (Mendes *et al*., 2012; Silva *et al*., 2013), demonstrating only one chromosome pair bearing these clusters in all species.

No cytogenetic information is available for physical mapping of 5S rDNA in the Rhamphichthyidae. Therefore, in an effort to collect more information about chromosomal diversity within the family Rhamphichthyidae, this study establishes the first cytogenetic description of *G. britskii* by classic and molecular cytogenetics techniques.

Material and Methods

Twenty-two (8 males, 9 females and 5 sex indeterminate) individuals of *G. britskii* from Dourado stream, Mato Grosso do Sul State, Brazil (23°51'04.9''S and 54°25'13.9''W) were analysed. This stream is a tributary of the right margin of the Iguatemi River, which belongs to the upper Paraná River basin (Fig. 1).

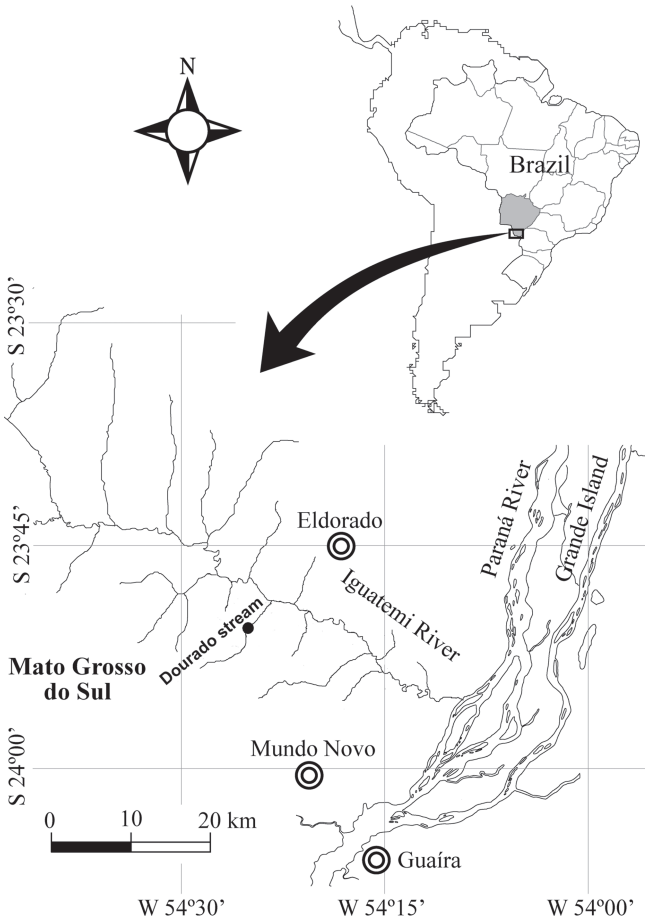

Fig. 1. Location of Dourado stream in the upper Paraná River basin where *Gymnorhamphichthys britskii* individuals were captured. The dark circle indicates the sampling spots.

Animals were captured with the permission of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio; number 64611). Voucher specimens were deposited in the fish collection of the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUPELIA), Universidade Estadual de Maringá, PR Brazil, as *Gymnorhamphichthys britskii* (NUP 16251) (Fig. 2).

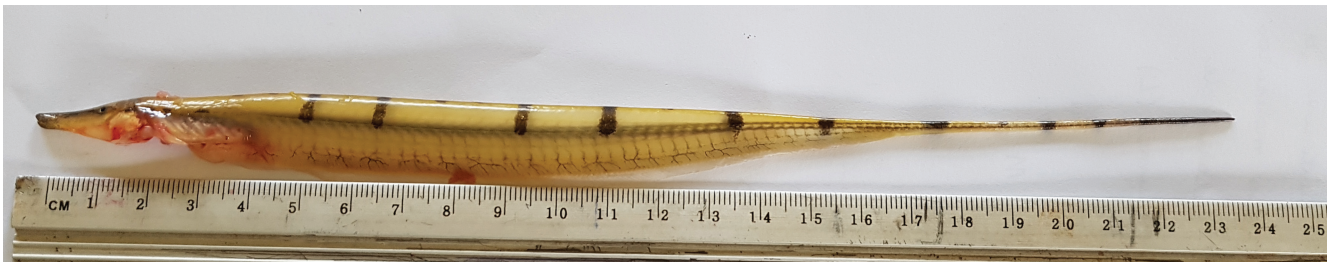

Fig. 2. *Gymnorhamphichthys britskii* sampled in the Dourado stream, Mato Grosso do Sul State, Brazil.

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, approved by the Committee on Ethics of Animal Experiments of the Universidade Estadual do Mato Grosso do Sul (License Number: Protocol 024/2018 — CEUA/UEMS). The experiments followed the ethical conduct, and before euthanasia, the fish were anesthetized by an overdose of clove oil (Griffiths, 2000). Metaphase chromosomes were obtained from anterior kidney cells using the air-drying technique (Bertollo *et al*., 1978). C-positive heterochromatin (C-bands) was visualized by the procedure of Sumner (1972), with minor adaptations. NORs were detected by means of silver nitrate staining (Ag-NORs), according to Howell, Black (1980).

At least 30 metaphases were analyzed for each individual and those with better chromosome morphology were used for the karyotype analysis. The chromosomes are classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) according to Levan *et al*. (1964). The fundamental number (FN) is calculated according to the chromosomal arm numbers (the chromosomes m, sm and st are considered to contain two arms —*p* and *q* arms— and the a with one arm —only *q* arm).

Physical mapping of the 5S and 18S rDNA was carried out by fluorescence *in situ* hybridization (FISH) according to Pinkel *et al*. (1986) and modifications suggested by Margarido, Moreira-Filho (2008), using DNA probes obtained from the genomes of *Megaleporinus elongatus* (Valenciennes, 1850) (Martins, Galetti, 1999) and *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka, Galetti, 2004), respectively. The probes were labelled through nick translation, with digoxigenin-11-dUTP (18S rDNA) and biotin-16-dUTP (5S rDNA) (Roche). Detection and amplification of the hybridization signal were carried out using avidin-FITC and anti-avidin biotin (Sigma) for probes labelled with biotin, and anti-digoxigenin rhodamine (Roche) for probes labelled with digoxigenin. Slides were counterstained with DAPI (4'6-Diamine-2'-phenylindole dihydrochloride) (50 µg ml^{-1}) and analyzed in epifluorescence microscope (Olympus BX61). The images were captured using the software DP controller (Media Cybernetics) and the image composition was carried out with Adobe Photoshop CS6.

Results

All the twenty-two individuals of *G. britskii* had 2n = 38 chromosomes and karyotype composed of 14 m, 8 sm, 2 st and 14 a, and FN = 62 for both sexes (Fig 3a). Heteromorphic sex chromosomes were absent. A secondary constriction was observed in the terminal region of the *p* arm of the sm pair number 9, which corresponds to the Ag-NORs signals (Fig. 3a, in box) and also had a clear size heteromorphism (Figs. 3a, c).

The heterochromatins were preferentially located in the centromeric and pericentromeric regions in various chromosomes. Large terminal blocks were evidenced on the *q* arm of the pairs number 13, 15, 16 (one homolog) and 17, and a large heterochromatic block on the *p* arm of the pair number 9 flanked by NOR (Fig. 3b).

Double FISH with 18S and 5S rDNA probes confirmed the Ag-NORs sites and did not detect any further inactive major ribosomal clusters (Fig. 3c); in addition, it showed that minor rDNA clusters occur interstitially in the acrocentric pair number 13 and do not co-localize with the major rDNA clusters (Fig. 3c).

Discussion

The lack of karyotype data for several fish groups impairs comparative analyzes on their evolutionary trends and chromosomal relationships. This is the case for the family Rhamphichthyidae for which chromosomal characteristics are known only for three genera: *Rhamphichthys*, *Hypopygus* and *Steatogenys*, and all species presented 50 chromosomes. In this sense, this study is the first one providing classical and molecular cytogenetic data for one of its representative species, *G. britskii*.

Both male and female specimens of *G. britskii* have the same karyotype structure, with 2n = 38 chromosomes (14m + 8sm + 2st + 14a), with no evidence of differentiated sex chromosomes. Diploid number of 38 chromosomes found in *G. britskii* differs from the findings for every other species of the same family. Compared to other rhamphichthyids that have 2n = 50, the karyotype of *G. britskii* suggested a reduction in the 2n, indicating that chromosome rearrangements, such as centric fusions that can alter the chromosome number may have occurred during the diversification of this group.

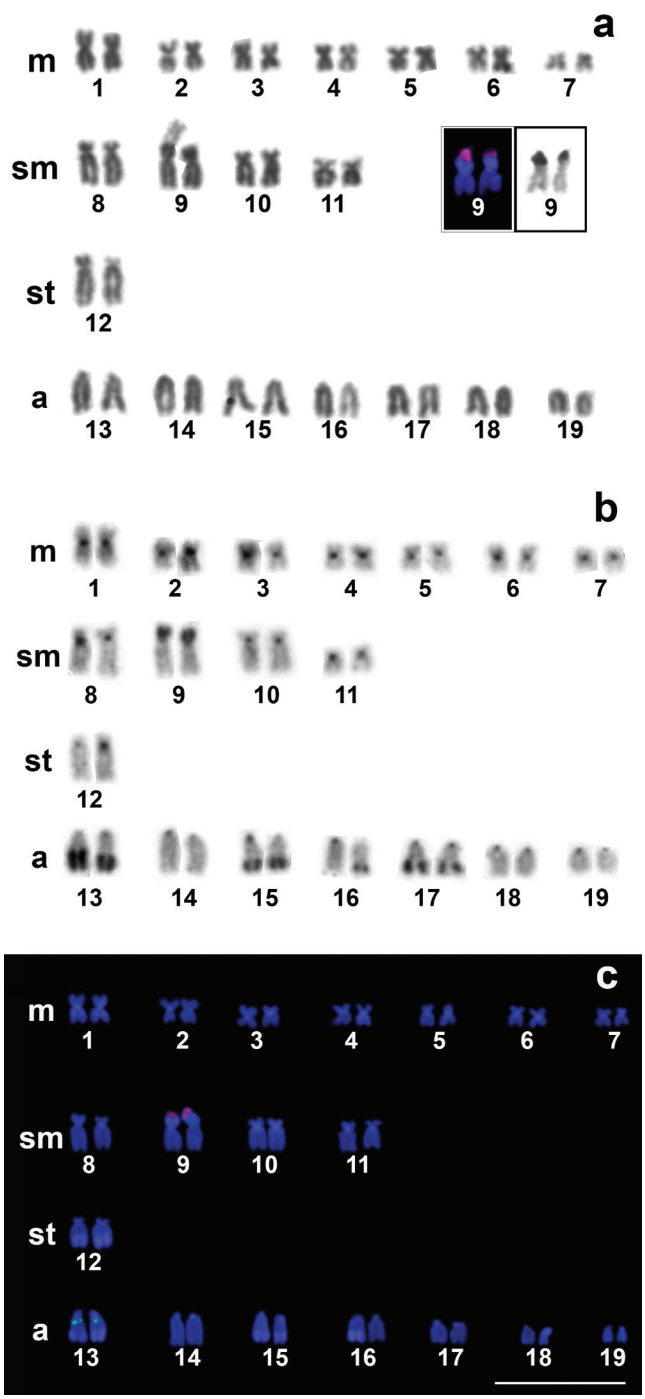

Fig. 3. Karyotypes of *Gymnorhamphichthys britskii* arranged **a.** from Giemsa stained; **b.** C-banded; and **c.** after double-FISH with 18S rDNA (red) and 5S rDNA (green) probes.. The NOR-bearing chromosomes (pair 9) are in the box. Note the size heteromorphism involving the NORs detected by the Ag-NOR and 18S rDNA-FISH techniques. Scales bar = 10 µm.

Chromosome fusions were identified as important events in the diversification of Hypopomidae (the sister group of Rhamphichthyidae), in which the diploid number varies between 36 chromosomes, as in *B. brevirostris*, and 48 chromosomes, as in *M. bilineatus* (de Jesus *et al*., 2016; Batista *et al*., 2017; Cardoso *et al*., 2018). In Rhamphichthyidae, the chromosomic data of *G. britskii* alter the paradigm of karyotype evolution characterized by 2n conservation (50 chromosomes) and chromosome inversions (Cardoso *et al*., 2011; Mendes *et al*., 2012; Silva *et al*., 2013). Nonetheless, more rhamphichthyid species need to be cytogenetically analyzed to confirm this hypothesis (diversification by chromosome fusions). Moreover, centric fusion is the mechanism proposed in the origin of multiple sex chromosome system found in Gymnotiformes species belonging each one to different families: *Eigenmannia* sp. 2 (Almeida-Toledo *et al*., 2000a), *E. trilineata* (Fernandes *et al*., 2010), *Brachyhypopomus gauderio* (Mendes *et al*., 2012), *B. pinnicaudatus* (Almeida-Toledo *et al*., 2000b), *Gymnotus pantanal* (cited as *Gymnotus* sp., Silva, Margarido, 2005), *G. coropinae* (da Silva *et al*., 2014). According to Silva, Margarido (2005), the presence of a same $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in six species seems to be a homoplasic event, and it is highly probable that this apomorphic character has arisen by independent events. Thus, centric fusions appear to be an important and common mechanism acting in the karyotype evolution and differentiation among Gymnotiformes species.

NORs were located in terminal position on the *p* arm number 9, as revealed by the Ag-NOR and 18S rDNA-FISH techniques (Fig. 3a, box), thus characterizing a simple NORs system in *G. britskii*. Similar pattern was observed in karyotypes of *S. duidae*, *S. elegans* (Cardoso *et al*., 2011), *R. hahni, R. pantherinus* and *R. rostratus* (Mendes *et al*., 2012; Silva *et al*., 2013). A single chromosome pair with NORs sites is thought to be a plesiomorphic characteristic of the Rhamphichthyidae. By contrast, multiple NORs sites have characterized the karyotypes of the Hypopomidae.

In *G. britskii*, size heteromorphism involving the NORs was detected by the Ag-NOR and 18S rDNA-FISH techniques. This characteristic can be explained mainly by unequal recombination or random duplication (Gornung, 2013). Events as these can be observed in chromosomes regions composed of repetitive DNA. In *G. britskii*, the NORs are flanked by heterochromatin. Heterochromatic regions and ribosomal genes are known to contain highly repetitive DNA. Therefore, our results reinforce the suggestion of Fernandes-Matioli *et al*. (1998) that heterochromatic regions bordering NORs tend to favor chromosome rearrangements in these particular chromosome areas.

The present study performed the first physical mapping of the 5S DNA in chromosomes of Rhamphichthyidae. In *G. britskii*, the 5S rDNA is located in a single chromosome pair and nonsyntenic with the 18S rDNA sites, a common characteristic of several fish groups (Pisano *et al*., 2007). Simple 5S rDNA sites were also observed in karyotypes of *M. bilineatus*, but in only one chromosome of the 12th pair in males showed synteny between the 18S rDNA and 5S rDNA sites (de Jesus *et al*., 2016). Moreover, in *M. bilineatus* the 5S rDNA cistrons are located in the terminal region, while in *G. britskii* these cistrons are located in the interstitial position.

The heterochromatin distribution follows the general pattern usually found in many other fish species, preferentially centromeric localization. In contrast, large telomeric heterochromatic blocks in acrocentric chromosomes were verified in *G. britskii*, but not observed in *Rhamphichthys* and *Steatogenys* (Cardoso *et al*., 2011; Mendes *et al*., 2012; Silva *et al*., 2013). Moreover, *G. britskii* had a conspicuous large heterochromatic block in the NOR-bearing chromosomes, adjacent to secondary constriction that can be used as cytogenetic markers for the species.

The results presented in this study allowed the first cytogenetic characterization of a species within the *Gymnorhamphichthys* genus, contributing to the karyotype knowledge of the family Rhamphichthyidae. Data from NORs confirmed the tendency of this family in presenting simple NORs sites, similar to the other Gymnotiformes clades. We also reported the presence of a large heterochromatic block in the NOR-bearing chromosome, adjacent to secondary constriction, which can be used as cytogenetic markers for *G. britskii*, and that centric fusions appear to be an important mechanism in the karyotype evolution and differentiation among Gymnotiformes species.

Acknowledgments

The authors thank Dr. Weferson Júnio da Graça for the taxonomic identification of the specimens. Besides, we are grateful to the Ministério do Meio Ambiente/ Instituto Chico Mendes de Conservação da Biodiversidade (MMA/ ICMBio – License number 64611) for authorizing the collection of the biological material.

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Submitted July 1, 2019
Accepted September 9, 2019 by Claudio Oliveira