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Cytogenetic Markers Reinforce the Redescription of the Armored Pleco *Hypostomus spiniger* (Loricariidae - Hypostominae), an Endemic Species in the Uruguay River Basin and Patos Lagoon System

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HIGHLIGHTS

- Determination of chromosomal characters with cytotaxonomic value.
- Cytogenetic analyzes are important in the diagnosis of *Hypostomus* species.
- The cytogenetic reinforces the valid status of *Hypostomus spiniger.*

Abstract: Among the Neotropical fish fauna, suckermouth armored catfishes (*Hypostomus* - Loricariidae) stands out as one of the most difficult groups to diagnose morphologically. So the use of different molecular markers, as is the case of cytogenetics, has been fundamental for a precise identification of some species. In the present study, we characterize the karyotypes of two allopatric *Hypostomus spiniger* populations, using classical and molecular cytogenetic methods. This species was described by Hensel (1870) but later synonymized with *Hypostomus commersoni*, and recently it was again recognized as a valid species. Taking to account this taxonomic problematic, the aim of this study is to determine chromosomal characters that may be useful to validate the taxonomic status of *H. spiniger* and to complement its diagnosis in relation to *H. commersoni* populations. The karyotype of *H. spiniger* is composed by 66 chromosomes (10m+16sm+14st+26a), few heterochromatin and a multiple nucleolar organizer regions (NORs) system. Despite, the currently geographic isolation among the samples collected in Forquetinha River (Patos Lagoon

basin) and Quadros Lagoon (Tramandaí River basin), both shared the same karyotypic structure, this cytogenetic evidence, suggest that both populations belongs to the same species. Additionally, our results clearly distinguish *H. spiniger* from *H. commersoni* populations collected along the Paraná River basin, that exhibited 2n=68 chromosomes and several divergences in heterochromatin and NORs pattern. In sum, the present study reinforces the valid status of *H. spiniger* and demonstrated the importance of basic cytogenetic analysis to understand conflictuous taxonomic matters.

Keywords: cytotaxonomy; heterochromatin; karyotypes; synonymy; 18S rDNA.

INTRODUCTION

The South America includes one of the most diversified ichthyofauna on Earth. This great biodiversity has aroused the interest of taxonomists, but it is still a great challenge to characterize and establish phylogenetic hypotheses in several groups, as is the case of *Hypostomus* (Loricariidae-Hypostominae). The suckermouth armored catfishes actually includes 152 species popularly known as cascudos, acaris, plecos, and bodós that occur in all the major hydrographic systems of South America, with most of the species occurring in rivers of the southeastern Brazil, in the Paraguay basin, and coastal drainages of Guiana and Suriname [1-4]. This hyperdiverse fish group, are recognized as a monophyletic assemblage by both morphological [5-8] and molecular [9-15] phylogenetic approaches. Recently, Jardim de Queiroz and coauthors [11], performed a most complete phylogeny of *Hypostomus* in terms of taxon sampling and geographical distribution. These authors sampled 206 representatives from all over the major Neotropical hydrographic systems and analyzed four nuclear and two mitochondrial markers. This results subdivided *Hypostomus* in four major lineages called super groups: *H. cochliodon*, *H. hemiurus*, *H. auroguttatus* and *H. plecostomus*.

The super group *Hypostomus plecostomus* is the second species-rich lineage among the suckermouth armored catfishes and include 38 morphospecies widespread along the major South America drainages. This super-group was subdivided into five well supported clades, named: *H. punctatus, H. carinatus*, *H. robinii*, *H. plecostomus* sensu strictu and *H. watwata*. The *Hypostomus punctatus* clade includes several undescribed species and some species with difficult taxonomic diagnosis, such as *H. ancistroides* Complex and *H. commersoni* [11, 16]. *Hypostomus spiniger* was considered a junior synonym of *H. commersoni* for more than 150 years, this species inhabits the Uruguay River and Dos Patos system, but the southernmost sampled locality was Gualeguaychú, Entre Ríos province in Argentina [17]. The taxonomic history of *H. spiniger* has undergone several changes since its description by Hensel (1870) that analyzed specimens from Cadea River, an important tributary of the Laguna dos Patos system in Rio Grande do Sul, Brazil. Only seven years after its description, Steindachner (1877) proposed *H. spiniger* as a junior synonym of *H. commersoni*. Later, Eigenmann and Eigenmann (1888:168) described *H. limosus*, and Malabarba (1989) restricted the type locality of *H. limosus* to the Patos Lagoon, and also considered this species a junior synonym of *H. commersoni*. Recently, Cardoso and coauthors [17], concluded that specimens described from the Patos Lagoon and Uruguay River correspond to *H. spiniger*, and *H. limosus* as its junior synonym.

The diversity of *Hypostomus* remains underestimated, with several species still not formally described, while others need revalidation [18]. Therefore, the development of complementary approaches becomes essential to better characterize the diversity and relationships among the species of the genus. In the last years, chromosome markers have proved to be an excellent tool for discriminating species with taxonomic problems [19-29]. Chromosome information about *Hypostomus* reveals a great chromosomal variability, diploid number ranges from 64 in *H. cochliodon*, *H. faveolus* [30] and *H. soniae* [31] to 84 in *H. perdido* (cited as *Hypostomus* sp. 2-rio Perdido NUP 4249) [32]. The physical mapping of ribosomal DNAs (rDNA) in *Hypostomus* reveals the presence of multiple NORs in the terminal position of subtelocentric or acrocentric chromosomes as the most frequent condition [33-36]. This set of features makes the suckermouth armored catfishes a promising model among the Neotropical Siluriformes to better understand the mechanisms involved in chromosome diversification [37-41].

In the present study we access the karyotypic structure of two geographically isolated populations of *Hypostomus spiniger* collected in Patos Lagoon system and Tramandaí River basin, both important hydrographic drainages situated in the Rio Grande do Sul – Brazil. We applied conventional cytogenetics techniques (Giemsa staining, C-band) and fluorescent *in situ* hybridization (FISH) using 18S rDNA probes, for understand the karyotypic diversification process among these two allopatric populations. Additionally, we compared the cytogenetic data of *H. spiniger* with *Hypostomus commersoni* populations from Paraná River basin, aiming to determine chromosomal markers that complements the diagnosis of *H. spiniger* and confirms its recent redescription, as a valid specie and not a synonymy of *H. commersoni*.

MATERIAL AND METHODS

Species and collection sites

We carried out chromosome analyses on 15 individuals identified as *H. spiniger*: 12 specimens (5 males and 7 females) collected from Forquetinha River in Forquetinha / Rio Grande do Sul – Brazil (29°21'43.5''S / 52° 07'39.6''W) and 3 specimens (2 males and 1 female) collected from Quadros Lagoon at Barra do João Pedro / Rio Grande do Sul (29°44'42.8''S /50°06'54.3''W) (Figure 1). The collection of specimens was performed under permit number SISBIO 11399-1 (ICMBio - Instituto Chico Mendes de Conservação da Biodiversidade). The specimens were deposited in the Museum of Zoology of Universidade Estadual de Londrina (MZUEL) under the voucher numbers MZUEL 8713 and MZUEL 4849. The experiments were performed according to Ethics Committee for Animal Use of the Universidade Estadual de Londrina, under the protocol number 60/2017.

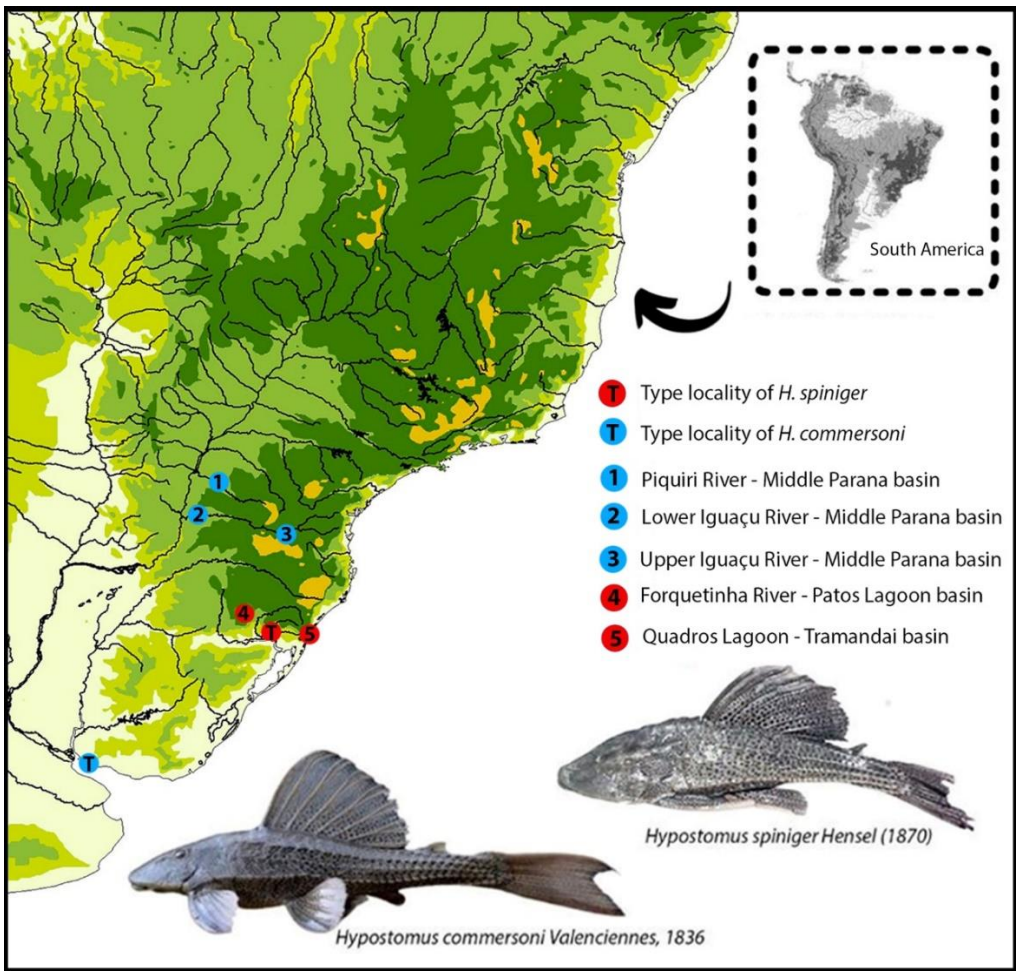

Figure 1. South American map with the collections sites of *H. spiniger* (analyzed in the present study) and *H. commersoni* populations that have been karyotyped so far. The *H. spiniger* image was obtained from Cardoso and coauthors [17], while the *H. commersoni* photo was obtained by Corrêa and coauthors [42].

Mitotic chromosomes preparations, chromosome banding and Fluorescence *in situ* hybridization (FISH)

Chromosome preparations were obtained from kidney cells according to the air-drying technique proposed by Bertollo and coauthors [43]. Previously, a mitotic stimulation with 300µL of Broncho-vaxom (bacterial lysate 0,2g/mL of water) was injected to trigger an inflammatory process and increase the number of renal cells in mitotic division [44]. We counted at last twenty-five metaphases plates per individual in order to define the diploid number using a Leica DM 2000 microscope equipped with a digital camera Moticam Pro 282B. The best metaphases were photographed through the software Motic Images Advanced 3.2. We measured each chromosome using the DRAWID v0.26 software [45], and the karyotype formulas were determined according to the ratio of arms proposed by Levan and coauthors [46]. The heterochromatin

pattern was detected in accordance with the protocol described by Sumner [47] with modification in the staining step [48].

The 18S rDNA probe was obtained by Mini-Prep (i.e., extraction of plasmidial DNA) from *Prochilodus argenteus* (Spix and Agassiz, 1829) [49] and labeled by nick translation (Roche®) according to the manufacturer's instructions using biotin-16-dUTP. FISH was performed under high stringency conditions on metaphase chromosome spreads of *H. spiniger*, as detailed in Pinkel and coauthors [50]. About 20 µl of the hybridization mixture (2.5 ng/µl probes, 2 µg/µl salmon sperm DNA, 50% deionized formamide, 10% dextran sulfate) were dropped on the slides, and the hybridization was performed for 24 h at 37 °C in a moist chamber containing distilled water. The signals were detected using avidin fluorescein isothiocyanate (Avidin-FITC) (Sigma, St. Louis, MO, USA). The propidium iodide was used as chromosome counterstaining in a concentration of 1.0 µg/ml in an anti-fading solution (Vector, Burlingame, CA, USA).

RESULTS

All specimens of *H. spiniger* collected from Quadros Lagoon and Forquetinha River, showed 2n=66 chromosomes, with karyotype formula: 10m+16sm+ 14st+26a, without differences among the sexes (Figure 2a). Regarding the heterochromatin distribution, both populations shared the same pattern, with few blocks detected in pericentromeric region of the pair 2m, on the short arm of the pairs 7sm and 14st, and on terminal position on the long arm of the pair 28a (Figure 2b). The FISH with 18S rDNA probes evidenced in all individuals from both localities, a multiple NORs system, however a numeric variation was detected, allowing the identification of two configurations, named: pattern A characterized by the presence of terminal sites on p arm of the pair 7sm and two terminal sites on q arm of the pair 28a (Figure 2c). This pattern was described in two individuals of Quadros Lagoon and two specimens collected in Forquetinha River). The pattern B, that was characterized by the presence of three NORs sites: two terminal sites on the p arm of pair 7sm and in q arm of only one chromosome 28a (Figure 2d). The pattern B was detected in the majority of sample here analyzed, being reported in one specimen collected in Quadros Lagoon and ten individuals from Forquetinha River.

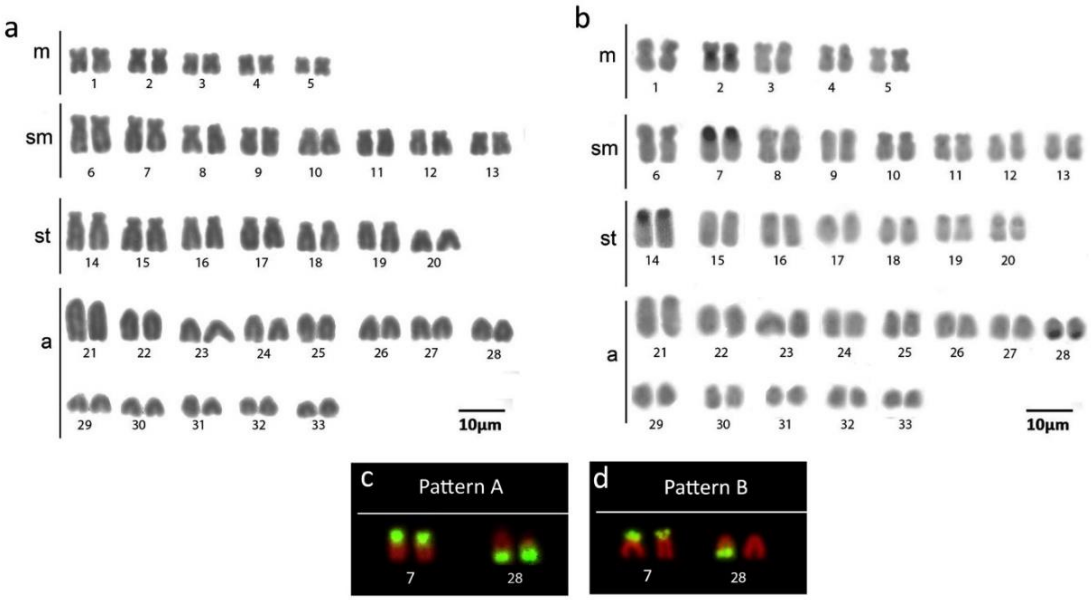

Figure 2. Karyotypes of *H. spiniger.* (a) Giemsa staining; (b) C-banding; (c,d) FISH with rDNA 18S probes. Note the presence of numerical variation in the NORs sites that was detected in both populations.

DISCUSSION

During its more than 50 years of history, Brazilian Fish Cytogenetics has revealed many particularities concerning the chromosomal diversity and evolution of several freshwater fish taxa. It is also an elucidative approach when combined with traditional and now molecular taxonomy for identification of new species [51,52] or understanding conflicting phylogenetic issues [24,25]. In the present study, we discussed the importance of cytogenetic data for understand the taxonomic problematic related to the *Hypostomus spiniger*. This species was described in the late 19th century, however a few years later it was synonymized with *H. commersoni*, remaining under this classification until 2019, when Cardoso and coauthors [17] redescribed, the species based on molecular markers. The cytogenetic analysis performed here, reveals in two allopatric

populations of *H. spiniger*, the same karyotype structure, with 2n=66 chromosomes (10m+16sm+14st+26a), few heterochromatin blocks and multiple NOR system. Similar results were reported by Rubert and coauthors [35], in specimens identified as *H. commersoni* from Forquetinha River. This karyological similarity together with the morphological diagnosis and geographic distribution determined by Cardoso and coauthors [17], confirms that such specimens are actually *H. spiniger* and not another population of *H. commersoni*. It's important to highlight that the minor divergences observed between the karyotypes of the specimens analyzed here with the sample studied by Rubert and coauthors [35], are due to the different criteria of chromosome classification, adopted by the authors, that can be affected the chromosomal formulae and the position of the chromosomes bearing the NORs sites.

According to Jardim de Queiroz and coauthors [11], *H. spiniger* is currently included in the "*Hypostomus punctatus* group" together with other species reported mainly from southeastern coastal basins (i.e., *H. interruptus, H. affinis, H. tapijara, H. scabriceps*) and from the Paraná River (*H. derbyi, H. ancistroides, H. commersoni*). The cytogenetic data available for species allocated in *H. punctatus* group, reveals the occurrence of species bearing 2n=66 chromosomes, as *H. spiniger*, *H. affinis* from Paraíba do Sul drainage, *H. tapijara* endemic specie of Ribeira de Iguape basin and some populations of the *H. ancistroides* Complex collected in the Upper Tibagi River and Upper Paranapanema River (Table 1). Interestingly, all these species with 2n=66 chromosomes occurs in different sub-basins from South Atlantic coastal hydrographic system. The exceptions would be only the specimens identified as *Hypostomus* aff. *ancistroides*, which, although collected in non-coastal regions, occur in locations close to watershed dividers [53,56]. The other species from the *H. punctatus* group have 2n=68 chromosomes, as observed in *H. commersoni, H. derbyi* and the majority of *H. ancistroides* Complex populations collected in the Tietê, Paranapanema, Ivaí and Piquiri Rivers, important sub-basins of the Parana River system (Table 1). From the karyoevolutive point of view, it is still extremely premature to estimate what would be the ancestral diploid number for the clade *Hypostomus punctatus*, even though the present evidences point to this being 2n=66 or 2n=68. Thus, the confirmation of such hypothesis will only be possible when more cytogenetic information on other species of the *H. punctatus* group is available, and especially when chromosome data are obtained from the other subgroups that are included in the *H. plecostomus* clade. Thus, the integration of these cytogenetic data with well representative phylogenies using ancestral character reconstruction (RCA) software seems to be the best alternative to define which would be the ancestral 2n for the *H. punctatus* group.

Hypostomus spiniger populations had only a few pairs of chromosome with heterochromatin, most of them associated with DNAr 18S, a condition frequently reported for some *Hypostomus* species of different clades [30]. Among the South American Loricariidae, *Hypostomus* stands out for exhibiting remarkable variability regarding the distribution [30], amount [63, 64] and composition [38, 62, 65] of heterochromatin segments. This extensive variation is a result of a complex process of karyotype diversification and has also been important to characterize and distinguish species that have taxonomic problems, such as *H. strigaticeps* [37], *H. ancistroides* [54] and *H. commersoni* [34,38]. The distribution of the heterochromatin was one of the characteristics that allowed the discrimination of *H. spiniger* from the other *H. commersoni* populations, not only because of the amount of blocks but also because of the position of these sites within the chromosomes.

Table 1. Cytogenetic data available for the "Hypostomus punctatus group"

Species	2n	Karyotype formula	NORs	Localities	Ref
H. affinis	66	12m+12m+14st+28a	6^{FISH}	Jacuí Creek (PRSB)	[40]
H.affinis	66	12m+12m+14st+28a	4^{FISH}	Paraíba do Sul River (PRSB)	[40]
Hypostomus aff. *ancistroides*	66 XX	16m + 12sm + 12st + 26a	3^{FISH}	Paranapanema River (PRB)	[53]
Hypostomus aff. *ancistroides*	66 XY	17m + 12sm + 12st + 25a	4^{FISH}	Paranapanema River (PRB)	[53]
Hypostomus aff. *ancistroides*	66	12m + 16sm + 10st + 28a	$4\text{-}6^{FISH}$	Tibagi River (PRB)	[54]
H. spiniger	66	10m +16sm + 14st + 26a	$3\text{-}4^{FISH}$	Quadros Lagoon (TRB)	[*]
H. spiniger	66	10m +16sm + 14st + 26a	$3\text{-}4^{FISH}$	Forquetinha River (PLB)	[*]
H. spiniger (cited as *H. commersoni*)	66	10m +16sm + 14st + 26a	3^{FISH}	Forquetinha River (PLB)	[35]
H. tapijara	66	14m + 24sm + 14st + 14a	6^{FISH}	Ribeira de Iguape River (RIB)	[55]
Hypostomus aff. *ancistroides*	68 ZZ	16m + 12sm + 22st + 18a	6^{FISH}	Keller River (PRB)	[56]
Hypostomus aff. *ancistroides*	68 ZW	16m + 13sm +22st +18a	6^{FISH}	Keller River (PRB)	[56]
H. ancistroides	68	16m + 18sm + 34st/a	6^{AGNOR}	Monjolinho Creek (PRB)	[57]
H. ancistroides	68	18m + 10sm + 12st + 28a	6^{AGNOR}	Araquá River (PRB)	[58]
H. ancistroides	68	14m + 14sm + 8st + 32a	2^{AGNOR}	Piquiri River (PRB)	[30]
H. ancistroides	68	14m + 14sm + 8st + 32a	4^{FISH}	Piquiri River (PRB)	[34]
H. ancistroides	68	10m + 26sm + 32st/a	4^{FISH}	Paranapanema River (PRB)	[59]
H. ancistroides	68	14m + 12sm + 18st + 24a	8^{AGNOR}	Dourados Stream (PRB)	[60]
H. ancistroides	68	16m + 12sm + 18st + 22a	4^{AGNOR}	Maringá Stream (PRB)	[60]
H. ancistroides	68	8m + 10sm + 18st + 32a	6^{AGNOR}	Ximbaúva Stream (PRB)	[60]
H. ancistroides	68	16m + 4sm + 16st + 32a	6^{AGNOR}	Corumbataí River (PRB)	[61]
H. ancistroides	68	10m + 20sm + 10st + 28a	6^{FISH}	Hortelã Creek ((PRB)	[62]
H. ancistroides	68	14m + 16sm + 22st + 16a	6^{FISH}	Lapa Stream (PRB)	[55]
H. commersoni	68	12m + 14sm + 14st + 28a	6^{FISH}	Piquiri River (PRB)	[34]
H. commersoni	68	12m + 14sm + 14st + 28a	6^{FISH}	Iguaçu River (PRB)	[34]
H. commersoni	68	12m + 12sm + 14st + 30a	8^{FISH}	Iguaçu River (PRB)	[38]
H. derbyi	68	12m + 12sm + 10st + 34a	2^{FISH}	Iguaçu River (PRB)	[38]

2n=diploid number; Ref=references; [*****]=Present study; m=metacentric; sm=submetacentric; st=subtelocentric; a=acrocentric; NORs=nucleolus organizer regions detected by silver nitrate impregnation (Ag-NOR) or FISH with 18S rDNA probes (FISH); (PRB) Paraná River basin; (PRSB) Paraíba do Sul River basin; (TRB) Tramandaí River basin; (PLB) Patos Lagoon basin; (RIB) Ribeira de Iguape River basin.

Multiple 18S rDNA sites in terminal position are the most common pattern described among *Hypostomus* species, however a conspicuous variation occurs on the chromosome pairs that bear this repetitive sequences [35]. According to the Table 1, most species included in the *Hypostomus punctatus* group exhibited multiple 18S rDNA sites, and generally showing a numeric variation of 4 or 6 sites. The exception was *H. derbyi* [38] and specimens of *H. ancistroides* collected from Piquiri River [34]. When compared the NOR pattern described for *H. spiniger* to the results obtained in *H. commersoni*, we detected numeric divergence: *H. spiniger* have four, while *H. commersoni* populations have six (Piquiri and Lower Iguaçu River) and eight sites (Upper Iguaçu River) (Table 1). Despite this numeric variability, both species exhibit terminal 18S DNAr sites on the long arm of a small acrocentric pair, we hypothesized that these chromosomes are homeologous, being therefore, another feature that reinforces the strict evolutionary relationship among *H. spiniger and H. commersoni*.

Hypostomus spiniger also showed an intra and interpopulational 18S rDNA numeric variation characterized by the presence of specimens with four of these sites while some individuals showed only three sites. These two patterns co-exist in both populations, but specimens with three sites of 18S rDNA were predominated in the Patos Lagoon System. According to Traldi and coauthors [55], the detection of rDNA sites by FISH in *Hypostomus* in only one of the homologue chromosomes is recurrent, possibly due to the limited resolution of the FISH technique for detecting very small sites [66]. Additionally, the origin of this numeric variation can be explained by non-reciprocal translocations involving terminal segments. During interphase, the chromatin fibers are less condensed and positioned in specific nuclear domains due to the interactions between telomeres and the nuclear envelope. This conformation, called Rabl's model,

guarantees a closer arrangement among non-homologous chromosomes and may promote translocations of terminal segments [67,68]. Another widely discussed possibility is the spreading of NORs among different chromosomes has often been related to the presence of mobile genetic elements, which may insert itself in regions of 18S rDNA and spread them to other chromosomal sites [69-72].

The taxonomic history of *H. spiniger* is marked by reallocations and uncertainties about its taxonomic status. This species was described by Hensel (1870), but for a long time, was considered a junior synonymy of *H. commersoni*, and only recently was redescribed by Cardoso and coauthors [17]. These authors performed an integrative taxonomic analysis, that showed clear morphological and molecular (mtD-Loop region) differences between the specimens previously identified as *H. commersoni* from Uruguay/Dos Patos Lagoon and Paraná/Paraguay/Río de La Plata Rivers. In fact, our cytogenetic analysis supports the valid status of *H. spiniger*, because it can be easily distinguished from *H. commersoni* by having 2n=66 chromosomes, few heterochromatin regions and only three or four NORs, whereas *H. commersoni* populations have 2n=68 chromosomes, a moderate amount of heterochromatin and six to eight NORs (Figure 3). Considering this divergences, we suggest that the main mechanisms involved in the diversification between these species are: (a) Robertsonian rearrangements, which decrease (centric fusion) or increase (centric fission) the diploid number, the precise direction of these events still remain unclear, so both scenarios (reduction or elevation) should be considered; (b) pericentric inversions promotes slight alterations in chromosome morphology and produce different karyotype formulas; (c) the dispersion of heterochromatic segments also played a key role in the diversification process between *H. spiniger* and *H. commersoni*.

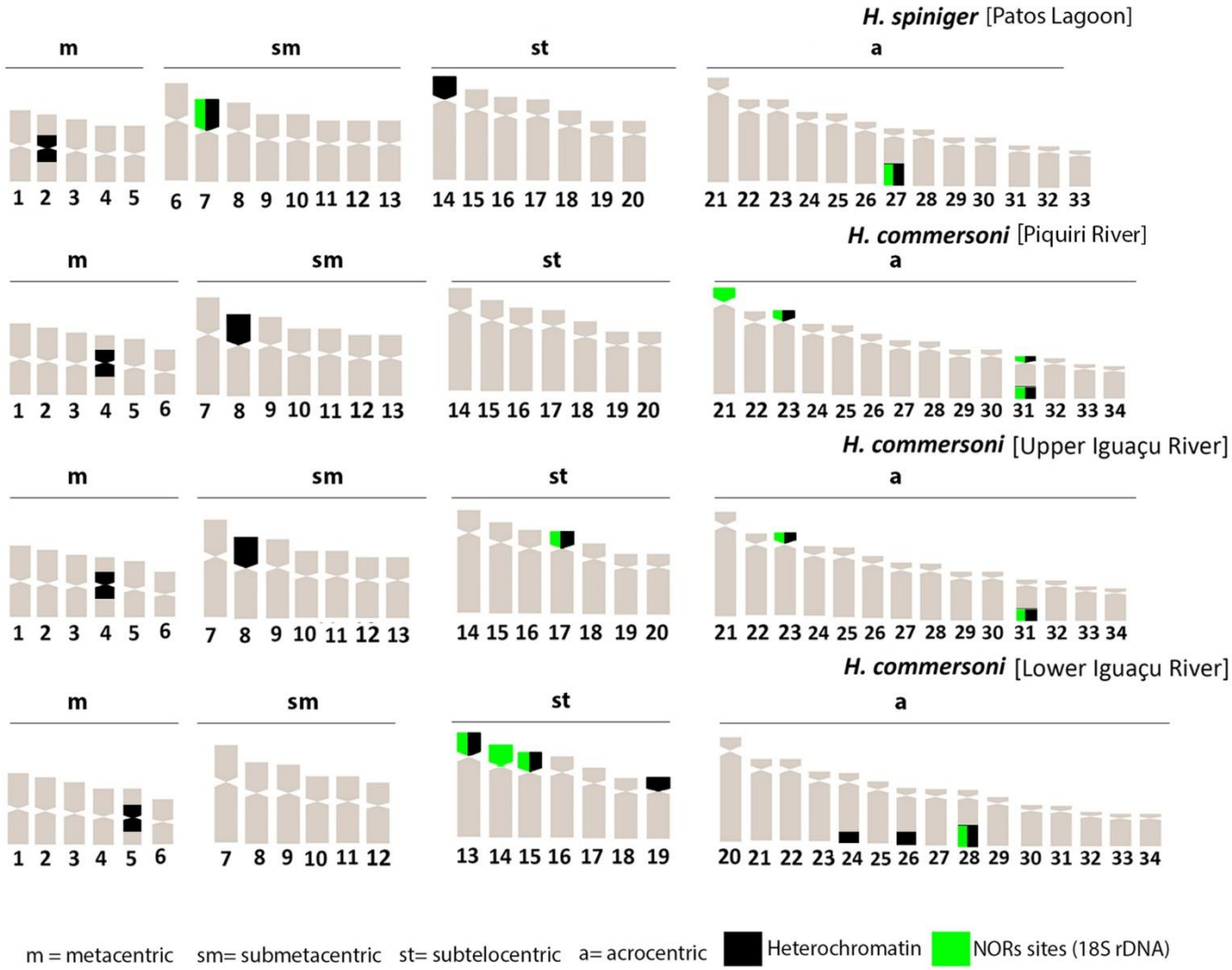

Figure 3. Comparative idiograms of *H. spiniger* and *H. commersoni* populations representing the chromosome variability reported for this species after cytogenetics techniques.

Hypostomus has shown to be a very interesting group of fish to unravel the role played by hydrogeological history in shaping the karyotype diversity in several species from different hydrographic systems in South America [37-41, 65]. Here, we characterized the karyotypes of two geographically isolated populations of *H. spiniger*, collected in Patos Lagoon and Tramandaí River basins, and no significant differences are observed when the karyotypes of these two allopatric populations are compared. The role of chromosomal rearrangements in the speciation process still remains a very ambiguous issue and requires further discussions [73-75]. However, among the freshwater fishes, a higher degree of karyotype variability is observed, it can be explained by the common topographic barriers in freshwater environments that would hamper the gene flow among populations, leading to fixation of macro-structural alterations in chromosomes [76]. Paradoxically, even though geographically isolated, populations of *H. spiniger* still share most of the karyotype characteristics, so we believe that the time of geographic isolation has not been enough to promote karyotype changes. This idea corroborates Jardim de Queiroz and coauthors [11] and Cardoso and coauthors [12], that suggests that the *H. punctatus* group may represent a very recent colonization and radiation into the Atlantic coastal rivers.

This study provides a set of chromosome markers with cytotaxonomic value that supports the reestablishment of *H. spiniger* and the previous morphological and molecular evidences [17]. Nowadays, the combined analysis consists in a resolute approach to species diagnoses and can assist in a more complete biodiversity characterization, a fundamental step for the development of conservation strategies, or evolutionary and phylogenetic studies [77]. For several authors, cytogenetic analysis is currently considered an outdated tool, which has been replaced by technologies derived from the genome sequencing analysis. However, we strongly believe that cytogenetics still has great relevance because it is a very resolute tool in some taxonomic matters, especially in groups with difficult morphological diagnosis, just as it has been demonstrated in several lineages of *Hypostomus* [25, 28, 29].

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Conflicts of Interest: The authors declare no conflict of interest.

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