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Chromosomal Evolution in Aspredinidae (Teleostei, Siluriformes): Insights on Intra- and Interspecific Relationships with Related Groups

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Keywords
Banjo catfish · Chromosomal rearrangements · Cytotaxonomy · DNA barcoding · Ribosomal DNA

Abstract
The family Aspredinidae comprises a clade of complex systematic relationships, both from molecular and morphological approaches. In this study, conventional and molecular cytogenetic studies coupled with nucleotide sequencing were performed in 6 Aspredininae species (*Amaralia hypsiura*, *Bunocephalus* cf. *aloikae*, *Bunocephalus amaurus*, *Bunocephalus* aff. *coracoideus*, *Bunocephalus verrucosus*, and *Platystacus cotylephorus*) from different locations of the Amazon hydrographic basin. Our results showed highly divergent diploid numbers (2n) among the species, ranging from 49 to 74, including the occurrence of an XX/X0 sex chromosome system. A neighbor-joining phylogram based on the cytochrome *c* oxidase I (*COI*) showed that *Bunocephalus coracoideus* is not a monophyletic clade, but closely related to *B. verrucosus*. The karyotypic data associated with *COI* suggest an ancestral karyotype for Aspredinidae with a reduced 2n, composed of bi-armed chromosomes and a trend toward chromosomal fissions resulting in higher diploid number karyotypes, mainly composed of acrocentric chromosomes. Evolutionary relationships were discussed under a phylogenetic context with related species from different Siluriformes families. The karyotype features and chromosomal diversity of Aspredinidae show an amazing differentiation, making this family a remarkable model for investigating the evolutionary dynamics in siluriforms as well as in fish as a whole.

Introduction

Aspredinidae fishes (Teleostei, Siluriformes) are commonly known as banjo catfish due to their body shape resembling such musical instruments: a very wide body on the scapular girdle, with a narrow caudal peduncle and depressed head [Myers, 1960]. They are endemic to South America, where their greatest diversity is found in the Amazon basin [Nelson et al., 2016].

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Nowadays, its 44 valid species are distributed in 13 genera, divided into 3 subfamilies: Aspredininae, Hoplomyzontinae, and Pseudobunocephalinae [Fernández-Yépez, 1950; Nelson et al., 2016; Carvalho et al., 2018; Fricke et al., 2019]. However, intra-family phylogenetic relationships are not well resolved yet [Friel, 1994; Cardoso, 2008; Carvalho et al., 2018]. Likewise, the relationships of Aspredinidae with other siluriform families are controversial. Available phylogenetic studies disagree among themselves regarding the position of some taxa in the tree and even about the validity of some genera. However, 2 hypotheses are currently accepted based on both molecular and morphological data, i.e., Aspredinidae as sister-group to (1) the Doradoidea superfamily, composed of Doradidae and Auchenipteridae, both Neotropical clades [Friel, 1994; Sullivan et al., 2006, 2008; Kappas et al., 2016; Arcila et al., 2017; Carvalho et al., 2018]; and (2) the Asian clade Sisoroidea (Amblycipitidae, Akysidae, Erethistidae, and Sisoridae) [Mo, 1991; Chen, 1994; Pinna, 1998; Britto, 2002; Diogo, 2004; Cardoso, 2010].

Cytogenetics has been a valuable tool to decipher the evolutionary relationships of several fish groups, owing to their specific chromosomal and genomic features [Cioffi et al., 2018]. Especially, repetitive DNA sequences, which constitute the most significant part of the eukaryotic genome, display an enormous potential for expanding the knowledge of karyotype differentiation [Cioffi and Bertollo, 2012]. Among the Aspredinidae and phylogenetically related families, although most chromosomal studies are restricted to diploid number findings (2n) and karyotype composition, they point to a huge chromosomal variation ranging from 2n = 24 in *Liobagrus marginatus* (Amblycipitidae) to 2n = 58 in several other species. Besides, all species analyzed so far display homomorphic sex chromosomes, except for *Bunocephalus coracoideus* (Aspredinidae) which presents an uncommon $X_1X_1X_2X_2/X_1Y_1X_2Y_2$ sex chromosome system [Ferreira et al., 2016]. This system probably originated from chromosomal fusions followed by paracentric inversions, and also implies the displacement of 5S rDNA sites [Ferreira et al., 2016].

Its puzzling systematics makes Aspredinidae an attractive group to investigate the role of the chromosomal features on its evolutionary history and relationships with closely related groups. In this sense, we analyzed 6 Aspredinidae species, connecting conventional and molecular cytogenetic data and nucleotide sequencing with its inferred phylogenetic tree to improve the knowledge on the evolution and relationships among some species of this family.

Materials and Methods

Individuals, Mitotic Chromosome Preparation, and Ag- and C-Banding

Six Aspredinidae species (*Amaralia hypsiura*, *Bunocephalus* cf. *aloikae*, *Bunocephalus amaurus*, *Bunocephalus* aff. *coracoideus*, *Bunocephalus verrucosus*, and *Platystacus cotylephorus*) were collected from distinct wild natural ecosystems of the Brazilian Amazonian region (Fig. 1). The number and sex of the analyzed individuals are presented in Table 1. All individuals were deposited in the Fish Collection of the National Institute of Amazonian Research (INPA). Mitotic chromosomes were obtained from anterior kidney by the conventional air-drying method [Gold et al., 1990]. The distribution of the C-positive heterochromatin blocks was obtained according to Sumner [1972], and the determination of the nucleolar organizer regions (NOR) was performed with silver nitrate staining (Ag-NOR) according to Howell and Black [1980]. Anesthesia with clove oil was conducted before sacrificing the animals. The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) or acrocentric (a) according to the arm ratios [Levan et al., 1964].

Fluorescence in situ Hybridization

Genomic DNA was extracted from liver tissues with Promega's Wizard® Genomic DNA Purification Kit following the manufacturer's instructions. The amplification of the 18S rDNA, 5S rDNA, and telomeric sequences $(TTAGGG)_n$ followed Gross et al. [2010], Martins and Galetti [1999], and Ijdo et al. [1991], respectively. FISH was performed according to Pinkel et al. [1986], with minor modifications. The 18S rDNA and telomeric probes were labeled with digoxigenin-11-dUTP, and the 5S rDNA with biotin-16-dUTP by nick translation (Roche, Mannheim, Germany), according to the manufacturer's instructions. Hybridization was performed for 16–18 h at 37°C in a moist chamber. After hybridization, the slides were washed for 5 min with 2× SSC and then rinsed quickly in 1× PBS. The detection of the probes was performed with streptavidin-FITC (Sigma) for the 5S rDNA probe and anti-digoxigenin-rhodamine (Roche) for the 18S rDNA and telomeric probe. The chromosomes were counterstained with DAPI (1.2 g/mL) in antifading solution (Vector Laboratories).

DNA Barcoding Analysis

Representatives of each cytogenetically analyzed population were used, in addition to some populations of *B. coracoideus* studied by Ferreira et al. [2016, 2017]. A sequence from GenBank of the species *Diplomystes nahuelbutaensis* was employed as an external group (Table 1). The liver and muscle tissues were stored in absolute ethanol, and genomic DNA was obtained with the Wizard® Genomic DNA Purification Kit. The GoTaq Colorless Master Mix (Promega) was used for PCR of the mitochondrial gene cytochrome *c* oxidase I (*COI*) with the primers VF1_t1 (TGT AAA ACG GCC AGT CAA CCA ACC ACA AAG ACA TTG G) and VR1_t1 (CAG GAA ACA GCT ATG ACT AGA CTT CTG GGT GGC CAA AGA ATC A) [Ivanova et al., 2006]. The PCR products were purified with 20% PEG [Kimura, 1980] and, for the sequencing reaction, the BigDye Sequence Terminator v.3.1 kit (Applied Biosystems) was used following the manufacturer's instructions. After that, the products were precipitated and sequenced (ABI PRISM 3100 Genetic Analyzer of Applied Biosystems/made by HITACHI).

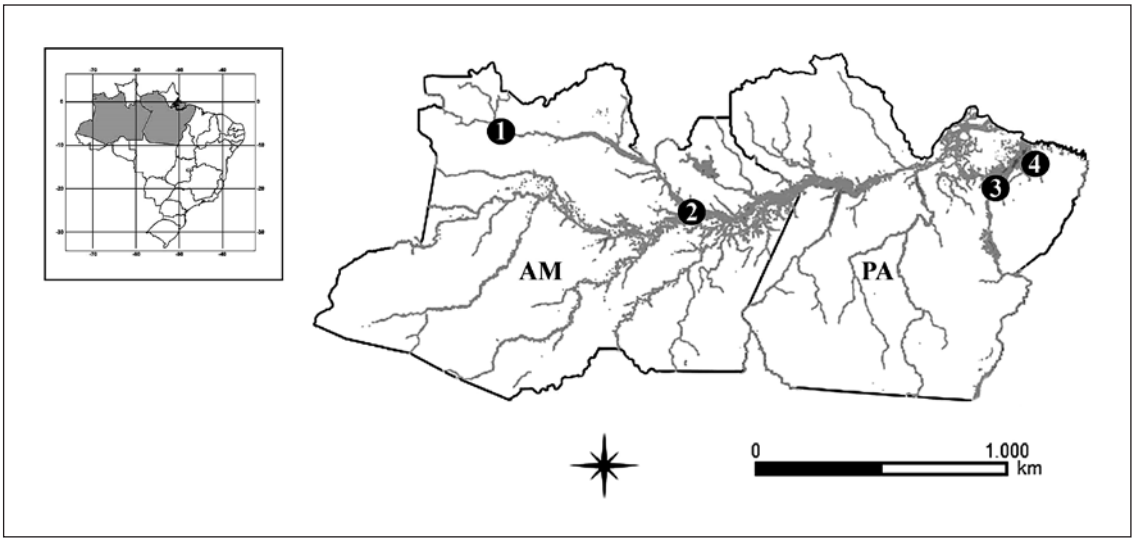

Fig. 1. Legal Brazilian Amazon map, highlighting the collection sites of the Aspredinidae species analyzed in this study. **1** *Bunocephalus* cf. *aloikae* – Igarapé Bucu, Curicuriari River, Negro River basin, AM. **2** *Bunocephalus verrucosus* – Igarapé do Sítio Santa Maria, Negro River basin, AM. **3** *Amaralia hypsiura* and *Platystacus cotylephorus* – Ilha Jutuba, Tocantins River basin, PA. **4** *B. amaurus*, *Bunocephalus* aff. *coracoideus* – Igarapé Apeú, Guamá River basin, PA. PA, Pará state; AM, Amazonas state, Brazil.

Alignment of Sequences and Phylogenetic Analysis

690-bp sequences were utilized to perform the barcode analysis, using the *COI* gene. The obtained sequences were submitted to the GenBank and Barcode Index Number (BIN) system of BOLD (Barcode of Life Data Systems). The sequences were aligned using the Geneious® 10.1.3 software, and Kimura's 2-parameter distance model [Kimura, 1980] was used to make a neighbor-joining dendrogram and bootstrap analysis [Felsenstein, 1985] with 1,000 repetitions. Every aligned sequence was translated into amino acids to detect eventual alignment mistakes.

Results

Chromosome Data

A great chromosomal variety was found among the analyzed species, with diploid numbers ranging from 44 to 74, and the following karyotype formulas and fundamental numbers (FN) were determined: *A. hypsiura* 2n = 74 (4m + 4st + 66a), FN = 82 (Fig. 2a); *Bunocephalus* cf. *aloikae* 2n = 44 (18m + 20sm + 6st), FN = 88 (Fig. 2b); *B. amaurus* 2n = 62 (4m + 14sm + 4st + 40a), FN = 84 (Fig. 2c); *Bunocephalus* aff. *coracoideus* 2n = 61 (5m + 12sm + 4st + 40a), FN = 82 (Fig. 2d); *B. verrucosus* 2n = 64 (4m + 16sm + 10st + 34a), FN = 94 (Fig. 2e); and *Platystacus cotylephorus* 2n = 50 (8m + 20sm + 14st + 8a), FN = 92 for females and 2n = 49 (7m + 20sm + 14st + 8a), FN = 90 for males (Fig. 2f), revealing the occurrence of a sex chromosomes system of the XX/X0 type.

A significant variation was also detected concerning the distribution of rDNA sites. Although only 1 pair carrying 18S rDNA sequences occurs in all species, there are significant interspecific variations concerning this chromosome, as well as in the position of the sites (Fig. 3). The Ag-NOR results agreed with the 18S rDNA mapping and also confirmed that every site was transcriptionally active in the precedent interphase (Fig. 2, boxes). Such differential distribution also applies to the 5S rDNA sites, plus additional number variations (Fig. 3). Notably, in *Bunocephalus* aff. *coracoideus*, the 5S sequences are located in distinct positions in the homologues of pair 8, indicating that a probable rearrangement has modified their previous position on this chromosome (Fig. 3d). Heterochromatin has a pericentromeric distribution in most chromosomes (Fig. 4), except for chromosome 1 in *Bunocephalus* aff. *coracoideus*, which has an interstitial block in its long arm (Fig. 4d). Telomeric sequences are located in the terminal regions of all chromosomes (Fig. 5). In all of these analyses, there were no differences between male and female specimens.

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Table 1. Aspredinidae species analyzed and the identification code of the exemplars

Voucher	Species	Locality	Hydrographic basin	Specimens, *n*	2n	FN	GenBank/BIN number	GPS data	References
INPA-ICT 052186	*Amaralia hypsiura*	Ilha Jutuba, Cametá, PA	Tocantins River	2♂4♀	74	82	MF416151–MF416155	02°14′46.5″S 49°24′59.7″W	Present study
INPA-ICT 049727	*Bunocephalus* cf. *aloikae*	Rio Curicuriari, Igarapé Bucu, São Gabriel da Cachoeira, AM	Negro River	2♂	44	88	MF416162–MF416163	0°14′39.0″S 67°03′31.1″W	Present study
INPA-ICT 052187	*Bunocephalus amaurus*	Igarapé Apeú, Castanhal, PA	Guamá River	2♂1♀	62	80	MF416200–MF416202	01°23′20.4″S 47°59′07.5″W	Present study
INPA-ICT 052189	*Bunocephalus* aff. *coracoideus*	Igarapé Apeú, Castanhal, PA	Guamá River	1♂	61	82	MF416156	1°23′20.4″S 47°59′07.5″W	Present study
INPA-ICT 053207	*Bunocephalus verrucosus*	Igarapé Sítio Santa Maria, Iranduba, AM	Negro River	8♂11♀	64	94	PEAM001-19	03°06′23″S 60°19′07″W	Present study
INPA-ICT 052190	*Platystacus cotylephorus*	Ilha Jutuba, Cametá, AM	Tocantins River	6♂12♂	49♂ 50♀	90♂ 92♀	PEAM002-19	02°14′46.5″S 49°24′59.7″W	Present study
INPA-ICT 052182	*Bunocephalus coracoideus*	Unidentified Igarapé, Barcelos, AM	Negro River	3♂2♀	42	61♂ 60♀	MF416157–MF416161	0°57′45.072″S 62°55′36.282″W	Ferreira et al. [2016]
INPA-ICT 053205	*Bunocephalus coracoideus*	Igarapé do banjo, Barcelos, AM	Demini River	2♂3♀	44	62	MF416168–MF416172	00°23.624′S 62°48.187′W	Ferreira et al. [2017]
INPA-ICT 052185	*Bunocephalus coracoideus*	Unidentified Igarapé, Tapauá, AM	Purus River	14♂13♀	40–46	54–62	MF416184–MF416199	5°37′21.7″S 63°15′01.5″W	Ferreira et al. [2017]
INPA-ICT 053204	*Bunocephalus coracoideus*	Igarapé Jundiá, Fazenda Dimona, AM (Reserva PDBFF[a])	Cuieiras River	3♂2♀	42	82	MF416164–MF416167	2°20′59.9″S 60°05′50.9″W	Ferreira et al. [2017]
INPA-ICT 052188	*Bunocephalus coracoideus*	Igarapé Apeú, Castanhal, PA	Guamá River	2♂9♀	56	78	MF416173–MF416183	1°23′20.4″S 47°59′07.5″W	Ferreira et al. [2017]
	Diplomystes nahuelbutaensis						EF014945.1		GenBank sequence

[a] Forest Fragment Biological Dynamics Project.

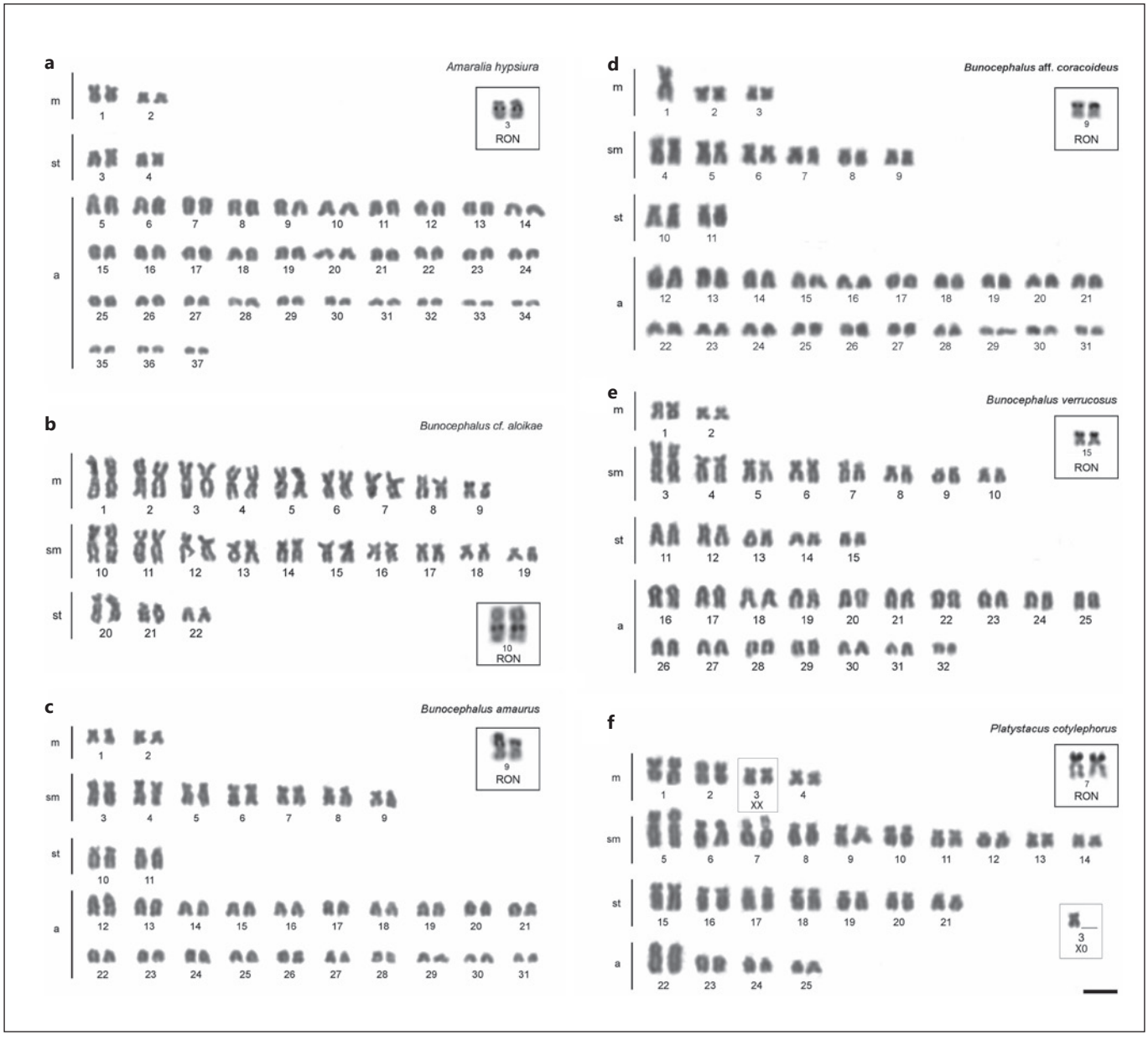

Fig. 2. Standard Giemsa-stained karyotypes of *Amaralia hypsiura* (**a**), *Bunocephalus* cf. *aloikae* (**b**), *Bunocephalus amaurus* (**c**), *Bunocephalus* aff. *coracoideus* (**d**), *Bunocephalus verrucosus* (**e**), and *Platystacus cotylephorus* (**f**). The Ag-NOR bearing pairs (RON) are shown in boxes. Scale bar, 10 μm.

DNA Barcoding Data

The dendrogram generated with the neighbor-joining algorithm indicated that all species are well structured with bootstrap values superior to 99%. The clade including *P. cotylephorus* was positioned as a basal one. *A. hypsiura* is a sister group of the clade encompassing *Bunocephalus* species. *B. amaurus* and *Bunocephalus* cf. *aloikae* are sister groups. However, the *B. coracoideus* population from the Cuieiras River basin [Ferreira et al., 2017] is more closely related to these species than to proper *B. coracoideus*, with 1 individual grouped alongside the individuals of the *Bunocephalus* cf. *aloikae* clade. Besides, there is another sister group constituted by *B. verrucosus* and 4 populations of *B. coracoideus.* It is noteworthy that

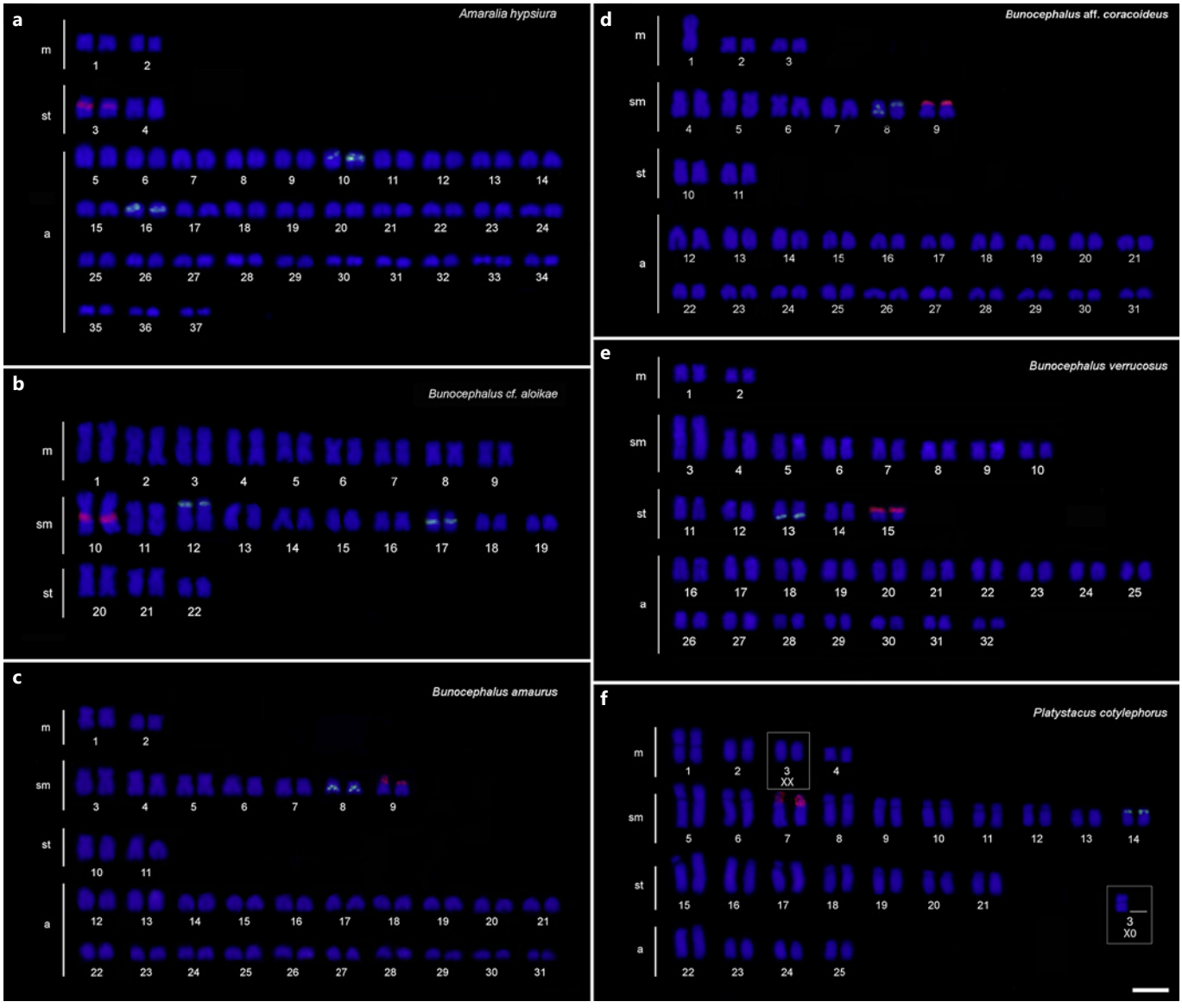

Fig. 3. Karyotypes of *Amaralia hypsiura* (**a**), *Bunocephalus* cf. *aloikae* (**b**), *Bunocephalus amaurus* (**c**), *Bunocephalus* aff. *coracoideus* (**d**), *Bunocephalus verrucosus* (**e**), and *Platystacus cotylephorus* (**f**) after dual-color FISH with 18S (red) and 5S (green) rDNA probes. Scale bar, 10 μm.

2 *B. coracoideus* populations, Negro River [Ferreira et al. [2016] and Purus River [Ferreira et al., 2017], are on the same clade, with 93.3% bootstrap.

Kinship configuration based on *COI* followed this arrangement: *P. cotylephorus*, [*A. hypsiura*, *Bunocephalus* cf. *aloikae*, (*B. amaurus*, *Bunocephalus* aff. *coracoideus*), (*B. coracoideus*, *B. verrucosus*)]. Clade structuration showed a close relationship with the chromosomal macrostructure (Fig. 6).

Discussion

Up to now, the karyotypic knowledge of Aspredinidae was restricted to only 2 species, *Bunocephalus doriae* [Fenocchio and Swarça, 2012] and B. coracoideus [Ferreira et al., 2016, 2017]. Thus, the present study extends chromosomal data to 6 other species, all of them belonging to the Aspredininae subfamily. A huge chromosomal diversity was found to occur within this fish group, re-

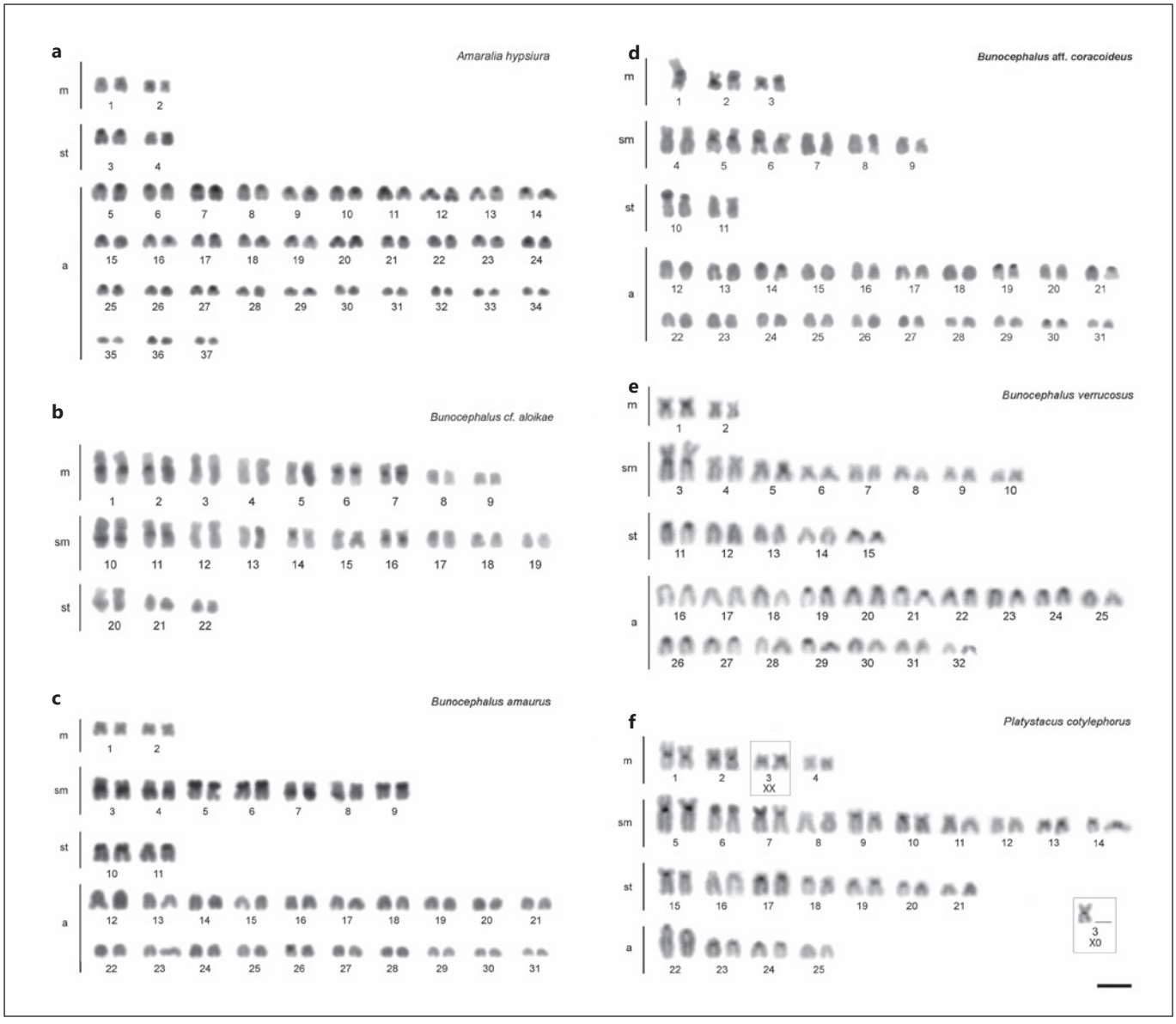

Fig. 4. Karyotypes of *Amaralia hypsiura* (**a**), *Bunocephalus* cf. *aloikae* (**b**), *Bunocephalus amaurus* (**c**), *Bunocephalus* aff. *coracoideus* (**d**), *Bunocephalus verrucosus* (**e**), and *Platystacus cotylephorus* (**f**) after C-banding. Scale bar, 10 μm.

vealing great differentiation in the diploid numbers and karyotypic formulas among species. *Bunocephalus* cf. *aloikae*, investigated here, presents a very similar karyotype to that of *B. coracoideus* from the Cuieiras River basin [Ferreira et al., 2017], and both species also share the interstitial NOR locus on the largest submetacentric pair. This is thought to be an apomorphic feature among Siluriformes [Ferreira et al., 2014], but an ancestral feature for Aspredinidae species or an evolutionary convergence, a somewhat similar pattern also found in Ancistrini species [Alves et al., 2006]. Thus, it is likely that *Bunocephalus* cf. *aloikae* and also *B. coracoideus* have the karyotype most similar to the ancestral one for Aspredinidae, due to its low 2n and karyotype macrostructure displaying mostly bi-armed chromosomes. On the other hand, *A. hypsiura* displays the largest diploid number (2n = 74) described so far for Aspredinidae. This seems to be a derived trait when compared to other aspredinids since most of the

chromosomes are acrocentric, unlike the plesiomorphic karyotype suggested for Siluriformes with mainly bi-armed chromosomes [Oliveira and Gosztonyi, 2000]. *B. amaurus* (2n = 62) and *B. verrucosus* (2n = 64) possibly followed the same evolutionary trend encompassing centric fissions and a large number of acrocentric chromosomes. *B. doriae* and *B. coracoideus* also share the same trait [Fenocchio and Swarça, 2012; Ferreira et al., 2016]. It is likely that, at first, the karyotype evolution in Aspredinidae followed a trend of reduction of the diploid number from its Siluriformes ancestors, maintaining bi-armed chromosomes in most species. Afterward, new rearrangements, such as centric fissions and pericentric inversions, increased the diploid number, as well as the number of acrocentric chromosomes in some species.

The mapping of the 18S and 5S rDNAs corroborates the chromosomal diversity within Aspredinidae. In fact, the species studied so far do not show a shared pattern. In each species, these markers are located in morphologically distinct chromosome pairs and/or in different chromosome regions, discarding homologies. The 5S sites also can present differentiations in number, although this does not apply for the 18S sequences. On the other hand, the heterochromatic regions are more conservative traits, maintaining the ancestral Siluriformes characteristics with few heterochromatin amounts preferentially located in the pericentromeric regions [Oliveira and Gosztonyi, 2000].

Noteworthy, cytogenetic and morphological data suggest that a male specimen of *Bunocephalus* aff. *coracoideus* resulted from a crossbreeding between *B. amaurus* (present study) and *B. coracoideus* [Ferreira et al., 2017], both collected in the same location. This individual has some morphological traits related to *B. coracoideus,* but its karyotype is closer to that of *B. amaurus*, a proximity also corroborated by the DNA barcoding data. Besides, the 5S rDNA mapping showed that there is no perfect pairing and homology between the chromosomes of the

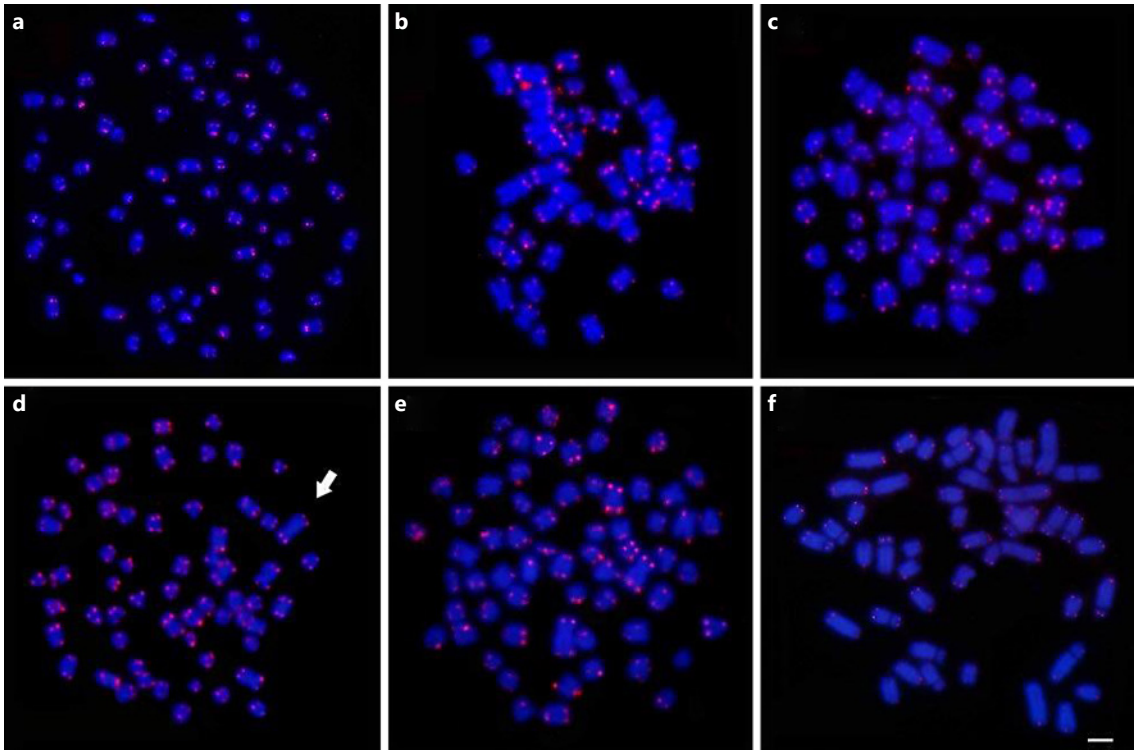

Fig. 5. Somatic metaphases of *Amaralia hypsiura* (**a**), *Bunocephalus* cf. *aloikae* (**b**), *Bunocephalus amaurus* (**c**), *Bunocephalus* aff. *coracoideus* (**d**), *Bunocephalus verrucosus* (**e**), and *Platystacus cotylephorus* (**f**) after FISH with the telomeric probe. The arrow indicates the single metacentric chromosome. Scale bar, 10 μm.

Fig. 6. Phylogram of Aspredinidae species based on neighbor-joining analysis for the *COI* mitochondrial gene. The bootstrap values for 1,000 replications are above the branches.

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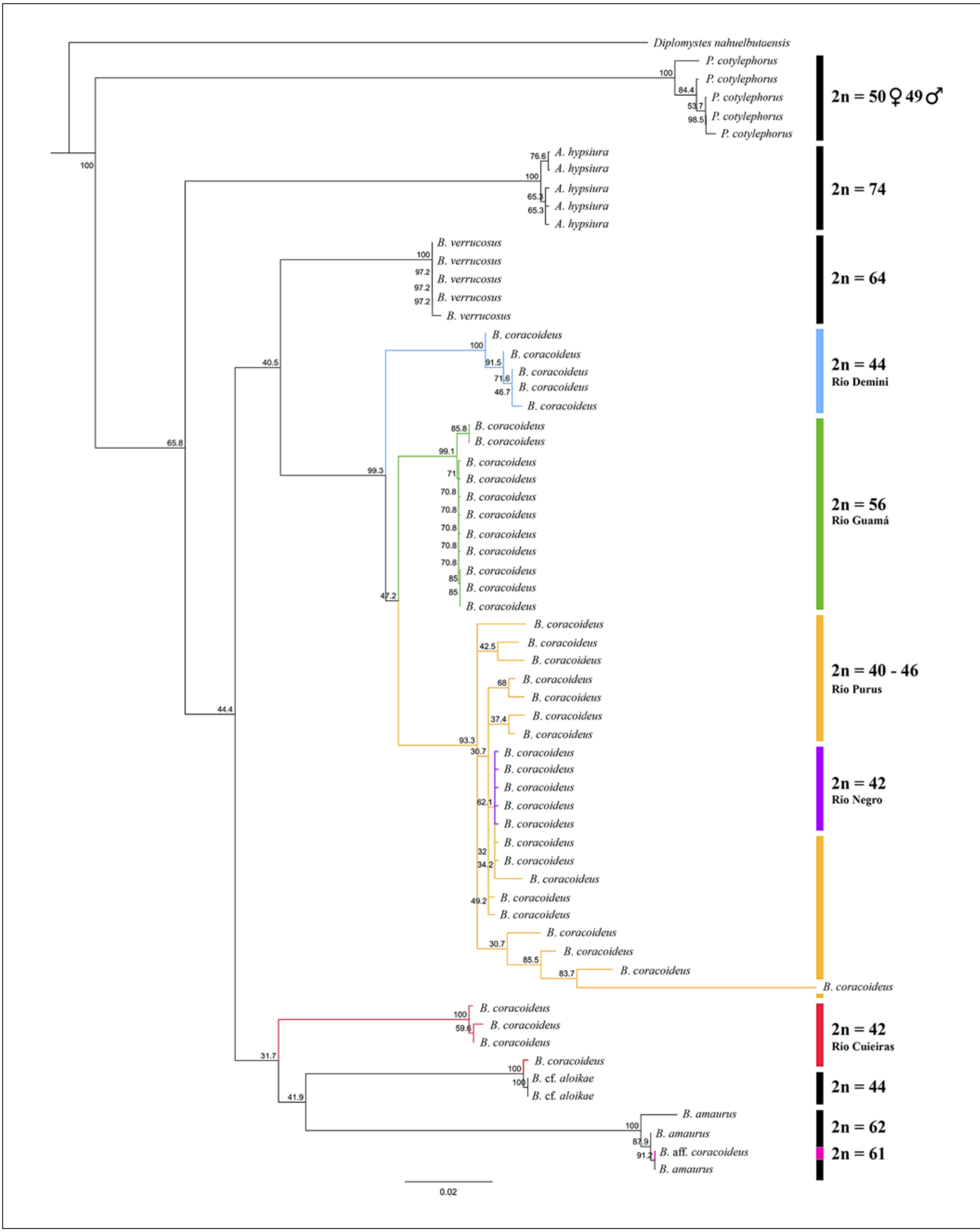

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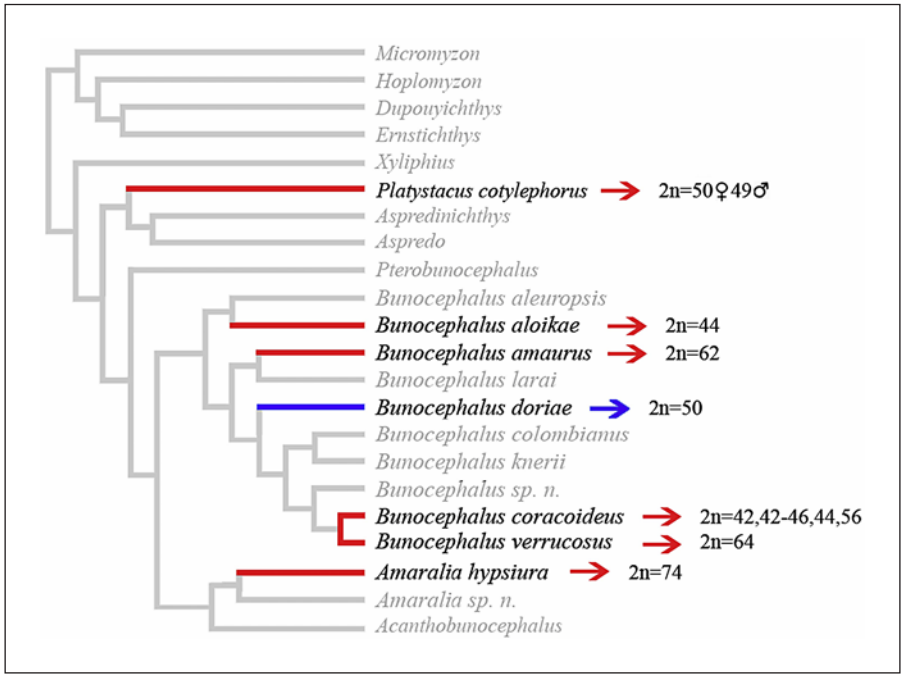

Fig. 7. Phylogram of Aspredinidae phylogenetic relationships according to Cardoso [2008], with overlap of available cytogenetic data.

pair carrying these sequences, thus appearing to be inherited from both species (*B. coracoideus* and *B. amaurus*). Furthermore, a single metacentric chromosome, which may have resulted from the fusion of 2 acrocentric chromosomes as a requirement for meiotic adjustments, reinforces this proposal. Although no interstitial telomeric sequences could be demonstrated, an interstitial heterochromatic region in the long arm suggests it is the remnant of a second chromosome (Fig. 4d).

Another distinguishing trait concerning Aspredinidae is the occurrence of differentiated sex chromosomes. *B. coracoideus*, previously described by Ferreira et al. [2016], has a multiple sex chromosome system of the $X_1X_1X_2X_2/X_1Y_1X_2Y_2$ type. In the present study, a distinct sex chromosome system of the XX/X0 type was identified in *P. cotylephorus*, constituting the second description of such a type among Siluriformes, while the first one has been found in an *Ancistrus* species [Alves et al., 2006]. An XX/X0 system emerges from the elimination of 1 male chromosome [Devlin and Nagahama, 2002], thus representing an uncommon event since it can produce genetic unbalances and meiotic problems. Anyway, differentiated sex chromosomes constitute a relevant evolutionary event, if we consider the rearrangements needed to their differentiation, as well as their role as speciation promoter [Faria and Navarro, 2010].

Our barcoding analysis of Aspredinidae corroborates the phylogeny proposed by Cardoso [2008], and is similar to that of Carvalho et al. [2018] if we consider only the species studied herein. However, additional information was now highlighted. The first one regards the *B. coracoideus* population from the Cuieiras River, which is not in the clade with the other populations of the same species. On the contrary, it groups with *Bunocephalus* cf. *aloikae* and *B. amaurus*. As previously discussed, this *B. coracoideus* population has some karyotype characteristics very close to those of *Bunocephalus* cf. *aloikae*, and the data from the mitogenome region corroborate the hypothesis that these species have a close evolutionary history and conserved *Bunocephalus* ancestral characters. Another interesting point are the relationships between *B. coracoideus* populations from Purus and Negro Rivers. Once more, karyotype and molecular data corroborate that they are the most derivative populations or lineages of this species. Altogether, the Negro River population, which presents a novel sex chromosome system among Siluriformes [Ferreira et al., 2016], and the Purus River population, which has a diploid number varying from 40 to 46 chromosomes [Ferreira et al., 2017], denote recent karyotype rearrangements, but still maintain some ancestral characteristics in the chromosomal microstructure.

The extent of chromosomal variation between the species of Aspredinidae, 2n = 42–74, indicates that this family has undergone major evolutionary rearrangements. To test our hypothesis of 2n reduction and further rearrangements, we overlapped the available diploid numbers

Table 2. Chromosomal data of Aspredinidae and phylogenetically related families

Clade	Family	Species	2n	Karyotypes	FN	Nor	Locality	References
	Aspredinidae	*Bunocephalus doriae*	50	6m + 10sm + 6st + 28a	72	11p, st, terminal	Argentina, Posadas	Fenocchio and Swarça [2012]
		Bunocephalus coracoideus	42♀	4m + 14sm + 24a	60	5p, sm, terminal	Brazil, AM	Ferreira et al. [2016, 2017]
			42♂	5m + 14sm + 23a	61	5p, sm, terminal	Brazil, AM	Ferreira et al. [2016, 2017]
		Amaralia hypsiura	74	4m + 4st + 66a	82	3p, st, proximal	Brazil, PA	Present study
		Bunocephalus cf. *aloikae*	44	18m + 20sm + 6st	88	10q, sm, proximal	Brazil, AM	Present study
		Bunocephalus amaurus	62	4m + 14sm + 4st + 40a	84	9p, sm, terminal	Brazil, PA	Present study
		Bunocephalus aff. coracoideus	61	5m + 12sm + 4st + 40a	82	9p, sm, terminal	Brazil, PA	Present study
		Bunocephalus verrucosus	64	4m + 16sm + 10st + 34a	94	15p, st, proximal	Brazil, AM	Present study
		Platystacus cotylephorus	50♀	8m + 20sm + 14st + 8a	92	7p, sm, terminal	Brazil, AM	Present study
			49♂	7m + 20sm + 14st + 8a	90	7p, sm, terminal	Brazil, AM	Present study
Sisoroidea	Amblycipitidae	*Amblyceps mangois*	36	4m + 12sm + 16st + 4a	52	–	India, Jammu/Kashmir	Sharma and Sharma [1995]
		Liobagrus andersoni	28	18m + 10sm	56	–	Korea, Han River	Son and Lee [1989]
		Liobagrus anguillicauda	34	20m + 12sm + 2st	68	–	China, Zheijiang	Li et al. [1983]
		Liobagrus marginatus	24	20m + 2sm + 2st	48	–	China, Sichuan	Li et al. [1981a]
		Liobagrus mediadiposalis	42	26m + 16sm	84	–	Korea, Nakdong	Son and Lee [1989]
		Liobagrus mediadiposalis	42	26m + 12sm + 4st/a	80	–	Korea, Hamyang	Kim et al. [1982]
		Liobagrus mediadiposalis	28	18m + 6sm + 4st	56	–	Korea, Gum River	Ueno [1985]
		Liobagrus obesus	20	20m	40	–	Korea, Kum River	Son and Lee [1989]
		Liobagrus reinii	38	26m + 8sm + 4st	76	–	Japan, Gifu	Ueno [1985]
	Erethistidae	*Pseudolaguvia ribeiroi*[a]	50	6m + 28sm + 16a	84	–	India, Assam	Khuda-Bukhsh et al. [1995]
	Sisoridae	*Euchiloglanis davidi*	36	8m + 6m + 22st/a	50	–	China, Sichuan	Li et al. [1981b]
		Euchiloglanis kishinouyei[b]	50	14+6sm + 30st/a	70	–	China, Sichuan	Li et al. [1981b]
		Glyptothorax telchitta	56	18m + 26sm + 2st + 10a	102	–	India, Bihar	Khuda-Bukhsh et al. [1986]
		Glyptothorax trilineatus	52	18m + 24sm + 10a	94	–	India, Assam	Khuda-Bukhsh et al. [1995]
		Gagata cenia	46	4m + 8sm + 8st + 26a	66	–	India, UP	Mishra [1998]
Doradoidea	Auchenipteridae	*Ageneiosus atronasus*[c]	56	16m + 16sm + 12st + 12a	100	sm, interstitial	Brazil, AM	Fenocchio and Bertollo [1992]
		Ageneiosus inermis[d]	56	20m + 16sm + 10st + 10a	102	terminal	Brazil, AM	Fenocchio and Bertollo [1992]
		Auchenipterus nuchalis	58	24m + 14sm + 10st + 10a	106	15p, sm, interstitial	Brazil, PR	Ravedutti and Julio [2001]
		Glanidium ribeiroi	58	28m + 16sm + 10st + 4a	112	17p, sm, interstitial	Brazil, PR	Ravedutti and Julio [2001]
		Glanidium ribeiroi	58	22m + 20sm + 10st + 6a	110	14p, sm, interstitial	Brazil, PR	Lui et al. [2015]
		Trachelyopterus galeatus[e]	58	22m + 12sm + 6st + 18a	98	23p, a, terminal	Brazil, PR	Ravedutti and Julio [2001]
	Doradidae	*Hassar orestis*	58	32+20+20sm + 6st	116	22p, sm, distal	Brazil, PA	Milhomem et al. [2008]
		Hassar cf. *orestis*	58	32m + 18sm + 8st	116	20p, sm, distal	Brazil, PA	Milhomem et al. [2008]
		Hassar wilderi	58	32m + 16sm + 10st	116	27p, st, terminal	Brazil, MG	Eler et al. [2007]
		Hassar wilderi	58	26m + 20sm + 12st	116	28p, st, terminal	Brazil, MT	Takagui et al. [2017a]
		Leptodoras cataniae	58	20m + 18sm + 16st + 4a	112	23p, st, terminal	Brazil, MT	Takagui et al. [2017a]
		Opsodoras ternetzi	58	44m + 12sm + 2a	114	24q, sm, distal	Brazil, PA	Milhomem et al. [2008]
		Ossancora punctata	66	12m + 8sm + 6st + 40a	92	–	Brazil, MT	Takagui et al. [2017b]
		Platydoras cf. *costatus*	58	26m + 16sm + 4st + 12a	104	20p, sm, distal	Brazil, PA	Milhomem et al. [2008]

Table 2 (continued)

Clade	Family	Species	2n	Karyotypes	FN	Nor	Locality	References
		Platydoras armatulus	58	22m + 14sm + 18st + 4a	112	–	Brazil, MT	Takagui et al. [2017b]
		Pterodoras granulosus	58	16m + 16sm + 14st + 12a	104	–	Brazil, MT	Takagui et al. [2017b]
		Oxydoras niger[f]	58	20m + 16sm + 8st + 14a	102	15p, sm, terminal	Brazil, PA	Fenocchio et al. [1993]
		Rhinodoras dorbignyi[g]	58	20m + 20sm + 4st + 14a	102	16p, sm, terminal	Brazil, SP	Fenocchio et al. [1993]
		Tenellus leporhinus	58	36m + 18sm + 4st	116	23q, sm, terminal	Brazil, MT	Takagui et al. [2017a]
		Tenellus trimaculatus	58	21m + 18sm + 11st + 8a 20m + 18sm + 12st + 8a	108	22p, st, terminal	Brazil, MT	Takagui et al. [2017a]
		Trachydoras paraguayensis	56	36m + 16sm + 4st	112	11q, m, interstitial	Brazil, PR	Baumgärtner et al. [2016]
		Trachydoras paraguayensis[h]	56	32m + 20sm + 4st	112	q, sm, interstitial	Brazil, PR	Fenocchio et al. [1993]
		Wertheimeria maculata	58	24m + 14sm + 8st + 12a	104	27p, a, terminal	Brazil, MG	Eler et al. [2007]

2n, diploid number; m, metacentric; sm, submetacentric; st, subtelocentric; a, acrocentric; p, short arm, q, long arm. [a] In karyotype paper reported as: *Laguvia rebeiroi*, [b] In karyotype paper reported as: *Coreglanis kishinouyei*, [c] In karyotype paper reported as: *Ageneiosus atronases*, [d] In karyotype paper reported as: *Ageneiosus brevifilis*, [e] In karyotype paper reported as: *Parauchenipterus galeatus*, [f] In karyotype paper reported as: *Pseudodoras niger*, [g] In karyotype paper reported as: *Rhynodoras d'orbignyi*, [h] In karyotype paper reported as: *Trachydoras paraguaiensis*.

to the most recent phylogeny of the family (Fig. 7), as described by Cardoso [2008], and over the phylogram resulting from the present study (Fig. 6). This shows that *P. cotylephorus* is the basal clade, and the genus *Amaralia* is a sister clade of *Bunocephalus*; however, according to cytogenetic data, *A. hypsiura* corresponds to a derived clade. The clade containing the *Bunocephalus* genus has the most information available, and morphological, mitochondrial, and chromosomal data keep *B. coracoideus* and *B. verrucosus* more closely related, although in a more derived position.

Our cytogenomic results also indicate that relationships between Aspredinidae and other Siluriformes families are controversial. Comparisons of chromosomal data of Aspredinidae and other suggested related families (Table 2) denote a greater similarity between Aspredinidae and the Asian clade Sisoroidea. As Aspredinidae, the Sisoridae, and also the Amblycipitidae and Erethistidae families do not have a conserved diploid number, with FN < 100 for most of their species. On the other hand, Auchenipteridae and Doradidae families share a modal number of 2n = 58, and FN > 100 for most of their species (Table 2). Although molecular studies [Sullivan et al., 2006, 2008; Kappas et al., 2016; Carvalho et al., 2018] associate Aspredinidae with the Doradoidea clade (Doradidae and Auchenipteridae), chromosome data corroborate that Aspredinidae is the sister group of the Sisoroidea Asian clade (Amblycipitidae, Akysidae, Sisoridae, and Erethistidae). Chromosomal data suggest that Sisoroidea and Aspredinidae experienced the same selective pressures, leading to several rearrangements and later fixation of their heterokaryotypes.

Both cytogenetic and molecular data are important tools to evidence species relationships. Chromosomal rearrangements shape genomes as part of the evolutionary process and play a fundamental role acting as genetic barriers to the gene flow and on the reduction of recombination rates, favoring genetic drift [Navarro and Barton, 2003]. However, how to explain the contradictions between molecular/morphological and cytogenetic data on Aspredinidae relationships? The answer might reside in the chromosomal rearrangements. It is known that rearranged genome regions may contribute to variable rates of molecular evolution, particularly to high rates of recombination around the rearrangement breakpoints and the rearrangements themselves [Rozas et al., 2001; Marques-Bonet and Navarro, 2005]. In fact, some studies suggest an association between chromosome rearrangements and genetic variability [Marques-Bonet and Navarro, 2005]. Then, in theory: the more rearrangements,

the more nucleotide replacement rates, and the more divergent species. Sisoroidea and Aspredinidae are characterized by heterokaryotypes and high molecular divergence. Thus, these traits explain why molecular studies do not associate them as sister groups. Therefore, we suggest that these groups became more divergent because they went through more chromosome rearrangements. Although cytogenetic data do not recover a phylogenetic history due to their homoplastic nature, they corroborate morphological phylogenies suggesting Aspredinidae and Sisoroidea as related clades, since they have karyotype similarities probably modeled by the same selective pressures. Then, why do Aspredinidae and Doradoidea show proximity in phylogenies based on morphological and molecular data? We hypothesize that both Aspredinidae and Sisoroidea species underwent similar high selective pressures on their chromosomes and so a high evolutionary rate at the molecular level, thus pointing to little homology between them in the recovered phylogenies. On the other hand, Doradoidea species would have suffered minor selective pressure on their chromosomes, thus keeping a more constant evolutionary rate for molecular sequences and an apparent evolutionary homology closer to Aspredinidae. Unfortunately, chromosomal data, based on the analyses currently available, are not yet able to indicate the time of divergence and the rates of evolution, which could explain when speciation occurred [Faria and Navarro, 2010]. However, the complete genome sequencing of a larger number of representatives from each one of these families may be an alternative proposal to provide substantial data on the divergence rates of these species [Yang, 2010]. In this case, the mapping of regions that have undergone rearrangements, as well as those that are more conserved, will allow better clarification of their interrelationships.

Another important result that emerged from this study are the interspecific relationships in the genus *Bunocephalus*. Carvalho et al. [2018] recovered the monophyly of this genus after excluding the species *Bunocephalus chamaizelus*, which gathered the status of sister group. However, despite indicating *Bunocephalus* and *Amaralia* as closely related sister groups, our results show that there are significant chromosomal divergences inside the *Bunocephalus* genus, thus indicating taxonomic problems to be solved.

Final Remarks

Karyotype data are excellent markers for biodiversity. In this study, the association of chromosomal and DNA barcode data was able to corroborate previous relationships considered for Aspredinidae. However, both markers point out that *B. coracoideus* is not a monophyletic clade, showing that some samples described as *B. coracoideus* deserve further taxonomic revision. In fact, their great karyotype divergence shows that huge biodiversity characterizes this nominal species. Besides, chromosomal data also suggest that Aspredinidae is phylogenetically related to the Asian clade Sisoroidea, rather than to the Doradoidea group as currently proposed.

Statement of Ethics

This work followed the Ethical Standards for Animal Research according to the Ethics Committee for Animal Use of the National Institute for Research in the Amazon under protocol number 010/2015. Authorization for the collection of individuals was granted by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA), in accordance with SISBIO license number 48795-1.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

M.F., I.S.J., and P.F.V. carried out the molecular cytogenetic analyses and drafted the manuscript. C.G., D.A.M., M.B.C., L.A.C.B., and E.F. helped in analysis and drafted the manuscript. E.F. coordinated the study, drafted and revised the manuscript. All authors read and approved the final version of the manuscript.

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