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First cytogenetic characterization of *Pseudoloricaria laeviuscula* (Valenciennes, 1840): a monotypic genus of Loricariidae (Loricariinae)

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ABSTRACT

This study presents the first cytogenetic characterization of *Pseudoloricaria laeviuscula*, a species widely distributed in the Amazon and Tocantins-Araguaia basins. Individuals collected from the Negro River, Amazonas, Brazil, exhibited a diploid chromosome number (2n) of 54, with a karyotypic formula of 14m + 10sm + 4st + 26a and a fundamental number of 82. The nucleolus organizer region (NOR) was interstitially located on a pair of subtelocentric chromosomes, confirmed via FISH using the 18S rDNA probe. Constitutive heterochromatin is preferentially located in centromeric regions, and 5S rDNA was mapped pericentromerically in a single acrocentric pair. The telomeric probe exclusively mapped the terminal regions of the chromosomes. The cytogenetic study revealed plesiomorphic characteristics of the Loricariidae family, such as the diploid number, NOR location and heterochromatin distribution. However, the localization of 18S and 5S rDNA sites on distinct chromosomes represents a derived feature. Comparative cytogenetic analysis of species in the *Loricariichthys* group indicates the occurrence of non-Robertsonian chromosomal rearrangements. Thus, the data from the present study expand our knowledge of the genus *Pseudoloricaria* and can serve as cytotaxonomic markers for a better understanding of the *Loricariichthys* group and its relationships within the subfamily Loricariinae.

KEYWORDS: Amazon, repetitive DNA, inversions, *Loricariichthys* group, rearrangements

Primeira caracterização citogenética de *Pseudoloricaria laeviuscula* (Valenciennes, 1840): um gênero monotípico de Loricariidae (Loricariinae)

RESUMO

Este estudo apresenta a primeira caracterização citogenética de *Pseudoloricaria laeviuscula*, espécie amplamente distribuída nas bacias Amazônica e Tocantins-Araguaia. Indivíduos coletados no Rio Negro, Amazonas, Brasil, exibiram um número cromossômico diploide de 2n=54, com fórmula cariotípica de 14m + 10sm + 4st + 26a e número fundamental igual a 82. A região organizadora do nucléolo (RON) localiza-se intersticialmente em um par de cromossomos subtelocêntricos, confirmada por FISH com a sonda de DNAr 18S. A heterocromatina constitutiva localiza-se preferencialmente em regiões centroméricas, e o DNAr 5S foi mapeado pericentromericamente em um único par acrocêntrico. A sonda telomérica mapeou exclusivamente as regiões terminais dos cromossomos. O estudo citogenético revelou características plesiomórficas da família Loricariidae, como o número diploide, a localização da RON e a distribuição da heterocromatina. No entanto, os sítios DNAr 18S e 5S se localizaram em cromossomos distintos, o que é uma característica derivada. A análise citogenética comparativa de espécies do grupo *Loricariichthys* indica a ocorrência de rearranjos cromossômicos não-Robertsonianos. Assim, os dados do presente estudo ampliam o nosso conhecimento sobre o gênero *Pseudoloricaria* e podem servir como marcadores citotaxonômicos para uma melhor compreensão do grupo *Loricariichthys* e suas relações dentro da subfamília Loricariinae.

PALAVRAS-CHAVE: Amazônia, DNA repetitivo, inversões, grupo *Loricariichthys*, rearranjos

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INTRODUCTION

Within the order Siluriformes, the family Loricariidae is the most diverse, and several studies have sought to elucidate its phylogenetic relationships (Montoya-Burgos *et al*. 1998; Schaefer 1998; Armbruster 2004; Cramer *et al*. 2011; Lujan *et al*. 2015; Covain *et al*. 2016; Roxo *et al*. 2019). Currently, with more than a thousand valid species, the family is organized into six subfamilies: Lithogeninae, Delturinae, Rhinelepinae, Loricariinae, Hypoptopomatinae and Hypostominae (Fricke *et al*. 2024).

The members of the Loricariinae, known as armored catfish, are distributed throughout the rivers of South America, from the affluents of La Prata River in the south to the coastal rivers of the Caribbean in the north (Ferraris Jr. 2003; Rapp Py-Daniel and Ohara 2013). Fishes of this subfamily are easily distinguished from other loricariids by their very depressed and elongated caudal peduncle, the absence of an adipose fin and the fact that they often have a depressed snout. In addition, they usually have variations in body shape, lip morphology and dentition (Covain and Fisch-Muller 2007; Rapp Py-Daniel and Ohara 2013). Nonetheless, considering the various taxonomic revisions in the systematics of Loricariinae, as evidenced by both morphological and molecular findings (Isbrücker 1979; Rapp Py-Daniel 1997; Covain *et al*. 2016; Londoño-Burbano and Reis 2021), the validity of several genera remains contentious, resulting in a degree of complexity within the taxonomy of this group (Covain *et al*. 2016; Londoño-Burbano and Reis 2021).

Covain *et al*. (2016) organized Loricariinae into two tribes: Harttiini and Loricariini. Within the tribe Loricariini, two subtribes were further delineated: Farlowelliina and Loricariina. Loricariina was diagnosed based on the dentition and structure of the lips, and it encompasses three morphological groups: *Loricaria-Pseudohemiodon*, *Loricariichthys,* and *Rineloricaria*.

The clade *Loricariichthys* comprises several genera, including *Loricariichthys* Bleeker 1862, *Furcodontichthys* Rapp Py-Daniel 1981, *Hemiodontichthys* Bleeker 1862, *Limatulichthys* Isbrücker & Nijssen 1979, and *Pseudoloricaria* Bleeker 1862. This clade is characterized predominantly by monotypic or poorly diversified genera (Covain and Fisch-Muller 2007; Covain *et al*. 2016). In this clade, the genus *Pseudoloricaria* has undergone some discussions. It was originally designated as a member of *Loricaria* Linnaeus 1758 with the species *Loricaria laeviuscula* Valenciennes, 1840, and was later established as *Pseudoloricaria* by Bleeker in 1862. At first, there were uncertainties regarding its validity as a genus or subgenus of *Loricaria*. Such doubts arose from the original description, which was made from a single specimen, without additional information on the collection locality. It was only known that it originated in South America (Bleeker 1862; Isbrücker and Nijssen 1976). Despite initial uncertainties, *Pseudoloricaria* is considered a valid genus that was redescribed and revised by Isbrücker and Nijssen (1976), who classified two species: *P. laeviuscula* and *Pseudoloricaria punctata* (Regan, 1904). Nonetheless, *P. punctata* was relocated to the genus *Limatulichthys* and it is currently referred to as the species *Limatulichthys petleyi* (Fowler 1940) (Covain and Fisch-Muller 2007). Thus, *Pseudoloricaria* is monotypic, containing only *P. laeviuscula*.

The true diversity of the genus *Pseudoloricaria* may be underestimated, since exclusive studies on the genus are scarce. Several decades after the studies of Isbrücker and Nijssen (1976), Ohara (2010) conducted a taxonomic review of the genera *Pseudoloricaria* and *Limatulichthys*, recognizing, based on samples from different locations in the Amazon basin, two new species within the genus: *Pseudoloricaria* sp. n. "mucajaí" and *Pseudoloricaria* sp. n. "madeira". Ohara's study was based only on morphological characters, and the author emphasized the need for more detailed investigations of the genus, demonstrating the possibility that *Pseudoloricaria* may not be monotypic. In other studies, it is possible to note the difficulty in identification, since some authors refer only to *Pseudoloricaria* sp. (Melo *et al*. 2004; Lujan *et al*. 2012) or *P.* aff. *laeviuscula* (Collins *et al*. 2015). Data are limited regarding distribution, and studies indicate the occurrence of the genus and *P. laeviuscula* in the Amazon and Tocantins-Araguaia basins (Covain and Sleen 2017; SiBBr 2024).

Cytogenetic studies regarding the *Loricariichthys* group remain limited, with existing data restricted to the genera *Loricariichthys* (Scavone and Júlio Jr. 1995; Fenocchio *et al*. 2003; Rodrigues 2010; Takagui *et al*. 2014, 2020) and *Hemiodontichthys* (Carvalho *et al*. 2018). The 2n within this group ranges between 46 and 56, with 2n=54 predominating. Heterochromatin is observed in a few discrete blocks, and the nucleolus organizer region (NOR) is characterized as simple (Scavone and Júlio Jr. 1995; Carvalho *et al*. 2018; Takagui *et al*. 2014, 2020). Additionally, the 18S and 5S ribosomal sites exhibit synteny in *Loricariichthys platymetopon* Isbrücker & Nijssen 1979, whereas they are located on different chromosomes in *Loricariichthys anus* (Valenciennes 1835) (Takagui *et al*. 2020). Considering the existing uncertainties surrounding the taxonomy and the deficiencies in the cytogenetic data for this subfamily, this study aimed to characterize *P. laeviuscula* through both conventional and molecular cytogenetic approaches to infer its evolutionary relationships within the *Loricariichthys* group.

MATERIAL AND METHODS

In this study, 20 individuals (seven males and thirteen females) of the species *Pseudoloricaria laeviuscula* (Figure 1) were collected manually using trawl nets, with nineteen obtained from the Cuieiras River (2°49'03.6" S, 60°29'09.6" W) and one individual from the Negro River, in the Anavilhanas

Figure 1. Specimen of *Pseudoloricaria laeviuscula*: **A**) Dorsal view, **B**) Ventral view (opened due to cytogenetic procedures), **C**) Lateral view (mirrored to the left, as it was the better-preserved side). Male individual measuring 260 mm, coloration in alcohol.

National Park (2°40'27.6" S 60°39'18.4" W), Amazonas, Brazil (Figure 2). The Brazilian Institute of the Environment and Renewable Resources (IBAMA) authorized the collections under a permanent license from the Biodiversity Information System (SISBio No. 28095-1). The experiments followed the ethical guidelines established by the National Council for the Control of Animal Experimentation (CONCEA) and were approved by the Ethics Committee for Animal Research at INPA (CEUA-INPA) under protocol number 030/2020, SEI 01280.001063/2020-07. The specimens were identified by Dra. Lúcia Helena Rapp Py-Daniel and deposited in the fish collection at INPA (INPA-ICT 059686 and INPA-ICT 060695).

Mitotic chromosomes were obtained according to the protocol of Gold *et al*. (1990). The heterochromatin pattern was determined using C-banding (Sumner 1972; Lui *et al*. 2012), and the nucleolus organizer regions were located via impregnation with silver nitrate (Ag-NOR) (Howell and Black 1980).

Genomic DNA extraction was performed using the muscle tissue and liver of the species under study, which were preserved in 100% ethanol, using the Wizard® extraction kit (Promega), following the manufacturer's recommendations. The repetitive sequences 18S and 5S rDNA, used as probes, were isolated via PCR, using the primers: 18Sf (5'-CCG CTT TGG TGA CTC TTG AT-3') and 18Sr (5'-CCG AGG ACC TCA CTA AAC CA-3') (Gross *et al*. 2010); 5Sf (5'-TAC GCC CGA TCT CGT CCG ATC-3') and 5Sr (5'- CAG GCT GGT ATG GCC GTA AGC-3') (Martins and Galetti Jr. 1999). For the detection of telomeric sequences, the primers (TTAGGG)5 and (CCCTAA)5 were used (Ijdo *et al*. 1991). The PCR products were labeled using nick translation with the labeling kit dUTP-ATTO-550 (red) for 18S rDNA and the telomeric sequence and dUTP-ATTO-488 (green) for 5S rDNA, following the manufacturer's instructions (Jena Bioscience). Fluorescent *in situ* hybridization (FISH) followed the protocol of Pinkel *et al*. (1986). The slides containing the chromosomes were denatured with 70% formamide and hybridized at 37 °C, overnight, with 77% stringency. Metaphases were stained with DAPI (0.8 ng/µL) in an antifading reagent (Vectashield®).

The slides that used fluorochromes (C-bands and FISH) were analyzed under an epifluorescence photomicroscope (Olympus, BX-51) with an appropriate filter. At least 30 metaphases per individual were analyzed, and the best ones had their image captured using the DPController image capture system and were processed using the DPManager program. To assemble the karyotypes, Adobe Photoshop 7.0 (version CS6) was used, via which the chromosomes in mitotic metaphase were cut, paired, measured in the DPManager program, and placed in descending order of size. The chromosomes were classified according to Levan *et al*. (1964).

RESULTS

Pseudoloricaria laeviuscula showed a diploid number equal to 54 chromosomes (14m + 10sm + 4st + 26a) and the fundamental number (FN) was equal to 82, with no evidence of chromosomal sexual heteromorphism (Figure 3a).

C-banding revealed blocks of constitutive heterochromatin in the pericentromeric and centromeric regions of most

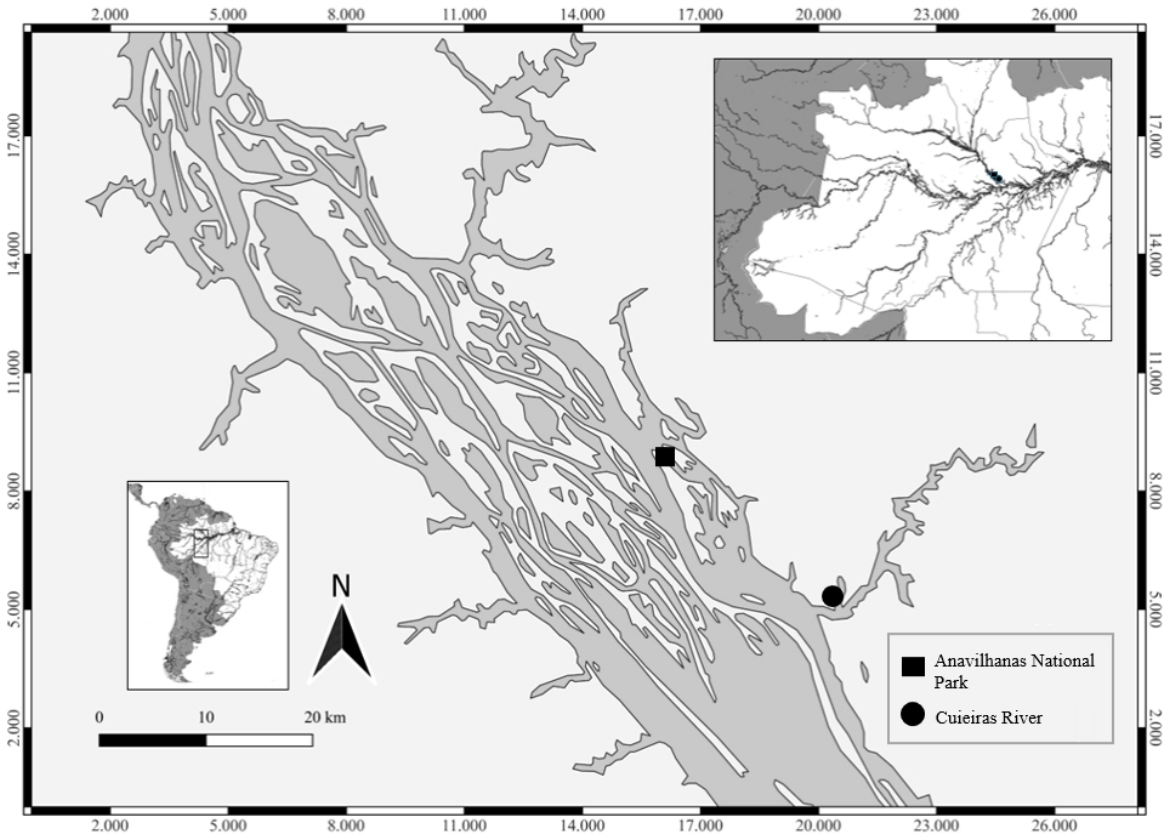

Figure 2. Sampling sites: in the circle, the Cuieiras River; in the square, the Anavilhanas National Park, both located in the state of Amazonas, Brazil.

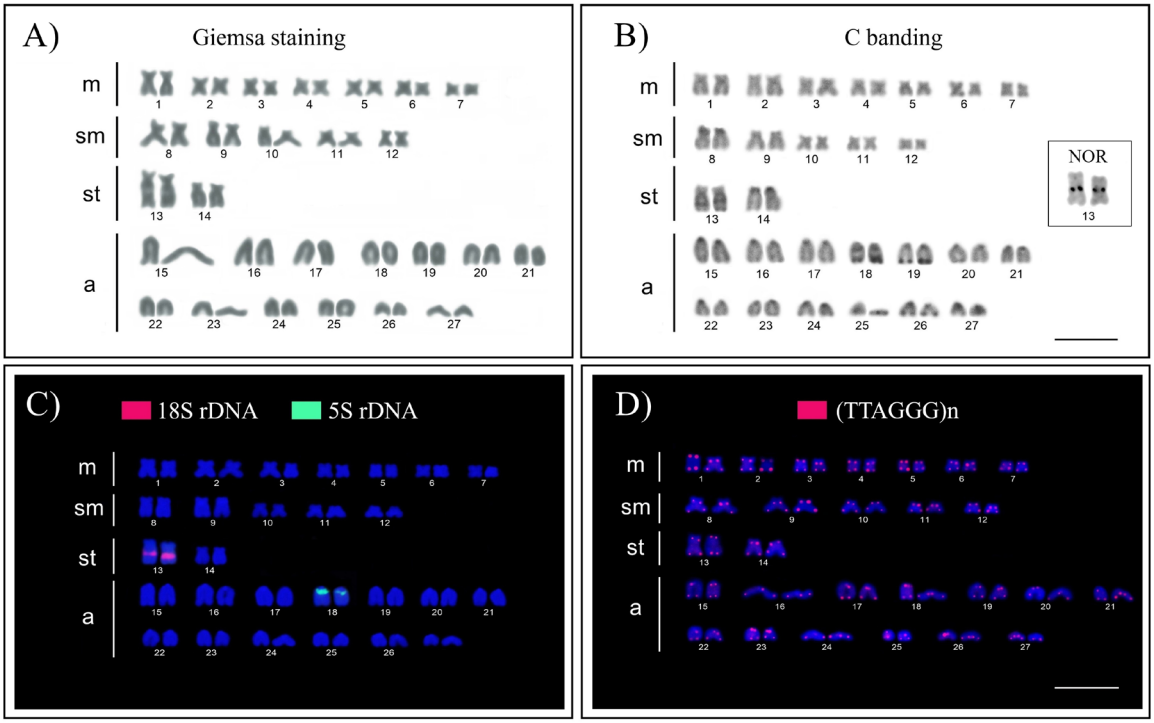

Figure 3. Karyotype of *Pseudoloricaria laeviuscula*: **A**) conventional staining with Giemsa; **B**) C-banding; Ag-NOR highlighted in pair 13; **C**) chromosome mapping using fluorescent in situ hybridization (FISH) of the 18S rDNA (red) and 5S rDNA (green); and **D**) telomeric sequence (red). Bar equal to 20 µm.

chromosomes. Additionally, a pair with heterochromatic short arms (pair 8), an interstitial block in the long arm (pair 13), and terminal markings in the long arms of pairs 18, 19 and 26 were observed (Figure 3b), highlighting conspicuous blocks in the centromeric region of pair 27. The NOR was evidenced in the long arms, in the interstitial position of pair 13, coincident with the secondary constriction and the C^+ band (Figure 3b) and confirmed via FISH with the 18S rDNA probe (Figure 3c), while the 5S rDNA site is in pair 18, in a pericentromeric position, coincident with heterochromatin (Figure 3c). The telomeric sequence was observed only in the terminal portions of all the chromosomes (Figure 3d).

DISCUSSION

The karyotype analysis of *P. laeviuscula* reinforces the idea of a plesiomorphic chromosomal arrangement in the family, which is characterized by a diploid number of 54 chromosomes. This chromosomal configuration is similarly observed in species of the genera *Harttia* Steindachner 1877 and *Loricariichthys* (Scavone and Júlio Jr. 1995; Blanco *et al*. 2017; Takagui *et al*. 2014, 2020; Sassi *et al*. 2020, 2021). Species of the other genera of Loricariinae present diverse karyotypic macrostructures, which result from Robertsonian and non-Robertsonian rearrangements (Rosa *et al*. 2012; Ferreira *et al*. 2014; Primo *et al*. 2016; Glugoski *et al*. 2018, 2023). Some studies consider 2n=54 to be the ancestral number, i.e., plesiomorphic of Loricariidae. This assertion is based on the observation that this chromosomal number occurs in species belonging to the sister group Trichomycteridae and the subfamilies Hypostominae, Hypoptopomatinae and Loricariinae (Artoni and Bertollo 2001; Kavalco *et al*. 2005; Alves *et al*. 2012; Ziemniczak *et al*. 2012; Takagui *et al*. 2020).

Within Loricariinae, the species belonging to the morphological group *Loricariichthys* have a predominance of 2n=54. An exception is noted for *Hemiodontichthys acipenserinus* (Kner 1853), which has been reported to possess two diploid numbers, specifically 2n=46 and 2n=58 (Carvalho *et al*. 2018). Additionally, *Loricariichthys maculatus* (Bloch 1794) is characterized by a 2n=56 (Fenocchio *et al*. 2003). Furthermore, *P. laeviuscula*, which also has a diploid number of 54, shares the same FN (82) as *L. anus* and *L. platymetopon* (Scavone and Júlio Jr. 1995; Takagui *et al*. 2014, 2020). However, the karyotypic formulas differ, indicating the presence of non-Robertsonian rearrangements. A striking difference is the presence of subtelocentric chromosomes in *P. laeviuscula*, which is absent in *L. anus* (Takagui *et al*. 2014, 2020), and in certain populations of *L. platymetopon* (Takagui *et al*. 2014, 2020). In contrast, a predominance of a higher number of acrocentric chromosomes is observed in all species of this group.

Considering that the ancestral diploid number for Loricariidae is established as 2n=54, pericentric inversions are believed to have played a significant role in the karyotypic diversification of the *Loricariichthys* group, thus originating different karyotypic formulas, but preserving the diploid number at 54 in the species *L. anus* (Takagui *et al*. 2014, 2020), *L. platymetopon* (Scavone and Júlio Jr. 1995; Takagui *et al*. 2014, 2020) and *P. laeviuscula*. On the other hand, centric fissions are thought to have resulted in karyotypes with 2n=56 in *L. maculatus* (Fenocchio *et al*. 2003) and 2n=58 in *H. acipenserinus* (Carvalho *et al*. 2018). Additionally, the fusion events are believed to give rise to the karyotype with 2n<54 in *H. acipenserinus* (2n=46) (Carvalho *et al*. 2018).

The heterochromatic pattern found in *P. laeviuscula* exhibits similarities to that which is observed for most of Loricariinae, characterized by a limited number of small heterochromatic blocks. This configuration is also considered plesiomorphic in Loricariidae, as evidenced in species of the genera *Harttia*, *Loricariichthys* and *Rineloricaria* Bleeker 1862, among others (Kavalco *et al*. 2005; Ziemniczak *et al*. 2012; Blanco *et al*. 2017; Takagui *et al*. 2014, 2020; Sassi *et al*. 2020). However, despite the few blocks, the distribution of heterochromatin in these species reveals distinct chromosomal characteristics, with each species presenting a unique and exclusive pattern, indicating the occurrence of chromosomal rearrangements. For instance, in *L. anus*, two populations sharing the same diploid number (2n = 54) and the same FN (82) have been found to exhibit pericentric inversions involving both metacentric and acrocentric chromosomes. These inversions, detected by the heterochromatin distribution pattern, account for the variations observed in karyotypic formulas within the same species (Takagui *et al*. 2014).

In *P. laeviuscula*, the presence of terminal heterochromatic blocks in three pairs (18, 19 and 26, acrocentric) corresponds to the pattern also found in some species of the clades Harttiini and Farlowellina, which are considered basal clades of Loricariinae (Kavalco *et al*. 2004; Fernandes *et al*. 2015, 2021; Blanco *et al*. 2014, 2017; Marajó *et al*. 2018; Sassi *et al*. 2021). This observation suggests that, despite *P. laeviuscula* being classified within a derived clade according to the phylogenetic analysis of Covain *et al*. (2016), it may still retain karyotype characteristics that are reminiscent of the basal groups of the subfamily.

Another character considered plesiomorphic in the family is the simple and interstitial NOR (Oliveira and Gosztonyi 2000; Kavalco *et al*. 2005; Alves *et al*. 2012; Ziemniczak *et al*. 2012). This character is also present in *P. laeviuscula*. However, there is a variation in the chromosome location and karyotype position of the NOR among species within the *Loricariichthys* group. Specifically, in *P. laeviuscula*, the NOR was observed in a subtelocentric chromosome pair, whereas in *L. anus*, and *L. platymetopon* (Takagui *et al*. 2014, 2020), the NOR is located on acrocentric chromosomes. This pattern is similar to that found in species of the clade Harttiini (Centofante *et al*. 2006; Blanco *et al*. 2012, 2014, 2017).

Nonetheless, when comparing the nucleolar pair of *P. laeviuscula* in relation to the characteristics of the *Loricariichthys* group, it is possible to suggest that the presence of an interstitial NOR in a subtelocentric pair may be a consequence of a pericentric inversion. This hypothesis is supported by the findings in some species of *Harttia*, such as *H. longipinna* Langeani, Oyakawa & Montoya-Burgos 2001, *H. gracilis* Oyakawa 1993, *H. punctata* Rapp Py-Daniel & Oliveira 2001, *H. torrenticola* Oyakawa 1993, and *H. carvalhoi* Miranda Ribeiro 1939, in which the NOR is located in the proximal region of the first pair of acrocentric chromosomes (Centofante *et al*. 2006; Blanco *et al*. 2012, 2014, 2017). In contrast, in *H. absaberi* Oyakawa, Fichberg & Langeani 2013 (Rodrigues 2010) and *H. kronei* Miranda Ribeiro 1908 (Blanco *et al*. 2017), the NOR is located on metacentric chromosomes. This difference in the NOR pattern is suggested to result from pericentric inversions.

In contrast to the syntenic arrangement of 18S and 5S rDNA, which is considered plesiomorphic within Loricariidae (Mariotto *et al*. 2011; Ziemniczak *et al*. 2012; Blanco *et al*. 2017; Takagui *et al*. 2020), and observed in species of *Harttia* and *Farlowella* Eigenmann & Eigenmann 1889 (Centofante *et al*. 2006; Blanco *et al*. 2013, 2017; Deon *et al*. 2020; Fernandes *et al*. 2021), *P. laeviuscula* exhibits the presence of 18S and 5S rDNA sites on distinct chromosomal pairs, indicating a derived character. The non-synteny of ribosomal genes in fishes is regarded as an evolutionary benefit, as it prevents the potential for detrimental rearrangements between these sites (Martins and Galetti Jr. 1999, 2001). Our results indicate that the 18S and 5S rDNA sites are associated with constitutive heterochromatin. This association may serve to protect these gene sequences from selective pressures, facilitating the differential evolution of various genomic regions (Gross *et al*. 2010; Favarato *et al*. 2019).

The 5S rDNA site in *P. laeviuscula* exhibits a simple configuration, located in a mid-acrocentric pair in the pericentromeric region. This pattern contrasts with those found in other genera of Loricariinae. In this subfamily, the 5S rDNA demonstrated considerable variability, both in the number of sites and in their chromosomes. It can be simple, as in *P. laeviuscula* (present work) and *H. kronei* (Blanco *et al*. 2017), or as multiple sites, as evidenced in *L. platymetopon*, *Rineloricaria cadeae* (Hensel 1868), *Rineloricaria pentamaculata* Langeani & de Araujo 1994 and *Farlowella hahni* Meinken 1937 (Porto *et al.* 2011; Takagui *et al*. 2020; Fernandes *et al.* 2021; Venturelli *et al*. 2021). In the context of Loricariinae, the 5S rDNA is significantly more variable and unstable compared to the 18S rDNA, which exhibits relative conservation in the number of sites. This characteristic suggests that the 5S rDNA may serve as a cytotaxonomic marker for *P. laeviuscula* and other species within Loricariinae. This marker has previously been employed to separate species of *Rineloricaria*, which are characterized by considerable karyotypic diversity (Venturelli *et al*. 2021). Regarding the telomeric sequence (TTAGGG)n, *P. laeviuscula* showed sites that are exclusively located in the terminal regions of its chromosomes.

Thus, cytogenetic analysis reveals that *P. laeviuscula* is a species with most of its chromosomal characteristics conserved, and the data presented here corroborate that this species is more closely related to the *Loricariichthys* group than to the *Loricaria-Pseudohemiodon* group (Rapp Py-Daniel 1997; Covain *et al*. 2016; Roxo *et al*. 2019). The diploid number found for Loricaria is 64 (Porto *et al*. 2014; Benitez *et al*. 2016; Takagui *et al*. 2020), while for the *Loricariichthys* group it is predominantly 2n=54 (Scavone and Júlio Jr. 1995; Rodrigues 2010; Takagui *et al*. 2014, 2020). This fact should be considered when discussing its taxonomic position in Loricariinae. Thus, we believe that *Pseudoloricaria* may still have its diversity underestimated, considering the possible new species (Ohara 2010) and uncertainties in identification (Melo *et al*. 2004; Lujan *et al*. 2012; Collins *et al*. 2015).

CONCLUSIONS

The analysis of the karyotypic macrostructure of *Pseudoloricaria laeviuscula* revealed the conservation of the diploid number (2n=54), the C-banding pattern and the NOR localization, placing the species in a plesiomorphic context within the family Loricariidae. However, the location of the 18S and 5S rDNA sites on distinct chromosomes represents a derived characteristic of the family. This study establishes a basis for future research, suggesting an integrative taxonomic revision across the entire distribution of the genus.

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