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Karyotypic diversity in a population of *Bryconamericus* aff. *iheringii* (Characidae)

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ABSTRACT. *Bryconamericus* comprises 56 species distributed into three groups, on the basis of the position and shape of the maxillary teeth: *B. exodon, B. microcephalus* and *B. iheringii* groups. Few cytogenetic data are available for this genus, but the diploid number of 52 chromosomes is quite common, although the karyotypic variability is extensive. This study aimed to characterize a population of *B.* aff. *iheringii* and thus contribute more cytogenetic information and better understanding of the structure and karyotypic evolution of this genus. We found 6 cytotypes with different NOR patterns: cytotype I showed a karyotype formula of 12m+10sm+16st+14a (FN = 90) and single NORs; cytotype II with 18m+14sm+10st+10a (FN = 94) and cytotype III with 20m+18sm+4st+10a (FN = 94), showing both single and multiple NORs; cytotype IV with 20m+14sm+12st+6a (NF = 98), cytotype V with 22m+18sm+8st+4a (FN = 100) and cytotype VI with 18m+24sm+6st+4a (FN = 100), all with multiple NORs. Cytotype I is the most different in relation to FN and NOR pattern, and can be regarded as belonging to another species of the genus *Bryconamericus*, living in sympatry in Três Bocas Stream. The remaining cytotypes may have been generated by crosses between them and by pericentric

inversions. Meiotic cells were also analyzed and showed that despite the high karyotypic variability, chromosome pairing occurred normally. The great variability found in *B.* aff. *iheringii* may be related to a high degree of polymorphism; nevertheless, the possibility of occurrence of more than one species in this location is not ruled out, demonstrating the need for conservation of the Três Bocas Stream.

Key words: C-banding; Cytotypes; Chromosomal rearrangements; Polymorphism

INTRODUCTION

The genus *Bryconamericus* is not taxonomically well defined, and many authors believe that it is not monophyletic, since there are no synapomorphies that bring the species together. This genus includes 56 valid species that inhabit a variety of freshwater ecosystems throughout Central and South America and is divided into three main groups, on the basis of the position and shape of the maxillary teeth: *B. iheringii* Boulenger 1887, *B. exodon* Eigenmann 1907 and *B. microcephalus* Miranda Ribeiro 1908 (Silva, 2004).

Despite the great karyotypic diversity reported for the genus, the diploid number of 52 is very conserved (Capistano et al., 2008; de Brito Portela-Castro et al., 2008), except for *Bryconamericus* sp E of the Avoadeira Stream, MT, which shows 54 chromosomes (Wasko and Galetti Jr., 1998). Furthermore, the pattern of multiple nucleolar organizer regions (NORs) is the most common within the genus (Capistano et al., 2008), as well as the association of these sites with positive signals for chromomycin A_3 (CMA_3^+) or mithramycin (MM^+) (Paintner-Marques et al., 2002; dos Santos et al., 2012), indicating that the association of the NORs with GC-rich base pair sequences is common.

On the other hand, the single NOR pattern has already been documented for one of the cytotypes of *B. ecai* Silva 2004 (dos Santos et al., 2012) and for *B.* aff. *iheringii* of Tatupeba Stream (Capistano et al., 2008) and Agua da Floresta River (Paintner-Marques et al., 2003), and the latter is the first species in the genus to have single NORs documented.

In the genus *Bryconamericus*, the heterochromatin is distributed mainly in terminal and pericentromeric regions (Paintner-Marques et al., 2003; de Brito Portela-Castro et al., 2008), and terminal heterochromatin in subtelocentric chromosomes was regarded by Paintner-Marques et al. (2003) as a chromosome marker for *B. iheringii*. Furthermore, the composition of heterochromatin may vary and is used to differentiate between cytotypes after staining with base-specific fluorochromes (dos Santos et al., 2012), as well as distinguishing heterochromatic types in the same karyotype by digestion with *Alu*I restriction enzyme (Paintner-Marques et al., 2003).

Ruiz (2007) performed a taxonomic analysis on individuals of the *Bryconamericus* species that occur in Tibagi River basin, PR, and identified a species that did not fall within any of the described species, and called it *Bryconamericus* sp and included it in the *B. iheringii* group. Its distribution includes the region of Middle Taquara River and Três Bocas Stream, both tributaries of Tibagi River, in the municipality of Londrina. The species of the present study was then called *B.* aff. *iheringii* and cytogenetically analyzed by different chromosome banding techniques, thereby contributing more data to the genus to get a better understanding of the evolutionary and phylogenetic relationships within the group.

MATERIAL AND METHODS

We analyzed sixteen specimens (9 females, 7 males) of *B.* aff. *iheringii* from Três Bocas Stream, Londrina, PR, Brazil (23°23'06.6"S, 51°04'35.8"W) (Figure 1). Specimens were deposited in the Museum of Zoology of Universidade Estadual de Londrina (MZUEL) under the numbers 5775 and 5776. The samples were collected with the permission of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), protocol No. 11399-1.

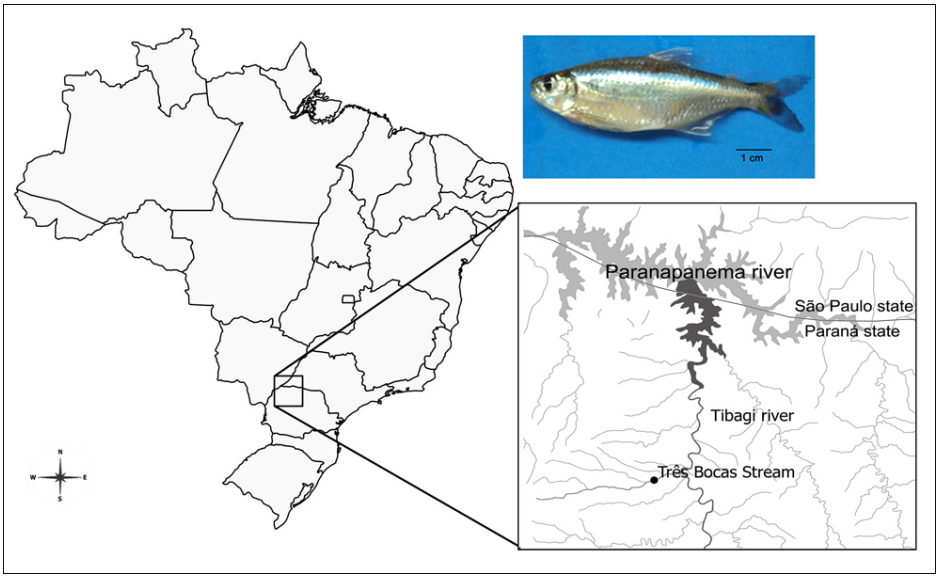

Figure 1. Map of Brazil indicating the collection sites in Três Bocas Stream, owned by Paranapanema Basin, Paraná State. Highlighted, a specimen of *Bryconamericus* aff. *iheringii*.

Mitosis was stimulated by injection of animals with a yeast suspension, as described by Lee and Elder (1980). Mitotic chromosomes were obtained by direct preparation, removing the anterior kidney and short term culture using solid tissues, according to Bertollo et al. (1978) and Fenocchio et al. (1991), respectively, then stained with 5% Giemsa in phosphate buffer, pH 6.8. Meiotic chromosomes were obtained from gonadal cells by the technique developed by Kligerman and Bloom (1977), with modifications. The chromosomes were classified according to Levan et al. (1964), with modifications. For determination of the fundamental number (FN), the metacentric (m), submetacentric (sm), and subtelocentric (st) chromosomes were considered biarmed, and the acrocentric (a), uniarmed.

The distribution of heterochromatin was analyzed by Giemsa C-banding after treatments with 0.2 N HCl, $Ba(OH)_2$ and 2X SSC (Sumner, 1972) and sequence staining with CMA_3 and 4'-6-diamino-2-phenylindole (DAPI). Silver nitrate staining of active NORs (Ag-NOR) was performed according to Howell and Black (1980). The GC- and AT-rich bands were detected with CMA_3 and DAPI, according to Schweizer (1980). The slides were stained

with 0.5 mg/mL CMA_3 for 1 h, washed in distilled water and sequentially stained with 2 µg/mL DAPI for 15 min. Slides were mounted with a medium composed of glycerol/McIlvaine buffer, pH 7.0, 1:1, plus 2.5 mM $MgCl_2$.

Fluorescence *in situ* hybridization (FISH) was performed according to Pinkel et al. (1986), with modifications. The 18S rDNA probe of *Prochilodus argenteus* Agassiz 1829 (Hatanaka and Gatetti, 2004) was labeled with biotin-14-dATP by nick translation. Slides were treated with 50 µL hybridization mixture containing 7.5 µL 100 ng labeled probe, 30 µL 50% formamide, 12 µL 50% dextran sulfate, 10.5 µL 20X SSC. The material was denatured at 80°C for 10 min, and hybridization was performed overnight at 37°C in a humidified chamber. Post-hybridization washes were carried out in 2X SSC for 5 min, in 1X PBS and 1X PBD (20X SSC, Triton-100, non-fat milk and distilled water qsp 100, pH 7), all at 45°C. The probe was detected with 5 µL FITC (1:100) and 45 µL BSA (5%) and 40 µL amplification solution (1 mL anti-avidin-biotin conjugate and 39 mL 1X PBD) were used to amplify the signals. Slides were mounted with 25 µL medium composed of 23 µL DABCO solution (1,4-diazabicyclo(2.2.2)-octane (2.3%), 20 mM Tris-HCl, pH 8.0 (2%) and glycerol (90%), in distilled water), 1 µL 50 mM $MgCl_2$ and 1 µL 50 µg/mL propidium iodide. All the images were acquired with a Leica DM 4500 B microscope equipped with a DFC 300FX camera and the Leica IM50 4.0 software, and optimized with the iGrafx Image software for best constrast and brightness.

RESULTS

Conventional staining

All specimens of *Bryconamericus* aff. *iheringii* showed 2n = 52 chromosomes, but different karyotypic formulae. The occurrence of six cytotypes (Figure 2) was observed, as shown in Table 1, along with their respective frequencies.

NORs and fluorochromes

Distinct NOR patterns were observed within and between the different cytotypes after impregnation with silver nitrate and FISH with a 18S rDNA probe (Figures 2 and 3, respectively). Each box in Figure 2 represents a NOR pattern. The two individuals of cytotype I had only single NORs with signals on the short arm of pair 15 (st) (Figure 2a).

Cytotypes II and III showed both single and multiple NOR patterns. One individual of cytotype II had terminal NORs on the short arm of pair 5 (m), and in one of the homologous, the NOR was double and coincident with a secondary constriction. The two other individuals of cytotype II had terminal signals on the long arm of pairs 4 (m) and 12 (sm) and on the short arm of pair 5 (m) (Figure 2b). An individual of cytotype III displayed terminal NORs on the short arm of pair 5 (m), and another showed signals on the long arm of pairs 4 (m) and 14 (sm) and on the short arm of pair 5 (m) (Figure 2c).

Cytotypes IV, V and VI exhibited a multiple NOR pattern, with variation in the number and position of those sites. One individual of cytotype IV showed terminal NORs on the short arm of pair 5 (m) and on the long arm of pair 19 (st); another individual showed terminal bands on the long arm of pair 12 (sm) and pair 19 (st) (Figure 2d).

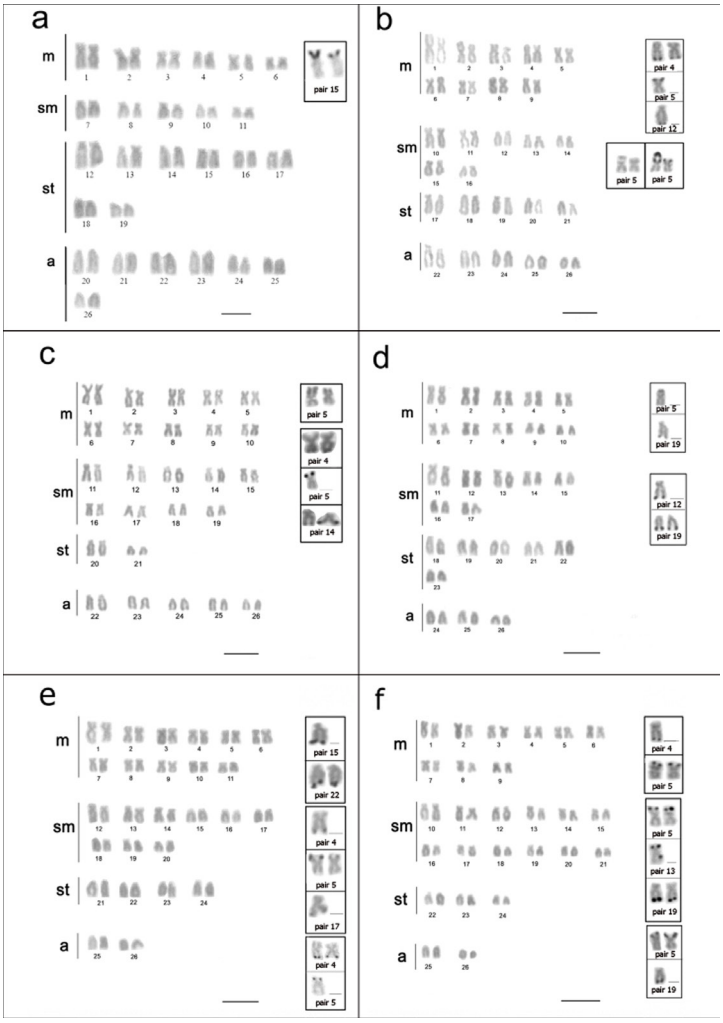

Figure 2. Cytotypes of *Bryconamericus* aff. *iheringii* from Três Bocas Stream: **a.** CytI, **b.** CytII, **c.** CytIII, **d.** CytIV, **e.** CytV, **f.** CytVI. In the boxes, there are Ag-NORs with different patterns found in each cytotypes. In cytotype II, the pair 5 is also evident with convencional staining to demonstrate secundary constriction in one of homologues. Scale bar = 5 µm.

Table 1. Cytotypes of *Bryconamericus* aff. *iheringii* from Três Bocas Stream.

Cytotypes		FN	No. of individuals	Frequency (%)
I	12m+10sm+16st+14a	90	2	12.5
II	18m+14sm+10st+10a	94	3	18.75
III	20m+18sm+4st+10a	94	2	12.5
IV	20m+14sm+12st+6a	98	2	12.5
V	22m+18sm+8st+4a	100	3	18.75
VI	18m+24sm+6st+4a	100	4	25

FN = fundamental number.

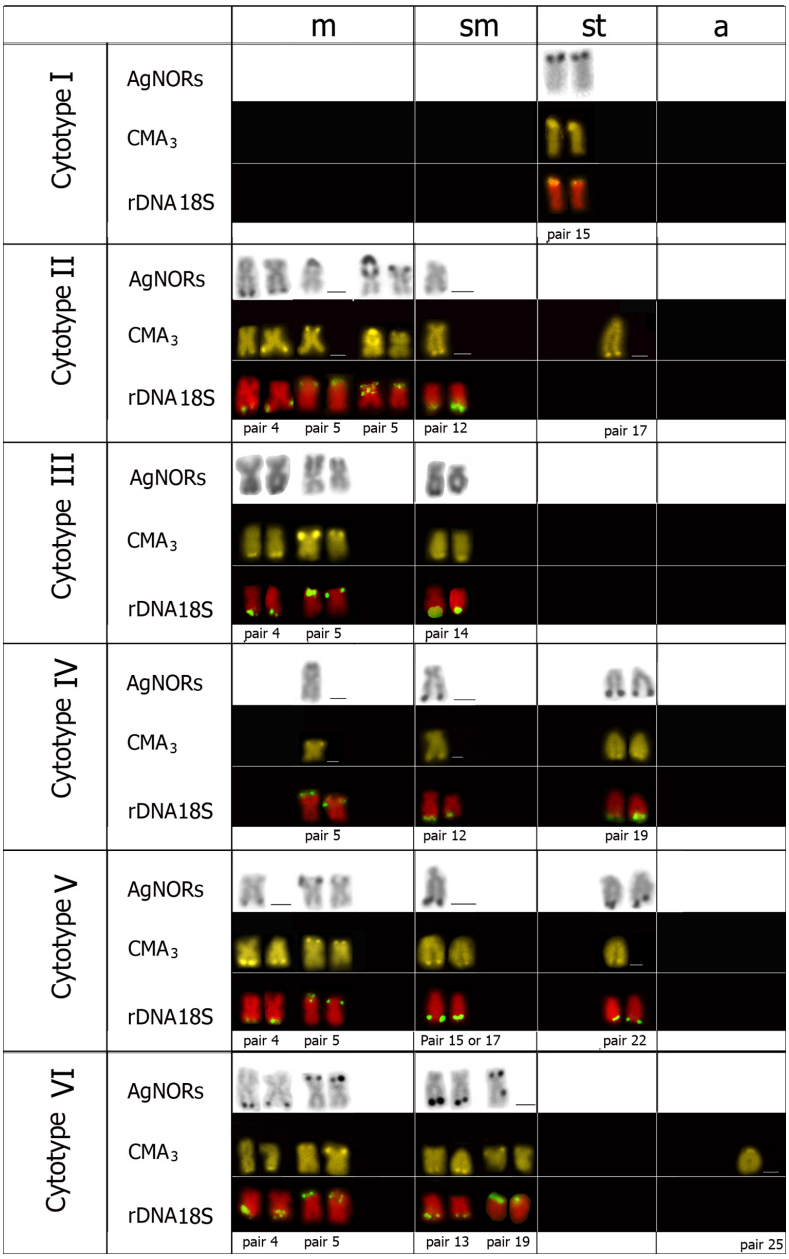

Figure 3. Chromosomes of *Bryconamericus* aff. *iheringii* submitted to treatment with silver nitrate, *in situ* hybridization with rDNA 18S probe and fluorochrome chromomycin A_3 in different cytotypes. Note that there are chromosomes in cytotypes II and VI which are CMA_3^+ but that have no association with NORs. In cytotype II, it is represented the pair 5 with a double NOR that only occurs in NORs simple standard and pair 5 without heteromorphism that occurs in multiple NORs standard.

Cytotype V showed three patterns: an individual with terminal NORs on the long arm of pairs 15 (sm) and 22 (st); another with terminal NORs on the short arm of pair 5 (m) and on the long arm of pairs 4 (m) and 17 (sm); and a third one with terminal NORs on the short arm of pair 5 (m) and on the long arm of pair 4 (m) (Figure 2e).

Cytotype VI also showed three patterns of multiple NORs: two individuals with terminal NORs on the long arm of pair 4 (m) and on the short arm of pair 5 (m); an individual with terminal NORs on the short arm of pairs 5 (m) and 13 (sm) and on the long arm pair 19 (sm); and a third individual with terminal NORs on the short arm of pair 5 (m) and on the long arm pair 19 (sm) (Figure 2f).

Fluorochrome staining revealed CMA_3^+ signals coincident with the NORs, while DAPI was homogeneous (Figure 3). Only 2 chromosomes were revealed to be CMA_3^+ without association with the NORs (one in cytotype II and another in cytotype VI).

C-band staining with Giemsa and fluorochrome

After C-banding, the heterochromatin proved to be poorly distributed in the pericentromeric region of most chromosomes in all cytotypes (Figures 4 and 5). Sequential staining with fluorochromes revealed that the pericentromeric heterochromatin was $DAPI^+$, whereas the terminal CMA_3^+ signals probably coincided with the NORs. It was possible to see a differentiation between the cytotypes: I and V showed very weak $DAPI^+$ signals; III, IV and VI displayed discrete signals; and cytotype II showed conspicuous $DAPI^+$ blocks (Figures 4 and 5).

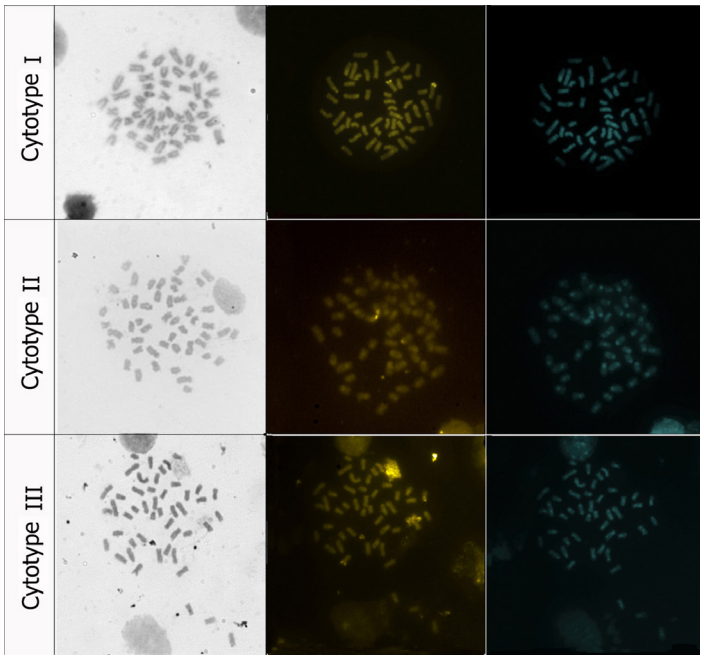

Figure 4. Somatic metaphases of cytotypes I, II and III of *Bryconamericus* aff. *iheringii* submitted to C-banding, with Giemsa staining, CMA_3 and DAPI. Cytotype III shows sequencial staining. Note that the pericentromeric heterochromatin is $DAPI^+$, while others sites are terminal CMA_3^+, probably corresponding to NORs.

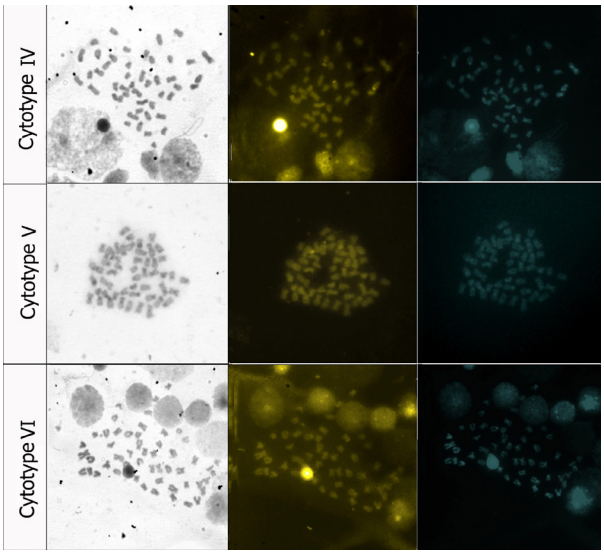

Figure 5. Somatic metaphases of cytotypes IV, V and VI of *Bryconamericus* aff. *iheringii* submitted to C-banding, sequential staining by Giemsa, CMA_3 and DAPI. Note that the pericentromeric heterochromatin is $DAPI^+$, while others sites are terminal CMA_3^+, probably corresponding to NORs.

Meiotic cells

Meiotic cells of male individuals were also analyzed, and the following phases detected: spermatogonial, with 52 chromosomes; zygotene; pachytene; diplotene; diakinesis; metaphase I, with 26 bivalents; and metaphase II with 26 chromosomes (Figure 6).

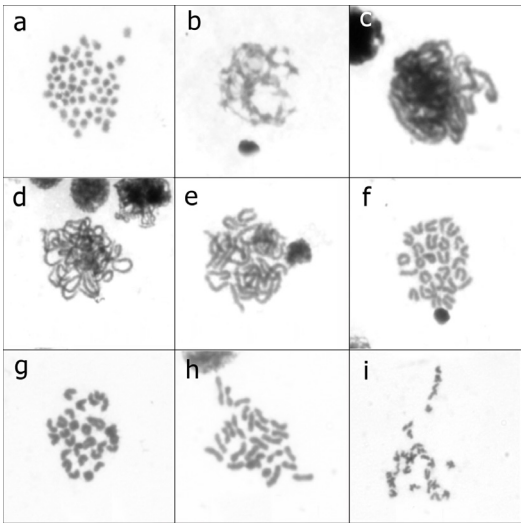

Figure 6. Meiotic stages of males gonadal cells of *Bryconamericus* aff. *iheringii* from Três Bocas Stream: **a.** espermatogonial, **b.** zygotene, **c.** pachytene initial, **d.** pachytene median, **e.** pachytene end, **f.** diplotene, **g.** diakinesis, **h.** metaphase I, **i.** metaphase II.

DISCUSSION

A diploid number of 52 is very conserved in the genus *Bryconamericus*, as observed in *Bryconamericus* aff. *iheringii* of Três Bocas Stream and in other species and populations (Capistano et al., 2008; de Brito Portela-Castro et al., 2008; dos Santos et al., 2012), except for *Bryconamericus* sp E of the Avoadeira Stream, MT, which exhibits 54 chromosomes (Wasko and Galetti Jr., 1998). However, a wide variability in karyotypic macrostructure has been a characteristic of this genus (Table 2). The occurrence of cytotypes in this species has already been documented by de Brito Portela-Castro et al. (2008), who observed two cytotypes in the population of Keller River: I - 12m+18sm+8st+14a (FN = 90) and II - 8m+28sm+6st+10a (FN = 94). Distinct karyotypic formulae have also been documented for *Bryconamericus* sp B of Piracicaba River (Wasko et al., 1996; Wasko and Galetti Jr., 1998; 1999), *B.* aff. *exodon* of Três Bocas Stream (Painter-Marques et al., 2002) and *B. ecai* of Forquetinha River (dos Santos et al., 2012).

Eberhardt et al. (2012) had previously reported four cytotypes for the population of Três Bocas Stream: cytotype I, with 12m+16sm+10st+14a (FN = 90); cytotype II, with 14m+18sm+10st+10a (FN = 94); cytotype III, with 10m+24sm+6st+12a (FN = 92); and cytotype IV, with 10m+14sm+8st+20a (FN = 84). It is worth noting that these four cytotypes are different from those found in this study, confirming the great karyotypic variation in this population. These results and those reported in the literature show that non-Robertsonian chromosomal rearrangements, such as pericentric inversions, which alter the karyotype formula but do not alter the diploid number, may be the mechanisms responsible for the chromosomal evolution in the genus *Bryconamericus*.

Among the six cytotypes of *B.* aff. *iheringii*, cytotype I is the most different in relation to FN, and besides, it has the exclusive presence of single NORs and can be regarded as belonging to another species of the genus *Bryconamericus,* living in sympatry in Três Bocas Stream. The other cytotypes may have been generated by crosses between them and by pericentric inversions. To help understand this hypothesis, the cytotypes were reorganized into groups of m/sm and st/a: cytotype II, with 32m/sm+20st/a; cytotype III, with 38m/sm+14st/a; cytotype IV, with 34m/sm+18st/a; cytotype V, with 40m/sm+12st/a; and cytotype VI, with 42m/sm+10st/a. As shown in Figure 7, the crossing between cytotypes IV and VI originated cytotype III, which, in turn, through an intersection of cytotype VI, yielded cytotype V. Therefore, cytotypes III and V would be the most recent in the population, and cytotypes II, IV and VI would the oldest and probably derived from pericentric inversions. However, it was not possible to determine the evolutionary line that originated cytotypes II, IV and VI, because a more parsimonious hypothesis was not available, since all possible orders had the same or very similar probability of having occurred.

Except for cytotype I, which has single NORs, a variability of Ag-NOR, 18S rDNA and CMA_3^+ sites between and within other cytotypes was observed. All 18S rDNA sites were associated with CMA_3^+ signals, meaning that there was an association of NORs with sequences rich in GC base pairs. However, we observed two chromosomes with fluorescent signals without any relationship to the ribosomal sites, indicating the existence of another type of repetitive DNA rich in CG not related to the NOR. The coincidence between CMA_3^+ or MM^+ signals and NOR sites have already been observed in *Bryconamericus* sp A, B, C and D (Wasko and Galetti Jr., 1999), *B.* aff. *exodon* (Paintner-Marques et al., 2002) and *B.* aff. *iheringii* (Paintner-Marques et al., 2003).

Table 2. Cytogenetic data on species of the genus *Bryconamericus*.

Species	Locality	2n	Karytotypic formula	FN	Ag-NORs	rDNA 18S, 28S or 45S	RF
Bryconamericus aff. *exodon*	Três Bocas Stream/PR	52	16m+12sm+6st+18a	86	2-5 (t. p or q m, sm)	8 (t. m, sm)	1
	Três Bocas Stream/PR	52	10m+24sm+6st+12a	92	2-5 (t. p or q m, sm)	8 (t. m, sm)	1
Bryconamericus aff. *iheringii*	Água da Floresta River/PR	52	8m+22sm+10st+12a	92	2 (t. p sm)	2 (t. p sm)	2
	Maringá Stream/PR	52	12m+18sm+8st+14a	90	2-4 (t. p sm)	6 (t. p sm)	3
	Keller River/PR	52	8m+28sm+6st+10a	94	2-4 (t. p st)	10 (t. p sm, st)	3; 4
	Tatupeba Stream/PR	52	8m+20sm+8st+16a	88	2 (t. p sm)	2 (t. p sm)	3
	Keller River/PR	52	12m+18sm+8st+14a	90	-	-	4
	Três Bocas Stream/PR	52	12m+16sm+10st+14a	90	-	-	5
	Três Bocas Stream/PR	52	14m+18sm+10st+10a	94	-	-	5
	Três Bocas Stream/PR	52	10m+24sm+6st+12a	92	-	-	5
	Três Bocas Stream/PR	52	10m+14sm+8st+20a	84	-	-	5
	Três Bocas Stream/PR	52	12m+10sm+16st+14a	90	2 (t. p st)	2 (t. p st)	6
	Três Bocas Stream/PR	52	18m+14sm+10st+10a	94	2-4 (t. p m, q. m, sm)	2-6 (t. p m, q. m, sm)	6
	Três Bocas Stream/PR	52	20m+18sm+4st+10a	94	2-5 (t. p m, q m, sm)	2-6 (t. p m, q. m, sm)	6
	Três Bocas Stream/PR	52	20m+14sm+12st+6a	98	2-3 (t. p m, q sm, st)	4 (t. p m, q sm, st)	6
	Três Bocas Stream/PR	52	22m+18sm+8st+4a	100	3-4 (t. p m, q sm, st)	4-6 (t. p m, q sm, st)	6
	Três Bocas Stream/PR	52	18m+24sm+6st+4a	100	3-5 (t. p m, q m, sm)	4-6 (t. p m, q m, sm)	6
Bryconamericus stramineus Eigenmann, 1908	Mogi-Guaçu River/SP	52	26m/sm+26st/a	78	-	-	7
Bryconamericus ecai Silva, 2004	Forquetinha River/RS	52	10m+10sm+8st+24a	80	2-4 (t. p m, sm, st, a; q sm)	4 (t. p m, a; q sm)	8
	Forquetinha River/RS	52	10m+14sm+12st+16a	88	2 (t. p st)	2 (t. p st)	8
	Forquetinha River/RS	52	14m+12sm+8st+18a	86	2-3 (t. p sm, a)	6 (t. p m, sm, st, a; q sm)	8
	Forquetinha River/RS	52	10m+24sm+14st+4a	100	2-3 (t. p sm, st)	2 (t. p m, st)	8
Bryconamericus sp A	Piracicaba River/SP	52	6m+30sm+6st+10a	94	2-3 (t. p sm, st)	-	9; 10; 11
Bryconamericus sp B	Piracicaba River/SP	52	10m+6sm+18st+18a	86	1-3 (t. p sm, st)	-	9
	Piracicaba River/SP	52	6m+10sm+20st+16a	88	1-3 (t. p sm, st)	-	10; 11
Bryconamericus sp C	Três Bocas Stream/PR	52	6m+18sm+14st+14a	90	1-4 (t. p m, sm, a, q sm, st)	-	10; 11
Bryconamericus sp D	Avoadeira Stream/MT	52	8m+14sm+16st+14a	90	1-4 (t. p st, a)	-	10; 11
Bryconamericus sp E	Avoadeira Stream/MT	54	10m+16sm+22st+6a	102	-	-	10

2n = diploid number, FN = fundamental number, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric, t. = terminal, p = short arm, q = long arm, RF = reference. 1 = Paintner-Marques et al. (2002); 2 = Paintner-Marques et al. (2003); 3 = Capistano et al. (2008); 4 = de Brito Portela-Castro et al. (2008); 5 = Eberhardt et al. (2012); 6 = Present study; 7 = Portela et al. (1988); 8 = dos Santos et al. (2012); 9 = Wasko et al. (1996); 10 = Wasko and Galetti Jr. (1998); 11 = Wasko and Galetti Jr. (1999).

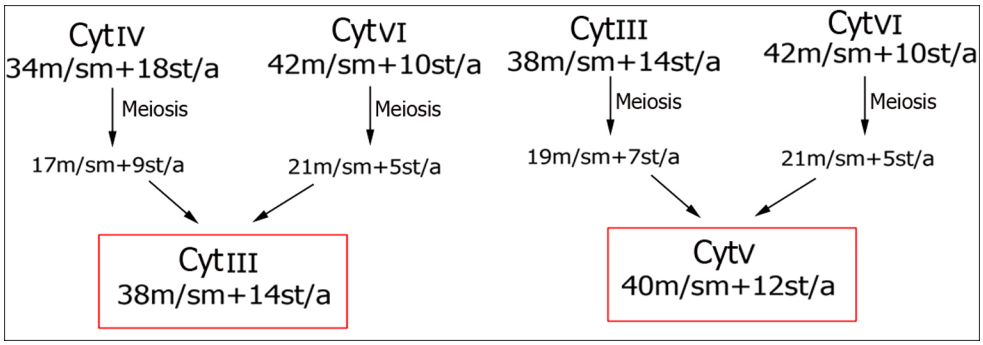

Figure 7. Representation of the possible origin of cytotypes III and V of *Bryconamericus* aff. *iheringii* of Três Bocas Stream, through crossings between cytotypes IV x VI and VI x III, respectively.

Although a pattern of multiple NORs was found in five cytotypes, differences in the location and number of NORs were observed, except for the fifth pair, which was conserved in these cytotypes. The variability of NOR sites may result from the dispersion of these genes in the karyotype, which probably occurred due to chromosomal rearrangements, such as transposition and/or translocation. Moreover, the existence of distinct karyotypic formulae suggests the evolutionary fixation of an extensive chromosomal diversification in the species, as already proposed by Paintner-Marques et al. (2003) and Capistano et al. (2008).

The pattern of multiple NORs is the most common within the genus, having been observed in *B.* aff. *exodon*, *B.* aff. *iheringii* and *B. ecai*, as shown in Table 2. The first report on single NORs was by Paintner-Marques et al. (2003) for *B.* aff. *iheringii* of Água da Floresta River, where a signal on the short arm of a large submetacentric pair was observed. Subsequently, the same result was found by Capistano et al. (2008) in *B.* aff. *iheringii* of Tatupeba Stream. dos Santos et al. (2012) also described single NORs in cytotype II of *B. ecai* of Forquetinha River, with bands on the short arm of a pair of subtelocentric chromosomes, which were the same type as those found in cytotype I of *B.* aff. *iheringii* analyzed here.

The genetic diversity in the genus *Bryconamericus* also includes variation in heterochromatin composition, and in this study it was possible to differentiate between cytotypes of *B.* aff. *iheringii*: the cytotypes I and V showed very subtle $DAPI^+$ signals in the heterochromatic regions; cytotypes III, IV and VI showed weak signals; and cytotype II displayed conspicuous $DAPI^+$ blocks, indicating a differentiation in relation to the ratio of AT base pairs.

Paintner-Marques et al. (2003) analyzed *B.* aff. *iheringii* of Água da Floresta River and detected different types of heterochromatin in the same karyotype even after treatment with *Alu*I restriction enzyme. dos Santos et al. (2012) found differences in the composition of heterochromatin between cytotypes of *B. ecai* after staining with base-specific fluorochromes.

In this genus, the heterochromatin is distributed mainly in the pericentromeric and terminal regions, and according to Paintner-Marques et al. (2003), terminal signals in subtelocentric chromosomes can be considered chromosomal markers for *B. iheringii*. However, this location was not detected in *B.* aff. *iheringii* of Três Bocas Stream, which may indicate a differentiation marker between these species, since they are so similar in morphology.

The high karyotypic diversity found in this study may be related to the environmental characteristics of Três Bocas Stream. Winkaler et al. (2001) analyzed physiological and pathological parameters of *A. altiparanae* Garutti and Britski 2000 and *A. fasciatus* Cuvier 1819 of

this stream and observed changes in the gill structure of the two species. According to these authors, the histopathological lesions occurred in response to the effect of toxic agents present in the water and sediments. Near that location there are some farms and stretches of land with little preserved riparian vegetation, allowing the inflow of pesticides and other agricultural effluents. These features make Três Bocas Stream an environment with intense selective pressure on the populations that live in it. Thus, the high genetic variability found may be the reason why this population of *Bryconamericus* remained over time in this region.

From Eberhardt et al. (2012) until the present study, the collections in Três Bocas Stream occurred at different times for approximately five years and during that period the number of cytotypes increased. It is worth noting that instead of documenting individuals with cytotypes already described, researchers have documented new specimens representing other cytotypes, suggesting that this point of Três Bocas Stream is transitional for *B*. aff. *iheringii*. This fact indicates that individuals from other nearby locations, such as small tributaries or even from Tibagi River, can temporarily be in Três Bocas Stream, either for breeding or feeding. Therefore, during some stage of their life cycle, these fish remain in this area, revealing the necessity of preserving this stream throughout its length, and consequently conserving the populations from other localities.

Data reported here for *B*. aff. *iheringii* corroborate the conservation of the diploid number in the genus, as well as its high karyotypic diversity, suggesting that chromosomal rearrangements, especially pericentric inversions and/or translocations, are important mechanisms in the chromosome evolution of this group of fish. Moreover, the crossing between cytotypes is also a source of karyotypic variability, which further increases polymorphism within the population. Meiotic analysis proved that these individuals have such great variation and that the formation of gametes occurs regularly, explaining why the different cytotypes are maintained. However, the occurrence of more than one species in Três Bocas Stream and the need for a taxonomic revision of the group are not discarded, since the phylogenetic relationships between the species of *Bryconamericus* are not well defined.

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