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Original article

Cytogenetic markers as tools in delimiting species of the highly diverse Neotropical fish *Bryconamericus* (Characiformes: Characidae)

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Bryconamericus is a highly diverse group of characid fishes, being cytogenetic a valuable tool for the delimitation of species. *Bryconamericus* aff. *iheringii* (Upper Uruguay/Lower Paraná), *B. coeruleus* (Upper Paraná), *B*. cf. *ecai* e *B*. cf. *eigenmanni* (Upper Uruguay) were studied cytogenetically, and presented 2n=52 chromosomes, with interpopulational/interspecific variation of karyotype and fundamental number. Heterochromatin was evidenced in pericentromeric, telomeric and interstitial regions, and it was shown to be an important cytogenetic marker. Single nucleolar organizing regions (NORs) were found in *B*. cf. *eigenmanni*, *B*. cf. *ecai* and *B*. aff. *iheringii* (Lower Paraná), and multiple in *B*. aff. *iheringii* (Upper Uruguay) and *B. coeruleus*, with occurrence of two patterns for the first species, and three for the second. The 5S/18S rDNA-FISH confirmed the location of the NORs and showed single 5S rDNA cistrons only in *B*. aff. *iheringii* (Lower Paraná), evidencing the dispersion of both genes, often co-located, in the karyotype of the others species. The data of this work contribute for the delimitation of the species of the genus. Co-localization of ribosomal genes may represent a plesiomorphic condition for the group, and their dispersion suggest the occurrence of duplication, pseudogeneization and transposition events mediated by mobile genetic elements.

Keywords: Chromosome rearrangements, Co-localization, Cytotaxonomy, Cytosystematics, Intra and interspecific variation, Ribosomal genes.

Bryconamericus é um grupo altamente diverso de caracídeos, sendo a citogenética uma valiosa ferramenta para a delimitação de espécies. *Bryconamericus* aff. *iheringii* (Alto Uruguai/Baixo Paraná), *B. coeruleus* (Alto Paraná), *B*. cf. *ecai* e *B*. cf. *eigenmanni* (Alto Uruguai) foram estudados citogeneticamente, e apresentaram 2n=52 cromossomos, com variação interpopulacional/interespecífica de cariótipo e número fundamental (NF). Heterocromatinas foram evidenciadas nas regiões pericentromérica, telomérica e intersticial, e mostrou-se um importante marcador citogenético. Regiões organizadores de nuclcéolos (RONs) simples foram encontradas em *B*. cf. *eigenmanni*, *B*. cf. *ecai* e *B*. aff. *iheringii* (Baixo Paraná), e múltiplas em *B*. aff. *iheringii* (Alto Uruguai) e em *B. coeruleus*, com a ocorrência de dois padrões de localização para a primeira espécie, e três para a segunda. A FISH-DNAr 5S/18S confirmou a localização das RONs e mostrou cístrons simples de DNAr 5S apenas em *B.* aff. *iheringii* (Baixo Paraná), evidenciando a dispersão de ambos os genes, muitas vezes co-localizados, no cariótipo das demais espécies. Os dados deste trabalho contribuem para a delimitação das espécies do gênero. A co-localização dos genes ribossomais pode representar uma condição plesiomórfica para o grupo, e sua dispersão sugere a ocorrência de eventos de duplicação, pseudogenização e transposição mediada por elementos genéticos móveis.

Palavras-chave: Citossistemática, Citotaxonomia, Co-localização, Genes ribossomais, Rearranjos cromossômicos, Variação intra e interespecífica.

Introduction

Bryconamericus Eigenmann (1907) is a diverse group with approximately 60 species (Fricke *et al.*, 2019) distributed by the cis and trans Andean basins from Panama in Central America to northern Argentina in South America (Jerep, Shibatta, 2017; Mirande, 2018). They are small fish, known to have morphologically similar species and high taxonomic and phylogenetic complexity.

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Despite the efforts, the phylogeny of *Bryconamericus* is quite controversial and currently no monophyletism evidence has been found. The genus was first recorded in Tetragonopterinae (Géry, 1977), after was classified as *incertae sedis* in Characidae (Lima *et al*., 2003), and in the same year it changed to "clade A" *sensu* Malabarba, Weitzman (2003). Using various morphological and osteological characters, Mirande (2009) reorganized *incertae sedis* genera into previously identified clades, including *Bryconamericus*, which became the Stevardiinae clade. Javonillo *et al*. (2010) kept *Bryconamericus* in *incertae sedis* within the so-called "clade A", which also contained two subfamilies of Characidae (Glandulocaudinae and Stevardiinae) and other *incertae sedis* genera. Mirande (2010, 2018), Oliveira *et al.* (2011) and Thomaz *et al.* (2015) propose *Bryconamericus* as a polyphyletic group within Stevardiinae, but only the phylogeny of Mirande (2018) restricts the genus to the Diapomini tribe.

Even with its representativeness in Stevardiinae and the high number of phylogenetic studies, few *Bryconamericus* species have been the target of cytogenetic and molecular studies. The data obtained are mostly restricted to the Upper Paraná River basin, where four species of this genus: *B. exodon* Eigenmann (1907) (type-species), *B. iheringii* (Boulenger, 1887), *B. turiuba* Langeani, Lucena, Pedrini & Tarelho-Pereira, 2005 and *B. coeruleus* Jerep & Shibatta, 2017, quoted above as *B*. aff. *iheringii* and found in the basins of the Ivaí, Piquiri and Tibagi rivers, syntopic with other Stevardiinae.

Cytogenetic studies in trans-Andean *Bryconamericus* species evidenced the maintenance of the diploid number of 52 chromosomes, with inter and intraspecific divergences regarding the karyotype formula and the fundamental number (FN) (Paintner-Marques *et al*., 2002a, 2002b, 2003; Capistano *et al*., 2008; Portela-Castro *et al*., 2008; among others). Likewise, other cytogenetic characters were shown to be variable, such as the distribution of heterochromatin and the number and location of the nucleolar organizer regions (NORs) (Eberhardt *et al*., 2012; Santos *et al*., 2012, 2017; Silva *et al*., 2014), with variation even among individuals of the same population. In relation to 5S rDNA ribosomal genes, the data are restricted to only four species, revealing simple cistrons in *B.* cf. *iheringii* (Piscor *et al*., 2013) and *Bryconamericus* sp. (Santos *et al*., 2017, Cambutá River), and multiple cistrons with intraspecific variation in *B. turiuba* (Piscor *et al*., 2013), *B. ecai* and *Bryconamericus* sp. (Vermelho stream) (Santos *et al.*, 2017), being that in some cytotypes these genes presented syntenic to 18S rDNA genes.

Due to the high complexity chromosomal and phylogenetic found in *Bryconamericus*, and the absence of broad studies of phylogeny including cytogenetic data, this work uses basic cytogenetic techniques (Giemsa, C-banding and AgNORs) and molecular (FISH with probes from the 5S and 18S rDNA). These techniques will be useful as markers in the differentiation of four *Bryconamericus* species, from the Upper Uruguay River, Upper Paraná River and Lower Iguaçu River. In this way, the present study aimed to expand the cytogenetic data for the genus in order to find for markers that may aid in the differentiation of these species and in the understanding of phylogenetic relationships into the group.

Material and Methods

Specimens of four species of *Bryconamericus* were collected: 4 males and 5 females of *B*. aff. *iheringii* from the Ijuí River, Upper Uruguay River basin (State of Rio Grande do Sul); 1 male and 3 females of *B.* aff. *iheringii* from the Iguaçu River, Lower Iguaçu River basin (State of Paraná); 13 males and 4 females of *B. coeruleus* of the Piquiri River, Upper Paraná River basin (State of Paraná); 1 male and 5 females of *B.* cf. *ecai*, and male of *B.* cf. *eigenmanni* from the Biguá Stream, Upper Uruguay River basin (State of Santa Catarina) (Tab. 1).

All specimens were anesthetized and euthanized by an overdose of clove oil (Griffiths, 2000), and deposited in the Coleção Ictiológica do Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUPELIA) of the Universidade Estadual de Maringá (UEM), Brazil. The chromosomal preparations followed the methodology proposed by Bertollo *et al.* (1978), and the NORs were evidenced by impregnation by silver nitrate, according to Howell, Black (1980). The C-banding was used to determine the heterochromatic regions following the technique proposed by Sumner (1972), with modifications suggested by Lui *et al*. (2012). Physical mapping of the 5S rDNA and 18S rDNA was carried out by fluorescence *in situ* hybridization (FISH) according to Pinkel *et al*. (1986) and modifications suggested by Margarido, Moreira-Filho (2008), using DNA probes obtained from *Megaleporinus elongatus* (Valenciennes, 1850) (Martins, Galetti-Junior, 1999) and from *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka, Galetti-Junior, 2004), respectively. Probes were labeled by nick translation method with digoxigenin11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA) (Roche®). Detection of signals was performed with antidigoxigenin-rhodamine (Roche®) for probe of 5S rDNA and amplified avidin-FITC with biotinylated antiavidin (Sigma-Aldrich) for probe of 18S rDNA, with the chromosomes counterstained with 4',6-diamidino-2-phenylindole (DAPI, 50 µg/mL). Metaphases were photographed using a BX 61 epifluorescence microscope, coupled with Olympus DP 72 digital camera (Olympus America, Inc.) with the Olympus cellSens software 2.1 for image processing. The homologous chromosomes were paired and classified in accordance to the ratio of arms (q/p) in metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a), as proposed by Levan *et al.* (1964). The fundamental number (FN) was calculated considering the chromosomes 'm', 'sm' and 'st' as having two arms, and the 'a' chromosomes as having only one chromosomal arm.

Table 1. *Bryconamericus* species collected and their collection sites. ♂ = Males, ♀ = Females, NUP = voucher numbers of the Coleção Ictiológica do Nupélia.

Species	Locality	Basin	Geographic Coordinates	♂	♀	NUP
Bryconamericus aff. *iheringii*	Ijuí River	Upper Uruguai River	28°18'06.3"S/53°53'33.6"W	4	5	15737
Bryconamericus aff. *iheringii*	Iguaçu River	Lower Iguaçu River	25°37'13.20"S/54°23'29.20"W	1	3	14930
Bryconamericus coeruleus	Piquiri River	Upper Paraná River	24°56'54"S/52°35'49"W	13	4	5923
Bryconamericus cf. *ecai*	Bigua Stream	Upper Uruguai River	26°53'22.5"S/53°23'03.1"W	1	5	21293
Bryconamericus cf. *eigenmanni*	Bigua Stream	Upper Uruguai River	26°53'22.5"S/53°23'03.1"W	1	-	21294

Results

The results are presented in Figs. 1–3, and summarized in Tab. 2 and Fig. 4. They are described below.

***Bryconamericus* aff. *iheringii* (Ijuí River).** The diploid number observed was 52 chromosomes (10m + 16sm + 14st + 12a, FN = 92). Silver impregnation revealed the presence of two NOR localization patterns: pattern I – 5 individuals who exhibited markings in the terminal region of the short arm of the pair of acrocentric chromosomes 26, and in one of the chromosomes of the acrocentric pair 25; and pattern II – 4 individuals who exhibited the third marking on the first chromosome of the acrocentric pair 21 (Fig. 1a, boxes). The location of AgNORs was confirmed by 18S rDNA-FISH for the two patterns (Fig. 3a). The C-banding showed heterochromatin in the pericentromeric region of almost all chromosomes, as well as conspicuous blocks associated with NORs, pale blocks in both arms of the metacentric pair 01, and subterminal/terminal heterochromatin in the long arm of the acrocentric pair 22 (Fig. 2a). It should be noted that pattern II presented a higher number of chromosomes with pericentromeric heterochromatic regions when compared to the pattern I (Fig. 4a). 5S rDNA-FISH showed simple cistrons in the terminal region of the short arm of the pair of acrocentric chromosomes 26, syntenic to the cistrons of 18S rDNA, for the individuals who presented the pattern II; and four cistrons of this gene for the individuals that presented the I pattern, three being syntenics to the 18S rDNA cistrons (pair of acrocentric chromosomes 25 and first chromosome of the acrocentric pair 21), and the other located in the terminal region of the short arm of the first chromosome of the submetacentric pair 7 (Fig. 3a).

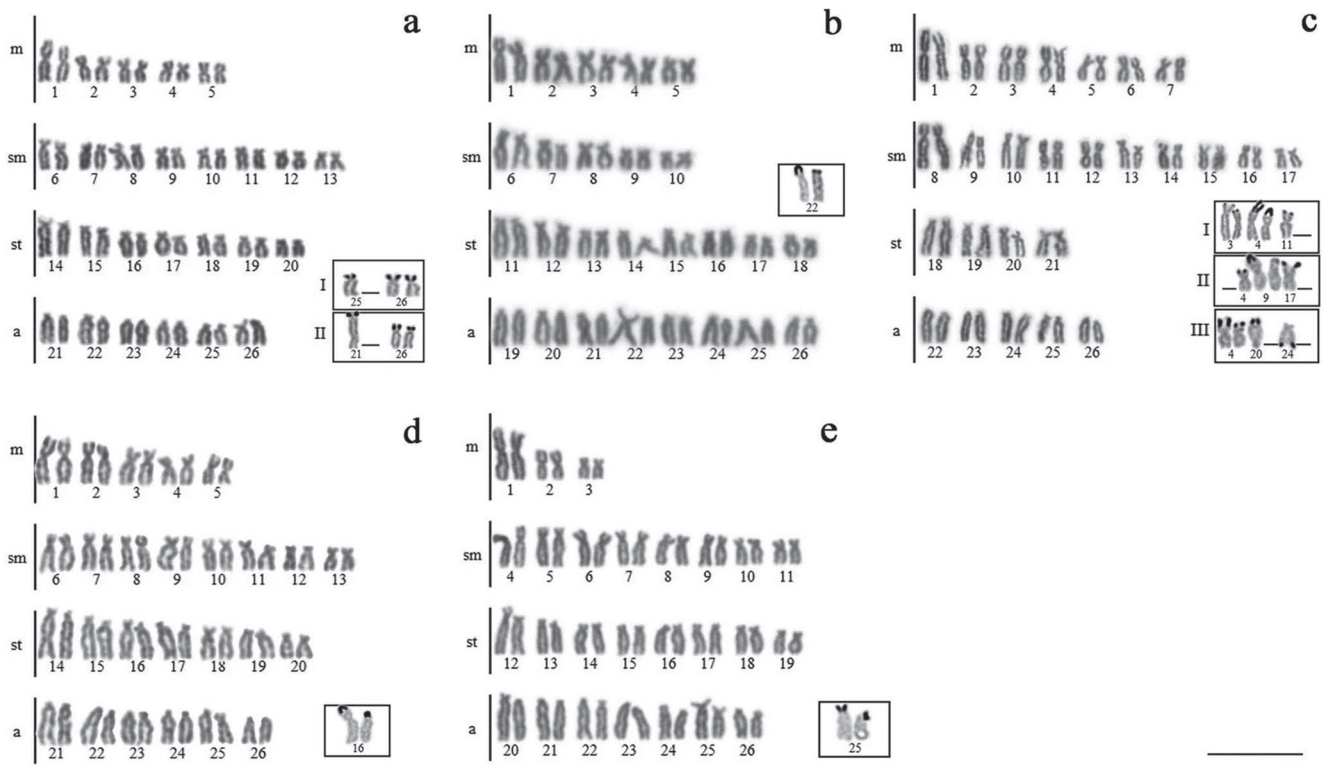

Fig. 1. Karyotypes arranged from Giemsa-stained chromosomes. Pairs of the AgNORs are in the boxes. **a.** *Bryconamericus* aff. *iheringii* (Ijuí River) - box I: pattern I; box II: pattern II; **b.** *B.* aff. *iheringii* (Iguaçu River); **c.** *B. coeruleus* - box I: pattern I; box II: pattern II; box III: pattern III; **d.** *B.* cf. *ecai*; **e.** *B.* cf. *eigenmanni*. Scales bar = 10 μm.

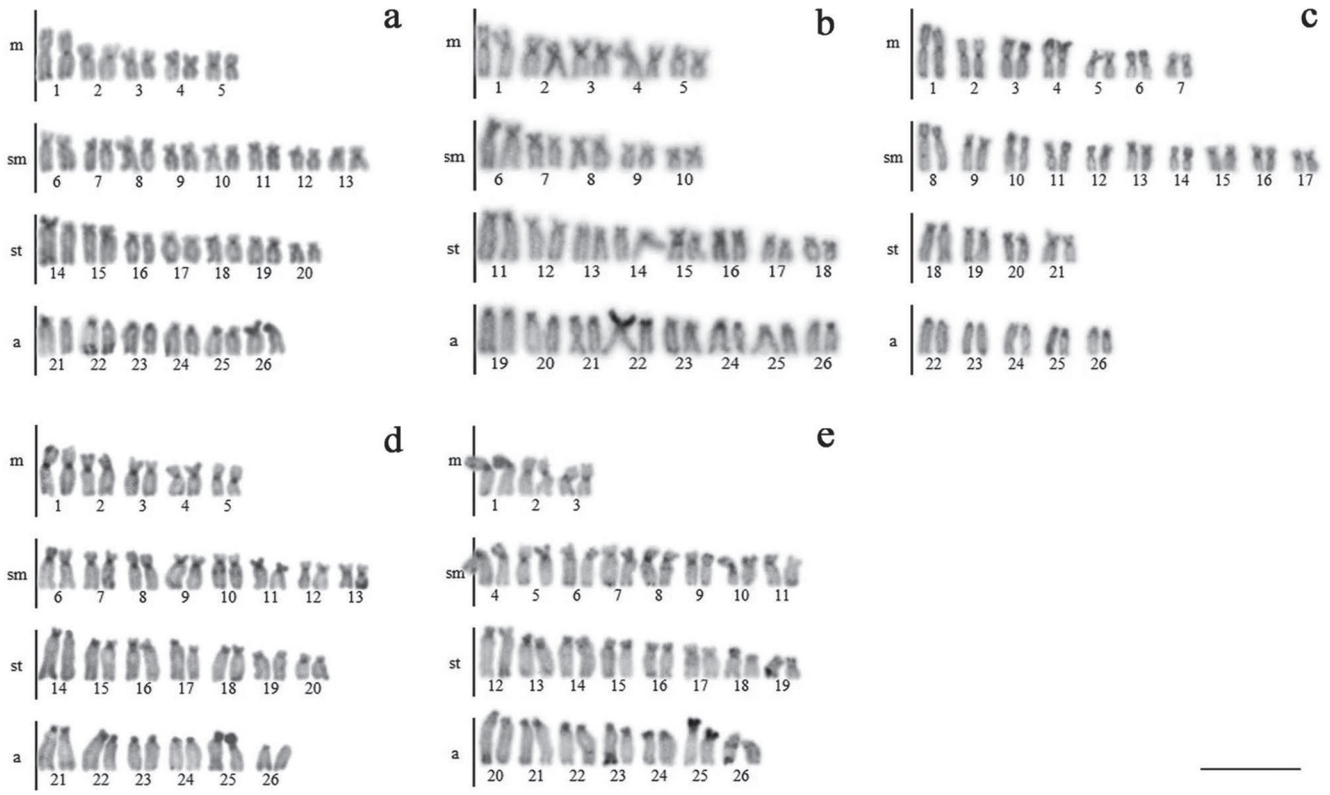

Fig. 2. Karyotypes arranged from C-banded chromosomes. **a.** *Bryconamericus* aff. *iheringii* (Ijuí River, pattern II); **b.** *B.* aff. *iheringii* (Iguaçu River); **c.** *B. coeruleus* (pattern II); **d.** *B.* cf. *ecai*; **e.** *B.* cf. *eigenmanni*. Scales bar = 10 µm.

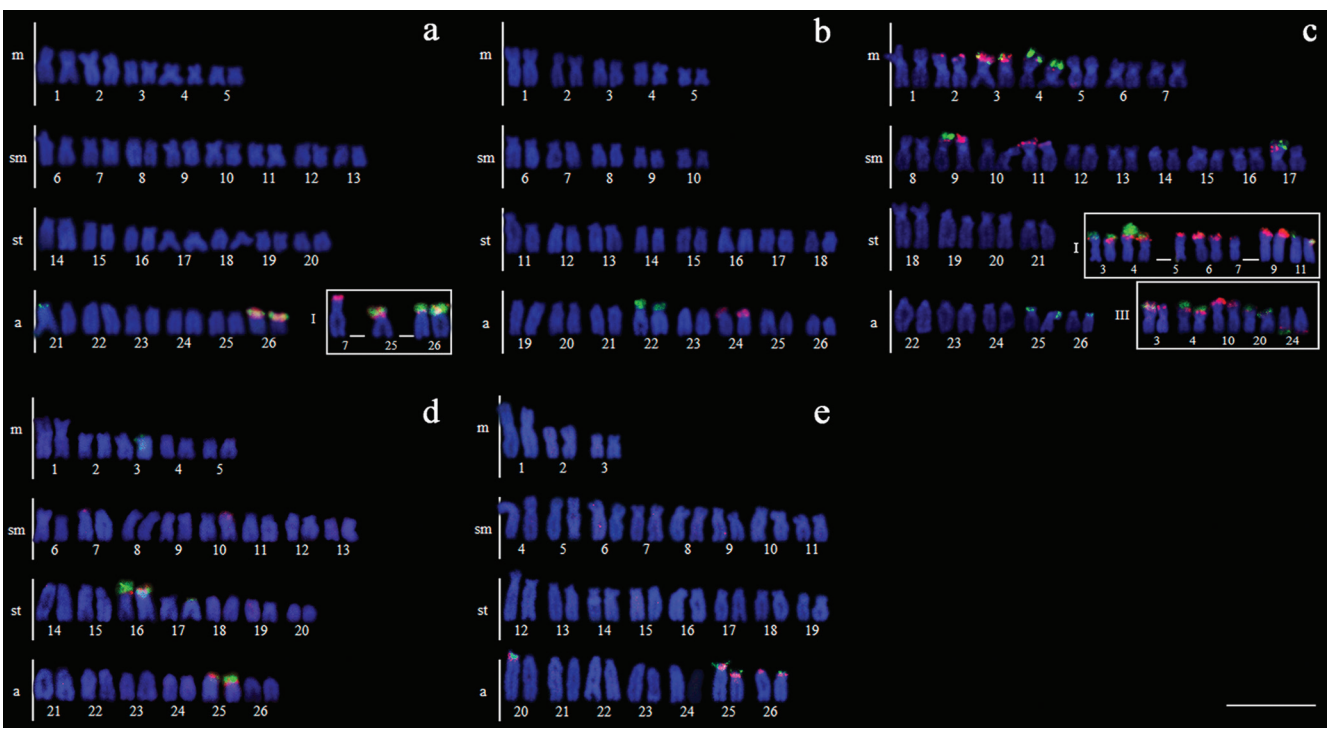

Fig. 3. Karyotypes after FISH with 5S rDNA probes (red) and 18S rDNA probe (green). In the boxes, the intra-population variations that represent distinct patterns of localization of ribosomal genes. **a.** *Bryconamericus* aff. *iheringii* (Ijuí River, pattern II) - box I: pattern I; **b.** *B.* aff. *iheringii* (Iguaçu River); **c.** *B. coeruleus* (pattern II) - box I: pattern I; box III: pattern III; **d.** *B.* cf. *ecai*; **e.** *B.* cf. *eigenmanni*. Scales bar = 10 µm.

***Bryconamericus* aff. *iheringii* (Iguaçu River).** The diploid number observed was 52 chromosomes (10m + 10sm + 16st + 16a, FN = 88), and the presence of single NORs, located in the terminal region of the short arm of the pair of acrocentric chromosomes 22 (Fig. 1b), and confirmed by 18S rDNA-FISH (Fig. 3b). The C-banding showed heterochromatin in the pericentromeric region of almost all chromosomes, in addition to revealing conspicuous blocks in the terminal region of the pairs of chromosomes corresponding to NORs, pale blocks in both arms of the metacentric pair 01, and interstitial heterochromatin in the long arm of the pair of subtelocentric chromosomes 15 (Fig. 2b). The 5S rDNA-FISH showed single cistrons, located in the terminal region of the short arm of the pair of acrocentric chromosomes 24.

***Bryconamericus coeruleus* (Piquiri River).** The diploid number observed was 52 chromosomes (14m + 20sm + 8st + 10a, FN = 94). At least three patterns of NORs were found for this population, being denominated as I (4 individuals), II (8 individuals) and III (5 individuals). The pattern I was characterized by the presence of at least five bearing chromosomes these regions (metacentric pairs 3 and 4, and one of the chromosomes of the subtelocentric pair 11 - Fig. 1c, box I), while the other two patterns presented four bearing chromosomes, which differed from each other (pair of submetaccentric chromosomes 9, and one of the chromosomes of the metacentric 4 and submetacentric 17 pairs to the pattern II, and in the pair of metacentric chromosomes 4 and one of the chromosomes pairs of the subtelocentric 20 and acrocentric 24 - long arm, in this case - for pattern III (Fig. 1c, boxes II and III, respectively)). The C-banding showed heterochromatin in the pericentromeric region of most chromosomes, and terminal pairs in addition to those corresponding to NORs, such as pairs 1 and 2 (both arms), 3, 6, 10, 11 (only on short arm) and 23 (only on long arm) of the individuals who presented the pattern I (Fig. 2c), differing from pattern II where only the metacentric chromosome pair 1 exhibited terminal heterochromatin. This pattern was also different due to the presence of a pericentromeric/ interstitial band in the long arm of the acrocentric chromosomes 26 pair (Fig. 4c-II). The pattern III showed heterochromatic regions similar to those of pattern I, except for heterochromatin associated with NORs, whose pairs of bearing chromosomes differed between the two patterns (Fig. 4c-III). The 18S rDNA-FISH confirmed the location of the NORs and showed an extra cistron for the pattern I, five extra cistrons for the pattern II and two extra cistrons for the pattern III, totaling six, ten and six 18S rDNA sites, respectively (Fig. 3c). The 5S rDNA-FISH revealed a pattern of dispersion of this gene in the karyotype, with intrapopulational variation of 10 to 11 cistrons, all restricted to subterminal/terminal region of the short arm, except for an individual who presented pattern III where a cistron of 5S rDNA was evidenced in the subterminal region of the long arm of the pair of acrocentric chromosomes 24. The three patterns exhibited various colocalized 5S/18S rDNA cistrons. There were four syntenic chromosomes in pattern I (both homologues of pairs 3 and 4), six in pattern II (both homologues of pairs 3 and 4, and one chromosome of pairs 9 and 17) and six in pattern III (both homologues of pairs 4 and 20, and one chromosome of pairs 3 and 24), plus six cistrons of 5S rDNA for pattern I and four for patterns II and III, totaling ten, eleven and ten cistrons of this gene, respectively (Figs. 3c, 4c).

***Bryconamericus* cf. *ecai* (Biguá Stream).** The diploid number observed was 52 chromosomes (10m + 16sm + 14st + 12a, FN = 92), and the presence of single NORs located in the terminal region of the subtelocentric chromosomes 16 pair (Fig. 1d). The C-banding revealed heterochromatin in the pericentromeric region of most chromosomes, interstitial in the long arm of the pair of submetacentric chromosomes 10, and terminal in the pairs of metacentric chromosomes 1 and 2 (both arms), submetacentric pair 10, subtelocentric pairs 14, 15 and 16, and acrocentric pairs 21, 24 and 26 (only in the long arm for all). In the pair of acrocentric chromosomes 25 the short arm was entire heterochromatic, unlike the pair of NORs, which in this species did not present heavily marked heterochromatic bands (Fig. 2d). The 18S rDNA-FISH confirmed the localization of the NORs, and evidenced extra cistrons in the terminal region of the short arm of the acrocentric chromosomes 25 pair, in both cases syntenic to 5S rDNA cistrons (Figs. 3d, 4d).

***Bryconamericus* cf. *eigenmanni* (Biguá Stream).** The diploid number observed was 52 chromosomes (6m + 16sm + 16st + 14a, FN = 90), and the presence of simple NORs located in the terminal region of the acrocentric chromosomes 25 pair (Fig. 1e). The C-banding revealed heterochromatin in the pericentromeric region of most chromosomes, interstitial in the long arm of the submetacentric chromosomes 8 and acrocentric 26 pairs, and terminal on the short arm of submetacentric pairs 4, 9 and 10 and subtelocentric pair 19, and in the long arm of submetacentric pairs 4, 5, 6, 7, 8 and 11, subtelocentric pair 16, and acrocentric pairs 20, 21, 23 and 26. The chromosomes pair bearing the NORs had the fully heterochromatic short arm (Figs. 2e, 4e). The 18S rDNA-FISH confirmed the localization of the NORs and showed two extra cistons in the short arm of one acrocentric chromosomes of the pairs 20 and 26. The 5S rDNA-FISH showed 5 cistrons of this gene, four of them syntenic to 18S rDNA genes, and the other located on the short arm of the first chromosome of the acrocentric pair 26 (Figs. 3e, 4e).

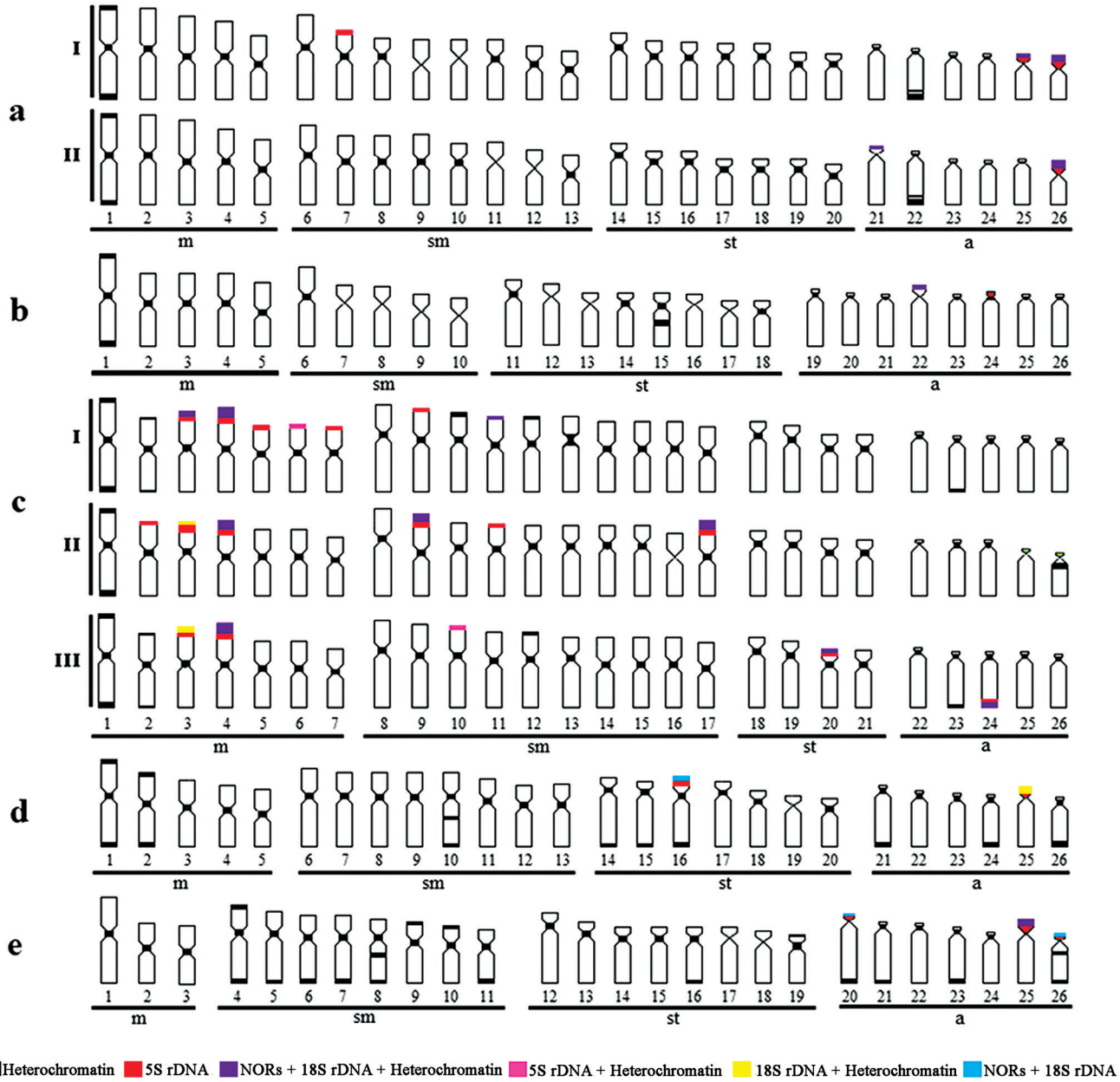

Fig. 4. Idiogram comparing the cytogenetic characteristics of the four *Bryconamericus* species. **a** *B.* aff. *iheringii* (Ijuí River): patterns I and II; **b** *B.* aff. *iheringii* (Iguaçu River); **c** *B. coeruleus*: patterns I, II and II; **d** *B.* cf. *ecai*; **e** *B.* cf. *eigenmanni*.

Discussion

This study presents the first cytogenetic data for the populations of *B.* aff. *iheringii* from Ijuí and Iguaçu River, *B. coeruleus* from Piquiri River, *B.* cf. *ecai* and *B.* cf. *eigenmanni* from Biguá Stream (being the first study for the last). Of the analyzed cytogenetic characters, the fundamental number and the karyotypic formula were effective in the delimitation of the species and in the differentiation of the two populations of *B.* aff. *iheringii*. The detection of interstitial heterochromatin showed to be a valuable species marker for *B.* cf. *ecai* (submetacentric pair 10) and *B.* cf. *eigenmanni* (submetacentric pair 8 and acrocentric pair 26), as well as a population marker for *B.* aff. *iheringii* from the Iguaçu River (subtelocentric pairs 15 and 18). The presence of single NORs (confirmed by 18S rDNA-FISH) and single 5S rDNA cistrons in individuals of Iguaçu River also differentiated them from *B.* aff. *iheringii* of Ijuí River. The number and distribution of the 5S-18S rDNA cistrons were shown to be relevant marker both for *B.* cf. *ecai* as in *B.* cf. *eigenmanni.*

The cytogenetic studies in *Bryconamericus* corroborate the diploid number of 52 chromosomes, invariable and considered ancestral for the genus (Wasko, Galetti-Junior, 1998). Added to that is found inter- and intrapopulation/ specific variation for the karyotypic macrostructure and the

FN, ranging from 80 in cytotype I of *B. ecai*, to 100 in the cytotype IV of *B. ecai* (Santos *et al*., 2012), and V and VI of *B. coeruleus* (Silva *et al.*, 2014) (Tab. 2). These characteristics were also verified for all the species analyzed here. According to Wasko, Galetti-Junior (1998), the variation in the FN without the change in the diploid number comprises, in evolutionary terms, the occurrence of chromosomal rearrangements, possibly of the pericentric inversion type, that act in the process of diversification of this group of fish. It is important to note that what occurs in *Bryconamericus* differs, for example, from that observed in *Astyanax*. In this genera the karyotype variation is accompanied by high intra/interspecific and interpopulational divergence of diploid number (Pazza *et al.*, 2018), due to events of fusion and centric fission, often accompanied by inversions and translocations, common in *Astyanax* spp. (Fernandes, Martins-Santos, 2005; Pazza *et al.*, 2008; Piscor *et al.*, 2019; among others).

Table 2. Review of cytogenetic data in *Bryconamericus*. 2n=Diploid number; FN=Fundamental number; CB=C-banding, 18S=18S rDNA cistrons number; 5S=5S rDNA cistrons number; PR = State of Paraná; RS = State of Rio Grande do Sul; SC = State of Santa Catarina; SP = State of São Paulo; *Cited as *B.* aff. *iheringii*; C = Centromeric, I = Interstitial, ST = Subterminal, T = Telomeric, T* = Terminal.

Species	Locality/State	2n	Karyotype formula	FN	CB	NORs	18S	5S	References
B. aff. *exodon* - I	Três Bocas Stream (PR)	52	16m+12sm+6st+18a	86	-	2-5	8	-	Paintner-Marques *et al*. (2002a,b)
B. aff. *exodon* - II	Três Bocas Stream (PR)	52	10m+24sm+6st+12a	92	-	2-5	8	-	
*B. coeruleus**	Água da Floresta River (PR)	52	8m+22sm+10st+12a	92	C, T	2	2	-	Paintner-Marques *et al*. (2003)
*B. coeruleus**- I	Keller River (PR)	52	12m+18sm+8st+14a	90	P, T	-	-	-	Portela-Castro *et al.* (2008)
*B. coeruleus**- II	Keller River (PR)	52	8m+28sm+6st+10a	94	P, T	-	-	-	
*B. coeruleus**	Tatupeba Stream (PR)	52	8m+20sm+8st+16a	88	-	2	2	-	
*B. coeruleus**	Maringá Stream (PR)	52	12m+18sm+8st+14a	90	-	2-4	6	-	Capistano *et al*. (2008)
*B. coeruleus**	Keller River (PR)	52	8m+28sm+6st+10a	94	-	2-4	10	-	
*B. coeruleus**- I	Três Bocas Stream (PR)	52	12m+16sm+10st+14a	90	-	3-5	-	-	
*B. coeruleus**- II	Três Bocas Stream (PR)	52	14m+18sm+10st+10a	94	-	2	-	-	Eberhardt *et al.* (2012)
*B. coeruleus**- III	Três Bocas Stream (PR)	52	10m+24sm+6st+12a	92	-	2-3	-	-	
*B. coeruleus**- IV	Três Bocas Stream (PR)	52	10m+14sm+8st+20a	84	-	0	-	-	
*B. coeruleus**- I	Três Bocas Stream (PR)	52	12m+10sm+16st+14a	90	P	2	2	-	
*B. coeruleus**- II	Três Bocas Stream (PR)	52	18m+14sm+10st+10a	94	P	2-4	2-6	-	
*B. coeruleus**- III	Três Bocas Stream (PR)	52	20m+18sm+4st+10a	94	P	2-5	2-6	-	Silva *et al*. (2014)
*B. coeruleus**- IV	Três Bocas Stream (PR)	52	20m+14sm+12st+6a	98	P	2-3	4	-	
*B. coeruleus**- V	Três Bocas Stream (PR)	52	22m+18sm+8st+4a	100	P	3-4	4-6	-	
*B. coeruleus**- VI	Três Bocas Stream (PR)	52	18m+24sm+6st+4a	100	P	3-5	4-6	-	
*B. coeruleus**	Ocoí River (PR)	52	12m+18sm+8st+14a	90	P	2	2	-	Nishiyama *et al.* (2015)
B. coeruleus	Piquiri River (PR)	52	14m+20sm+8st+10a	94	P, T	4-5	6-9	10-11	Present study
B. aff. *iheringii*	Ijuí River (RS)	52	10m+16sm+14st+12a	92	P, ST, T*	3	3	2-4	Present study
B. aff. *iheringii*	Iguaçu River (PR)	52	10m+10sm+16st+16a	88	P, T*, I	2	2	2	Present study
B. cf. *iheringii*	Tributary of Corumbataí River (SP)	52	10m+14sm+18st+10a	94	P	2	2	2	Piscor *et al*. (2013)
B. ecai - I	Forquetinha River (RS)	52	10m+10sm+8st+24a	80	P, T	2-4	4	-	
B. ecai - II	Forquetinha River (RS)	52	10m+18sm+8st+16a	88	P	2	2	-	Santos *et al.* (2012)
B. ecai - III	Forquetinha River (RS)	52+B	14m+14sm+6st+18a	86	P	2-3	6	-	
B. ecai - IV	Forquetinha River (RS)	52	10m+24sm+14st+4a	100	P, T, I	2	2	-	
B. ecai - V	Forquetinha River (RS)	52	8m+16sm+14st+14a	90	-	3	4	6	
B. ecai - VI	Forquetinha River (RS)	52	10m+16sm+8st+18a	86	-	3	13	8	Santos *et al.* (2017)
B. ecai - VI	Forquetinha River (RS)	52	8m+18sm+10st+16a	88	-	3	10	7	
B. cf. *ecai*	Bigua Stream (SC)	52	10m+16sm+14st+12a	92	P, T*, I	2	4	4	Present study
B. cf. *eigenmanni*	Bigua Stream (SC)	52	6m+16sm+16st+14a	90	P, T*, I	2	4	5	Present study
B. turiuba	Tributary of Passa-Cinco River (SP)	52	8m+10sm+14st+20a	84	P	2	4	4	Piscor *et al*. (2013)
Bryconamericus. sp. - Group 1	Vermelho River (PR)	52	16m+14sm+10st+12a	92	-	2	4	6	
Bryconamericus. sp. - Group 2	Vermelho River (PR)	52	16m+14sm+10st+12a	92	-	5	16	8	Santos *et al.* (2017)
Bryconamericus sp.	Cambutá River (PR)	52	2m+10sm+20st+20a	88	-	3	6	2	

The two populations of *B*. aff. *iheringii* analyzed presented different karyotypic macrostructure among themselves and compared to other populations of the same species. Likewise, *B*. cf. *ecai* differed from other populations already studied (Santos *et al*., 2012, 2017). This indicates a high degree of chromosomal rearrangements, common in other Stevardiinae genera such as *Piabina* (Fernandes *et al*., 2010; Pazian *et al*., 2012; Piscor *et al*., 2018) and *Piabarchus* (Fernandes *et al*., 2010, cited as *Bryconamericus*), and in other complex groups of Characidae, such as the *Serrasalmus* (Martins-Santos *et al*., 1994; Centofante *et al*., 2002), *Cheirodon* (Soto *et al*., 2018) and *Astyanax* (Yano *et al*., 2014; Paiz *et al*., 2015; Nishiyama *et al*., 2016, among others).

Cytogenetic studies in *Bryconamericus* by Paintner-Marques *et al*. (2003) have involved the detection of heterochromatic regions through C-banding, revealing relevant characteristics in comparative analyzes. In the species analyzed here, the predominance of heterochromatin in the pericentromeric region of most chromosomes was obseerved, a common pattern for the genus, but with variation in chromosomes with interstitial, subterminal and terminal heterochromatin. Conspicuous heterochromatic blocks in the interstitial region of chromosomes were found only in cytotype IV of *B. ecai* (Santos *et al.,* 2012), and in the present study in *B.* aff. *iheringii* (Iguaçu River), *B.* cf. *ecai* and *B.* cf. *eigenmanni* (Figs. 2, 4b, d, e), allowing to distinguish these species among themselves and to differentiate the two populations of *B.* aff. *iheringii*. In *B.* aff. *iheringii* from the Ijuí River, the presence of subterminal heterochromatins on a pair of acrocentric chromosomes was observed in both patterns, revealing that this is a conserved characteristic in the population, and also allows distinguishing it from the population of the Iguaçu River. The *B. coeruleus* pattern II differed from the others by the presence of a pericentromeric/interstitial heterochromatic block on the long arm of the last pair of acrocentric chromosomes (Fig. 4c-II).

The number of chromosomes pairs with terminal heterochromatin also made it possible to differentiate the species and populations of *Bryconamericus* (Figs. 2, 4), with the exception of terminal heterochromatin located in both arms on the metacentric chromosome pair 1, which were shared by all species and patterns found, except in *B.* cf. *eigenmanni*. In this species, the same pattern of heterochromatin was evidenced in the first pair of submetaccentric chromosomes (pair 4), suggesting that these chromosomes may have been metacentric chromosomes that underwent pericentric inversion, so that all possibly share the same sequences in these regions. As proposed by Wasko, Galetti-Junior (1998), the *Bryconamericus* species do not seem to reveal a general trend in relation to the heterochromatin distribution, so that each species can be characterized by a specific C-band pattern, as also observed by Piscor *et al*. (2013) and in the present study. However, the variation in the distribution of these regions is also visible at interpopulation (Portela-Castro *et al*., 2008; present study) and intrapopulation levels (Santos *et al.*, 2012; present study, in *B.* aff. *iheringii* from the Ijuí River and in *B. coeruleus*, especially regarding heterochromatins associated with NORs).

Although silver impregnation revealed single NORs in three species of this study (Fig. 1b, d, e, boxes), 18S rDNA-FISH confirmed single sites only in *B*. aff. *iheringii* (Iguaçu River) (Figs. 1, 4b, d, e). *Bryconamericus* aff. *iheringii* from the Ijuí River exhibited two patterns of multiple NORs, both with three chromosomes bearing these regions (Figs. 1, 4a), whereas in *B. coeruleus* it was possible to identify three distinct patterns (pattern I: five bearing chromosomes; patterns II and III: four bearing chromosomes) (Figs. 1, 4c). The presence of single NORs is less recurrent in *Bryconamericus* than the occurrence of multiple NORs, as evidenced in Table 2. According to Machado *et al.* (2017), the presence of multiple 18S rDNA sequences dispersed in the genome may reflect the amplification and dispersion of these genes mediated by their association with mobile genetic elements (transposons and/or retrotransposons).

The analysis of the chromosomes carrying the NORs allows the diagnosis of intra-individual heteromorphism of region size between the homologous chromosomes in *B.* aff. *iheringii* from the Iguaçu River, *B. coeruleus* (patterns I and III: pair 4; pattern II: pair 9), and *B.* cf. *ecai* of the present study (Fig. 1b, c, d). These data were confirmed by 18S rDNA-FISH, revealing that in these species there is, in fact, variation in the number of copies of this ribosomal gene, due to events of duplication or deletion occurred during meiosis. Similar considerations were made by Piscor *et al*. (2013) in *B.* cf. *iheringii*, and Santos *et al.* (2017) in *Bryconamericus* sp. (Group 1), but in both cases 18S rDNA-FISH detected sites of similar size, indicating that the variation visualized by silver impregnation could refer to differences in the degree of condensation between homologous chromosomes, and differential gene activity rDNA segments (45S).

The 5S rDNA-FISH revealed the presence of these genes always in the terminal region of the chromosomes, varying between single sites in *B.* aff. *iheringii* from the Iguaçu River and pattern II of the Ijuí River population (Figs. 3, 4a, b), and multiple sites in pattern I of this population and in the other species analyzed. With the exception of *B.* aff. *iheringii* from the Iguaçu River, the other species and the other population presented 5S rDNA sites located in the short arm of pairs bearing 18S rDNA genes, configuring the syntenic location. Data for 5S rDNA are scarce for the genus, and are restricted to some populations of *B.* cf. *iheringii*, *B. turiuba*, *B. ecai* and *Bryconamericus* sp. (Piscor *et al*., 2013; Santos *et al.*, 2017), revealing a variation in the number and location of these cistrons, as well as the frequent sinteny to 18S rDNA genes, as verified in the species of the present study. The 5S-18S rDNA syntenic localization has also been reported in other groups, such as *Prochilodus lineatus* (Valenciennes, 1837) (Vicari *et al.*, 2006), *Hypostomus commersoni* Valenciennes, 1836 (Bueno *et al.*, 2014), *Astyanax altiparanae* Garutti & Britski, 2000, *Astyanax lacustris* (Lütken, 1875), *Astyanax*

fasciatus (Cuvier, 1819), *Astyanax schubarti* Britski, 1964 and *Astyanax paranae* Eigenmann, 1914 cited as *Astyanax scabripinnis* (Jenyns, 1842) (Almeida-Toledo *et al*., 2002), *Solea senegalensis* Kaup, 1858 (Cross *et al*., 2006), and *Corydoras carlae* Nijssen & Isbrücker, 1983 (Rocha *et al*., 2016) and marine species *Thalassoma noronhanum* (Boulenger, 1890), *Halichoeres penrosei* Starks, 1913, *H. poeyi* (Steindachner, 1867), *H. radiatus* (Linnaeus, 1758) and *H. brasiliensis* (Bloch, 1791) (Amorim *et al.*, 2016).

The ribosomal DNA genes in eukaryotes comprise two multigenic families: 45S rDNA, transcribed in the nucleolus, and 5S rDNA, transcribed outside the nucleolus, suggesting that the differential functioning of both families requires physical detachment (Martins, Galetti-Junior, 2001), avoiding the occurrence of disruptive interferences, such as translocations from 5S to 45S and vice-versa, capable of affecting the dynamics of both (Martins, Galetti-Junior, 1999). On the other hand, as the *double*-FISH techniques have been performed, the greater the number of fish species with a syntenic location of these sequences. According to Schweizer, Loidl (1987), the telomeric regions are susceptible to equi-local transfers, even between non-homologous chromosomal arms, according to their proximity in the interphase nucleus promoted by the orientation of the chromosomes according to the Rabl model. Thus, we can infer that in all species analyzed in this work (except *B*. aff. *iheringii* from the Iguaçu River that did not display sinteny), the transfer events between the two multigenic families may have been facilitated.

The large variation in the number of 5S rDNA cistrons, especially in *B*. *coeruleus*, may suggest the occurrence of pseudogeneization events or the insertion of mobile genetic elements in the 5S rDNA intergenic spacers, promoting the detection of "extra" sites of this gene through FISH, in addition to those effectively functional, as observed in *Erythrinus erythrinus* (Bloch &Schneider, 1801) (Cioffi *et al*., 2010) and *Gymnotus mamiraua* Albert & Crampton, 2001 (Silva *et al*., 2016).

The cytogenetic data of this study (diploid number, karyotype formulas and the fundamental numbers) corroborate the hypothesis of the occurrence of chromosomal rearrangements, possibly pericentric inversions. That demonstrate the complexity involved in the evolutionary dynamics of the group and, although *Bryconamericus* does not present a general trend of heterochromatin distribution, specific heterochromatic blocks proved to be important cytogenetic markers in the identification of some species and in the differentiation of the two analyzed populations. The dispersed location of ribosomal genes (5S-18S rDNA) suggests the occurrence of amplifications of these sequences, associated with events of duplication, pseudogeneization and transposition mediated by mobile genetic elements, and their co-localization in all species may represent a plesiomorphic condition in *Bryconamericus*, although this group lacks cytogenetic and molecular data to better understand the evolutionary dynamics of these genes.

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