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The Complete Mitochondrial Genome of Two Armored Catfish Populations of the Genus *Hypostomus* (Siluriformes, Loricariidae, Hypostominae)

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INTRODUCTION

Hypostomus Lacépède (1803) is the dominant genus of armored catfish (Siluriformes, Loricariidae, Hypostominae) in Brazilian rivers (Britski, 1972). This group presents a wide interspecific color and morphology variation (Oyakawa et al., 2005), which hinders the identification of some species. Likewise, the existence of various cytogenetic phenotypes, including different chromosomal numbers, karyotype formulas, and location of ribosomal genes (Rocha-Reis et al., 2020), reinforces the need for more appropriate methodologies for species identification.

The Alto Paraná river basin concentrates the largest number of studies on freshwater fish in Brazil (Agostinho et al., 2007). This drainage basin is the habitat of 25 species of the genus *Hypostomus* (Weber, 2003), suggesting a complex evolutionary history for the taxon in this region. *Hypostomus ancistroides* Ihering (1911), which has a natural distribution in the basins of the rivers Tietê, Ribeira de Iguape, and Alto Paraná (Fricke et al., 2020), is considered a complex of cryptic species due to the different morphotypes found in its area. In fact, this complex of cryptic species has representatives with small morphological variations among populations, different chromosomal numbers, unique karyotype formulas for each population, polymorphisms related to the number and location of Nucleolus Organizer Regions, ribosomal DNAs, constitutive heterochromatin patterns, and even the (occasional) existence of sex chromosome systems (Rocha-Reis et al., 2020).

A previous study conducted by Rocha-Reis et al. (2018) showed that a population of this complex collected in the Paranapanema river (Alto Paraná basin, Brazil) had highly differentiated traits when compared to other groups. Although morphologically similar to *H. ancistroides*, the specimens presented distinct karyotypic data, characterized by lower chromosome numbers and the presence of a neo-XY sex chromosome system in which X chromosomes constitute a pair of small acrocentric chromosomes in females with only one chromosome of this type in males (Rocha-Reis et al., 2018). The Y chromosome, on the other hand, is the largest metacentric of the karyotype complement, only present in males.

Species delimitation within a complex is often difficult because lineages might not show large morphological differences, despite being reproductively isolated (Lane, 1997). Consequently, Evolutionary Significant Units (ESU) and species can be both identified using alternative approaches, such as genetic techniques (Rocha-Reis et al., 2018).

The mitochondrial genome (mtDNA) of vertebrates is a circular and closed molecule, with conserved gene content (Billington and Hebert, 1991). It is 16–20 kb in length and consists of a light and a heavy chain with a non-coding control region and 37 genes: 13 protein-coding genes (PCGs), 22 RNA transfer genes (tRNAs), and 2 ribosomal RNA genes (rRNAs) (Boore, 1999; Taanman, 1999; Bernt et al., 2013). Features such as high evolutionary rate, low recombination, and maternal inheritance make mtDNA a powerful molecular marker and a useful tool for biological identification studies, phylogenetic analyses, and population genetics (Harrison, 1989; Caccone et al., 2004; Ma et al., 2012; Hirase et al., 2016).

Here, we describe the complete mitochondrial genome of two *Hypostomus* populations of the *H. ancistroides* species complex. In addition, we seek to elucidate the phylogenetic positioning of these populations based on their mitogenomes.

MATERIALS AND METHODS

Specimen Collection and DNA Extraction

We collected one male specimen of *H. ancistroides* from the Tietê river basin, near the municipality of Conchas, São Paulo, Brazil (22°59′57.84″S; 48°0′16.09″O), and two individuals of *Hypostomus* aff. *ancistroides*, one of each sex, from a stream near the municipality of São Miguel Arcanjo, São Paulo, Brazil (23°54′44.58″S; 47°57′40.50″O). The identification of their sex was performed either through morphological observation, since many species of *Hypostomus* have sexual dimorphism, or through the presence of gonads. We extracted heart and liver to obtain DNA, which were stored in a freezer at −20°C. We deposited the tissue and DNA samples from individuals under accession numbers in the Tissue, Cell Suspension, and DNA Bank in the Laboratory of Ecological and Evolutionary Genetics, Federal University of Viçosa—*Campus* Rio Paranaíba. Sampling and euthanasia procedures were conducted in accordance with the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

We extracted the total genomic DNA from liver and heart samples according to the instructions of Invitrogen's DNA extraction and purification kit and checked DNA quality and concentration on a 1% agarose gel, with subsequent measurement using a NanoDrop 2000 spectrophotometer and a Qubit fluorometer.

Library Construction and Sequencing

The Whole Genome Sequencing was performed by Novaseq 6000 (Illumina, San Diego, CA) by the company Novogene, UK. A total amount of 1.0 μg of DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® DNA Library Prep Kit following the manufacturer's recommendations and indices were added to each sample. The genomic DNA was randomly sheared to 350-bp fragments. These DNA fragments were then end-polished, A-tailed, and ligated with the NEBNext adapter for Illumina sequencing, and further PCR enriched by P5 and indexed P7 oligos. The PCR products were purified (AMPure XP system) and the resulting libraries were analyzed for size distribution through Agilent 2100 Bioanalyzer and quantified using real-time PCR. The qualified libraries were input into Illumina sequencers after pooling according to their effective concentration and expected data volume.

Sequence Assembly and Analysis

For the *de novo* mitogenome assembly, we used NovoPlasty 3.7 (Dierckxsens et al., 2017) with 2 × 150 raw reads and three distinct kmers (19, 21, and 23).

We annotated the mitochondrial sequences obtained in MitoAnnotator (Iwasaki et al., 2013) present in MitoFish. We performed the analyses as a base composition with Fasta Statistics 1.0.1 (Seemann and Gladman, 2012), available at The Galaxy Project (https://usegalaxy.org/) (Afgan et al., 2018). The complete mitochondrial genomes of the two populations are available at GenBank under accessions MT066232 for *H. ancistroides*, MT081402 for the male, and MT396945 for the female of *Hypostomus* aff. *ancistroides*.

Phylogenetic Analysis

We estimated the phylogenetic relationships of *H. ancistroides* and *H.* aff. *ancistroides* by comparison with three Hypostominae species: *Hypostomus francisci* (sequence obtained through personal communication with the author, Pereira et al., 2019), *Hypostomus plecostomus* (NC025584, Liu et al., 2016), and *Pterygoplichthys disjunctivus* (NC015747, identical to AP012021, Nakatani et al., 2011). Additionally, we included a species of the family Hypoptopomatinae, *Hypoptopoma incognitum* (NC028072, Moreira et al., 2015), as the outgroup. We used ClustalW (Thompson et al., 1994), implemented by MEGA 7.0.21 software (Kumar et al., 2018), with all the default parameters to align the complete mitogenome sequences.

We performed Bayesian Analysis with the concatenated sequences using the 13 PCGs. Each gene was divided into three partitions, allowing the selection of the best model for each segment individually. The analysis of the model choice was performed in PartitionFinder 2.1.1 (Lanfear et al., 2016) using the Bayesian Information Criterion (BIC), which resulted in five partitions with the following models: SYM + I, HKY + I, GTR + G, HKY + I, and HKY. After 10 million samples of Markov Chain Monte Carlo in MrBayes 3.2.7a (Ronquist et al., 2012), the length of the sampling chain was calculated every 1,000 generations using Tracer 2.6.2 (Rambaut et al., 2018) to estimate the effectiveness of sample size and chain convergence. We visualized the tree in the FigTree 1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/) and edited the images with Inkscape (https://www.inkscape.org) and GNU Image Manipulation Program (GIMP) 2.10.14 (https://www.gimp.org/).

RESULTS AND DISCUSSION

Organization of Mitochondrial Genomes

The organization of the mitogenomes of the two populations was very similar to other ostariophysan fishes (Satoh et al., 2016) and, consequently, to other *Hypostomus* already described: 13 PCGs, 22 tRNA genes, 2 rRNA genes, and a partial control region (D-loop) (**Figure 1**). However, we found different sizes for the

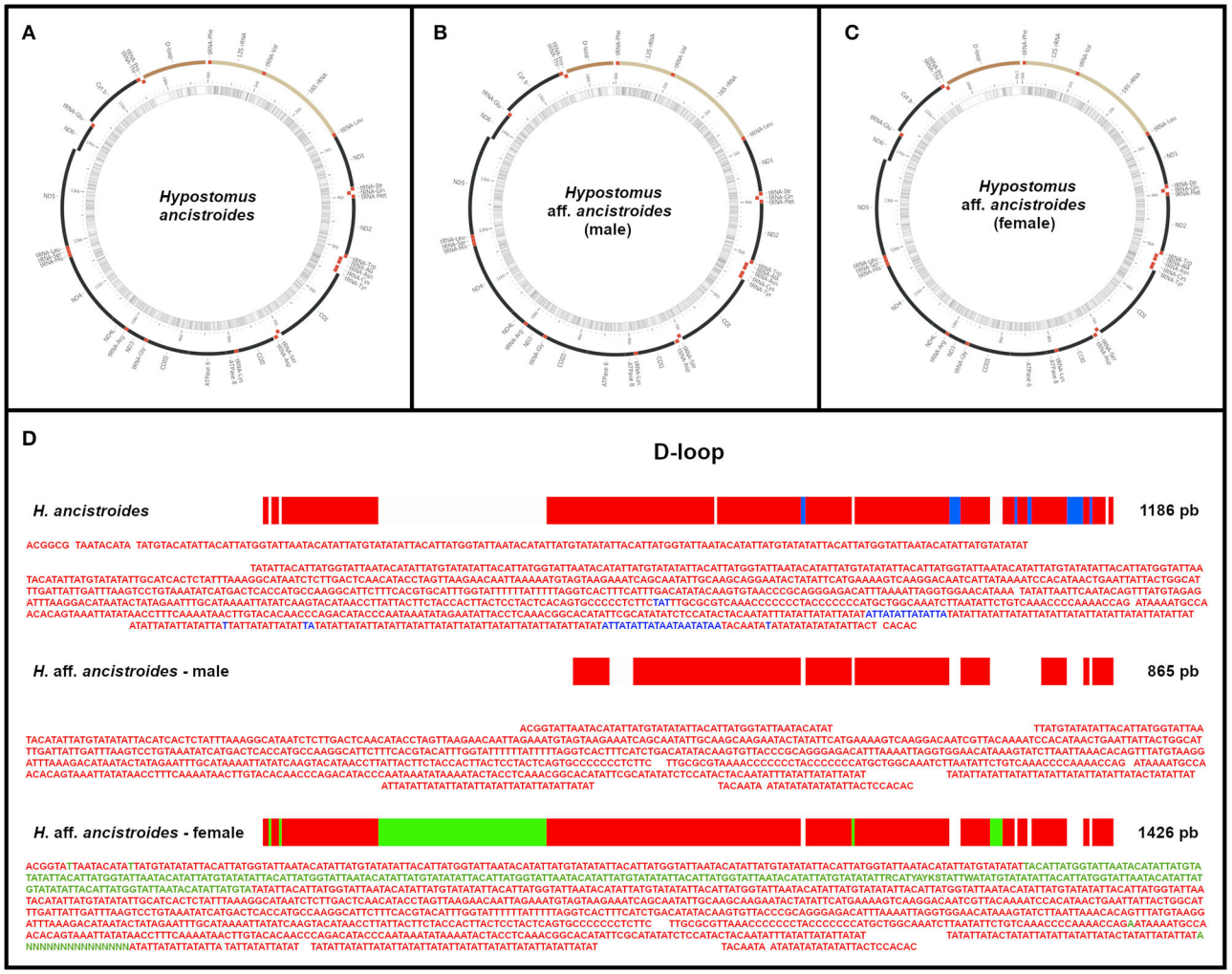

FIGURE 1 | The complete mitochondrial genomes of *Hypostomus ancistroides* **(A)** and male **(B)** and female **(C)** of *Hypostomus* aff. *ancistroides*. The mitogenomes of the two populations have a similar organization, with small variations in the start and stop positions of genes. In **(D)**, a comparison between the control regions (D-loop): homologous bases among individuals are represented in red, bases exclusive to *H. ancistroides* are in blue, and bases exclusive to the female of *H.* aff. *ancistroides* are in green. Blanks represent absent regions.

three mitochondrial genomes: *H. ancistroides* presented 16,826 bp, while the male of *H.* aff. *ancistroides* was 16,505 bp and the female was 17,066 bp (**Figure 1**, **Supplementary Table 1**). The difference in sizes is due to the control region.

The base composition of the mitochondrial genome of *H. ancistroides* was estimated as 31.7% A, 26.6% C, 14.6% G, and 26.9% T. For the *H.* aff. *ancistroides* male, we found 31.5% A, 26.5% C, 14.7% G, and 27.1% T, while for the female, we found 31.6% A, 25,89% C, 14,51% G, and 27,87% T. In all mitogenomes, most genes are in the heavy chain, with only eight tRNAs and a PCG found in the light chain (**Figure 1**, **Supplementary Table 1**).

Protein-Coding Genes

The three mitogenomes analyzed were the same size for PCGs. *Hypostomus* aff. *ancistroides* individuals showed identical sequences, although they may have varied the starting and ending position of PCGs within the genome when compared to *H. ancistroides* (**Supplementary Table 1**). The mitochondrial genomes have thirteen PCGs: *atp6* (683 bp), *atp8* (168 bp), *cox1* (1551 bp), *cox2* (691 bp), *cox3* (784 bp), *cytb* (1138 bp), *nad1* (975 bp), *nad2* (1045 bp), *nad3* (349 bp), *nad4* (1381 bp), *nad4L* (297 bp), *nad5* (1827 bp), and *nad6* (522 bp), representing about 70% of the mitogenomes.

There was an overlapping coding region between *atp8* and *atp6*, *nad4L* and *nad4*, and *nad5* and *nad6*. Except for *cox1*, which starts with GTG, the other PCGs use the ATG codon for initiation. The stop codons identified were TAA (for *atp8*, *nad4L*, *atp6*, *nad1*, *cox1*, and *nad5*), TAG (for *nad6*), and incomplete T-stop (for *nad3*, *cox2*, *cox3*, *nad2*, *cytb*, and *nad4*). Except for *nad6*, all the other PCGs were in the heavy chain (**Figure 1**, **Supplementary Table 1**).

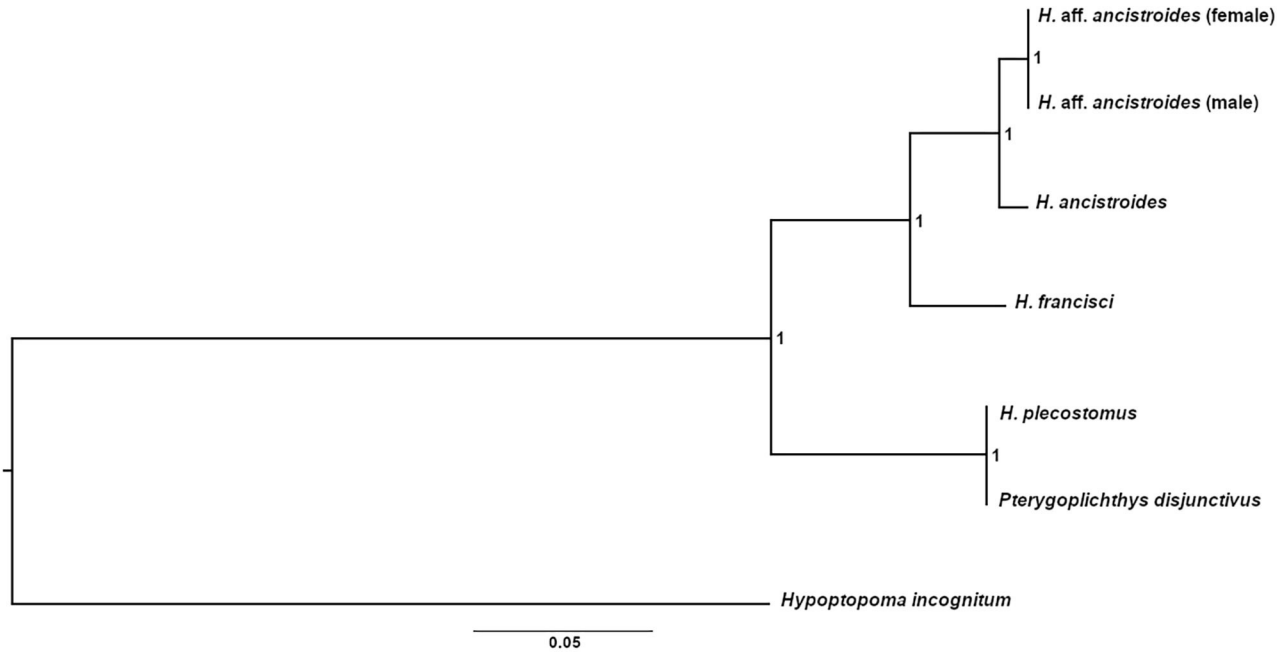

FIGURE 2 | Bayesian phylogenetic tree for mitogenomes of *Hypostomus* and other Loricariidae species using *Hypoptopoma incognitum* as an external group. The analysis was performed with the concatenation of the 13 PCGs after choosing the best nucleotide replacement model for each gene. The analysis was carried out using four independent chains with 10,000,000 generations. The numbers on each node represent the posterior probabilities obtained.

Ribosomal and Transfer RNA Genes

We found two rRNA genes, 12S rRNA (954 bp) and 16S rRNA (1677 bp), located between tRNA-Phe and tRNA-Leu, separated by tRNA-Val (**Figure 1**, **Supplementary Table 1**), as commonly observed in other vertebrates (Inoue et al., 2000).

There were 22 tRNAs in both mitogenomes, with sizes ranging from 67 to 75 bp. There was a single tRNA size difference between the two populations: tRNA-Lys has 74 bp in *H. ancistroides* and 73 bp in *H.* aff. *ancistroides*. There are only eight tRNAs in the light chain: tRNA-Gln, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, tRNA-Ser, tRNA-Glu, and tRNA-Pro (**Supplementary Table 1**). Some tRNAs had atypical codons, such as tRNA-Leu (UAA), tRNA-Ser (UGA), tRNA-Ser (GCU), and tRNA-Leu (UAG).

Control Region

As presented before, we found different sizes for the three mitochondrial genomes: *H. ancistroides* presented 16,826 bp, while the male of *H.* aff. *ancistroides* had 16,505 bp and the female had 17,066 bp (**Figure 1**, **Supplementary Table 1**). This difference was observed in the control region (D-loop): *H. ancistroides* presented 1186 bp, whereas males of *H.* aff. *ancistroides* had 865 bp and females had 1426 bp in this same region (**Figure 1**, **Supplementary Table 1**). We compared the D-loop sequences of the three individuals and found some regions that are exclusive in *H. ancistroides* and others that are exclusive in the female of *H.* aff. *ancistroides* (**Figure 1D**). This exclusivity in each individual, probably due to a 35-bp tandem repeat unit, is responsible for the significant difference in the total size of the control regions and may consequently reflect the difference in the size of their genomes. Repeated regions are a well-known problem for sequence assembly algorithms. Therefore, it was hard to assemble the D-loop region with extensive repeated units. Consequently, repetitive sequences and their repetitions resulted in different sequence length by NGS (Hahn et al., 2013; Tang et al., 2014). D-loop size variations have also been observed in other mitogenomes of different species (Wilkinson and Chapman, 1991; Miracle and Campton, 1995; Shan et al., 2014; Xue et al., 2015; Zhang et al., 2016), and differences in the size of this region between sexes were also observed in *Meretrix lamarckii* (Bettinazzi et al., 2016). Except for the gap in the D-loop region, all mitogenomes were assembled successfully in their entirety. Nevertheless, further studies on this variation in the D-loop are still necessary.

Phylogenetic Analysis

Almost all representatives of *Hypostomus* were grouped in the same clade in the tree topology obtained through Bayesian Analysis (**Figure 2**), except for *H. plecostomus*. This species position as a sister group of *Pterygoplichthys* may suggest an introgression of mitochondrial genes, a misidentification of the individual, or a result from the possible paraphyletic characteristic of *Hypostomus* (Armbruster, 2004).

Furthermore, females and males of *H.* aff. *ancistroides* form a well-supported clade (**Figure 2**). Despite being part of the same population, individuals of both sexes have sufficient differences in the PCGs that allow their separation into two distinct branches. However, they remain in a single clade, which presents itself as a

sister group of *H. ancistroides*. Besides the proximity of these two populations, these data reinforce how different *H.* aff. *ancistroides* is when compared to *H. ancistroides*.

In conclusion, based on the morphological, cytogenetic, genetic, and mitochondrial differences here presented, this scenario supports *H.* aff. *ancistroides* as another species of the complex (Rocha-Reis et al., 2018), which needs to be formally described and named as a taxonomically valid species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, MT066232; https://www.ncbi.nlm.nih.gov/genbank/, MT081402; https://www.ncbi.nlm.nih.gov/genbank/, MT396945.

ETHICS STATEMENT

The animal study was reviewed and approved by Conselho Nacional de Controle de Experimentação Animal (CONCEA).

AUTHOR CONTRIBUTIONS

DR-R, FM, RP, and KK contributed to the conceptualization, investigation and design of the study. DR-R, RP, and FM performed the statistics analysis and were responsible for data validation. JH-H, TS, RP and KK provided resources to carry out the work. RP and KK were responsible for the administration and supervision of the research project. DR-R wrote the first draft of the manuscript. RP, KK, FM, JH-H, and TS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.579965/full#supplementary-material

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