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Mitochondrial multilocus data suggests fine-scale influences of Pleistocene events on the spatial distribution of lineages of a fish species among neighboring sub-basins in the southern upper Paraná River

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Fragmentations and reconnections events in river systems during Pleistocene climatic oscillations have been associated with lineage diversification and subsequent secondary contacts of the Neotropical armored catfish *Hypostomus ancistroides* along the upper Paraná River basin (UPRB). However, this information remains unknown for several drainages in the southern portion of the UPRB, including the Cinzas River sub-basin, where some evidence suggests the predominance of a distinct lineage compared with those predominant in other areas of the UPRB, even among geographically close sub-basins, supporting the hypothesis that fine-scale influences of Pleistocene events may have occurred between these drainages. This hypothesis was tested by analyzing three mitochondrial DNA segments (D-loop region, ATPase and COI genes) of *H. ancistroides* from the Cinzas River sub-basin and other sub-basins of the Paranapanema River and the UPRB. Divergence-time estimates indicated lineage diversification during the Pleistocene, and the main hypothesis was supported by the heterogeneous distribution of distinct lineages among neighboring sub-basins, including a particular lineage predominant in the Cinzas River sub-basin, as well as evidence of variations in historical levels of connectivity, use of distinct colonization routes, and headwater capture areas.

Keywords: ATPase, Cinzas River, Climatic oscillations, D-loop, *Hypostomus ancistroides*.

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Eventos de fragmentação e reconexões em sistemas fluviais durante as oscilações climáticas do Pleistoceno têm sido associados à diversificação de linhagens e aos contatos secundários subsequentes do cascudo Neotropical *Hypostomus ancistroides* ao longo da bacia do alto rio Paraná (BARP). No entanto, essas informações ainda são desconhecidas para diversas drenagens na porção sul da BARP, incluindo a sub-bacia do rio das Cinzas, onde algumas evidências sugerem a predominância de uma linhagem distinta em comparação com aquelas predominantes em outras áreas da BARP, mesmo entre sub-bacias geograficamente próximas, o que sustenta a hipótese de que influências em pequena escala de eventos do Pleistoceno podem ter ocorrido entre essas drenagens. Essa hipótese foi testada por meio da análise de três segmentos de DNA mitocondrial (região D-loop, genes ATPase e COI) de *H. ancistroides* provenientes da sub-bacia do rio Cinzas e de outras sub-bacias dos rios Paranapanema e BARP. As estimativas de tempo de divergência indicaram diversificação de linhagens durante o Pleistoceno, e a hipótese principal foi corroborada pela distribuição heterogênea de linhagens distintas entre sub-bacias vizinhas, incluindo uma linhagem particular predominante na sub-bacia do rio das Cinzas, bem como evidências de variações nos níveis históricos de conectividade, uso de rotas de colonização distintas e áreas de captura de cabeceiras.

Palavras-chave: ATPase, D-loop, *Hypostomus ancistroides*, Oscilações climáticas, Rio das Cinzas.

INTRODUCTION

The South American freshwater ichthyofauna, the most diverse in the world, began its diversification even before the separation of this continent from Africa in the Late Cretaceous, about 110 million years ago (Mya) (Lundberg *et al*., 1998; Albert, Reis, 2011; Reis *et al*., 2016). It has been influenced by the interaction of multiple factors throughout history, including geomorphological and climatic changes, repeated marine incursions and regressions, changes in river courses, and historical connections (Lundberg *et al*., 1998; Hubert, Renno, 2006; Albert, Reis, 2011). In this scenario, while some major endemic clades emerged in the Early Cenozoic or Late Cretaceous, many higher-level taxa (genera and above) appeared during the Paleogene period, and the ichthyofauna was already largely modern by the end of the Miocene (~11.6–5.3 Mya) (Lundberg *et al*., 1998; Albert *et al*., 2020). The upper Paraná River basin (UPRB), in particular, although it appears to have maintained its main physiographic structure over the last nine million years (Albert, Reis, 2011), has exhibited evidence of influence from multiple geological and climatic events that occurred from the Miocene to the Pleistocene. These include marine incursions, climatic oscillations (Hubert, Renno, 2006; Hollanda Carvalho *et al*., 2016; Cardoso *et al*., 2021), as well as inter-basin connections (Lundberg *et al*., 1998; Serra *et al*., 2007; Roxo *et al*., 2014) and intra-basin headwater capture events (Lucena *et al*., 2012; Frota *et al*., 2016; Cavalli *et al*., 2018; Ferreira *et al*., 2023), which have driven diversification among fish populations and species, including members of the armored catfish genus *Hypostomus* Lacepède, 1803 (Hollanda Carvalho *et al*., 2016; Cardoso *et al*., 2021).

Hypostomus is among the most species-rich catfish genera, with 144 currently recognized species within the family Loricariidae (Siluriformes) (Fricke *et al*., 2025). Like other Loricariidae members, this genus includes fish species commonly known as "suckermouth armored catfishes" (Reis *et al*., 2003). They occur across major Neotropical hydrographic systems, such as the Amazon, Paraguay-Paraná-Plata, and São Francisco River basins (Britski *et al*., 1988; Weber, 2003; Cardoso *et al*., 2012), where they inhabit the bottoms of streams, large rivers, and lowland lagoons (Oyakawa *et al*., 2005; Cardoso *et al*., 2012). The genus *Hypostomus* originated in the Amazon/Orinoco ecoregion (~20 Mya, see Cardoso *et al*., 2021) and subsequently dispersed throughout tropical South America and the eastern Andes, undergoing great diversification in body size and shape, color pattern and habitat diversity, mainly due to inter-basin dispersal and intra-basin speciation events (Montoya-Burgos, 2003; Cardoso *et al*., 2012; Hollanda Carvalho *et al*., 2016).

In the Paraguay-Paraná-Plata system (the second largest in South America), *Hypostomus* forms a polyphyletic group, apparently established through multiple independent colonization events (four or more) during the Middle Miocene, possibly involving unrelated ancestral lineages from the Amazon basin (Montoya-Burgos, 2003; Silva *et al*., 2016; Cardoso *et al*., 2012, 2021). Following inter-basin dispersal events (*e.g*., headwater captures), the UPRB may have acted as a "biogeographic corridor", facilitating the dispersal of the genus throughout the Paraguay-Paraná-Plata system, as well as into the São Francisco River and southern Brazilian coastal drainages (Cardoso *et al*., 2021). Within the UPRB, influences of Pleistocene climatic events on *Hypostomus* have been demonstrated through mitochondrial data (ATP synthase gene) obtained from *Hypostomus ancistroides* (Ihering, 1911), which suggest that climatic oscillations and geomorphological processes during this epoch shaped overlapping phylogeographic patterns across the basin. These processes likely involved alternating cycles of prolonged isolation followed by subsequent mergers of different populations (Hollanda Carvalho *et al*., 2016). In this context, while isolation contributed to the evolution of distinct genetic lineages along the UPRB, subsequent dispersal opportunities (*e.g*., flooding of barriers and headwater captures events) would have promoted secondary contact among many of these lineages along sub-basins of the drainage system. In the southern UPRB, for example, recent mitochondrial D-loop data also indicate secondary contact between different lineages of *H. ancistroides* in the lower section of the Laranjinha River, the main tributary of the Cinzas River, Paranapanema River basin (Apolinário-Silva *et al*., 2021). Although not included in previous phylogeographic analyses (*e.g*., Hollanda Carvalho *et al*., 2016), these data suggest that the Cinzas River sub-basin harbors a predominant genetic lineage distinct from those in adjacent basins, raising several questions about the evolutionary history of *H. ancistroides* along the Paranapanema River basin.

In fact, if neighboring tributaries exhibit the predominance of different genetic lineages, it is plausible to hypothesize that the genetic distributions of *H. ancistroides* along the Paranapanema River basin, and within the Cinzas River sub-basin, could represent fine-scale evidence of influences attributed to Pleistocene events, including factors that affected historical connectivity, altered colonization routes, and shaped opportunities for dispersal and secondary contact. In addition, the hypothesis of the predominance of a particular lineage along the Cinzas River sub-basin would also highlight this drainage as "an area harboring a distinct fraction of the genetic diversity"

of *H. ancistroides*, reinforcing arguments that consider this basin a Priority Area for Biodiversity Conservation (MMA, 2016). The present study tested these hypotheses using a mitochondrial multilocus approach, analyzing sequences from the D-loop region, the ATP synthase (ATPase) gene, and the cytochrome oxidase subunit I (COI) gene in samples of *H. ancistroides* obtained from Cinzas River sub-basin, other Paranapanema River tributaries, and additional drainages across the UPRB.

MATERIAL AND METHODS

Sampling, DNA extraction, amplification and sequencing. The upper Paraná River basin (UPRB) drains around 900,000 km^2 of South American territory (Fig. 1), encompassing all drainages connected to the Paraná River upstream of the Itaipu Hydroelectric Power Plant (Castro *et al*., 2003). The Paranapanema River, in turn, is the largest tributary (929 km long) in the southern portion of the UPRB, comprising several important sub-basins, such as the Cinzas River, a watershed that drains 9,658.8 km^2 within Paraná State, Brazil (Britto *et al*., 2003; Vianna, Nogueira, 2008). With regard to dispersal barriers that are still apparent in the study area, three waterfalls (Fig. 1) should be highlighted: i - Salto Grande Falls (about 20 m high) in the Paranapanema River main channel, a waterfall that is currently submerged by a dam (UHE Salto Grande) (Leuzzi *et al*., 2004) and appears to have produced historical genetic differences in ichthyofauna occurring upstream and downstream of the obstacle (Almeida *et al*., 2003; Ferreira *et al*., 2022); ii - Salto Cavalcante Falls (about 15 m high) in the Cinzas River main channel, which separates the upper section from the rest of the sub-basin (Vianna, Nogueira, 2008); iii - Cachoeirinha Falls (over 20 m high) in the middle section of the Cachoeirinha Stream (S1^{CI}), a left bank tributary of the Cinzas River (Fig. 1).

To test the hypothesis that different lineages occur between sub-basins of the UPBR, the mitochondrial DNA data were first analyzed from a populational-level standpoint using the D-loop region. For this purpose, D-loop sequences from 1,405 individuals of *H. ancistroides* (only five obtained from databases) were analyzed, including: data from the Cinzas River sub-basin (CI) obtained by Apolinário-Silva *et al*. (2021) along the Laranjinha River main channel (LA^1-LA^7) and eight tributary streams (S1^{LA}-S8^{LA}); as well as individuals collected in the present study along the Cinzas River main channel (CI^1-CI^2 upstream and CI^3-CI^5 downstream of Salto Cavalcante Falls) and six tributary streams (S1^{CI}-S6^{CI}). Data were also obtained from the Paranapanema River, including the middle section of its main channel (PP^M) and one tributary stream (S1^{PP}), as well as from other sub-basins, including the Tibagi River (TB^1 in the main channel, CO in the Congonhas River, and S1^{TB}-S4^{TB} in tributary streams), the Pirapó (PI), Jaguariaíva (JA), and Itapetininga (IT) rivers. Additional specimens were collected from other UPRB tributaries, including the Ivaí (IV) and Piquiri (PQ^1 - upper and PQ^2 - lower) rivers, and tributary streams of the Itaipu Reservoir (IR^1 and IR^2). Individuals from the Iguaçu River (IG), which is not part of the UPRB, were also included. Although reliable D-loop sequences of *H. ancistroides* in databases are scarce, the dataset also included five sequences available on GenBank, from the Grande (GR = 1), Tietê (TI = 3) and Ribeira de Iguape (RG = 1) rivers (Fig. 1; Tab. **S1**).

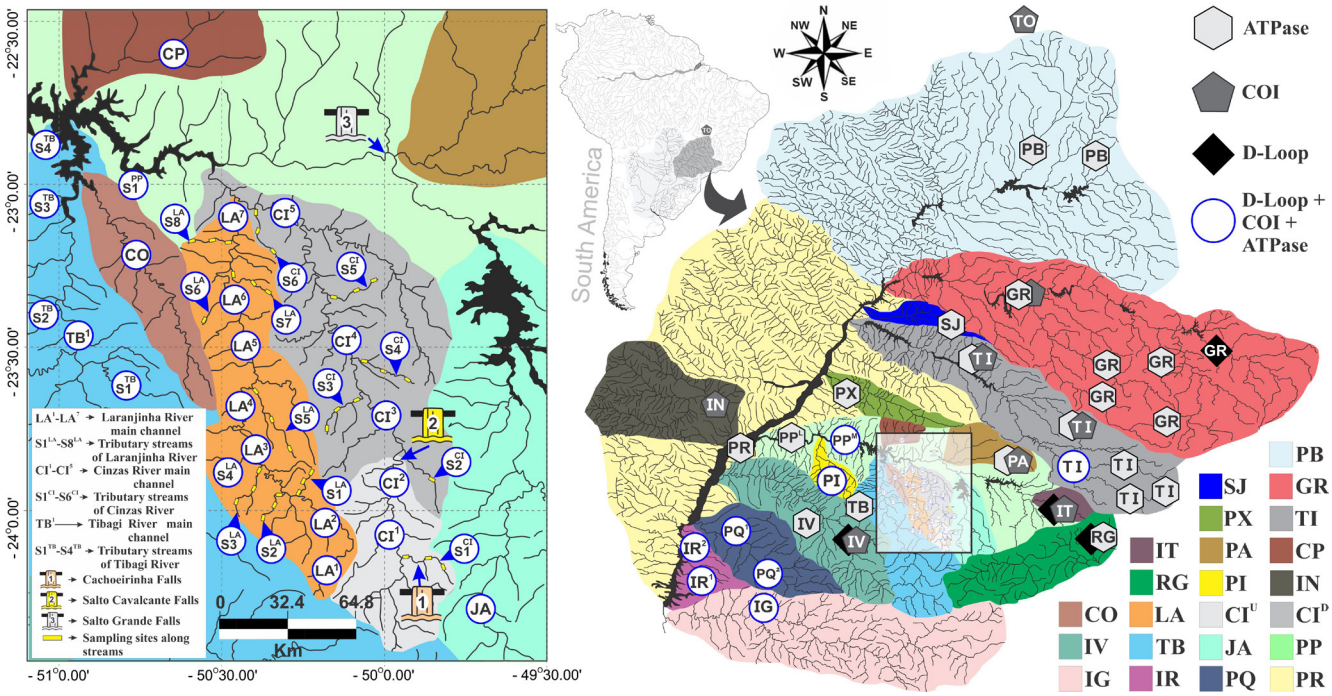

FIGURE 1 | Sampling locations for *Hypostomus ancistroides* along the Paranapanema River sub-basins and in other locations along the upper Paraná River basin, including sites where all three mitochondrial regions were analyzed (circle) and sites where only one or two of them were analyzed (D-loop - diamond; ATPase - hexagon; COI - pentagon). Enlarged on the left, sampling sites along the Cinzas River sub-basin, including the Laranjinha River main channel (LA^1-LA^7) and its tributary streams ($S1^{LA}$-$S8^{LA}$) and Cinzas River main channel (CI^U: CI^1 and CI^2 - upstream and CI^D: CI^3 to CI^5 - downstream of the Salto Cavalcante waterfall) and its tributary streams ($S1^{CI}$-$S6^{CI}$), as well as the Tibagi River main channel (TB^1) and its tributaries (including streams, $S1^{TB}$-$S4^{TB}$, and the Congonhas River - CO), the Jaguariaíva River (JA), a tributary of the Paranapanema River ($S1^{PP}$) and the Capivara River (CP). On the right, other locations investigated in the Paranaíba (PB), Grande (GR), São José dos Dourados (SJ), Tietê (TI), Peixe (PX), Paranapanema (PP^M - middle; PP^L - lower), Itapetininga (IT), Pardo (PA), Ivinhema (IN), Pirapó (PI), Ivaí (IV), Piquiri (PQ^1 - upper; PQ^2 - lower) rivers and in the tributary streams of the Itaipu Reservoir (IR^1 and IR^2), as well as in the Iguaçu (IG), Ribeira de Iguape (RG) and Tocantins (TO) rivers, which are not part of the UPRB. PR indicates a location in the Paraná River main channel, as well as the coverage of areas not investigated in the present study (light yellow) (Source: modified from Google Earth, 2024 https://www.google.com.br/maps).

Most tributary streams of the Cinzas and Laranjinha rivers were sampled in three longitudinal stretches (upper, middle and lower), generally with more than 20 individuals per site; however, sample sizes varied across the studied drainages (Tab. **S1**). The fish sampling was performed using fishing sieves, cast nets, and trawl nets along a 300 m stretch at each site (delimited with 2 mm mesh nets), with a minimum effort of 1 h per site. Voucher specimens were euthanized using eugenol and subsequently subjected to taxonomic confirmation (based on morphology) by specialists (supported by the COI gene analyses in the present study), following identification keys for the UPRB (Dias, Zawadzki, 2018; Ota *et al.*, 2018). Some voucher specimens were deposited at the Museu de Zoologia, Universidade Estadual de Londrina (catalog numbers MZUEL 19310–19339).

In a second step, one individual representing each distinct D-loop haplotype was used to analyze the mitochondrial DNA segment that encodes subunits 6 and 8 of the ATP synthase enzyme (ATPase gene), allowing estimates of divergence time between lineages and comparisons with ATPase data previously obtained by Hollanda Carvalho *et al*. (2016) in several drainages across the UPRB (NCBI accession numbers in Tab. **S1**), including the Paranaíba (PB), Grande (GR), São José dos Dourados (SJ), Tietê (TI), and Ivaí (IV) rivers; the Paraná River main channel (PR); the lower section of the Paranapanema River (PP^L) and some of its tributaries (Capivara – CP, Pardo – PA, Tibagi – TB and Pirapó – PI rivers); as well as the Ribeira de Iguape River (RG), which is not part of the UPRB. Finally, a representative of each D-loop haplotype was also used to analyze part of the cytochrome c oxidase subunit I (COI) gene, enabling divergence-time estimates using concatenated sequences (COI + D-loop + ATPase), phylogenetic analyses, and comparisons with BOLD (The Barcode of Life Data Systems) and GenBank sequences (NCBI and BOLD accession numbers in Tab. **S1**) obtained from the Grande (GR), Tietê (TI), Pardo (PA), Itapetininga (IT), Ivinhema (IN) and Tocantins (TO) river basins (Fig. 1).

The mitochondrial DNA segments analyzed in the present study were amplified via polymerase chain reaction (PCR) using the primers DLA-III 5'-TATTTAAAGRCATAATCTCTTGAC-3' and HygDL-R 5'-WTGCKARTATGTGCCGYYTG-3' (Cardoso *et al*., 2012) for the D-loop region; 8.2_L8331 5'-AAAGCRTYRGCCTTTTAAGC-3' and CO3.2 H9236 5'-GTTAGTGGTCAKGGGCTTGGRTC3' (Hollanda Carvalho *et al*., 2016) for the ATPase gene; and Fish F1 5′TCAACCAACCACAAAGACATTGGCAC-3′ and Fish R1 5′-TAGACTTCTGGGTGGCCAAAGAATCA -3′ (Ward *et al*., 2005) for the COI gene. PCR reactions were performed in a final volume of 15 µL, containing 1X PCR Master Mix (Promega), 0.65 µM of each primer (forward and reverse), and 15 ng of DNA. Amplifications were carried out in a thermal cycler with an initial denaturation of 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s (standardized for D-loop, ATPase and COI) and 72 °C for 1 min, with a final extension of 10 min at 72°C. PCR products were purified using ExoSAP IT® (Prodimol) and prepared for bidirectional Sanger sequencing using BigDye Terminator v. 3.1 kit (Applied Biosystems), according to the manufacturer's instructions. The readings were carried out on an ABI-PRISM 3500 XL automated sequencer (Applied Biosystems), followed by editing and alignment in MEGA 11 (Tamura *et al*., 2021). Sequences of all identified haplotypes were deposited in the GenBank (NCBI accession numbers in Tabs. **S2**, **S3**, **S4**).

Haplotype analysis, phylogenetic relationships, and diversification through time. DnaSP v. 5 (Librado, Rozas, 2009) was used to identify the different haplotypes in the D-loop, ATPase and COI data, as well as in the concatenated data. For D-loop data, Arlequin v. 3.5.1.3 (Excoffier, Lischer, 2010) was employed just on samples with more than 15 individuals (N > 15, aiming to minimize sampling bias) to estimate the haplotype diversity (*h*), nucleotide diversity (π), Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) statistics, pairwise mismatch distributions, and pairwise Φ_{ST} values - using significant estimates based on 10,000 permutations.

Haplotype networks based on the median-joining algorithm (Bandelt *et al*., 1999) were constructed using Network v. 4.6.1.1 (Fluxus Technology Ltd, http://www.fluxus-engineering.com), including data from the three mitochondrial regions (analyzed separately and concatenated). The D-loop haplotype network was constructed using sampling sites specified individually (in rivers and streams), whereas the ATPase and COI networks were constructed by grouping each river with its respective tributary streams.

Phylogenetic relationships were inferred for each mitochondrial marker (D-loop region, and ATPase and COI genes), as well as for the concatenated dataset, using Bayesian inference (BI). In general, concatenated analyses included only individuals sequenced in the present study, and MEGA 11 (Tamura *et al*., 2021) was used to concatenate the D-loop, ATPase, and COI sequences from a representative of each distinct D-loop haplotype. Sequences were summarized into haplotypes, including *H. ancistroides* samples from UPRB as reported in previous studies and outgroups selected according to Hollanda Carvalho *et al*. (2016) and the species placed outside the *H. ancistroides* clade by Cardoso *et al.* (2021) (see NCBI and BOLD accession numbers in Tab. **S1**; Figs. **S5–S7**). Analyses were performed in BEAST v. 2.7.7 (Bouckaert *et al*., 2019) under a strict molecular clock model and a nucleotide substitution model independently estimated for each gene (TN93+G+I for D-loop, ATPase and COI; TIM+G+I for concatenated dataset) using bModelTest (Bouckaert, Drummond, 2017). Divergence times (from concatenated dataset and ATPase data only) were estimated using two independent strategies: (i) an ATPase-based analysis calibrated with a fixed mutation rate of 0.013 substitutions per site per million years (equivalent to 1.3% per million years; Bermingham *et al*., 1997) and conducted under the standard Yule tree prior; and (ii) a D2a-calibrated analysis based on the divergence interval reported by Cardoso *et al*. (2021). The D2a calibration was implemented as a uniform prior in Ma (mega-annum), with offset = 7.09 Ma and upper bound = 19.71 Ma, and was run under the Calibrated Yule tree prior to avoid distortion of the calibrated-node distribution (Heled, Drummond, 2012). For both strategies, four independent MCMC runs of 10 million generations were performed, sampling every 1,000 generations, and convergence was assessed in Tracer v. 1.7.2 (Rambaut *et al*., 2018), ensuring Effective Sample Size (ESS) > 200 for all parameters. Runs were combined and summarized in a maximum clade credibility tree using TreeAnnotator (BEAST v. 2.7.7), with a 10% burn-in and posterior probability threshold of 0.7, and visualized in FigTree v. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Population genetics and phylogenetic relationships. Analysis of 553 base pairs (bp) of the mitochondrial DNA D-loop region revealed 96 different haplotypes, of which only four were from GenBank data, and 268 variable sites (NCBI accession numbers in Tab. **S2**). Considering the Bayesian phylogenetic inference (Fig. **S6**), our ATPase and COI results and the findings of Hollanda Carvalho *et al.* (2016), a total of 12 haplogroups (HG1–HG12) were assigned to the D-loop haplotypes (H^D), including five detected along the Paranapanema River basin: HG3 predominant in the Cinzas River sub-basin (CI+LA); HG7 with one representative in LA; HG1 predominant in the Tibagi River

sub-basin (TB+CO) and other drainages to the west (PI and PP); HG5 predominant in the Jaguariaíva River (JA); and HG6 detected in the Itapetininga and Laranjinha rivers (Figs. 2, **S6**; Tab. **S1**). The remaining seven haplogroups were distributed throughout the Ivaí (HG9), Piquiri (HG8, HG4, HG10 and HG11), Iguaçu (HG4 and HG8), Tietê (HG2) and Grande (H12) rivers, and tributary streams of the Itaipu Reservoir (HG8, HG10 and HG11).

In the genetic diversity estimates (samples N > 15), the *h* ranged from 0.131 (LA^{2}) to 0.905 (CI^{5}), while the π ranged from 0.01% ($S1^{LA}$) to 10.60% ($S6^{CI}$). Tajima's *D* values were significant (P value < 0.05) in nine samples (LA^{1}, LA^{2}, LA^{3}, LA^{4}, LA^{6}, $S5^{LA}$, $S7^{LA}$, CO and PP^{M}), five samples (LA1, LA^{2}, LA3, $S1^{LA}$, and $S5^{LA}$) showed significant Fu's *Fs* values, and the mismatch distribution was unimodal in nine samples ($S3^{CI}$, $S4^{CI}$, LA^{1}, LA^{2}, LA^{3}, LA^{5}, $S1^{LA}$, $S2^{LA}$, $S5^{LA}$) (Tab. 1; Fig. **S5**). Most of pairwise Φ_{ST} values were significant (P value < 0.05), ranging from 0.010 (LA^{1} x $S1^{LA}$) to 0.967 ($S1^{LA}$ x CO) (Tab. 2).

Analysis of 841 bp of the ATPase gene, as well as 617 bp of the COI gene, revealed 27 different ATPase haplotypes (H^{A}) and 13 different COI haplotypes (H^{C}) (NCBI accession numbers in Tabs. **S3**, **S4**) among representatives of the 92 D-loop haplotypes generated

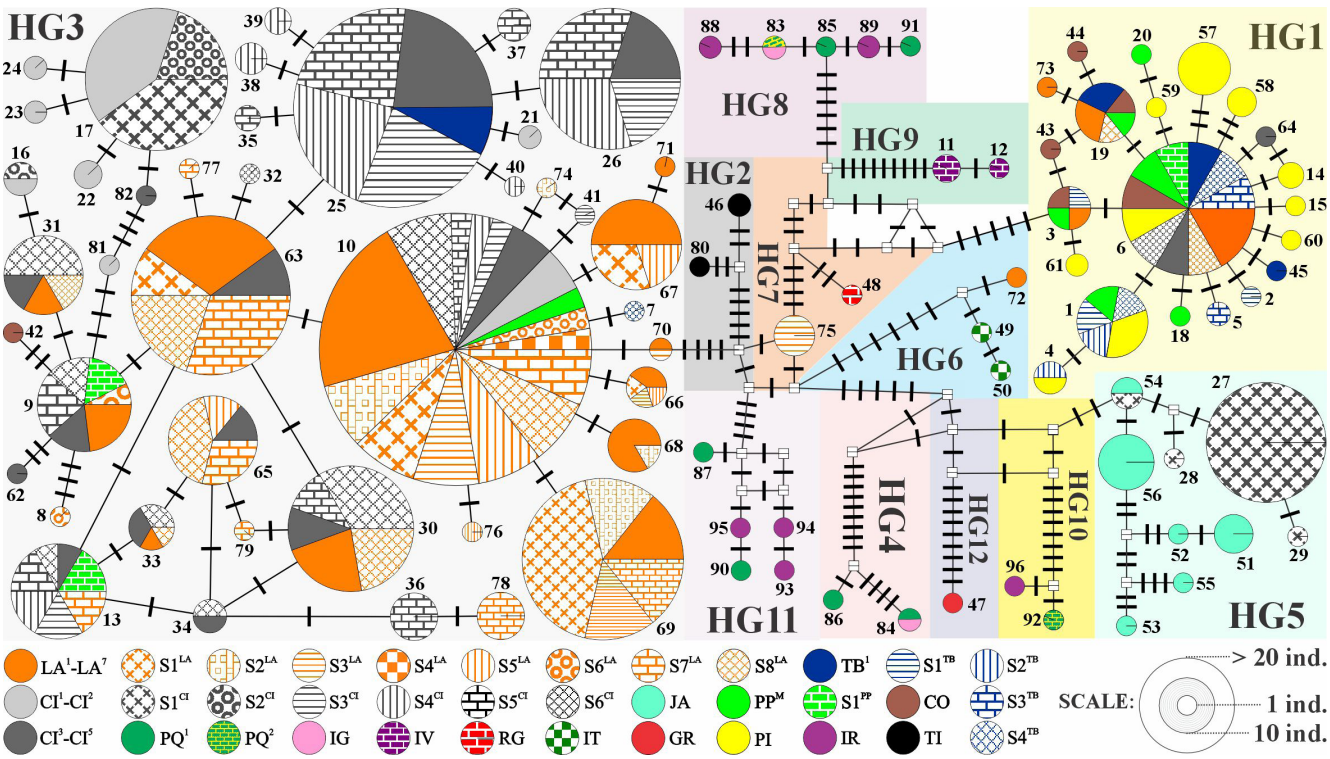

FIGURE 2 | Haplotype network comprising 96 haplotypes (92 haplotypes generated in the present study and four from GenBank) obtained from part of the D-loop region (mtDNA) for *Hypostomus ancistroides* specimens sampled across sub-basins of the Paranapanema River and additional sites within the upper Paraná River basin. Circle sizes are proportional to the haplotype frequency (scale: ind. = individuals) and black traces represent mutation steps. According to previous data and phylogenetic analyses, 11 haplogroups are assigned (HG1-HG11). The dataset is shown for: Laranjinha River main channel (LA^{1}-LA^{7}) and its tributary streams ($S1^{LA}$-$S8^{LA}$); Cinzas River main channel (CI^{1}-C5) and its tributary streams ($S1^{CI}$-$S6^{CI}$), Tibagi River main channel (TB^{1}) and its tributaries ($S1^{TB}$-$S4^{TB}$ and the Congonhas River - CO); Paranapanema River main channel (PP^{M}) and a tributary stream ($S1^{PP}$); tributary streams of the Itaipu Reservoir (IR); as well as the Pirapó (PI), Jaguariaíva (JA), Itapetininga (IT), Piquiri (PQ^{1} - upper; PQ^{2} - lower), Ivaí (IV), Iguaçu (IG), Ribeira de Iguape (RG), Grande (GR) and Tietê (TI) rivers.

TABLE 1 | Genetic diversity (D-loop) estimated for samples (N > 15) of *Hypostomus ancistroides* in the sub-basins of the Paranapanema River, upper Paraná River. N: Number of individuals analyzed, *H*: number of haplotypes found, *h*: haplotype diversity, π: nucleotide diversity, *D*: Tajima's neutrality test (Tajima, 1989), *Fs*: Fu neutrality test (Fu, 1997) and pattern (unimodal or multimodal) in the Mismatch distribution test. Samples N > 15 were obtained in the main channel (CI^{2}, CI^{4} and CI^{5}) and in tributary streams ($S1^{CI}$, $S3^{CI}$-$S6^{CI}$) of the Cinzas River; in the main channel (LA^{1}-LA^{7}) and in tributary streams ($S1^{LA}$-$S3^{LA}$, $S5^{LA}$, $S7^{LA}$, $S8^{LA}$) of the Laranjinha River, in the Congonhas River (CO), in the Paranapanema River main channel (PP^{M}), in the Pirapó River (PI), and in the Jaguariaíva River (JA). *Significant values P ≤ 0.05.

Samples N >15	N	*H*	*h*	ϖ	*D*	*Fs*	Mismatch
CI^{2}	33	6	0.583	0.60%	-0.865	1.528	multimodal
CI^{4}	18	6	0.680	0.30%	-1.593	-0.580	multimodal
CI^{5}	22	12	0.905	0.90%	-1.454	-1.125	multimodal
$S1^{CI}$	63	4	0.513	1.30%	2.780	14.388	multimodal
$S3^{CI}$	76	5	0.286	0.10%	-0.544	-0.882	unimodal
$S4^{CI}$	91	6	0.595	0.10%	-0.301	-0.966	unimodal
$S5^{CI}$	94	9	0.703	0.20%	0.505	-0.779	multimodal
$S6^{CI}$	85	12	0.812	10.60%	0.934	0.011	multimodal
LA^{1}	31	4	0.187	0.04%	-1.731*	-3.436*	unimodal
LA^{2}	30	3	0.131	0.02%	-1.507*	-2.355*	unimodal
LA^{3}	28	4	0.267	0.05%	-1.527*	-2.610*	unimodal
LA^{4}	30	4	0.434	0.36%	-2.439*	2.561	multimodal
LA^{5}	39	5	0.435	0.10%	-1.079	-1.855	unimodal
LA^{6}	24	7	0.736	0.81%	-1.580*	1.994	multimodal
LA^{7}	33	10	0.805	1.08%	-1.199	1.640	multimodal
$S1^{LA}$	85	4	0.092	0.01%	-1.308	-3.049*	unimodal
$S2^{LA}$	92	5	0.293	0.08%	-0.513	-0.840	unimodal
$S3^{LA}$	79	5	0.252	0.28%	-0.654	1.254	multimodal
$S5^{LA}$	89	5	0.280	0.06%	-1.641*	-2.679*	unimodal
$S7^{LA}$	76	8	0.698	0.53%	-1.538*	1.501	multimodal
$S8^{LA}$	87	8	0.507	0.23%	-0.549	-1.108	multimodal
CO	19	5	0.637	0.54%	-2.135*	1.445	multimodal
PP^{M}	16	7	0.833	0.71%	-2.226*	0.563	multimodal
PI	36	9	0.832	0.60%	-0.610	-1.536	multimodal
JA	28	7	0.698	0.33%	-1.371	-0.882	multimodal

in present study (excluding four from the database), totaling 69 ATPase haplotypes (102 variable sites) and 24 COI haplotypes (56 variable sites) in conjunction with GenBank and BOLD data (Fig. 3), including data from Hollanda Carvalho *et al.* (2016). ATPase data showed almost all the same haplogroups assigned to D-loop data, except HG11 and HG12 (D-loop), which clustered in HG2 of ATPase data (Figs. 3, **S7**). In the case of COI data, the main haplogroups obtained from the other loci (HG1, HG3, HG4, HG5,

TABLE 2 | Pairwise genetic differentiation (Φ_{ST} – D-loop) among samples (N > 15) of *Hypostomus ancistroides* obtained along the sub-basins of the Paranapanema River (upper Paraná River basin). Cinzas River main channel (CI^{2}, CI^{4} and CI^{5}) and its tributary streams ($S1^{CI}$, $S3^{CI}$-$S6^{CI}$), Laranjinha River main channel (LA^{1}-LA^{7}) and its tributary streams ($S1^{LA}$-$S3^{LA}$, $S5^{LA}$, $S7^{LA}$, $S8^{LA}$), Congonhas River (CO), Paranapanema River main channel (PP^{M}), Pirapó River (PI), and Jaguariaíva River (JA). *Significant values P ≤ 0.05. Non-significant values are shown in bold.

	CI^{2}	CI^{4}	CI^{5}	$S1^{CI}$	$S3^{CI}$	$S4^{CI}$	$S5^{CI}$	$S6^{CI}$	LA^{1}	LA^{2}	LA^{3}	LA^{4}
CI^{2}												
CI^{4}	0.436*											
CI^{5}	0.354*	0.156*										
$S1^{CI}$	0.496*	0.542*	0.488*									
$S3^{CI}$	0.664*	0.060*	0.390*	0.692*								
$S4^{CI}$	0.613*	**0.009**	0.375*	0.682*	0.097*							
$S5^{CI}$	0.557*	**0.007**	0.256*	0.658*	0.063*	0.057*						
$S6^{CI}$	0.136*	0.081*	0.072*	0.228*	0.157*	0.171*	0.164*					
LA^{1}	0.536*	0.523*	0.104*	0.590*	0.691*	0.577*	0.384*	0.094*				
LA^{2}	0.537*	0.519*	0.101*	0.588*	0.689*	0.575*	0.381*	0.092*	**-0.017**			
LA^{3}	0.453*	0.297*	**0.050**	0.552*	0.521*	0.477*	0.332*	0.091*	**0.023**	**0.024**		
LA^{4}	0.516*	0.476*	0.091*	0.578*	0.665*	0.560*	0.372*	0.089*	**0.014**	**-0.010**	**0.033**	
LA^{5}	0.546*	0.469*	0.119*	0.605*	0.643*	0.560*	0.385*	0.104*	**0.042**	**0.015**	**0.020**	**0.013**
LA^{6}	0.391*	0.213*	**0.001**	0.502*	0.455*	0.426*	0.300*	0.080*	**0.051**	**0.048**	**0.002**	**0.038**
LA^{7}	0.354*	0.169*	**0.016**	0.487*	0.376*	0.370*	0.270*	0.088*	0.066*	0.064*	0.024*	**0.057**
$S1^{LA}$	0.677*	0.693*	0.221*	0.697*	0.763*	0.660*	0.474*	0.147*	0.010*	**0.002**	**0.100**	0.017*
$S2^{LA}$	0.591*	0.702*	0.447*	0.696*	0.776*	0.719*	0.602*	0.171*	0.665*	0.663*	0.507*	0.622*
$S3^{LA}$	0.517*	0.324*	0.095*	0.617*	0.481*	0.459*	0.345*	0.140*	**0.050**	**0.049**	**0.041**	**0.042**
$S5^{LA}$	0.647*	0.567*	0.192*	0.692*	0.675*	0.609*	0.442*	0.150*	**0.003**	**0.007**	**0.021**	**0.041**
$S7^{LA}$	0.477*	0.282*	0.057*	0.610*	0.432*	0.434*	0.319*	0.129*	0.150*	0.149*	0.130*	0.144*
$S8^{LA}$	0.564*	0.376*	0.105*	0.659*	0.516*	0.501*	0.345*	0.142*	0.072*	0.071*	0.087*	0.070*
CO	0.834*	0.884*	0.778*	0.666*	0.949*	0.933*	0.908*	0.272*	0.934*	0.933*	0.879*	0.926*
PP^{M}	0.816*	0.866*	0.750*	0.641*	0.944*	0.927*	0.900*	0.254*	0.925*	0.923*	0.863*	0.914*
PI	0.859*	0.900*	0.823*	0.706*	0.948*	0.935*	0.914*	0.320*	0.934*	0.933*	0.894*	0.927*
JA	0.836*	0.891*	0.803*	0.375*	0.946*	0.928*	0.898*	0.272*	0.935*	0.934*	0.884*	0.927*

TABLE 2 | (Continued)

	LA^{5}	LA^{6}	LA^{7}	$S1^{LA}$	$S2^{LA}$	$S3^{LA}$	$S5^{LA}$	$S7^{LA}$	$S8^{LA}$	CO	PP^{M}	PI
CI^{2}												
CI^{4}												
CI^{5}												
$S1^{CI}$												
$S3^{CI}$												
$S4^{CI}$												
$S5^{CI}$												
$S6^{CI}$												
LA^{1}												
LA^{2}												
LA^{3}												
LA^{4}												
LA^{5}												
LA^{6}	0.061*											
LA^{7}	0.081*	**-0.025**										
$S1^{LA}$	0.100*	0.130*	0.142*									
$S2^{LA}$	0.620*	0.458*	0.386*	0.723*								
$S3^{LA}$	0.071*	**0.027**	**0.048**	0.088*	0.466*							
$S5^{LA}$	0.029*	0.113*	0.130*	0.055*	0.648*	0.087*						
$S7^{LA}$	0.163*	0.104*	0.078*	0.222*	0.444*	0.162*	0.200*					
$S8^{LA}$	0.093*	0.093*	0.094*	0.113*	0.506*	0.104*	0.099*	0.082*				
CO	0.930*	0.819*	0.770*	0.967*	0.954*	0.901*	0.955*	0.864*	0.921*			
PP^{M}	0.920*	0.795*	0.746*	0.963*	0.949*	0.892*	0.951*	0.852*	0.914*	**-0.011**		
PI	0.931*	0.852*	0.812*	0.962*	0.952*	0.908*	0.953*	0.877*	0.925*	0.279*	0.151*	
JA	0.929*	0.837*	0.788*	0.965*	0.950*	0.894*	0.952*	0.859*	0.914*	0.874*	0.858*	0.886*

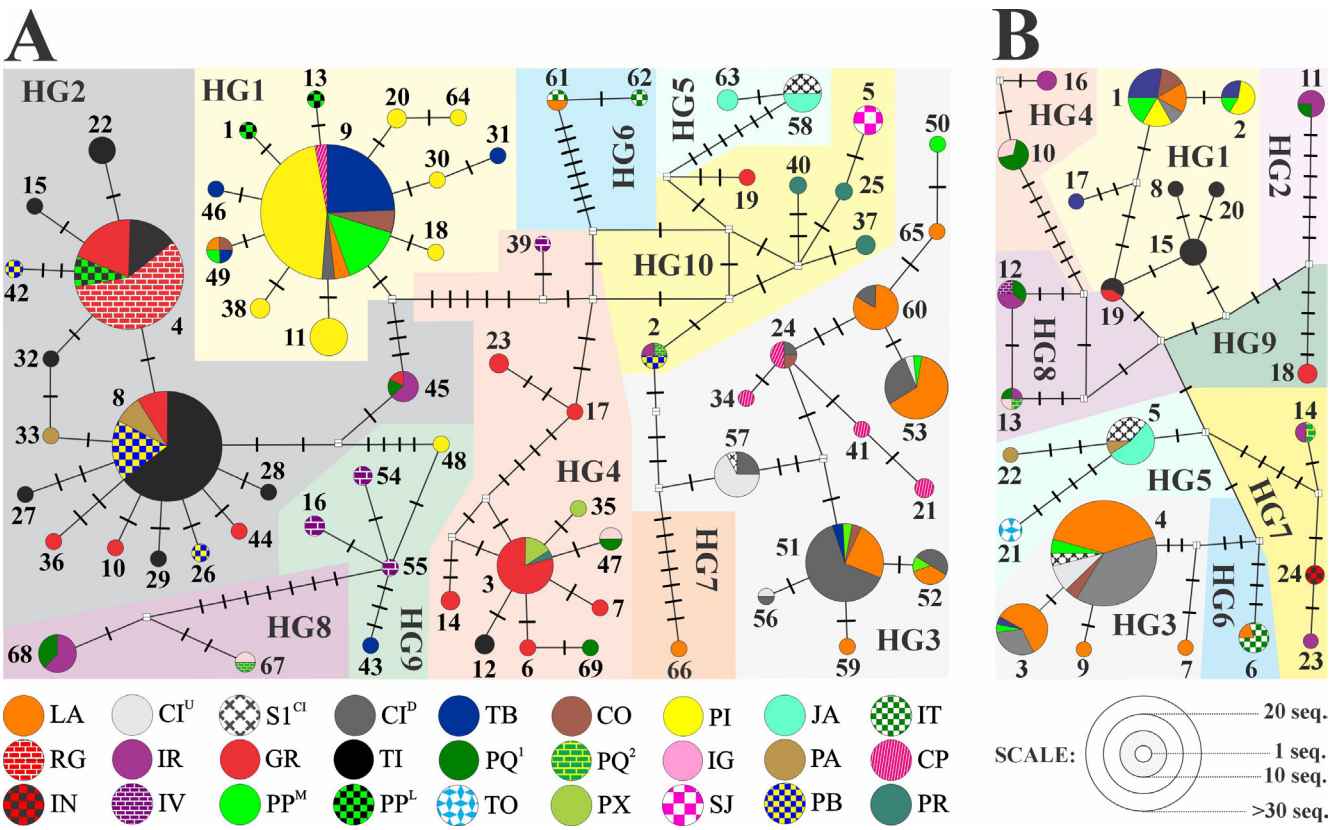

FIGURE 3 | Haplotype network obtained from the sequencing of **A.** ATPase (69 haplotypes) and **B.** COI (24 haplotypes) genes for samples of *Hypostomus ancistroides* investigated along sub-basins of the Paranapanema River and other locations in the upper Paraná River basin. Circle sizes are proportional to the haplotype frequency (scale: seq. = sequences from representatives of each D-loop haplotype and from databases) and black traces represent mutation steps. The main haplogroups (HG1, HG3, HG4, HG5 and HG6) kept the same names in the analysis of the three loci. In this analysis, the samples were organized as follows: TB - Tibagi River and its tributary streams; CO - Congonhas River; LA - Laranjinha River and its tributary streams; CI^U and CI^D - Cinzas River and its tributary streams (upstream and downstream of Salto Cavalcante waterfall, respectively); $S1^{CI}$ - Cachoeirinha Stream (due to headwater captures); tributary streams of the Itaipu Reservoir (IR); Paranapanema Rive main channel (middle - PP^M, lower - PP^L); as well as the Pirapó (PI), Jaguariaíva (JA), Itapetininga (IT), Pardo (PA), Capivara (CP), Piquiri (PQ^1 - upper; PQ^2 - lower), Ivaí (IV), Iguaçu (IG), Ribeira de Iguape (RG), Grande (GR), São José dos Dourados (SJ), Ivinhema (IN), Peixe (PX), Tietê (TI), Paranaíba (PB), Paraná (PR) and Tocantins (TO) rivers.

HG6 and HG8) were also detected and kept the same names (Figs. 3, **S8**). However, due to the lower variation and the smaller amount of GenBank and BOLD sequences, only nine haplogroups were assigned to COI haplotypes (HG1-HG9), so that some haplogroups assigned to D-loop and ATPase data had no representatives or merged, receiving other names in the COI data: $HG2^{ATPase}$ was encompassed by $HG1^{COI}$; $HG7^{D\text{-}loop/ATPase}$ was encompassed by $HG3^{COI}$; $HG10^{D\text{-}loop/ATPase}$ received the name of $HG7^{COI}$; $HG9^{D\text{-}loop/ATPase}$ was encompassed by $HG8^{COI}$; $HG9^{COI}$ was assigned to a haplotype from the Grande River (Tab. **S1**). On the other hand, the concatenated data (D-loop 553 bp, plus ATPase 841 bp, plus COI 617 bp = 2011 bp) also show patterns (Figs. **S9-S10**) very similar to those obtained from the D-loop data.

Phylogenetic relationship and divergence time among lineages. Phylogenetic analysis using Bayesian statistical methods showed congruent topologies from all mitochondrial data (D-loop, ATPase and COI data separately and concatenated data set), indicating a clear separation between the branches that include HG3, which is predominant along the Cinzas River sub-basin, and HG1, which is predominant in the western portion of the Paranapanema River basin, including its main channel and the Pirapó and Tibagi sub-basins (Figs. **S6-S8**, **S10**). Considering the divergence time among lineages, both Bayesian approaches, ATPase-based (Fig. **S11**) and D2a calibration (Cardoso *et al*., 2021), showed highly time divergence estimations, therefore only the D2a calibrated tree is shown (Fig. 4). The lineages of *H. ancistroides* were split in two highly supported groups, the first including (HG1, HG2, HG9, HG11, HG8 and HG4) and a second one including (HG3, HG7, HG5, HG10, and HG6) (also supported in ATPase only tree; Figs. **S12**A, B), with divergence time estimated in 1.52 [1.16–1.96] Mya (middle Pleistocene). In the two lineages, several further splits occurred in the mitochondrial lineages between 1.5 and 0.5 Mya to originate the recovered haplogroups.

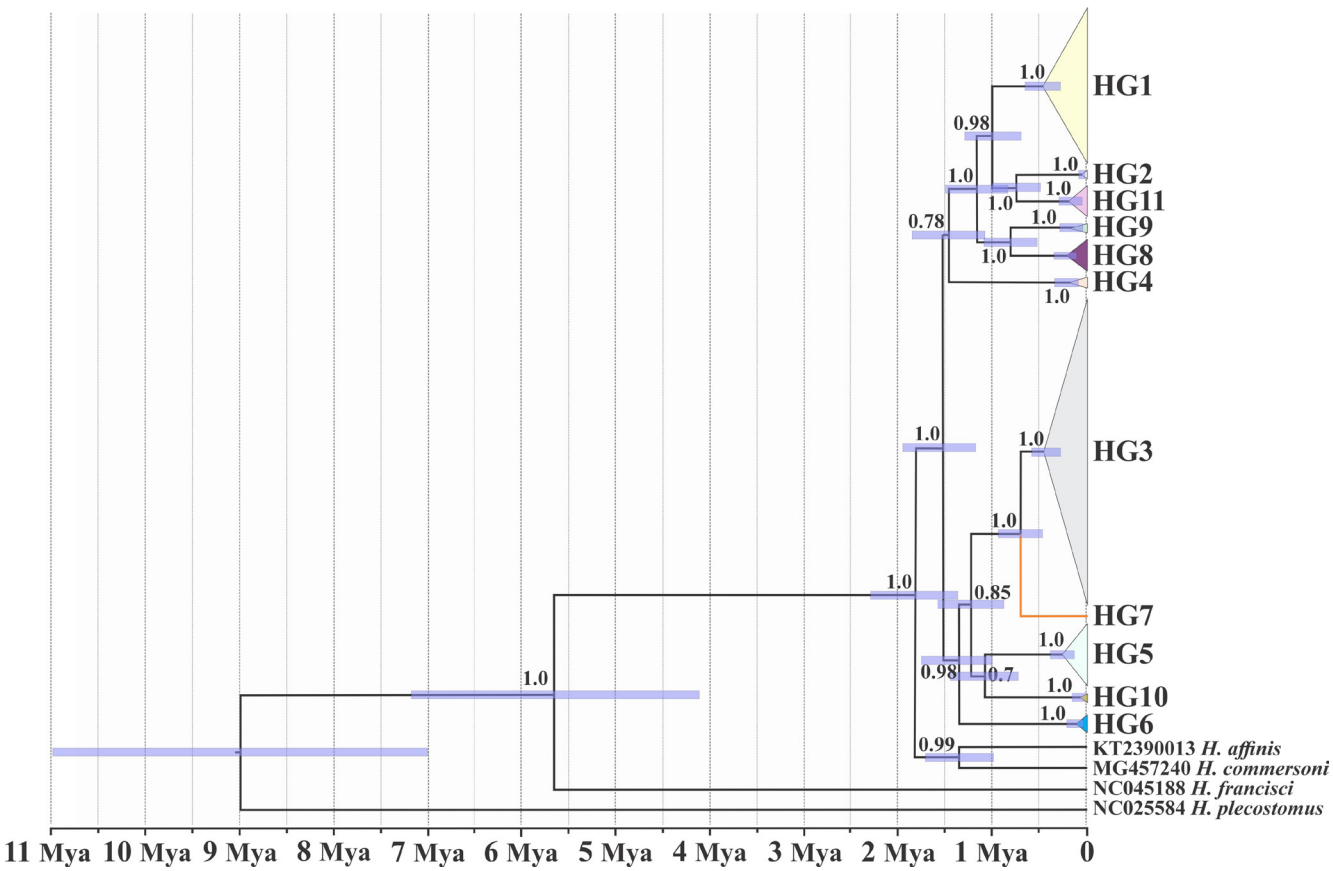

FIGURE 4 | Divergence time among lineages (haplogroups) of *Hypostomus ancistroides* estimated from concatenated mitochondrial data (D-loop+ATPase+COI data) using the Cardoso *et al*. (2021) time estimations for D2a clade [7.86–15.67] (Mya - millions of years ago). Branches within each haplogroup are collapsed. Haplogroups are numbered and colored as in Fig. 2. Posterior probability values are given next to the nodes. Blue bar indicates 95% credibility intervals.

DISCUSSION

Three mitochondrial DNA regions analyzed corroborate the hypothesis that the lineage of *H. ancistroides* predominating in the Cinzas River sub-basin (haplogroup HG3) is not the same lineage that predominates in other large neighboring sub-basins within the Paranapanema River basin, including the Tibagi River sub-basin (predominated by HG1), which confluence is geographically very close (~ 70 km). These differences were also corroborated by population data (from D-loop haplotypes), which indicated high genetic structuring levels among samples from different sub-basins, supporting that the Cinzas River sub-basin may represent "an area harboring a specific fraction of the genetic diversity" of *H. ancistroides* along the UPRB. Additionally, the divergence-time estimates indicate that *H. ancistroides* lineages likely diversified during the Pleistocene, consistent with the findings of Hollanda Carvalho *et al.* (2016), supporting the hypothesis that events during this period greatly influenced the evolutionary history of *H. ancistroides* along the Cinzas River sub-basin (even at a fine-scale), as well as throughout the Paranapanema River basin.

Lineage distribution and divergence time. Geomorphological changes in the upper Paraná River during the Pleistocene have been associated with climatic and tectonic variations that influenced processes of sedimentation, erosion and landscape reorganization (Riccomini, Assumpção, 1999; Stevaux, 2000; Sanches *et al*., 2024). The UPRB tectonism, although more subtle during this period, includes evidence of ancient fault reactivations, determining vertical displacements and relief adjustments that may have contributed, for example, to drainage captures and river course redirections (Riccomini, Assumpção, 1999; Fortes *et al*., 2007; Sowinski *et al*., 2024). In the Cinzas River sub-basin, particularly, the reactivation of these faults has been associated with watershed asymmetry patterns, alterations in the topographic surface, anomalies in the drainage network patterns and disturbances in the longitudinal profiles (Sanches *et al*., 2024). Extreme climatic oscillations of the Pleistocene, in turn, determined profound influences on the relief, river dynamics and sedimentation patterns of the UPRB, reflecting the alternation between long arid and semi-arid periods (glacial, drier) and long periods of increased humidity (interglacial, warmer) (Stevaux, 1994, 2000; Albert, Reis, 2011; Albert *et al*., 2020). Considering their influences on ichthyofauna, extreme reductions in water levels during long drought periods resulted in geographic barriers (*e.g*., drought zones and disconnections between hydrographic systems), promoting isolation of populations in the sub-basins (contraction of the available habitat to fishes), thus contributing to the establishment of lineages or even allopatric speciation (Lundberg *et al*., 1998; Albert, Reis, 2011). On the other hand, large increases in the water level of hydrographic systems during more humid periods would have contributed to watershed expansions and increased connectivity between drainages, allowing dispersal events and genetic exchange between populations (Hubert, Renno, 2006).

Genetic evidence suggesting influences of Pleistocene climate oscillations has already been obtained for different Neotropical fish species (Ochoa *et al*., 2015; Lima *et al*., 2016; Costa *et al*., 2019), including species from the Paraguay-Paraná-Plata system (Artoni *et al*., 2006; Mondin *et al*., 2018; Ferreira *et al*., 2023). In the case of *H. ancistroides*, Hollanda Carvalho *et al.* (2016) argued that habitat fragmentation and reconnection during

this period likely determined population isolation and the establishment of different lineages of *H. ancistroides* throughout the UPRB, as well as the subsequent merging of populations and secondary contact between these lineages. Our data corroborates this interpretation and indicates scenarios of past fine-scale connectivity limitations that may have contributed to distinct colonization patterns between neighboring sub-basins. Based on phylogenetic data, the separation between the branches that gave rise to the haplogroups detected in the Paranapanema River basin (HG1 compared with HG3, HG5 and HG6 - 1.52 [1.16–1.96] Mya) would predate the colonization process of *H. ancistroides* along this hydrographic system, since HG1, HG3, HG5 and HG6 are close to very distinct haplogroups occurring farther north in the UPRB, which is likely the origin center of the species (Cardoso *et al*., 2021). In this scenario, it seems plausible that the different lineages arrived in the Paranapanema River basin at different times and via different colonization routes, one from the west and another from the east.

HG1 occupied the western portion of the Paranapanema River basin, spreading along the main channel and tributaries (*e.g*., Pirapó and Tibagi sub-basins) until reaching some past obstacle in the stretch between the Tibagi and Cinzas confluences, which would have blocked the dispersal of HG1 to upstream areas, including the Cinzas River sub-basin. Consequently, HG1 became predominant in western drainages (PI, TB, CO, PP^{L} and PP^{M}) and rare in the Cinzas River sub-basin. In the case of HG3, ancestors appear to have originated from the upper Paranapanema River (east portion), dispersing downstream through the Salto Grande Falls, as supported by the greater similarity of HG3 to haplogroups (HG5 and HG6) occurring in eastern drainages (IT and JA), as well as by the presence of an HG6 haplotype ($H^{D}72/H^{A}61/H^{C}06$) in the Cinzas River sub-basin. While HG3 colonized and diversified along the Cinzas River sub-basin, its dispersal to western drainages would have been limited by the obstacle between the Tibagi and Cinzas confluences, and its dispersals to eastern drainages limited by the Salto Grande Falls (~ 20 m high) (Leuzzi *et al*., 2004). Since Salto Grande Falls tend to isolate the upper section of the Paranapanema River basin, it seems plausible that colonization of *H. ancistroides* upstream of these waterfalls occurred through past connections (*e.g*., headwater captures) between upper Paranapanema River drainages and headwaters of neighboring watersheds, enabling dispersal of individuals from distinct lineages from those that occupied western drainages. For example, the Pardo River (located upstream of Salto Grande Falls) contains HG5 (COI data) and HG2 (ATPase data), the latter common in the Tietê River basin, suggesting past connectivity. In fact, inter-basin dispersal through headwater captures is an important aspect of the evolutionary history of *Hypostomus* throughout Neotropical systems (Cardoso *et al*., 2021). In *H. ancistroides*, these events may have facilitated the dispersal from the UPRB to the Ribeira de Iguape coastal basin (Hollanda Carvalho *et al*., 2016), possibly involving colonizers from the Tietê River basin.

Currently, the stretch between the Tibagi and Cinzas confluences (~ 70 km) is flooded by the Capivara Reservoir (Leuzzi *et al*., 2004) and no longer represents a barrier. Before flooding, this reach was described as a long segment of large rapids without major waterfalls (Vianna, Nogueira, 2008), suggesting that some gene flow would have been possible. However, during extreme Pleistocene droughts, drastic reductions in water level may have produced extremely unfavorable conditions for species such as *H. ancistroides* in long-rapids stretches, potentially creating disconnections. *H. ancistroides*

is a benthic detritivore fish (bottom scraper) (Suzuki *et al*., 2000; Oliveira, Bennemann, 2005) that, unlike many other species of the genus, prefers lentic, deeper, and turbid waters rich in suspended sediments and nutrients (Uieda *et al*., 1997; Casatti *et al*., 2005; Teresa, Casatti, 2013; Cardoso *et al*., 2021), feeding at night by foraging on branches, leaves and submerged wood (Casatti *et al*., 2005). Thus, long rapids with extremely low water volume (at sub-basins mouths and in the stretchs between them) could have limited both dispersal and gene flow after population establishment. Although challenging to reconstruct, similar scenarios (together with other barriers) may also have occurred elsewhere in the UPRB, preventing certain haplogroups from entering the Paranapanema River through the Paraná River main channel. Some haplogroups rare within Paranapanema drainages occur in sympatry to the south and north of the river mouth (*e.g*., HG4, HG8, and HG10 – ATPase data), both in the Paraná River main channel and in its tributaries (PR, IR, PB, SJ, PQ^{1}, PQ^{2}, and IV).

Secondary contacts - reconnections and headwater captures. Although it is difficult to determine what may have limited the past dispersal of HG3 (westwards) and HG1 (eastwards) in the Paranapanema River, it seems clear that the subsequent elimination of this obstacle likely enabled secondary contacts between these haplogroups, as evidenced by the low frequency of HG1 in the Cinzas River sub-basin (only in the lower section) and the rare occurrence of HG3 in the Tibagi River sub-basin drainages and adjacent areas of the Paranapanema River, *i.e*., the dispersal of these haplogroups into drainages where they are not predominant is apparently more recent. In the case of the "long rapids" hypothesis, after a long period of extremely low water levels due to Pleistocene droughts, the subsequent occurrence of a prolonged humid and flooding period (Albert *et al*., 2020) would have raised water levels and eliminate obstacles, favoring gene flow and the exchange of individuals from different haplogroups. This scenario would have been possible, for example, during the last interglacial period (ending about 0.1 Mya) (Kukla *et al*., 2002), which is considered the most humid and warm interval of the Late Pleistocene (0.126–0.012 Mya) (Albert, Reis, 2011) and includes evidence of population expansions for fish species from the Paraguay-Paraná-Plata system (Mondin *et al*., 2018). At the same time, dispersal opportunities after the Last Glacial Maximum (LGM ~ 0.026–0.020 Mya) (Clark *et al*., 2009) also seem plausible, since drainage systems would have been returning to higher water levels, enabling reconnections. In any case, the more recent dispersal of HG3 and HG1 toward drainages where they are not predominant is corroborated both by the haplotype distributions and by populational genetic data (D-loop), mainly from samples obtained in the lower section of the Cinzas and Tibagi river sub-basins (CI^{5}, $S6^{CI}$, LA^{6}, LA^{7}, $S7^{LA}$, and CO), which showed patterns associated with encounter between distinct lineage, including $h > 0.5$ combined with $\pi > 0.5$ % (Grant, Bowen, 1998) and a multimodal mismatch distribution (Rogers, Harpending, 1992).

Indeed, the extent of this secondary contact between HG1 and HG3 also corroborates the scenario of more recent dispersal after reconnections, since the coexistence of these haplogroups seems limited to the lower sections of the Cinzas and Tibagi River sub-basins and adjacent areas in the Paranapanema River (including the Capivara River – CA), not extending to drainages further west, such as PI, PP^{L} or PR, nor to the middle and upper sections of the Cinzas River sub-basin. Since the last reconnection must have

occurred thousands of years ago (possibly during the last interglacial period or after LGM), the patterns obtained (extension of secondary contact, $h > 0.5$ combined with $\pi > 0.5$ % and multimodal mismatch distribution) also suggest a relationship between the speed of the dispersal process and biological characteristics of the species. Indeed, *H. ancistroides* is a non-migratory fish that shows limited movement range throughout its life (Apolinário-Silva *et al*., 2021), which suggests slower dispersal and gene flows, involving several successive generations.

According to Apolinário-Silva *et al.* (2021), genetically structured populations of this species can occur even over short geographic distances, showing isolation by distance and gene flow consistent with the Stepping-Stone model (Kimura, Weiss, 1964). In this context, the data obtained allow us to infer the occurrence of two dispersal waves in the Cinzas River sub-basin: i - the main colonization by HG3 ancestors, which dispersed in the mouth-to-source direction throughout the watershed, evolving and diversifying along the main channel and tributaries. This process also overcame the obstacle imposed by Salto Cavalcante Falls (about 15 m high), so that HG3 is also predominant in the upper Cinzas River section, showing specific haplotypes that diversified upstream of the obstacle; ii - the more recent dispersal after reconnection, due to the period of humidity and flooding (Albert *et al*., 2020), already discussed for HG1. In this context, similar processes may also have occurred for the Tibagi River sub-basin, with HG1 representing the first dispersal wave and HG3 the second. However, although this second dispersal wave is referred to as "more recent," it is important to note that a very large number of generations would have been required for the establishment of the geographical extent of the secondary contacts reported here (both between HG1 and HG3, and between HG3 and HG5), including the potential formation of new haplotypes after secondary contact (*e.g.*, $H^{D}64$ and $H^{D}73$ in Cinzas River sub-basin), which also requires a long time. In this scenario, influences of contemporary anthropogenic actions (*e.g.*, aquarium trade) do not seem parsimonious when considering the time required by the evolutionary dynamics of mtDNA (haploid uniparental inheritance, absence of genetic recombination, slow mutation rate) (Avise, 2004) and the gene-flow pattern of *H. ancistroides* (Stepping-Stone model).

Secondary contacts from headwater captures were also detected, evidencing another important smaller-scale influence of Pleistocene events. Since almost all drainages analyzed in the upper Cinzas River sub-basin showed the predominance of HG3 haplotypes, except $S^{C}1$ (the Cachoerinha Stream, a right-bank tributary of the upper Cinzas River), in which the HG5 (separated from HG3 about 1.2 Mya) was the most frequent haplogroup, this scenario appears to be clear genetic evidence of headwater captures between the Cinzas and Jaguariaíva river sub-basins, since HG5 was the only haplogroup detected in the Jaguariaíva River. Although Cinzas and Jaguariaíva river sub-basins occur in different sections of the Paranapanema River (upstream and downstream of Salto Grande Falls), their headwaters show marked geographical proximity, particularly between tributaries of the Cachoeirinha Stream and the Jaguariaíva River (Fig. **S13**), which could have facilitated past connections.

Evidence and discussions regarding potential headwater captures between neighboring sub-basins in the upper Paraná River are not unprecedented (Lucena *et al*., 2012; Lippert *et al*., 2014; Frota *et al*., 2016) and constitute parsimonious explanations for faunal exchanges between adjacent drainages, such as those reported among the Iguaçu,

Piquiri, Ivaí, Ribeira de Iguape rivers and tributaries of the Paranapanema river (Ribeiro, 2006; Frota *et al*., 2016; Cavalli *et al*., 2018; Morais-Silva *et al*., 2018). Such events have been commonly associated with intense geological activities and reactivation of ancient faults during the Cenozoic, particularly in areas influenced by the Serra da Esperança and the Ponta Grossa Arch (Ribeiro, 2006; Frota *et al*., 2016). In the case of the Cinzas River, they highlight the tectonic influence of the Ponta Grossa Arch on the Piraí Depression (in the Central Southeastern Brazilian Rift), which includes the headwaters of the Cinzas, Tibagi and Itararé river sub-basins (encompassing the Jaguariaíva River), as well as the Ribeira de Iguape coastal basin (Santos *et al*., 2022). According to morphometric, morphological and sedimentological evidence (Sordi *et al*., 2022), headward erosion has caused drainage rearrangements (including drainage captures) across the Piraí Depression since at least the Pliocene, and includes, in particular, a Cinzas River headwater area (near the Cachoeirinha Stream) that appears to have been captured from the Jaguariaíva River sub-basin, consistent with our genetic results. For ichthyofauna, surveys by Frota *et al*. (2020) and genetic data from Ferreira *et al*. (2023) had already suggested headwater captures as dispersal route and exchange for species between the Cinzas and Itararé river sub-basins during the Pleistocene, considering tectonic events in the Ponta Grossa Arch, such as those occurring around 1.7 Mya (Morais-Silva *et al*., 2018).

The analyses also suggest headwaters captures in other areas of the UPPR, including headwaters of the Ivaí and Tibagi rivers ($H^{A}43$), as well as headwaters of tributaries of the Piquiri (PQ^{2}) and Iguaçu (IG) rivers ($H^{D}83/H^{A}67/H^{C}14$) in the area influenced by Serra da Esperança; however, the dataset is not sufficient for firm conclusions. Finally, the presence of $H^{C}21$ (COI) in headwaters of the Tocantins River basin likely reflects human-mediated introduction (aquariophilia), since *H. ancistroides* is not native to this basin (Reis *et al*., 2003) and $H^{C}21$ belongs to the predominant haplogroup in the eastern Paranapanema River basin (HG5).

Considering the topics discussed above, it seems plausible that Pleistocene events played a major role in shaping the complex evolutionary history of widely distributed non-migratory fish in the UPRB, such as *H. ancistroides*, leaving signatures of fragmentation, reconnections, and headwater captures that can be detected even at small spatial scales from heterogeneous distributions of mitochondrial lineages among very close neighboring sub-basins of the Paranapanema River basin. This scenario appears to reflect the use of different colonization routes by distinct lineages along a hydrographic system, as well as the presence of natural obstacles and discontinuities in the drainage system during Pleistocene droughts, which may have contributed to the predominance of a lineage in the Cinzas River sub-basin that differs from those predominant in other Paranapanema River sub-basins and in the UPRB, raising questions about the occurrence of similar patterns in other species with comparable ecological behavior. Despite evidence of secondary contact, the predominance of a lineage that evolved within the Cinzas River sub-basin highlights this drainage as "an area harboring a specific fraction of the genetic diversity along the UPRB", reinforcing its importance as a sub-basin designated as a Priority Area for Biodiversity Conservation (APCB) by the Brazilian Ministry of the Environment (MMA, 2016).

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All procedures followed environmental authorization SISBIO/MMA nº 23315–1 and were approved by the Ethics Committee of the Universidade Estadual de Londrina (CEUA nº 28522.2013.89).

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and in the supplementary material of this article.

AI STATEMENT

The authors did not use any AI-assisted technologies in the creation of this manuscript or its figures.

COMPETING INTERESTS

The authors declare no competing interests.

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PEER REVIEW

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