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Cytogenetic and molecular identification of *Chaetostoma bifurcum* (Siluriformes: Loricariidae) from the Pacific Coast of Ecuador

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This study presents an integrated molecular and cytogenetic characterization of *Chaetostoma bifurcum* from Ecuador, which belongs to the Hypostominae subfamily. Morphological identification was confirmed by molecular data using cytochrome c oxidase I and cytochrome b sequences. Karyotype analysis was performed with conventional staining and fluorescence *in situ* hybridization (FISH). The karyotype comprises 48 metacentric/submetacentric and 6 subtelocentric/acrocentric chromosomes, maintaining the chromosome number (2n = 54) considered ancestral in Loricariidae, though uncommon in Hypostominae. No differences were observed between female and male karyotypes, suggesting absence of heteromorphic sex chromosomes. Faint interstitial telomeric sequences (ITSs) were detected on a submetacentric pair, whose origin remains unclear and may represent remnants of translocations resulting from DNA repair mechanisms or be part of satellite DNA. The 18S rDNA and 5S rDNA loci are localized on distinct chromosomes (pair 11 and 6, respectively), deviating from the plesiomorphic synteny observed in Loricariidae. This pattern may reflect both evolutionary history but also the effect of ecological pressures. Thus, comparative analyses across other *Chaetostoma* species are essential to determine whether these karyotypic traits reflect their phylogenetic position or specific adaptive processes since this study corresponds to the first characterization for the genus.

Keywords: Chromosomes, Fish, Phylogenetics, Ribosomal genes, Rubbernose plecos.

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Este estudo apresenta uma caracterização molecular e citogenética integrada de *Chaetostoma bifurcum* do Equador, pertencente à subfamília Hypostominae. A identificação morfológica foi confirmada por dados moleculares utilizando sequências dos genes da citocromo c oxidase subunidade I e citocromo b. A análise do cariótipo foi realizada com coloração convencional e hibridização *in situ* por fluorescência (FISH). O cariótipo é composto por 48 cromossomos metacêntricos/submetacêntricos e 6 subtelocêntricos/acrocêntricos, mantendo o número diploide (2n = 54) considerado ancestral na família Loricariidae, embora incomum em Hypostominae. Não foram observadas diferenças entre os cariótipos de fêmeas e machos, sugerindo ausência de cromossomos sexuais heteromórficos. Sequências teloméricas intersticiais (ITSs) tênues foram detectadas em um par submetacêntrico, cuja origem permanece incerta, podendo representar remanescentes de translocações decorrentes de mecanismos de reparo do DNA ou de DNA satélite. Os loci de rDNA 18S e 5S estão localizados em cromossomos distintos (pares 11 e 6, respectivamente), divergindo da sintenia plesiomórfica observada em Loricariidae. Esse padrão pode refletir tanto a história evolutiva quanto os efeitos de pressões ecológicas. Assim, análises comparativas com outras espécies do gênero *Chaetostoma* são essenciais para determinar se essas características cariotípicas refletem a posição filogenética ou processos adaptativos específicos, considerando que este estudo representa a primeira caracterização para o gênero.

Palavras-chave: Cascudos narigudos, Cromossomos, Filogenética, Fish, Genes ribossômicos.

INTRODUCTION

Loricariidae, commonly known as armored catfishes, represents a large family of freshwater fishes distributed across South and Central America. They are distinguished by their bony plates and suckermouths, which are specially adapted for feeding in fast-flowing, rheophilic environments (Covain, Fisch-Muller, 2007; Bressman *et al*., 2020). These fishes play a crucial ecological role, acting as specialized herbivores and consumers of organic matter. By controlling algae overgrowth, they help maintain the environmental balance within their habitats. Some Loricariidae species have been introduced into non-native freshwater ecosystems due to their popularity in the aquarium trade, where they can have negative impacts (Nico, Martin, 2001; Owsley *et al*., 2017; Orfinger, Goodding, 2018; Borzone Mas *et al*., 2019; Quintana *et al*., 2023). There are currently 1068 valid species within the Loricariidae, which is divided into six subfamilies: Lithogeninae, Delturinae, Rhinelepinae, Loricariinae, Hypoptopomatinae, and Hypostominae (Fricke *et al*., 2025). Hypostominae is the largest subfamily and comprises different tribes/clades whose relationships are still debated (Armbruster, 2004; Lujan *et al*., 2015a; Roxo *et al*., 2019). Hypostominae exhibited significant diversity in chromosome numbers and in the presence of differentiated sex chromosome systems (Fig. **S1**), although this variation is not uniformly distributed across tribes and

genera (Sassi *et al.*, 2024). Whitin Hypostominae, fishes of the genus *Chaetostoma* Tschudi 1846, generally attributed to the tribe Ancistrini and commonly known as rubbernose plecos, are described as the "*Chatestoma* clade" (Lujan *et al.*, 2015a). This informal clade is sister of all other Hypostominae. Beyond species of *Chaetostoma* it contains a monophyletic sister taxon composed of species of *Cordylancistrus* Isbrücker, 1980, *Dolichancistrus* Isbrücker, 1980, and *Leptoancistrus* Meek & Hildebrand, 1916, distributed primarily in the northern Andes (Lujan *et al.*, 2015b). *Chaetostoma* includes about 47 species, although its real diversity is likely underestimated (Lujan *et al.*, 2015b) as demonstrated by recent identification and descriptions of new species (Urbano-Bonilla, Ballen, 2021; Meza-Vargas *et al.*, 2022). These fishes are distributed along the Atlantic and Pacific slopes of the Andes Mountains, spanning from Panama to southern Peru, as well as in the Coastal Mountains of Venezuela, and various drainages within the Guiana and Brazilian shields (Lujan *et al.*, 2015b).

As is the case with the ichthyofauna inhabiting streams and rivers of Andean Piedmont, *Chaetostoma* is threatened by the hydroelectric development (Finer, Jenkins, 2012). As a result, their preservation and conservation, together with their habitats, are of utmost importance to safeguard the ecological balance and maintain the rich biodiversity in these imperiled aquatic environments. According to the International Union for Conservation of Nature (IUCN), 15 *Chaetostoma* species are threatened of extinction. Of these, eight are designated as Endangered (EN) and seven as Vulnerable (VU) (IUCN, 2024).

Chaetostoma bifurcum Lujan, Meza-Vargas, Astudillo-Clavijo, Barriga Salazar & López-Fernández, 2015 (Fig. 1) is found in the Pacific Coast drainages of western Ecuador and northwestern Peru in piedmont elevations ranging from approximately 100 to 650 meters above sea level. Its distribution includes the Esmeraldas, Guayas, Santa Rosa, and Tumbes River drainages, from north to south (Lujan *et al.*, 2015a). Unfortunately, the country's aquatic ecosystems in this area are seriously threatened and circumstances are deteriorating (Aguirre *et al.*, 2021). The species has not been evaluated by the IUCN and is unexplored in many biological and genetic aspects. In addition, there is no available data on any *Chaetostoma* species among the about 300 records (142 species) on chromosomal data for Hypostominae subfamily. Here we report the first cytogenetic data on *C. bifurcum*. Our objective is to ascertain whether chromosome number and karyotype structure exhibit the ancestral traits of the family/subfamily or, conversely, exhibit chromosomal rearrangements, as well as to investigate the presence of heteromorphic sex chromosomes in this species.

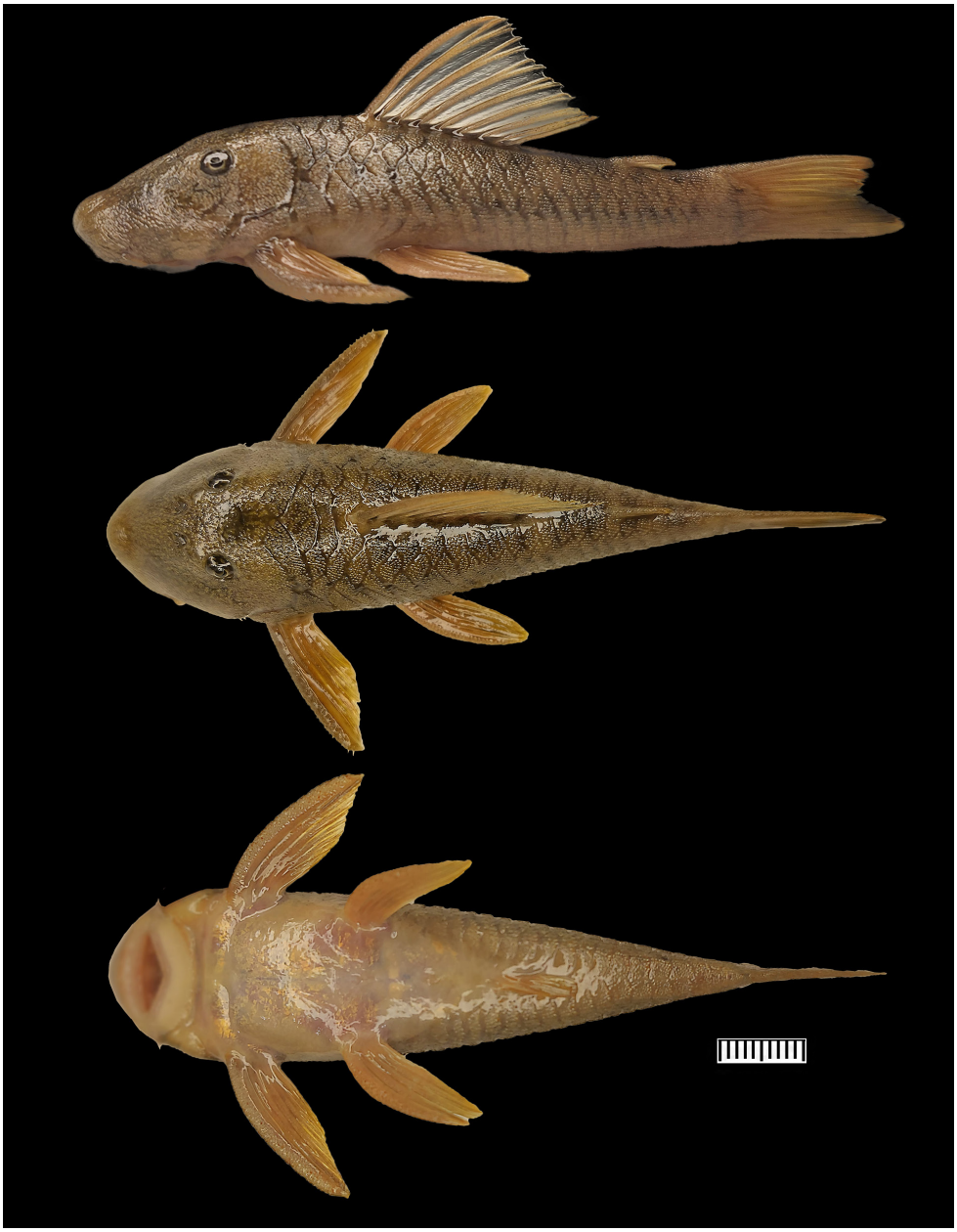

FIGURE 1 | *Chaetostoma bifurcum*: dorsal (top), lateral (middle), and ventral (bottom) views. The bony plates covering the body and the distinct suckermouth, characteristic of the genus *Chaetostoma*, are visible, illustrating the adaptations of the species for adhering to rocky substrates in fast-flowing rivers. Scale bar = 10 mm.

MATERIAL AND METHODS

Specimen collection. Twenty-four individuals of *Chaetostoma bifurcum* (9 males, 11 females, and 4 undetermined) were collected using a seine net in the Dos Bocas River (Cantón Pasaje), Ecuador. Fish were transported in sealed plastic bags, with the upper two-thirds of each bag filled with pure oxygen, ensuring optimal well-being to the Laboratories of Universidad Técnica de Machala until processing. The specimens were preliminarily identified based on external morphology (Lujan *et al.*, 2015b) (Fig. 1), and this attribution was later validated on a subset of 11 specimens through molecular analysis. Sex determination was performed during dissection; sexually mature individuals could be reliably sexed based on gonadal morphology, whereas immature individuals were recorded as undetermined. Voucher specimens were fixed in a 10% formalin solution and deposited in the collection of the Instituto Nacional de Biodiversidad de Ecuador, under catalogue number MECN-DP 4962 (Tab. **S2**).

Specimen molecular identification. DNA extraction and polymerase chain reaction (PCR) amplification followed the procedures reported in Nirchio *et al.* (2023) using the Fish F1 and Fish R1 for cytochrome c oxidase I, COI (Ward *et al.*, 2005) and cytbFa and cytbRa for cytochrome b, Cytb (Lujan *et al.*, 2015a). Amplicons were purified and sequenced (Sanger method) using an external service (www.microsynth.ch). Sequences were aligned using Clustal X (Thompson *et al.*, 2002), and the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to explore for Cytb and COI sequence similarity on the GenBank database and the Identification Request for COI sequences in BOLD System (https://v3.boldsystems.org).

Cytogenetic Analysis. Mitotic chromosome preparations were performed according to Nirchio *et al.* (2023). Chromosome were stained with Giemsa for morphology and the karyotype analysis. Metacentric (m) or submetacentric (sm) chromosomes were classified as bi-armed, and subtelocentric (st) or acrocentric (a) chromosomes as uniarmed (Levan *et al.*, 1964). The C-banding procedure (Sumner, 1972) was applied for heterochromatin visualization; the silver staining method (Howell, Black, 1980) was used to identify nucleolus organizer regions (NORs).

Fluorescence *in situ* hybridization experiments. Genomic DNA from *Chaetostoma bifurcum* samples was isolated from the fin tissue preserved in 95% ethanol with the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. Subsequently, DNA was amplified via PCR using specific primers corresponding to the 5S rDNA (Pendas *et al.*, 1995), 18S rDNA (Utsunomia *et al.*, 2016), and telomeric probe (Ijdo *et al.*, 1991). Using the nick-translation protocol (Jena Bioscience, Germany), the 5S rDNA and telomeric PCR-amplified sequences were labeled with Atto-550-dUTP and the 18S rDNA with Atto-425-dUTP. The FISH experiments followed the conditions described in Nirchio *et al.* (2023). To validate the results of *in situ* hybridization, at least 30 metaphase spreads were examined. Metaphases were captured in an Axioplan II fluorescence microscope (Carl Zeiss Jena GmbH, Germany) with the ISIS software (MetaSystems, Silver Spring, MD, USA).

RESULTS

Molecular identification of specimens. COI sequences of 650 base pair (bp) corresponding to a single haplotype were obtained and deposited in GenBank (OR237843) (Nirchio *et al*., 2023). BLAST search (September 27, 2024) identified 98.45% similarity with *Chaetostoma bifurcum*, and similarity < 96% with several *C. fisheri*. In the BOLD System (accessed on September 27, 2024) there were no available sequences of *C. bifurcum* yet, and the highest similarity percentage was observed with *C. fisheri*. Cytb sequences (complete sequence, 1048 bp, a single haplotype. PV030915) confirmed species attribution and results of COI (99.7–99.5% similarity with *C. bifurcum* and < 96% with other congeneric species).

Cytogenetic analyses. Both males and females of *Chaetostoma bifurcum* possess a diploid complement of 54 chromosomes, with a karyotype composed of 48 m-sm + 6 st-a, and a fundamental number (FN) of 102 (Fig. 2). Silver nitrate impregnation revealed a unique pair of transcriptionally active nucleolus organizer regions (NORs), present at a distinct secondary constriction located on the short arm of the submetacentric pair 11 (Fig. 2A, boxed). C-banding revealed blocks of constitutive heterochromatin in all chromosomes, located in the centromeric and pericentromeric regions (Fig. 2B). A distinct heterochromatic block was consistently observed on the short arm of chromosome pair 3. No differences were found between males and females concerning their heterochromatin distribution.

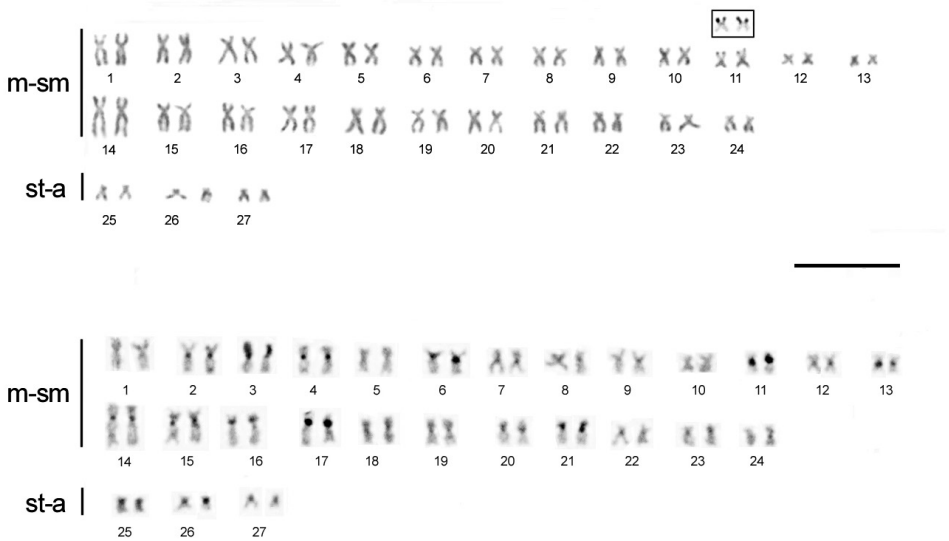

FIGURE 2 | Karyotype of *Chaetostoma bifurcum* stained with Giemsa (**A**), and after C-banding (**B**). The chromosome pair showing the nucleolus organizer region, after silver staining, is shown in the inset. m-sm, metacentric or submetacentric chromosomes; st-a subtelocentric or acrocentric chromosomes. Scale bar = 10 µm.

The fluorescence *in situ* hybridization (FISH) assay using the 18S rDNA probe confirmed the existence of a single large ribosomal cluster within the secondary constriction of the m-sm pair 11 (Fig. 3), which coincides with the NOR region. Moreover, the 5S rDNA probe revealed the presence of the minor ribosomal cistrons interstitially on the short arms of a middle-sized m-sm chromosome, likely pair 6. FISH with a telomeric probe shows positive signals in the terminal region of all chromosomes; in addition, a small interstitial telomeric sequence (ITS) was observed on a submetacentric chromosome pair (Fig. 4). No differences were recorded for any cytogenetic markers, between females and males.

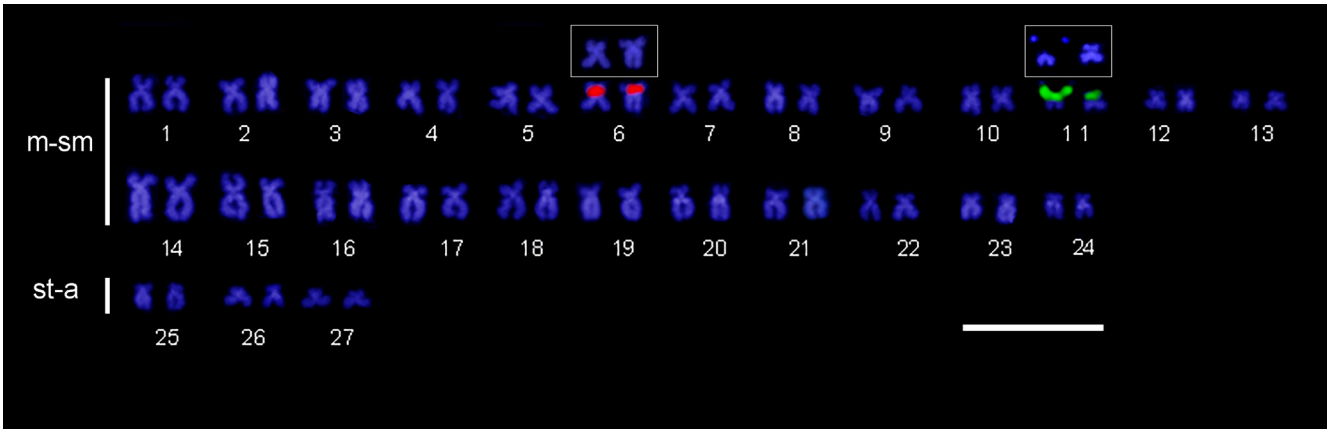

FIGURE 3 | *Chaetostoma bifurcum* karyotype after double fluorescence *in situ* hybridization with 18S rDNA (green) and 5S rDNA (red) probes. Box highlights the labeled pairs and their aspect in DAPI. Scale bar = 10 µm.

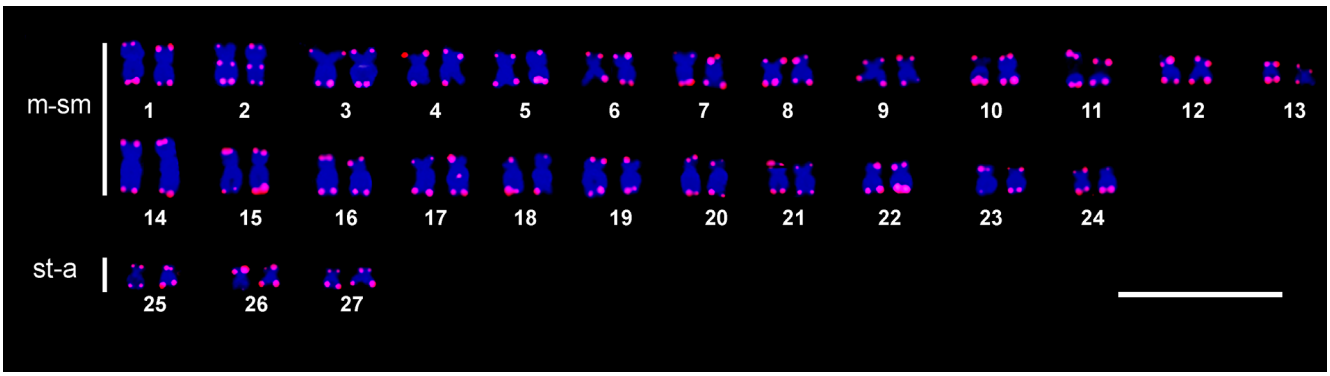

FIGURE 4 | *Chaetostoma bifurcum* karyotype after fluorescence *in situ* hybridization using telomeric probes TTAGGGn. Faint ITSs are visible on chromosome pair number 2. Scale bar = 10 µm.

DISCUSSION

Integrating molecular and cytogenetic approaches in teleost research has proven to be instrumental in uncovering the mechanisms underlying karyotype diversity and evolutionary pathways. These methodologies provide a framework to explore how genetic and evolutionary factors influence chromosomal rearrangements and diversification of species (Nirchio *et al*., 2014; Martinez *et al*., 2015; Supiwong *et al*., 2019; Moraes *et al*., 2021). In this study, we present the first cytogenetic data for *Chaetostoma bifurcum,* extending the number of Hypostominae species/genera analyzed. While our study focuses primarily on karyotypic patterns and their evolutionary implications, it sets the stage for future research aimed at exploring potential ecological correlates of karyotypic diversity within this group.

In Hypostominae, chromosome number ranges from 2n = 34 to 2n = 82 (Fig. **S1**). The tribes within the subfamily are not uniformly represented regarding species diversity, available research, or chromosome number (Fig. **S3**). For instance, the tribe Ancistrini is one of the two most studied, with 126 records spanning 16 genera, while Hypostomini includes 158 records from just two genera (*Aphanotorulus* Isbrücker & Nijssen, 1983 and *Hypostomus* Lacepède, 1803). In contrast, Pterygoplichthini includes 13 records for the single genus, *Pterygoplichthys* Gill, 1858. The broad chromosome number range in Hypostominae is largely driven by two genera: *Ancistrus* Kner, 1854, with 2n ranging from 34 to 54 chromosomes (2n = 52 being the most common) and *Hypostomus* with 2n ranging from 66 to 82 (2n = 72 being the most common). Other genera and species, regardless of their tribe, typically share 2n = 52–54 chromosomes. This suggests that chromosome changes in the subfamily have not occurred uniformly during the evolutionary diversification of its genera (Cereali *et al*., 2008; Mariotto *et al*., 2011). In this context, although the phylogeny of *Chaetostoma* remains debated (Armbruster, 2004; Lujan *et al*., 2015a; Roxo *et al*., 2019) the genus is sister to all remaining Hypostominae, and the cytogenetic features of *C. bifurcum* are consistent with this interpretation. The species retains the ancestral diploid number proposed for Loricariidae (2n = 54) (Artoni, Bertollo, 2001), although this is not the most frequent chromosome number observed within Hypostominae (reviewed in Sassi *et al*., 2024). Further analyses of other *Chaetostoma* species within the genus are needed to determine whether this chromosome number is a general characteristic of the genus or a specific trait of *C. bifurcum*. Regarding sex chromosomes, none were detected in *C. bifurcum*, which is not unexpected given the apparent randomness of sex chromosome differentiation across Hypostominae even among closely related species (Glugoski *et al*., 2020).

Regarding the heterochromatin distribution, a conspicuous block was consistently observed on the short arm of chromosome pair 3. This block, clearly visible under conventional C-banding, may represent a species-specific cytogenetic feature. In other Ancistrini, extensive heterochromatic blocks have been linked to the accumulation of microsatellite repeats, such as $(AC)_{15}$ and $(GT)_{15}$, which are often interspersed within or adjacent to heterochromatic regions (Takagui *et al*., 2025). Moreover, certain microsatellites have been shown to interact with other repetitive DNA elements, such as rDNA sites, contributing to the establishment of evolutionary breakpoint regions that favor chromosomal rearrangements (Glugoski *et al*., 2018; Deon *et al*., 2022). Thus, we might hypothesize that the block observed in *C. bifurcum* reflects a

preferential accumulation of microsatellites in that region. This hypothesis warrants further investigation through the physical mapping of specific microsatellite motifs on the karyotype of this species.

Different mechanisms may explain the presence of the ITSs, detected in *C. bifurcum*, which are absent in other Hypostominae: (a) ITSs are often interpreted as remnants of chromosomal rearrangements such as fusions or inversions (Ocalewicz, 2013). In *C. bifurcum*, where the diploid number (2n = 54) is the highest reported within Ancistrini, a fusion origin can be reasonably excluded, as fusions typically lead to a reduction in chromosome number. However, chromosomal inversions, which do not alter the chromosome count, remain a plausible explanation. (b) Another possible mechanism involves the generation of interstitial telomeric repeats through DNA repair processes. Comparative analyses of vertebrate genomes have shown that these sequences can arise independently of chromosomal rearrangements, as part of double-strand break repair pathways (Faravelli *et al*., 2002; Teixeira *et al*., 2016; Sola *et al*., 2021). This mechanism could account for the random presence of ITSs across closely related species, and thus be compatible with the absence of ITSs in other Ancistrini. (c) An additional source of ITSs are chromosomal rearrangements mediated by satellite DNA (Garrido-Ramos *et al*., 1998), although no information is currently available on the satellite DNA content of *C. bifurcum*, limiting the evaluation of this hypothesis. Although the ITS signal observed in *C. bifurcum* is relatively strong, even stronger than telomeric signals in the terminal regions of other chromosomes, its evolutionary and functional significance remains speculative. It is also possible that in related species, the absence of detectable ITSs results from technical limitations in identifying short interstitial sequences (Lund *et al*., 2009; Downs *et al*., 2012). Together, these findings emphasize the need for further comparative cytogenetic and genomic studies to better understand the origin and relevance of these chromosomal features.

Finally, we reflect on the rDNA localization pattern, which has long been recognized as a significant cytotaxonomic marker (Venere *et al*., 2008). *Chaetostoma bifurcum* possesses a single chromosome pair bearing 18S rDNA, a feature observed in the vast majority of Hypostominae, Loricariidae, and other teleost groups (Gornung, 2013), along with a single 5S rDNA site located on a different chromosome. This arrangement deviates from the plesiomorphic syntenic condition proposed for Loricariidae (Ziemniczak *et al*., 2012) but aligns with a more derived condition observed in Hypostominae, where 18S and 5S rDNA loci are commonly located on distinct chromosomes. On the other hand, rDNA positioning is also influenced by NOR-associated heterochromatin remodeling, which can drive interchromosomal exchange (Gornung, 2013). This indicates that rDNA mapping does not simply reflect lineage history but may also be shaped by additional factors. For example, differences in rDNA localization may arise from adaptation to environmental conditions (Silva *et al*., 2019), allowing species to optimize protein synthesis in response to ecological pressures (Cayuela *et al*., 2020), or by regulating rRNA and ribosome production (Kenmochi *et al*., 2001; Symonová, 2019; Hori *et al*., 2023). Furthermore, the adaptive potential of rDNA extends to others, extra ribosomal, functions: it may act as an early indicator of genomic instability or stress (Salim, Gerton, 2019). Therefore, the rDNA localization pattern observed in *C. bifurcum* likely results from both evolutionary divergence and local adaptive responses.

Since Arai's (2011) comprehensive compilation, the number of karyotyped fish species has significantly increased, reflecting the growing application of cytogenetics in ichthyological research. This expansion has not only enhanced our understanding of chromosomal evolution across diverse lineages but has also proven instrumental in identifying cryptic species and uncovering hidden biodiversity (Cioffi *et al*., 2018). These advances underscore the continued significance of cytogenetic research in reconciling molecular and morphological methodologies, providing a more comprehensive understanding of the evolutionary history of teleost fishes.

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ETHICAL STATEMENT

All procedures executed within this study were conducted with the proper authorization granted by the Institutional Research Project (2024/UTMACH-PR-GEN-278). Furthermore, all activities involving experimental animals were conducted in strict accordance with the ethical standards outlined by the Universidad Técnica de Machala's Ethics Committee on Animal Experimentation, as indicated by the official process number UTMACH-CEEA-014/2024.

DATA AVAILABILITY STATEMENT

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

COMPETING INTERESTS

The authors declare no competing interests.

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