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ANA CAMILA PRIZON

Contribuições ao status taxonômico de *Ancistrus* Kner, 1854 (Loricariidae, Ancistrini) da bacia do Rio Paraná, Brasil baseados em marcadores citogenéticos e moleculares

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Orientadora: Prof[a] Dr[a] Ana Luiza de Brito Portela Castro.
Coorientador: Prof Dr. Daniel Pacheco Bruschi

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BIOGRAFIA

Ana Camila Prizon nasceu em 30 de maio de 1989, em Paraíso do Norte, Paraná. Em 2008 ingressou no curso de Ciências Biológicas da Universidade Estadual de Maringá, realizou estágio no laboratório de Citogenética de Peixes, realizando atividades em projetos de pesquisa e iniciação científica. Ao final do ano letivo de 2011 obteve o título de bacharel em Ciências Biológicas pela referida instituição. Ingressou no Programa de Pós-Graduação em Ciências Biológicas – Área de Concentração em Biologia Celular e Molecular da Universidade Estadual de Maringá no ano de 2012, e obteve o título de Mestre em Ciências Biológicas no início do ano letivo de 2014 e em seguida ingressou no Doutorado pelo mesmo Programa de Pós-Graduação em Ciências Biológicas, onde atua na área de Citogenética de Peixes.

Dedico este trabalho aos meus pais Anélio Prizon e Vera Lucia T. Prizon, e ao meu noivo Jonyson pelo amor, apoio e confiança.

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Sumário

Apresentação

Esta Tese é composta por dois capítulos. O capítulo I compreende um artigo sobre análises citogenéticas e moleculares realizadas em diferentes populações de *Ancistrus* da bacia do alto rio Paraná, intitulado de: "**Diversidade escondida nas populações de *Ancistrus* Kner, 1854 (Loricariidae, Hypostominae) da bacia do rio Paraná, reveladas por dados moleculares e citogenéticos".** O segundo capítulo trata-se de um artigo sobre o mapeamento de sequencias de DNA repetitivas e suas implicações na diversidade cariotípica de populações de *Ancistrus* sp. da bacia do rio Paraná, intitulado: "**Mapeamento de repetições de microssatélites e retrotransposon não-LTR revela sua interação na diversificação dos cariótipos das populações de *Ancistrus* da bacia do Paraná no Sul da América do Sul**". De acordo com o regulamento do Programa de Pós-Graduação em Ciências Biológicas, os artigos foram redigidos de acordo com as normas das revistas às quais foram e serão submetidos, conforme a seguir:

Capítulo I - Prizon AC, Bruschi DP, Borin-Carvalho LA, Cius A, Barbosa LM, Ruiz HB, Zawadzki CH, Fenocchio AS, Portela-Castro ALB. Hidden Diversity in the Populations of the Armored Catfish *Ancistrus* Kner, 1854 (Loricariidae, Hypostominae) from the Paraná River Basin Revealed by Molecular and Cytogenetic Data. Front Genet 2017; 8:185. doi: 10.3389/fgene.2017.00185.

Capítulo II – Prizon AC; Bruschi DP, Portela-Castro ALB. Mapping of microsatellite repeats and non-LTR retrotransposons reveals their interplay in the diversification of the karyotypes of the *Ancistrus* populations from the Paraná basin in southern South America. Zebrafish.

Resumo Geral

Ancistrus Kner, 1854 é um dos grupos mais especiosos da tribo Ancistrini, compreendendo 69 espécies descritas. Atualmente são conhecidos os cariótipos de 33 espécies de *Ancistrus*, embora a maioria destas ainda não tenha sido formalmente identificada. Os dados cariotípicos disponíveis para este gênero indicam uma diversidade cromossômica considerável, com números diploides variando de 2n = 34 a 2n = 54. Além da diversidade numérica, tem sido relatado variação na estrutura cromossômica, incluindo cariótipos com cromossomos sexuais, distintos padrões de distribuição de heterocromatina e de sequências de DNA repetitivos, com diferenças no número e localização dos sítios de DNA ribossômicos, especialmente dos sítios de DNAr 5S. As descrições cariotípicas abrangem principalmente as espécies provenientes das bacias do Paraguai, nos estados do Mato Grosso e da Amazônia. Sob o ponto de vista taxonômico, apenas uma espécie de *Ancistrus* foi registrada para a bacia do alto rio Paraná, identificada como *Ancistrus cirrhosus* por Valenciennes 1836, coletada na região de Missiones, Argentina. No entanto, a ampla variação encontrada na morfologia e coloração das populações amostradas nos afluentes do Rio Paraná-PR, tornou-se difícil estabelecer o *status* taxonômico dos espécimes de *Ancistrus*, sugerindo que *Ancistrus cirrhosus* não seja a única espécie encontrada nesta bacia. A inclusão de dados genéticos em estudos de taxonomia e evolução teve uma profunda influência na compreensão da diversidade de espécies para a região neotropical e a aplicação de métodos taxonômicos integrativos, que combinam diferentes linhas de evidência (seqüências de DNA, dados cromossômicos e características morfológicas) alterou nossa percepção da diversidade biológica da região e contribuiu para o aumento da taxa de descoberta de novas espécies, especialmente em linhagens crípticas. Neste contexto, testes de delimitação de espécies que usam sequências de DNA podem ser altamente informativos, permitindo a documentação mais sistemática da diversidade de espécies. Além disso, diante da extensa variação cariotípica encontrada para o gênero *Ancistrus*, é possível que alguns elementos repetitivos possam ser responsáveis por grande parte da variação dos cariótipos dessas espécies, uma vez que seu envolvimento na diversificação cariotípica já tem sido reportado. Entre as diversas seqüências de DNA repetitivas que estão presentes no genoma eucariótico, os elementos transponíveis (TEs) são os mais representativos. Eles são capazes de se mover dentro do genoma, inserindo-se em novos sítios, próximos, ou até mesmo dentro de sequências gênicas. Já as sequências de DNA repetitivas organizadas em *tandem*, estão dispostas em uma série seqüencial de unidades repetidas, variando de 150-180 a 300-360 bps e os microsatélites (ou repetições em tandem curto) são constituídos por sequências curtas de um a seis nucleotídeos. Dessa forma, dada à suposição de que existe diversidade críptica nas populações de *Ancistrus* dos rios e córregos da bacia do Alto Paraná, nesse estudo combinamos dados cromossômicos e de sequências de DNA para avaliar o *status* taxonômico dessas populações. Ao mesmo tempo, com o intuito de formular hipóteses sobre os mecanismos que levaram a diversificação cariotípica entre elas, incluímos também o mapeamento físico de sequências repetitivas em tandem (repetições teloméricas e microssatélites), e dispersas no genoma (elemento retrotransponível *Rex*-3), para uma melhor compreensão da organização do genoma dos *Ancistrus* desta bacia. Em nossa análise combinada, recuperamos cinco linhagens distintas entre as populações analisadas, claramente observadas em nossas análises moleculares (inferências filogenéticas e pelo método de delimitação de espécies - GYMC). Apesar de todas as linhagens apresentarem fórmulas cromossômicas distintas, todos os espécimes apresentaram 2n=50 cromossomos. Assim, os dados sugeriram pelo menos quatro espécies candidatas de *Ancistrus* as quais podem estar presentes na bacia do Paraná,

além de *A. cirrhosus*. Dentre os cariótipos encontrados para cada linhagem, destacamos as populações do Córrego 19 e Rio Keller, que apresentaram 12m+18sm+12st+8a (fêmeas) e 11m+18sm+13st+8a (machos), consistente com um sistema sexual XX/XY. O cromossomo X (metacêntrico) das populações variou em quantidade e distribuição de blocos de heterocromatina e não pôde ser distinguido do cromossomo Y (subtelocêntrico) pela banda C. Clusters de DNAr 18S foram observados em um único par de cromossomos em sete populações de *Ancistrus* analisadas, mas em diferentes posições, em alguns casos, em sintenia com os sítios de DNAr 5S. Múltiplos sítios de 5S foram observados em todas as populações. O mapeamento físico utilizando como sonda o elemento TE *Rex*-3 isolado de um espécime de *Ancistrus* sp. do rio Keller revelou sinais fluorescentes dispersos pelos cromossomos de todas as sete populações de *Ancistrus* analisadas, estando alguns sinais associados a blocos heterocromáticos e a sítios de rDNA. A presença deste elemento distribuído por todo o genoma poderia explicar a presença dos múltiplos sítios de DNAr 5S, assim como a quebra da sintenia entre os sítios de DNAr 18 e 5S detectada em algumas populações, sendo a condição sintênica basal para o gênero *Ancistrus*. As sequências de nucleotídeos do elemento *Rex*-3 revelaram alta semelhança com as seqüências do mesmo elemento depositado em bancos de dados BLAST/CENSOR online. O alinhamento da sequência de aminoácidos presumida com *X. maculatus* revelou domínios de transcriptase reversa altamente conservados, sugerindo que esse elemento encontra-se potencialmente ativo nesse genoma e pode ter contribuído para a disseminação das cópias detectadas por FISH. A hibridação fluorescente *in situ* (FISH) dos microssatélites $(CA)_{15}$ e $(GA)_{15}$ forneceu padrão de bandas preferencial nas regiões subterminais e intersticiais da maioria dos braços cromossômicos, com alguns sinais aparecendo mais fortes e mais estendidos do que outros. Sinais de hibridização foram observados nas seqüências repetitivas acumuladas no par sexual heteromórfico (XY) do *Ancistrus* sp. do Rio Keller e Córrego 19, revelando um possível envolvimento dessas sequencias repetitivas (*Rex*-3 e CA/GA) na diferenciação dos cromossomos sexuais heteromórficos. A diversidade cariotípica detectada em *Ancistrus* pode ser uma conseqüência da presença de elementos repetitivos (*Rex*-3 e microssatélites GA/CA), que foram espalhados por todo o complemento cromossômico, indicando que essas duas classes de seqüências de DNA repetitivas têm desempenhado um papel importante na diferenciação do genoma deste taxon. Assim, as inferências obtidas no presente estudo a partir de uma abordagem combinada de análises moleculares de DNA e citogenéticas foi especialmente importante devido à falta de características diagnósticas na morfologia desses peixes, a qual nos permitiu diferenciar cinco linhagens distintas de *Ancistrus*, reforçando a hipótese da presença de pelo menos quatro espécies candidatas na bacia do Alto Paraná, além de *Ancistrus cirrhosus*, anteriormente descrito.

Palavras-Chave: *Ancistrus*, delimitação de espécies, sequencias de DNA repetitivas, citotaxonomia, diversidade cariotípica.

Abstract

Ancistrus Kner, 1854 is one of the most specious groups of the Ancistrini tribe comprising 69 described species. Currently, karyotypes are known for 33 *Ancistrus* species, although most of these have not yet been formally identified. The karyotype data available for this genus indicate considerable chromosome diversity, with diploid numbers varying from 2n = 34 to 2n = 54. In addition to numerical diversity, chromosome structure variation has been reported, including karyotypes with sex chromosomes, distinct heterochromatin distribution patterns and repetitive DNA sequences, with differences in the number and location of ribosomal DNA sites, especially the 5S rDNA sites. The karyotypic descriptions cover mainly the species from the basins of Paraguay, in the states of Mato Grosso and the Amazon. From the taxonomic point of view, only one species of *Ancistrus* was recorded in the upper Paraná River basin, identified as *Ancistrus cirrhosus* by Valenciennes 1836, collected in the region of Missiones, Argentina. However, the wide variation found in the morphology and color of the populations sampled in the tributaries of the Paraná River (PR), became difficult to establish the taxonomic *status* of the *Ancistrus* specimens, suggesting that *Ancistrus cirrhosus* is not the only species found in this basin. The inclusion of genetic data in taxonomy and evolution studies had a profound influence on the understanding of species diversity for the neotropical region and the application of integrative taxonomic methods, combining different lines of evidence (DNA sequences, chromosome data and morphological characteristics) altered our perception of the region's biological diversity and contributed to the increased rate of discovery of new species, especially in cryptic lineages. In this context, species delimitation tests using DNA sequences can be highly informative, allowing more systematic documentation of species diversity. Besides that, due to the extensive karyotype variation found for the genus Ancistrus, it is possible that some repetitive elements may be responsible for much of the karyotype variation of these species, since their involvement in karyotype diversification has already been reported. Among the several repetitive DNA sequences that are present in the eukaryotic genome, the transposable elements (TEs) are the most representative. They are able to move within the genome, inserting themselves into new sites, close, or even within gene sequences. The tandem-organized repetitive DNA sequences are arranged in a sequential array of repeating units, ranging from 150–180 bps to 300–360 bps and microsatellites (or short tandem repeats) are made up of short sequences of one to six nucleotides. Thus, given the assumption that there is cryptic diversity in the *Ancistrus* populations of the rivers and streams of the Upper Paraná basin, in this study we combine chromosome and DNA sequence data to evaluate the taxonomic *status* of these populations. At the same time, in order to formulate hypotheses about the mechanisms that led to karyotypic diversification among them, we also included the physical mapping of repetitive tandem sequences (telomeric and microsatellite repeats) and dispersed in the genome (*Rex*-3 retrotransposable element), for a better understanding of the organization of the *Ancistrus* genome of this basin. In our combined analysis, we recovered five distinct lineages among the analyzed populations, clearly observed in our molecular analyzes (phylogenetic inferences and by the species delimitation method - GYMC). Although all lineages presented distinct chromosomal formulas, all the specimens presented 2n = 50 chromosomes. Thus, the data suggested at least four candidate species of *Ancistrus* which may be present in the Paraná basin, in addition to *A. cirrhosus*. Among the karyotypes found for each lineage, we highlight the populations of 19 Stream and Keller River, which presented 12m+18sm+12st+8a (females) and 11m+18sm+13st+8a (males), consistent with a XX/XY sexual system. The X (metacentric) chromosome of the populations varied in

amount and distribution of heterochromatin blocks and could not be distinguished from the Y chromosome (subtelocentric) by the C-band. Clusters of 18S rDNA were observed on a single pair of chromosomes in seven analyzed *Ancistrus* populations, but in different positions, in some cases, in synteny with the 5S rDNA sites. Multiple sites of 5S were observed in all populations. The physical mapping using as probe the *Rex*-3 element isolated from a specimen of *Ancistrus* sp. of the Keller River revealed fluorescent signals scattered across the chromosomes of all seven *Ancistrus* populations analyzed, with some signals associated with heterochromatic blocks and rDNA sites. The presence of this element distributed throughout the genome could explain the presence of the multiple sites of 5S rDNA, as well as the synteny break between the sites of rDNA 18 and 5S detected in some populations, being the basal condition for the genus *Ancistrus*. The nucleotide sequences of *Rex*-3 element revealed high similarity to sequences from the same element deposited in the online BLAST/CENSOR database. Alignment of the presumed amino acid sequence with *X. maculatus* revealed highly conserved reverse transcriptase domains, suggesting that this element is potentially active in this genome and may have contributed to the dissemination of the copies detected by FISH. Fluorescent *in situ* hybridization (FISH) of the microsatellites $(CA)_{15}$ and $(GA)_{15}$ provided preferential band pattern in the subterminal and interstitial regions of most chromosome arms, with some signs appearing stronger and more extended than others. Hybridization signals were observed in the repetitive sequences accumulated in the heteromorphic sexual pair (XY) of *Ancistrus* sp. of the Keller River and 19 Stream, revealing a possible involvement of these repetitive sequences (*Rex*-3 and CA/GA) in the differentiation of heteromorphic sex chromosomes. The karyotypic diversity detected in *Ancistrus* may be a consequence of the presence of repetitive elements (*Rex*-3 and GA/CA microsatellites), which were scattered throughout the chromosomal complement, indicating that these two classes of repetitive DNA sequences have played an important role in the differentiation of the genome of this taxon. Thus, the inferences obtained in the present study from a combined approach of molecular and cytogenetics analyzes was especially important due to the lack of diagnostic features in the morphology of these fishes, which allowed us to differentiate five distinct lineages of *Ancistrus*, reinforcing the hypothesis of the presence of at least four candidate species in the upper Paraná basin, besides of the *Ancistrus cirrhosus*, previously described.

Keywords: *Ancistrus*, species delimitation, repetitive DNA sequences, cytotaxonomy, karyotypic diversity.

CAPÍTULO I

ARTIGO PUBLICADO:

Diversidade escondida nas populações de *Ancistrus* Kner, 1854 (Loricariidae, Hypostominae) da bacia do rio Paraná, reveladas por dados moleculares e citogenéticos

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Hidden Diversity in the Populations of the Armored Catfish *Ancistrus* Kner, 1854 (Loricariidae, Hypostominae) from the Paraná River Basin Revealed by Molecular and Cytogenetic Data

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Only one species of armored catfish, *Ancistrus cirrhosus* Valenciennes 1836, has been historically described in the basin of the Paraná River, from Misiones (Argentina). However, the ample variation found in the morphology and coloration of the populations sampled in the tributaries of the Brazilian state of Paraná makes it difficult to establish the real taxonomic status and evolutionary history of the *Ancistrus* specimens, suggesting that *A. cirrhosus* is not the only species found in this basin. By combining data on mitochondrial DNA (COI gene) and chromosomal markers from different *Ancistrus* populations, totaling 144 specimens, in the tributaries of the Paraná, and specimens from Misiones (type-locality of *A. cirrhosus*), we detected five distinct evolutionary lineages. All the specimens were 2n = 50, but had four distinct karyotype formulae. The results of the Generalized Mixed Yule Coalescent (GYMC) and the genetic distances (uncorrected *P*-values) between lineages ranged from 3 to 5%. Clusters of 18S rDNA were observed in a single chromosome pair in seven populations of *Ancistrus,* but at different positions, in some cases, in synteny with the 5S rDNA sites. Multiple 5S sites were observed in all populations. Overall, the cytogenetic data reinforce the genetic evidence of the diversification of lineages, and indicate the existence of candidate species in the study region. The evidence indicates that at least four candidate species of the *Ancistrus* may coexist in the Paraná basin besides *A. cirrhosus*. Overall, our results provide a comprehensive scenario for the genetic variation among *Ancistrus* populations and reinforce the conclusion that the true diversity of the freshwater fish of the Neotropical regions has been underestimated.

Keywords: *Ancistrus* genus, cytotaxonomy, species delimitation, candidate species, chromosomal evolution

INTRODUCTION

The inclusion of genetic data in studies of taxonomy and evolution has had a profound influence on our understanding of the unique diversity of species in the Neotropical region (Pereira et al., 2011, 2013). The combination of approaches has altered our perceptions of the region's biological diversity and contributed to an increase in the rate of discovery of new species, especially in cryptic lineages. In this context, the delimitation of species using DNA sequences can be highly efficient, enabling the more systematic documentation of species diversity (e.g., Yang and Rannala, 2010; Ence and Carstens, 2011; Fujisawa and Barraclough, 2013). For example, the Generalized Mixed Yule Coalescent (GMYC) method has been designed to delimit potential lineages using information on a single locus, that is, this method considers that the mutations arising in one species cannot spread readily into another species (Ahearn and Templeton, 1989; Barraclough et al., 2003; De Queiroz, 2007). The premise of the GMYC method is that independent evolution leads to the emergence of distinct genetic clusters, separated by longer internal branches, optimizing the set of nodes that defines the transition between inter- and intra-specific processes (Barraclough et al., 2003).

Fish are an excellent candidate group for the application of integrative taxonomical methods, which combine different lines of evidence (DNA sequences, chromosomal data, and morphological features) to define taxonomic status. Most surveys of Neotropical freshwater fish have focused on major river basins, even though a large proportion of the total diversity comprises small species, found in minor rivers and streams. These species are often highly endemic and occupy a wide variety of microhabitats, providing enormous potential for diversification (Viana et al., 2013). Castro (1999) referred to the identification of the cryptic diversity of small freshwater fish as a major challenge for Neotropical Ichthyology. The reliable definition of species and their ranges is also essential to conservation strategies (Angulo and Icochea, 2010). In this context, the rivers and streams of the Paraná River basin provide an interesting study area for the evaluation of the degree to which the taxonomic diversity of these environments has been underestimated.

The Ancistrini is composed of 29 genera, with a total of 217 recognized species (Fisch-Muller, 2003). With 69 species, *Ancistrus* Kner, 1854 is one of the most diverse Ancistrini groups, the second richest in species of the Loricariidae (Ferraris, 2007; Bifi et al., 2009; Froese and Pauly, 2017). Cytogenetic data on *Ancistrus* are still scarce, and restricted to species found in the basins of the Paraguay River in Mato Grosso, and the Amazon, in Manaus (de Oliveira et al., 2009; Mariotto et al., 2011, 2013; Favarato et al., 2016; Prizon et al., 2016). While the cytogenetics of *Ancistrus* species from other river basins are still unknown, considerable variability has been found in this genus, with diploid (2n) numbers of 34, 38, 40, 42, 44, 48, 50, and 54 chromosomes. Surveys of the upper Paraná River have revealed the presence of a single species of *Ancistrus* in this basin, identified as *Ancistrus cirrhosus* (Langeani et al., 2007). However, the considerable variation in the morphology and coloration observed in the specimens collected in the tributaries of the Paraná River hampers the reliable identification of the *Ancistrus* species found in this region. Thus, given the karyotypic diversity of *Ancistrus* and the assumption that cryptic diversity exists in the rivers and streams of the Upper Paraná River basin, we combine chromosomal data and DNA sequences to evaluate the taxonomic status of these populations.

MATERIALS AND METHODS

Biological Samples

A total of 144 *Ancistrus* specimens were collected in ten rivers of the Paraná River basin (**Table 1**, **Figure 1**). Specimen collection was authorized by the Brazilian Environment Ministry through its Biodiversity Information and Authorization System (SISBIO), under license number 36575-1. The protocols used in this study were submitted to the Ethics Committee on the use of animals in research (CEUA) of the Universidade Estadual de Maringá (UEM) and approved under case number 013/2009. Voucher specimens were deposited in the ichthyological collection of the Limnology, Ichthyology and Aquaculture Research Center (Nupélia) at Universidade Estadual de Maringá, Paraná, Brazil. The catalog numbers are provided in **Table 1**.

TABLE 1 | Details of the *Ancistrus* populations and specimens sampled in the study area of the upper Paraná basin.

Code	Molecular sample	Cytogenetic sample	River	Locality/State or Province/Country	Geographical coordinates	NUP
L1	1♂+1♀	13♂+36♀	Mourão	Campo Mourão/Paraná/Brazil	25°04′46″S, 53°54′45″W	11,993
L2	2♂	7♂+4♀	19 Stream	Paraiso do Norte/Paraná/Brazil	52°38′17″S, 23°16′08″W	13,646
L3	2♂+2♀	2♂+2♀	Keller	Marialva/Paraná/Brazil	23°38′48″S, 51°52′51″W	18,794
L4	1	–	Patos	Prudentópolis/Paraná/Brazil	25°09′59″S, 50°56′29″W	15,537
L5	1	–	São João	Prudentópolis/ Paraná/Brazil	25°05′10″S, 51°00′11″W	15,812
L6	1♂+1 Immature	2♂+2 Immature	São Francisco Verdadeiro	Toledo/Paraná/Brazil	24°46′50″S, 53°43′00″W	15,146
L7	1	–	Arroyo Iguaçu	Marechal Cândido Rondon/ Paraná/Brazil	24°25′18″S, 54°01′09″W	15,250
L8	4	2♂+10♀	Arroyo San Juan	Misiones/Posadas/Argentina	27°22.623′S, 55°53.571′W	18,795
L9	1♂+1 Immature	10♀+8♂+3 Immature	Ocoí	Medianeira/Paraná/Brazil	25°15′12″S, 54°01′55″W	4,729
L10	1♂+1♀	10♂+30♀	São Francisco Falso	Vera Cruz do Oeste/Paraná/Brazil	25°04′46″S, 53°54′45″W	15,145

NUP, catalog number of the voucher specimen in the Nupélia collection; ♂, male; ♀, female.

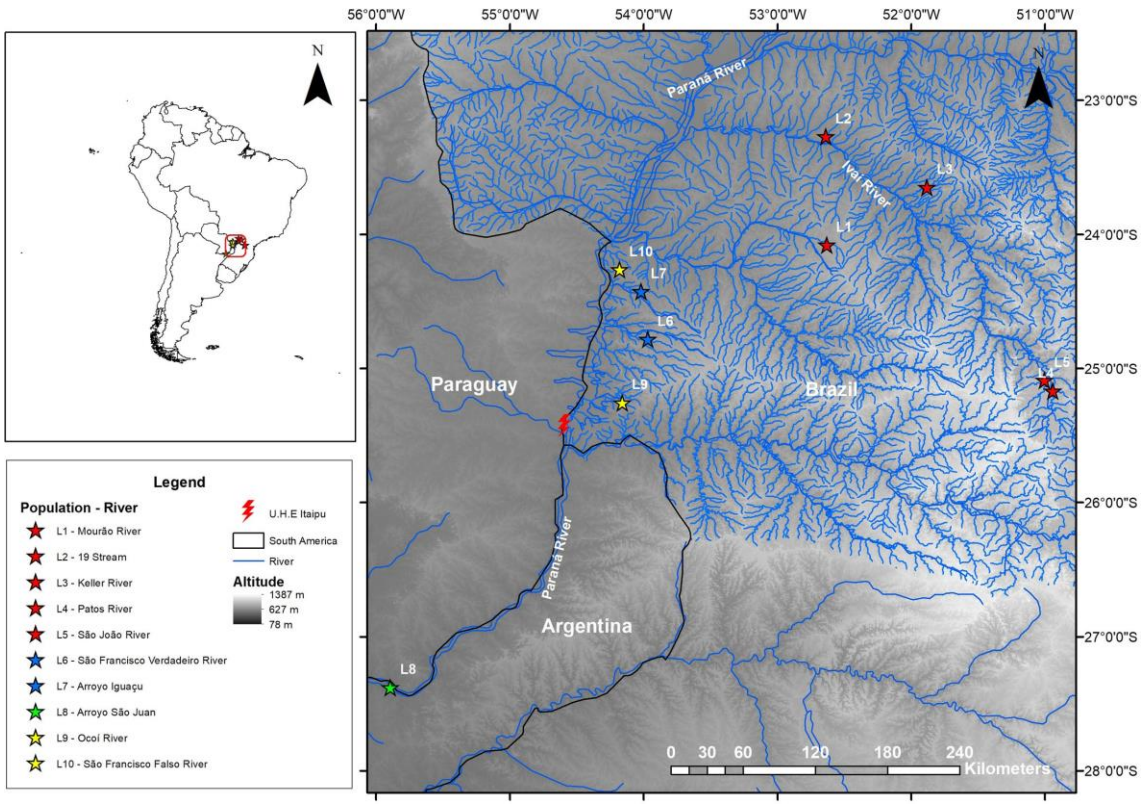

FIGURE 1 | Topographic map showing the sites for the collection of specimens from the *Ancistrus* populations sampled in the Paraná River basin, in the present study. Each color represents a different clade recovered in our phylogenetic inferences (see Figure 2): red star, populations in clade I; blue star, populations in clade II; green star, populations in clade IV; and yellow star, populations in clade V. L1, Mourão River; L2, 19 Stream; L3, Keller River; L4, Patos River; L5, São João River; L6, São Francisco Verdadeiro River; L7, Arroyo Iguaçu; L8, *Ancistrus cirrhosus*; L9, Ocoí River; L10, São Francisco Falso River.

Isolation, Amplification, and Sequencing of the DNA

Genomic DNA was extracted from the liver, muscle tissue, or a cell suspension of a subset of the sample (**Table 1**) using the TNES method, as applied by Bruschi et al. (2012). A fragment of the mitochondrial cytochrome C oxidase subunit I (COI) gene was amplified by polymerase chain reaction (PCR) using the primers: FishF1 (5′-TCAACCAACCACAAAGACATTGGCAC- 3′), FishR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) (Ward et al., 2005). The solution for the amplification reaction included 20 ng/μl of the DNA template, 7 pmol of the forward and reverse primers, 10 mM of dNTPs, 1 U *Taq* DNA Polymerase, 1.5 mM $MgCl_2$, and 1x PCR buffer (200 mM Tris, pH 8.4, 500 mM KCL). The amplification protocol was 5 min−94°C/(94°C/30 s−60°C/1 min−72°C/30 s) 35 cycles/10 min−72°C. The amplified products were purified using Exonuclease I (10 units) and SAP (1 unit), incubated for 45 min 37°C, followed by denaturation at 85°C for 10 min (Applied Biosystems, Santa Clara, CA, USA), as recommended by the manufacturer. The samples were then used directly as templates for sequencing in an automatic ABI/Prism DNA sequencer (Applied Biosystems, Foster City, CA, USA) with the BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA), as recommended by the manufacturer. The DNA samples were sequenced bidirectionally and were edited in Bioedit version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/page2.html) (Hall, 1999).

Phylogenetic Inferences and the Delimitation of Species

The phylogenetic relationships among the populations were inferred from the matrix of the 459-bp sequence of the COI gene. The dataset was complemented with 35 sequences of *Ancistrus* and one sequence of the sister group *Lasiancistrus* available in GenBank (Supplementary File 1). The outgroup was *Pseudolithoxus* sp., which was chosen based on the arrangement reported by Lujan et al. (2015). The sequence was aligned using Clustal W in BioEdit, version 7.2.5.0 (Thompson et al., 1994). The initial alignments were checked visually and adjusted wherever necessary. The dataset was used for phylogenetic reconstruction by Bayesian inference (BI) and the Maximum Parsimony (MP) approach.

Bayesian Inference (BI) method was applied to the dataset, which were divided into three partitions according to codon position for *mit*-COI. The best model of nucleotide evolution for each nucleotide partition was determined using Akaike

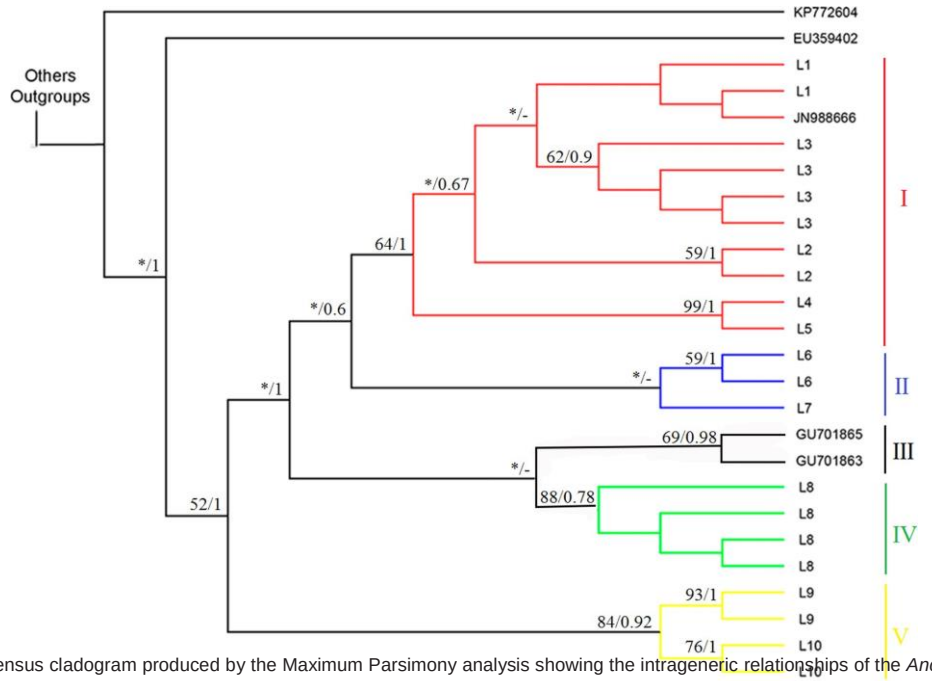

FIGURE 2 | Strict consensus cladogram produced by the Maximum Parsimony analysis showing the intrageneric relationships of the *Ancistrus* species from the Paraná basin, based on a 459-bp sequence of the COI gene. The numbers above the branches show the bootstrap support and Bayesian posterior probabilities, respectively. Each color represents one of the five evolutionary lineages recovered in the analysis. The terminal species with alphanumerical identifiers were obtained from GenBank (Supplementary File 1). The asterisks indicate nodes with bootstrap values of <50. The trace indicates that the node has not been recovered by Bayesian inference. Codes: L1, Mourão River; L2, 19 Stream; L3, Keller River; L4, Patos River; L5, São João River; L6, São Francisco Verdadeiro River; L7, Arroyo Iguaçu; L8, *Ancistrus cirrhosus*; L9, Ocoí River; L10, São Francisco Falso River.

Information Criterion (AIC) with the software jModelTest v2.1.6 (Guindon and Gascuel, 2003; Darriba et al., 2012). The BI was performed with the software Mr. Bayes 3.2.6 (Ronquist and Huelsenbeck, 2003), as available in the CIPRES Science Gateway 3.1 (Miller et al., 2010). BI was implemented using two independent runs, each starting from random trees, with four simultaneous independent chains, and performed 10,000,000 generations, keeping one tree every 1,000th generation. Of all trees sampled, 20% were discarded as burn-in and checked by the convergence criterion (frequencies of average standard deviation of split <0.01) with Tracer v.1.6 (Rambaut et al., 2014), while the remaining were used to reconstruct a 50% majority-rule consensus tree and to estimate Bayesian posterior probabilities (BPP) of the branches. A node was considered to be strongly supported if it had a BPP ≥ 0.95, while moderate support was considered when BPP ≥ 0.9.

The MP analysis was implemented in TNT v1.1 (Goloboff et al., 2003) using a heuristic search method with tree bisection-reconnection (TBR) swapping and 100 random additional replicates. The bootstrap values of the branches inferred in this analysis were calculated with 1,000 non-parametric pseudoreplicates.

The genetic distances among and within species were calculated using the Kimura-2-Parameter (K2P) and *p*-distance model (Kimura, 1980) implemented in MEGA v 6.0. A neighbor-joining (NJ) tree of K2P distances was created to provide a graphic representation of the patterning of divergence between species with the software MEGA v 7.0 (Kumar et al., 2016). We also applied the General Mixed Yule-coalescent (GMYC) method to delineate species using single-locus sequence data. The GMYC requires a fully resolved and ultrametric tree as input for the analysis and combines a coalescence model of intraspecific branching with a Yule model for interspecific branching to estimate species boundaries and provide statistical confidence intervals to evaluate the sequences of the clusters recovered. Ultrametric trees were constructed by a BI tree in BEAST2 2.4.0 (Drummond et al., 2006; Drummond and Rambaut, 2007). We conducted three independent runs using different priors, that is, the Yule, relaxed clock, and constant coalescent models. An ultrametric gene tree was obtained for each prior. An XML file was produced using the BEAUti2 v2.4.5 interface with the following settings: GTR+G+I substitution model, previously inferred by MrMODELTEST (Nylander et al., 2004), empirical base frequencies, four gamma categories, all codon positions partitioned with unlinked base frequencies and substitution rates. The MCMC chain was 10 million generations long, and was logged every 1,000 generations. The Estimated Sample Sizes (ESS) and trace files of the runs were

TABLE 2 | Uncorrected pairwise distances between the mitochondrial COI sequences of the *Ancistrus* populations from the Paraná basin.

Populations*	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)	9 (%)	10 (%)	11 (%)	12 (%)
1. *Ancistrus* sp. L1												
2. *A.cirrhosus* JN988666	0.5											
3. *Ancistrus* sp. L2	0.5	0.4										
4. *Ancistrus* sp. L3	0.5	0.4	0.4									
5. *Ancistrus* sp. L4	1.6	1.8	1.8	1.8								
6. *Ancistrus* sp. L5	1.6	1.8	1.8	1.8	0.0							
7. *Ancistrus* sp. L6	1.8	2.0	1.8	2.0	2.7	2.7						
8. *Ancistrus* sp. L7	2.2	2.5	2.5	2.5	3.3	3.3	2.0					
9. *Ancistrus cirrhosus* L8	2.5	2.7	2.7	2.7	2.7	2.7	2.2	3.1				
10. *A.cirrhosus* GU701865	2.2	2.4	2.4	2.4	2.7	2.7	1.8	2.7	2.2			
11. *A.cirrhosus* GU701863	2.0	2.2	2.2	2.2	2.9	2.9	2.0	2.9	2.4	1.3		
12. *Ancistrus* sp. L9	3.2	3.4	3.4	3.0	3.4	3.4	2.8	3.7	3.2	2.6	2.8	
13. *Ancistrus* sp. L10	2.7	2.9	2.9	2.9	3.1	3.1	2.4	3.3	2.5	2.4	2.5	1.2

**Codes: L1, Mourão River; L2, 19 Stream; L3, Keller River; L4, Patos River; L5, São João River; L6, São Francisco Verdadeiro River; L7, Arroyo Iguaçu; L8, Ancistrus cirrhosus; L9, Ocoí River; L10, São Francisco Falso River. JN988666, GU701865, and GU701865: GenBank Access Number.*

evaluated in Tracer v1.6. The resulting logs were analyzed in TREEANNOTATOR 2.4.4, with 25% burn-in, maximum clade credibility trees with a 0.5 posterior probability limit, and node heights of the target tree. The splits package (https://r-forge. r-project.org/R/?group_id=333) in R was used for the GMYC calculations, using the single-threshold strategy and default scaling parameters.

Cytogenetic Analysis

All specimens were anesthetized and euthanized by an overdose of clove oil (Griffiths, 2000). Mitotic chromosomes were obtained from kidney cells according to Bertollo et al. (1978). The AgNORs were revealed by the silver nitrate impregnation technique (Howell and Black, 1980). The regions of heterochromatin were determined by the C-banding technique (Sumner, 1972) and stained with propidium iodide according to the method of Lui et al. (2012). Physical mapping of the 5S rDNA and 18S rDNA sequences was carried out by fluorescence *in situ* hybridization (FISH) according to Pinkel et al. (1986), with probes obtained from *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti, 1999) and *Prochilodus argenteus* Spix et Agassiz, 1829 (Hatanaka and Galetti, 2004). We also isolated and cloned the rDNA 5S gene of the *Ancistrus* sample from Keller River using a more specific DNA probe for this gene. The genomic DNA extracted for the molecular analyses was used as the template for the amplification reaction, using the primers 5S-A (5′-TACGCCCGATCTCGTCCGATC-3′) and 5S-B (5′-CAGGCTGGTATGGCCGTAAGC-3′) (Pendas et al., 1994). The products of this amplification were isolated in the 1.5% agarose gel, then purified with an Quick Gel EasyPure® Extraction kit. These sequences were inserted into a linearized cloning vector (pJET1.2/blunt) by the CloneJET PCR Cloning kit (Thermo Scientific) and cloned in *Escherichia coli*. Ten clones with the insert containing the 5S rDNA sequence were selected for sequencing. The 5S rDNA nucleotide sequences were edited using BioEdit software and compared with sequences from GenBank database (www.ncbi.nlm.nih.gov).

Hybridization was conducted under high stringency conditions (77%), and the probes were labeled by nick translation with digoxigenin-11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA). The 5S rDNA probes obtained from the recombinant plasmids were labeled by PCR using the fluorochrome digoxigenin-11-dUTP. The solution for labeling reaction included 20 ng/μl of the DNA template, 7 pmol of the forward and reverse primers, 4 mM of dNTPs, 1 mM of dig-11-dUTP, 1 U *Taq* DNA Polymerase, 1.5 mM $MgCl_2$, and 1x PCR buffer (200 mM Tris, pH 8.4, 500 mM KCL). The hybridization signals were detected using anti-digoxigenin-rhodamine for the 5S rDNA probe and avidin-FITC (fluorescein isothiocyanate) for the 18S rDNA probe. The chromosomes were counterstained with DAPI. Double staining was carried out with chromomycin A3 (CMA3) and DAPI, according to Schweizer (1976). The metaphases were photographed using an epifluorescence microscope and adjusted for best contrast and brightness using the Adobe Photoshop CS6 software.

RESULTS

Phylogenetic Inferences and Delimitation of Species

The phylogenetic reconstructions based on the MP and BI approaches produced the basic topology of the dataset (**Figures 2**, **3**). Topology inferred from the Bayesian analysis performed with the software Mr. Bayes 3.2.6. and from Neighbor-Joining were presented in the supplementary material (**Figures S1**, **S2**, respectively). These analyses recovered five clades from the *Ancistrus* populations of the Paraná basin. The first clade (clade I) comprises five populations: *Ancistrus* sp. "Mourão River" (L1) + *Ancistrus* sp. "19 Stream" (L2) + *Ancistrus* sp. "Keller River" (L3) + *Ancistrus* sp. "Patos River" (L4) + *Ancistrus* sp. "João River" (L5). The genetic distance

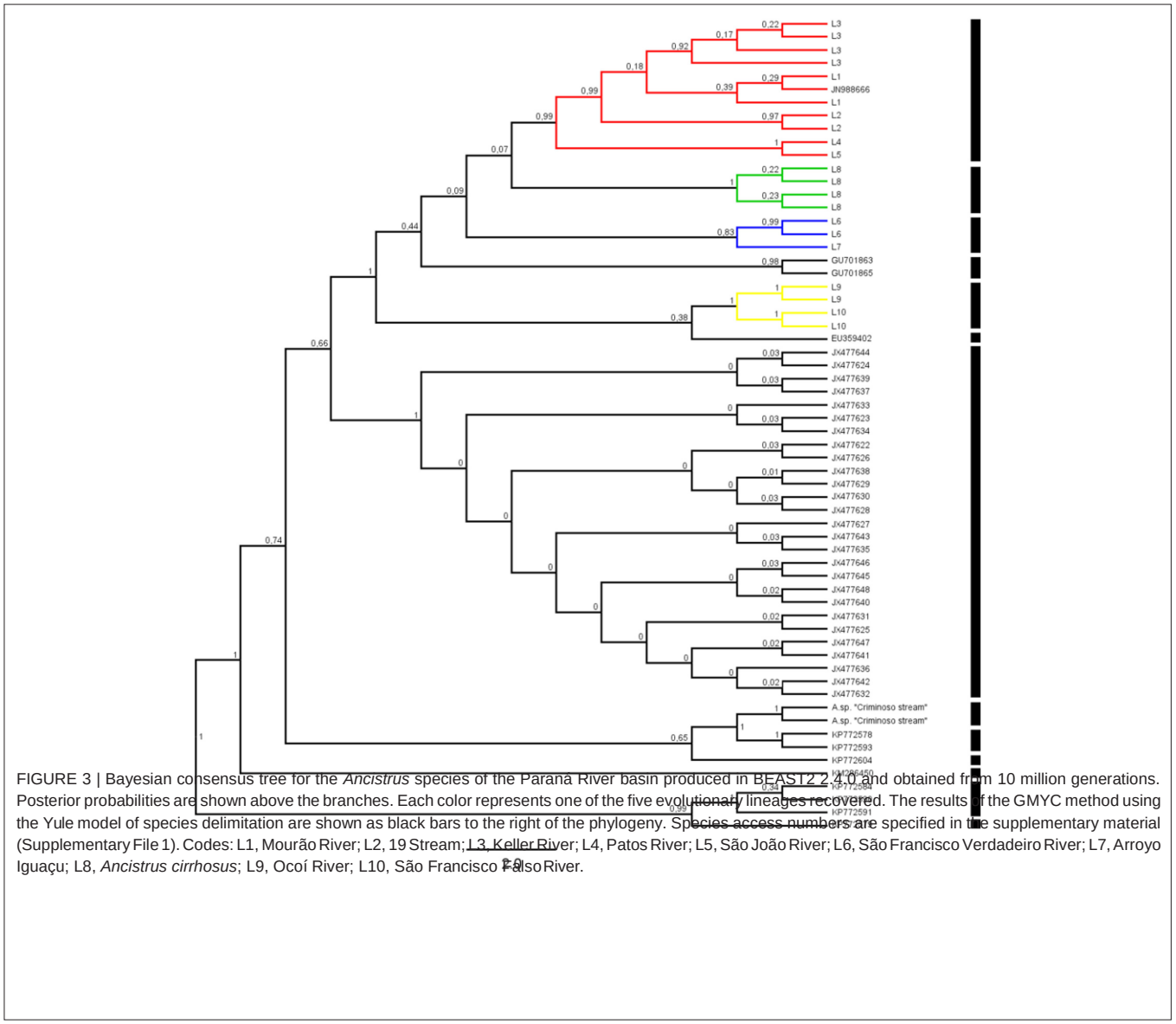

FIGURE 3 | Bayesian consensus tree for the *Ancistrus* species of the Paraná River basin produced in BEAST2 2.4.0 and obtained from 10 million generations. Posterior probabilities are shown above the branches. Each color represents one of the five evolutionarily lineages recovered. The results of the GMYC method using the Yule model of species delimitation are shown as black bars to the right of the phylogeny. Species access numbers are specified in the supplementary material (Supplementary File 1). Codes: L1, Mourão River; L2, 19 Stream; L3, Keller River; L4, Patos River; L5, São João River; L6, São Francisco Verdadeiro River; L7, Arroyo Iguaçu; L8, *Ancistrus cirrhosus*; L9, Ocoí River; L10, São Francisco Falso River.

analysis returned low uncorrected *P*-distances among these populations, ranging from 0.0 to 1.8% (**Table 2**). The second clade (clade II) included the *Ancistrus* populations from São Francisco Verdadeiro River (L6) and *Ancistrus* sp. from Arroyo Iguaçu (L7), separated by a genetic distance of 2.0% (**Table 2**). Two sequences deposited in Genbank as *A.* "*cirrhosus*" included in our dataset were recovered in the clade III. Clade IV included only the population from Arroyo San Juan (L8), Argentina, and was considered to represent the nominal *A. cirrhosus* due to its proximity to the type-locality of this species. Clade V consisted of the *Ancistrus* sp. "Ocoí River" (L9) + *Ancistrus* sp. "São Francisco Falso River" (L10) populations. The uncorrected *P*- distance between these populations (1.2%) was also relatively low (**Table 2**). In all cases, by contrast, the genetic distances (uncorrected *P*-values) between clades were at least 3%, being 3% between clades I–II, I–III, II–III, II–IV, III–IV; 4% between clades I–IV, II–V, III–V, IV–V and finally a genetic distance of 5% between clades I–V.

The ultrametric trees obtained using the Yule and Constant Coalescent priors were congruent in the GMYC analysis, recognizing 13 separate entities and identifying nine clusters in our dataset (including outgroup sequences). Considering only the *Ancistrus* populations, the focus of the present study, the sequences were grouped into five well-supported clusters (**Figure 3**), which corresponded exactly to the major clades recovered in our phylogenetic inferences (BI and MP). The Yule and Constant Coalescent priors of the branching rates indicated that the likelihood of the null model (i.e., that all the sequences belong to the same species) was 412.5381 and 421.5504, respectively, with a likelihood of 421.9409 and 430.6968, respectively, for the GMYC model (i.e., the existence of distinct species). The difference is highly significant, indicating

TABLE 3 | Cytogenetic parameters of the *Ancistrus* populations sampled in the different rivers of the Paraná basin.

Ancistrus population	Clade	Karyotype formula	Ag-NOR	rDNA sites	
				18S	5S
Mourão River (L1)	I	12m+18sm+12s+8a	12 (sm)	12 (sm)	1 (m), 14 (sm), 19 (st)**, 20 (st)
Stream 19 (L2)	I	12m+18sm+12s+8a-♀	12 (sm)	12 (sm)*	1 (m), 12 (sm)*, 15 (sm), 20 (st), 25(a)
		11m+18sm+13s+8a-♂			
Keller River (L3)	I	12m+18sm+12s+8a-♀	12 (sm)	12 (sm)*	1(m), 12(sm)*, 15(sm), 20(st), 22(a), 25(a)
		11m+18sm+13s+8a-♂			
São Francisco Verdadeiro River (L6)	II	14m+16sm+14st+6a	18 (st)	18 (st)*	1 (m), 15 (sm), 18 (st)*, 21 (st)
Arroyo San Juan (L8)	III	10m+14sm+12st+14a	17 (st)	17 (st)	1 (m), 18 (st), 23(a)
Ocoí River (L9)	IV	10m+18sm+16st+6a	18 (st)	18 (st)*	18 (st)*, 21 (st), 22 (st)
São Francisco Falso River (L10)	IV	10m+18sm+16st+6a	18 (st)	18 (st)*	11 (sm), 14 (sm)18 (st)*, 19 (st)

**Synteny between the 18S and 5S rDNA sites; **in only one homolog; m, metacentric; sm, submetacentric; st, subtelocentric; a, acrocentric; ♂, male; ♀, female.*

the presence of more than one species in our sample. The analysis based on the Relaxed clock priors presented low scores in Tracer and did not produce a reliable interpretation of the phylogenetic relationships among the populations, and was not considered in the species delimitation tests.

Cytogenetic Analysis

Cytogenetic data were obtained for the populations from the Mourão, Keller, São Francisco Verdadeiro, São Francisco Falso, and Ocoi Rivers, and 19 Stream (Brazil), and Arroyo San Juan (Argentina). All the specimens analyzed presented a diploid number of 2n = 50 chromosomes, although four distinct karyotype formulae were detected (**Table 3**, **Figure 4**), together with variation in the location of the 18S and 5S rDNA sites (**Figure 5**). Interestingly, these formulae corresponded strongly with the major clades recovered in our genetic analysis, with exception of the clade III that have no karyotype available. A chromosome heteromorphism found in all the males of Stream 19 (L2) and Keller River (L3) populations were consistent with an XX/XY system. The X chromosome is a large metacentric (second pair) while the Y chromosome is a small subtelocentric (**Figure 4**).

The analysis of the heterochromatin revealed C-positive blocks at the pericentromeric and subterminal positions in a number of different chromosomal pairs of the populations allocated to clade I, with a conspicuous block coinciding with the NOR site of pair 12 in all karyotypes (**Figures 6A–C**). The X chromosome (pair 2, metacentric) from 19 Stream had a C-positive block in the pericentromeric region, while we detected heterochromatin blocks in the pericentromeric, interstitial, and subterminal regions of the X chromosome from Keller River. The Y chromosome of the Keller River population had a weak heterochromatin block in the subterminal region of the long arm, which was not found in the Y chromosome from 19 Stream (**Figures 6B, C**). In clades II, IV, and V, considerable variation was found in the amount and distribution of constitutive heterochromatin, which was concentrated primarily in the pericentromeric and subterminal regions of the chromosomes (**Figures 6D–G**). In the population from the São Francisco Falso River (clade V), in addition, we detected an interstitial heterochromatin block in pair 17, as well as a much larger amount of heterochromatin distributed throughout the chromosomes in comparison with the other populations (**Figure 6G**). Conspicuous blocks of heterochromatin were also found in the nucleolar pairs of all the populations analyzed in clades II and IV (pair 18) (**Figures 6D,E**). The base-specific fluorochrome staining enhanced the distinctive composition of some heterochromatin blocks. In particular, staining with Chromomycin A3 revealed a richness of G and C in the subterminal and pericentromeric heterochromatin blocks of all the populations analyzed, and provides additional features for the comparative analysis. As expected, conspicuous C-positive areas were detected in the NOR-bearing chromosomes (pair 12, 17, and 18) (**Figure S3**).

DISCUSSION

Molecular Phylogenetic Inferences Reveal a Number of Distinct Lineages in the Paraná Basin

The phylogenetic reconstructions and cytogenetic analyses presented in this study both detected the presence of five major clades among the *Ancistrus* populations surveyed, pointing to the existence of at least five lineages within the Paraná basin in Brazil previously undetected. Historically, all *Ancistrus* from the Paraná River basin were assigned to a single species, *A. cirrhosus*, which was described by Valenciennes (1836) from specimens collected in the Province of Misiones and Buenos Aires (Argentina), although the diagnostic traits are weakly defined (Langeani et al., 2007). Despite the morphological variations and wide geographic distribution, the *Ancistrus* populations of the Paraná basin have been identified invariably as *A. cirrhosus.* This fact has intrigued the ichthyologists with regard to the taxonomic status of many specimens, using only morphological and meristic characters. Therefore, the data obtained in the present study recovered at least five independent lineages (clades I–V) in Paraná basin. Given the proximity of the Arroyo San Juan to the type-locality of *A. cirrhosus*, this population (clade IV) was identified as nominal species *A. cirrhosus*. In this context, the other four clades (I, II, III, and V) can be categorized as "candidate species" in the terminology of Vieites et al. (2009). This interpretation is also

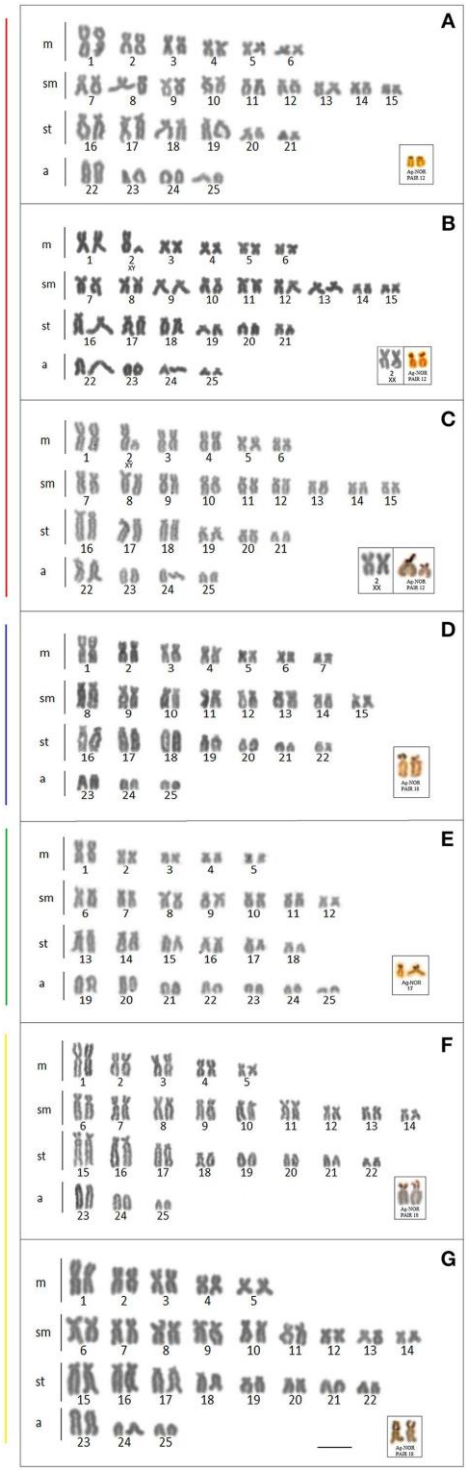

FIGURE 4 | Karyotype of the *Ancistrus* populations sampled in different rivers of the Paraná basin, stained with Giemsa. The configuration of the silver nitrate-stained nucleolar organizing regions (Ag-NORs) are shown in the box. Each color of the side bars represents one of the evolutionary lineages recovered in the analysis, according to Figure 2. (A) L1: Mourão River; (B) L2: 19 Stream; (C) L3: Keller River; (D) L6: São Francisco Verdadeiro River; (E) L8: *Ancistrus cirrhosus*; (F) L9: Ocoí River; (G) L10: São Francisco Falso River. Bar = 10 µm.

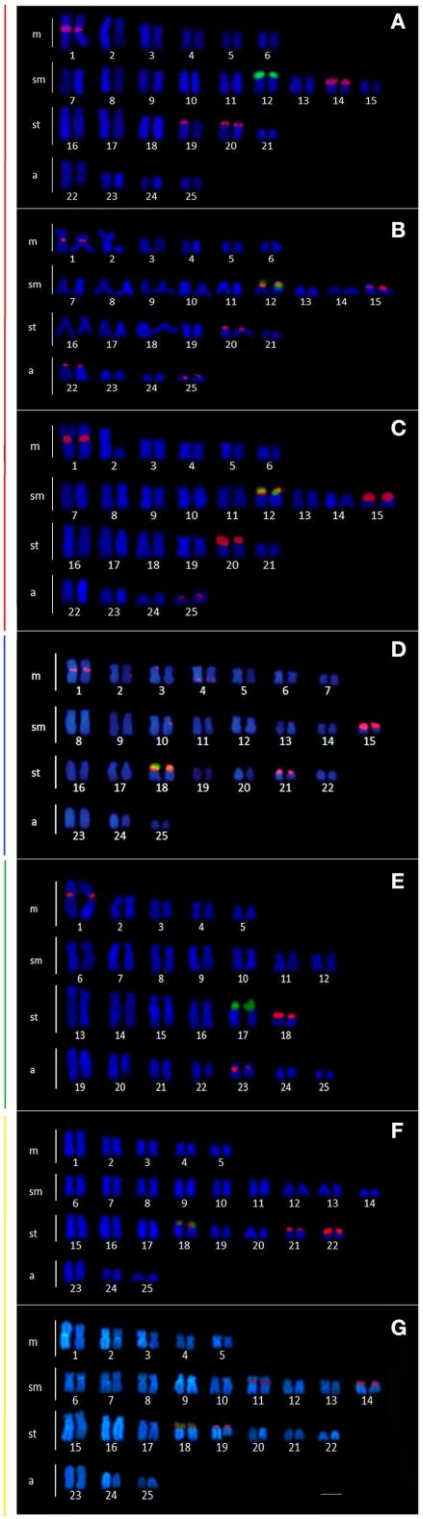

FIGURE 5 | Chromosomes of the *Ancistrus* populations of species after dual color-FISH showing the 5S rDNA (red) and 18S rDNA (green) sites. Each color of the side bars represents one of the evolutionary lineages recovered in the analysis, according to Figure 2. (A) L1: Mourão River; (B) L2: 19 Stream; (C) L3: Keller River; (D) L6: São Francisco Verdadeiro River; (E) L8: *Ancistrus cirrhosus*; (F) L9: Ocoí River; (G) L10: São Francisco Falso River. Bar = 10 µm.

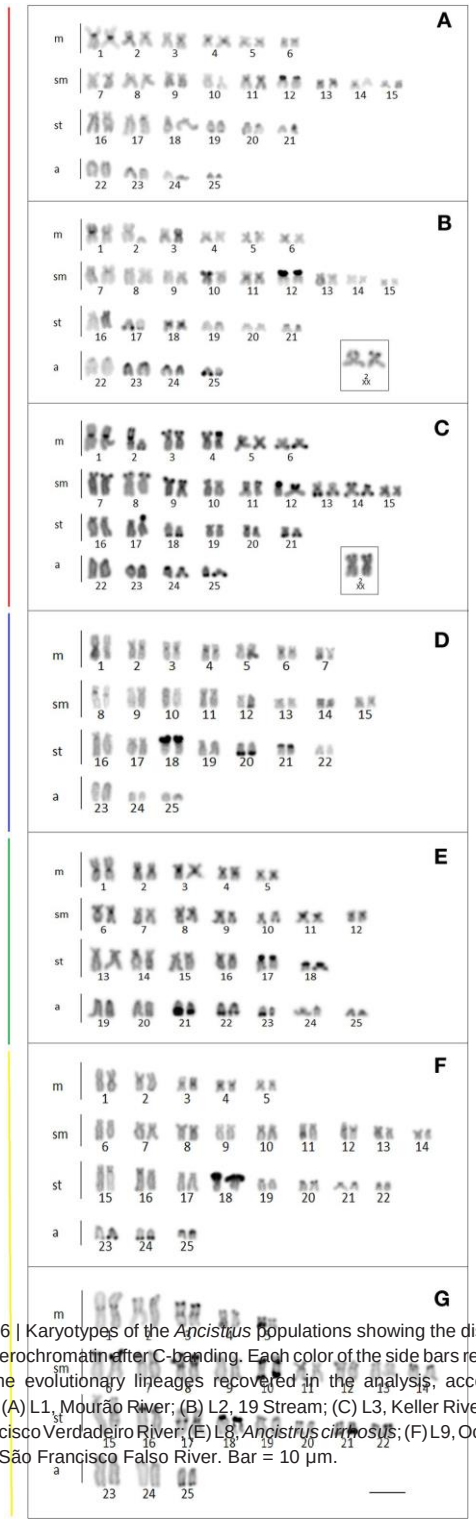

FIGURE 6 | Karyotypes of the *Ancistrus* populations showing the distribution of the heterochromatin after C-banding. Each color of the side bars represents one of the evolutionary lineages recovered in the analysis, according to Figure 2. (A) L1, Mourão River; (B) L2, 19 Stream; (C) L3, Keller River; (D) L6, São Francisco Verdadeiro River; (E) L8, *Ancistrus cirrhosus*; (F) L9, Ocoí River; (G) L10, São Francisco Falso River. Bar = 10 µm.

supported by the results of the GMYC method, and the genetic distance detected among populations, considering a 2% threshold for interspecific differentiation.

The GMYC method has become one of the most popular tools for the delimitation of species based on single-locus data, and has been applied to the analysis of a number of poorly-known groups of organism (Barraclough et al., 2009; Monaghan et al., 2009; Marshall et al., 2011; Vuataz et al., 2011; Roxo et al., 2015). The GMYC method uses an ultrametric tree derived from the sequences to identify shifts in the branching rate from the Yule model (species) to the coalescent (population) process, with the algorithm computing the probability of splits between lineages in relation to speciation rates, thus identifying a cutoff value, at which species and populations split from one another (Powell, 2012). This approach was highly effective in the present study, allowing us to delimit five groups, which four corresponded exactly with the chromosomal data (unfortunately, no chromosomal data available to clade III), reinforcing the hypothesis that four "candidate species" exist in the Paraná River basin, besides *A. cirrhosus* as previously reported.

Armbruster (2004) found a sister group relationship between the Ancistrini and Pterygoplichthini tribes, which both belong to the subfamily Hypostominae. *Ancistrus* is the most species- rich genus of the tribe Ancistrini, although phylogenetic analyses of this genus are still scarce. Lujan et al. (2015) adopted a broad approach to the phylogenetic relationships of the Loricariidae, a Neotropical catfish family, but included few *Ancistrus* species, which reinforces the need for further research. Studies in systematics based on osteological (Schaefer, 1987) and molecular (Montoya-Burgos et al., 1998) data show that *Ancistrus* constitutes a monophyletic group of species. The lack of any robust phylogenetic tree for *Ancistrus* still limits our understanding of its intrageneric relationships, and more detailed analyses, with a more representative dataset, are needed to provide a more comprehensive understanding of the phylogenetic relationships of this genus.

The Chromosomal Data Reinforce the Hypothesis of Complete Lineage Divergence

Cytogenetic studies, together with molecular and biochemical analyses, may be useful for the identification of cryptic species (Nakayama et al., 2001; Milhomen et al., 2007). In the present study, in fact, the inclusion of a cytogenetic approach was crucial to the recognition of the "candidate species," given that the distinct chromosomal formulae found in the four lineages (clades I, II, IV, and V) emphasizes their reciprocal monophyly and the lack of gene flow between them. The results obtained also complement the available cytogenetic data to the *Ancistrus* species, since these studies are currently restricted to the taxa found in the Paraguay (Mato Grosso) and Amazon (Manaus) basins (de Oliveira et al., 2007, 2008, 2009; Mariotto et al., 2011,
2013; Favarato et al., 2016; Prizon et al., 2016).

In the present study, a diploid number of 2n = 50 chromosomes was recorded in all the samples from the Paraná River basin. This number is within the range recorded for the

genus, which vary from 2n = 34 in *Ancistrus cuiabae* to 2n = 54 in *Ancistrus claro* (Mariotto et al., 2009, 2013; Favarato et al., 2016; Prizon et al., 2016). Despite the homogeneity of the diploid number, the karyotype formula varied considerably among populations. The number of acrocentric chromosomes, for example, varied from three to four pairs in the populations of clades I, II, and V, to seven pairs in clade IV (Arroyo San Juan). This points evidences to the occurrence of chromosomal rearrangements, such as translocation and pericentric inversions, which did not affect the diploid number. Unfortunately, while we were able to identify these features, we were unable to trace the pathways of the transformations due to the lack of resolution in the internal topology of the *Ancistrus* lineages recognized here. In this case, further phylogenetic studies, based on a larger set of characters and a multi-locus dataset, may provide more definitive insights into the evolution of this group. Among all the ancistrinis species studied so far, the karyotypic evolution of *Ancistrus* is invariably associated with chromosomal rearrangements, which typically involve variation in the diploid number, given that this number ranges from 34 to 54 in this genus. As Artoni and Bertollo (2001) considered 2n = 54 to be the ancestral diploid number of the Loricariidae, karyotypic evolution in *Ancistrus* appears to have been associated with a reduction in the chromosome number. In fact, de Oliveira et al. (2009) suggested that the karyotypic evolution of this genus was predominantly involves by centric fusions.

In *Ancistrus* the occurrence of heteromorphic sex chromosomes has been well-documented in some species, including simple systems (Mariotto et al., 2004; Alves et al., 2006; Mariotto and Miyazawa, 2006) and multiples (de Oliveira et al., 2007, 2008) which also contributed to the karyotype evolution in the genus (Favarato et al., 2016). One peculiar feature observed in clade I was the heteromorphic sex chromosomes found in the populations of 19 Stream (L2) and the Keller River (L3), which are consistent with an XX/XY system. Surprisingly, however, this feature was not observed in the specimens from the Mourão River (L1), which were included in clade I and separated by low genetic distances from the populations of Stream 19 and the Keller River. The karyotypic formula of the Mourão River population differs from these two other populations only by the absence of the pair of sex chromosomes, and the GYMC analysis also identified these populations as a unique taxonomical unit. A similar result was obtained by Henning et al. (2011), combining cytogenetic and molecular data for different species of *Eigenmannia*, included two populations of *E. virescens* (Mogi-Guaçu and Tietê rivers), whose karyotypes with 2n = 38 differ by the presence of a pair sexual XX/XY (Tietê river population). The acrocentric X chromosome possesses a heterochromatinized distal region (Almeida-Toledo et al., 2001) and according to Henning et al. (2011), both populations (Mogi-Guaçu e Tietê rivers) were considered sister species. Furthermore, these authors concluded that seems likely that suppression of recombination in the homologous pair of acrocentric chromosomes and accumulation of heterochromatin on the X chromosome occurred after a recent geographical separation. In this context, we hypothesized that the heteromorphic sex chromosome found in the *Ancistrus* populations of 19 Stream and the Keller River represent a recent event which may have occurred after the geographical isolation of these populations from that of the Mourão River, together with the behavioral characteristics of these fish, which occupy specific microhabitats, form territories, and do not normally migrate (Power, 1984, 1990; Buck and Sazima, 1995). All these characteristics favor the fixation of chromosomal rearrangements and could be contribute to allopatric speciation on a micro scale (de Oliveira et al., 2009).

The hypothesis of the recent differentiation of the sex chromosome pair in the *Ancistrus* populations of clade I was also supported by the C-banding data. Heterochromatin is widely used in the identification of the sex chromosomes, and the addition or deletion of heterochromatin or the occurrence of a pericentric inversion involving one of the chromosomes have been postulated as important mechanisms in the origin of simple sexual chromosome systems in Neotropical fish (Almeida-Toledo et al., 2000). If we compare the Y chromosome of males from 19 Stream (L2) and Keller River (L3), it is possible to detect a discreet heterochromatin block in the subterminal region of the long arm of the Keller River males, which was not detected in the Y chromosomes from 19 Stream. The presence of the heteromorphic pair in the *Ancistrus* population of 19 Stream and Keller River suggests that chromosomal rearrangements (inversions), the loss of chromosomal material and, in the specific case of the Keller River, the presence of constitutive heterochromatin in the heteromorphic pair, may all be evidence of the recent origin of the Y chromosome, derived from a large metacentric, similar to the X chromosome. Pericentric inversions, followed by a loss of chromosomal material, have been suggest as a mechanism to explain the origin of the ZZ/ZW chromosome system in *Ancistrus* sp. Piagaçu (de Oliveira et al., 2007) and the XX/XY chromosome systems in *Ancistrus* sp. Purus and *Ancistrus* sp. Macoari (de Oliveira et al., 2009), given absence of the heterochromatin blocks in either the X or Y chromosomes.

The NOR mapping provided an excellent marker in the present study, being found in a single chromosome pair in each clade, a condition shared with most other *Ancistrus* species (Medeiros et al., 2016). The variation among clades in the NOR-bearing chromosome may be the result of chromosomal rearrangements occurring during chromosomal evolution. We also recorded synteny between the 18S and 5S rDNA sites in most populations of clades I, II, and V, except for the population from Mourão River (clade I). This synteny of the rDNA may represent the basal condition for the genus (Mariotto et al., 2011), as it is found in *A. claro* (2n = 54), although synteny of the ribosomal sites was not found in the population from Arroyo San Juan (clade IV). The position and distribution of the 5S rDNA sites varied considerably among the four clades, as they do in other *Ancistrus* species, occupying multiple sites in pericentromeric, interstitial, or terminal positions. This variation is considered to be an important reflection of the enormous karyotypic diversity found in the *Ancistrus*, which is seen as evidence of the apomorphic condition of this group (Medeiros et al., 2016). This variability, together with the disjunction of the ribosomal sites caused by rearrangements or mobile genetic elements appears to be a common condition among

Neotropical fish species (de Oliveira et al., 2009). We observed heterochromatin in association with the ribosomal DNA sites (18 and 5S), recurrent characteristic in the karyotypes of Neotropical fishes (Vicari et al., 2003). The presence of heterochromatin, which contains large quantities of transposable elements (Dimitri et al., 2009) may facilitate transposition events, moving ribosomal genes around the genome (Moreira-Filho et al., 1984; Vicari et al., 2008; Gross et al., 2009, 2010). This may be one of the factors responsible for the presence of the multiple 5S ribosomal DNA sites found in the present study.

CONCLUSION

The cytogenetic data available for the genus *Ancistrus* indicate a highly heterogeneous pattern of chromosome evolution, marked by Robertsonian and non-Robertsonian rearrangements. While we do not have an exact understanding of the mechanisms that determine these rearrangements in natural populations, their fixation may either initiate or contribute to the divergence process, with specific implications for the utility of chromosomal characters for phylogenetic inference (Sites and Kent, 1994). As in other fish groups, the sex chromosomes, present in some *Ancistrus* species, may have contributed to high rates of evolution. The inferences obtained in the present study from a combined approach of molecular and cytogenetic analyses further corroborate the taxonomic complexity of this genus. This approach was especially important due to the lack of diagnostic features in the morphology of these fishes. The hidden diversity of the study populations was nevertheless decoded successfully by the combined approach, which allowed us to differentiate five distinct lineages of *Ancistrus*, reinforcing the hypothesis of the presence of at least four candidate species in the upper Paraná River basin, besides of the *A. cirrhosus*, previously described. Finally, our findings reinforce the observation that the true diversity of the freshwater fish of the Neotropical has been underestimated and improve our understanding of a regional diversity within *Ancistrus* genus.

AUTHOR CONTRIBUTIONS

AP: provided chromosomal and molecular data, and drafted the manuscript; DB: designed and coordinated the study of molecular data and helped draft the manuscript; HR and CZ: collected specimens from Paraná state and helped to identify the specimens; AF: collected and processed material of specimens from Arroyo San Juan, Argentina; LB-C, AC, and LB: assisted in the execution and analysis of chromosomal banding; AdBP-C: designed and coordinated the study of cytogenetic data and helped draft the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2017.00185/full#supplementary-material

Figure S1 | Topology inferred from the Bayesian analysis performed with the software Mr. Bayes 3.2.6. posterior probabilities are shown at each node. Scale bar represents the number of substitutions per site. The terminal species with alphanumerical identifiers were obtained from GenBank (Supplementary File 1). Codes: L1, Mourão River; L2, 19 Stream; L3, Keller River; L4, Patos River; L5, São João River; L6, São Francisco Verdadeiro River; L7, Arroyo Iguaçu; L8, *Ancistrus cirrhosus*; L9, Ocoí River; L10, São Francisco Falso River.

Figure S2 | Chromosomes of the *Ancistrus* populations of species after CMA3 staining. Each color of the side bars represents one of the evolutionary lineages recovered in the analysis, according to Figure 2. (a) L1, Mourão River; (b) L2, 19 Stream; (c) L3, Keller River; (d) L6, São Francisco Verdadeiro River; (e) L8, *Ancistrus cirrhosus*; (f) L9, Ocoí River; (g) L10, São Francisco Falso River. Bar = 10 μm.

Figure S3 | NJ dendrograma of the *Ancistrus* specimens. Node values = bootstrap test (1,000 pseudo replicas) are shown next to the branches. The terminal species with alphanumerical identifiers were obtained from GenBank (Supplementary File 1). Codes: L1, Mourão River; L2, 19 Stream; L3, Keller River; L4, Patos River; L5, São João River; L6, São Francisco Verdadeiro River; L7, Arroyo Iguaçu; L8, *Ancistrus cirrhosus*; L9, Ocoí River; L10, São Francisco Falso River.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary file 1 (S1): Details (species, voucher number, sample locality, accession number and authors) of the sequences obtained in this work and from GenBank used for phylogenetic inferences.

Species	Voucher	Locality	Access	Reference
Ancistrus cirrhosus	LBP-35987	Brazil, Paraná, Ervalzinho/Marquinho	GU701865	Pereira et al. 2013
Ancistrus cirrhosus	LBP-35959	Brazil, Paraná, Nova Laranjeiras	GU701863	Pereira et al. 2013
Ancistrus cirrhosus	LBPV-34759	Brazil, Paraná, Marabá	JN988666	Pereira et al., 2013
Ancistrus sp.	Ex51H11	Germany	KM286450	Knebelsberger et al. 2015
Ancistrus brevipinnis	MCP 21246		EU359402	Cramer et al. 2007
Ancistrus sp.			JX477648	Carvalho D.C. 2012
Ancistrus sp.			JX477647	Carvalho D.C. 2012
Ancistrus sp.			JX477646	Carvalho D.C. 2012
Ancistrus sp.			JX477645	Carvalho D.C. 2012
Ancistrus sp.			JX477644	Carvalho D.C. 2012
Ancistrus sp.			JX477643	Carvalho D.C. 2012
Ancistrus sp.			JX477642	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477641	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477640	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477639	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477638	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477637	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477636	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477635	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477634	Carvalho D.C. 2012
Ancistrus sp.			JX477633	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477632	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477631	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477630	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477629	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477628	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477627	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477626	Carvalho D.C. 2012
Ancistrus cryptophthalmus			JX477625	Carvalho D.C. 2012
Ancistrus sp.			JX477624	Carvalho D.C. 2012
Ancistrus sp.			JX477623	Carvalho D.C. 2012
Ancistrus sp.			JX477622	Carvalho D.C. 2012
Ancistrus sp	INPA 43862	Brazil, Pará, Lower Nhamundá River	KP772604	Collins R.A. et al. 2015
Ancistrus dolichopterus	INPA 43877	Brazil, Pará, Lower Nhamundá River	KP772593	Collins R.A. et al. 2015
Ancistrus dolichopterus	INPA 43877	Brazil, Pará, Lower Nhamundá River	KP772578	Collins R.A. et al. 2015
Ancistrus sp. "córrego Criminoso"	NUP 12018	Brazil, Mato Grosso do Sul, Coxim		Prizon et al. 2016
Ancistrus sp. "rio Mourão"	NUP 11993	Brazil, Paraná, Campo Mourão		Present study
Ancistrus sp. "córrego 19"	NUP 13646	Brazil, Paraná, Paraíso do Norte		Present study
Ancistrus sp. "rio Keller"	NUP 18794	Brazil, Paraná, Marialva		Present study
Ancistrus sp. "rios dos Patos"	NUP 15537	Brazil, Paraná, Prudentópolis		Present study
Ancistrus sp. "rio São João"	NUP 15812	Brazil, Paraná, Prudentópolis		Present study
Ancistrus sp. "rio SFVerdadeiro"	NUP 15146	Brazil, Paraná, Toledo		Present study
Ancistrus sp. "Arroyo Iguaçu"	NUP 15250	Brazil, Paraná, Marechal Cândido Rondon		Present study
Ancistrus cirrhosus	NUP 18795	Brazil, Misiones, Posadas		Present study
Ancistrus sp. "rio Ocoí"	NUP 4729	Brazil, Paraná, Medianeira		Present study
Ancistrus sp. "rio SFFalso"	NUP 15145	Brazil, Paraná, Vera Cruz do Oeste		Present study
Lasiancistrus schomburgkii	INPA 43886	Brazil, Pará, Lower Nhamundá River	KP772579	Collins R.A. et al. 2015
Pseudolithoxus sp.	INPA 43889	Brazil, Pará, Lower Nhamundá River	KP772591	Collins R.A. et al. 2015
Pseudolithoxus sp.	INPA 43889	Brazil, Pará, Lower Nhamundá River	KP772590	Collins R.A. et al. 2015
Pseudolithoxus sp.	INPA 43889	Brazil, Pará, Lower Nhamundá River	KP772584	Collins R.A. et al. 2015

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Fig. S1

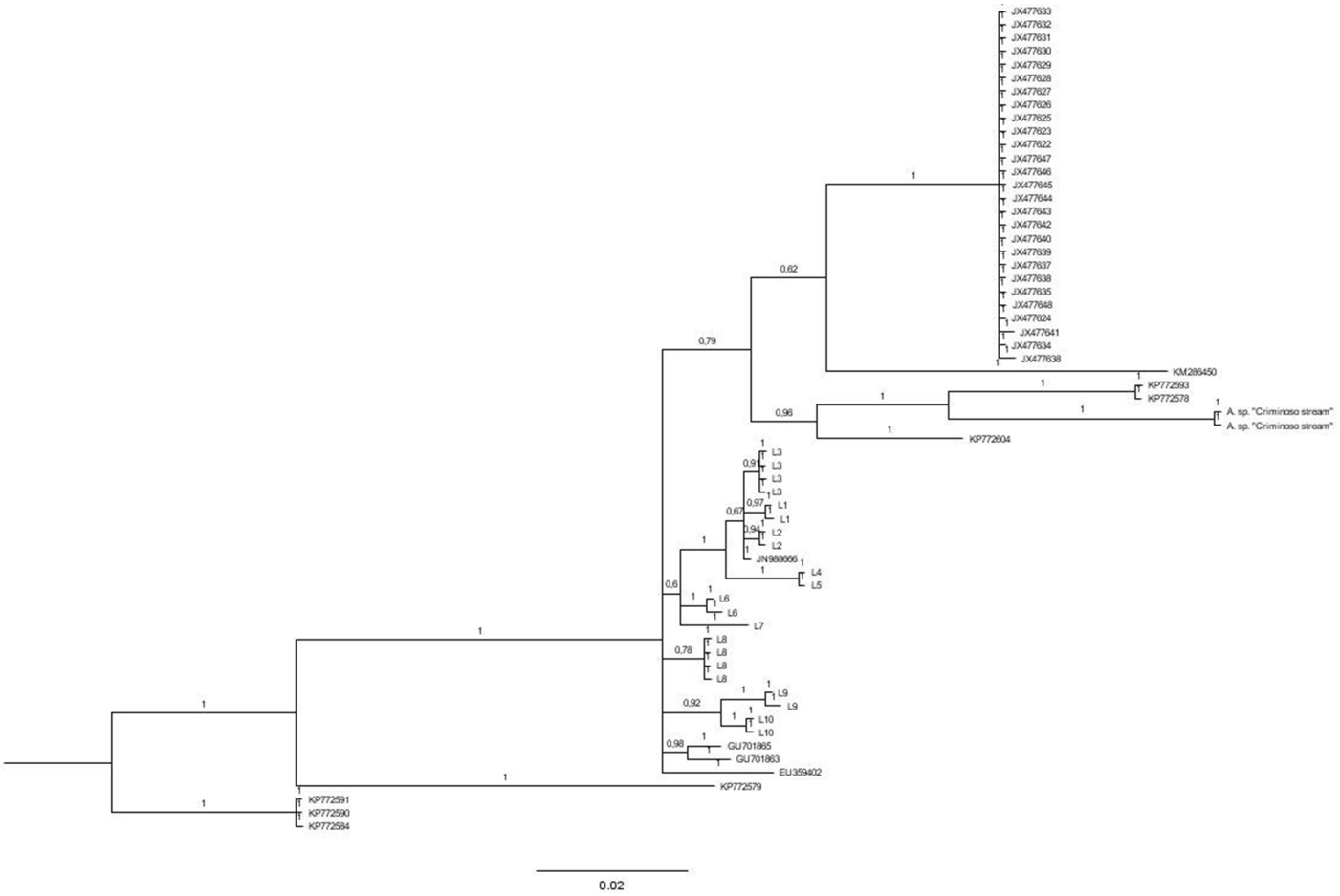
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Fig. S2

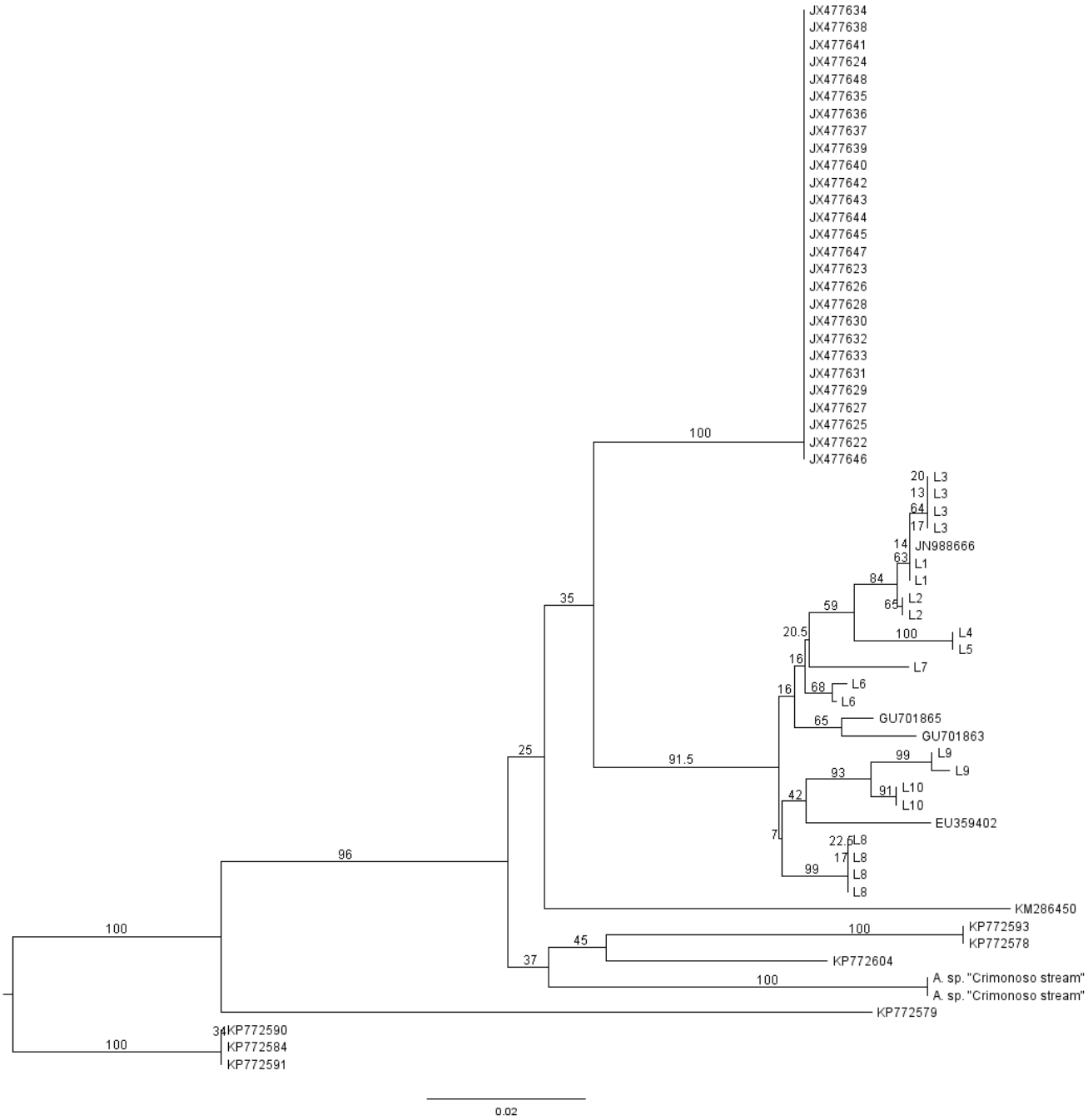
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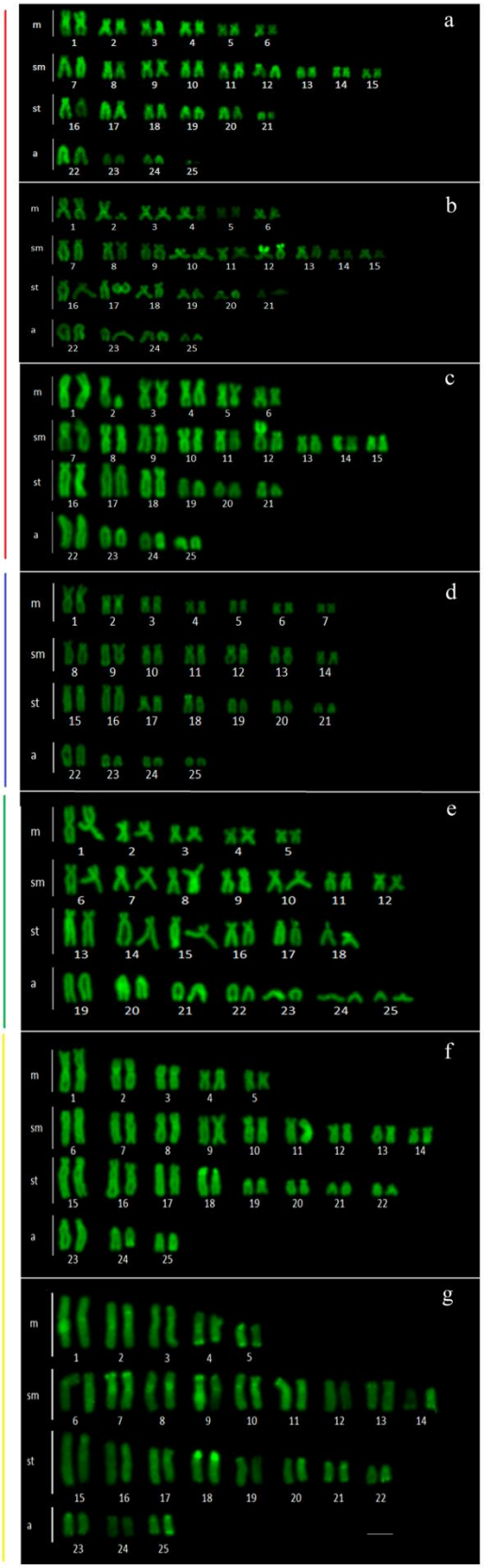
Fig. S3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st 16 17 18 19 20 21
a 22 23 24 25
c
m 1 2 3 4 5 6
sm 7 8 9 10 11 12 13 14 15
st 16 17 18 19 20 21
a 22 23 24 25
d
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sm 8 9 10 11 12 13 14
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sm 6 7 8 9 10 11 12
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a 19 20 21 22 23 24 25
f
m 1 2 3 4 5
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CAPÍTULO II

ARTIGO:

Mapeamento de repetições de microssatélites e retrotransposon não-LTR revela sua interação na diversificação dos cariótipos das populações de *Ancistrus* da bacia do Paraná no Sul da América do Sul

Este artigo será submetido ao periódico Zebrafish.

Mapping of microsatellite repeats and non-LTR retrotransposons reveals their interplay in the diversification of the karyotypes of the *Ancistrus* populations from the Paraná basin in southern South America

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Abstract
Repetitive DNA sequences represent a large portion of the eukaryotic genome, and are abundant in the fish genome. These elements play a significant role in the evolution of the eukaryote genome, and in the diversification of karyotypes. The catfish genus *Ancistrus* is an interesting candidate for the investigation of karyotype evolution, given its extensive chromosomal variation. The present study investigated the distribution and behavior of the non-LTR retrotransposable element *Rex*-3, and the $(CA)_{15}$ and $(GA)_{15}$ microsatellites in *Ancistrus*, with seven populations being sampled in different rivers of the Paraná basin in Brazil and Argentina. The transposable element (TE) was amplified by PCR using heterologous primers for *Rex*-3, which generated fragments of approximately 500 bps. The physical mapping using the *Rex*-3TE as a probe revealed fluorescent signals scattered throughout the chromosomes of all the *Ancistrus* specimens analyzed, while the $(CA)_{15}$ and $(GA)_{15}$microsatellite probes hybridized preferentially in the subterminal and interstitial regions of most chromosome arms, although these two classes of repetitive DNA were co-located in some chromosome pairs. Clusters of repetitive DNA elements were observed in some chromosomal pairs, associated with heterochromatin blocks and rDNA sites. In the X chromosome, large numbers of hybridization signals were detected, scattered throughout the chromosome in the specimen from the Keller River, whereas weaker signals were detected in the specimen from 19 Stream. The analysis of the nucleotide sequences of the *Rex*-3 TE from isolated copies of the *Ancistrus* genome, representing the different populations, revealed a high degree of similarity with sequences of the same element deposited in the online CENSOR/BLAST database. The alignment of the presumed amino acid sequence with that of *Xiphophorus maculatus* revealed highly conserved reverse transcriptase domains, which indicates that this element is potentially active in this genome, and may have contributed to the dispersal of the copies detected by FISH. Considerable variation was found among the different *Ancistrus* populations in the distribution of the heterochromatin, and the presence of *Rex*-3 TEs in these blocks and at 5S rDNA sites, reflecting their role in the dispersion of these sites. Our findings provide important insights into the mechanisms of karyotype diversification in the genus *Ancistrus*, which involve these repetitive sequences.

Keywords: Ancistrini, repetitive DNA, retrotransposable element, chromosomal mapping, karyotype diversity.

Introduction

Tandem repeats, including satellite DNA, minisatellite and microsatellite repeats, and transposons and retrotransposons, are the most abundant repetitive DNA components in the eukaryote genome.[1] A number of recent studies have provided increasing evidence of the active role of these sequences in the evolution of the eukaryotic genome.[2-4] In contrast with the more traditional assumption, that they are merely a structural component of the chromosomes,[5,6] these elements are now known to participate in cell metabolism through the regulation of gene expression, for example. [2, 7-9] These sequences are frequently involved in chromosomal rearrangements, such as deletions, duplications, inversions and translocations, which are responsible for much of the variation found in the karyotypes of many groups of organisms.[10-12] The presence of highly repetitive DNA at the fragile sites of certain genomic regions is consistent with the existence of chromosome recombination hotspots, which may affect the structure of the chromosomes and be fundamental to the speciation.[13]

Repetitive DNA sequences are often associated with the heterochromatin regions that can be detected by traditional C-banding techniques or base-specific fluorochromes. These methods have a reduced potential for discrimination, however, use of the molecular cytogenetic tools, based on the Fluorescence *in situ* hybridization (FISH) technique, have helped advance the understanding of the hidden diversity of the repetitive sequences that compose the heterochromatin blocks in the fishes genome.[14-16] The mapping of these specific sequences in chromosomes has provided new perspectives for the analysis of chromosomal variation and the understanding of their role in chromosomal evolution.[16-18]

Cytogenetic research on fish has been enhanced by the analysis of repetitive sequences, which have clearly shaped the chromosomal diversification among groups.[19-21] Transposable elements (TEs) are the most common component of the repeated DNA fraction of the fish genome and are assigned to two main classes, I–TEs that are moved and amplified by RNA intermediates (retrotransposons), and II–TEs in which transposition is mediated by DNA molecules or DNA transposons.[9,10,22] Retrotransposons can be further divided according to the presence (LTR) or absence (non-LTR) of long terminal repeats (LTR). Two main classes of non-LTR retrotransposons can usually be found in eukaryotic genomes: the short (SINE) and long interspersed elements (LINE), which can be distinguished by their length and sequence structure.[1, 23-26] The *Rex*-3 elements, which are non-LTR retrotransposons, were first isolated from the melanoma fish model *Xiphophorus* and have been widely reported in the fish genome.[14-16, 27-29]

The satDNAs are a class of tandem repeats arranged in a sequential array of repeating units, ranging from 150–180 bps to 300–360 bps, while microsatellites (or short tandem repeats) are made up of short sequences of one to six nucleotides repeated in tandem.[30,31] Studies in the fish genome show an accumulation of microsatellites preferentially in the sex chromosomes and in subterminal and centromeric regions of the chromosomes (usually heterochromatic regions), which are being interesting markers for the study of the chromosomal diversification of different fish groups.[32-34] In addition, a number of studies have also reported the co-location of different classes of repetitive elements (satDNA and TEs, for example), which indicates the existence of an interrelationship.[35-37]

Prizon et al.[21] recently identified high levels of cryptic diversity within the *Ancistrus* populations of the Paraná River basin, based on a combined molecular and cytogenetic approach. While all the populations shared the same chromosome number,

five distinct evolutionary lineages were recognized, based on their different karyotype formulae, heterochromatin patterns, and the presence/absence of heteromorphic sex chromosomes.[21] This indicates the occurrence of intra-chromosomal rearrangements, such as translocations and pericentric inversions, which did not affect the diploid number, even though the chromosomal evolution of the *Ancistrus* group as a whole is based on rearrangements, in particular fusions, which result in variation in the diploid number.[38,39] As the evolutionary lineages from the Paraná basin have been established through intra-chromosomal rearrangements with no modifications of the chromosome number, but variation in the location and distribution of heterochromatin blocks, breaks in synteny between 5S and 18S rDNAs, and multiple 5S rDNA sites,[21] we would expect the repetitive DNA content to have played an important role in the karyotype diversification of these lineages. Based on this, we evaluated the potential role of three repetitive sequence classes (the *Rex*-3 non-LTR element, microsatellite repeats, and telomeric motifs) in this process of karyotype diversification.

Materials and Methods

Biological samples

Specimens of *Ancistrus* collected from 10 rivers of the Paraná basin and cytogenetics data analyzed initially by Prizon et al.[21] are summarized in the Table 1. The collection of specimens was authorized by the Brazilian Environment Ministry through its Biodiversity Information and Authorization System (SISBIO), which conceded license number 36575-1.The protocols used in this study were submitted to the Ethics Committee on the use of animals in research (CEUA) of Maringá State University, and approved under case number 013/2009. Voucher specimens were deposited in the ichthyological collection of the Limnology, Ichthyology and Aquaculture Research Center (Nupélia) at Maringá State University, Paraná, Brazil. The catalog numbers of the specimens are provided in Table 1.

Table 1- Collection sites, geographic coordinates, and catalog numbers of the voucher specimens of *Ancistrus* obtained from the populations of the upper Paraná River basin.

River	Locality/State or province/Country	Geographical coordinates	Karyotype formula	NUP
Mourão	Campo Mourão/Paraná/Brazil	25◦04′46″S, 53◦54′45″W	12m+18sm+12s+8a	11993
19 Stream	Paraiso do Norte/Paraná/Brazil	52º38'17"S, 23º16'08"W	12m+18sm+12s+8a-♀ 11m+18sm+13s+8a-♂	13646
Keller	Marialva/Paraná/Brazil	23º38'48"S, 51º 52'51"W	12m+18sm+12s+8a-♀ 11m+18sm+13s+8a-♂	18794
Patos	Prudentópolis/Paraná/Brazil	25º09'59"S, 50º56'29"W	---	15537
São João	Prudentópolis/ Paraná/Brazil	25º05'10"S, 51º00'11"W	---	15812
São Francisco Verdadeiro	Toledo/Paraná/Brazil	24◦46′50″S, 53◦43′00″W	14m+16sm+14st+6a	15146
Arroyo Iguaçu	Marechal Cândido Rondon/ Paraná/Brazil	24º25'18"S, 54º01'09"W	---	15250
Arroyo San Juan*	Misiones/Posadas/Argentina	27º22.623'S, 55º53.571'W	10m+14sm+12st+14a	18795
Ocoí	Medianeira/Paraná/Brazil	25º15'12"S, 54º01'55"W	10m+18sm+16st+6a	4729

São Francisco Falso	Vera Cruz do Oeste/Paraná/Brazil	25°04'46"S, 53°54'45"W	10m+18sm+16st+6a	15145

---, *Ancistrus* populations that were included only in the molecular analyses; NUP, catalog number of the voucher specimen in the Nupélia collection; ♂, male; ♀, female. *population of Arroyo San Juan nominal *A. cirrhosus* by Prizon et al.[21]

Rex-3 sequence isolation, cloning and sequencing

The genomic DNA was extracted from the liver or muscle tissue of the specimens collected from the *Ancistrus* populations (Table 1) using the TNES method, as applied by Bruschi et al.[40] One individual from each population was selected for analysis, except in the case of the populations with heteromorphic sex chromosomes (Stream 19 and the Keller River), when one individual of each sex was selected. A partial sequence of the *Rex*-3 retrotransposon was amplified by PCR using the heterologous primers *Rex*-3F (5'-CGGTGAYAAAGGGCAGCCCTG-3') and *Rex*-3R, 5'-TGGCAGACNGGGGTGGTGGT-3'.[27] The amplification solution contained 20 ng/µl of the DNA template, 7 pmoles/uL of the forward and reverse primers, 10 mM of dNTPs, 1 U *Taq* DNA polymerase, 1.5 mM $MgCl_2$, and 1x PCR buffer (200 mM Tris, pH 8.4, 500 mM KCl). The amplification protocol was 5 min - 96°C / (1 min - 96°C/1 min - 60°C/2 min - 72°C) 35 cycles/ 8 min - 72°C. The amplified products were purified using Exonuclease I (10 units) and SAP (1 unit), incubated for 45 minutes at 37ºC, followed by denaturation at 85ºC for 10 minutes (Applied Biosystems, Santa Clara, CA, USA), as recommended by the manufacturer. The samples were then used directly as templates for sequencing in an automatic ABI/Prism DNA sequencer (Applied Biosystems, Foster City, CA, USA) with the BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA), as recommended by the manufacturer. The DNA samples were sequenced bidirectionally and then edited in Bioedit version 7.2.5, http://www.mbio.ncsu.edu/bioedit/page2.html.[41]

To identify taxa, the edited sequences were compared with those available for other organisms in the Basic Local Alignment Search Tool (BLAST) database at the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/blast/) and the CENSOR software at the Genetic Information Research Institute, GIRINST (http://www.girinst.org/censor/index.php). The analysis of the DNA sequences, genetic diversity statistics, nucleotide variability (π) and the number of haplotypes (h), were computed in DnaSP v.5.10.01.[42]

The amplified products were also purified using Wizard SV Gel and the PCR Clean-up system (Promega), and inserted into the cloning vector (pJET1.2/blunt) by the CloneJET PCR Cloning kit (Thermo Scientific), which was used to transform the *Escherichia coli* Top10 cells. The cloned fragments were amplified by PCR using the primers pJETF and pJETR, and the amplicons were purified using Wizard SV Gel and the PCR Clean-up System (Promega), before being sequenced in an automatic ABI/Prism DNA sequencer (Applied Biosystems, Foster City, CA, USA) with the BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA), as recommended by the manufacturer.

Fluorescence *in situ* hybridization (FISH)

Mitotic chromosomes were obtained from kidney cells following Bertollo et al.[43] and the constitutive heterochromatin was identified by the C-banding technique[44] and stained with propidium iodide.[45] Fragments of element *Rex*-3 were labeled by PCR, using 11-digoxigenin-dUTP. The probes were precipitated in calf thymus DNA (100 ng/µL) and resuspended in hybridization medium (50% formamide, 20xSSC, 10%

dextran sulfate) The sequences of the *Rex*-3 element were mapped physically by FISH, following Traut et al.[46], under high stringency conditions (77%).

We mapped the chromosomal sites of the $(CA)_{15}$ and $(GA)_{15}$ microsatellites using oligonucleotide probes, which were acquired commercially and labeled directly with Cy5-fluorochrome at the 5' end during synthesis (Sigma Aldrich). The FISH experiments were conducted according to the protocol of Kubat et al.[47]

Finally, the general vertebrate telomeric (TTAGGG)n sequence probe was obtained by amplification and labeling in the following reaction solution: 1x Taq reaction buffer, 40 µm dATP, dGTP and dCTP, 28 µm dTTP, 12 µm digoxygenin- 11 dUTP, 0.2 µm $(TTAGGG)_5$ primer, 0.2 µm $(CCCTAA)_5$, 2 mM $MgCl_2$, and 2U Taq DNA polymerase. The first amplification was run at low stringency: 4 min at 94°C, 12 cycles of 1 min at 94°C, 45 s at 52°C, and 90 s at 72°C; followed by 35 cycles at high stringency: 1 min at 94°C, 90 s at 60°C, and 90 s at 72°C. The telomeric (TTAGGG)n sequences were mapped by Fluorescence *in situ* Hybridization (FISH), following Pinkel et al.[48]

Results

Rex-3 sequences and the physical mapping of repetitive sequences

We obtained fragments of the *Rex*-3 element of approximately 500 bps from the genomic DNA of the specimens collected from all the *Ancistrus* populations sampled, irrespective of the sex of the individual. Nucleotide divergence between *Ancistrus* populations ranged from 0% to 1%, while a divergence of 3% was found in comparison with *Xiphophorus maculatus* (reference sequence –Volff et al., 1999). We found reduced nucleotide variability (π) (0.00317) and a small number (4) of haplotypes in the *Rex*-3 fragments sequenced in the *Ancistrus* specimens. The analysis of the nucleotide sequences revealed a high degree of similarity with the non-LTR *Rex*-3 retrotransposon sequences deposited in the online BLAST database and confirmed by CENSOR. The comparative analysis of the cloned *Rex*-3 of the *Ancistrus* specimens from the Keller River revealed a high degree of similarity with the sequences of the catfish species *Corumbataia cuestae* (94%) and *Pseudotocinclus tietensis* (84%), both members of the Siluriformes, and those of other fish orders, such as the Cypriniformes (80–84%), Perciformes (79–83%), Tetraodontiformes (82–86%), Esociformes (84%), Beloniformes (81%), and Cyprinodontiformes (80–82%), including *Xiphophorus maculatus*, which is a Cyprinodontiformes.

Predictive amino acid sequences of the *Rex*-3 fragments isolated from *Ancistrus* sp. allowed us to identify the regions corresponding to the conserved domains 1, 2, 2A, A, and B of the reverse transcriptase of *X.maculatus*, as identified by Volff et al. 1999 (Fig. 1). While some substitutions were observed, they did not involve the conserved amino acids described by Xiong and Eickbush (1990) and Malik and Eickbush (1998) (see Figure 1).

The physical mapping of *Rex*-3 element probes revealed fluorescent signals throughout the chromosomes of the specimens of all *Ancistrus* populations (seven) analyzed (Fig. 2), while the $(CA)_{15}$ and $(GA)_{15}$ microsatellites probes produced hybridization signals mainly in the subterminal and interstitial regions of most chromosome arms, with some signals appearing stronger and more extended than others (Fig. 3, 4).

Based on the distribution of the chromosomal pairs bearing the 18S, 5S rDNA sites and heterochromatin blocks of the *Ancistrus* populations analyzed previously by Prizon et al. (2017), a partial association was observed between these regions with the

repetitive sequences analyzed in this study, as shown in the figure 5 (columns I, II and VI). Hybridization signals from the *Rex*-3 element (Fig. 5, column III) were detected in association with the 18S rDNA sites only in pair 18 of the Ocoi and São Francisco Falso populations while the 5S rDNA sites were shown in pair 20 of the 19 Stream specimens, and in pairs 15 and 21 of the São Francisco Verdadeiro River specimens.

The location of the (CA) and (GA) microsatellites mostly coincided in the chromosomes, either co-located or adjacent to one another (Fig. 3, 4). These repeats also coincided with heterochromatic regions for the most chromosomes in according to pattern of C-banding showed in our studies with *Ancistrus*. Interestingly, we detected the same (CA/GA) signals coinciding with the 5S rDNA sites of some pairs (pair 19 from the Mourão River; pairs 15, 20 and 25 in the Keller River specimens; pair 15 of the *Ancistrus* sp. "São Francisco Verdadeiro River"; pairs 18 and 23 for *Ancistrus* from Arroyo San Juan and pair 21 from Ocoí River). However, we did not detect (CA) and (GA) repeats coinciding with the 18S rDNA sites in any population (Fig. 5).

The Fig. 6 represents the heteromorphic sexual pair (XY) of the 19 Stream and Keller River populations after cytogenetic mapping using microsatellites (GA/CA) and *Rex*-3 element probes and the distribution of the heterochromatin pattern after C-banding. Interspersed hybridization signals of *Rex*-3 element were observed in large numbers distributed throughout the X chromosome from the Keller River specimens, with weaker signals being found in the X chromosome in specimens from 19 Stream. The heteromorphic sexual pairs from both 19 Stream and Keller River presented signals of (CA) and (GA) scattered throughout the X and Y chromosomes, with a greater accumulation of (CA) repeats in the subterminal region of the short arm of the X chromosome. The X chromosome of the Keller River has an interstitial (GA) block in the short arms.

As expected, the FISH with the telomeric (TTAGGG)n probe revealed hybridization signals in each telomere of all the chromosomes, while no interstitial telomeric sites (ITSs) were found anywhere (Fig 1- Supplementary material).

Discussion

The results of the present study confirm that the sequences isolated from the *Ancistrus* genome using heterologous primers represent the fragment of a non-LTR retroelement of the *Rex*-3 family, as found in other fish groups.[14,15,29,51] This is supported clearly by the high similarity values obtained from comparisons with the BLAST and CENSOR databases. The *Rex*-3 element isolated in the *Ancistrus* populations presented considerable similarities with the sequences recorded in other groups of fish,[14,15,52,53] which indicates that these elements are largely conserved in the different fish orders. The platyfish *X. maculatus* was used as a reference,[27] and detected low levels of nucleotide variation (3%) between the fragments found in widely divergent species. This finding was further validated when we compared the presumed amino acids sequences of the reverse transcriptase (RT) segment between species. Five conserved RT domains (1, 2, 2A, A, and B) were also recognized in the fragment, as described previously by Malik and Eickbush[50] and Volff et al.[27] Together, this evidence confirms that the elements isolated in *Ancistrus* represent the non-LTR-retroelement *Rex*-3, or at least sequences derived from it,[27] and that this element may have been transposed in the studied populations, indicating that it is potentially active in the genome. Despite the recognition of the protein domains of the RT-coding region in the fragments, future research on the complete sequence may provide further insights into the integration of this element into the *Ancistrus* genome.

The physical mapping of the *Rex*-3 clone in the *Ancistrus* karyotypes revealed a pan-genomic distribution, highlighted by the hybridization signal scattered throughout the karyotype. This dispersed pattern of the TEs in the fish genome, as observed in all the cases analyzed in the present study, has also been recorded in other fish groups, such as the Hypoptomatinae, in which three species (*Pseudotocinclus tietensis, Hisonotus leucofrenatus, Corumbataia cuestae*) were analyzed by Ferreira et al.[14], species of the genus *Leporinus,*[29] *Erythrinus erythrinus,*[17] and in several Antarctic ice-fishes, in which hybridization signals were distributed homogeneously among the chromosomes.[51] Even so, the genomic organization of the *Rex*-3 element found here in the *Ancistrus* populations from the Paraná basin diverges from that of Amazonian *Ancistrus*.[16] In the Amazonian species, the *Rex*-3 probe mapped primarily terminal and pericentromeric blocks, in both heterochromatic and euchromatic regions.[16] In contrast with the pattern expected for other chromosomal markers (i.e. rDNA), these relatively mobile molecular genetic elements may be far more variable in closely-related species due their independent evolutionary dynamics, as found here in *Ancistrus*.

Variation in the distribution pattern of the *Rex* element family has also been reported in other fish groups. Valente et al.[28] detected differences in the number of copies and the chromosomal distribution of the TE signals (scattered *vs.* grouped in pericentromeric regions) both in related species and within the same species in fishes of the family Cichlidae. Ozouf-Costaz et al.[51] reported another example in Antarctic fish species, with differences in the accumulation of these elements, also related to the number of copies and distribution, being found in different species of the same suborder. Overall, then, this variation in the organization and chromosomal distribution of the TEs in the genomes of different groups of fish reinforces the conclusion that the TEs evolve independently during the evolutionary history of each species.[28]

The association of the TEs with heterochromatic regions is a common feature of most genomes, and was detected in the karyotypes of all the specimens analyzed here (compared with the data on heterochromatic patterns provided by Prizon et al.[21]). A number of studies have shown a preference of these retroelements for heterochromatic regions,[55,56] which regulate the expression and dispersal of these sequences without suffering major consequences. This suggests that epigenetic mechanisms may influence these repetitive elements to restrict their excessive propagation in other regions of the genome.[57,58] While TEs are also found in euchromatic regions, which are rich in genes, the presence of these elements in these regions can lead to mutations, chromosomal rearrangements, and alterations in gene regulation, resulting in greater genetic variability. The configuration of these sequences thus determines the potential genetic diversity of a group of organisms.[59,60] In this case, the euchromatic hybridization signals observed in our analyses indicate the massive accumulation of repeats of the *Rex*-3 element (or at least sequences derived from it) in the *Ancistrus* genome. Favarato et al.[16] also described the presence of *Rex*-1, *Rex*-3, and *Rex*-6 distributed in small clusters, detected in the euchromatic regions of all the *Ancistrus* specimens analyzed from the Amazon basin.

Data obtained here reveal that a significant fraction of the *Ancistrus* genome has been invaded by mobile genetic elements, such as the non-LTR retransposon identified. We expected the analysis of repetitive content to provide important insights into the marked diversity of karyotypes – in particular in their structure and sex chromosome systems – known to exist in *Ancistrus*. Based on the distribution of *Rex*-3 in the *Ancistrus* populations of Paraná River basin, we suggest these elements are shaping karyotype structure without altering the chromosome number. Prizon et al.[21] used a combined cytogenetic and molecular approach to analyze the *Ancistrus* populations of

the Paraná basin, and found a conserved diploid number (2n = 50) in all specimens, but five distinct lineages based on the karyotype formula, the presence of heteromorphic sex chromosomes, and the distribution of heterochromatin and ribosomal sites. The lineages were confirmed by the analysis of the mitochondrial COI gene, which indicated the existence of a number of candidate species in the study region. In this complementary analysis, we provided a more comprehensive scenario of the potential source of the karyotype diversity found among these evolutionary lineages. In this context, the TEs stand out because of their capacity to generate evolutionary change through a number of different processes of chromosomal rearrangement, such as deletions, duplications, inversions, and translocations.[10]

The presence of the *Rex*-3 TEs in the *Ancistrus* populations analyzed in the may account for some of the specific features of the the karyotype profile detected in the group, such as the rupture of synteny between the 18S and 5S rDNA genes, and the multiple 5S rDNA sites identified by Prizon et al.[21] In support of this conclusion, the co-location of the 5S rDNA sites with the *Rex*-3 element suggests that the dispersion of the 5S rDNA was facilitated by the transposition of *Rex*-3 to other sites in the genome, generating multiple 5S sites and rupturing the 18S/5S synteny, which are both derived conditions in this genus.[61]

The chromosomal mapping of the (CA) and (GA) microsatellite repeats in the *Ancistrus* specimens analyzed in the present study revealed regions that coincided with the TE *Rex*-3 element. There are a number of reports of the co-location of these two classes of repetitive DNA (revision in Meštrović et al.[37]), indicating the existence of an interrelationship between them. This co-location may be accounted for by two alternative hypotheses. One is based on the findings of Volff et al.[27], who isolated the *Rex*-3 retrotransposon in the platyfish, *X. maculatus*: the authors found that the 3'end of this *Rex*-3 sequence presented two (GAA) repeats followed by a GATG tandem repeat (between 8 and 17 repeats), depending on the copy. As the pan-genomic distribution of the *Rex*-3 element coincides with the (GA) hybridization pattern, it is possible that the microsatellite markers correspond to the 3' end of the *Rex*-3 element. An alternative hypothesis can be derived from the human genome study of Ahmed and Liang[35], which showed that tandem repeat elements (TRs), including microsatellites, minisatellites, and satellites, may be derived from transposable elements (TEs), based on the similarity of their sequences and overlapping genomic positions. While the origin of the tandem replication can be accounted for in only 6% of the TEs found in the human genome, based on the known mechanisms of replication, the authors suggested that, in some cases, the TEs may be active not only for transposition, but also for the generation of TRs. A similar situation may thus be found in the fish genome, resulting from the expansion of TRs derived from the adjacent *Rex*-3 TEs, which would account for the scenario detected in the present study.

In the chromosomes of all the *Ancistrus* species analyzed in the present study, the (CA)/(GA) microsatellites were well dispersed, but were accumulated primarily in the subterminal regions. A similar configuration was recorded by Vanzela et al.[33], with a marked accumulation of $(GA)_{15}$ and $(A)_{30}$ microsatellites in the subterminal regions of the chromosomes of the Siluriformes species *Imparfinis schubarti*, *Steindachneridion scripta* and *Rineloricaria latirostris*. The same general distribution pattern was found in both the male and female specimens from the Keller River and 19 Stream, where Prizon et al.[21] recorded a XX/XY sex chromosome system. The heteromorphic sex chromosomes presented the di-nucleotide (CA)/(GA) markers in subterminal and some interstitial regions, while the hybridization signal of the *Rex*-3 element was spread throughout the heteromorphic XY pair in the specimens from both populations. Even

so, we cannot rule out altogether the possible presence of other repetitive elements in these sex chromosomes, given that other types of TE and microsatellite have been found in the sex chromosomes of other fish species.[29,32]

The heteromorphic (XY) chromosomes of the populations from 19 Stream and the Keller River also diverge in their heterochromatic patterns, as detected by C-banding. The X chromosome from the Keller River also presents an exclusive (GA) cluster in the interstitial short arm. The differentiation of the sex chromosomes in allopatric populations may often be based on a distinct content of repetitive DNA, as reported by Cioffi et al.[32] These features likely depend on the differential evolutionary trajectory of the chromosomes in allopatric populations, in particular because the suppression of recombination favors the local accumulation of mutations, and can lead to the deletion of inactivated genes, and the consequent accumulation of repetitive DNA sequences, leading to structural and molecular differentiation during the early stages of the evolution of one of the sexual chromosomes. Thus, the accumulation of transposable elements may be initial effects on the differentiation of sex chromosomes[62]. The sex chromosomes of the *Ancistrus* populations studied here may still be an early stage of development, considering that pericentric inversions followed by loss of chromosomal material and presence of low constitutive heterochromatin in the sex chromosomes of the populations of the 19 Stream and Keller River may be evidence of the recent differentiation between X and Y.[21] In addition, the presence of the *Rex*-3 element and microsatellites GA and CA across the length of both sexual pairs corroborates the hypothesis that they may be contributing to the differentiation of the same.

Given the whole scenario involving chromosomal rearrangements detected in the *Ancistrus* genome, the analysis using the telomeric (TTAGGG)n probe was aimed at discovering regions prone to pericentric inversions, one of the rearrangements suggested for differentiation of the Y sexual chromosome, from a large metacentric. However, no evidence of ITSs was observed, with hybridization signals present only in the telomeres of the chromosomes (SFig. 1).

Conclusion

The karyotype diversity detected in the genus *Ancistrus* may be a consequence of the presence of repetitive elements, which were scattered throughout the chromosomal complement, indicating that these two classes of repetitive DNA sequences (TE and microssatelites) have played an important role in the differentiation of the genome of this taxon, including the heteromorphic sex chromosomes. The co-location of the two repetitive DNA classes detected in the *Ancistrus* genome reinforces the strong link between them in the eukaryote genome. Overall, then, the results of the present study provide new insights into the chromosomal constitution of the autosomal and heteromorphic sex chromosomes of the different *Ancistrus* lineages, indicating a major contribution of these repetitive elements to the diversification detected previously in the group. This provides a more detailed understanding of the role of these repetitions in karyotype evolution.

Figure Legends

Fig. 1: Amino acid sequence inferred from the nucleotide sequences of the *Ancistrus* sp. populations from ParanáRiver basin. Point sites are the conserved amino acids described by Xiong and Eickbush (1990) and Malik and Eickbush (1998). The bars indicate the 1-B domains of the *Rex*-3 RT of *Xiphophorusmaculatus*, as determined by Volff et al. (1999).

Fig. 2: Cytogenetic mapping of the *Rex*-3 retrotransposon probe (red signals) in seven population of *Ancistrus* sp. from Paranábasin in southern South America. The chromosomes were counterstained with DAPI. (a) Mourão River; (b) 19 Stream; (c) Keller River; (d) São Francisco Verdadeiro River; (e) Arroyo San Juan; (f) OcoíRiver; (g) São Francisco Falso River. Scale bar = 10 μm.

Fig. 3: *In situ* fluorescence hybridization using microsatellite $(CA)_{15}$ DNA probes (red signals) in seven populations of *Ancistrus* sp. from the Paraná River basin in southern South America. Chromosomes were counterstained with DAPI. (a) Mourão River; (b) 19 Stream; (c) Keller River; (d) São Francisco Verdadeiro River; (e) Arroyo San Juan; (f) Ocoí River; (g) São Francisco Falso River. Scale bar = 10 μm.

Fig. 4: *In situ* fluorescence hybridization using microsatellite $(GA)_{15}$ DNA probes (red signals) in seven populations of *Ancistrus* sp. from the Paraná River basin in southern South America. Chromosomes were counterstained with DAPI. (a) Mourão River; (b) 19 Stream; (c) Keller River; (d) São Francisco Verdadeiro River; (e) Arroyo San Juan; (f) OcoíRiver; (g) São Francisco Falso River. Scale bar = 10 μm.

Fig. 5: Two classes of repetitive DNA found in the chromosomes carrying the 18S (column I) and 5S (column II) rDNA sites in the *Ancistrus* sp. populations of the Paraná River basin, previously reported by Prizon et al. (2017). Column III shows the *Rex*-3 TE, column IV shows the locations of the CA microsatellites, and column V, the GA microsatellites. Column VI shows the pairs with heterochromatic blocks (C-banding) presented by Prizon et al. (2017). CH= constitutive heterochromatin.

Fig. 6: Heteromorphic sex chromosomes (XY, pair 2) of the *Ancistrus* populations from 19 Stream and the Keller River, highlighting the presence of *Rex*-3 ETs and the CA and GA microsatellites. CH= constitutive heterochromatin.

Supplementary Material

SFig. 1: Metaphases of *Ancistrus* sp from(a) Mourão River; (b) 19Stream; (c) Keller River; (d) São Francisco Verdadeiro River; (e) Arroyo San Juan; (f) Ocoí River; (g) São Francisco Falso River, subjected to fluorescence *in situ* hybridization with probe of (TTAGGG)*n*.

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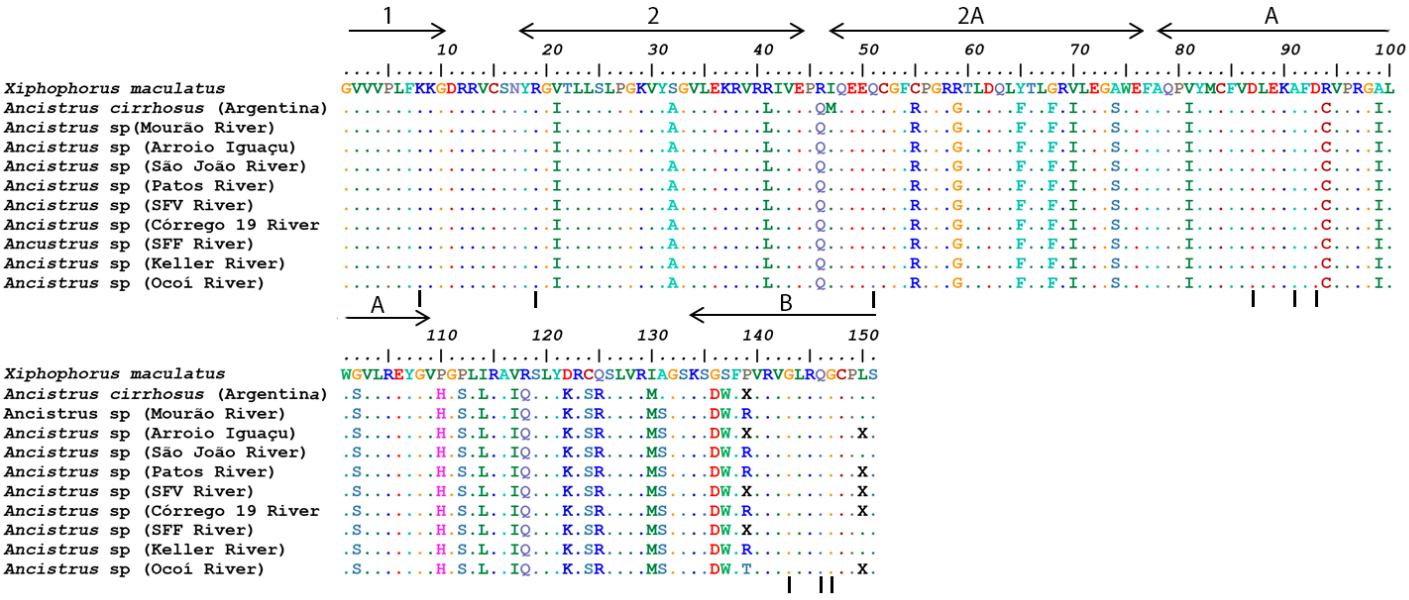
Fig 1.


1
2
2A
A
10
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30
40
50
60
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80
90
100
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Ancistrus cirrhosus (Argentina)
Ancistrus sp(Mourão River)
Ancistrus sp (Arroio Iguaçu)
Ancistrus sp (São João River)
Ancistrus sp (Patos River)
Ancistrus sp (SFV River)
Ancistrus sp (Córrego 19 River
Ancustrus sp (SFF River)
Ancistrus sp (Keller River)
Ancistrus sp (Ocoí River)
A
B
110
120
130
140
150
Xiphophorus maculatus WGVLREYGVPGPLIRAVRSLYDRCQSLVRIAGSKSGSFPVRVGLRQGCPLS
Ancistrus cirrhosus (Argentina)
Ancistrus sp (Mourão River)
Ancistrus sp (Arroio Iguaçu)
Ancistrus sp (São João River)
Ancistrus sp (Patos River)
Ancistrus sp (SFV River)
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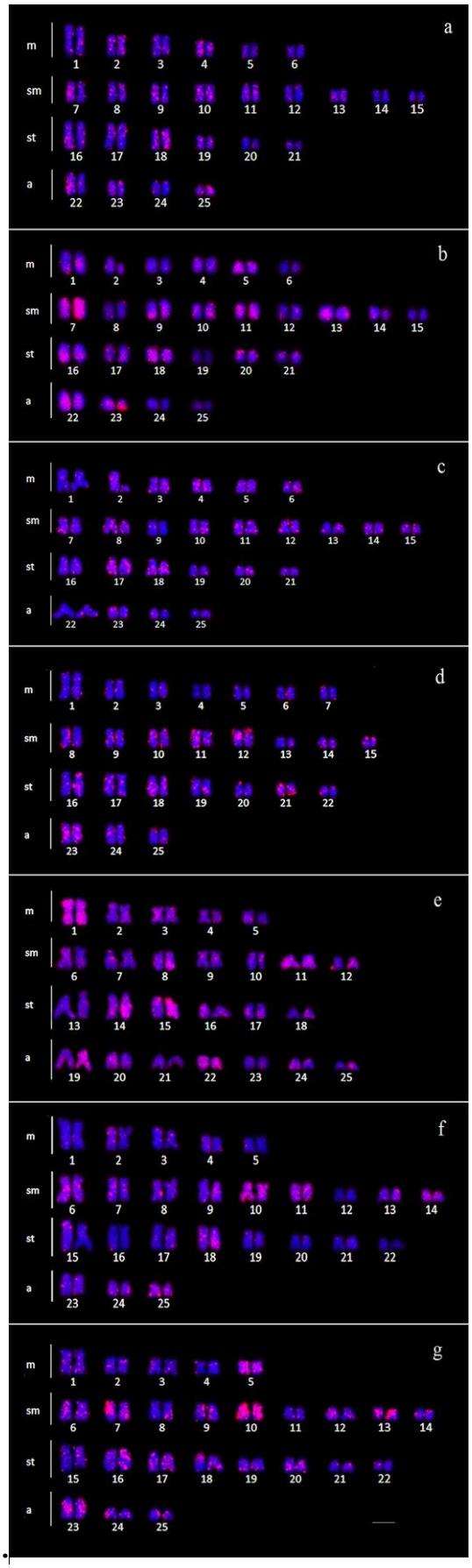
Ancistrus sp (Keller River)
Ancistrus sp (Ocoí River)

Fig. 2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sm 7 8 9 10 11 12 13 14 15
st 16 17 18 19 20 21
a 22 23 24 25
b
m 1 2 3 4 5 6
sm 7 8 9 10 11 12 13 14 15
st 16 17 18 19 20 21
a 22 23 24 25
c
m 1 2 3 4 5 6
sm 7 8 9 10 11 12 13 14 15
st 16 17 18 19 20 21
a 22 23 24 25
d
m 1 2 3 4 5 6 7
sm 8 9 10 11 12 13 14 15
st 16 17 18 19 20 21 22
a 23 24 25
e
m 1 2 3 4 5
sm 6 7 8 9 10 11 12
st 13 14 15 16 17 18
a 19 20 21 22 23 24 25
f
m 1 2 3 4 5
sm 6 7 8 9 10 11 12 13 14
st 15 16 17 18 19 20 21 22
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g
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a 23 24 25

Fig. 3

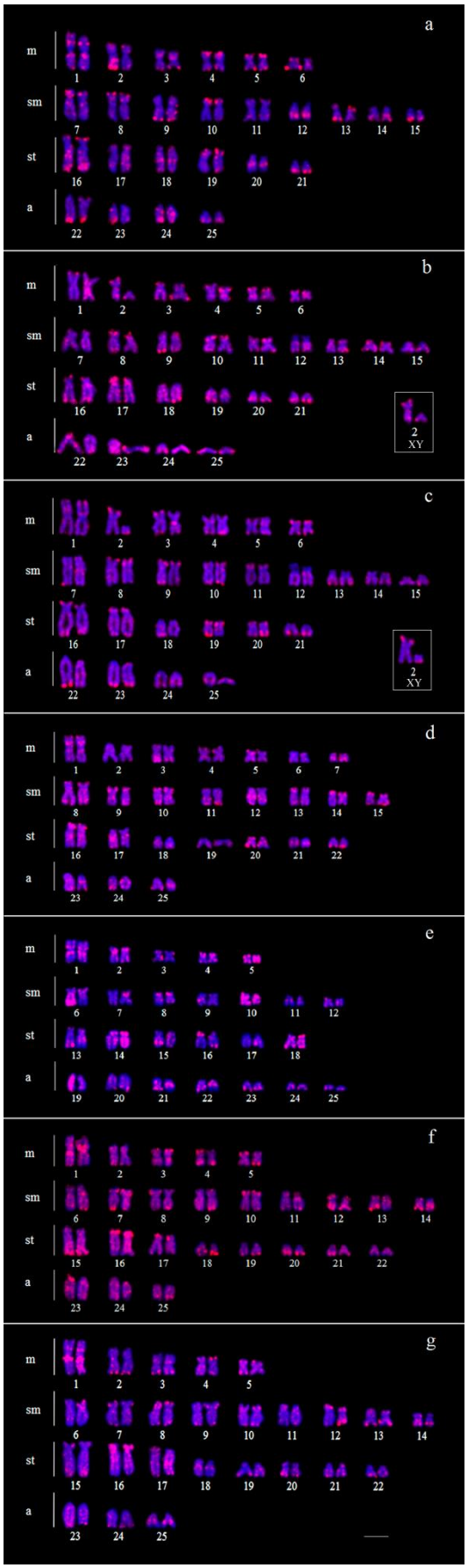
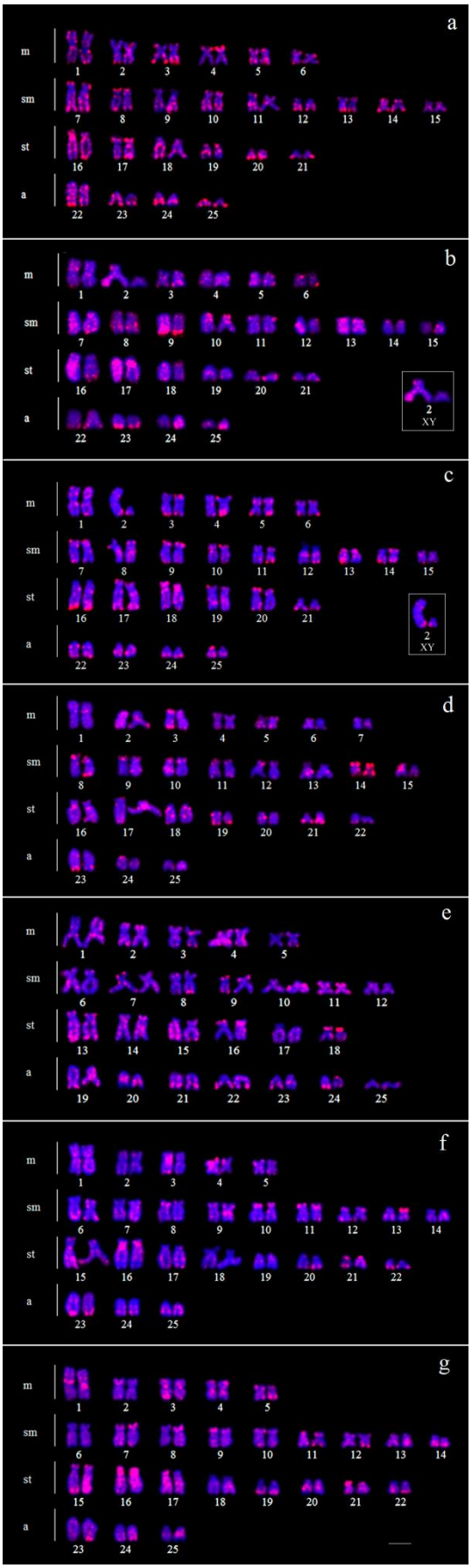


Fig. 4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sm
7 8 9 10 11 12 13 14 15
st
16 17 18 19 20 21
a
22 23 24 25
2
XY
c
m
1 2 3 4 5 6
sm
7 8 9 10 11 12 13 14 15
st
16 17 18 19 20 21
a
22 23 24 25
2
XY
d
m
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sm
8 9 10 11 12 13 14 15
st
16 17 18 19 20 21 22
a
23 24 25
e
m
1 2 3 4 5
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6 7 8 9 10 11 12
st
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a
19 20 21 22 23 24 25
f
m
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sm
6 7 8 9 10 11 12 13 14
st
15 16 17 18 19 20 21 22
a
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g
m
1 2 3 4 5
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6 7 8 9 10 11 12 13 14
st
15 16 17 18 19 20 21 22
a
23 24 25

Fig. 5

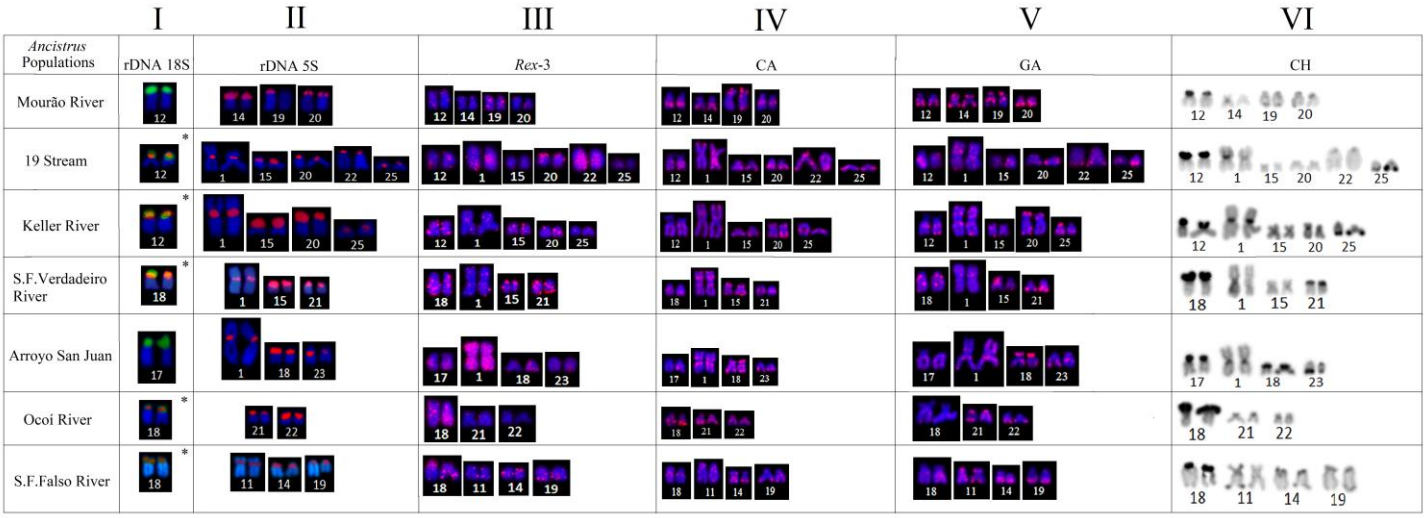
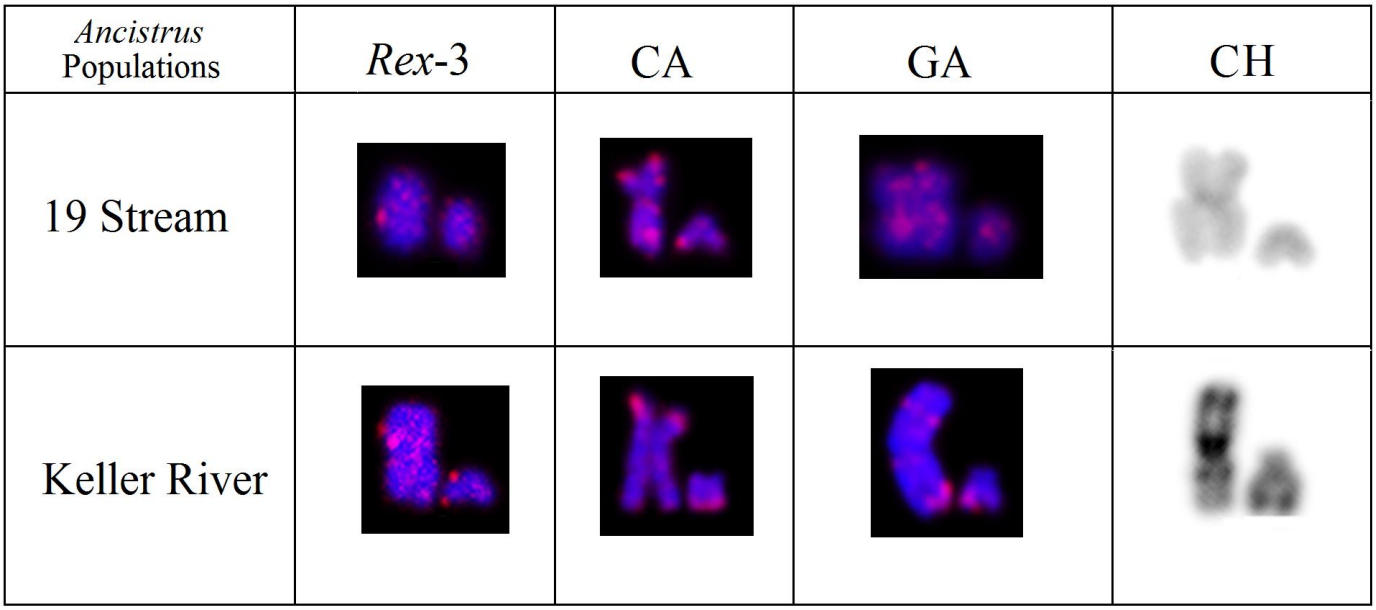

I
II
III
IV
V
VI
Ancistrus Populations
rDNA 18S
rDNA 5S
Rex-3
CA
GA
CH
Mourão River
19 Stream
Keller River
S.F.Verdadeiro River
Arroyo San Juan
Ocoí River
S.F.Falso River

Fig. 6


Ancistrus Populations
Rex-3
CA
GA
CH
19 Stream
Keller River

SFig. 1- Supplementary Material