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Phylogenetic and Phylogeographic Relationships among Lineages of the Armored Catfish *Ancistrus* Kner, 1854 (Loricariidae: Ancistrini), from the Amazon and Paraguay Basins

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Abstract

Ancistrus is one of the most diverse genera in the Ancistrini tribe, with 64 nominal species. The group is characterized by high cytogenetic variability; the diploid number of chromosomes ranges from 2n = 34 to 2n = 54. *Ancistrus* is widely distributed in the basins of the Uruguay, Paraguay, and Amazonian rivers; the latter two regions show the greatest diversity of *Ancistrus* species and karyotypes. Despite these characteristics, the group includes species for which taxonomic identification is difficult, and phylogenetic relationships and phylogeographic patterns, especially in the Paraguay and Amazon basins, have not yet been revealed. In this study, we determined the phylogenetic and phylogeographic relationships among the *Ancistrus* lineages in these regions. In particular, 93 concatenated sequences of mitochondrial ATPase 6/8 and COI as well as nuclear Rag2 were used for a phylogenetic analysis, and ATPase 6/8 were used for a phylogeographic analysis. The topology generated by the Bayesian method included three distinct clades subdivided into 21 groups. The clades indicated a monophyletic relationship among the lineages from the Amazon and Paraguay basins. The 21 groups had a high average genetic distance (8.4%) and were structured genetically. In the haplotype network, eight large groups were observed, seven belonging to the Paraguay basin and one corresponding to the Amazon basin, and no haplotypes were shared between the two basins. These results indicate that *Ancistrus* lineages form a monophyletic unit in the Paraguay and Amazon basins, and these lineages have a high level of divergence and genetic isolation. These results corroborate the existence of cryptic species in the region and emphasize the need for a taxonomic revision of the genus in these basins.

Keywords: Catfish; Hypostominae; Genetic structure; Distance-based tree; Taxonomy

Introduction

The Amazon and Paraguay basins are the main water tributaries of Brazil and are responsible for a significant portion of the hydrological drainage of South America [1,2]. These flood regions also harbor great diversity and rich ichthyology, sheltering species with different trophic structures, sizes, and patterns of migration and locomotion [3,4].

The *Loricariidae* superfamily represents one of the largest groups among the neotropical species observed in most Brazilian rivers, with 1078 estimated species and 931 nominal species [5-7]. The genus *Ancistrus* includes 69 nominal species; it is the most diverse group within Ancistrini and the second most species-rich genus in *Loricariidae* [5,8,9]. The genus is characterized by the absence of plates and odontodes on the leading edge of the snout and the presence of tentacles and well-developed protractile interopercular odontodes [10].

The Amazon and Paraguay basins in the states of Amazonas and Mato Grosso, harbor the largest number and greatest diversity of *Ancistrus* species [11]. Several species in the genus have already been described in this region, and others are in the process of being identified. Based on cytogenetical analyses, these species show substantial variation in the diploid number (2n = 34 to 2n = 54), unlike the species found in the Paraná river basin, which do not show any variation (2n = 50) [12]. Species from the Amazon and Paraguay basins also have other interesting cytogenetics features, including the presence of sex chromosome systems (ZZ/ZW and XX/XY), polymorphisms in the nucleolar organizing region, and the occurrence of rearrangements involving pericentric and paracentric inversions [12-23].

Molecular tools, including the sequencing of multiple genes, have helped to identify phylogenetic and phylogeographic patterns of fish species [24-28]. Recent molecular and cytogenetic analyses have enabled the discrimination of cryptic species in the genus *Ancistrus* and have revealed the higth diversity of this genus in the Amazon, Paraguay, and Paraná basins [27,29]. Despite an increase in cytogenetic studies of this group, taxonomic assignments, phylogenetic relationships, and species distributions in the genus remain unclear, particularly in the Amazon and Paraguay basins [30,31]. In this study, we analyzed the concatenated sequences of nuclear and mitochondrial markers to identify the phylogenetic relationships and phylogeographic patterns of *Ancistrus* lineages from the Amazon and Paraguay basins.

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Materials and Methods

Sample collection and DNA amplification

Samples were obtained from 146 *Ancistrus* spp. in 23 populations, including eight populations from the Amazon basin and 15 from the Paraguay basin (Figure 1). All individuals had known diploid numbers, and Hypostomus commersoni and Rhamdia quelen were used as outgroups. The specimens were deposited in the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUPELIA), and the Instituto Nacional de Pesquisas da Amazônia (INPA). Specimen collection was authorized by the Brazilian Environment Ministry though its Biodiversity Information and Authorization System (SISBIO), under license number 42144-1, and the protocols used in this study were submitted to the Ethics Committee on the Use of Animals in Research (CEUA) of the Universidade Estadual Paulista (UNESP) and approved under protocol number 7913. A total of 91 sequences from different *Ancistrus* species were analyzed; 34 of these sequences represented the Amazon basin, and 112 represented the Paraguay basin. DNA was extracted from the muscles and flippers using the phenol-chloroform-isoamyl alcohol technique [32] and the NucleoSpin® Tissue Kit (Macherey-Nagel, Duren, Germany).

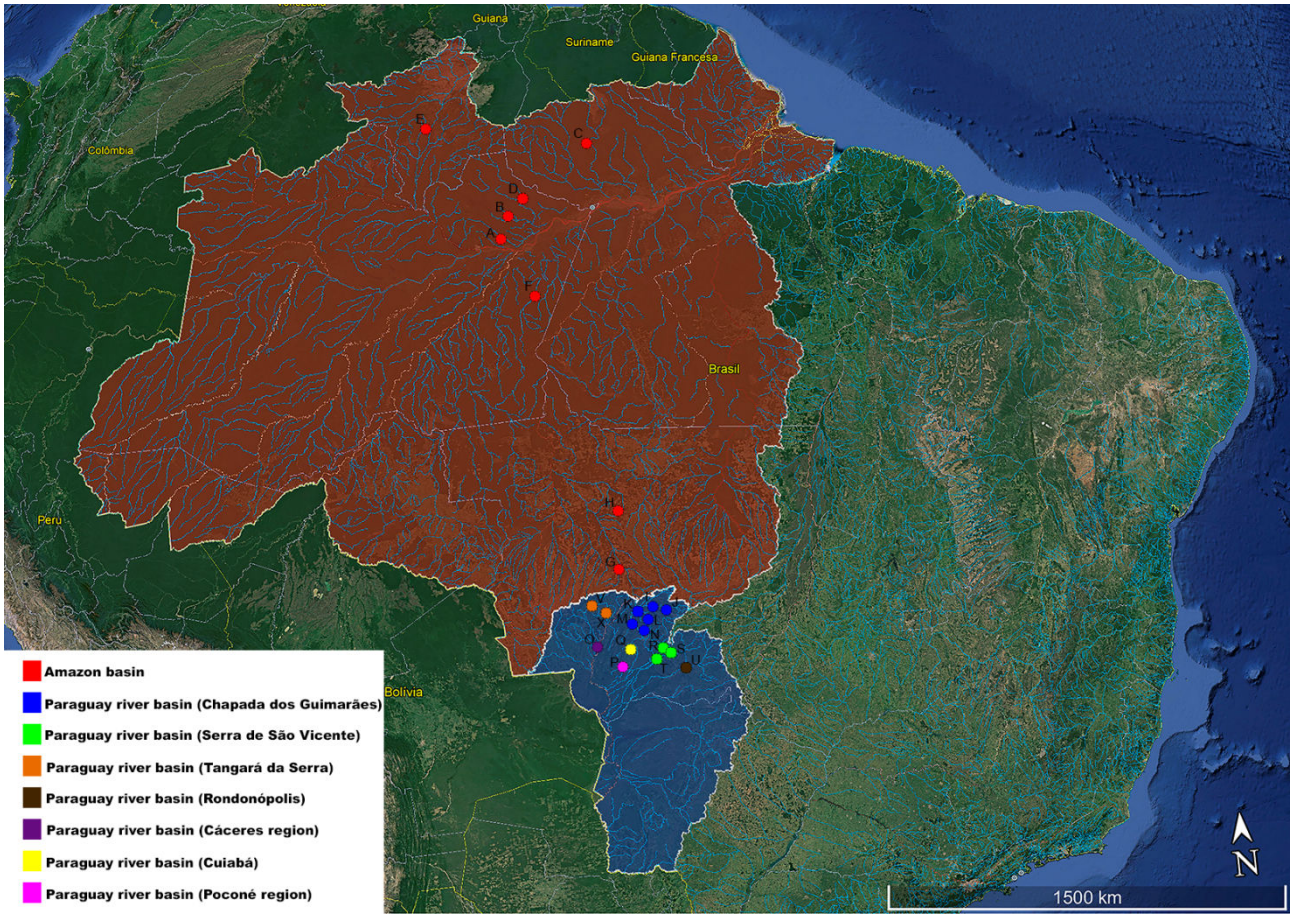

Figure 1: Map of the Amazon and Paraguay hydrographic systems showing the collection points (colorful circles). Amazon basin: A (Catalão), B (Igarapé Dimeni), C (Igarapé Trombetas), D (Igarapé Barretinho), E (Igarapé Mocoari), F (Juma river), G (Matrinxã stream) and H (Preto river). Paraguay basin: I (Coxipó stream), J (Rio do Peixe), K (Soberbo stream), L (Mutuca stream), M (Sagradouro stream), N (Pari river), O (Flecha stream), P (Baia do Arrombado), Q (Santa Cruz stream), R (Cupim stream), S (Macaco stream), T (Tamanduá stream), U (Vermelho stream), V (Tangará stream) and X (Currupira stream). The red shading delimits the Amazonian hydrographic region and the blue shading delimits the Paraguay hydrographic region.

The mitochondrial genes ATP synthase (ATPase) subunits 6 and 8 and cytochrome oxidase subunit I (COI) and the nuclear gene Rag2 were analyzed (Table 1). Polymerase chain reaction (PCR) was conducted using a 13.5 µL solution containing 6.25 µL of PCR Mix (Qiagen, Hilden, Germany), 5.25 µL of Milli Q water (Millipore, Billerica, MA, USA), 0.5 µL of primer F (10 µM), 0.5 µL of primer R (10 µM), and 1.0 µL of template DNA (200 ng). PCR was performed using a thermocycler (Eppendorf Mastercycler) and consisted of an initial cycle of denaturation at 94°C for 40 s, followed by 35 cycles of 94°C for 30 s, annealing temperature (Table 1) for 40 s, and chain extension at 68°C, and a final extension at 72°C for 5 min. For the sequence analysis, the amplified DNA was purified with the EXOSAP enzyme and subsequently sent for sequencing (MacroGen, Seoul, Korea).

Primers	Sequences of primers	Ta (ºC)	References
L8331 – F (ATPase)	AA GCR TYR GCC TTT TAA GC	55	Perdices et al. 2002
H92326 – R (ATPase)	GTT AGT GGT CAK GGG CTT GGR TC		
VF1 (COI)	TTC TCA ACC AAC CAC AAA GAC ATT GG	50	Ivanova et al. 2007
VR1 (COI)	TAG ACT TCT GGG TGG-3		
Rag2 F	TGY TAT CTC CCA CCT CTG CGY T	55	Hardman 2004
Rag2 R	TCA TCC TCC TCA TCK TCC TCW TT		

Table 1: Sequences of primers used in the samples amplification. Ta: Annealing temperature; F: Forward; R: Reverse.

Phylogenetic, phylogeographic, and population analyses

The sequences were aligned using ClustalW in BioEdit Sequence Alignment Editor v7.0.5.3 [33-35] and DAMBE [36]. For phylogenetic analyses, genetic distances were estimated separately for each data set (ATPase, COI, and Rag2) using the neighbor-joining (NJ) method implemented in MEGA v.6 [37]. Concatenated sequences were obtained using Geneious [38], and a Bayesian Markov chain Monte Carlo (MCMC) approach was utilized to estimate tree topologies using MrBayes v. 3.1.2 [39]. Four simultaneous MCMC analyses were run for 2 million generations under the general time-reversible (GTR) model of evolution, with a sampling frequency of 100 generations and a heating temperature of 0.2. Log-likelihood stability was reached after approximately 80,000 generations (excluding the first 80,000 trees). The remaining trees were used to compute a 50% majority-rule consensus tree, and the posterior probability values were calculated to determine the level of support for the Bayesian topology.

Phylogeographic and population analyses were performed using the mitochondrial gene ATPase. Haplotype and nucleotide diversity (π) were calculated using DNASP v.5.10.01 [40]. The median-joining algorithm in Network 4.6.1.1 (fluxus-engineering) was used to examine the relationships among haplotypes. Population structure and genetic variation (FST and AMOVA) were characterized using Arlequin v.3.5 [41].

Results

Molecular characterization

In total, 146 sequences were obtained (ATPase, COI, and Rag2). The ATPase gene was 868 bp, with a nucleotide composition of 28.8% (T), 29.1% (C), 30.1% (A), and 12% (G). The COI gene was 675 bp with a nucleotide composition of 29.4% (T), 24.2% (C), 26.9% (A), and 19.5% (G). The Rag2 nuclear gene was 894 bp with a nucleotide composition of 2.4% (T), 24.1% (C), 26.9% (A), and 24.2% (G).

The concatenated sequence analysis included 91 sequences, of which 64 corresponded to specimens from the Paraguay basin and 27 to specimens from the Amazon basin. The sequences were 3623 bp in total, with a mean nucleotide composition of 26.9% (T), 25.4% (C), 28.5% (A), and 19.2% (G). There were 1825 variable sites and 918 conserved sites.

Phylogenetic analysis

Independent analyses of mitochondrial and nuclear genes generated topologies similar to those observed in the analysis of concatenated sequences.

Using concatenated sequence data, three clades (I, II, and III) were observed, and these were divided into 21 groups (G1–G21) (Figure 2). Clades I and III were formed exclusively by samples from the Paraguay river basin, and clade II was formed mostly by samples from the Amazon basin, except for three groups that belonged to two localities in the Paraguay basin (Chapada dos Guimarães and Poconé) (Figure 2A and Supplementary Figure S1). The three groups were well-supported, with a posterior probability value of 100%, and clades I and II formed a monophyletic unit (Figure 2B). Clade III, formed by samples from Cuiabá, Cáceres, and Chapada dos Guimarães (Paraguay basin), exhibited a basal position in the topology when compared with clades I and II.

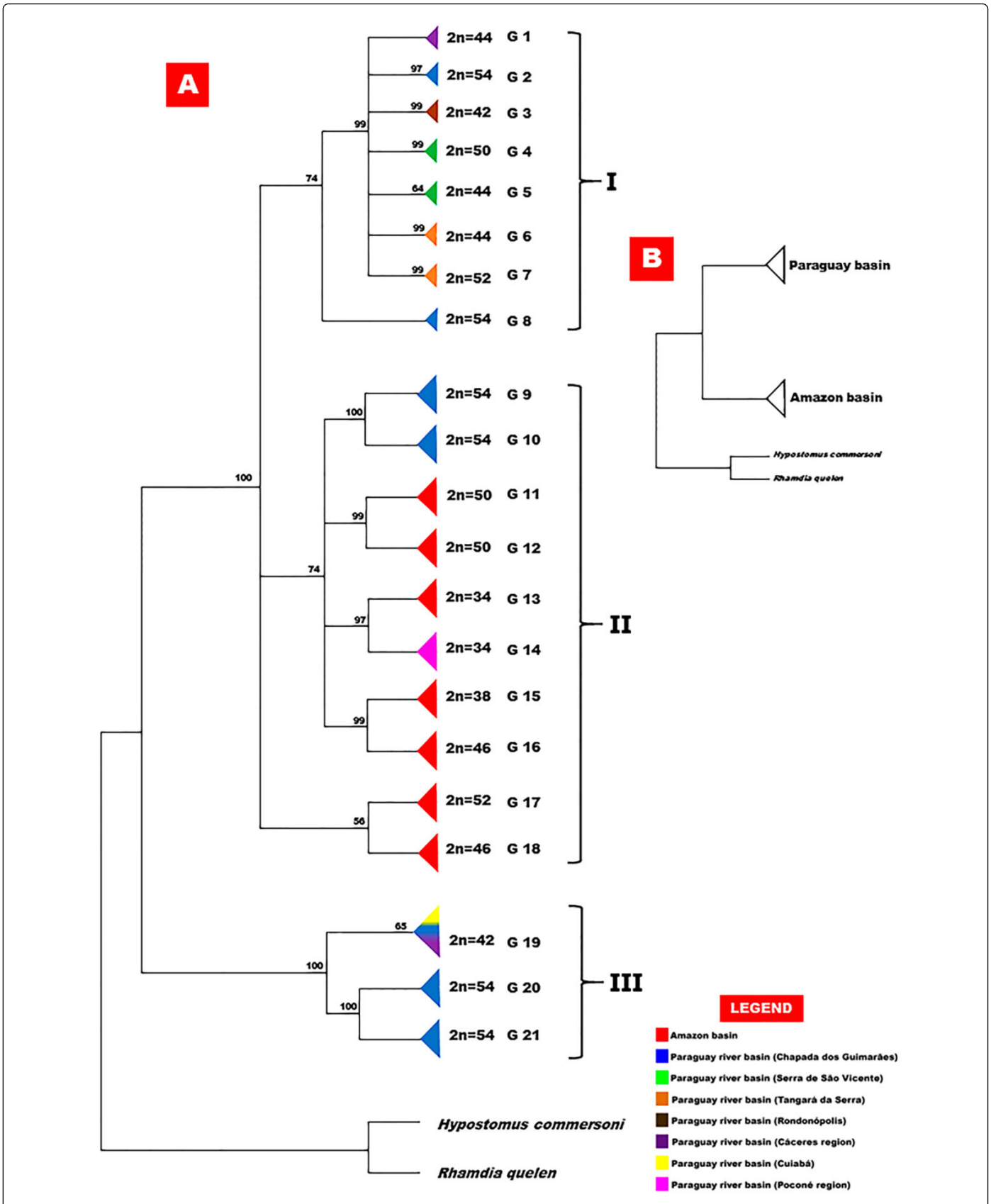

Figure 2: A) Phylogenetic relationship among Ancistrus lineages based on Bayesian analysis of concatenated sequences (ATPase, COI and Rag2). B) Monophyletic relationship among Paraguay and Amazon basins lineages.

Group	Diploid number	Collect points	Basin
G 1	2n=44	Flecha stream	Paraguay
G 2	2n=54	Pari stream	Paraguay
G 3	2n=42	Vermelho river	Paraguay
G 4	2n=50	Cupim/Macaco stream	Paraguay
G 5	2n=44	Tamanduá stream	Paraguay
G 6	2n=44	Currupira stream	Paraguay
G 7	2n=52	Tangará stream	Paraguay
G 8	2n=54	Peixe stream	Paraguay
G 9	2n=54	Pari/Mutuca stream	Paraguay
G 10	2n=54	Mutuca stream	Paraguay
G 11	2n=50	Matrinxã river (*Ancistrus tombador*)	Amazon
G 12	2n=50	Preto river (*Ancistrus tombador*)	Amazon
G 13	2n=34	Catalão	Amazon
G 14	2n=34	Arrombado	Amazon
G 15	2n=38	Trombetas stream	Amazon
G 16	2n=46	Igarapé Macoari (*Ancistrus maximus*)	Amazon
G 17	2n=52	Iguarapé Dimona (*Ancistrus dolichopterus*)	Amazon
G 18	2n=46	Iguarapé Barritinho (*Ancistrus dubius*)	Amazon
G 19	2n=42	Sangradouro/Santa Cruz/Flecha stream	Paraguay
G 20	2n=54	Soberbo/Peixe stream	Paraguay
G 21	2n=54	Soberbo/Coxipo stream	Paraguay

Table 2: Relation of groups (G 1 to G 21) with diploid number and collection points.

The smaller groups (G1-G21) in the topology were consistent with different collection points within each of the studied basins, i.e., G1 to G10 and G19 to G21 corresponded to points in the Paraguay basin and G11 to G18 corresponded to points in the Amazon basin. The diploid number for individuals in each group was determined (Table 2). The mean genetic distance (p distance) was 0.4% within groups and 8.4% between groups.

Phylogeographic and population analyses

Using mitochondrial ATPase gene sequences, a network of 60 haplotypes formed by individuals from the two basins (Amazon and Paraguay) was generated (Figure 3). The most frequent haplotype (H_3) was detected in 14 individuals from the Paraguay basin. This haplotype was shared by samples from three collection points in the region of Chapada dos Guimarães (Coxipó stream, Peixe river, and Mutuca stream) with a diploid number of 2n = 54. The other most frequent haplotypes were H_13 of the Amazon basin, detected in ten individuals (2n = 50), H_4 of the Paraguay basin, detected in eight individuals (Serra de São Vicente region) (2n = 50), and H_5 of the Paraguay basin, detected in eight individuals (Cáceres region) (2n = 44).

Haplotype sharing was not observed among individuals from the Amazon and Paraguay basins; accordingly, both locations exhibited a high level of isolation in the network, with the exception of haplotype H_32, which was allocated to the Amazon basin and included samples from the Poconé region (Paraguay basin), with a diploid number of 2n = 34. In the Paraguay basin network, there was separation among the Chapada dos Guimarães, Serra de São Vicente, and Cáceres regions, with no haplotype sharing among these regions (Figure 3).

FST values were obtained for all 21 groups identified in the phylogenetic tree (G1-G21) to investigate genetic structure. The FST indexes for populations in both the Amazon and Paraguay basins varied widely from 0 to 1 (Supplementary Table 1), and these values were statistically significant ($p < 0.05$) for most of the groups in both basins.

An analysis of molecular variation (AMOVA) indicated that there is differentiation among groups I, II, and III, and these groups correspond to the three clades observed in the phylogenetic tree (I, II, and III). AMOVA indicated a total genetic variance of 0.49, and negative values for the variance between groups, with the greatest proportion of total variance explained by variation within three groups, 0.33% and 67.16%, respectively (Supplementary Table 1). In addition, the observed differentiation among groups was significant ($p < 0.05$).

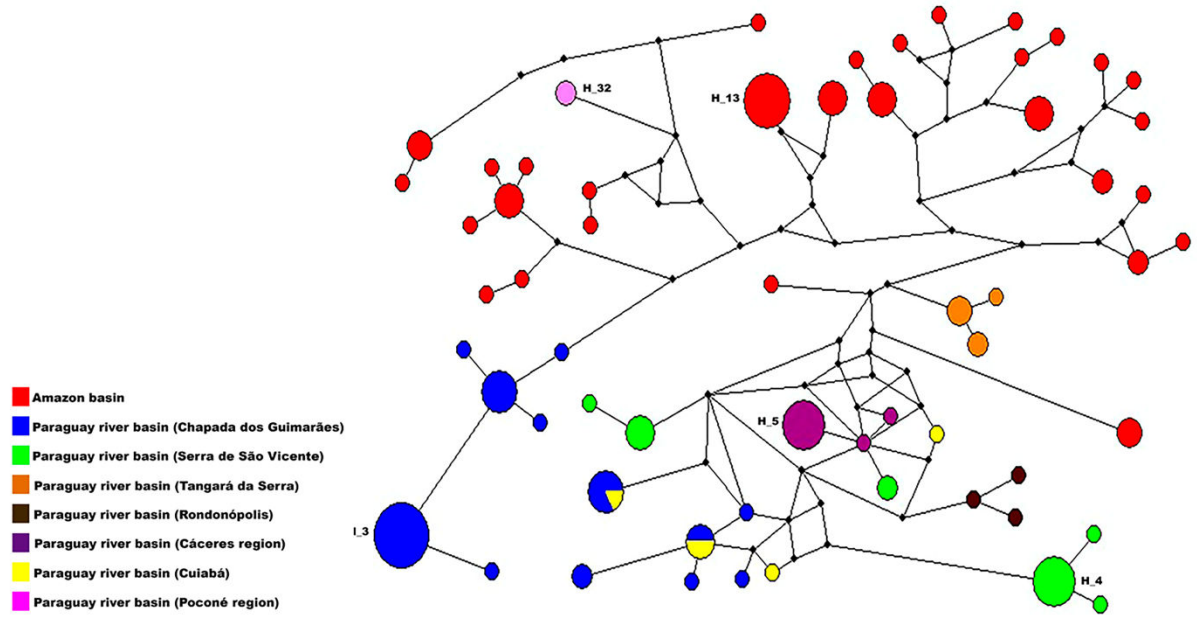

Figure 3: Network haplotypes depicting the relationship of Ancistrus lineages from Paraguay and Amazon basins.

Discussion

The genealogy generated using the concatenated sequences of three genes (ATPase, COI, and Rag2) exhibited a topology similar to that observed using mitochondrial genes (ATPase and COI) independently. These topologies offered a higher resolution than that of topologies generated using the nuclear gene Rag2. This difference can be explained by differences in mutation rates, which are lower for nuclear genes than for mitochondrial genes [42].

In the topology inferred from the concatenated sequences, three clades (I, II, and III) were clearly detected. Clades I and II formed a monophyletic unit. Clade I was formed exclusively by samples from the Paraguayan basin, while clade II was formed mostly by samples from the Amazon basin. It is possible to demonstrate the occurrence of monophyly among the *Ancistrus* strains present in the two basins, a condition already described for other traditional groups of Siluriformes, which also represent monophyletic groups [28,43-45]. Phylogenies inferred from multiple gene sequences also indicate that the Loricariidae family and some subfamilies (Hypostominae) are monophyletic groups [46,47]. Other studies of loricariids based on osteological and molecular characters suggest that *Ancistrus* is a monophyletic group within the Ancistrini tribe [48,49]. Thus, our results obtained using concatenated sequences corroborate the hypothesis of monophyly for the group.

Clade III exhibited a basal position in the phylogeny. This clade consisted exclusively of representatives of the Paraguay basin and was formed by groups G19, G20, and G21; individuals in the last two groups have 2n = 54 chromosomes. In Loricariidae, species with a diploid number 2n = 54 and metacentric and submetacentric chromosomes are considered basal [20,50-53]. The basal position of the clade with 2n = 54 chromosomes reinforces the hypothesis that this diploid number is a plesiomorphic characteristic for *Ancistrus*.

The groups that form clades I and II in the topology were characterized by a wide variety of karyotypes; in clade I, diploid numbers of chromosomes ranged from 2n = 42 to 2n = 54, and in clade II, they ranged from 2n = 34 to 2n = 54. The great variation in diploid numbers is an interesting feature of *Ancistrus* lineages from the Paraguay and Amazon basins [13,16,17,18,20]. This large karyotypic variability may be a reflection of the high genetic distance observed among the lineages and groups in this study.

Recently, Prizon et al. [29] detected five lineages of *Ancistrus* in the Paraná basin using chromosomal and molecular data. These lineages do not constitute monophyletic groups in the topology and have the same diploid number (2n = 50). These results indicate that genetic and chromosomal variation in the group may be related to the geographical distribution of taxa in the studied basins, and the substantial genetic and chromosomal variation observed in the Amazon and Paraguay basins can be attributed to the high isolation among the *Ancistrus* lineages in these regions.

The haplotype network results were consistent with the results of the phylogenetic analysis, indicating clear separation and a high degree of isolation between *Ancistrus* lineages from the Paraguay and Amazon basins. The network showed separation among the three major groups observed in the phylogeny, clades I, II, and III. The haplotype sub-network corresponding to clade III in the phylogeny was composed of the haplotype H_3 (2n = 54), which was the most frequent haplotype in the network and can be considered the ancestral haplotype for the group, reinforcing the hypothesis that the lineages belonging to clade III are the basal lineages for these localities.

The high genetic distances, high degree of isolation according to both the phylogeny and the haplotype network, genetic structure (as determined by FST and AMOVA), and extensive karyotype variation for the *Ancistrus* lineages provide insight into the biological, ethological, and biogeographic aspects of the group. Fish from the *Ancistrus* group use microhabitats in their life cycle. In this way, they establish territories that consequently reduce their vagility [54,55]. These characteristics may favor the emergence of isolation mechanisms or barriers to gene flow, which contribute to the occurrence of micro-allopatric speciation [56]. Such events have already been characterized in other fish species, such as African cichlids, which are endemic to the great lakes of East Africa and possess a high degree of genetic and geographic isolation [57,58].

Geomorphological aspects of the region may have contributed to the observed isolation among lineages. The regions of Serra de São Vicente and Chapada dos Guimarães have rugged relief features, with steep slopes [59,60]. These characteristics are associated with a high level of biological diversity in various ecosystems [61,62] and may explain, in part, the chromosomal and genetic differentiation of *Ancistrus* lineages in this region.

In conclusion, our molecular data suggest that the *Ancistrus* lineages form a monophyletic unit in the Paraguayan and Amazonian basins and the diploid number of 54 is a plesiomorphic character for the group. The high genetic distances and genetic structure may reflect the history of physical and biological isolation of the studied *Ancistrus* lineages.

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