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Chromosomal Diversity in Two Allopatric Populations of *Farlowella hahni* Meinken 1937 (Teleostei: Siluriformes): Cytogenetics and Cytochrome *b* Analyses

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Abstract

Farlowella is the second richest genus in Loricariinae, broadly distributed in freshwater streams and rivers of South America. In this article, we aimed to expand on the cytogenetic and molecular data available for two allopatric populations of *Farlowella hahni*. Both populations had diploid chromosome number 58, but with karyotype differences, indicative of chromosomal rearrangements. C-banding showed large heterochromatic blocks at telomeric regions in acrocentric chromosomes in both populations. Fluorescence *in situ* hybridization (FISH) revealed a single 18S rDNA site in both populations and a single 5S rDNA site for individuals from lower Paraná River basin (native region) and multiple 5S rDNA sites for individuals from upper Paraná River basin (non-native region). Mitochondrial sequence analyses did not separate the two *F. hahni* populations. The cytogenetic and molecular data obtained are relevant in a preliminary study and suggested the existence of cryptic diversity and the hypothesis that at least two *Farlowella* lineages may coexist in the Paraná basin.

Keywords: mitochondrial sequence, ribosomal DNA, C-banding, chromosomal rearrangements, fish cytogenetics

Introduction

Among the Loricariinae, *Farlowella* is the second richest genus in the subfamily with 30 valid species,[1] broadly distributed in freshwater streams and rivers of the Amazon, Orinoco, Maracaibo, Paraná, and coastal drainages of the Guiana shield,[2] being recently recorded for the Magdalena-Cauca system.[3] These catfish have an extremely thin and elongated body, a bony snout, and prominent tail filaments reaching a length of only 265 mm. The wood-like appearance and elongated shape of these animals has earned them the common name of twig catfish, being utilized as ornamental fish.[2,4] *Farlowella hahni* is a non-native species from the upper Paraná River (native from lower Paraná River basin), cited previously as *F. amazonum* for this part Paraná River, and its occurrence can be associated with the filling of the Itaipu Reservoir and the consequent inundation of the Sete Quedas Falls.[5]

Available cytogenetic data for Loricariinae show that the diploid number ranges from $2n = 36$ in *Rineloricaria latirostris* (Boulenger, 1900)[6] to $2n = 74$ in *Sturisoma* cf. *nigrirostrum* Fowler, 1940.[7] Cytogenetic studies in *Farlowella* are rare and restricted to *F. hahni* (cited previously as *F. amazonum*), *Farlowella* cf. *amazonum* and *F. schreitmuelleri*, which have showed a diploid number of 58 chromosomes, but with minor karyotype differences between three species analyzed.[8–10]

In Loricariinae, physical mapping of 18S rDNA were detected in three chromosomes for *Rineloricaria lanceolata*,[11] in two chromosomes for *Harttia loricariformis*[12] and individual females of *Harttia punctata*,[13] in one chromosome of individual males *H. punctata*.[13] Physical mapping of 5S rDNA in this subfamily were detected in *H. punctata*, located in two chromosomes for males or females.[13] In *Farlowella*, cytogenetic studies on the distribution of ribosomal RNA (rRNA) genes are scarce, with single 18S rDNA sites in *F. schreitmuelleri* and *Farlowella* cf. *amazonum* and single 5S rDNA sites in *Farlowella* cf. *amazonum* and multiple 5S rDNA sites in *F. schreitmuelleri*.[10]

In this article, we aimed to expand on the cytogenetic and molecular data available for *F. hahni*, contributing to the first record of mapping of ribosomal DNA (5S and 18S rDNA)

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and cytochrome *b* (*Cyt b*) analyses in this species, including in the analyses, individuals of region native (lower Paraná basin) and individuals from non-native region (upper Paraná basin) of this species.

Materials and Methods

Six individuals of *F. hahni* were collected from populations of Paraná River basin (Fig. 1): two males and three females of *F. hahni* from the Dourado stream, Upper Paraná River basin (Mundo Novo- MS; 23°51′04,9″S and 54°25′13,9″W) and one individual of *F. hahni* from the Iguassu River, Middle Paraná River basin (preservation area of the Iguassu National Park-PR; 25°38′18.72″S; 54°28′4.74″W).

Animals were captured with the permission of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio; number 45442 Voucher specimens were deposited in the fish collection of the Núcleo de Pesquisas em

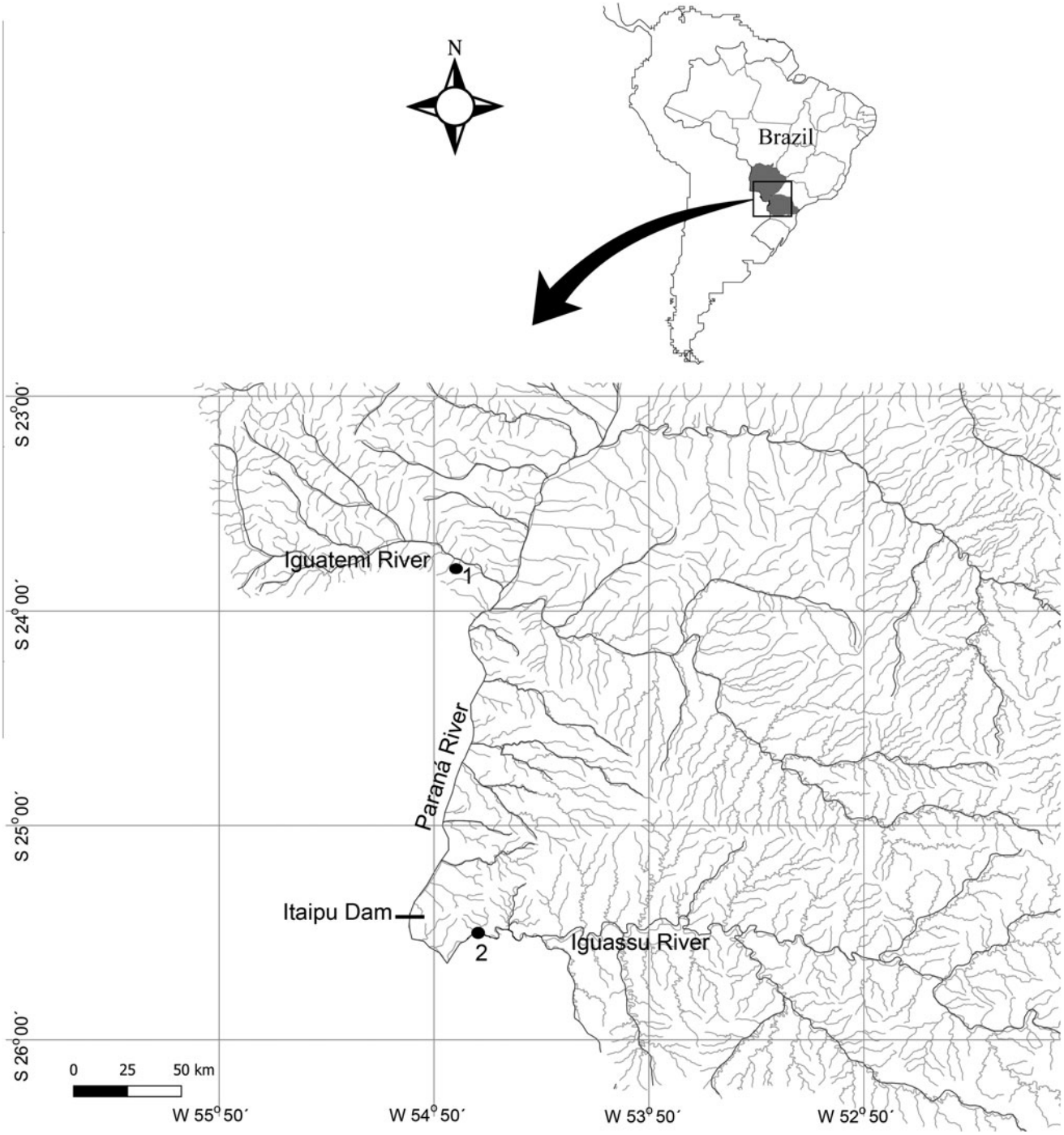

FIG. 1. Location of (1) Dourado stream (upper Paraná River basin) and (2) Iguassu River (middle Paraná River basin) where *Farlowella hahni* individuals were captured. *Dark circle* indicates the sampling spots.

Limnologia, Ictiologia e Aquicultura (NUPELIA), Universidade Estadual de Maringá, PR Brazil, as *F. hahni* from Dourado stream (NUP 22695), and *F. hahni* from Iguassu River (NUP 22696).

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.[14]

Cytogenetics analyses

Metaphase chromosomes were obtained from anterior kidney cells using the air-drying technique.[15] The C-positive heterochromatin (C-bands) visualized by the procedure of Sumner,[16] with some minor adaptations.[17]

At least 30 metaphases were analyzed for each individual and those with better chromosome morphology were used for the karyotype analysis. The chromosomes were classified as metacentric (*m*), submetacentric (*sm*), subtelocentric (*st*), and acrocentric (*a*) according to Levan *et al.*[18] The fundamental number (FN) was calculated according to the chromosomal arm numbers (the chromosomes *m*, *sm*, and *st* were considered to contain two arms—*p* and *q* arms—and the *a* with one arm—only *q* arm).

The location of the 5S and 18S rDNA sites in the chromosomes was performed by fluorescence *in situ* hybridization (FISH)[19] with modifications,[20] using probes from the genome of *Megaleporinus elongatus* (Valenciennes, 1850)[21] and *Prochilodus argenteus* Spix and Agassiz, 1829,[22] respectively. The probes were labeled through nick translation, with digoxigenin-11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA) (Roche). Detection and amplification of the hybridization signal were carried out using avidin-FITC and anti-avidin biotin (Sigma) for probes labeled with biotin, and anti-digoxigenin rhodamine (Roche) for probes labeled with digoxigenin. Chromosomes were counterstained with DAPI (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 with Adobe Photoshop CS6.

Molecular analyses

For molecular analyses of mitochondrial data we used six specimens of *F. hahni*. *Farlowella platoryncha* (GenBank: DQ133779.1) and *Rineloricaria fallax* (GenBank: DQ133780.1) were used as outgroup. Extraction of total DNA from hepatic tissue preserved in 100% ethanol was performed with the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following manufacturer's recommendations. The genomic DNA was quantified on the nanospectrophotometer NanoK (Kasvi) and then diluted to a concentration of 10 ng/μL. For amplification of mitochondrial genes *Cyt b* we used the primers GluDGF[23] and H16460R.[24] The final polymerase chain reaction (PCR) products were purified using the Wizard Kit SV Gel and PCR Clean-up System (Promega), according to the manufacturer's guidelines. Samples were sequenced by Sanger Method using BigDye® Terminator v3.1 Cycle Sequencing Kit and performed on an ABI 3730 DNA Analyzer at the Centro de Pesquisa do Genoma Humano, Universidade de São Paulo, Brazil.

Sequences were then edited with BioEdit[25] and aligned with MUSCLE algorithm[26] implemented on MEGA X.[27] Saturation signal was checked by DAMBE v7.0.28,[28] and the best-fitting model was chosen using the Akaike Information Criterion using jModelTest2.[29] The evolutionary history was inferred by using the neighbor-joining (NJ) based on the Kimura 2-parameter method and the maximum likelihood (ML) method based on the Hasegawa–Kishino–Yano model with Gamma distribution (five categories [+G, parameter = 0.2988]), with 1000 bootstrap replicates, were conducted in MEGA X.[27] Species delimitation was performed using the ABGD model (Automatic Barcode Gap Discovery)[30] at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html, using as input a *fasta* file; the Kimura distance model (K80) and the simple distance were analyzed in the result.

Results

Cytogenetics analyses

F. hahni—Iguassu River (lower Paraná River basin). Diploid number was 58 chromosomes (12*m* + 20*sm*+22*st*+4*a*, FN = 112) (Fig. 2a). C-banding showed large heterochromatic blocks at telomeric region in the long arm of pair 29 and interstitial position proximal to the telomeric region in the long arm of pair 28, and at pericentromeric regions in the long arm of pairs 22, 28, and 29, besides centromeric markings in some chromosomes (Fig. 2c). FISH revealed a single 5S rDNA site in pericentromeric position in the *st* pair 22 and a single 18S rDNA site in terminal position on the short arm of the *a* pair 28 (Fig. 2e).

F. hahni—Dourado stream (upper Paraná River basin). Diploid number was 58 chromosomes (12*m* + 30*sm*+10*st*+6*a*, FN = 110) for males and females (Fig. 2b). C-banding showed large heterochromatic blocks at telomeric regions in the long arm of pairs 27 and 28 and at pericentromeric regions in the long arm of pairs 8, 14, 24, 27, and 28, besides centromeric markings in some chromosomes (Fig. 2d). FISH revealed multiple 5S rDNA sites in pericentromeric position in the *st* pairs 24 and 27, and in the subterminal/terminal position of *a* pairs 27 and 28, respectively, and a single 18S rDNA site in terminal position on the *q* arm of the *a* pair 27, with a clear size heteromorphism (Fig. 2f).

Molecular analyses

Sequences are available in the GenBank with the following accession numbers: MW269961–MW269966. The *Cyt b* matrix contains six terminals and 986 bp with one variable site, further two terminals as outgroup (Supplementary File S1). The overall mean and pairwise genetic distances between the two populations of *F. hahni* were statistically insignificant. Both trees resulting from the phylogenetic analysis (NJ and ML) evidenced that specimens analyzed in this study formed a monophyletic group with high bootstrap values (Fig. 3). The ABGD model (Supplementary File S2) for species delimitation showed the same arrangement as that of NJ and ML.

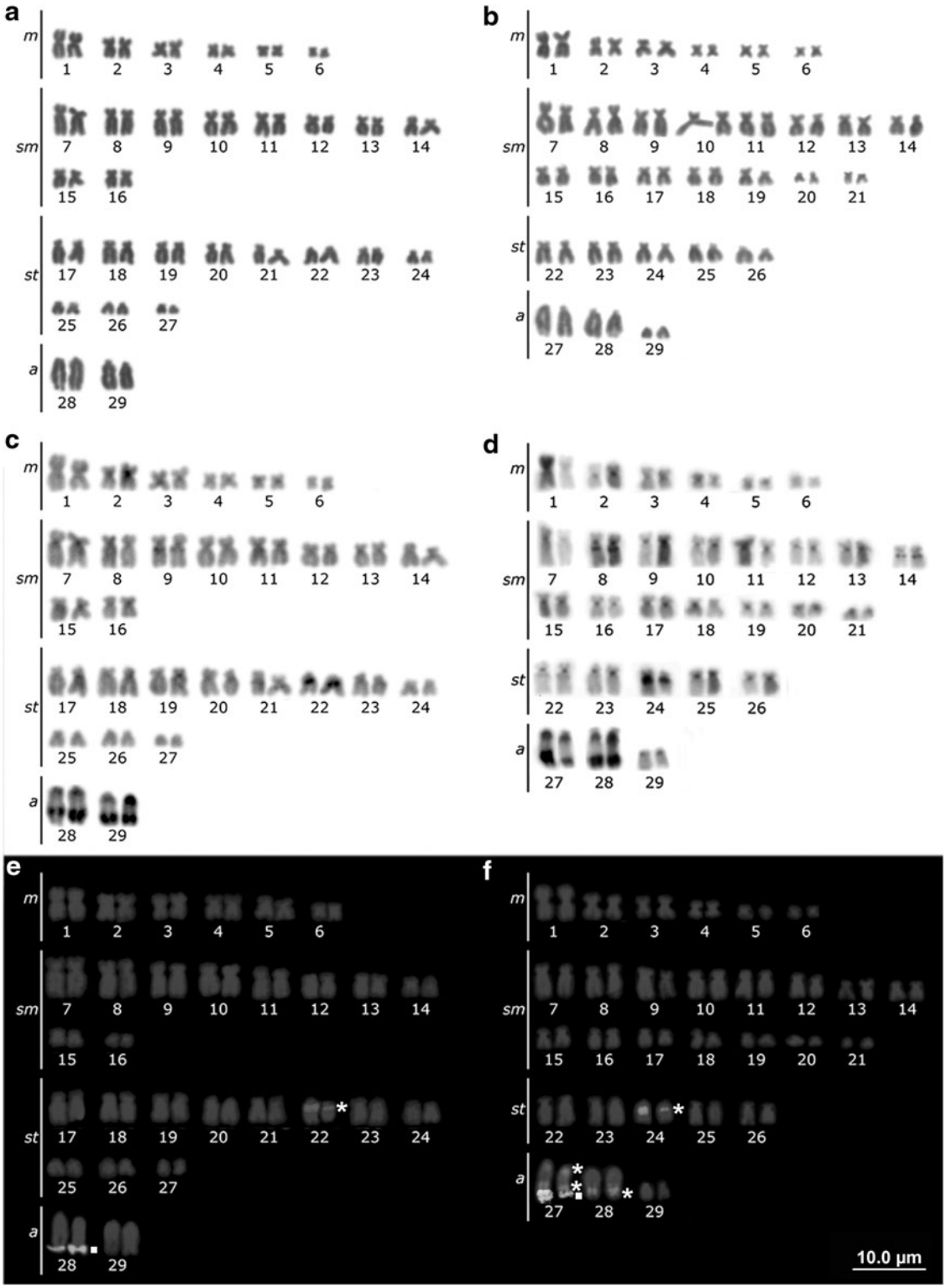

FIG. 2. Karyotypes **(a, c, e)** *Farlowella hahni* from Iguassu River, **(b, d, f)** *F. hahni* from Dourado stream. Karyotypes **(a, b)** stained with Giemsa, **(c, d)** C-banded and **(e, f)** after double FISH with 5S rDNA probes (*asterisk*) and 18S rDNA (*square*). FISH, fluorescence *in situ* hybridization.

Discussion

A diploid number of 58 chromosomes has been identified invariably in *Farlowella* species, including *Farlowella* cf. *amazonum*, *F. schreitmuelleri*[10] and different *F. hahni* populations as shown in Fernandes *et al.*[8,9] and the present study. A number of chromosomal rearrangements, such as pericentric inversions and/or deletions are found in the genomes of *F. hahni* analyzed in this study, given that, although they have the same 2*n*, the FN value and the karyotypes are

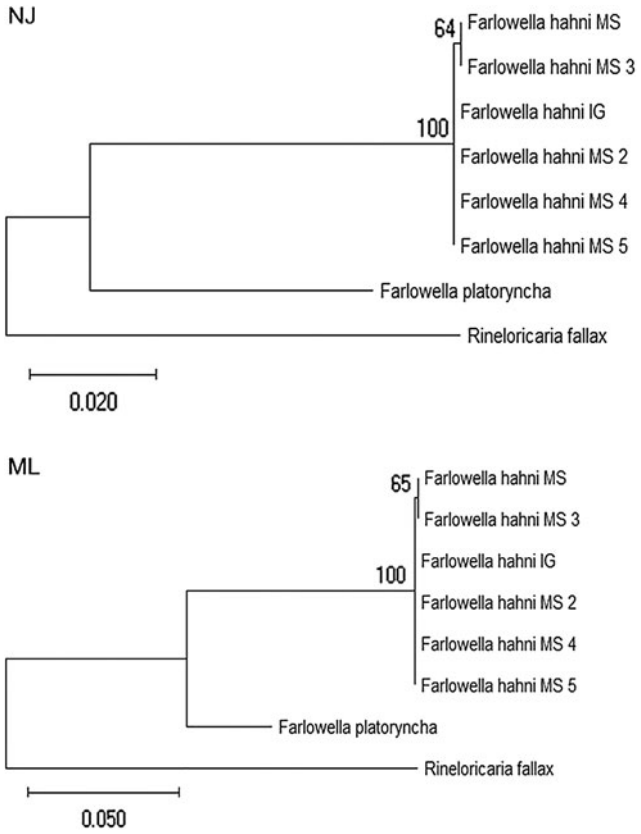

FIG. 3. Relationship cladograms performed with cytochrome *b* sequences generated by NJ and ML analyses. Bootstrap values are represented in the branches. *Farlowella platorynchus* and *Rineloricaria fallax* were used as outgroup. ML, maximum likelihood; NJ, neighbor-joining.

different, which the separation geographic and reduced vagility of species, which may lead to the formation of small isolated populations, may have facilitated the establishment of this karyotype variation due to the restriction of gene flow between them.

In the *Farlowella* species analyzed up to now, the nucleolar organizer region (NOR) phenotype is simple, as confirmed by the 18S rDNA probe as shown in Fernandes *et al.*[8,9] and Marajó *et al.*,[10] and the present study. In the karyotype of *F. hahni* under study, whereas in those of *F. schreitmuelleri*[10] and *F. hahni* previous studies,[8,9] the NORs are found on the long arms of the first acrocentric pair, which appears to be a conserved pattern in this genus, except in *Farlowella* cf. *amazonum*[10] the NOR site is located in the pericentromeric region of the long arms of the first metacentric pair.

In this study, size heteromorphism involving the NORs was detected by 18S rDNA-FISH only in the individuals of *F. hahni* from upper Paraná River basin. These different-sized regions between homologs may be the result of differences in the copy numbers of ribosomal genes.[31,32] This characteristic can be explained mainly by unequal recombination or random duplication in ribosomal cluster.[33]

Physical mapping of 5S rDNA in genome of *F. hahni* showed differences between populations analyzed. The 5S rDNA is located in a single chromosome pair for individual from Iguassu River, whereas for individuals from Dourado stream is located in three chromosome pairs. The large variation in the number of 5S rDNA cistrons may suggest the occurrence of pseudogeneization events or the insertion of mobile genetic elements (TEs) in the 5S rDNA intergenic spacers in *F. hahni* from upper Paraná basin, promoting the detection of ''extra'' sites of this gene through FISH. In both populations studied here, the 5S rDNA sites were associated with constitutive heterochromatin. The presence of sequences 5S rDNA in heterochromatic regions, and their association with TEs, are considered hotspots of genomic alterations, given that repetitive sequences are more susceptible to rearrangement, due to their intrinsic structural organization.[34,35] The TEs inside 5S rDNA nontranscribed sequences (NTS) are well described in fish genomes.[36–39]

The situation where two or more 5S rRNA gene clusters are localized on the same chromosome is quite rare; however, it was reported for fish species, including *Upsilodus* sp.,[40] *Hippoglossus hippoglossus* (Linnaeus 1758),[41] *Trachydoras paraguayensis* (Eigenmann and Ward 1907),[42] *Apteronotus albifrons* (Linnaeus 1766),[43] and *Sternopygus macrurus* (Bloch and Schneider 1801).[44] Double 5S rDNA sites (both in *q* arms) in the same chromosome observed in the karyotype of *F. hahni* from upper Paraná River basin, indicated that paracentric inversion might have caused the breakage of the 5S rDNA cluster, then transferring part of the site to the same arm of the same chromosome. This type of signal was found in the individuals *F. hahni* from upper Paraná River basin may represent species-specific chromosome marker.

This study showed an accentuated accumulation of heterochromatin, with large conspicuous bands, found in acrocentric chromosome pairs in both *F. hahni* populations. This pattern was also observed in *F. schreitmuelleri*,[10] whereas *Farlowella* cf. *amazonum*[10] have less heterochromatin in comparison with aforementioned species. Thus, the distribution of heterochromatin can be useful as a cytotaxonomic marker among *Farlowella*, separating at least two groups, those with large heterochromatic blocks in acrocentrics (*F. hahni* and *F. schreitmuelleri*) and those with little heterochromatin (*Farlowella* cf. *amazonum*) spread throughout the genome.

Molecular data indicate the existence of one clade de *F. hahni* from Paraná River basin with low genetic differentiation. Although the molecular characters (e.g., *Cyt b* and cytochrome oxidase subunit I—*COI*—genes) are considered efficient for the identification of fish species, with effective results in several studies,[45] this tool is less conclusive when compared with chromosomal data. Similar results were found in other studies in *Astyanax*.[46–48] and in other complex groups, such as *Rineloricaria*[49] and *Prochilodus*,[50] indicating that this gene may be less efficient for taxonomically complex clades with recent speciation.

According Júlio Júnior *et al.*,[51] fish species have invaded in the upper part of the Paraná River basin after the Itaipu Reservoir inundated Sete Quedas Falls, a natural and effective barrier that was the limit of two hydrographic ecoregions (upper Paraná River basin and lower Paraná River basin). The role of Sete Quedas Falls as a limit of these ichthyofaunistic regions was recognized by several authors.[52–54] The Itaipu Dam, located 150 km downstream from Sete Quedas, impounded the River Paraná in 1982 and completely flooded the falls. Therefore, several species endemic to the lower Paraná River basin successfully colonized and spread over the upper Paraná River. This is case of *F. hahni*, a native species from lower Paraná River basin.[4,55] Thus, the fact of *F. hahni* to colonize the upper

Paraná River recently may have contributed to this chromosomal differentiation between the populations analyzed in this study. Therefore, we can conclude that chromosomal evolution in *F. hahni* is not followed by the *Cyt b* gene variation, making it increasingly important to use integrative tools that include chromosomal markers in phylogenetic studies.

Cytogenetic data, such as karyotype formula and 5S rDNA sites were efficient to separate *F. hahni* from the upper Paraná River of *F. hahni* from the lower Paraná River. Overall, the cytogenetic and molecular data obtained are relevant in a preliminary study and suggested the existence of cryptic diversity and the hypothesis that at least two *Farlowella* lineages may coexist in the Paraná basin.

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Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary File S1
Supplementary File S2

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