



Cytogenetic and COI-based analyses support a polytypic status of *Astrodoras* (Doradidae: Siluriformes)

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Astrodoras is a monotypic genus of thorny catfishes inhabiting major drainages of the Amazon River system, currently represented by a single recognized species, *A. asterifrons*. However, recent evidence suggests that this genus may not be monotypic and could encompass at least two undescribed species. In this study, we integrated molecular and cytogenetic approaches to investigate the genetic diversity within *A. asterifrons*. Twelve individuals were collected from two locations in the Negro River basin: (a) Negro River, near the Anavilhanas fluvial archipelago, and (b) Negro River, at the Barcelos sandbanks. Our analyses revealed three distinct karyomorphs: karyomorphs A and B (co-occurring near Anavilhanas) and karyomorph C (restricted to Barcelos, ~500 km upstream). Morphological analysis confirmed that karyomorphs A and B correspond to *A. asterifrons sensu stricto*, while karyomorph C fits the diagnosis of the *Astrodoras* sp. “sem fulcro”, an undescribed species identified by previous studies. DNA barcoding and species delimitation algorithms identified these three karyomorphs as independent Molecular Operational Taxonomic Units (MOTUs), along with another undescribed species previously reported as *Astrodoras* sp. Therefore, *Astrodoras* comprises at least four species inhabiting the Amazon basin, three of which remain undescribed, corroborating the previously suggested polytypic status of the genus.

Keywords: Cryptic species, ITS, Karyomorphs, MOTUs, U2 snDNA.

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Astrodoras é um gênero monotípico de bagres que habita as principais drenagens do sistema do rio Amazonas, atualmente representado por uma única espécie reconhecida, *A. asterifrons*. No entanto, evidências recentes sugerem que esse gênero pode não ser monotípico e pode incluir pelo menos duas espécies não descritas. Neste estudo, integramos abordagens moleculares e citogenéticas para investigar a diversidade genética dentro de *A. asterifrons*. Doze indivíduos foram coletados em duas localidades da bacia do rio Negro: (a) rio Negro, próximo ao arquipélago fluvial de Anavilhanas, e (b) rio Negro, nos bancos de areia de Barcelos. Nossas análises revelaram três cariomorfos distintos: cariomorfo A e B (coexistindo próximos a Anavilhanas) e cariomorfo C (restrito a Barcelos, aproximadamente 500 km rio acima). As análises morfológicas confirmaram que os cariomorfos A e B correspondem a *A. asterifrons sensu stricto*, enquanto o cariomorfo C se enquadra no diagnóstico de *Astrodoras* sp. “sem fulcro”, uma espécie não descrita identificada em estudos anteriores. O DNA *barcoding* e os algoritmos de delimitação de espécies reconheceram esses três cariomorfos como Unidades Taxonômicas Operacionais Moleculares (MOTUs) independentes, juntamente com outra espécie não descrita previamente reportada como *Astrodoras* sp. Portanto, *Astrodoras* é composto de pelo menos quatro espécies que habitam a bacia amazônica, corroborando o status politípico previamente sugerido para o gênero.

Palavras-chave: Cariomorfos, Espécies crípticas, ITS, MOTUs, U2 snDNA.

INTRODUCTION

The Doradidae family comprises over 96 valid species of freshwater fish, widely distributed across most hydrographic systems in South America (Sabaj, Arce, 2021; Fricke *et al.*, 2025). Both molecular (Moyer *et al.*, 2004; Arce *et al.*, 2013; Sabaj, Arce, 2021) and morphological evidence (Higuchi, 1992; Birindelli, 2014) support the monophyletic status of Doradidae, which is currently subdivided into six subfamilies: Acanthodoradinae, Agamyxinae, Astrodoradinae, Doradinae, Rhinodoradinae, and Wertheimerinae (Sabaj, Arce, 2021). Astrodoradinae was first recognized by Higuchi *et al.* (2007) and included the genera *Anadoras*, *Astrodoras*, *Scorpiodoras*, *Amblydoras*, *Physopyxis*, *Merodoras*, and *Hypodoras*. A few years later, Sousa (2010) conducted a taxonomic review of Astrodoradinae, which he considered to include two other genera, *Acanthodoras* and *Agamyxis*. Subsequently, based on mitochondrial and nuclear genes, Arce *et al.* (2013) also corroborated the monophyly of Astrodoradinae *sensu* Higuchi *et al.* (2007), thus excluding *Acanthodoras* and *Agamyxis*. In addition, *Merodoras* was considered a junior synonym of *Amblydoras* (Arce *et al.*, 2013; Sabaj, Arce, 2021). More recently, Sabaj, Arce (2021) classified *Acanthodoras* and *Agamyxis* into their own subfamilies, Acanthodoradinae and Agamyxinae, further upholding the monophyly of Astrodoradinae as proposed by Higuchi *et al.* (2007). Currently, Astrodoradinae is composed of six genera: *Anadoras*, *Astrodoras*, *Scorpiodoras*, *Physopyxis*, *Hypodoras*, and *Amblydoras*.

Astrodoras is a monotypic genus represented solely by *A. asterifrons*, originally described by Kner (1853, 1855) from specimens collected in the Negro River near Manaus and the Guaporé River. Based on Kner's (1853) line drawing of the disembodied gas bladder of the type-specimen, *A. asterifrons* is distinguished by having the gas bladder with terminal diverticula completely united via longitudinal septum, effecting attenuate structure with a single distal tip. Eigenmann (1925) was first to document intraspecific variation in *A. asterifrons* by noting that the gas bladder in his specimens from Santarém resembled Kner's *A. asterifrons*, but that a single specimen from Jutáí had a gas bladder with a terminal diverticulum "consisting of two sausage-shaped tubes directed sideways" (Eigenmann, 1925:324). Roa-Fuentes *et al.* (2010) used Eigenmann's (1925) character (*i.e.*, terminal diverticulum with divergent tips) to identify a new and undescribed species of *Astrodoras* in the Colombian Amazon.

Sousa (2010) conducted an extensive taxonomic revision of *Astrodoras* and identified two putative undescribed species: *Astrodoras* sp. "sem fulcro" and *Astrodoras* sp. "fulcro", both remain undescribed. These species occur sympatrically in several locations in the Amazon River basin, including the Catalão Lake and the Negro River at the Anavilhanas fluvial archipelago. According to Sousa (2010), these two undescribed species can be distinguished by the degree of development of the fulcra (*i.e.*, bony plates framing the caudal peduncle, formed by the dorsal and ventral expansions of the procurrent caudal-fin rays), the shape of the upper edge of the orbit, and the shape of the gas-bladder terminal diverticula (*e.g.*, singular in *A. asterifrons* and *A. sp.* "sem-fulcro" *vs.* with divergent tips in *A. sp.* "fulcro"). Sabaj, Arce (2021) similarly described variation in the size, shape, and articulation of the fulcra in *Astrodoras* and demonstrated the enlarged fulcra to be the fusion of at least two procurrent elements. Nevertheless, the diversity within *Astrodoras* remains unresolved, particularly regarding the status of these putative new species, which warrants further investigation using complementary approaches.

Genetic data have proven to be powerful tools in exploring species diversity (Souza-Shibatta *et al.*, 2013; Carvalho *et al.*, 2018; Argolo *et al.*, 2020). Among these, DNA barcoding (*i.e.*, the 600 bp fragment of the COI) has demonstrated high effectiveness for taxonomic identification and resolution of species-level boundaries across a wide array of taxa (Hebert *et al.*, 2003; Ward *et al.*, 2005; Souza-Shibatta *et al.*, 2018). Notably, combining the DNA barcoding with cytogenetics has also provided valuable information for species delimitation in groups with complex taxonomy, cryptic species, or underestimated diversity (Prizon *et al.*, 2017; Carvalho *et al.*, 2018; Anjos *et al.*, 2019). Chromosomal studies have emerged as powerful tools for characterizing species and understanding the genetic mechanisms underlying diversification. Since the late 1970s, they have provided key insights into numerous Neotropical fish groups (*e.g.*, Bertollo *et al.*, 1978; Moreira-Filho, Bertollo, 1991; Tenório *et al.*, 2013; Paiz *et al.*, 2015; Takagui *et al.*, 2017a, 2020; Ferreira *et al.*, 2017; Santos *et al.*, 2021). Within Doradidae, cytogenetic data have contributed to resolving complex taxonomic questions, such as the potential placement of *Franciscodoras marmoratus* within the Wertheimerinae subfamily (Takagui *et al.*, 2019), and support for *Anadoras* sp. "araguaia" (*sensu* Sousa, 2010) as a distinct species (Takagui *et al.*, 2022). However, the only sequences available for *Astrodoras* are restricted to two mitochondrial and one nuclear marker that were applied in phylogenetic reconstructions (Arce *et al.*, 2013; Sabaj, Arce, 2021), and to date no chromosomal data have been published for *Astrodoras* (Tab. S1).

Based on the putative cryptic diversity revealed by Eigenmann (1925) and Sousa (2010), *Astrodoras* may not represent a monotypic genus. Therefore, in this study, we investigate this hypothesis by applying a combination of molecular (DNA barcoding) and cytogenetic analyses (Giemsa staining, C-banding, and physical mapping of 18S rDNA, 5S rDNA, U2 snDNA, and telomeric sequences) to samples of *Astrodoras* collected near the Anavilhanas fluvial archipelago and at the Barcelos sandbanks, both located in the Negro River basin. Through this approach, we aim to enhance our understanding of the taxonomic status of *Astrodoras*, as well as to expand the knowledge of chromosomal diversity within Doradidae.

MATERIAL AND METHODS

Species identification and collection sites. We analyzed 12 individuals collected from two localities along the Negro River. Eight individuals (four males and four females) (Fig. 1A), collected near the fluvial Anavilhanas archipelago, Amazonas State, Brazil ($02^{\circ}39'28.1''\text{S}$ $60^{\circ}39'02.4''\text{W}$), possess the procurrent caudal-fin rays well developed and the lateral border of the cranium above the orbit distinctly elevated, and thus fit the diagnosis of the nominal *Astrodoras asterifrons* (*sensu* Sousa, 2010). The remaining four individuals (three males and one female) (Fig. 1B) from the upper Negro River in the Barcelos municipality, Amazonas State, Brazil ($00^{\circ}57'27.41''\text{S}$ $62^{\circ}55'10.67''\text{W}$) show the procurrent caudal-fin rays not expanded and the lateral border of the cranium above the orbit only slightly elevated, and thus fit the diagnosis of the *A. sp.* “sem fulcro” (*sensu* Sousa, 2010), being herein identified as *Astrodoras sp.* The gas bladders of these specimens were removed during preparation and not preserved for specimen identification. The specimens are deposited in the Museu de Zoologia at the Universidade Estadual de Londrina (MZUEL) under the voucher numbers MZUEL 17800 and 17813 (Tab. S2).

Mitotic chromosome preparations and chromosomal banding. Metaphasic chromosomes were obtained from the kidney cells using the air-drying technique described by Bertollo *et al.* (1978). Briefly, specimens were treated with a 0.02% aqueous colchicine solution (1 ml/100 g) for 30–60 min, followed by euthanasia through clove oil overdose (Griffiths *et al.*, 2000). Subsequently, small pieces of kidney tissue were obtained using surgical scissors and tweezers, homogenized in a KCl solution (0.075 M) with a glass syringe, and incubated for 30 min at 37°C . After hypotonic treatment with KCl, the cells were treated with a fixative solution composed of methanol and acetic acid (3:1) and stored at -20°C . For karyotype analyses, chromosomes were stained with Giemsa 5% diluted in phosphate buffer, pH = 6.8, and classified according to arm ratio as acrocentric, subtelocentric, submetacentric, or metacentric (Levan *et al.*, 1964). The constitutive heterochromatin (CH) pattern was detected using the acid/base/salt method described by Sumner (1972), with modifications in the staining process: the chromosomes were stained with a solution composed of 0.7 μL of propidium iodide (50 $\mu\text{g}/\text{mL}$) and 20 μL of antifading solution (Vector, Burlingame, CA, USA), according to Lui *et al.* (2012).

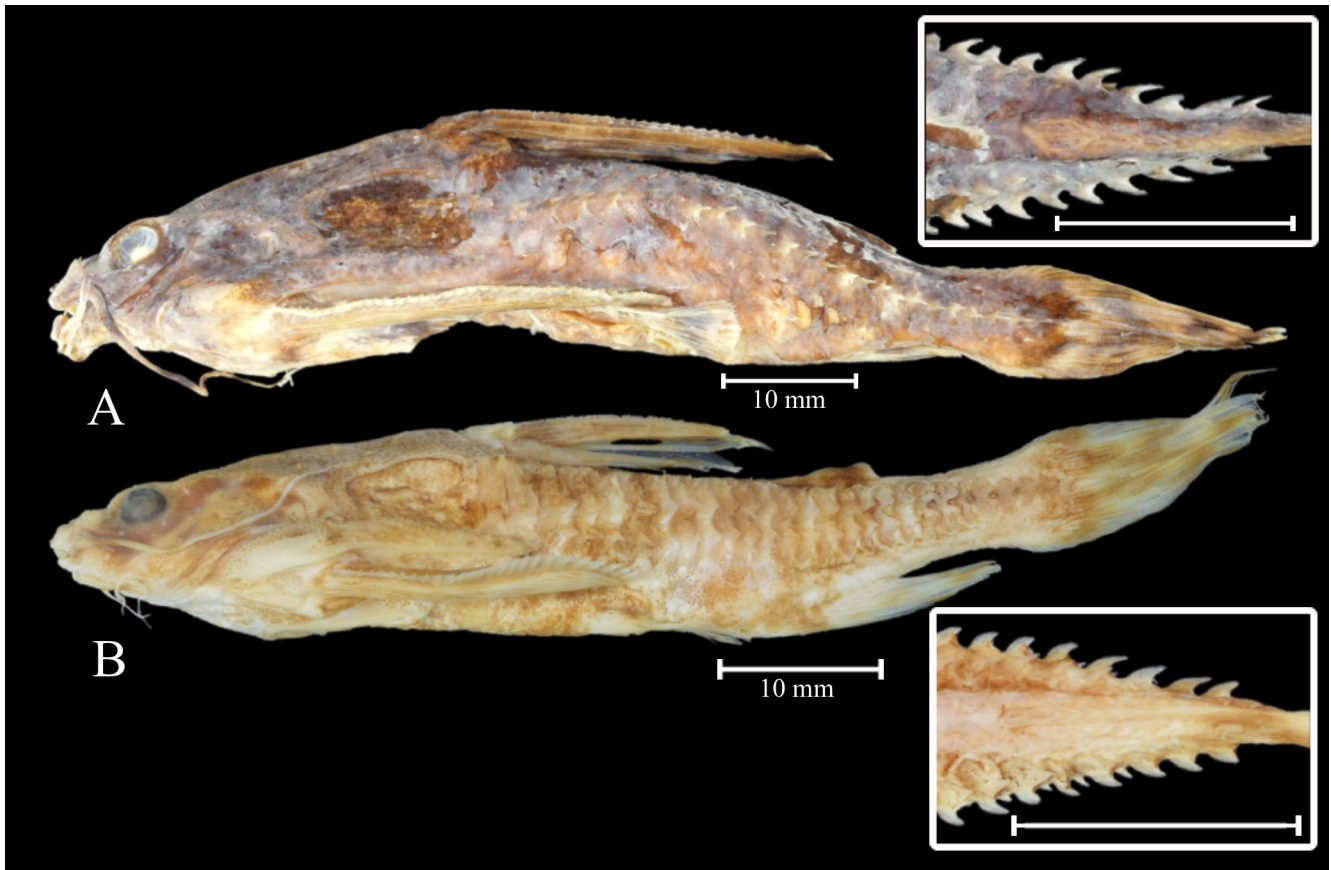


FIGURE 1 | Samples used in this study: **A.** *Astrodoros asterifrons*, MZUEL 17813, 72.7 mm SL, from Negro River, near Anavilhanas; **B.** *Astrodoros* sp., MZUEL 17800 57.9 mm SL, Negro River at Barcelos. The caudal peduncle in dorsal view is presented in the boxes.

Probes for fluorescent *in situ* hybridization. We investigated the distribution of four repetitive DNA sequences: two rDNAs, one snDNA, and telomeric motifs (TTAGGG)_n. The 18S rDNA probes were obtained from a mini-prep of *Prochilodus argenteus* (Hatanaka, Galetti, 2004) and labeled with biotin-16-dUTP (Bio-Nick-Translation mix, Roche Diagnostics, Mannheim, Germany). The 5S rDNA probes were obtained from a mini-prep of *Megaleporinus elongatus* (= *Leporinus elongatus* Valenciennes, 1850) (Martins, Galetti, 1999) and labeled with digoxigenin-11-dUTP (Dig-Nick-Translation mix, Roche Diagnostics). Telomeric probes were obtained through a polymerase chain reaction (PCR) using a pair of self-complementary primers (Ijdo *et al.*, 1991) and labeled with tetramethyl-rhodamine-5-dUTP (red; Roche Diagnostics). The U2 snDNA probes were obtained through PCR using the *Trachelyopterus galeatus* total DNA (extracted from liver tissues) and the primers described by Bueno *et al.* (2013). The PCR products were sequenced and deposited in GenBank under the sequence ID: OK166555.1 (see Haerter *et al.*, 2022). For FISH experiments, U2 snDNA probes were labeled by PCR using fluorescein-12-dUTP (Roche Diagnostics).

Fluorescence *in situ* hybridization (FISH) experiments. FISH experiments were adapted from Yano *et al.* (2017a). Briefly, slides pretreatment included the following steps: (a) two washes in 2x SSC solution for 5 min at room temperature, (b)

treatment with RNase solution (100 µg/mL in 2x SSC) for 1 h at 37°C in a dark, moist chamber, (c) two washes with 2x SSC solution for 5 min at room temperature, and (d) dehydration in an ethanol series (70% and 100%) at -20°C. Chromosome denaturation was performed using 70% deionized formamide in 2x SSC at 70°C, while denaturation of the hybridization mixture (200 ng of each probe, 2x SSC, 50% deionized formamide, 10% dextran sulfate) was conducted at 90°C in a dry block for 10 min. Hybridization was performed for 24 h at 37°C in a dark, moist chamber. Post-hybridization stringent washes included once in 15% deionized formamide/2x SSC at 42°C for 10 min and twice in 0.5% tween/4x SSC (SSCT) at room temperature for 5 min each. The 5S rDNA probe signals were detected using anti-digoxigenin-rhodamine (red; Roche Diagnostics), while the 18S rDNA probe signals were detected using avidin fluorescein isothiocyanate (green; avidin-FITC) (Roche Diagnostics). Chromosome counterstaining was performed with fluorochrome 4',6'-diamidino-2-phenylindole (blue; DAPI) in antifading solution (Vector).

DNA extraction and molecular analyses. Genomic DNA was extracted from the muscle tissue of *Astrodoras* species using the phenol-chloroform method described by Almeida *et al.* (2001). The mitochondrial cytochrome C oxidase subunit I (COI) gene was amplified by PCR using the primers described by Ward *et al.* (2005). Each PCR reaction contained 50 ng of genomic DNA, 0.25 µM of each primer, GoTaq® Master Mix (Promega), and deionized water to fill the final volume. The amplification protocol included: (a) 2 min at 94°C; (b) 35 cycles of 1 min at 94°C, 30 sec at 54°C, and 90 sec at 72°C; and (c) a final extension of 5 min at 72°C. Amplicons were checked in a 1% agarose gel electrophoresis. For the sequencing reaction, we used the Big Dye Sequence Terminator kit (Applied Biosystems), according to the manufacturer's instructions. The amplification conditions consisted of 25 cycles at 96°C for 30 sec, 15 sec at 50°C, and 4 min at 60°C. After the reaction, the amplicons were precipitated. The reading was performed with the sequencer model ABI PRISM 3500 XL Genetic Analyzer from Applied Biosystems made by HITACHI.

Sequence alignment and molecular delimitation analysis. In this study, four sequences were generated and deposited on the Barcode of Life Data Systems (BOLD) platform under the project "Species delimitation of thorny catfishes (Doradidae-Astrodoradinae): an integrated analysis based on Chromosomal and DNA barcoding - DORBC". These sequences were automatically assigned to a barcode index number (BIN) (*i.e.*, a group of sequences corresponding to a single taxon), following the analytical procedures of Ratnasingham, Hebert (2013). Additionally, three sequences of *Astrodoras* and 16 sequences from other Astrodoradinae species previously deposited on BOLD were included in the analysis. Sequences from *Wertheimeria maculata* (Wertheimerinae) and *Platydoras armatulus* (Valenciennes, 1840) (Doradinae) were used as outgroups (Tab. S2).

The dataset was aligned on BioEdit Sequence Alignment Editor v. 7.1.9. (Hall, 1999) with the ClustalW tool (Thompson *et al.*, 1994). Intra- and interspecific genetic distance matrices were generated in MEGA v. 6 (Tamura *et al.*, 2011). The Neighbor-Joining (NJ) tree was generated based on the Kimura-2-Parameter (K2P) nucleotide substitution model (Kimura, 1980) with 1,000 bootstrap replicates. Phylogenetic reconstructions

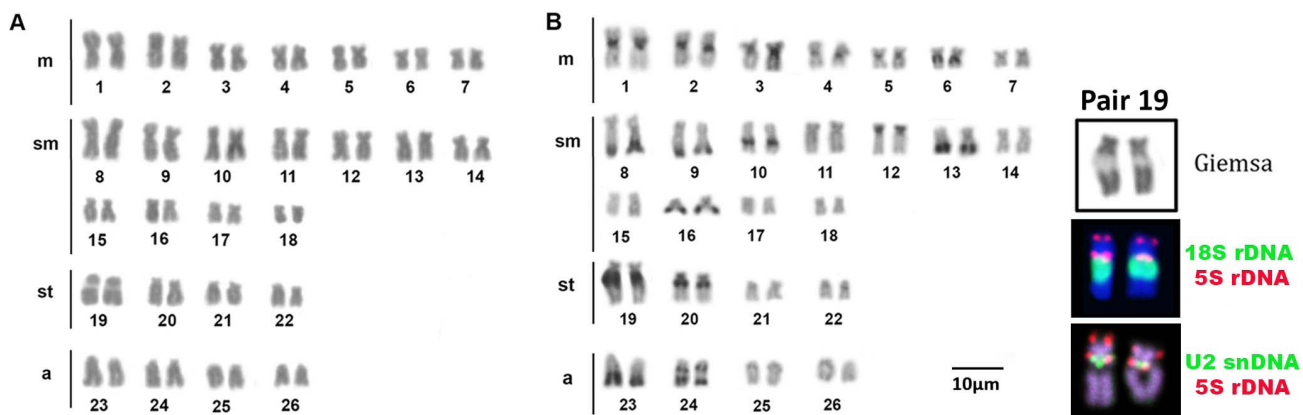
were performed using maximum likelihood (ML) and Bayesian inference (BI) methods in RAxML v. 8.2 (Stamatakis, 2014) and MrBayes v. 3.2.6 (Ronquist, Huelsenbeck, 2003), respectively, through the CIPRES Science Gateway v. 3.3 (Miller *et al.*, 2010). For BI, we used two rows of four chains (10×10^6 generations) with sampling at each 1,000 trees, assuming 10% of burn-in. Branch support values were calculated using posterior probabilities (BI) and 1,000 bootstrap replicates (ML).

The barcodes obtained in the present study were submitted to the analysis of three algorithms based on single-locus data commonly employed for species delimitation: barcode index numbers (BIN) (Ratnasingham, Hebert, 2013), single General Mixed Yule-Coalescent analysis (GMYC) (Pons *et al.*, 2006), and Bayesian Poisson Tree Process (bPTP) (Zhang *et al.*, 2013). BIN analyses were performed by comparing the input taxa with all others that share identical BINs, including those uploaded by different users based on the Refined Single Linkage (RESL) algorithm. This system divided the COI sequences uploaded in the BOLD systems into Molecular Operational Taxonomic Units (MOTUs) independently of their predefined taxonomic classification, providing a framework for assessing congruence between DNA barcodes and species designations (Ratnasingham, Hebert, 2013). The sGMYC analysis was performed for mtDNA lineages delimitation (Fujisawa, Barraclough, 2013), available on <https://species.h-its.org/gmyc/>, and using an ultrametric tree as input, obtained in the BEAST v. 1.8.2 software (Drummond *et al.*, 2012). For bPTP analysis, we used a maximum likelihood phylogenetic tree constructed in the RAxML-HPC BlackBox v. 8.2.10 as the input file. The bPTP adds Bayesian inference support values to the branches of the inputted tree, enhancing reliability (<https://species.h-its.org/>).

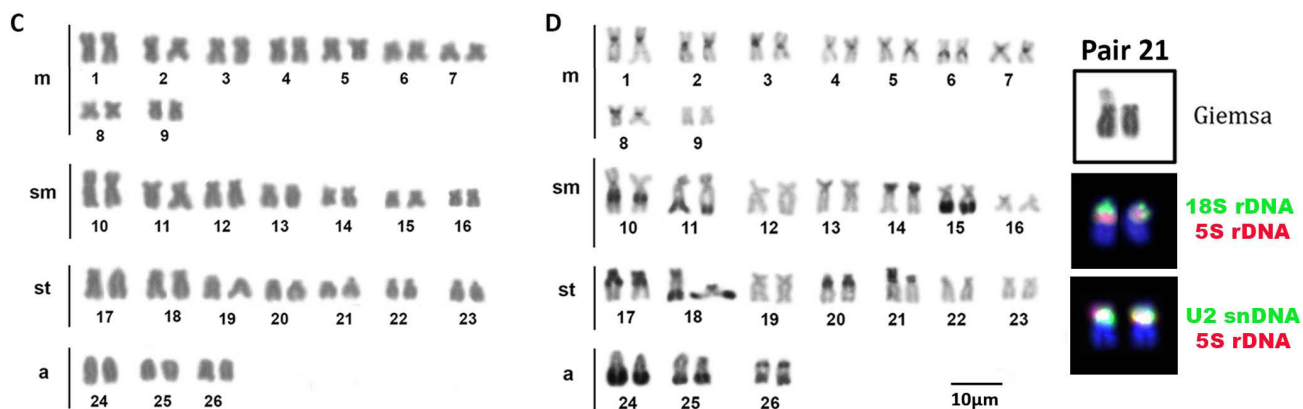
RESULTS

***Astrodoras asterifrons*.** Three of the eight specimens collected in the Negro River, near the Anavilhanas archipelago, exhibited $2n = 52$ chromosomes and a karyotype composed of $14m + 22sm + 8st + 8a$ chromosomes for both sexes. These individuals were designated as Karyomorph A in this study (Fig. 2A). C-banding revealed CH blocks in pericentromeric regions of chromosome pairs 1m, 2m, 3m, 4m, and 5m; interstitial blocks on the long arm of the chromosome pairs 6m, 10sm, 19st, and 20st; terminal blocks on the short arm of chromosome pair 12sm; and terminal blocks on the long arm of chromosome pairs 8sm, 9sm, 13sm, 16sm, and 23a. The chromosome pair 24a exhibited interstitial CH on the long arm (Fig. 2B). FISH with 18S rDNA probes revealed a single site on the long arm of chromosome pair 19st (Fig. 2B - box). FISH with 5S rDNA probes revealed two sites on chromosome pair 19st: one site on the terminal position of the short arm and another site on the proximal position of the long arm (Fig. 2B - box). FISH with telomeric probes $(TTAGGG)_n$ showed fluorescent signals at the terminal positions of all chromosomes. Additionally, interstitial telomeric sites (ITSs) were observed in the chromosome pairs 2m, 8sm, 9sm, and 10sm (Fig. 3A). The U2 snDNA sites were detected in the long arm of the chromosome pair 19st (Fig. 2B - box).

KARYOMORPH A



KARYOMORPH B



KARYOMORPH C

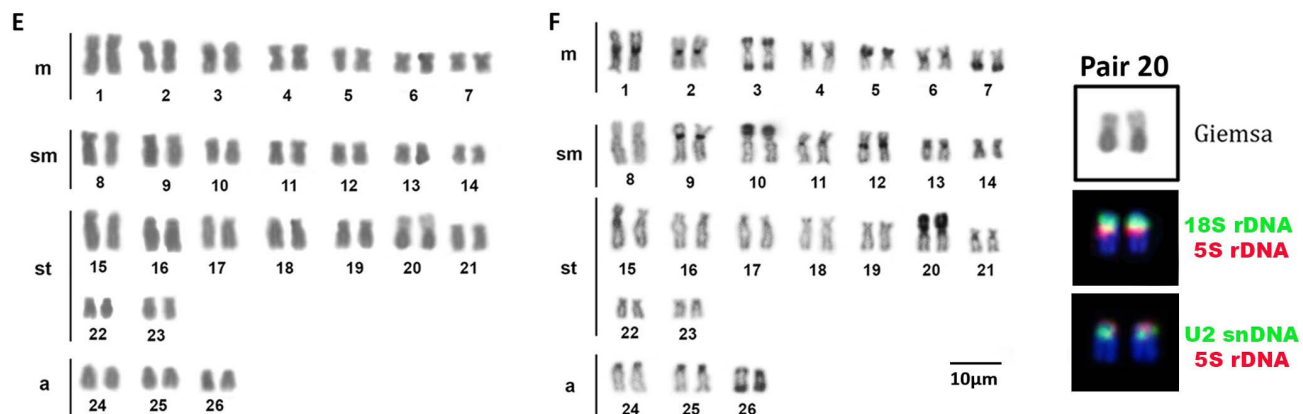


FIGURE 2 | Karyotypes of *Astrodoras asterifrons sensu lato* after conventional and molecular cytogenetics methods: karyomorph A after (A) Giemsa staining (B) C-banding; karyomorph B after (C) Giemsa staining (D) C-banding; karyomorph C (E) Giemsa staining (F) C-banding. The boxes contain the chromosome pairs with the 18S (green) and 5S rDNA sites (red), and with the U2snDNA (green) and 5S rDNA sites (red).

The remaining five specimens also exhibited $2n = 52$ chromosomes, with a karyotype composed of $18m + 14sm + 14st + 6a$ chromosomes, and no sexual dimorphism in chromosome number or morphology was observed. These specimens were designated as Karyomorph B in this study (Fig. 2C). C-banding revealed CH blocks in pericentromeric regions of pairs: 1m, 2m, 3m, 4m, 5m, 6m, 7m, and 8m; interstitial blocks on the long arm of chromosome pairs: 10sm, 17st, and 20st; terminal blocks on the short arm of chromosome pairs 14sm, 18sm, 17st, and 21st; and terminal blocks on the long arm of chromosome pairs: 11sm, 15sm, 18st, 24a, and 25a. The pair 26 exhibited proximal and terminal CH on the long arm (Fig. 2D). FISH with 18S rDNA probes revealed two terminal sites on the short arm of pair 21st (Fig. 2D - box). 5S rDNA probes

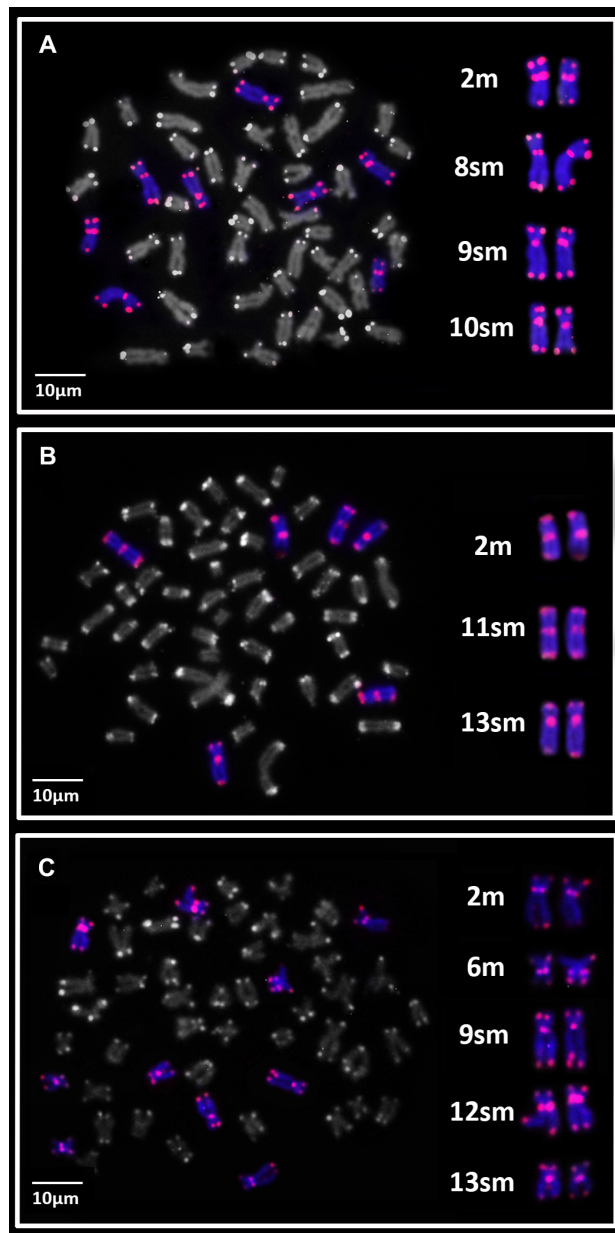


FIGURE 3 | Metaphase plates of *Astrodoros asterifrons sensu lato* showing the (TTAGGG)_n distribution pattern (red). A. karyomorph A; B. karyomorph B; C. karyomorph C.

revealed a single proximal site in the short arm of pair 21st (Fig. 2D - box). FISH with telomeric probes (TTAGGG)_n evidenced fluorescent signals in terminal positions of all chromosomes and ITSs in the pairs 2m, 11sm, and 13sm (Fig. 3B). The U2 snDNA sites were detected on the long arm of the chromosome pair 21st, the same chromosomes carrying 18S and 5S rDNA sites (Fig. 2D - box).

***Astrodoras* sp.** All specimens from the Negro River in Barcelos exhibited $2n = 52$ chromosomes, with a karyotype composed of 14m + 14sm + 18st + 6a for both sexes (Fig. 2E). These individuals were designated as Karyomorph C in this study. C-banding revealed CH blocks on pericentromeric regions of the chromosome pairs: 1m, 2m, 4m, 6m, 9sm, 11sm, 12sm, 13sm, 21st, 24a, and 25a; terminal blocks on the long arm of the chromosome pairs 5m, 10sm, and 20st; and terminal CH blocks on the long arm of the chromosome pair 7m. The chromosome pair 3m had CH blocks in terminal position of both chromosomal arms, while the chromosome pair 26a exhibited proximal and terminal CH blocks on the long arm (Fig. 2F). FISH with 18S rDNA probes revealed single, terminal sites on the short arm of chromosome pair 20st (Fig. 2F - box). FISH with 5S rDNA probes also revealed sites on the short arm of chromosome pair 20st but in a proximal position (Fig. 2F - box). FISH with telomeric probes (TTAGGG)_n evidenced fluorescent signals in terminal positions of all chromosomes and ITSs on the chromosome pairs 2m, 6m, 9sm, 12sm, and 13sm (Fig. 3C). The U2 snDNA sites were detected on the long arm of the chromosome pair 20st, which also carries the 18S and 5S rDNA clusters (Fig. 2F - box).

Molecular species delimitation analysis based on a fragment of the mtDNA COI gene. The COI sequences of obtained from seven specimens of *Astrodoras* resulted in a 604 bp dataset, with no deletions, insertions, or stop codons. The NJ, ML, and BI methods produced similar topologies, revealing seven major clades with high support values (above 90% bootstrap and 0.9 posterior probability): *Wertheimeria* and *Platydoras* (outgroups), *Scorpiodoras*, *Physopyxis*, *Anadoras*, *Amblydoras affinis*, *Amblydoras* sp., and *Hypodoras* + *Astrodoras*. The *Astrodoras* specimens from our sample were subdivided into three well-supported clades, corresponding to the three karyomorphs identified in cytogenetic analyses (A, B, and C). The specimens of *Astrodoras asterifrons* from near Anavilhanas correspond to Karyomorph A and B, while *Astrodoras* sp. corresponds to Karyomorph C.

To estimate genetic distances, two grouping approaches were applied based on morphological classification and molecular analyses results. The first approach considered all sequences of *A. asterifrons* of our sample and from available databases as a single group that was named as *A. cf. asterifrons*, revealing an intraspecific distance of 0.055 (5.5%) (Tab. S3). A second morphological grouping (named as *A. asterifrons*), excluding one sequence from the Madeira River (05° 48'33.0"S 61° 18'01.1"W) identified as *Astrodoras* sp. (*sensu* Roa-Fuentes *et al.*, 2010) in Sabaj, Arce (2021), demonstrated an intraspecific distance of 0.046 (4.6%).

Using the second grouping approach, based on the subdivisions identified in the phylogenetic reconstructions, which also correspond to the karyomorphs identified in our sample through cytogenetic analyses, *Astrodoras* samples were divided into three groups: group 1 (karyomorph A + KP772601), group 2 (karyomorph B +

KC555597), and group 3 (karyomorph C). The interspecific genetic distances ranged from 2.9% (between groups 2 and 3) to 6.5% (between groups 1 and 3) and 7% (between groups 1 and 2). The intraspecific distances ranged from 0.7% in group 3 and 0.9% in group 1 to 1.4% in group 2 (Tab. S3).

Species delimitation algorithms yielded slightly different MOTU counts: BIN and GMYC identified 20 MOTUs, whereas bPTP identified 19. Despite this variation, all algorithms consistently recovered the three karyomorphs (A, B, and C) as distinct clades with strong support (above 90%; Fig. 4).

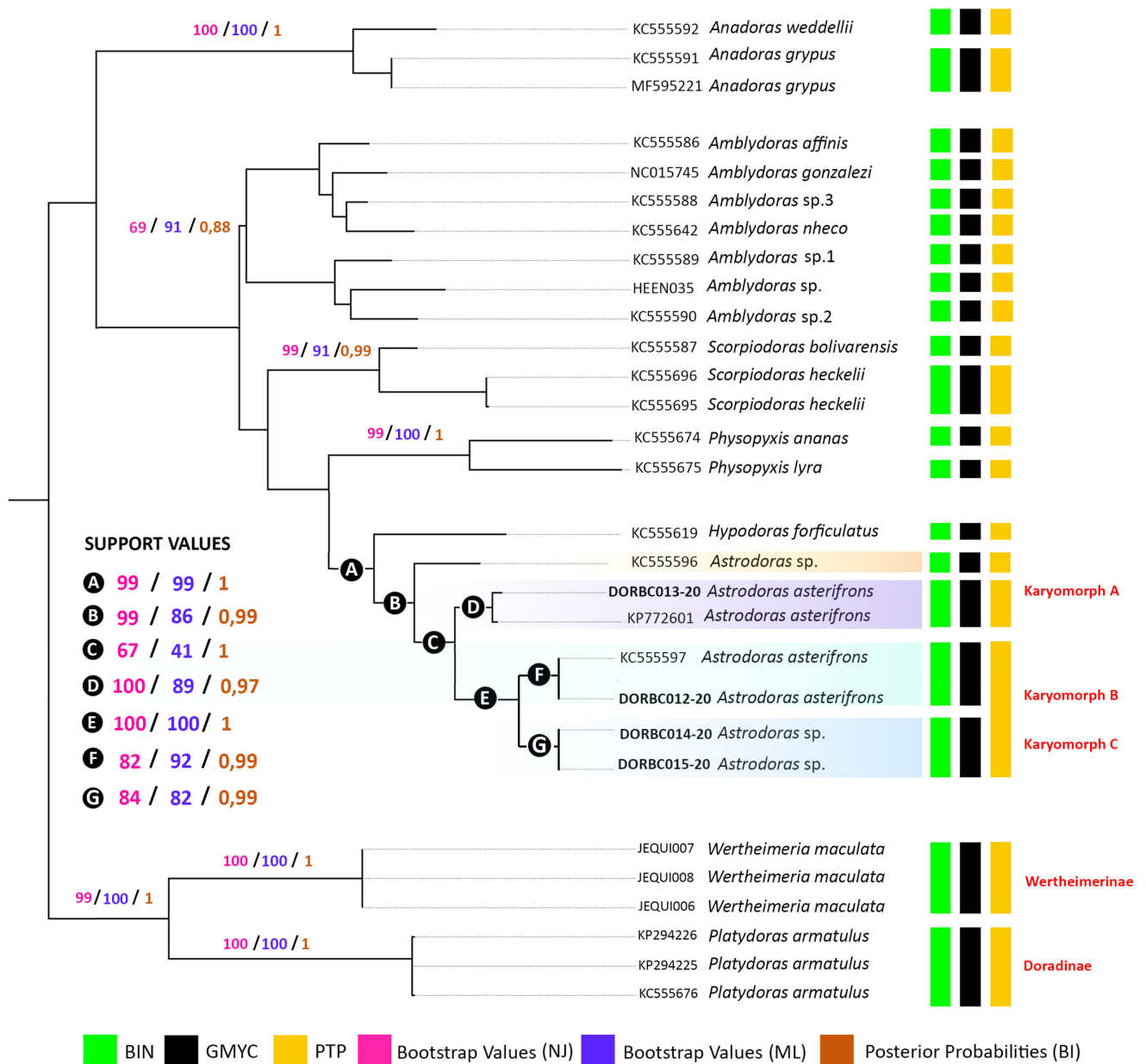


FIGURE 4 | Consensus tree (NJ neighbor-joining, ML maximum likelihood and BI Bayesian inference) based on COI sequences from samples of *Astrodoras asterifrons* sensu lato (the same specimens cytogenetically analyzed), species of Astrodoradinae, *Wertheimeria maculata* and *Platydoras armatulus* (outgroup). The vertical-colored bars represent the results obtained after the species delimitation analyses.

DISCUSSION

Karyotypic data for Doradidae have been predominantly reported for species within the subfamilies Doradinae (Milhomem *et al.*, 2008; Takagui *et al.*, 2021) and Wertheimerinae (Eler *et al.*, 2007; Takagui *et al.*, 2019). In contrast, cytogenetic information for Astrodoradinae remains scarce, limited to the three species of *Anadoras*, all presenting $2n = 56$ chromosomes (Baumgärtner *et al.*, 2018; Takagui *et al.*, 2022). Our study expands the chromosomal dataset to *A. asterifrons*, revealing a $2n = 52$ chromosomes, the lowest reported to date for Doradidae. Moreover, we identified three distinct karyomorphs within *Astrodoras*. Karyomorphs A and B were collected in sympatry in the Negro River, near Anavilhanas, while Karyomorph C was sampled in the Negro River near Barcelos City (500 km upstream from Anavilhanas). All karyomorphs exhibited $2n = 52$ chromosomes, with variations in karyotype composition, CH distribution, rDNA and U2 snDNA site organization, and the number of interstitial telomeric sequences (ITS). Species delimitation analyses based on molecular data support the interpretation of these karyomorphs as independent evolutionary units, thus reinforcing the hypothesis of a polytypic status for *Astrodoras*.

Ancestral state reconstruction suggests that $2n = 58$ constitutes the plesiomorphic condition for Doradidae. Thus, chromosome diversification in the thorny catfishes can be explained by three main evolutionary pathways: (a) maintenance of the ancestral $2n = 58$ chromosomes; (b) reduction in $2n$ via chromosome fusions; and (c) increase in $2n$ through chromosome fissions (see Takagui *et al.*, 2024). The specimens of *A. asterifrons* in our sample exhibit the smallest $2n$ reported among thorny catfishes so far ($2n = 52$), corresponding to the chromosomal diversification pathway (b) $2n$ reduction through chromosome fusions. This type of rearrangement may occur in two situations: loss of the capping function while telomeric repeats are still present or the loss of telomeric repeats that maintain the telomere functional (Slijepcevic, 1998; Murnane, 2006). According to Ocalewicz (2013), pericentromeric and interstitial telomeric sites may represent relicts of the ancient chromosomal fusion events and can be classified into six types: heterochromatic (het-ITSs), short (s-ITSs), large ITSs in restricted euchromatic regions (restricted eu-ITSs), long subtelomeric ITSs, fusion ITSs, and pericentromeric ITSs (Lin, Yan, 2008; Ruiz-Herrera *et al.*, 2008; Schmid, Steinlein, 2016).

In *Astrodoras*, the hypothesis of chromosomal fusion is further corroborated by FISH analyses using telomeric probes, which revealed ITSs in all examined specimens, varying from three sites in karyomorph B and four sites in karyomorph A to five sites in karyomorph C. Despite this variation, all karyomorphs share at least three chromosome pairs (metacentrics and submetacentrics) carrying ITSs, suggesting that these chromosomal fusions might represent an ancestral event in *Astrodoras*. However, karyomorphs A and C exhibit additional ITSs, which might represent remnants of other types of structural rearrangements, such as pericentric inversions (reviewed in Vicari *et al.*, 2022). Alternatively, some of these additional ITSs could also represent Het-ITS, that is, TTAGGG repeats associated with heterochromatic regions (*e.g.*, Milhomem *et al.*, 2008; Ruiz-Herrera *et al.*, 2008; Scacchetti *et al.*, 2011; Marajó *et al.*, 2018; Campos *et al.*, 2020; Viana *et al.*, 2020; Vicari *et al.*, 2022).

Chromosomal fusions have been reported only in a few species of Doradidae. For instance, Baumgärtner *et al.* (2016) identified two proximal ITSs on the long arm of the chromosome pair 19 in *Trachydoras paraguayensis* (Eigenmann & Ward, 1907) ($2n = 56$),

suggesting that the $2n$ reduction in this species was the result of the fusion of acrocentric and submetacentric chromosomes. In contrast, *Anadoras* species lacks ITSs despite their reduced $2n = 56$ chromosomes, making it difficult to determine the type of fusion (in tandem or centric) or to identify the fused chromosome pairs (Takagui *et al.*, 2022). In the sister family Auchenipteridae, ITSs have been identified in three species so far: *Ageneiosus inermis* (Linnaeus, 1766) which has one ITS (Lui *et al.*, 2013); *Centromochlus schultzi* Rösse, 1962, which has two ITSs (Kowalski *et al.*, 2024); and *Centromochlus heckelii* (De Filippi, 1853) which exhibits several ITSs (Haerter *et al.*, 2025). In *A. inermis*, the ITS was attributed to a single end-to-end chromosomal fusion, while in *C. schultzi*, the heterochromatic ITSs (het-ITSs) might be either a result of pericentric inversions or repetitive DNA amplification (Kowalski *et al.*, 2024). Interestingly, the origin of ITSs in *C. heckelii* is possibly related to multiple mechanisms, such as chromosomal fusions, inversions, transpositions, or association with satellite DNA (Haerter *et al.*, 2025).

C-banding revealed distinct CH distribution patterns across the *Astrodoras* karyomorphs. They share certain chromosomal pairs, including the chromosome pairs carrying the 18S rDNA, a submetacentric pair with CH in the entire short arm (pairs 12, 13, and 10), and an acrocentric chromosome pair with two CH blocks on the long arm (pairs 24, 26, and 26). However, Karyomorph C exhibited fewer CH blocks compared to the other two karyomorphs. Thus, the CH distribution may constitute an important chromosomal marker to differentiate them. Variation in CH has been fundamental to understanding cytotaxonomic issues and some diversification pathways in Doradidae (Baumgärtner *et al.*, 2018; Takagui *et al.*, 2019, 2021, 2022). For instance, in the subfamily Wertheimerinae, CH arrangements are highly divergent among the three studied species, likely influenced by the saltatory activity of retroelements, particularly from the Rex3 group (Takagui *et al.*, 2019). In the Doradinae, the CH distribution is crucial to karyotypically discriminate *Centrodoras brachiatus* (Cope, 1872), *Pterodoras granulatus* (Valenciennes, 1821), and *Oxydoras niger* (Valenciennes, 1821), which share the same diploid number, karyotype composition, and rDNA distribution patterns (Takagui *et al.*, 2021). Furthermore, C-positive regions were crucial during the differentiation of the ZZ/ZW sex chromosome system reported in *Nemadoras trimaculatus* (= *Tenellus trimaculatus* (Boulenger, 1898)) (*sensu* Sabaj, Arce, 2021) from the Araguaia River (Takagui *et al.*, 2017a) and in the evolution of supernumerary chromosomes (B chromosomes) of *Doras punctata* (= *Ossancora punctata* (Kner, 1853)), *Platydoras armatulus*, and *Pterodoras granulatus* (Takagui *et al.*, 2017b).

Physical mapping with 18S and 5S rDNA probes showed sites in the same chromosome pair across all *Astrodoras* karyomorphs but with some notable differences in the arrangement of these repetitive DNAs. In karyomorphs B and C, the rDNA loci are syntenic on the short arm of the chromosome pairs 20/21st. On the other hand, in Karyomorph A, the 18S rDNA locus is also located on a subtelocentric pair, but in an interstitial position on the long arm. It is noteworthy that simple, terminal 18S rDNA sites represent the putative plesiomorphic condition in Doradidae, inferred through ancestral state reconstruction (Takagui *et al.*, 2021). Similarly, most Doradidae species exhibit the 5S rDNA in a syntenic arrangement with the 18S rDNA cluster (Baumgärtner *et al.*, 2018; Takagui *et al.*, 2019, 2021, 2022), a feature that is absent in most Auchenipteridae species (see Kowalski *et al.*, 2024). Thus, the rDNA pattern observed in Karyomorph A might represent an apomorphic trait, likely resulting from a

pericentric inversion. In this scenario, the inversion would have encompassed the entire 18S rDNA cluster and part of the 5S rDNA cluster, leading to a fragmented 5S rDNA site. As a result, one portion of the 5S rDNA cluster remains at the terminal position of the short arm, while the other portion was relocated to an interstitial position on the long arm alongside the 18S rDNA site. This inversion hypothesis is also supported by the U2 snDNA mapping, which revealed interspersed U2/5S rDNA sites on the short arms of the chromosome pair 20 and 21 of karyomorphs B and C, respectively, whereas the karyomorph A exhibits the U2 snDNA loci associated only with the 5S fraction situated on the long arm, near to the 18S rDNA sites.

The organization and structure of snDNAs remain poorly understood in Neotropical fishes. In Doradidae, cytogenetic mapping of U2 snDNA has been conducted in two species, *Acanthodoras* cf. *polygrammus* (Linnaeus, 1758) and *Acanthodoras cataphractus* (Linnaeus, 1758), which possess two and three sites, respectively (Takagui *et al.*, 2024). Both species exhibit a syntenic arrangement of the U2 snDNA and 5S rDNA loci, a pattern consistent with that observed in *Anadoras* species in this study. Among neotropical fishes, this syntenic arrangement is relatively common and has been reported in characiforms *Piabarchus stramineus* (Eigenmann, 1908) (Piscor *et al.*, 2018), *Triportheus* (Yano *et al.*, 2017b), and ten species of *Characidium* (Scacchetti *et al.*, 2015), as well as the gymnotiform *Eigenmannia* aff. *trilineata* (Araya-Jaime, 2017). Interestingly, this syntenic arrangement is absent in Auchenipteridae species (see Haerter *et al.*, 2022). As noted by Takagui *et al.* (2024), such differences may serve as important cytotaxonomic markers, offering insights into karyotype differentiation and evolutionary processes in Doradidae and Auchenipteridae.

The taxonomic diagnosis of the *Astrodoras* species remains challenging. Although previous studies (Eigenmann, 1925; Roa-Fuentes *et al.*, 2010; Sousa, 2010) identified putative undescribed taxa within *Astrodoras*, these species have not yet been formally described. Our cytogenetic analyses revealed three distinct karyomorphs within *Astrodoras*, characterized by differences in CH distribution, the positions of 18S rDNA, 5S rDNA, and U2 snDNA sites, and the number of chromosomes carrying ITSs. The species delimitation analyses indicate that these karyomorphs represent independent evolutionary units, supported by high phylogenetic support values (NJ, BI, and ML). These units are also recognized by BIN and GMYC, two of the most reliable algorithms for species delimitation and the identification of MOTUs in fish (*e.g.*, Costa-Silva *et al.*, 2015; Carvalho *et al.*, 2018; Anjos *et al.*, 2019; Argolo *et al.*, 2020). Additional evidence for the distinction of these karyomorphs is provided by the absence of hybrids between karyomorphs A and B, despite their sympatric occurrence.

The intraspecific genetic distance for the morphology-based grouping was high (4.6%), exceeding by more than twice the commonly accepted threshold value (2%) for fish species delimitation using the DNA barcoding method (Pereira *et al.*, 2011, 2013). When considering the three MOTUs in *Astrodoras*, the interspecific distances (2.9%, 6.5%, and 7%) are consistent with previously reported values for congeneric species (Pereira *et al.*, 2011, 2013) (Tab. S3). Despite including only two specimens, phylogenetic analyses performed by Arce *et al.* (2013) and Sabaj, Arce (2021) had already recognized the non-monotypic status of *Astrodoras*, identifying at least two distinct taxonomic units. Considering all available *Astrodoras* sequences, including those from Arce *et al.* (2013) and Sabaj, Arce (2021), the molecular analyses recovered at least

four distinct lineages: (1) '*Astrodoras* sp. (*sensu* Roa-Fuentes *et al.*, 2010)' from Arce *et al.* (2013) and Sabaj, Arce (2021); (2) karyomorph B + '*A. asterifrons*' from Arce *et al.* (2013) and Sabaj, Arce (2021); (3) karyomorph A + *A. asterifrons* (KP772601); and (4) karyomorph C. Thus, integration of cytogenetic markers and COI-based species delimitation analyses further supports the polytypic status of *Astrodoras*.

Currently, it is widely known that the Amazon drainage system exhibits seasonal oscillations of water levels that induce the emergence and disappearance of many landscape formations, such as rivers, lakes, streams, beaches, floodplains, and flooded forests (Albert, Reis, 2011). This vast hydrographic system has an old and complex geomorphological history, marked by successive episodes of geomorphological changes and marine incursions (Hoorn *et al.*, 2010, 2017; Wesselingh, Hoorn, 2009; Cassemiro *et al.*, 2023). This highly complex scenario favors vicariant events and allopatric speciation (Dagosta, de Pinna, 2017), which is congruent with some Doradidae genera that were already the focus of taxonomic revisions, such as *Physopyxis* (Sousa, Rapp Py-Daniel, 2005) and *Scorpidoras* (Sousa, Birindelli, 2011). The region where these *A. asterifrons* samples were collected has historically served as a basin divider, particularly due to the presence of the Purus Arch (Lundberg *et al.*, 1998). Consequently, vicariant events associated with past geomorphological shifts in the region may underlie the chromosomal and molecular divergence observed among the karyomorphs. In this scenario, the sympatric occurrence in the Central Amazon River floodplains might be a result of the Late Miocene rise of the Vaupes structural arch and concomitant breaching of the Purus arch, which allowed the secondary contact among several freshwater fish species (Ivanyisky, Albert, 2014). Thus, it is possible that the distinct sympatric lineages that compose *Astrodoras* emerged through allopatric speciation, giving rise to sympatric populations by secondary contact.

The integration of cytogenetic and molecular evidence supports the hypothesis of a polytypic status of *Astrodoras*. The specimens analyzed in this study exhibited significant chromosomal and molecular divergence, even in cases of sympatric occurrence within the Central Amazon floodplains. These findings underscore the urgency of re-evaluating the taxonomic framework of *Astrodoras* through the inclusion of additional molecular markers, expanded geographic sampling, and integrative approaches to fully resolve its diversity and evolutionary history.

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AUTHORS' CONTRIBUTION

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ETHICAL STATEMENT

The experiments followed ethical conducts according to the Ethics Committee for Animal Use of the Universidade Estadual de Londrina, under the protocol number 60/2017. The specimens were collected under the authorization of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio; permit number 85306–1).

DATA AVAILABILITY STATEMENT

The cytogenetic data that support this study are partially included in the article. Unfortunately, the raw data with full metaphase plates hybridized with 5S rDNA, 18S rDNA, and U2 snDNA probes are no longer accessible. Further details can be requested from the corresponding author. The genetic datasets are deposited in the BOLD system and listed in the supplementary material.

AI STATEMENT

The authors declare that no artificial intelligence was used in this study.

SUPPLEMENTARY MATERIAL

Supplementary material S1
Supplementary material S2
Supplementary material S3

COMPETING INTERESTS

The authors declare no competing interests.

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