Cytogenetics of two *Farlowella* species (Loricariidae: Loricariinae): implications on the taxonomic status of the species

Leandro Marajó¹, Patrik F. Viana¹, Milena Ferreira¹, Lúcia H. Rapp Py-Daniel² and Eliana Feldberg¹

*Farlowella* is one of the most diverse genera of the Loricariinae, restricted to South America rivers. The taxonomic and phylogenetic relationships among its species are contentious and, while genetic studies would contribute to the understanding of their relationships, the only available datum refer to the karyotype description of only one species. In the present study two Amazonian species, *Farlowella cf. amazonum* and *F. schreitmuelleri*, were analyzed using conventional and molecular cytogenetic procedures. Both species had diploid chromosome number 58, but different fundamental numbers (NF) 116 and 112, respectively, indicative of chromosomal rearrangements. C-banding is almost poor, especially in *F. cf. amazonum*, and occurs predominantly in the centromeric and in some telomeric regions, although genome of *F. schreitmuelleri* possessed a much larger heterochromatin amount then those of *F. cf. amazonum*. The chromosomes bearing the NOR sites were likely the same for both species, corresponding to the 1⁰ metacentric pair in *F. cf. amazonum* and to the 28⁰ acrocentric in *F. schreitmuelleri*. The location of the 5S rDNA was species-specific marker. This study expanded the available cytogenetic data for *Farlowella* species and pointed the remarkable karyotype diversity among species/populations, indicating a possible species complex within genus.

**Key words:** Amazon region, Chromosomal markers, Karyotypic characterization, Ribosomal DNA, Twig catfish.

*Farlowella* é um dos gêneros mais diversos de Loricariinae, restrito aos rios da América do Sul. As relações taxonômicas e filogenéticas entre suas espécies são contenciosas e, enquanto os estudos genéticos contribuem para a compreensão dessas relações, o único dado disponível refere-se à descrição cariotípica de apenas uma espécie. No presente estudo, foram analisadas duas espécies amazônicas *Farlowella cf. amazonum* e *F. schreitmuelleri*, empregando procedimentos citogenéticos convencionais e moleculares. Ambas as espécies apresentaram número diploide igual a 58 cromossomos, mas com números fundamentais diferentes (NF) de 116 e 112, respectivamente, indicando rearranjos cromossômicos. Bandas C são poucas, especialmente em *F. cf. amazonum*, e ocorrem predominantemente nas regiões centroméricas e em algumas regiões teloméricas, embora *F. schreitmuelleri* apresenta uma quantidade de heterocromatina muito maior que *F. cf. amazonum*. Os cromossomos carreadores dos sítios da NOR foram provavelmente os mesmos para ambas as espécies, correspondo ao primeiro par metacentrico em *F. cf. amazonum* e ao 28º acrocentrico em *F. schreitmuelleri*. A localização do DNAr 5S foi espécie-específico. Este estudo expandiu os dados citogenéticos disponíveis para espécies de *Farlowella* e apontou uma remarcável diversidade cromossômica entre espécies/populações, indicando um possível complexo de espécies neste gênero.

**Palavras-chave:** Bagre-vara, Caracterização cariotípica, DNA ribossômico, Marcadores cromossômicos, Região amazônica.

**Introduction**

The Loricariidae is one of the world’s most diverse families of freshwater fish (Vani, Malabarba, 1998; Nelson et al., 2016; Reis et al., 2016). Endemic to the Neotropical region, approximately 970 valid loricariid species are known to exist, in eight recognized subfamilies, the Lithogeneinae, Delturninae, Rhinelepisinae, Hypoptopomatinae, Neoplecostomiinae, Hypostominae, Otothyrinae, and Loricariinae, and two incertae sedis lineages (Chiachio et al., 2008; Roxo et al., 2014; Lujan et al., 2015; Reis et al., 2016; Eschnmeyer, Fong, 2017) where Rhinelepisinae and Otothyrinae were created based on molecular data only.

The Loricariinae is a group of armored catfishes divided into two tribes, Harttiini and Loricariini (Covain et al., 2016), with a total of 34 recognized genera, of which, 15 are

¹Laboratório de Genética Animal, Programa de Pós-Graduação em Genética, Conservação e Evolução, Instituto Nacional de Pesquisas da Amazônia, Av. André Araújo 2936, Petrópolis, 69067-375 Manaus, AM, Brazil. (LM) biologomarajo@hotmail.com, https://orcid.org/0000-0002-4788-112X (corresponding author) (PFV) patrik.biologia@gmail.com (MF) milena_fro@hotmail.com, (EF) feldberg@inpa.gov.br

²Coleção de Peixes, Coordenação de Biodiversidade, Instituto Nacional de Pesquisas da Amazônia, Av. André Araújo, 2936, Petrópolis, 69067-375 Manaus, AM, Brazil. lucia.rapp@gmail.com

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monotypic. The genera *Loricaria* (17 species), *Loricariichthys* (18 species), *Hartia* (23 species), *Farlowella* (29 species), and *Rineloricaria* (63 species) are the most diverse, but also the most contentious in taxonomic and phylogenetic terms (Covain, Fisch-Muller, 2007; Ballen et al., 2016).

The species of the genus *Farlowella* are widely distributed in the rivers of South America, including the Amazon, Orinoco, Paraná/Paraguay, and Essequibo basins, and the coastal basins of the Guianas (Ferraris, 2003). These catfish are relatively small, reaching a length of only 26.5 cm, with an extremely thin and elongated body, a bony snout, and prominent tail filaments. The elongated shape and wood-like appearance of these animals has earned them the common name of twig catfish, and their unique morphology makes them extremely popular as ornamental fish (Berra, 2001; Ferraris, 2003; Covain, Fisch-Muller, 2007).

Cytogenetic studies in *Farlowella* species are still scarce. Only one of the 29 recognized species, *F. amazonum*, has been analyzed up to now, with individuals from two locations on the Paraguayan River being studied using conventional cytogenetic procedures. In the present study, we describe the karyotype and other chromosomal markers of two Amazonian species of the genus *Farlowella*.

**Material and Methods**

**Sampling sites.** We analyzed five individuals of *Farlowella cf. amazonum* from the paraná do Piloto in the municipality of Barcelos, Amazonas, Brazil, Negro River basin (0°56’04.8”S, 62°58’01.6”W). We also analyzed 14 individuals of *Farlowella schreitmuelleri* collected from the Jundiá Stream in the municipality of Manaus, Amazonas, Brazil, Cuieiras River basin (2°19’43.8”S, 60°04’40.4”W).

The collection of individuals was authorized by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA: Instituto Brasileiro do Meio Ambiente and Recursos Naturais Renováveis) through SISBIO license number 28095-1. Voucher specimens were deposited in the fish collection of the Instituto Nacional de Pesquisas da Amazonia (INPA-ICT 057606, INPA-ICT 057615). This study followed the ethical guidelines established by the INPA Ethics Committee for Animal Research, which approved the research through protocol number 006/2016.

**Cytogenetic analyses.** The mitotic chromosomes were obtained using the *in vitro* protocol of Gold et al. (1990), with the RPMI medium (Cultilab). The chromosomes were stained with Giemsa to determine the diploid number and the chromosome morphology. The chromosomes were classified according to Levan et al. (1964). Were applied the C-banding technique (Sumner, 1972) to characterize the heterochromatic band pattern, and silver nitrate impregnation (Ag-NOR) to visualize the nucleolus organizer regions (Howell, Black, 1980).

The probes used to locate the ribosomal genes were obtained by extracting the total DNA from the *Farlowella* muscle tissue using a Wizard® Genomic Purification kit (Promega), according to the manufacturer’s protocol. We used a GoTaq Colorless Master Mix kit (Promega) for the Polymerase Chain Reaction (PCR), and the amplification of the 18S rDNA (Gross et al., 2010) and 5S rDNA (Martins, Galetti, 1999) genes, and the (TTAGGG)n telomeric sequences (Ijdo et al., 1991). The fluorescence *in situ* hybridization (FISH) was based on Pinkel et al. (1986), with modifications. The 18S rDNA and telomeric probes were marked with digoxigenin-11 dUTP using a DIG-Nick Translation Mix kit (Roche), while the 5S rDNA probe was marked with biotin-14-dATP using a Biotin-Nick Translation Mix kit (Roche). The FISH had a stringency of 77%. We counterstained the chromosomes with 4’, 6-5 diamidino-2-phenylindole dihydrochloride (DAPI, 2 mg/ml) in VECTAShIELD® Mounting Media (Vector).

**Results**

*Farlowella cf. amazonum* (Günther, 1864). *Farlowella cf. amazonum* had diploid chromosome number 58 and karyotype composed of 14metacentric+30submetacentric+14subtelocentric, with fundamental number (FN) = 116 (Fig. 1a). No evidence was found of the presence of differentiated sex. The first chromosome pair had a secondary constriction on the long arm with a subtle difference between the two homologs. This region corresponded to the NOR (Fig. 1c) and the 18S rDNA sites (Fig. 2c).

C-bands are pale, distributed mainly in the centromeric position, although differing in amount among chromosomes. Additionally, some other conspicuous signals were also found, such as in the long arms of the 1st pair and the short arms of the 20th pair that were completely heterochromatic, and in both telomeric regions of pairs No. 13, 21, 26, 27, and 28 (Fig. 1b).

Hybridization with the 5S rDNA probe revealed signals only on the short arms of sm pair No. 20, which was completely heterochromatic (Fig. 2d). The mapping of the telomeric sequences revealed signals in the terminal regions of all the chromosomes, in addition to an accumulation of these sequences in the terminal portion of the long arms of pairs No. 16, 19, 21, 22, 26 and 27, no Interstitial Telomeric Sequences (ITS) were observed (Fig. 2a).

*Farlowella schreitmuelleri* Arnold, 1936. *Farlowella schreitmuelleri* also had a diploid chromosome number 58 and karyotype composed of 10m+30m+14st+4a, and FN = 112 (Fig. 1d). A secondary constriction was observed in the distal region of the long arms of the acrocentric pair No. 28, together with size heteromorphism, corresponding to the Ag-NOR (Fig. 1f), the ribosomal 18S DNA (Fig. 2e), and positive C-banding sites.

The genome of this species possessed a much larger quantity of heterochromatin compared to *Farlowella cf. amazonum*, located in the centromeric region of all chromosomes, where some bands were more conspicuous than others. Inter-
stitial bands also occurred in the long arms of pairs Nos. 7, 9, 14, 22, and 23. Pairs Nos. 8, 24, 25, and 28 also had conspicuous bands in the terminal region of the long arms (Fig. 1e).

Multiple 5S rDNA sites were observed, in sm pair No. 16 (in only one of the homologs) and st pair No. 26. Both these sites were positive for C-banding (Fig. 2f). Hybridization with telomeric probes revealed terminal signals in all chromosomes and Interstitial Telomeric Sequences (ITSs) in pairs Nos. 7, 14, 25, and 28, all of which corresponded with heterochromatin blocks. In pair No. 28, the ITS was observed in only one of the homologs (Fig. 2b).

**Discussion**

The hypothetical ancestral 2n suggested for the Loricariinae is 2n = 54 (Artoni, Bertollo, 2001). However, the
Loricariinae is characterized by a high degree of chromosomal diversity, in terms of both 2n and karyotype structure, as exemplified by the genus *Rineloricaria*, which has 2n varying from 36 to 70, indicating a large number of chromosomal rearrangements (Giuliano-Caetano, 1998; Alves et al., 2003; Mendes-Neto, 2008; Maia et al., 2010; Rodrigues, 2010; Porto et al., 2011; 2014; Rosa et al., 2012; Venturelli, 2014). While scarce, the cytogenetic data available for the genus *Farlowella* indicate a relative uniformity of the 2n = 58 chromosomes. However, the chromosomal markers, i.e., Ag-NOR sites, C-banding pattern, and karyotypes, does vary considerably among populations (Gindri, 2009; Fernandes et al., 2012; 2015; present study).

Three different karyotypes have been described in *Farlowella amazonum* from the Paraguay River basin, of Mato Grosso do Sul: 18m+20sm+12st+8a from the Ribeirão Stream, a tributary of the Taquari River (Gindri, 2009); 6m+38sm+8st+6a from the Água Boa Stream on the Iguatemi River (Fernandes et al., 2012); and 12m+30sm+10st+6a from the Dourado Stream, also in the Iguatemi basin (Fernandes et al., 2015). In the present study, we described a fourth karyotype from the Negro River, in the Amazon basin.

However, the fourth karyotype was identified as *F. cf. amazonum*, and is distinct from the *F. amazonum* populations of the Paraguay basin, due primarily to the absence of acrocentric chromosomes, although the karyotype of the populations from the Paraguay basin is highly similar to that described for *F. schreitmuelleri*, given that it has an acrocentric pair, which contains Ag-NOR sites. This pair seems to be homeologous between these species/populations.

A number of chromosomal rearrangements are found in the genomes of *Farlowella* species analyzed in the present study, given that, while they have the same 2n, the FN value and the karyotypes are different. The reduction of the number of metacentric chromosomes and the appearance of acrocentric chromosomes in genome of *F. schreitmuelleri* indicates a probably process of pericentric inversion, in which the 1st metacentric pair (pair No. 1) of *F. cf. amazonum* would have undergone a pericentric inversion to become an acrocentric, corresponding to the 1st acrocentric pair of *F. schreitmuelleri* (pair No. 28), or vice versa.

This proposed chromosomal rearrangement is supported by the presence of a secondary constriction and other cytogenetic markers (NOR, C-banding and the 18S rDNA) in these chromosome pairs. In addition, the karyotypes of two species have the same number of sm and st chromosomes, which are consistent with the view that centric fusion and fission events, associated with the inversions, played an important role in the karyotype diversification observed in *Farlowella* in our study, and in the Loricariinae in general (Giuliano-Caetano, 1998; Alves et al., 2003; Rosa et al., 2012; Porto et al., 2014; Takagui et al., 2014; Blanco et al., 2017; present study).

Most of the species of *Farlowella* present reduced vagility and inhabit specific portions of the river, which may lead to the formation of small, isolated populations, in which independent processes of genetic differentiation may result in speciation. This type of process has been observed in both sympatric and allopatric populations of *Bunocephalus coreodeus*, for example, which presents innumerable cytotypes, resulting from chromosomal rearrangements fixed in small populations (Ferreira et al., 2017).

The morphological parameters of the individuals examined in the present study are not fully consistent with those of *F. amazonum*. This implies two possibilities, that either (i) the specimens identified as *F. cf. amazonum* in the present study are in fact representatives of a distinct species or (ii) the *F. amazonum* specimens analyzed by Gindri (2009) and Fernandes et al. (2011; 2014) are actual members of a different species. In particular, Retzer, Page (1997) considered *Farlowella amazonum* (Günther, 1864), *F. gladiolus* (Günther, 1864), *F. carinata* Garman, 1889, *F. oliveira* Miranda-Ribeiro, 1939, *F. paraense* Meinken, 1937 (incorrectly spelled paranaensis), *F. pleurotaenia* Miranda-Ribeiro, 1939, and *F. pseudogladiolus* Steindachner, 1910 to be junior synonyms of *F. amazonum*.

*Farlowella amazonum* was described from Santarém, Pará state, Brazil. *Farlowella paraense* is the only junior synonym of *F. amazonum* from the Paraguay River, whereas all the others are from the Amazon basin. That certainly raises questions about the correct identity of the species found in the Paraguay River. Retzer (in Retzer, Page, 1997) remarks that he only examined one specimen of *F. paraense* in Museu de Zoologia da USP – MZUSP (from the upper Paraguay River). Based on the karyotype diversity observed so far, we are most certainly dealing with different species. However, the taxonomy of this genus is not well resolved, and detailed comparative morphometric studies are needed to understand the variation observed in *F. amazonum*.

In the *Farlowella* species analyzed up to now, the NOR phenotype is simple, as confirmed by the 18S rDNA probe (Fernandes et al., 2011, 2014; present study). A single chromosome pair with NOR sites is thought to be a plesiomorphic characteristic of the Loricariinae (Ziemniczak et al., 2012).

While there is a certain amount of consistency in the number of NOR sites, there is considerable variation in the position and types of chromosome that carry these sites in the different *Farlowella* species/populations. In the karyotype of *Farlowella cf. amazonum*, the NOR site is located in the pericentromeric region of the long arms of the 1st metacentric pair, whereas in those of *F. schreitmuelleri* and the other species described in the literature, the NORs are found on the long arms of the 1st acrocentric pair, which appears to be a conserved pattern in this genus. The presence of these sites in these chromosome pairs reinforces the conclusion that the origin of the different karyotypes in the *Farlowella* species/populations has originated from pericentric inversions.

In the Loricariinae, most of the cytogenetic studies that have focused on the physical mapping of the 18S ribosomal gene have demonstrated the presence of these sequences in a single chromosome pair, which has thus been identi-
fied as a basal character for this group, given that in all the Harttia species analyzed, the 18S rDNA was restricted to a single pair (Kavalco et al., 2005; Centofante et al., 2006; Rodrigues, 2010; Blanco et al., 2017). By contrast, the 5S rDNA are much more variable in number and location (Venturelli, 2014; Blanco et al., 2017).

In Farlowella cf. amazonum, the 5S rDNA is located in a single chromosome pair, whereas F. schreitmuelleri had three signals, including one site located on one of the homologs of the submetacentric pair No. 16, and the other two in subtelocentric pair No. 26. The absence of a 5S rDNA site on one of the homologs of pair 16 may have been the result of unequal crossing-over or the result of the displacement of the ribosomal sequence from pair 26 by mobile transposable elements, as observed in a number of different fish groups (Gross et al., 2010; Ferreira et al., 2011; Silva et al., 2011; 2016; Schneider et al., 2013).

In both species studied here, the 5S rDNA and 18S rDNA sites were associated with constitutive heterochromatin. The presence of these sequences in heterochromatic regions, and their association with transposable elements, were considered by Silva et al. (2014) to represent “hotspots” of chromosomal rearrangement, given that repetitive segments are more susceptible to rearrangement, due to their intrinsic structural organization (Ferreira et al., 2014). This implies that the heterochromatin is an important element for the diversification of the genome, in particular in isolated populations (Elgin, 1996; Schneider et al., 2013). In the present study, C-banding is almost poor, especially in F. cf. amazonum, and occurs an accentuated accumulation of heterochromatin in F. schreitmuelleri, with large, conspicuous bands, found in a number of different chromosome pairs was observed.

Comparing the distribution of the heterochromatin in genomes of the different Farlowella species, it is clear that Farlowella cf. amazonum (present study), F. amazonum (Fernandes et al., 2015), and F. amazonum (Gindri, 2009) have less heterochromatin in comparison with F. schreitmuelleri. The distribution of the C-bands in F. cf. amazonum is also different from the patterns described by Gindri (2009) and Fernandes et al. (2015), with a much larger quantity of heterochromatin found in the former one. These differences may be associated with environmental factors, given that different environmental stressors may mediate epigenetic processes of heterochromatin regulation, although it seems more likely that these differences are related to the lack of migration in these species, which favors the accumulation of differential sequences in isolated populations (Guimarães et al., 2017).

Present study performed the first physical mapping of the telomeric DNA in chromosomes of Farlowella. Whereas a stronger signal was recorded in some chromosome pairs in Farlowella cf. amazonum, F. schreitmuelleri presented more homogeneous telomeric signals, but also ITSs, which were not recorded in F. cf. amazonum. This variation in the intensity of the telomeric DNA signals is generally related to the variation in the length of the telomeric sequences (Pathak et al., 1994, 1996; Multani et al., 1999) and, the presence of ITSs may indicate the past occurrence of rearrangements such as fusions, inversions and translocations, during the genome evolution of this genus (Holmquist, Dancis, 1979; Hastie, Allshire, 1989; Meyne et al., 1990).

ITSs have been recorded in a number of different loricarid genera, including Harttia, Loricaria, Loricarichthys, and Rineloricaria (Rodrigues, 2010; Rosa et al., 2012; Porto et al., 2014; Blanco et al., 2017; Primo et al., 2017), as well as in Farlowella schreitmuelleri (present study), in which four chromosome pairs (Nos. 7, 14, 25 and 28) had ITSs in the pericentromeric region. In pair No. 28, the ITS was observed in only one of the homologs. In F. schreitmuelleri, the ITSs were observed in association with heterochromatin in all other pairs except pair No. 14. An association between intra-chromosomal telomeric DNA and constitutive heterochromatin has been recorded in many vertebrates, and this association is considered a component of the satellite DNA (Meyne et al., 1990; Multani et al., 2001; Rovatsos et al., 2015; Viana et al., 2016). These signals may also represent remnants of ancient chromosomal rearrangements (Metcalfe et al., 2004), indicating potential hotspots of rearrangement (Scouarne, Gribble, 2012).

Overall, then, the Farlowella species analyzed up to now present an uniform karyotype macrostructure in terms of the 2n, although the chromosomal markers indicated remarkable and dynamic evolutionary processes of their genomes. The presence of ITSs indicates the chromosomal rearrangements, which may have contributed to the diversification of their karyotype. These findings highlight the potential of the genus for further, integrated studies of taxonomy, ecology and genetics to a better knowledge of the systematics and evolutive relationships among the species of Farlowella and other Loricariidae.

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