

# Identification of cryptic species in allopatric populations of *Hypostomus tietensis* (Siluriformes: Loricariidae) through cytogenetics analyses

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*Hypostomus* is the most specious genus of Hypostominae, composed of several species with high intraspecific morphological and color pattern variation, making their identification a complex issue. One of the species with problematic identification is *Hypostomus tietensis* that was described from a single specimen, resulting in uncertainties about its color pattern and correct identification. To assist in this context, cytogenetic analyzes were carried out in three putative populations of *H. tietensis* from the Upper Paraná River basin, one of them from the type locality. The three populations showed considerable cytogenetic differences, with  $2n = 72$  chromosomes for the population from the type locality and  $2n = 76$  chromosomes for the others. Terminal NORs were detected (Ag- and 18S rDNA-FISH), being simple for the type locality population (acrocentric pair 23, long arm) and the Pirapó River (subtelocentric pair 11, short arm), and multiple for Do Campo River (subtelocentric pairs 11 and 12, short and long arm, respectively). C-banding was efficient in differentiating the type locality population from the others. Cytogenetic data revealed that populations from Pirapó and Do Campo rivers, although treated until now as *Hypostomus* aff. *tietensis*, represent a cryptic species, and those morphological analyses are necessary to differentiate and for describing this new species.

**Keywords:** Catfish, Chromosomal variation, Cytogenetics, Neotropical fishes, Upper Paraná River basin.

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*Hypostomus* é o gênero mais especioso de Hypostominae, composto por várias espécies com uma alta variação tanto morfológica, como no padrão de coloração intraespecífica, tornando sua identificação uma questão complexa. Uma das espécies com identificação complexa é *Hypostomus tietensis*, a qual foi descrita a partir de um único espécime, resultando em incertezas sobre o seu padrão de cor e identificação. Para auxiliar nesse contexto, análises citogenéticas foram realizadas em três populações putativas de *H. tietensis* da bacia do Alto rio Paraná, sendo uma delas da localidade tipo. As três populações apresentaram diferenças citogenéticas consideráveis, com  $2n = 72$  cromossomos para a população da localidade tipo e as demais com  $2n = 76$ . RONS terminais foram detectadas (Ag- e FISH-DNAr 18S), sendo simples para a população da localidade tipo (par acrocêntrico 23, braço longo) e do rio Pirapó (par subtelocêntrico 11, braço curto) e múltiplas para rio Do Campo (pares subtelocêntricos 11 e 12, braço curto e longo, respectivamente), confirmado pela FISH-DNAr 18S. O bandamento C foi eficiente em diferenciar a população da localidade tipo das demais. Os dados citogenéticos revelaram que as populações dos rios Pirapó e do rio Do Campo, embora tratadas até agora como *Hypostomus* aff. *tietensis*, representam uma espécie críptica, e que análises morfológicas são necessárias para diferenciar e descrever esta nova espécie.

**Palavras-chave:** Cascudos, Variação cromossômica, Citogenética, Peixes Neotropicais, Bacia do Alto Rio Paraná.

## INTRODUCTION

Loricariidae is the largest family in number of species within the order Siluriformes, and one of the largest in relation to all fish families in the world, with 163 genera and more than 1,020 species (Fricke *et al.*, 2022). The phylogeny of the family is not well established and, despite its monophyletism, many conflicts still permeate the taxonomy and phylogeny of its subfamilies, which have been constantly revised (Armbruster, 2004; Reis *et al.*, 2006; Chiachio *et al.*, 2008; Cramer *et al.*, 2011; Lujan *et al.*, 2015; Pereira, Reis, 2017; Roxo *et al.*, 2019).

*Hypostomus* Lacepède, 1803, belongs to the Hypostominae subfamily and is one of the most diverse and complex groups of fish, whose distribution extends from Central America to southern South America. The more than 150 species that make up the genus have various shades, which vary from light to dark tones, and may also have spots of different shapes spread over the body (Azevedo *et al.*, 2021). The great intraspecific variability, in relation to color and morphology patterns, has made the taxonomic identification of species extremely difficult, contributing to several uncertainties within the genus (Weber, 2003; Armbruster, 2004).

One of the species of the genus with problematic identification is *Hypostomus tietensis* (Ihering, 1905) (Fig. 1), which was described by Ihering (1905) from a single specimen from the Upper Tietê River. However, there is a problem regarding the definition of its color pattern, since Ihering (1905) defined *H. tietensis* as having the upper part of the head with dark stripes or vermiculations, and the body with dark spots, and in a study

carried out with species from the Upper Paraná River, Jerep *et al.* (2007) divided the main bulk of *Hypostomus* species from the Upper Paraná River into two groups based on color pattern, and included *H. tietensis* in the group of species with light rounded spots. Jerep *et al.* (2007) analyzed the holotype and several specimens from the Upper Tietê River basin, which showed irregularly spaced light stripes on a dark background. They further suggest that Ihering (1905) may have made a mistake, as defining the color pattern is complex and difficult task to do with just one specimen. As a result, several populations have been designated by taxonomists as *Hypostomus* aff. *tietensis*, or *Hypostomus* cf. *tietensis* in other regions of the Upper Paraná, such as in the flood area and basins of the Paranapanema and Piquiri rivers (Reis *et al.*, 2020), indicating the existence of variations in relation to the described species from the type locality.

In view of the difficulties in identifying several species of *Hypostomus* and its unclear phylogeny, cytogenetics has proved itself as an important tool in understanding the systematics of the genus (Artoni, Bertollo, 2001; Kavalco *et al.*, 2005; Alves *et al.*,



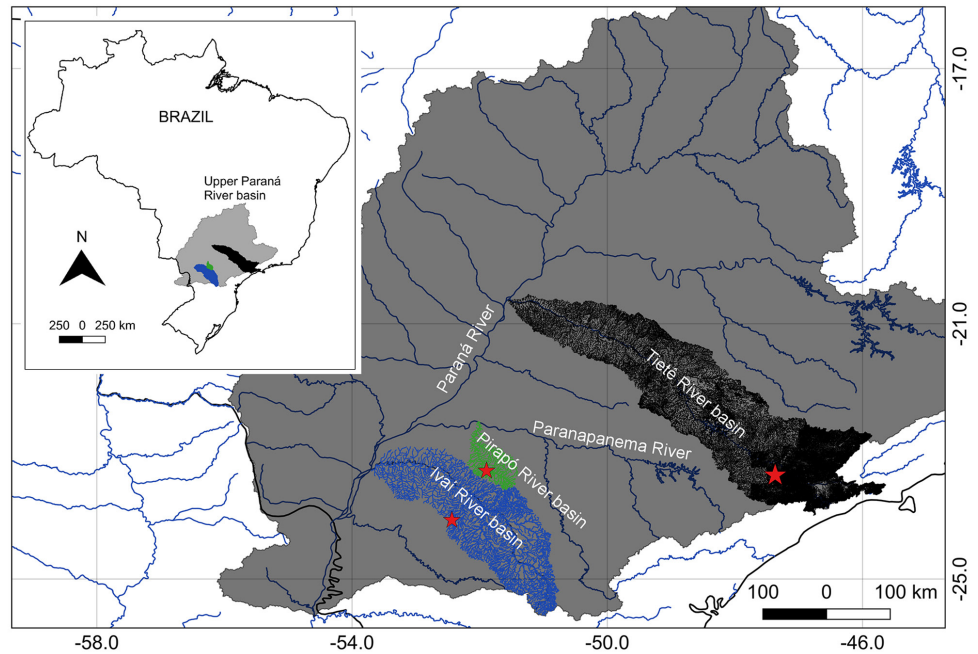
**FIGURE 1** | Holotype of *Hypostomus tietensis*, 160 mm SL, Tietê River, São Paulo, Brazil (<https://data.nhm.ac.uk/object/a9a46e32-84d2-4996-9e69-71e8a2a8aca2/1633478400000>, catalog number NHMUK 1905.6.9.1).

2006; Bueno *et al.*, 2012, among). Therefore, the objective of the present study was to cytogenetically analyze allopatric populations of *H. tietensis*, including that from the type locality and from two other localities in the Upper Paraná River basin where the occurrence of *H. tietensis* were previously recorded (Coleção Ictiológica do Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura - NUPELIA), in order to test the hypothesis that there is cryptic diversity and to collaborate in the identification and characterization of the species.

## MATERIAL AND METHODS

Three populations samples of *Hypostomus tietensis* were collected in the Upper Paraná River basin: 1- Pirai River a putative “type locality” (NUP 21903, a tributary of the upper stretches of the Tietê River basin, 23°22'22”S 47°22'13”W) with eight males and three females, 2- Pirapó River (NUP 2830, a tributary of Paranapanema River basin, 23°18'15”S 51°53'40”W) with 10 males and seven females, 3- Do Campo River (NUP 3025, a tributary of Ivaí River basin 24°04'41”S 52°26'12”W) with 13 males and five females (Fig. 2). The specimens were deposited in the Coleção Ictiológica do Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUP), Universidade Estadual de Maringá, Maringá.

All specimens were euthanized by an overdose of clove oil (Griffiths, 2000), and chromosome preparations were obtained from cells of anterior region of kidney (Bertollo *et al.*, 2015). Metaphase chromosomes were obtained by the air-drying method and classified and organized into metacentric, submetacentric, subtelocentric, and acrocentric (Levan *et al.*, 1964). The fundamental number (FN) was established considering the metacentric, submetacentric and subtelocentric chromosomes with two arms and the acrocentric chromosome with only one arm. Nucleolar organizing regions (AgNORs) were evidenced by silver nitrate impregnation (Howell, Black, 1980). Heterochromatin were determined following the C-banding technique and stained with propidium iodide (Sumner, 1972; Lui *et al.*, 2012). The physical mapping of the 18S sequences was carried out by Fluorescence *in situ* Hybridization (FISH) according to Pinkel *et al.* (1986), using DNA probes obtained from *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka, Galetti, 2004). Probes were labeled by nick translation method with biotin-16-dUTP (Roche®). Detection of signals was performed with avidin-FITC with biotinylated anti-avidin (Sigma-Aldrich), with the chromosomes counterstained with 4',6-diamidino-2-phenylindole (DAPI, 50 µg/mL). Metaphases were photographed using a BX 51 epifluorescence microscope, coupled with Olympus DP 71 digital camera (Olympus America, Inc.) with the Olympus DP Controller software 3.2.1.276.



**FIGURE 2 |** Map of Brazil showing the Upper Paraná River basin. Red stars represent *Hypostomus tietensis* populations, from Tietê River basin “type locality” (in black), from Pirapó River (in green), and from Do Campo River, tributary of Ivaí River basin (in blue).

## RESULTS

### *Hypostomus tietensis* – Pirai River “type locality”

The diploid number was 72 chromosomes, with a karyotype formula of  $8m+8sm+20st+36a$  and a fundamental number (FN) equal to 108 for the male and the female (Fig. 3A). A single pair of NORs was revealed with silver nitrate staining, located in the terminal regions of the long arms of the acrocentric pair 23 (Fig. 3A, highlighted above) confirmed by 18S rDNA-FISH (Fig. 3A, highlighted below). The heterochromatin detected by the C-banding showed large conspicuous terminal blocks in the long arm of chromosomes *a* (19, 20, 21 and 22) and pairs *a* 24, 32, 33, 34 (Fig. 3B).

### *Hypostomus aff. tietensis* – Pirapó River

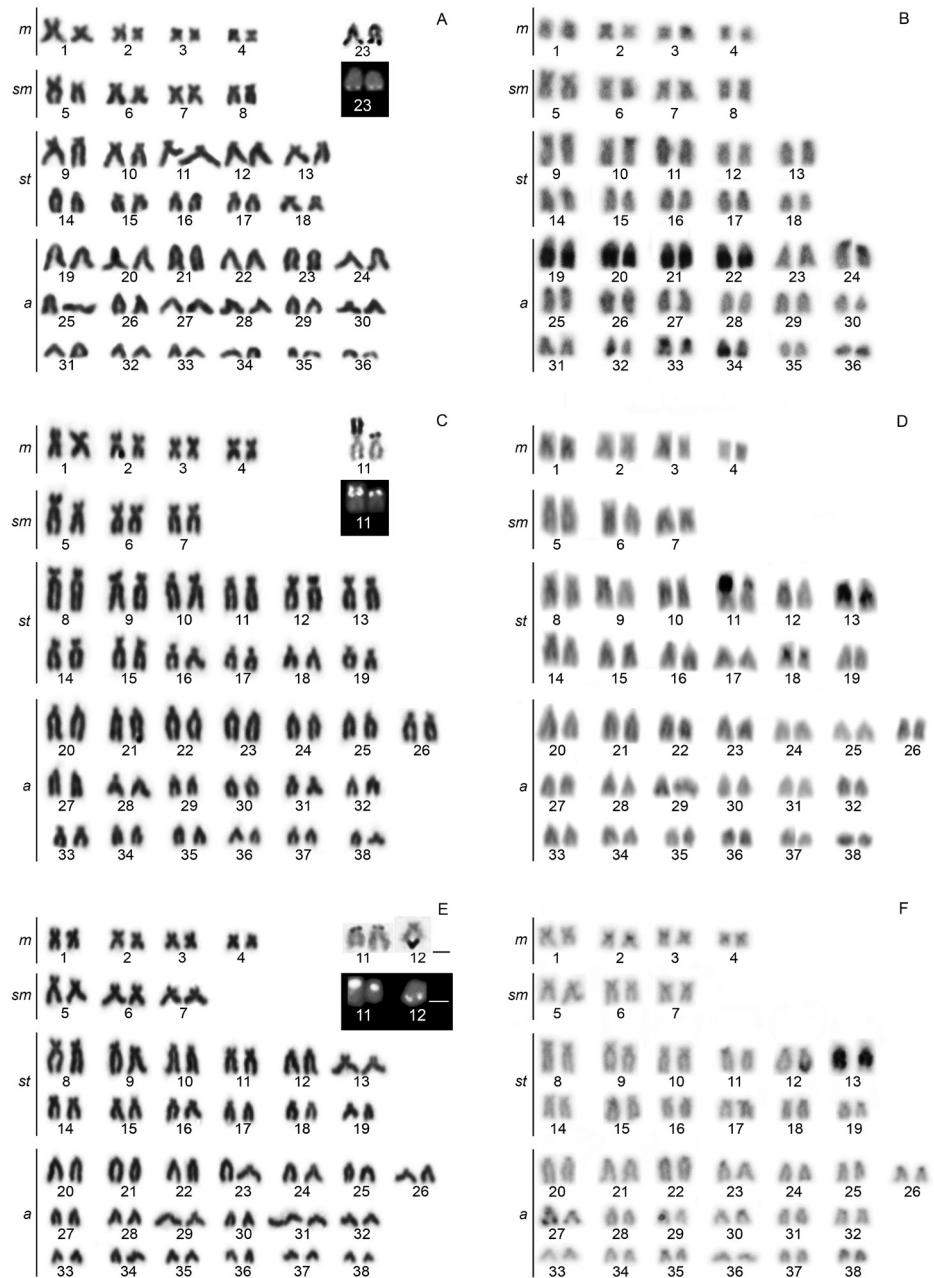
The diploid number found was 76 chromosomes, with a karyotype formula of  $8m+6sm+24st+38a$  and a fundamental number (FN) equal to 114 for males and females (Fig. 3C). A single pair of NORs was observed, located in terminal regions of the short arms of submetacentric pair 11 (Fig. 3C, highlighted above) confirmed by 18S rDNA-FISH (Fig. 3C, highlighted below). Heterochromatin were coincident with NORs and more evident in the submetacentric pairs 13 (Fig. 3D).

### *Hypostomus aff. tietensis* – Do Campo River

The diploid number found was 76 chromosomes, with a karyotype formula of  $8m+6sm+24st+38a$  and a fundamental number (FN) equal to 114 for males and females (Fig. 3E). Multiple NORs were found in terminal regions of the short arms



of subtelocentric pair 11 and long arm of only one subtelocentric chromosome of pair 12 (Fig. 3E, highlighted above) confirmed by 18S rDNA-FISH (Fig. 3E, highlighted below). Heterochromatin were coincident with NORs and more evident in the subtelocentric pair 13 (Fig. 3F).



**FIGURE 3 |** Karyotypes of *Hypostomus tietensis* (A, B) Pirai River “type locality”, (C, D) Pirapó River, and Do Campo River (E, F) with conventional Giemsa-staining (A, C, E), AgNORs (highlighted above) and 18S rDNA-FISH (highlighted below); C-banding (B, D, F). m = metacentrics; sm = submetacentrics; st = subtelocentrics; a = acrocentrics. Scale bars = 10 µm.

## DISCUSSION

This is the first paper about cytogenetic characterization of *Hypostomus tietensis*, once available cytogenetic data for *H. tietensis* included data from abstracts presented on the Brazilian Symposiums (for review, Bueno *et al.*, 2012). Even so, recently, considering these cytogenetic data, a study carried out on *Hypostomus* species from southeastern Brazil, using molecular and associated to cytogenetics data, subdivided the genus into subgroups according to their diploid number, including *H. tietensis* within the subgroup of species that they have  $2n = 66-68$  chromosomes (Rocha-Reis *et al.*, 2021).

Based on the results obtained in this study, the populations from Pirapo and Do Campo rivers showed considerable cytogenetic differences in relation to the population of the type locality (such as diploid number, karyotypic formula, AgNORs and heterochromatin distribution pattern) (Tab. 1), which can significantly contribute to the diagnosis of the species. The population of *H. tietensis* from the type locality had  $2n = 72$  chromosomes with FN = 108, unlike the other two *H. aff. tietensis* populations that present  $2n = 76$  chromosomes with FN = 114. Karyotypic differences were also observed in other populations of *H. aff. tietensis* from the Paranapanema River and Araquá River, where both had  $2n = 68$  chromosomes, with different karyotype formulas,  $14m + 12sm + 42st/a$  (Oliveira, Foresti, 1993) and  $18m + 10sm + 12st + 28a$  for *H. aff. tietensis* (Ishida *et al.*, 2002). In a cytogenetic study carried out in seven species of the tribe Hypostominae, Alves *et al.* (2006) obtained the same result of  $2n = 68$ , with a karyotype formula  $18m + 10sm + 12st + 28a$  for *Hypostomus ancistroides* (Ihering, 1911) from the Araquá River, which may be the same species, with possible errors in the identification of *H. aff. tietensis*, once they belong to same morphological group and may be misidentified. It is worthy to note that the range of  $2n = 64$  to  $2n = 68$  chromosomes is the diploid values to species pertaining to the super-group *Hypostomus plecostomus* (Linnaeus, 1758) *sensu* Queiroz *et al.* (2020): *H. ancistroides*, *H. boulengeri* (Eigenmann & Kennedy, 1903), *H. commersoni* Valenciennes, 1836, among others. In the Upper Paraná River basin, species lacking keels along lateral series of flanks, presenting no evident crests on compound-pterotic and having abdominal region somewhat naked, usually belongs to the super-group *Hypostomus auroguttatus* Kner, 1854. Species of the *H. auroguttatus* super-group present  $2n = 72$ ,  $2n = 74$  or  $2n = 76$  chromosomes (Bueno *et al.*, 2012). Therefore, although *H. tietensis* were not in the bulk of species from Queiroz *et al.* (2020) it is very probable the species to belong to this super-group. In addition, up to now, the only species to the super-group *H. plecostomus* naturally inhabiting upper stretches of the upper Paraná River basin is the *H. ancistroides* complex. Then, it is very probable that the previous *H. tietensis* identifications by cytogenetic works in fact refers to some population of the *H. ancistroides* species complex.

The karyotypic numbers found are within the expected range for *Hypostomus* species from the southern basins of South America proposed by Bueno *et al.* (2013), where the high diploid number with several acrocentric chromosomes is considered a derived characteristic within the group, since  $2n = 54$  chromosomes is the putative plesiomorphic condition for Loricariidae, being the centric fissions responsible for playing an important role in the karyotypic evolution of the genus (Artoni, Bertollo, 2001). In *Hypostomus*, chromosome numbers range from  $2n = 64$  in *H. faveolus* Zawadzki, Birindelli & Lima, 2008, *H. cochliodon* Kner, 1854, and *H. soniae* Hollanda Carvalho & Weber, 2005 to  $2n =$

**TABLE 1** | Cytogenetic data in *Hypostomus tietensis*. 2n = diploid number, KF = karyotypic formula, FN = fundamental number, m = metacentrics, sm = submetacentrics, st = subtelocentrics, a = acrocentrics.

Populations	2n	KF	FN	AgNORs	18S rDNA-FISH
Pirai River	72	8m+8sm+20st+36a	108	Simple	Simple
Pirapó River	76	8m+6sm+24st+38a	114	Simple	Simple
Do Campo River	76	8m+6sm+24st+38a	114	Multiple	Multiple

84 in *H. perdido* Zawadzki, Tencatt & Froehlich, 2014 (Artoni, 1996; Cereali *et al.*, 2008; Bueno *et al.*, 2013, 2014; Becker *et al.*, 2014; Oliveira *et al.*, 2019), with 2n = 72 and 2n = 76 chromosomes being one of the most frequent conditions, observed in more than 30% of the species analyzed cytogenetically (Bueno *et al.*, 2012). Although Mezzomo *et al.* (2020) consider the chromosome number range from 52 to 84, the 2n = 52 belongs to *H. chrysostiktos*, which the current status is *Pterygoplichthys chrysostiktos* (Birindelli, Zanata & Lima, 2007) (Fricke *et al.*, 2022).

AgNORs and 18S rDNA-FISH were also efficient in differentiation of the type locality population (terminal, long arm, acrocentric pair 23) from the other two populations (terminal, short arm, subtelocentric pair 11). Although in the Do Campo River population some individuals presented an extra chromosome bearing NOR (long arm of chromosome *st* 12), variation in the number of NORs is common in *Hypostomus*, both among populations of the same species and within populations (Bueno *et al.*, 2012). The presence of multiple NORs located in terminal regions is frequent in *Hypostomus* species (Artoni, Bertollo, 1996; Alves *et al.*, 2006; Rubert *et al.*, 2008; Martinez *et al.*, 2011), being derived in the Hypostominae group, where the presence of a single site is considered basal in Loricariidae and in fish in general (Artoni, Bertollo, 1996, 2001; Alves *et al.*, 2005). The most frequent locations of AgNORs are on the *st/a* chromosomes, corresponding to regions of heterochromatin in many populations, as has been reported in other species (Kavalco *et al.*, 2005; Rubert *et al.*, 2008; Traldi *et al.*, 2013). This association may contribute to the evolution of the group, as it allows the dispersion of NORs sites throughout the genome through transposable elements, which may carry complete or partial NORs (Moreira-Filho *et al.*, 1984; Vicari *et al.*, 2008; Schmid *et al.*, 2017).

The heterochromatin analysis was also efficient to differentiate the chromosomal structure among the three populations. Individuals from the type locality population showed large conspicuous blocks on acrocentric chromosomes pairs 19, 20, 21 and 22. In the populations of the Pirapó River and Do Campo River, heterochromatins were more evident in interstitial position on chromosome *st* par 13, and terminal blocks coincident with NORs in each population. In *H. aff. tietensis* studied by Ishida *et al.* (2002), the distribution was different, with the presence of heterochromatin in centromeric and pericentromeric positions, which suggests that this population should not represent *H. tietensis*.

Genetic variation does not immediately generate a phenotypic differentiation in the external morphology of an organism, revealing the presence of cryptic species (Bickford *et al.*, 2007; Endo *et al.*, 2012). The morphological similarity and diversity of karyomorphs in allopatric populations of *Hypostomus* represent cryptic species and species complexes (Lorscheider *et al.*, 2015). *Hypostomus paulinus* (Ihering, 1905), for



example, has populations with  $2n = 72$  to  $2n = 76$  chromosomes, being considered a species complex, as well as *H. iheringii* (Regan, 1908) ( $2n = 76$  and 80), *H. topavae* (Godoy, 1969), *H. nigromaculatus* (Schubart, 1964), *H. ancistroides*, and *H. regani* (Ihering, 1905) that have karyomorphs with distinct karyotype formulas (Endo *et al.*, 2012; Lorscheider *et al.*, 2015; Rubert *et al.*, 2016).

There are no strong morphological differences among the three populations analyzed in this work. The three populations present pale marks (blotches, spots or vermiculations) on a darker background, short to moderate jaw rami, flattened body, and comparatively to congeners, small eyes. However, the cytogenetic characters allowed to evidence a notable difference among the population of the type locality and the other populations, such as diploid number, karyotypic formula, NORs and heterochromatin distribution pattern, showing that the populations of the Pirapó River and Do Campo River evolved in a way independent in each basin, in relation to the population of the type locality, the valid *H. tietensis*. The data suggest the possible presence of cryptic species within this taxon, considering the populations of the Pirapó River and Do Campo River as *Hypostomus* aff. *tietensis*. Furthermore, given the range of karyomorphs already found for the species ( $2n = 68$  to 76), *H. tietensis* can eventually be considered a species complex.

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#### ETHICAL STATEMENT

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 the Ethics of Animal Experiments of the Universidade Estadual de Maringá (License number: Protocol 1968010719 – CEUA/UEM; License number: SISBIO 69480–2).

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The authors declare no competing interests.

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