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Do multiple karyomorphs and population genetics of freshwater darter characines (*Apareiodon affinis*) indicate chromosomal speciation?

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ABSTRACT

The role of chromosomal rearrangements in the evolution of species is controversial, and the term chromosomal speciation results from the observation that heterokaryotypes are often infertile. Parodontidae species (a Neotropical fish family) are distinguished only by a few subtle diagnostic morphological characteristics, leading to uncertainty over the inter-relationships and monophyly of the genera. *Apareiodon affinis* was originally described in the scientific literature as a species isolated from the la Plata river (Buenos Aires, Argentina) and subsequently found in all tributaries of the Rio Paraná basin. In this study, we compared the karyotypic organization, the number of cusps in premaxillary teeth and, nucleotide divergence using DNA barcode for six different demes identified morphologically as *A. affinis* from different hydrographic subsystems in the Paraná basin (upper Paraná – three demes, Paraguay, Cuiabá, and Uruguay rivers). Cytogenetic data revealed chromosomal differences among the *A. affinis* demes studied. Chromosomal rearrangements (e.g., translocations, pericentric inversion, and/or, centromere repositioning), differentiation of the ZZ/ZW_1W_2 multiple sex chromosome system, and accumulation of DNA repeats were proposed to explain the cytogenetic divergence among *A. affinis* demes. In addition, despite the absence of morphological variation, the molecular analysis demonstrates gene flow restriction among Paraguay, Cuiabá, and Uruguay *A. affinis* demes and, genetic isolation between upper Rio Paraná basin population from those of the lower Rio Paraná basin demes. Our results demonstrated that different *A. affinis* karyomorphs have differentiated in subsystems in the Paraná basin and, parapatric populations accumulate chromosomal divergences, supporting the hypothesis of an emerging younger species and/or chromosomal speciation in progress.

1. Introduction

Chromosomal rearrangements can act to supress recombination in heterokaryotypes, thus promoting species evolution (King, 1993; Faria and Navarro, 2010; Mezzasalma et al., 2014, 2017). Mechanical pairing problems in rearranged chromosomal segments inhibit crossing-over (Davisson and Akeson, 1993; Navarro and Ruiz, 1997; Faria and Navarro, 2010) or produce unbalanced gametes by irregular crossing over events in rearranged regions (Walsh, 1982; Spirito,

Abbreviations: rDNAs, ribosomal DNAs; PCR, polymerase chain reaction; DOP-PCR, degenerate oligonucleotide primed-polymerase chain reaction; DAPI, 4,6-diamidino-2-phenylindole; FISH, Fluorescence *in situ* hybridization; m, metacentric; sm, submetacentric; st, subtelocentric; a, acrocentric; FN, fundamental number; COI, *cytochrome c oxidase subunit I*; K2P, Kimura-2-Parameters.

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1998). Hence, chromosomal rearrangements can decrease the fitness of any resulting hybrids, deepening reproductive barriers within the hybrid zone (King, 1993; Navarro and Barton, 2003).

Parodontidae Eigenmann (1910) is represented by a group of neotropical fish (32 valid species) traditionally divided into three genera (*Parodon* Cuvier and Valenciennes, 1849; *Saccodon* Kner, 1863; and *Apareiodon* Eigenmann, 1916). These three genera are traditionally characterized by the variation of two characters: number of undivided rays in the pectoral fins (one ray in *Parodon* and *Apareiodon* and two rays in *Saccodon*) and the absence of teeth in the latero-posterior region of the jaw in *Apareiodon* and *Saccodon* (Pavanelli and Britski, 2003; Pavanelli, 2006). However, the taxa assignments within Parodontidae are controversial because family members lack diagnostic morphological traits reliable enough to support accurate phylogenetic analysis (Pavanelli, 2003).

Chromosome analyses on the genera *Apareiodon* and *Parodon* have revealed a conserved diploid number of 54 chromosomes (Bellafronte et al., 2011; Traldi et al., 2016), with different karyotypic formula and a variation in the location and frequency of DNA repeats (Schemberger et al., 2011, 2014). The most frequent DNA repeats observed in the chromosomes of Parodontidae are: 1) rDNAs (ribosomal DNAs), 2) the satellite DNA sequence pPh2004 of *Parodon hilarii* Reinhardt, 1866 (Vicente et al., 2003), 3) W*Ap* sequences identified in the heterochromatic fraction of the W sex chromosome in *Apareiodon* sp. (Vicari et al., 2010), 4) microsatellite repeats (Ziemniczak et al., 2014), and 5) the transposable element *Tc1-Mariner* (Schemberger et al., 2016). However, the major focus of chromosome studies in Parodontidae has been the differentiation of sex chromosome systems in morphology and in DNA repeats content, in which species are either sex-homomorphic (i.e., lacking a differentiated sex chromosome system) or sex-heteromorphic (i.e., possessing a differentiated sex chromosome system, ZZ/ZW or ZZ/ZW_1W_2; Schemberger et al., 2011).

The hydrographic system of the Paraná river is formed by tributaries of the rivers Paraná, la Plata, Uruguay, and Paraguay (Júlio et al., 2009). The Sete Quedas waterfalls represented an important geographic barrier for species dispersal, isolating species of the upper Rio Paraná basin from those of the lower Rio Paraná basin, la Plata, Paraguay, and Uruguay rivers (Fig. 1). With the building of the Itaipu Dam approximately 250 Km downstream, the Sete Quedas waterfalls were flooded and currently, this dam is considered to be the division point between the upper and lower Rio Paraná regions (Júlio Jr. et al., 2009). *Apareiodon affinis* (Steindachner, 1879) is found in all tributaries of the Rio Paraná basin, but originally was described as a species isolated from the la Plata river (Buenos Aires, Argentina). Specimens of *A. affinis* from the lower Paraná river have 2n = 54 chromosomes, comprising a variable number of acrocentric chromosomes and no karyological differences between males and females (Jesus et al., 1999; Jorge and Moreira-Filho, 2000, 2004; Calgaro et al., 2004). Prior to this study, *A. affinis* from the upper Rio Paraná basin (Table A1) was the only species that had been shown to carry the ZZ/ZW_1W_2 arrangement of sex chromosomes (Moreira-Filho et al., 1980; Leite and Maistro, 2004).

Integrative taxonomy/phylogenetics is a contemporary strategy addressed to questions in which single data-based approaches reveal less consistent conclusions rather than those by combined multisource evidences in terms to detect the species richness within supposedly homogeneous taxa (Padial et al., 2010; Schilick-Steiner et al., 2010; Grković et al., 2017). At this reasoning several current integrative approaches have detected a striking hidden diversity at several different taxa worldwide (Ruane, 2015; Gąsiorek et al., 2017) especially in Neotropical fishes (Travenzoli et al., 2015). The Neotropical fish diversity is quite diverse and relatively recent researches have demonstrated that this diversity might be higher than currently accepted (Santos et al., 2006; Torres et al., 2008; Craig et al., 2009; Pereira et al., 2013).

In this study, we aimed to compare the karyotypic organization, genetic parameters and molecular systematics of *A. affinis* from six drainages in the Rio Paraná basin to understand the mechanisms of chromosome differentiation and to assess data about species molecular identification and gene flow. As a result, we present an unusual case of high karyotypic variability compatible with complete speciation and a putative parapatric chromosomal speciation in progress.

2. Material and methods

Apareiodon affinis specimens (n = 122) were sampled between March 2011 and March 2016 from various counties of the Rio Paraná basin (Fig. 1) and cytogenetically analyzed. The specimens were identified based on the combination of the following features: coloration pattern with one regular, black longitudinal stripe, shape and number of cusps of the tooth adjacent to the premaxillary symphysial tooth (cutting border straight with rounded corners and 12–16 cusps) and at least 29.5 pre-anus scales (Pavanelli, 2003). The sampled data and voucher numbers of the specimens were deposited in the Coleção Ictiológica of Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia) of Universidade Estadual de Maringá and are summarized in Table A2. The sample collection procedures were in accordance with the Ethics Committee on Animal Experimentation (process number: 13/2014) of the Universidade Estadual de Ponta Grossa (Brazil).

The specimens were anesthetized with clove oil (Griffiths, 2000). The extraction of symphysial teeth was performed in solution (sodium hypochlorite 2,5% 1 min, hydrogen peroxide 10% 3 min and, alcohol 70 v/v 10 days), after cusps counting of the *A. affinis* specimens were performed in microscope. Chromosome preparations were obtained from renal cells by the air-drying method (Bertollo et al., 1978), with modifications (Blanco et al., 2012). Chromosome preparations were subjected to conventional Giemsa staining, for the determination of the diploid number and chromosome formula, and C-banding, for the identification of heterochromatin (Sumner, 1972).

Four kinds of DNA sequences were used for chromosome mapping: (1) an 18S rDNA sequence isolated from the total DNA of *Prochilodus argenteus* Spix and von Agassiz, 1829 (Hatanaka and Galetti, 2004); (2) a 5S rDNA sequence amplified by polymerase chain reaction (PCR) with the following primers: (forward primer 5Sa 5′-TACGCCCGATCTCGTCCGATC-3′, reverse primer 5Sb 5′-CAGGCTGGTATGGCCGTAAGC-3′) (Pendás et al., 1994), (3) the *P. hilarii* satellite DNA pPh2004 (Vicente et al., 2003), and (4) the *Apareiodon* sp. WAp repeat sequences of the W chromosome (Vicari et al., 2010). The sequences of the rDNA 5S and satellite pPh2004 were labeled with biotin 16-dUTP by nick translation (Biotin Nick Translation mix, Roche Applied Science, Mannheim, Germany) and the rDNA 18S sequence was labeled with digoxigenin 11-dUTP by nick translation (Dig Nick Translation mix, Roche Applied Science), according to the manufacturers' instructions. The WAp repeat DNA sequences were digoxigenin-labeled by degenerate oligonucleotide primed-PCR (DOP-PCR) according to Schemberger et al. (2011).

Fluorescence *in situ* hybridization (FISH) was performed under high stringency conditions (i.e., 2.5 ng/μL probe, 50% formamide, 2 × SSC, and 10% dextran sulfate for 18 h at 37 °C) according to the protocol described by Pinkel et al. (1986). Signal detection was performed using an anti-streptavidin antibody conjugated to Alexa Fluor 488 (Molecular Probes, Carlsbad, CA, USA) and an anti-digoxigenin antibody conjugated to rhodamine (Roche Applied Science). The chromosomes were counterstained with 0.2 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector, Burlingame, CA, USA) and analyzed using an

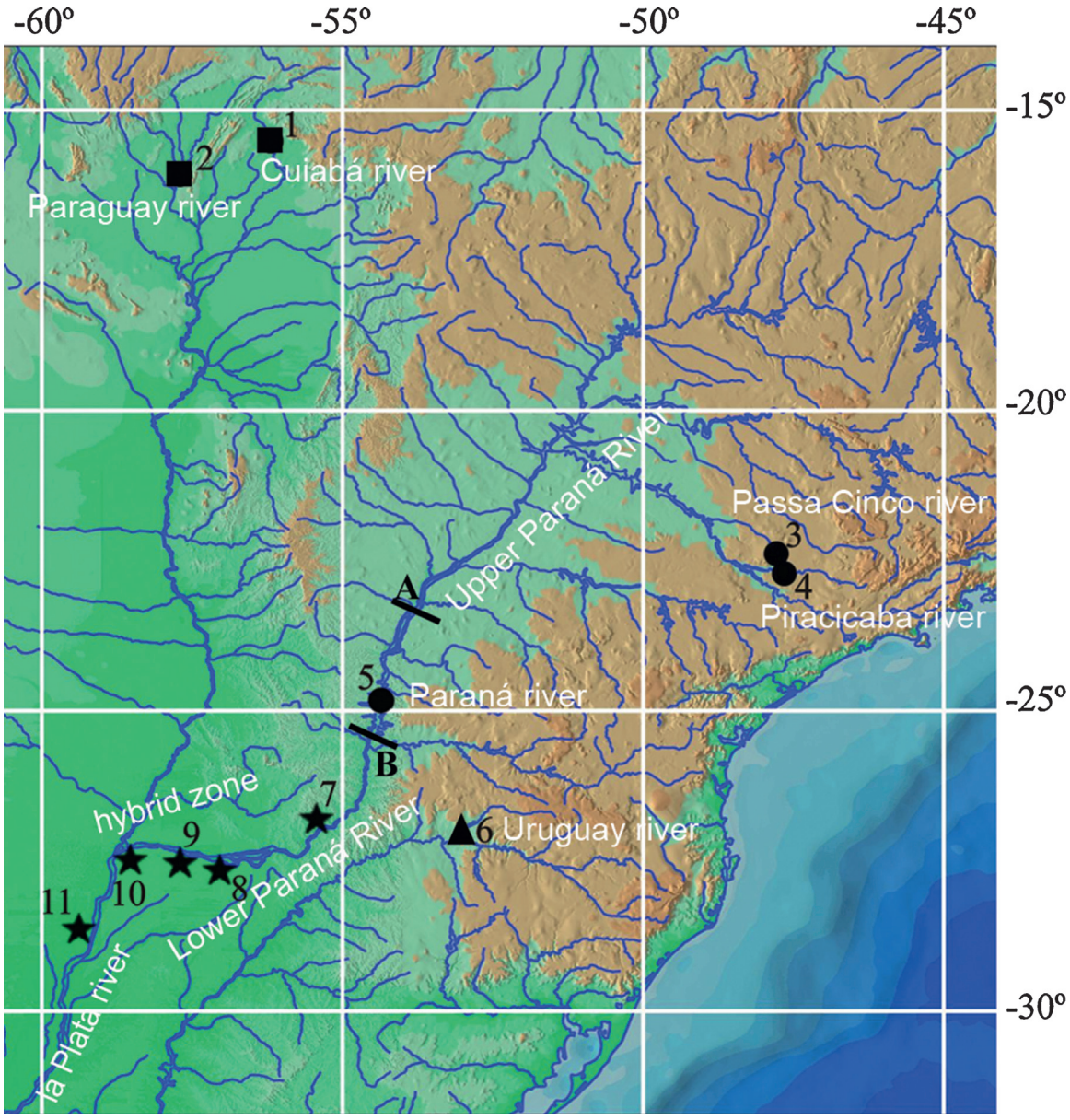

Fig. 1. Partial map of South America, especially the Paraná River basin. In A, region of flooded Sete Quedas waterfalls; B, Itaipu Dam. The localities sampled of *Apareiodon affinis* in Brazil. The squares represent the upper Paraguay river (1) and Cuiabá river (2); the circles, the upper Paraná river basin: Passa Cinco river (3), Piracicaba river (4), Itaipu reservoir, Paraná river (5) and; the triangle the upper Uruguay river (6). Samples 7–11 were selected from literature in the lower Paraná river basin (see Table 1) and constitutes a hybrid zone. In (7) Posadas, (8) Ituzaingó, (9) Itá Ibaté, (10) Corrientes and (11) Reconquista, Argentina.

Olympus BX41 epifluorescence microscope equipped with a DP71 digital image capture system (Olympus, Tokyo, Japan). The chromosomes were identified using the system proposed by Levan et al. (1964) and classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a).

Thirty specimens of *A. affinis* (five from each locality sampled) were selected randomly in order to obtain the *cytochrome c oxidase subunit I* (COI) sequences. DNA was extracted from muscle and liver samples by using a modified protocol of Doyle and Doyle (1990). COI amplicons were obtained using the primers set Fish F1 e Fish R1 according to procedures indicated by Ward et al. (2005). COI sequences were obtained by using the automated sequencer ABI-Prism 3500 Genetic Analyzer (Applied Biosystems). All sequences obtained in this study were deposited in the GeneBank (KY205721 – KY205740).

Obtained sequences were certified by using the blastn procedure at the NCBI website in order to avoid the using of numts (Nuclear mitochondrial sequences) in the analyses. The COI sequences were then aligned with the ClustaW algorithm implemented in the Bioedit v. 5.0.9 (Hall, 1999) with 15 and 0.3 as the penalties for gap openings and gap extensions respectively (Hall, 2001). The alignment was exported as a nexus file in order to obtain the phylogenetic and the population genetic data. Pairwise analyses of Kimura-2-Parameters (K2P) and neighbour joining tree were inferred using MEGA 7 (Tamura et al., 2011) with 500 replications for bootstrap support.

In order to test for the best molecular evolutionary model explaining the site variances we used jModelTest v. 2.0 (Guindon and Gascuel, 2003; Darriba et al., 2012). The population phylogeny was obtained using MrBayes v. 3.0 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) according to the model's parameters obtained by jModeltest.

The files for haplotype network and AMOVA analyses were obtained using DnaSP v. 5.10.1 (Librado and Rozas, 2009). The

haplotype network and AMOVA analyses were obtained using Network v. 4.613 (www.fluxus-engineering.com) and Arlequin v.3.5 (Excoffier and Lischer, 2010) respectively. An analysis of Bayesian population structuring was also developed using the software BAPs 2 (Corander et al., 2004).

3. Results

3.1. Morphological analyses

Counting of cusps number of the symphysial teeth in the *A. affinis* demes from the upper Rio Paraná evidenced broad variation: 11–15 cusps in specimens from the Passa Cinco River; 10–13 cusps in the Piracicaba River; 12–13 cusps in the Paraná River. Demes from the lower Rio Paraná system presented a cusp number with variation of: 10–13 in the Cuiabá River; 11–14 in the Paraguay River; 13–17 in the Uruguay River (Fig. S1 in Supplementary material).

3.2. Karyotypic data

Cytogenetic analyses showed four karyomorphs in *A. affinis* from the Rio Paraná basin.

3.2.1. Karyomorph A

This karyomorph was detected in the specimens of *A. affinis* from the upper Paraná river (Passa Cinco and Piracicaba rivers and Itaipu reservoir). Specimens from this region have 2n = 54 chromosomes in males and 2n = 55 chromosomes in females (Fig. 2). The chromosome formula and fundamental numbers (FN) show 50 m/sm + 4 st (FN = 108) in males and 49 m/sm + 6 st (FN = 110) in females (Fig. 2). This difference in the chromosome number was due to the presence of the multiple sex chromosome system, which provides sex chromosome genotype ZZ for males and ZW_1W_2 for females (Fig. 2). The Z sex chromosome was found to be the largest metacentric chromosome, whereas chromosomes W_1 and W_2 were subtelocentric and half the size of the Z chromosome (Fig. 2). The C-banding analysis showed heterochromatic blocks in pericentromeric regions of the all chromosomes (Fig. 2a). FISH using an 18S rDNA probe revealed that this sequence is located in the terminal region of the long arm of the st pair 26 and 5S rDNA was detected in the proximal region of the short arm of the m chromosome pair 8 (Fig. 2b). Three pairs of chromosomes were found to contain pPh2004 satellite DNA: m pair no. 2 in both terminal regions and sm pairs no. 5 and no. 23 in the terminal region of the long arm. In contrast, *A. affinis* specimens from the Rio Paraná (Itaipu Reservoir) region possess an additional pPh2004 satellite in the terminal region of the long arm of the sm pair no. 14 (Fig. 2c). FISH using the W*Ap* probe in *A. affinis* specimens from the upper Rio Paraná displayed several scattered terminal signals on autosomes. In addition, the Z, W_1, and W_2 chromosomes showed W*Ap* sites (Fig. 2d).

3.2.2. Karyomorph B

Karyomorph B was identified in *A. affinis* individuals from the upper Uruguay river (São Carlos, Santa Catarina State, Brazil) with 2n = 54 chromosomes, which are sex-homomorphic. These specimens were also found to have interindividual karyotype variations, with 70.6% of the specimens comprising 46 m/sm + 4 st + 4 a (FN = 104) and 29.4% of the specimens comprising 47 m/sm + 4 st + 3 a (FN = 105) (Fig. 3a). C-bands were located in the pericentromeric region of the chromosomes, in the interstitial region of chromosome pair 9, and in the terminal regions of a few chromosome pairs (Fig. 3a). 18S rDNA sites were located in the st pair 24 and in the a pair 26, whereas 5S rDNA was localized in the proximal region of the short arm of m pair 8 (Fig. 3b). FISH using the pPH2004 probe showed that *A. affinis* specimens from the upper Rio Uruguay did not carry pPh2004 satellite DNA (Fig. 3c), whereas WAp sites were scattered in the autosomes of this karyomorph (Fig. 3d).

3.2.3. Karyomorph C

Karyomorph C was assigned to *A. affinis* individuals from the upper Cuiabá river (Cuiabá, Mato Grosso State, Brazil). These individuals had 2n = 54 chromosomes, were sex-homomorphic, showed interindividual variation, and had chromosome formula variation. 63.7% of the specimens were 42 m/sm + 2 st + 10 a (FN = 98) and 36.3% were 43 m/sm + 2 st + 9 a (FN = 99) (Fig. 4a). The heterochromatin was located in the pericentromeric region of all chromosomes and in the terminal regions of a few chromosome pairs (Fig. 4a). FISH using an 18S rDNA probe revealed the presence of this sequence in the terminal region of the long arm of the st pair 22, whereas FISH using a 5S rDNA probe showed a chromosome variation, i.e., the 5S rDNA sites were located in the heteromorphic sm/a pair or in the homomorphic condition in the pair acrocentric 26 (Fig. 4b). In this karyomorph, six pairs (five a and one st) arranging to a total of eight sites were hybridized for pPh2004 (Fig. 4c), whereas dispersed autosomal signals were visualized with W*Ap* probe (Fig. 4d).

3.2.4. Karyomorph D

This karyomorph was detected in the *A. affinis* specimens from the upper Paraguay river (Cáceres, Mato Grosso State, Brazil). These specimens had 2n = 54 chromosomes, were sex-homomorphic, and comprised 36 m/sm + 2 st + 16 a, FN = 92 (Fig. 5), with chromosome pair 1 having a size heteromorphism in 31.25% of the specimens (three males and two females). The C-banding method showed pericentromeric bands in almost all chromosomes of the complement and little interstitial banding in the a pairs 24, 26 and 27 (Fig. 5a). 18S rDNA was present in the terminal region of the long arm of the st pair 19, and 5S rDNA sites were localized to the proximal region of the a pair 22 (Fig. 5b). 10 sites of pPH2004 were detected across eight acrocentric chromosome pairs (Fig. 5c), whereas WAp sites were scattered at terminal chromosome regions (Fig. 5d).

3.3. Molecular analyses

The jModelTest revealed that the best evolutionary model for the dataset was HKY + I (-lnL = 1511.6164, k = 67, p-inv. = 0.5290) after corrections from Akaike criterion. Bayesian population phylogeny showed two distinct clades with very high posterior probability supports (Fig. 6) in a total of 1,500,000 generations of Markov chains (standard deviation = 0.007). One of the clades comprised samples from Paraná, Passa Cinco, and Piracicaba rivers. Other clade grouped samples from Uruguay, Paraguay and Cuiabá rivers (Fig. 6). In this last grouping all samples from the Cuiabá River grouped together and all samples from the Paraguay formed another internal grouping with part of the samples from the Uruguay River with satisfactory branch supports.

The K2P intraspecific average genetic distance was 0.2% and interspecific was 5.2%. The neighbour joining tree (K2P distances) showed the same topology obtained by Bayesian analysis, with a clade containing samples from the Upper Paraná River (Paraná, Passa Cinco and Piracicaba rivers) and a distinct clade grouped samples from the Lower Paraná River (Cuiabá, Paraguay and Uruguay rivers) with higher values for bootstrap support (Fig. 6).

It was observed a total of eight different haplotypes and the haplotype diversity of 0.7883. The haplotype network revealed the close relationship among haplotypes from the Paraná, Passa Cinco, and Piracicaba rivers (haplotypes 6–8). The haplotype 6 is different in three and in 7 step mutations off the haplotypes 7 and 8, respectively. Sequences varied from 0.4 to 1.28% within this haplogroup (Fig. 7a,b).

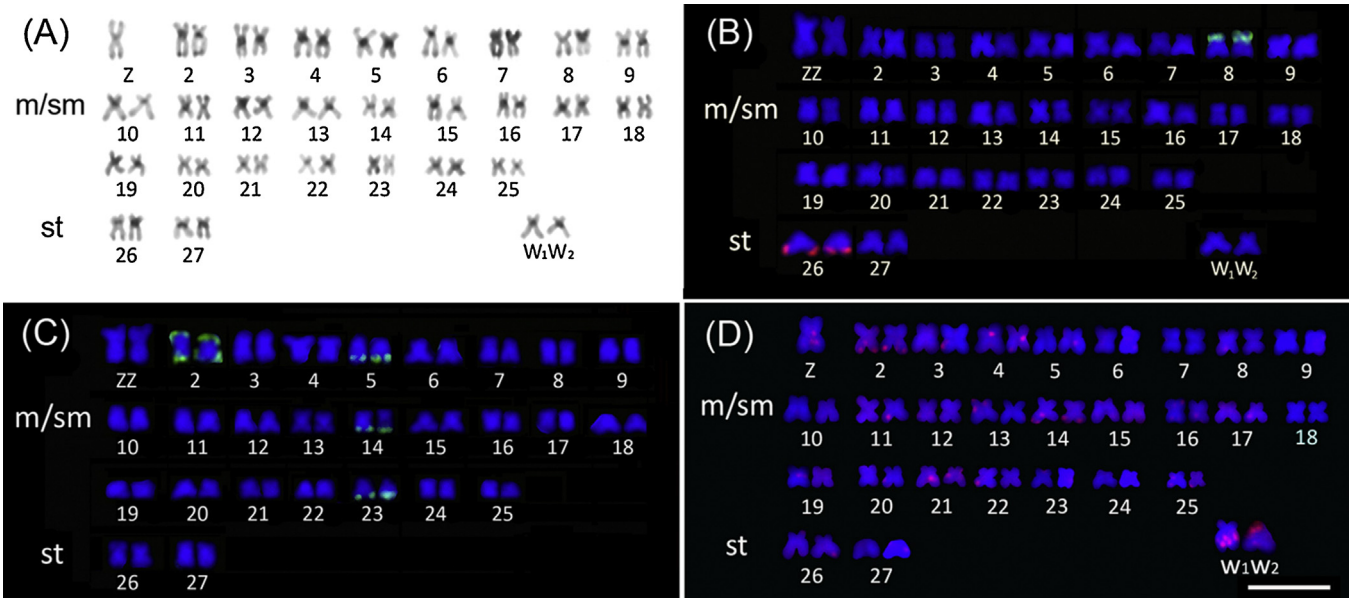

Fig. 2. Karyomorph A of the *A. affinis* from the upper Paraná river. In (A) C-band, in a female specimen, (B) chromosome localization of the 18S rDNA (red) and 5S rDNA (green) sites, in male specimen, in detail, W_1 and W_2 chromosomes of the female (C) chromosome localization of the satellite DNA pPh2004 sites (green) in a male specimen and, (D) FISH with WAp probe in a female specimen (red). Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

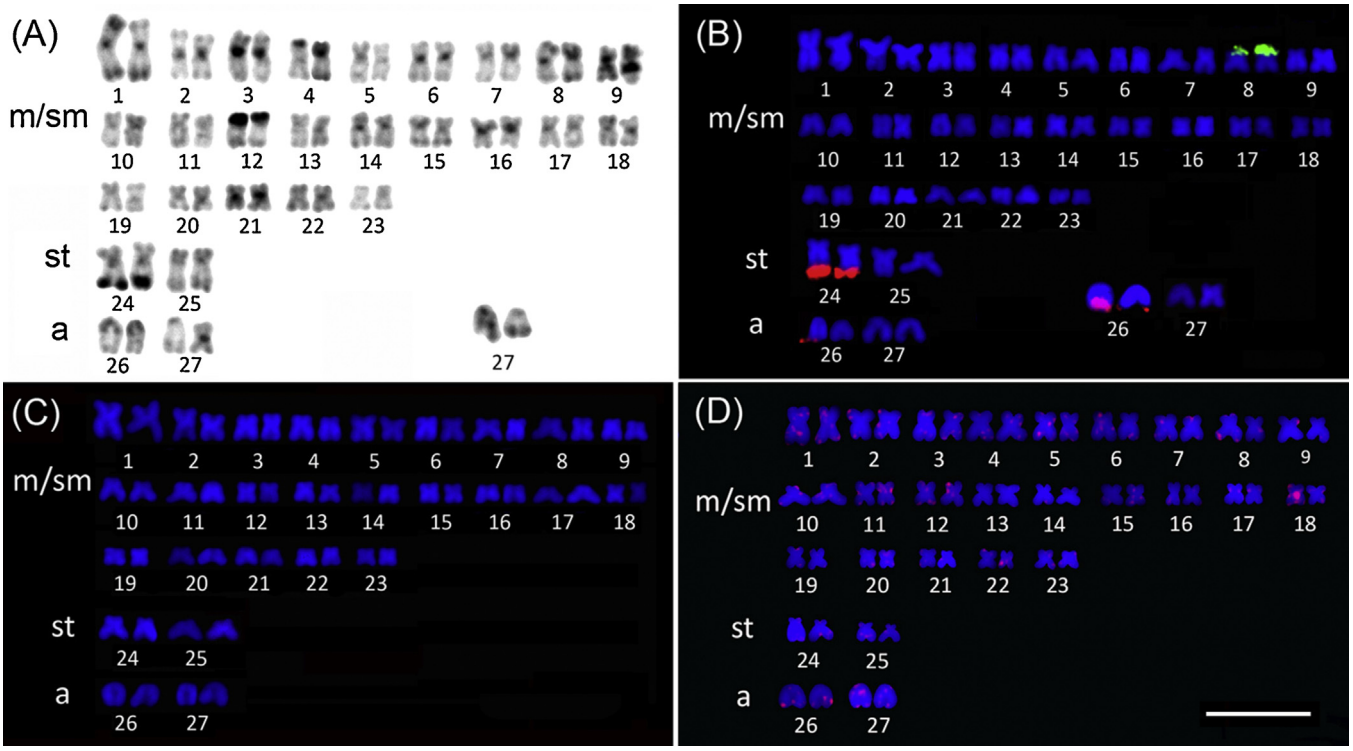

Fig. 3. Karyomorph B of the *A. affinis* from the upper Uruguay river. In (A) C-band, in detail, pair a 27 heteromorphism, (B) chromosome localization of the 18S rDNA (red), in detail, pair a 26 with rDNA size heteromorphism and 5S rDNA (green) sites, (C) chromosome localization of the satellite DNA pPh2004 sites (green) and, (D) scattered autosomal signals of FISH with WAp probe (red). Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cuiabá River has a single exclusive haplotype (H1) and it is closely related (one step mutation) to one of the haplotypes from the Uruguay River (H2). Another haplotype from the Uruguay River showed to be closely related (one step mutation) to those haplotypes from the Paraguay River. Sequences varied in 0.16% within this haplogroup. The haplogroup comprising Paraná, Passa Cinco, and Piracicaba rivers differs in 18 step mutations (2.88% of variation) of the haplogroup from the Cuiabá, Paraguay and Uruguay rivers (Fig. 7a, b).

AMOVA showed that 45.99% of the variance is among groups and 54.01% within groups, with and a ΦST = 0.459 ($p < .01$; Table 1). The structure tested by AMOVA (two groups: Paraná, Passa Cinco, and Piracicaba rivers one group, and Cuiabá, Paraguay and Uruguay rivers another group) revealed a ΦST = 0.533 ($p < .01$; Table 2).

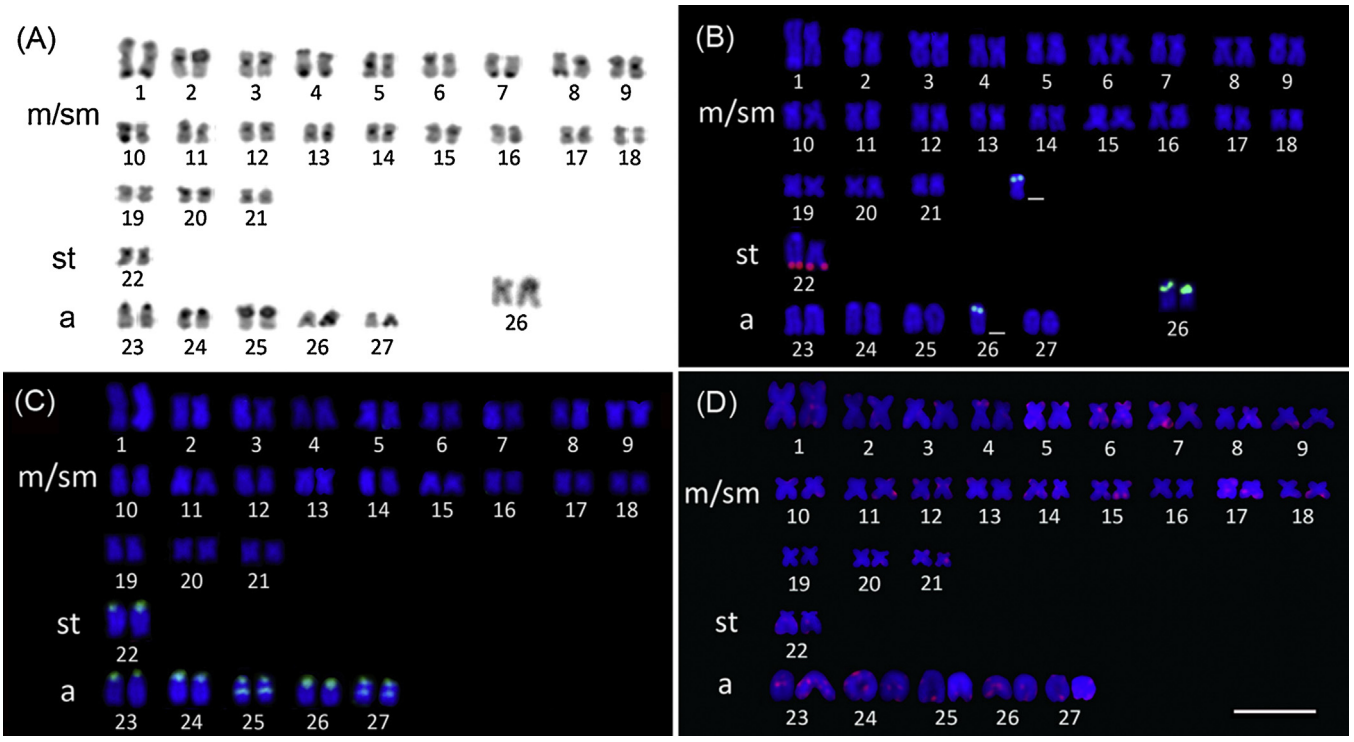

Fig. 4. Karyomorph C of the *A. affinis* from the upper Cuiabá river. In (A) C-band, in detail, pair heteromorphic 26, (B) chromosome localization of the 18S rDNA (red) and 5S rDNA (green) sites, in detail, pair heteromorphic 26, (C) chromosome localization of the satellite DNA pPh2004 sites (green) and, (D) scattered autosomal signals of FISH with WAp probe (red). Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

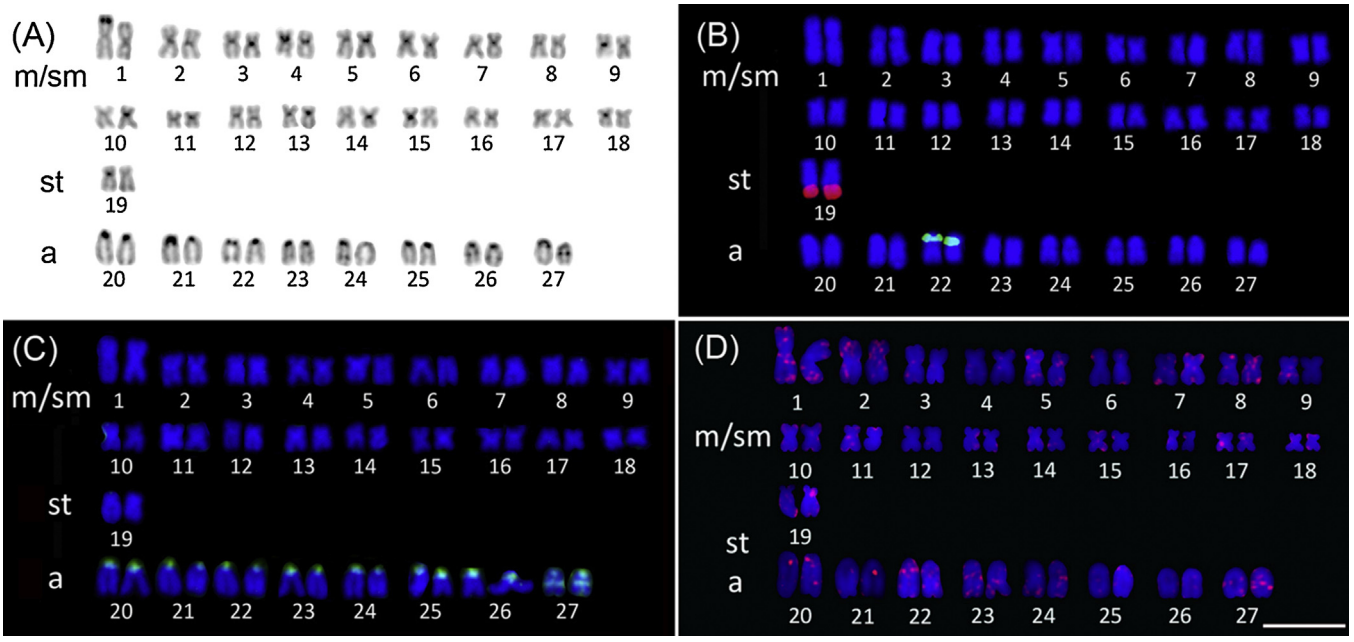

Fig. 5. Karyomorph D of the *A. affinis* from the upper Paraguay river. In (A) C-band, (B) chromosome localization of the 18S rDNA (red) and 5S rDNA (green) sites (C) chromosome localization of the satellite DNA pPh2004 sites (green) and, (D) scattered autosomal signals of FISH with WAp probe (red). Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
AMOVA results for all demes considered as a single group. ($p < 0.01$).

Source of variation	Degrees of freedom	Sum of squares	Variance components	% of variation
Among populations	5	6.133	0.19867Va	45.99
Within populations	24	5.600	0.23333Vb	54.01
Total	29	11.733	0.43200	
Fixation index	$\Phi_{ST} = 0.459$			

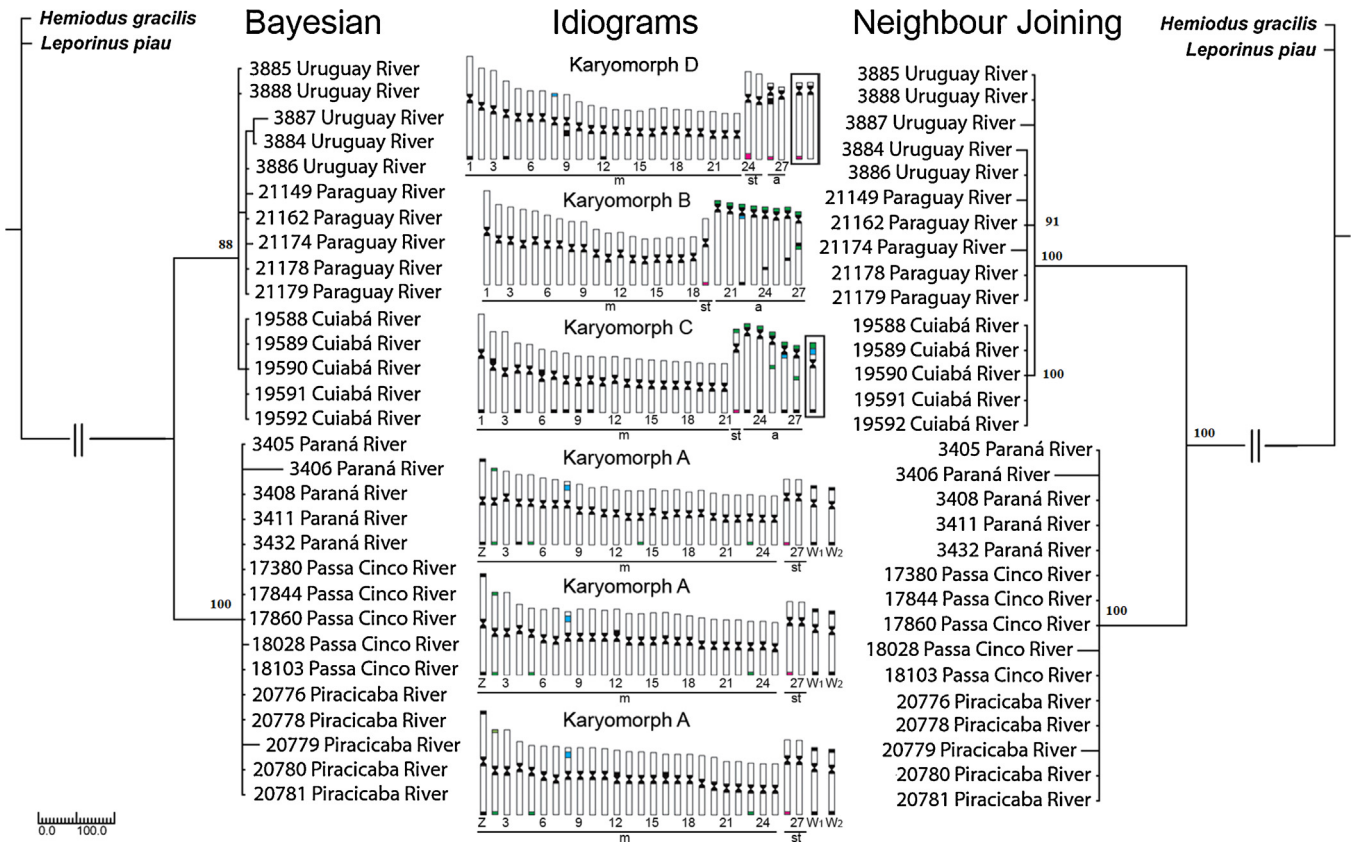

Fig. 6. Integrative molecular and karyological evidences to *A. affinis* species complex. In left, Bayesian topology (The numbers on the branches are posterior probability for Bayesian species tree). In middle, representative ideograms from the karyological data: the colours represent the chromosome localization of the cytogenetic markers: black (heterochromatin), green (pPh2004 satellite DNA), blue (5S rDNA) and, pink (18S rDNA). In right, Neighbour Joining tree (The numbers on the branches are bootstrap values). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Amova results for populations considered into two groups: Cuiabá+ Uruguay + Paraguay rivers/Paraná + Passa Cinco + Piracibaba rivers ($p < 0.01$).

Source of variation	Degrees of freedom	Sum of squares	Variance components	% of variation
Among groups	1	3.267	0.17000Va	34.00
Among populations within groups	4	2.867	0.09667Vb	19.33
Within populations	24	5.600	0.23333Vc	46.67
Total	29	11.733	0.50000	
Fixation index	$\Phi_{SC} = 0.292$ $\Phi_{ST} = 0.533$ $\Phi_{CT} = 0.340$			

Pairwise F_{ST} comparisons showed values varying from −0.11 to 0.8 among studied demes of *A. affinis* (Table A3 in Supplementary material). This analysis also showed striking minor signals for substructurings among Paraná, Passa Cinco, and Piracicaba rivers rather than those substructuring signals showed among Cuiabá, Uruguay, and Paraguay rivers. The Bayesian analysis of population structure revealed k = 2 genetic profiles divided into two clear genetic clusters (Fig. 7c).

4. Discussion

This comparative genetic study reveals karyotype differentiation and gene flow restriction among parapatric subpopulations of *A. affinis* from the upper Paraná, Paraguay, Cuiabá, and Uruguay rivers. Despite of some morphological variation, analyses of cusps of symphysial teeth were not effective to characterize the population differentiation among the hydrographic systems. It is important to say that this character has been used to help in distinction of closely related well-established species, eg. *A. affinis* and *Apareiodon piracicabae* (Eigenmann and Ogle, 1907). These species are morphologically discriminated only by the coloration pattern, number of pre-anus scales and cusps and form of teeth, and although those diagnostic characters may slightly overlap, these were considered as valid species (Pavanelli, 2003; Bellafronte et al., 2013). Although morphological analyses have failed to show consistent differences among *A. affinis* populations (Pavanelli, 2003), chromosome rearrangements (e.g., translocations and pericentric inversions) had been previously proposed for the Z, W_1, and W_2 chromosomes in populations from the upper Rio Paraná basin (Moreira-Filho et al., 1980; Schemberger et al., 2011). The chromosomal rearrangements reported herein confirm the existence of at least four different karyomorphs among *A. affinis* subpopulations of the upper and lower Rio Paraná basins (Table A1 in Supplementary material) suggesting some degree of isolation among populations.

In Parodontidae, majority of the species studied possess biarmed chromosomes (Vicari et al., 2006). The sister group, Hemiodontidae, have a similar karyotype organization to Parodontidae, with 2n = 54 chromosomes that are mostly m/sm with a few st chromo-

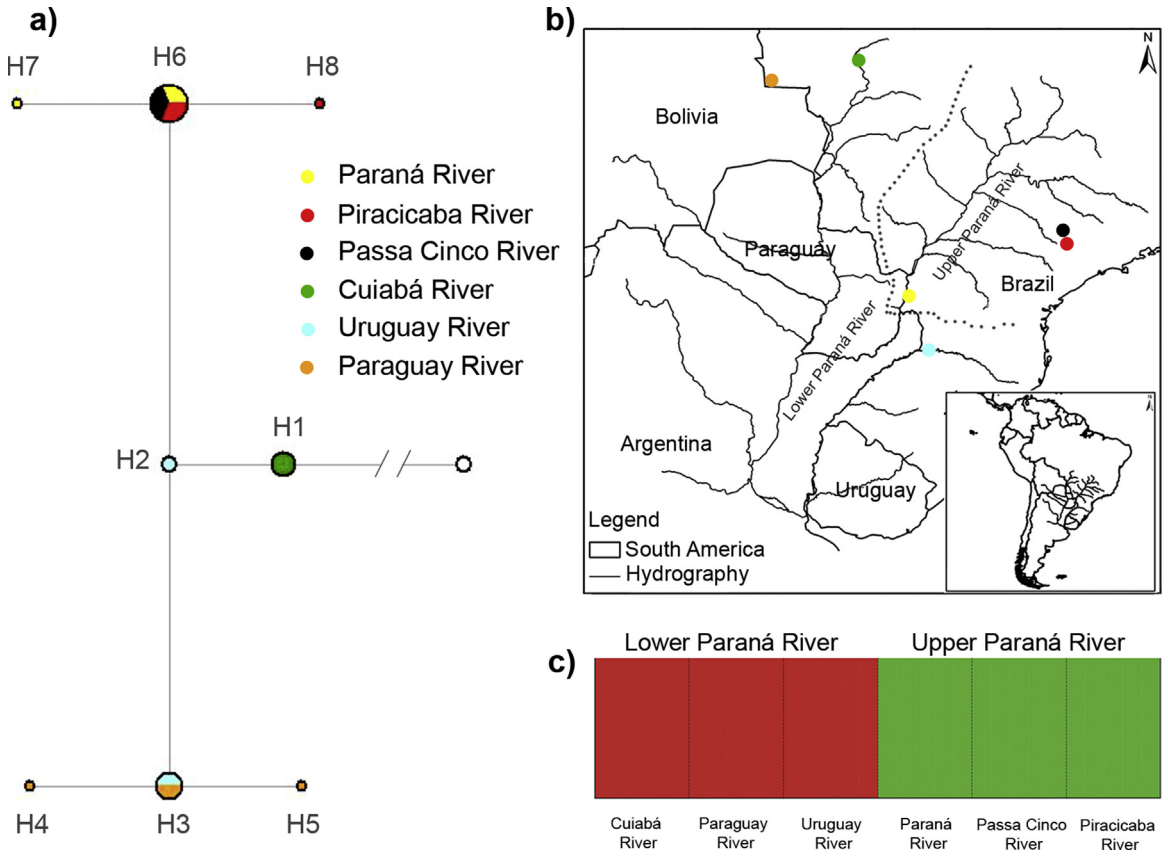

Fig. 7. Molecular data of *A. affinis* from the Paraná Basin. In (a), haplotype network and relationship among haplotypes of *A. affinis* from the Paraná (yellow), Piracicaba (red), Passa Cinco (black), Cuiabá (green), Uruguay (blue) and, Paraguay (orange) rivers. In (b), geographic localization map of the Paraná hydrographic basin in South America emphasizing sampled *A. affinis* localization. In (c) Distruct plot for the six *A. affinis* demes showing isolated clusters to Upper x Lower Paraná regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

somes (Porto et al., 1993). In addition, Schemberger et al. (2011) considered the 2n = 54 chromosomes (all m or sm) a putative basal karyotype formula in Parodontidae. Because we have identified acrocentric chromosomes in *A. affinis* demes from the Cuiabá (karyomorph C), Paraguay (karyomorph D), Lower Paraná (Jorge and Moreira-Filho, 2000), and Uruguay (karyomorph B) rivers, it is likely that this is a derived phenotype in Parodontidae. This may originate from pericentric inversions or centromere repositioning of m/sm chromosomes, as indicated by the maintenance of 2n = 54 and the variation in the FN (from 92 to 108).

Heterokaryotypes resulting from pericentric inversions or centromere repositioning can be partially or totally infertile, either due to segregation problems or due to their recombinant products originating from unbalanced gametes (Noor et al., 2001; Faria and Navarro, 2010). In our study, the *A. affinis* demes (Uruguay, Cuiabá, and Paraguay rivers, karyomorphs B, C and D, respectively) exhibit chromosome formula divergence due to pericentric inversions or centromere repositioning. From this, we may infer that individuals of *A. affinis* with heterokaryotypes represent an opportunity for natural selection by a reinforced decrease in hybridization. This would lead to population isolation and fixation of different karyomorphs. In this hypothesis, demes of *A. affinis* had diversification in parapatric conditions.

The use of rDNA chromosome markers in various *A. affinis* demes revealed homeologous chromosomal localizations (despite different pairs numbers in the karyotypes) for the 18S rDNA sites, which has also been observed in other *Apareiodon* species (Bellafronte et al., 2009). Additional 18S rDNA sites were detected for specimens from the Uruguay river (karyomorph B) in comparison to others karyomorphs, suggesting rDNA transpositions and chromosome divergence. The 5S rDNA has only been detected in a homeologous m pair among Parodontidae species (Bellafronte et al., 2011) and this has also been found among *A. affinis* demes from the upper Paraná river (karyomorph A). However, specimens from the upper Paraguay river (karyomorph D) indicated repositioning of the 5S rDNA to acrocentric chromosomes, likely due to pericentric inversions, promoting chromosomal diversification among others *A. affinis* demes.

Repetitive DNA sequences are important for promoting chromosome rearrangement and genomic evolution (Medrado et al., 2015; Pucci et al., 2016; Barbosa et al., 2017). Mapping of pPh2004 sites revealed its abundance in chromosomes of *A. affinis* from the upper Paraná (karyomorph A), Cuiabá (karyomorph C), and Paraguay (karyomorph D) rivers. In contrast, these were not detectable in specimens from the Uruguay river (karyomorph B). Among *A. affinis* specimens from Paraguay and Cuiabá rivers, an increased number of pPh2004 satellite DNA sites were observed compared to those from the upper Rio Paraná basin, especially in acrocentric chromosomes. This suggest that the pPh2004 satellite DNA probably originated in the founder population of the *A. affinis* demes from the upper Paraná, Paraguay, and Cuiabá rivers, thereby influencing the karyotype structure. The concerted evolutionary pattern of satellite DNA sequences (Hamilton et al., 1992; Barros et al., 2017) can explain the equidistant chromosome localization of the pPh2004 satellite in *A. affinis* st/a chromosomes. In heterokaryotypes, heterochromatin blocks can act to suppress chromatin crossing over or this can occur by irregular meiosis (Aprea et al., 2007; Mezzasalma et al., 2017). Therefore, the accumulation of chromosomal incompatibilities among populations connected by

gene flow was reinforced by satellite DNA differentiation and subsequent formation of heterochromatin blocks.

It is known that the Lower Paraná River fish population can disperse through the Lower Paraná route (La Plata – Uruguay – Paraguay rivers), which remains unchanged in the last 10 million years (Sivasundar et al., 2001). Connections among Paraguay – La Plata – Uruguay rivers basins have been postulated based by the occurrence of several fish species (see; Bonato and Ferrer, 2013; Paiz et al., 2014), as well as by geomorphological evidence (Boneto, 1994). However pairwise F_{ST} comparisons from AMOVA indicated an alternative hypothesis of restricted gene flow in *A. affinis* within the upper Paraguay basin (Cuiabá River – karyomorph C) to the lower Rio Paraná system (Plata and Uruguay rivers – karyomorphs B and D). The Bayesian topology also showed a striking and well-supported clade comprising specimens from the Cuiabá River, reinforcing the population division, and combined with the high F_{ST} values suggested a long-term gene flow break. At this sense both karyological (karyomorphs B, C, and D) and molecular evidences can support the hypothesis of a clear evolutionary isolation among those mentioned demes.

The data indicate gene flow between *A. affinis* from Paraguay and Uruguay rivers (karyomorphs B and D). The Bayesian topology reinforced the gene flow between those *A. affinis* populations, suggesting ancient river basin connections despite the different karyomorphs. Thus, it is possible that the karyological differentiation observed in isolated populations can present gene flow and interbreeding, promoting the chromosomal polymorphism in middle stretches of the Lower Paraná River, as observed in the confluence of regions of Paraná – La Plata – Paraguay rivers (Calgaro et al., 2004; Jorge and Moreira-Filho, 2004).

In a previous cytogenetic analysis of four *A. affinis* counties from the lower Paraná river region, Argentina (Jorge and Moreira-Filho, 2000), a probable hybrid zone was described (Fig. 1), which established several karyotypic forms and heterokaryotypes (Table A1 in Supplementary material). This region is close to a convergent point of the lower Paraná and Paraguay rivers that subsequently turns into the la Plata river (Fig. 1). In a detailed assessment, it may be possible to infer that the chromosomal structural polymorphism identified here was formed by different gamete combinations of the specimens originating from the three stable karyotypes: 50 m/sm + 4 st, 50 m/sm + 4 a, and 48 m/sm + 6 a. Our data supported the structural chromosomal polymorphisms observed by Jorge and Moreira-Filho (2000), which are evident in the mixture of parapatric *A. affinis* populations studied here. The occurrence of a possible hybrid zone demonstrates that speciation is not complete and natural selection, together with reinforcement, can be acting to prevent genetic introgression. This is evident from the maintenance of stable karyomorphs in *A. affinis* at the initial river stretches and low values to genetic divergence among them (upper Uruguay, upper Cuiabá, and upper Paraguay rivers, Karyomorphs B, C and D, respectively).

In Parodontidae, the DNA repeat sequences of the W*Ap* probe have been used to help with the identification of the W sex chromosome (Schemberger et al., 2011). *Apareiodon affinis* from the upper Paraná river (karyomorph A) has a multiple sex chromosome system in addition to chromosome formula divergence. This is consistent with the proposal by Schemberger et al. (2011) that W*Ap* sequences may participate in chromosome rearrangements leading to the differentiation of the Z, W_1, and W_2 sex chromosomes. In contrast, species without heteromorphic sex chromosomes contain scattered W*Ap* signals along their chromosomes. Sex chromosomes are considered important meiotic barriers to prevent genetic introgression (Qvarnström and Bailey, 2009; Pucci et al., 2014). Our study suggested that the chromosomal formula and sex chromosome system may contribute toward chromosomal speciation in *A. affinis* from the upper Paraná river population.

Yet, the absence of gene flow between *A. affinis* from the upper Rio Paraná (karyomorph A) and lower Rio Paraná (karyomorphs B, C and D) populations was demonstrated by karyological, AMOVA pairwise and K2P genetic distances. The K2P average genetic distances (average = 6,34%), and intrapopulational x interpopulational between the upper and lower Rio Paraná populations of *A. affinis* (Table A3 in Supplementary material), observed also by Bellafronte et al. (2013) are considered high divergent values and proper to separation of fish species (Ward et al., 2005). Primarily this finding could be explained by a typical allopatric isolation due to Sete Quedas Water falls (today submerged by the flooding waters from Itaipu Dam), indicating speciation.

Chromosome speciation is a controversial concept due to the inability of chromosomal rearrangements to constitute strong barriers to gene flow and thus unlikely to cause speciation (Faria and Navarro, 2010). However, chromosomal speciation has been described is several groups (Aprea et al., 2007; Mezzasalma et al., 2014, 2015, 2016, 2017; Pucci et al., 2014; Barbosa et al., 2017). Our data corroborated gene flow isolation between *A. affinis* from the upper Rio Paraná basin with karyomorph A and demes from the lower Rio Paraná basin with karyomorphs B, C, and D. In addition, parapatric demes possessing the karyomorphs B (upper Uruguay river), C (upper Cuiabá river), and D (upper Paraguay river) accumulate chromosomal divergences and low genetic distance values. According to Noor et al. (2001) younger species are expected to have a higher proportion of chromosomal rearrangements relative to molecular divergence when compared with species that originated long ago. These data supporting the hypothesis of an emerging younger species and/or chromosomal speciation in progress in *A. affinis* in lower Rio Paraná basin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jcz.2017.12.006.

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