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DEPARTAMENTO DE BIOTECNOLOGIA, GENÉTICA E BIOLOGIA CELULAR
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ANDRÉA CIUS

Análise dos rearranjos cromossômicos no grupo *Rineloricaria* aff *lima* (bacia do rio Iguaçu, PR) elucidado por dados moleculares e marcadores cromossômicos.

Maringá
2018

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Tese apresentada à Universidade Estadual de Maringá, como requisito parcial para a obtenção do título de doutora.

Orientadora: Dr.ª Ana Luiza de Brito Portela Castro

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Universidade Estadual de Maringá
Centro de Ciências Biológicas
Programa de Pós-Graduação em Ciências Biológicas

PARANÁ

ATA DA BANCA EXAMINADORA DA DEFESA DE TESE DE DOUTORADO DA PÓS-GRADUANDA ANDRÉA CIUS. Aos vinte e três dias do mês de fevereiro de dois mil e dezoito, sexta-feira, realizou-se no bloco H67, sala 5, no campus universitário, a sessão pública da defesa de tese intitulada: "Análise dos rearranjos cromossômicos no grupo *Rineloricaria* aff. lima (bacia do rio Iguaçu, PR) elucidado por dados moleculares e marcadores cromossômicos", apresentada pela pós-graduanda **Andréa Cius**, Licenciada em Ciências Biológicas pela Faculdade Estadual de Filosofia, Ciências e Letras de União da Vitória-Paraná, que concluiu os créditos exigidos para obtenção do grau de "Doutora em Ciências Biológicas". Os trabalhos foram instalados às 8:30, pela *Profa. Dra. Ana Luiza de Brito Portela Castro*, Presidente da Banca Examinadora, constituída pelos seguintes professores: Dra. Lucia Giuliano Caetano, Dr. Daniel Pacheco Bruschi, Dra. Andrea Beatriz Mendes Bonato e Dra. Luciana Andréia Borin de Carvalho como membros. A Banca Examinadora, tendo se decidido a aceitar a tese, passou à arguição pública da candidata. Encerrados os trabalhos de arguição às 12:45 horas, os examinadores deram parecer final, considerando a tese Aprovada. Proclamado o resultado pela Presidente da Banca Examinadora, foram encerrados os trabalhos e lavrada a presente ata que vai assinada pelos membros da Banca Examinadora. Maringá, aos vinte e três dias do mês de fevereiro de dois mil e dezoito.

Ana Luiza B. P. Castro
Profa. Dra. Ana Luiza de Brito Portela Castro

Lucia Giuliano Caetano
Profa. Dra. Lucia Giuliano Caetano

Prof. Dr. Daniel Pacheco Bruschi

ABBonato
Profa. Dra. Andrea Beatriz Mendes Bonato

Profa. Dra. Luciana Andréia Borin de Carvalho

ANDRÉA CIUS

ANÁLISE DOS REARRANJOS CROMOSSÔMICOS NO GRUPO *RINELORICARIA* AFF. LIMA (BACIA DO RIO IGUAÇU, PR) ELUCIDADO POR DADOS MOLECULARES E MARCADORES CROMOSSÔMICOS

Tese apresentada como requisito parcial para obtenção do grau de Doutora em Ciências Biológicas, do Programa de Pós-Graduação em Ciências Biológicas, da Universidade Estadual de Maringá, sob a apreciação da seguinte banca examinadora:

Aprovada em 23 de fevereiro de 2018.

Profa. Dra. Ana Luiza de Brito Portela Castro
(Presidente-Orientadora)

Profa. Dra. Lucia Giuliano Caetano
(Membro examinador externo convidado – UEL)

Prof. Dr. Daniel Pacheco Bruschi
(Membro examinador externo convidado - UFPR)

Profa. Dra. Andréa Beatriz Mendes Bonato
(Membro examinador – DBC/UEM)

Profa. Dra. Luciana Andréia Borin de Carvalho
(Membro examinador– DBC/UEM)

Maringá
2018

Ao meu pai (*in memorium*) e minha mãe, palavras se tornam pequenas diante do tamanho da minha gratidão.

AGRADECIMENTOS

Seguindo o pensamento de Epicuro: “as pessoas felizes lembram o passado com gratidão, alegram-se com o presente e encaram o futuro sem medo”, percebo que cada pessoa que passou em minha vida, cada experiência (boa ou ruim) me trouxeram até aqui, ao final desses cinco anos de pós-graduação, sou resultado de tudo isso, vivendo um presente feliz e animada com o que está por vir.

Meus pais foram, sem dúvidas, meu maior exemplo sobre buscar ser alguém melhor todos os dias. Tenho em minha mãe modelo de independência, força feminina, coração acolhedor, beleza além do que se externa. Meu pai, tenho e sempre terei, modelo de bondade, altruísmo, simpatia, aconchego. Em 2016 a vida infelizmente nos separou fisicamente, mas sou muito grata a todos os ensinamentos, aconchegos e acalantos. Isso nada separa.

Minha irmã, que me deu o presente mais lindo da minha vida: Clara. Diante de todas as nossas diferenças o amor nos une, e faz com que nossa relação seja movida por respeito e admiração, de ambos os lados.

Ana Luiza, minha gratidão eterna por todos os ensinamentos profissionais e pessoais. Você se mostrou compreensiva e humana, o que por vezes falta em nosso meio. Sempre acolhedora e uma grande profissional. Obrigada!

Carla, Daniel, Luciana, Mateus, profissionais incríveis aos quais me inspirei e continuo me inspirando e, que por sorte minha posso chamar amigos, aos quais não tenho dúvidas que posso contar. Obrigada por tudo!

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Maze, ou melhor Mãeze, conselhos, carinhos diários, conversas,

troca. Obrigada por todo o carinho que sempre teve por mim e que demonstra por todos. Isabelle, que se tornou uma amiga muito querida com que dividi extensas teorias sobre series, sorrisos e palavras de apoio.

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Ana Camila, uma princesa, um doce e uma das pessoas mais especiais e lindas que conheci. Ela me ensinou a ver o lado bom de tudo, sempre otimista, sempre apoiando. Nos desesperamos juntas, sorrimos e choramos juntas, mas o mais importante sempre estivemos uma ao lado da outra. Sempre vibramos com as conquistas uma da outra. Obrigada por tudo, te levarei para vida.

Aos meus queridos amigos dos “campos gerais”, Pati, Claudinha, John, Felipe, Ramiro, Cris, Meri, perto de vocês eu sempre me senti em casa. Aos queridos amigos do Nupélia, Louise, Bia, João Victor, Toé, que me acolheram e tornaram meus dias por aqui muito mais emocionantes. Ao meu irmão de coração Diogo, obrigada por tudo, pelos cuidados, pelo carinho, você foi a família que escolhi aqui.

Aos meus amigos de infância Wander, Jéssica e Edi, quanto amor tenho por vocês. Obrigada por sempre entenderem minhas ausências e mostrarem que a distância não é nada quando se existe algo verdadeiro. Felipinho, meu querido amigo, companheiro, sempre presente independentemente da situação. Marcel, meu querido amigo e confidente, a pessoa que me inspirou a mudar minha visão sobre muitas coisas. Obrigada!

Ao Pedro, partilhamos quatro desses cinco anos que foi minha pós.

Acompanhamos as mudanças um do outro, nos apoiamos e seguimos sempre fazendo parte um da vida do outro. A vida sempre teve mais cor ao seu lado. Obrigada pelas visões de mundo , carinho e memórias boas.

"- Por favor, qual é o seu nome? – perguntou ao gato. – Olha, sou Coraline. Tá? O gato bocejou lenta e cuidadosamente, revelando uma boca e uma língua de um rosa impressionante. - Gatos não têm nomes – disse.- Não? – perguntou Coraline.- Não – respondeu o gato. – Agora, vocês pessoas tem nomes. Isso é porque vocês não sabem quem vocês são. Nós sabemos quem somos, portanto não precisamos de nomes."
(Coraline – Neil Gaiman)

APRESENTAÇÃO

Esta tese é composta por dois capítulos. O capitulo I compreende um artigo sobre analise filogenética e citogenética em *Rineloricaria* intitulado de **"Phylogenetic and cytogenetic data: an evidence of the three lineages of *Rineloricaria* aff *lima* group (Loricariidae, Loricariinae) from Iguaçu River. "** O capítulo II trata-se de um artigo sobre DNA repetitivo e polimorfismo cromossômico numérico e estrutural em *Rineloricaria* intitulado: **"Distribution of DNA repetitive: a contribution to the understanding of the chromosomal rearrangements in three lineages of the *Rineloricaria* (Loricariidae, Loricariinae) from Iguaçu River, Paraná state, Brazil. "** De acordo com o regulamento do Programa de Pós-graduação em Ciências Biológicas, os artigos foram redigidos de acordo com as normas das revistas as quais serão submetidos, conforme a seguir:

Capitulo I – Andréa Cius, Ana Luiza de Brito Portela Castro. "**Phylogenetic and cytogenetic data: an evidence of the three lineages of *Rineloricaria* sp from Iguaçu River.**" Plos One.

Capitulo II - Andréa Cius, Ana Luiza de Brito Portela Castro. **Distribution of DNA repetitive: a contribution to the understanding of the chromosomal rearrangements in three lineages of the *Rineloricaria* sp from Iguaçu River, Paraná state, Brazil."** Zebrafish.

RESUMO GERAL

Rineloricaria (Bleeker, 1862), é o gênero mais rico em espécies da subfamília Loricariinae, apresentando cerca de 65 espécies e amplamente distribuídas do Panamá na América Central até o nordeste da Argentina. Apesar dessa ampla diversidade de espécies e habitats, estudos a respeito da taxonomia e filogenia do grupo são ainda escassos. *Rineloricaria* apresenta uma extensa diversidade cariotípica, com número diploide variando de 36 a 70 cromossomos. Além disso rearranjos do tipo fusão e/ou inversão estão intimamente ligados a carioevolução do gênero. No presente trabalho foram analisadas espécies de *Rineloricaria* coletadas no médio Rio Iguaçu (Município de União da Vitória-PR). Analises filogenéticas resgataram três linhagens de *Rineloricaria* para este local. Através de estudos citogenéticos, em duas das três linhagens, evidenciamos um extenso polimorfismo cromossômico. O primeiro clado apresentou sete cariótipos prováveis (A-G) variando para 2n = 65 a 67 cromossomos com uma grande diversidade de fórmulas cariotípica. O segundo clado evidenciou 2n = 64 com a fórmula do cariótipo 3m + 61st / a (Cariótipo H). O terceiro clado agrupou quatro cariótipos gerais (I-L) variando a 2n = 65 a 66 cromossomos com ampla diversidade nas fórmulas cariotípicas, além disso, dois espécimes (cariótipos J e L) deste clado apresentaram variações sobre o número diploide e a fórmula do cariótipo, respectivamente. Em estudo relacionado a região organizadora de nucléolo com emprego das técnicas de Ag-NOR, e 18S rDNA FISH, evidenciamos um sistema de NOR simples para as três linhagens. O padrão de heterocromatina constitutiva esteve distribuído modestamente ao longo de regiões centroméricas e terminais, revelando blocos conspícuos associados ao par da NOR. Mapeamento físico de 5S rDNA, localizou padrões diferentes para essas três linhagens, para o primeiro clado quatro sítios, seis para o segundo e três sítios para o terceiro clado. O mapeamento físico das sequências teloméricas revelou a presença de sítios intersticiais teloméricos (ITS) na região centromérica de duas linhagens de *Rineloricaria*, e ambas apresentaram ITS coincidindo com a NOR. As sondas de microssatélites $(CA)_{15}$ e $(GA)_{15}$ hibridaram preferencialmente nas regiões subterminal e intersticial, associados a blocos heterocromáticos e 18S rDNA. Também, estudos de células meióticas foram realizados para duas das três linhagens de *Rineloricaria* devido ao extenso polimorfismo cromossômico. Dados citogenéticos combinados com dados filogenéticos indicaram a existência de um alto nível de rearranjos de

cromossomos e apoiam a hipótese de que essas linhagens divergiram recentemente, sendo um estudo fundamental para entender a complexa evolução cariotípica desse grupo.

Palavras-chave: Polimorfismo cromossômico. *Rineloricaria*. Rearranjos cromossômicos. Filogenia.

ABSTRACT

Rineloricaria (Bleeker, 1862), is the species-richest genus of the subfamily Loricariinae, presenting about 65 species and widely distributed from Panama in Central America to northeastern Argentina. Despite the wide diversity of species and habitats, studies on the taxonomy and phylogeny of the group are still scarce. *Rineloricaria* presents an extensive karyotype diversity, with a diploid number varying from 36 to 70 chromosomes. In addition, fusion and / or inversion type rearrangements are closely linked to karyoevolution of the genus. In the present work were analyzed species of *Rineloricaria* collected in the middle Iguaçu River (Municipality of União da Vitória-PR). Phylogenetic analyzes rescued three lineages of *Rineloricaria* to this location. Through cytogenetic studies, in two of the three lineages, we showed an extensive chromosomal polymorphism. The first clade showed seven probable karyotypes (A-G) ranging from 2n = 65 to 67 chromosomes with a great diversity of karyotype formulas. The second clade showed 2n = 64 with the formula of the karyotype 3m + 61st / a (Karyotype H). The third clade grouped four general karyotypes (I-L) ranging from 2n = 65 to 66 chromosomes with wide diversity in the karyotype formulas; in addition, two specimens (J and L karyotypes) of this clade showed variations on the diploid number and the karyotype formula, respectively. In a study related to the nucleoli organizing region using Ag-NOR and 18S rDNA FISH techniques, we showed a simple NOR system for the three lineages. The constitutive heterochromatin pattern was distributed modestly along centromeric and terminal regions, revealing conspicuous blocks associated with the NOR pair. Physical mapping of 5S rDNA, located different patterns for these three lineages, for the first clade four sites, six for the second and three sites for the third clade. The physical mapping of the telomeric sequences revealed the presence of telomeric interstitial sites (ITS) in the centromeric region of two lines of *Rineloricaria* and both presented ITS coinciding with the NOR. Microsatellite probes $(CA)_{15}$ and $(GA)_{15}$ hybridized preferentially in the subterminal and interstitial regions, associated with heterochromatic blocks and 18S rDNA. In addition, meiotic cell studies were performed for two of the three lineages of *Rineloricaria* due to the extensive chromosomal polymorphism. Cytogenetic data combined with phylogenetic data indicated the existence of a high level of chromosome rearrangements and support the

hypothesis that these lineages diverged recently, being a fundamental study to understand the complex karyotype evolution of this group.

Keywords: Chromosomal polymorphism. *Rineloricaria*. Chromosomal rearrangements. Phylogeny.

SUMÁRIO

CAPITULO I

Phylogenetic and cytogenetic data: an evidence of the three lineages of *Rineloricaria* aff *lima* group (Loricariidae, Loricariinae) from Iguaçu River.

Este artigo será submetido ao periódico Plos One.

Phylogenetic and cytogenetic data: an evidence of the three lineages of *Rineloricaria* aff *lima* group (Loricariidae, Loricariinae) from Iguaçu River.

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Abstract

Rineloricaria is one of the Neotropical freshwater fish genera with the highest distribution, occupying most of the main drains from Panama to Argentina, besides occupying a great variety of habitats. The species of the *Rineloricaria* have intriguing cytogenetic features because descriptions of numerical and structural variations are relatively common in this genre. Cytogenetic studies in *Rineloricaria* from Iguaçu River reveled an extensive numerical and structural polymorphism. This feature raised the hypothesis that there could be more than one species in the area. By combining data on mitochondrial DNA (COI gene) and chromosomal markers from *Rineloricaria* in the Iguaçu River, we detected three distinct evolutionary lineages. The first clade rescued seven probable karyotypes (A-G) ranging to 2n=65 to 67 chromosomes with diversity karyotypes formula. The second clade showed 2n=64 with karyotype formula 3m+61st/a (Karyotype H). The third clade grouped four general karyotypes (I-L) ranging to 2n=65 to 66 chromosomes with diversity karyotypes formula, in addition, two specimens (karyotypes J and L) from this clade showed variations about diploid number and karyotype formula, respectively. Phylogenetic analyses, ABGD methods results and genetic distance value (cutoff 2%) also aided in the separation of the three lineages. Clusters of 18S rDNA in three clades of *Rineloricaria* from Iguaçu River were observed in a single chromosome pair. Multiple 5S rDNA sites were observed in all clades, within first clade present four sites, second clade six and third clade 3 sites. Overall, the cytogenetic data indicate the existence of a high level of chromosomes rearrangements and phylogenetic analyses support the hypothesis that these species have recently diverged.

Introduction

Neotropical freshwater ichthyofaunal is extremely large and considered the one more richest in the world (Schaefer 1998), in view of the vast diversity, studies on genetics and evolution have proved to be an important tool for increasing knowledge of the expressive diversity of specimens of this region (Pereira, et al., 2013). Most of the work on a Neotropical ichthyofaunal base to large river environments, but about 50% of this diversity is composed of small species from small rivers or streams. These species are high in endemism and occupy a wide variety of specific microhabitats, being less and less studied than larger species (Viana, et al, 2013). Castro (1999) argues that a major challenge for South American ichthyology does not exist in the study of the systematics, evolution and biology of small freshwater fish. Thus, environments of rivers and streams in the Iguaçu River become interesting study sites with high potential to determination how much species richness remains underestimation.

For more than 40 years, molecular methodologies, including phylogenetic analysis have been employed in the delimitation and signaling of cryptic speciation (Manwell and Baker, 1963; Ward et al., 2009). Bickford et al. (2007) point out that in the last two decades there has been an exponential increase in the identification and recognition of cryptic species with the advancement and use of molecular tools. An effective and simple instrument to delimit potential lineages is the Automatic Barcode Gap Discovery (ABGD) that consist in a method to automatically find the distance where the barcode gap is located, in other words, the barcode gap within the same species is smaller than that among organisms from different species. The premise of ABGD method is a standard definition of the barcode gap and can be used even when the two distributions overlap to partition the data set into candidate species, proposes the grouping of the input sequences into several hypothetical species by the sole use of pairwise differences (i.e. a distance matrix) (Puillandre et al. 2012) .

The family Loricariidae, allocated into six subfamilies (Armbruster 2004, Reis et al. 2006), is the most species-rich family of catfishes, containing over 800 valid species (Eschmeyer, 2014) and likely several hundred undescribed species. The Loricariinae is composed of genera, totaling 716 species described (Ferraris 2007). Cytogenetic studies of this family revealed high karyotype diversity, with diploid numbers ranging from 2n = 34 (Oliveira et al. 2009) to 2n = 96 (Kavalco et al. 2005). In addition, fusion-type rearrangements, centric fission, pericentric inversion and/or translocations may be

involved in the karyotype evolution of this family (Kavalco et al., 2005). The genus *Rineloricaria,* one of the most specious genera among the Loricaridae, presents a high chromosomal variability, being 2n = 36 chromosomes in Rineloricaria latirostris a 2n = 70 chromosomes in *Rineloricaria* sp., *Rineloricaria lima* and *Rineloricaria* cf. (Alves et al., 2003). Another important characteristic of this genus is the great amount of inter- and intra-population variation (Alves et al., 2005). Descriptions of numerical and structural variations are relatively common in this genre. Right similar morphological patterns of the specimens collected in Iguaçu River makes it difficult to identify the *Rineloricaria* species. This fact stimulated us to verify genetic and cytogenetic variability in a *Rineloricaria* from Iguaçu River. Preliminary cytogenetic studies performed by Cius (2015) in *Rineloricaria* from Iguaçu River that showed very similar morphological characteristics revealed an extensive karyotype diversity governed by several chromosomal events. Based on these results, we hypothesized that it could be more than one species, thus combining chromosomal and DNA sequence analysis (both ABGD and phylogenetic) to evaluate a possible evolutionary pathway for the *Rineloricaria* from Iguaçu River.

Materials and Methods

Biological samples

We analyzed cytogenetically 30 specimens, and for phylogenetic analyzes 14 specimens of *Rineloricaria* aff *lima* group from Iguaçu River/ União da Vitória/PR/Brazil (26º14’21.06”S/51º7’3.73”O). The protocols used in this study were submitted and reviewed by the Ethics Committee in Animal Experimentation (Protocol: 07/2011) of the State University of Ponta Grossa. Voucher specimens were deposited in the ichthyological collection of the Limnology, Ichthyology and Aquaculture Research Center (Nupélia) at Maringá State University, Paraná, Brazil.

Cytogenetic analysis

All specimens were anesthetized and sacrified by an overdose of clove oil (Griffiths 2000). The protocols used in this study were submitted and reviewed by the

Ethics Committee in Animal Experimentation (Protocol: 07/2011) of the State University of Ponta Grossa.

Mitotic chromosomes were obtained from kidney cells according to Bertollo et al. (1978). AgNORs were evidenced by silver nitrate impregnation technique (Howell and Black, 1980). Heterochromatin regions were determined following 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 Jr. 1999) and from *Prochilodus argenteus* Spix et Agassiz, 1829 (Hatanaka and Galetti Jr. 2004) and from *Ancistrus* sp "Keller River" (Prizon et al. 2017).

Hybridization was performed under high stringency conditions (77%). Probes were labeled by nick translation with digoxigenin-11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA). 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. The metaphases were photographed using an epifluorescence microscope and optimized for best contrast and brightness with Adobe Photoshop CS6 software. Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) or acrocentric (a) according to their arm ratios (Levan et al. 1964).

Isolation, amplification, and sequencing of DNA

Genomic DNA was extracted from liver or from cell suspension of a subset of sample using the TNES method as applied by Bruschi et al. 2012. The mitochondrial cytochrome C oxidase subunit I (COI) fragment gene was amplified by polymerase chain reaction (PCR) using the primers: FishF1 (5'-TCAACCAACCACAAAGACATTGGCAC–3'), FishR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Ward et al. 2009). The amplification reaction set up with 20 ng/µl of the DNA template, 7 pmol of the forward and reverse primer = 10 mM of dNTPs, 1U *Taq* DNA Polymerase, 1.5 mM $MgCl_2$, 1x PCR buffer (200 mM Tris, pH 8.4, 500 mM KCL). The amplification program set up: 5 min - 94°C /(30 s -94°C/30 s - 60°C/1 min - 72°C)$_{35\ cycles}$/ 10 min - 72°C. The amplified PCR products were purified using Exonuclease I (10 units) and SAP (1 unit), with a 60-min incubation

at 37ºC and a 15 min denaturation at 80ºC, 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 analysis

Fourteen new sequences of mit-COI were generated during this study, and combined with the sequences retrieved from Genbank, were used to construct the dataset which includes XX putative species. DNA sequences were aligned in MAFFT v7 (Katoh & Standley 2013) following the G-INS-I. Sequences were then manually corrected using the software MEGA 7 (Tamura et al. 2013). The final alignments (as well the final topologies) were logged in TreeBASE (http://www.treebase.org/treebase/index.html) under ID. New sequences generated for this work were included in Genbank (Sayers et al. 2009).

Maximum Likelihood (ML) and Bayesian Inference (BI) methods were 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 BIC (Bayesian Information Criterion) with the software jModelTest v2.1.6 (Guindon & Gascuel 2003; Darriba et al. 2012). ML analyses were performed using RAxML v. 8.2.4 software (Stamatakis 2014). The analysis first involved 100 ML searches, each starting from one randomized stepwise addition parsimony tree, under a GTRGAMMA model with all other parameters estimated by the software. To access the reliability of the nodes, multi-parametric bootstrapping replicates under the same model were computed, allowing the program halt bootstrapping automatically with the autoMRE option. The BI was performed with the software Mr. Bayes 3.2.6 (Ronquist & Huelsenbeck 2003), and it 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 1000th generation. Four rate categories were used to approximate the gamma distribution. 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 and/or BS ≥ 90%, while moderate support was considered when BPP ≥ 0.9 and/or BS ≥ 70%. *Ancistrus brevipinnis* authors and *Hypostomus cochliodon* authors were defined as outgroup based on a previous study that recovered it as a sister group of *Rineloricaria* (Lujan et al. 2015).

Barcode gap analysis and genetic distance

Barcode gap discovery was carried out using the ABGD online version (Automatic Barcode Gap Discovery), as available at abi.snv.jussieu.fr/public/abgd/abgdweb. The parameters used during the analyses were: Pmin=0.001; Pmax=0.1; number of steps=10; relative gap width=1.5. The model distance used was Kimura (K80). We calculated the barcode gap only between clade 1 and clade 3 (see results), since the clade 2 did not present enough sample. The Simple Genetic distance was obtained through MEGA software, by calculating the pairwise genetic distance (p value) (Tamura, Dudley, Nei, and Kumar 2007).

Results

Cytogenetic analysis

Cytogenetic data were obtained for the *Rineloricaria* aff *lima* group from Iguaçu River. The diploid number in the clade 1 was polymorphic and karyotypes ranging from 65 to 67 chromosomes (Fig 1). All the analyzed karyotypes demonstrated C-positive heterochromatic bands in conspicuous NOR sites and centromeric regions of few chromosomes (Fig 1). The chromosomes markers permit establishment of the seven general karyotypes (A-G) clearly identified based on the diploid number, karyotype structures, FN, heterochromatin distribution, number and location of the 5S rDNA sites. The *Clade 2* has one specimen with the general karyotype H (Fig 2) present simple NOR sites and heterochromatic band showed in almost chromosomes blocks in centromeric and some telomeric position (Fig 2) also block NOR sites conspicuous (Fig 2). The diploid number in the clade 3 was polymorphic and karyotypes ranging from 65 to 66 chromosomes (Fig 3). All the analyzed karyotypes demonstrated C-positive heterochromatic bands in conspicuous NOR sites and centromeric regions of few

chromosomes (Fig 3). The chromosomes markers permit establishment of the four general karyotypes (I-L) clearly identified based on the diploid number, karyotype structures, FN, heterochromatin distribution, number and location of the 5S rDNA sites. The diploid number and karyotype formula of the specimens of the same clade differs somewhat from individual to individual and, for this reason, they are best dealt showed in Table 1.

Table 1. Details of the three lineages of *Rineloricaria* from Iguaçu River.

Species	Molecular sample	Cytogenetic sample	2n	Karyotypic formula	NF	Ag-NOR and rDNA 18S	rDNA 5S	F
Clade 1								
Karyotype A	1♂	1♂+1♀	65	3m+62st/a	68	2ºst pair	8 and 13 pairs	2
Karyotype B	1♀	4♀	65	4m+61st/a	69	2ºst pair	8 and 23 pairs	4
Karyotype C	1♀	1♂+3♀	67	3m+64st/a	70	1ºst pair	8 and 13 pairs	3
Karyotype D	1♀	1♂+1♀	66	3m+63st/a	69	1ºst pair	8 and 23 pairs	2
Karyotype E	1♂	3♂	65	4m+61st/a	69	2ºst pair	8 and 23 pairs	3
Karyotype F	1♂	2♂+1♀	67	2m+65st/a	69	1ºst pair	8 and 13 pairs	3
Karyotype G	1♀	1♂+1♀	67	1m+66st/a	68	1ºst pair	8 and 23 pairs	2
Clade 2								
Karyotype H	1♂	1♂	64	3m+61st/a	67	2ºst pair	8, 13 and 23 pairs	1
Clade 3								
Karyotype I	1♂	1♂+1♀	66	2m+64st/a	68	2ºst pair	One homologous of the pair 8 and 13 pair	2
Karyotype J1	1♀	1♂+1♀	66	3m+63st/a	69	1ºst pair	One homologous of the pair 8 and 13 pair	1
J2	1♀	1♂+1♀	65	4m+61st/a	69	2ºst pair	One homologous of the pair 8 and 13 pair	
Karyotype K	1♂	1♂+1♀	65	3m+62st/a	68	2ºst pair	One homologous of the pair 8 and 13 pair	4
Karyotype L1	1♂	1♂+1♀	65	3m+62st/a	68	2ºst pair	One homologous of the pair 8 and 13 pair	1
L2	1♂	1♂+1♀	65	4m+61st/a	69	1ºst pair	One homologous of the pair 8 and 13 pair	
L3	1♂	1♂+1♀	65	6m+59st/a	71	1ºst pair	One homologous of the pair 8 and 13 pair	

F= frequency found in our collection.

Phylogenetic inferences and Barcode analysis

We obtained sequences for 14 specimens with more than 500 bp. The phylogenetic reconstruction based on the ML and BI approaches produced the similar topologies (Fig 8, Fig S1 respectively). The analyses recovered three clades within *Rineloricaria* from Iguaçu River. The first clade (clade 1) comprises seven general karyotypes ranging from A to G. The genetic interspecific distance analysis returned low uncorrected P-distances among these lineage, ranging from 0.0 to 1.0% (Table 2). The second clade (clade 2) comprise only general karyotype (Karyotype H) with a genetic distance of 2.0% (Table 2). Lastly, the third clade (clade 3) comprise four general karyotypes ranging from I to L and separated by a genetic distance of 0% (Table 1).

The ABGD method (Fig 9) between clade 1 and 3, reveals interspecific distance significant and did not showed intraspecific distance significant, supporting the hypothesis that clade 1 and 3 correspond to two distinct species. The second clade (clade 2) included by only one representative (for reasons of unavailability of collection), thus did not possible realized ABGD method, as well interspecific distance. The ABGD method and genetic distance was corresponded exactly to the major clades recovered in our phylogenetic inferences (BI and ML).

Table 2. Uncorrected pairwise intraspecific distances between the mitochondrial COI sequences of the three lineages of *Rineloricaria* from the Iguaçu River.

Clades*	E(%)	G(%)	F(%)	A(%)	C(%)	D(%)	H(%)	I(%)	J(%)	K(%)	L(%)
1 Karyotype E											
2 Karyotype E	0%										
3 Karyotype G	0%	0%									
4 Karyotype F	0%	0%	0%								
5 Karyotype A	1%	1%	1%	1%							
6 Karyotype C	1%	1%	1%	1%	0%						
7 Karyotype D	1%	1%	1%	1%	0%	0%					
8 Karyotype H	2%	2%	2%	2%	2%	2%	2%				
9 Karyotype I	3%	3%	3%	3%	3%	3%	3%	4%			
10 Karyotype J	3%	3%	3%	3%	3%	3%	3%	4%	0%		
11 Karyotype K	3%	3%	3%	3%	3%	3%	3%	4%	0%	0%	
12 Karyotype L	3%	3%	3%	3%	3%	3%	3%	4%	0%	0%	0%

*Codes: karyotype E-D clade 1; karyotype H (clade 2); karyotype I-L (clade 3).

Discussion

Chromosomal dada reinforce hypothesis of the lineages divergence

Starting of the cytogenetic approaches, we evidenced an extensive numerical and structural polymorphism in *Rineloricaria* from Iguaçu River, due to this wide-ranging diploid number (64 to 67), these data was crucial to start point to hypothesize that *Rineloricaria* has with more than one species. Thus, the use of the cytochrome c oxidase I gene (COI) was of great value to acknowledge "candidate species" because it shows a greater range of phylogenetic signal than any other mitochondrial gene (Hebert et al. 2003). These results also increasing cytogenetic data to *Rineloricaria* lineages showed an incredible diversity karyotype found in this genus. Karyotype studies in *Rineloricaria* have revealed a great chromosome diversity, both structural and numerical, with a diploid number varying from 2n = 36 to 70 chromosomes, being this the highest value described in the genus (Giuliano-Caetano, 1998; Alves et al., 2003; Rodrigues e Almeida–Toledo, 2008; Rosa et al., 2012). Of the three clades rescued by phylogenetic analysis, the clade 2 has particular features (Table 1) about diploid number and FN, which was lower in relation to the other clades, karyotype formula, and heterochromatic band in relation the clade 1 and clade 3. Indeed, Phylogenetic analysis also recovered this specimen as a unique taxonomic unit.

An interesting feature among the clades 1 and 3 was observed: numerical and structural polymorphism. If we observed specimens with 65 chromosomes in both clades, we realized a little difference between karyotypes formulas. Clade 1, in majority, showed karyotype formula with 4m+61st/a (except Karyotype A/3m+6st/a), while clade 3, 3m+62st/a (except Karyotype J2 and L2, both 4m+61st/a). Interesting condition occurs with karyotype D and karyotype J1, both shared the same diploid number (66 chromosomes) and karyotype formula (3m+63st/a), though belonging a different clades. It must be highlighted that even if chromosomal changes are required for speciation in some cases, there must be other conditions in which they are not, because speciation can occur without any significant change, purely genetic or behavioral factors can produce reproductive barriers between species (Sumner, 2003). In addition, Bayesian analysis and ABGD method also recuperated these specimens as two distinct taxonomic unit.

Specimens with 67 chromosomes were found only clade 1 and all individuals diverge in karyotype formula, when metacentric chromosomes number increase acrocentric chromosomes number decreases (Table 1). This characteristic supports rearrangements chromosomal events, already reported in this genus. Numerical and structural polymorphism also observed in two allopatric populations of *Rineloricaria lima* from Ribeira River and Açungui River, found a variation in chromosome number 2n=66 to 70. The authors suggested the 2n=70 chromosomes a start point of the current polymorphism, such as first hypothesis that one lineage of *Rineloricaria* diversified from a primitive karyotype with 2n=54 through centric fissions to reach a stage characterized by 2n=70. After these fissions events, chromosomal rearrangements such as fusions may operate to the appearance of all the other karyotypes (2n=69, 68 and 66). In addition, through chromosome markers, fusions, inversions and translocations events support this karyotypic diversity (Rosa, et al., 2012).

A particular chromosomal feature was reported in Karyotypes J and L, both belonging to clade 3. Karyotype J showed two different karyotypes formulas (Table 1), reveled a structural and numerical variation, characterized by increase metacentric chromosomes number and decreases acrocentric chromosomes number, however in both the FN maintain 69. This feature matches with Robertsonian rearrangements that consisting to either increase or decrease the chromosome number but maintain the FN (Meyne et al. 1990, Slijepcevic 1998). Similar condition was found in *Trichomycterus davisi*, showed diploid number ranging 53 to 56 chromosomes. The authors suggested may have originated due to a post-zygotic nondisjunction and fission in the first divisions after non-disjunction was the principal events for this mosaic. In addition, *T. davisi* also from Iguaçu River proposed the hypothesis that chemical and physical agents are influencing this intra-individual variation due to the increasing pollution of the river (Borin and Martins-Santos, 2000), in our study, we also did not rule out the influence by means of these agents for the peculiar intraindividual variations found in these two specimens. On the other hand, karyotype L presented three different karyotype formula (Table 1), characterized by increase metacentric chromosomes number and decreases acrocentric chromosomes number, however maintain the same diploid number and differing in FN was 68, 69 and 71. In this case, we suggest types of rearrangements of translocations and inversions, because there was only maintenance of the diploid number.

Rineloricaria from Iguaçu River and two allopatric populations of *Rineloricaria lima* (Rosa, et al., 2012) both presented numerical and structural polymorphism, presence

a high number of acrocentric in relation to metacentric chromosomes, and Karyotypes D and J1 (present study) resembles with Karyotype H in *R. lima* (Rosa, et al., 2012), both showed 2n=66 chromosomes. However, R. *lima* revealed higher diploid numbers (2n= 66 to 70) in relation to the three lineages of the present study (Clade 1: 2n= 65 to 66 and Clade 3: 2n=65 to 67) and the authors stipulate variant chromosomes between the karyotypes. Also, suggested that a start point for this polymorphism as of a basal karyotype with a higher diploid number. Here in present study was not possible establish variants chromosomes and a start point of the current polymorphism, however, the hypothesis of a basal karyotype with an even higher number cannot be ruled out.

Gamete combination between specimens with different diploid number and/or karyotype formula could lead maintenance these extensive polymorphism in clade 1 and 3. Using Karyotype I (2n=66/ 2m+64st/a) and Karyotype K (2n=65/ 3m+62st/a) as an example and crossing gametes: 1m+32st/a (Karyotype I) and 2m+31st/a (karyotype K) result in individual with 2n=66 chromosomes and 3m+63st/a karyotype formula, corresponding to Karyotype J1 (Table 1). Though meiosis in these individual may be perturbed, the gametic combination have maintained this polymorphism situation. Thus, studies involving chromosome pairing in meiosis is fundamental.

The banding C in two lineages of *Rineloricaria* (clade 1 and 3) showed pattern similar to that found in three different populations of *R. pentamaculata* analyzed by Errero Porto, et al. (2011), as well as for two populations analyzed by Venturelli (2014). However, the karyotype H (clade 2) showed a larger number of blocks in centromeric region of almost all chromosomes, similar to the heterochromatic pattern found in *Rineloricaria lima* (Rosa, et al., 2012). In addition, another characteristic commonly reported for the genus (Giuliano-Caetano, 1998; Errero Porto, et al., 2011) is the association of heterochromatin and NOR and has been interpreted as heterochromatic blocks interspersed between sites ribosomes (Pendás et al., 1993).

Contrary to the extensive chromosomal variation, the chromosome banding in the genus *Rineloricaria* shown a little more conserved with some punctual variations. In general, this genus present simple NOR in terminal position, most time, in only one pair of the st/a group. In addition, NOR heteromorphism has been constantly reported and is a shared feature for most species in the genus, as a in *R. lima, R. lanceolata, R. pentamaculata R. striglata, R. kronei, R. cadeae* e *R.* n.sp (Rosa, et. al, 2012; Errero-Porto, et. al, 2011 e 2014; Venturelli, 2014; Rodrigues 2010) and present study to the all clades. This heteromorphic feature, often, are structural modifications of NORs relate to

mechanisms involving segments of homologous chromosomes, such as uneven crossing-over, transpositions or rearrangements as deletions and / or duplications (Galleti-Jr et al., 1995; Castro et al.,1998). Variations about the NORs multiples sites distributions was reported only in *R. pentamaculata* (Errero-Porto, et al., 2011).

The physical mapping of the 5S rDNA showed notable difference between of clades, clade 1 present four sites (two patterns), clade 2 six sites and clade 3 three sites (Table 1), this feature corroborates with the three lineages revealed by analyses phylogenetic. In present study, multiple sites of 5S rDNA was revealed in terminal position and in subtelocentric and acrocentric chromosomes, similar condition was revels in *R. lima* (seven to ten sites), however, sites centromeric position in metacentric was found. The authors suggested this features could be involving fusion events, thus, evidenced by the presence of traces of ITSs in this region suggested that fusion of chromosomes carrying 5S rDNA in terminal region, forming metacentric chromosome (Rosa et al. 2012). Galetti and Martins (2004), suggested that the physical mapping of 5S rDNA sequences in most fish is located near the centromere, apparently as a general feature of the group. However, the presence of multiple sites of 5S rDNA suggests instability in the genome of fish species, which has been largely related to the presence of transposable elements associated with these genes, thus increasing the dispersion of these copies. As a suggested by Cioffi et al. (2010) in study with *Erythrinus erythinus* that showed 21-22 sites and the dispersion of copies of this gene would be associated with the Rex3 retrotransposon, considered as the main mechanism of dispersion of this gene.

Molecular phylogenetic inferences suggest three new lineages in Iguaçu River

Cytogenetic data reveals an expressive chromosome diversity with a wide chromosomal polymorphism in *Rineloricaria* from the Iguaçu River. Due to high variability, we hypothesis development that in this population could be present more than one species. Through assistance of phylogenetic reconstructions and cytogenetic analysis, we confirmed the presence of three clades among of the *Rineloricaria* from Iguaçu River (single point of collection). *Rineloricaria* is the richest genus in the Loricariinae and presents unsolved taxonomical issues within the subfamily. Historically, this group was described along with *Hemiloricaria* by Bleeker (1862), having as type species *Rineloricaria lima*, however there is no concrete information about the locality type. The

distribution of *Rineloricaria* occurs in most of the main drainage from Panama to Argentina, in addition to occupying a wide variety of habitats. Due to the variety of habitats in which the species of the genus are distributed, species of the genus have a great variety of forms, colors and strategies of life. In addition, some species of *Rineloricaria* have very similar morphological characteristics (Rodriguez and Reis, 2008), which often makes it difficult to describe the species. Here, combining molecular and cytogenetic evidences, we supported leastways three independent lineages. Ours data shown that remaining clades recovered here (1, 2 and 3) should categorize as "candidate species", according to classification proposed by Vieites et al. (2009). Our approaches also are supported by ABGD (Automatic Barcode Gap Discovery) results and genetic distance value considering cutoff 2%, according barcode researchers which have been used this value for species delimitation (Ward 2005; Pereira et al. 2011; Pereira et al. 2013).

Based on the phylogenetic tree drawn to *Rineloricaria*, these three "candidate species" reinforcing the hypothesis that these lineages have recently diverged. The ABGD method has become a popular tool for the delimitation of species and has been applicate in different organisms (Puillandre et al. 2012). The ABGD is efficient and performs well for standard prior maximum intraspecific divergences, except for one data set where less than three sequences per species were sampled, as a case to clade 2. The gap through the ABGD method between clade 1 and clade 3 correspond exactly to the major clades recovered in our phylogenetic inferences (BI and MP). The gap reveal a difference is highly significant, indicating the presence of more than one species in our sample. Considering *Rineloricaria* specimens from the same collection point belong to the Iguaçu River, scope of this study, the sequences were grouped into three clusters with high support values showed above the branches (Fig 1). The barcode gap was clearly identified even with the low distance values presented in table 1.

Recent phylogenetic and taxonomy integrative studies in *Rineloricaria* conducted by Costa-Silva et al. (2015) showed that the species differentiation limits, in some cases, the morphological limits appeared before the genetic limits, in others the genetic limit preceded the morphological, as we suggested in our study. In addition, cryptic species present in the same ecoregion showed a considerable genetic distance and the authors suggested that species probably could not interchange genes due reproductive isolation, as discussed by Kekkonen et al. (2014), which characterized these species as distinct species by biological concepts. Even though our approach involves cytogenetic studies

and phylogenetic analyzes differ somewhat, our data are congruent to the data presented by Costa-Silva (2015) for the genus.

In the present recovered phylogenies, we identified at least three independent lineages based on several specimens first though to represent only one specie. In the view of the rich species variability, wide distribution and peculiar events of chromosomal rearrangements make phylogenetic studies and identification taxonomic an indispensable tool for the understanding the evolution of the peculiar group such *Rineloricaria*.

Conclusion

The cytogenetic data obtained so far, for *Rineloricaria* from Iguaçu River show a fascinating pattern of chromosomal evolution, marked by translocations, inversions, fusions and rearrangements of fusions, revealing an extensive chromosomal polymorphism in two of the three lineages recovered by phylogenetic analysis and ABGD method. Evidences for a more robust hypothesis about exact understanding of which chromosomes are involved in these rearrangements need more deep studies. However, the data obtained suggest that this wide karyotype diversity can initiate and/or contribute to the divergence process, with specific implications for the usefulness of chromosomal characters for phylogenetic inference (Sites and Kent, 1994). *Rineloricaria* is acknowledged to report of chromosomal polymorphisms, as well as a confused genus regarding the taxonomy of this study, which combines an approach of molecular and cytogenetic analyzes, essential in the group's karyoevolutionary history. This approach was especially important because the lack of diagnostic characteristics in the morphology of these fish. In addition, a single point of collection revealed a high diversity until then hidden, in which our studies allowed to identify three lineages of *Rineloricaria*. Overall, the cytogenetic data combined phylogenetic data indicate the existence of a high level of chromosomes rearrangements and support the hypothesis that these lineages have recently diverged, this study being a "photograph" of the evolution of the *Rineloricaria* from Iguaçu River.

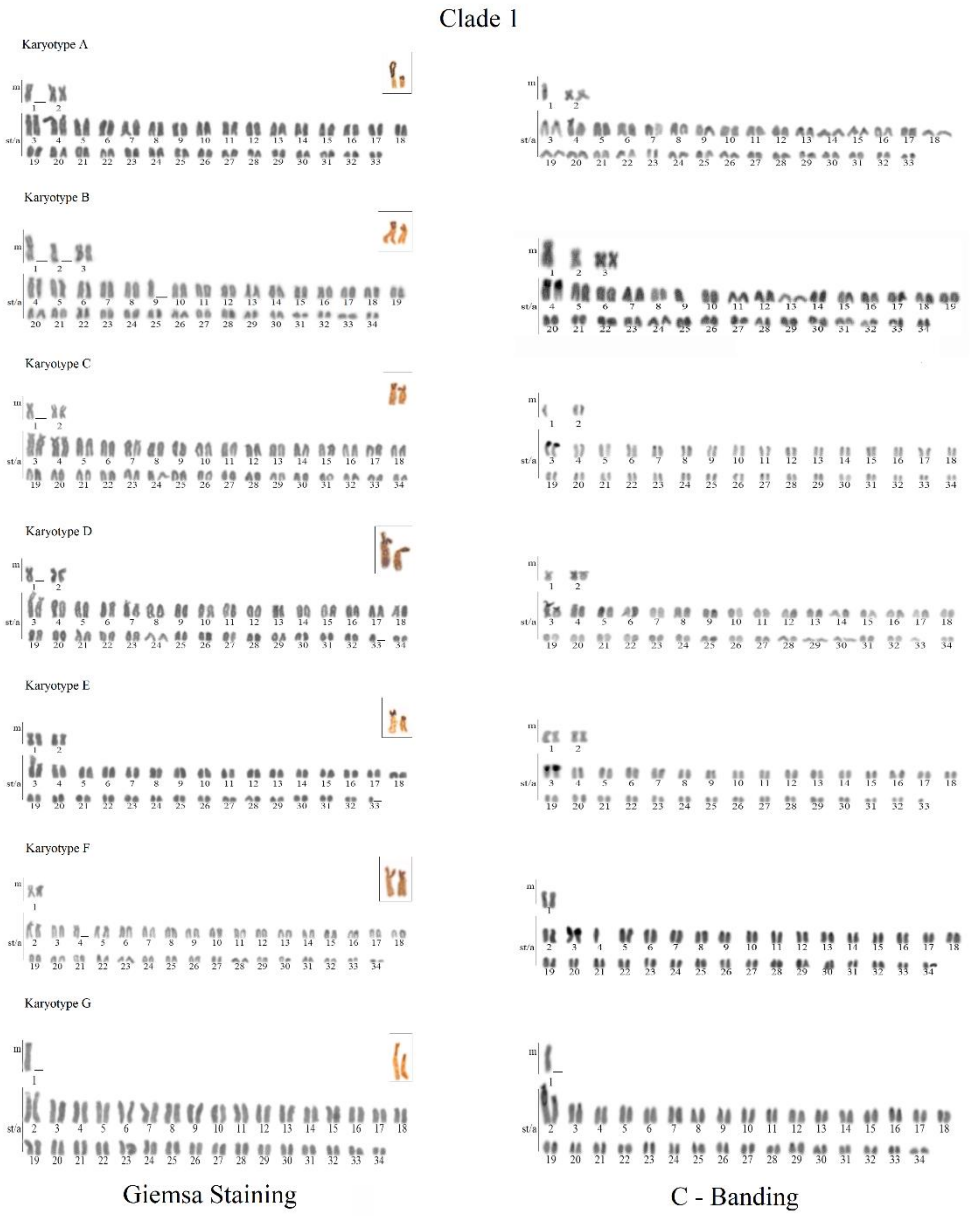


Figure 1. Karyotypes of the *Rineloricaria* clade 1 A-G arranged from Giemsa stained (left) and C-banded chromosomes (right). The configuration of the silver nitrate-stained nucleolar organizing regions (Ag-NORs) are shown in the box.

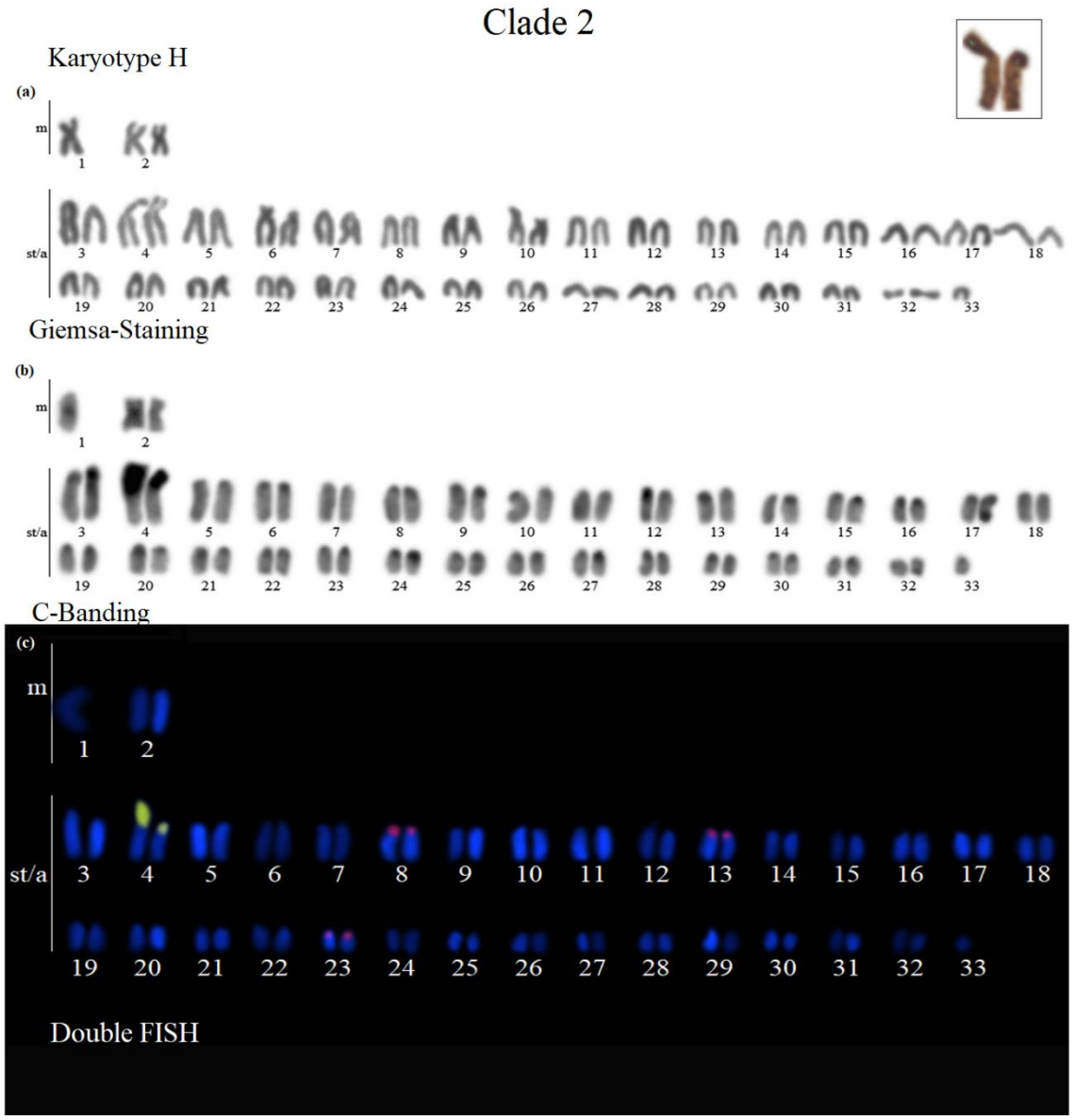


Figure 2. Karyotypes of the *Rineloricaria*, clade 2, corresponding Karyotype H. (a) Giemsa stained (b) C-banded chromosomes (c) dual color-FISH showing the 5S rDNA (red) and 18S rDNA (green) sites. The configuration of the silver nitrate-stained nucleolar organizing regions (Ag-NORs) are shown in the box.

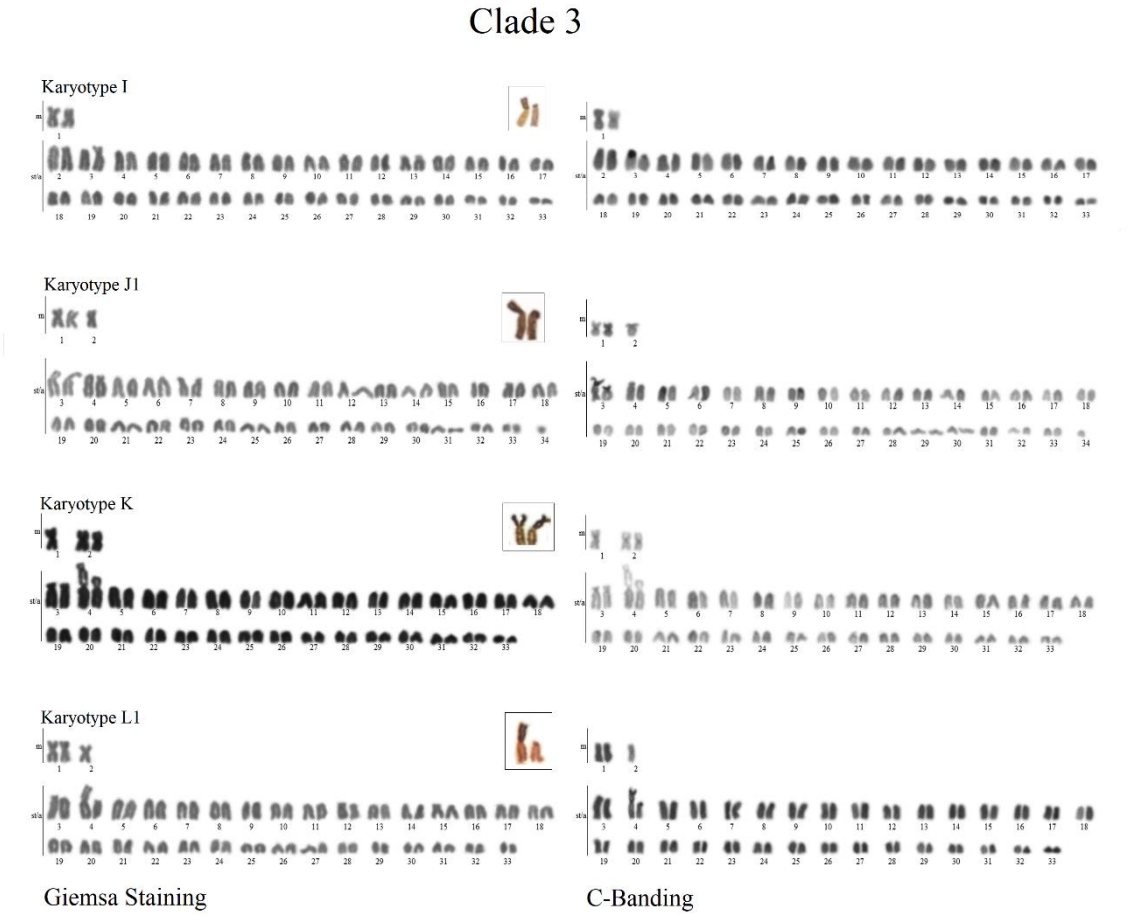


Figure 3. Karyotypes of the *Rineloricaria*, clade 3 I-L arranged from Giemsa stained (left) and C-banded chromosomes (right). The configuration of the silver nitrate-stained nucleolar organizing regions (Ag-NORs) are shown in the box.

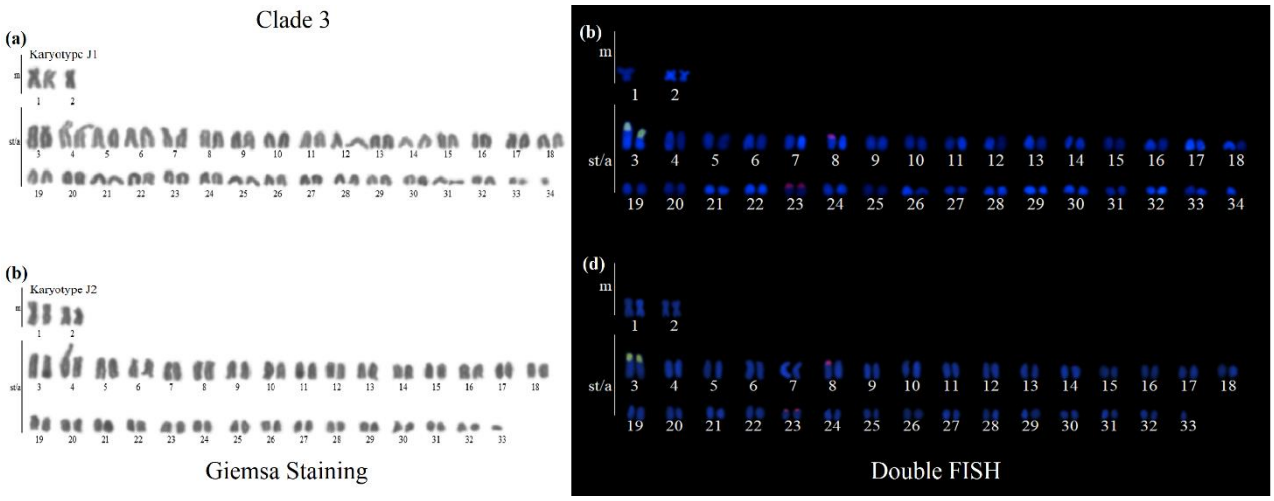


Figure 4. Karyotypes of the *Rineloricaria*, clade 3, corresponding Karyotypes J1 and J2. (a) Giemsa stained in Karyotype J1 (b) dual color-FISH in Karyotype J1 showing the 5S rDNA (red) and 18S rDNA (green) sites (c) Giemsa stained in Karyotype J2 (d) dual color-FISH in Karyotype J2 showing the 5S rDNA (red) and 18S rDNA (green) sites.

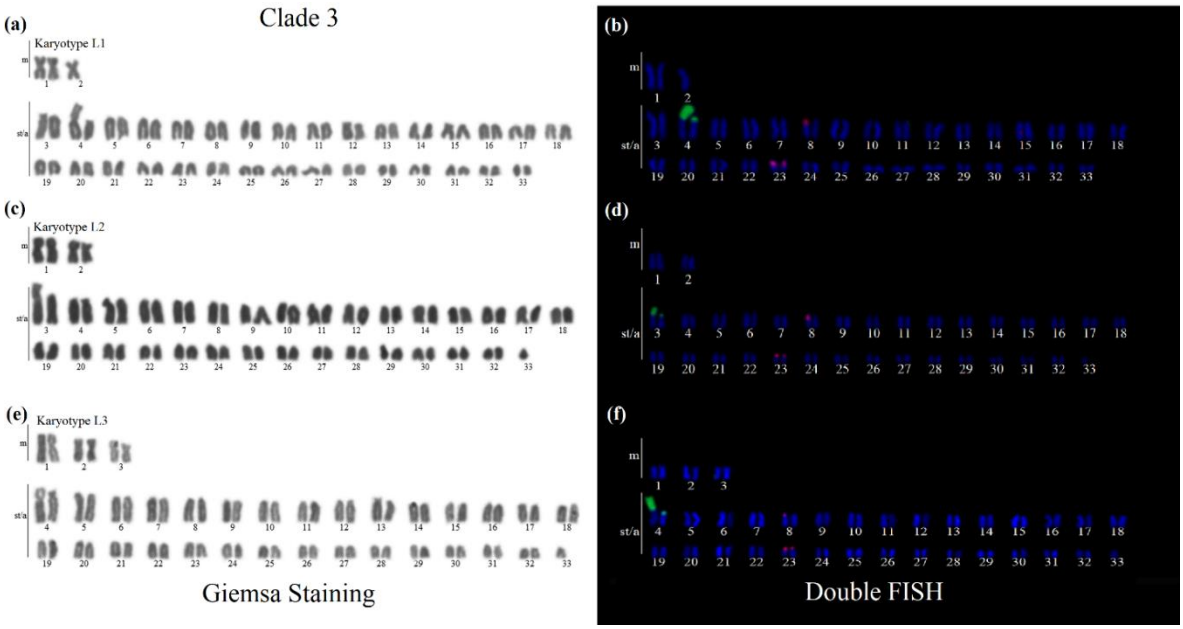


Figure 5. Karyotypes of the *Rineloricaria*, clade 3, corresponding Karyotypes L1, L2 and J3. (a) Giemsa stained in Karyotype L1 (b) dual color-FISH in Karyotype L1 showing the 5S rDNA (red) and 18S rDNA (green) sites (c) Giemsa stained in Karyotype L2 (d) dual color-FISH in Karyotype L2 showing the 5S rDNA (red) and 18S rDNA (green) sites (e) Giemsa stained in Karyotype L3 (f) dual color-FISH in Karyotype L3 showing the 5S rDNA (red) and 18S rDNA (green) sites.

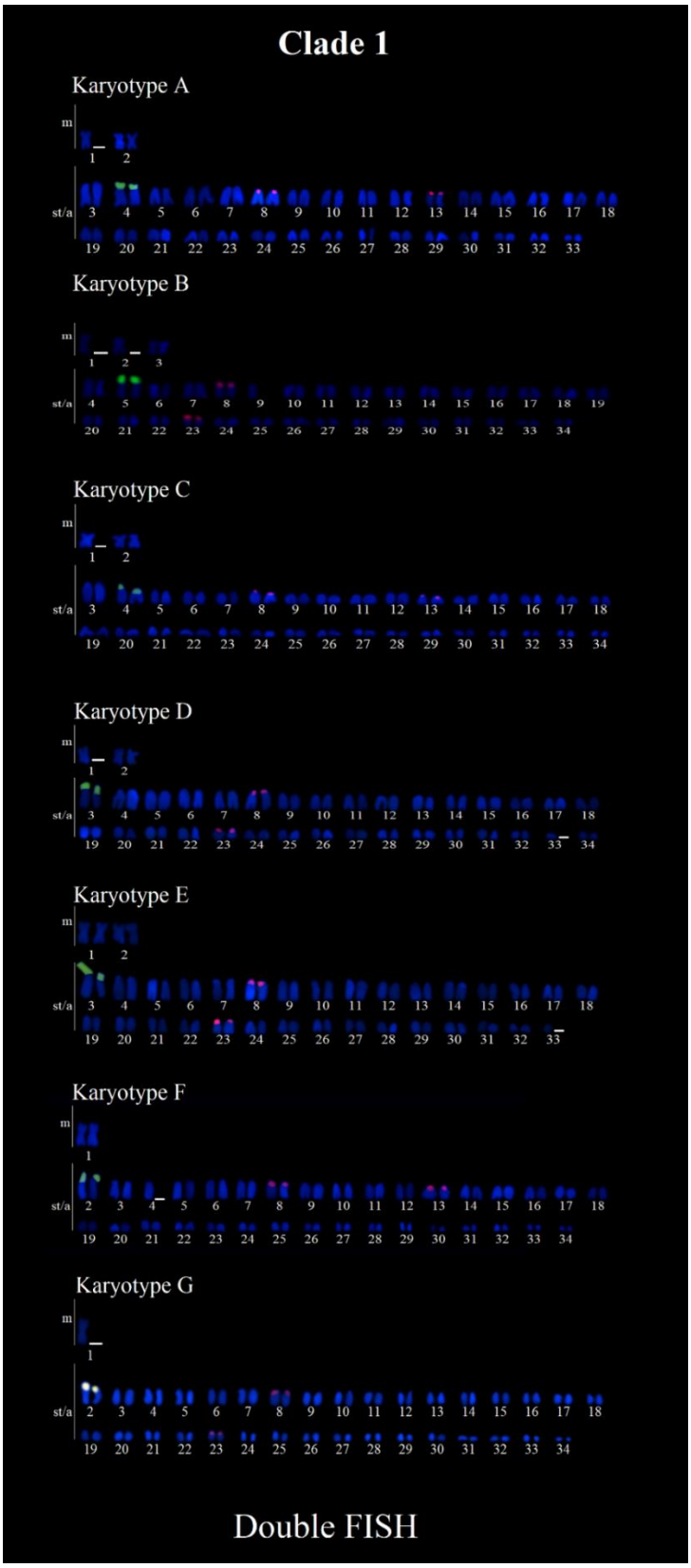


Figure 6. Karyotypes of the *Rineloricaria* from Iguaçu River A-G of species corresponding clade 1 showing the 5S rDNA (red) and 18S rDNA (green) sites after dual color-FISH analyses.

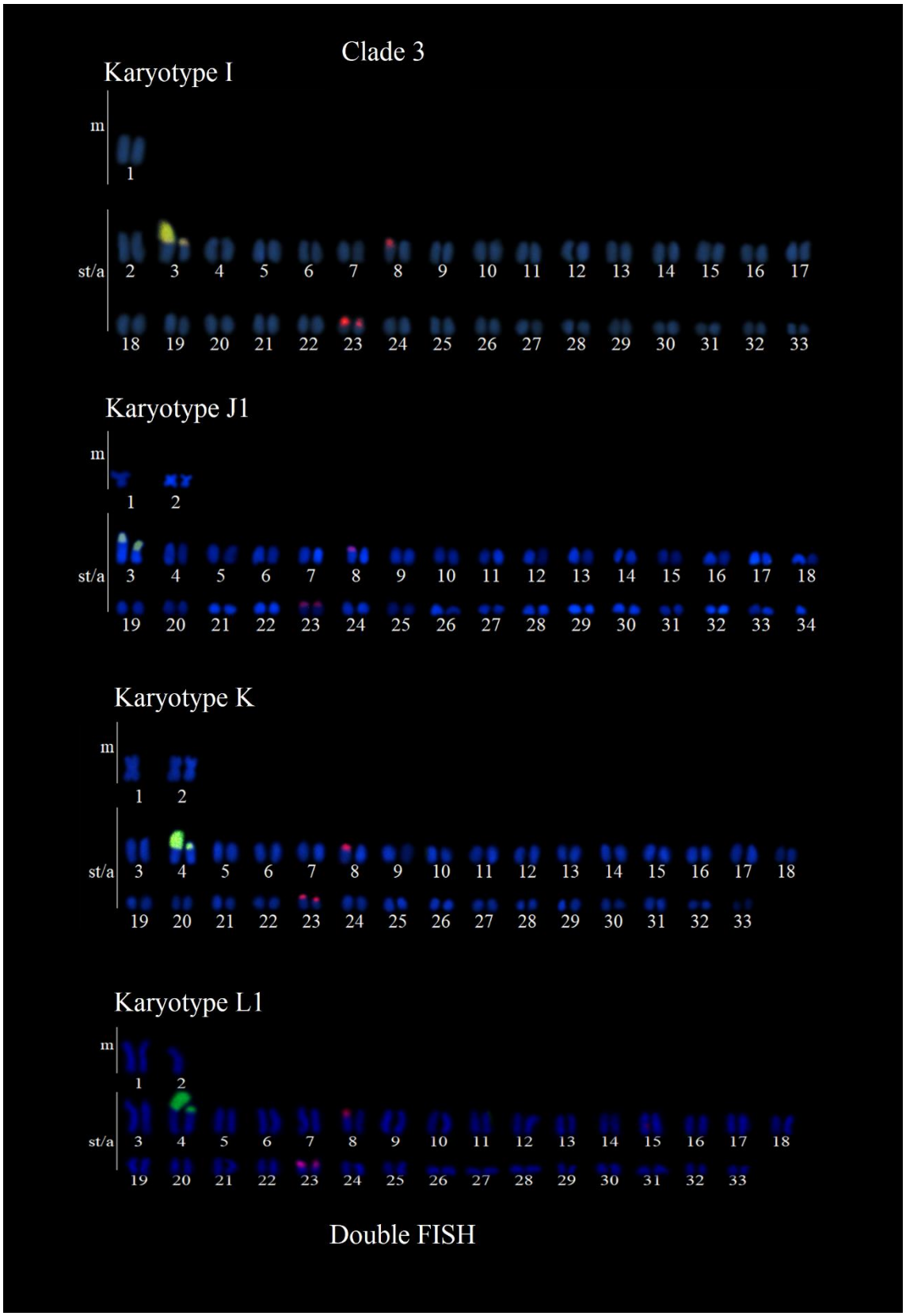


Figure 7. Karyotypes of the *Rineloricaria* from Iguaçu River I-L of species corresponding clade 3 showing the 5S rDNA (red) and 18S rDNA (green) sites after dual color-FISH analyses**.**

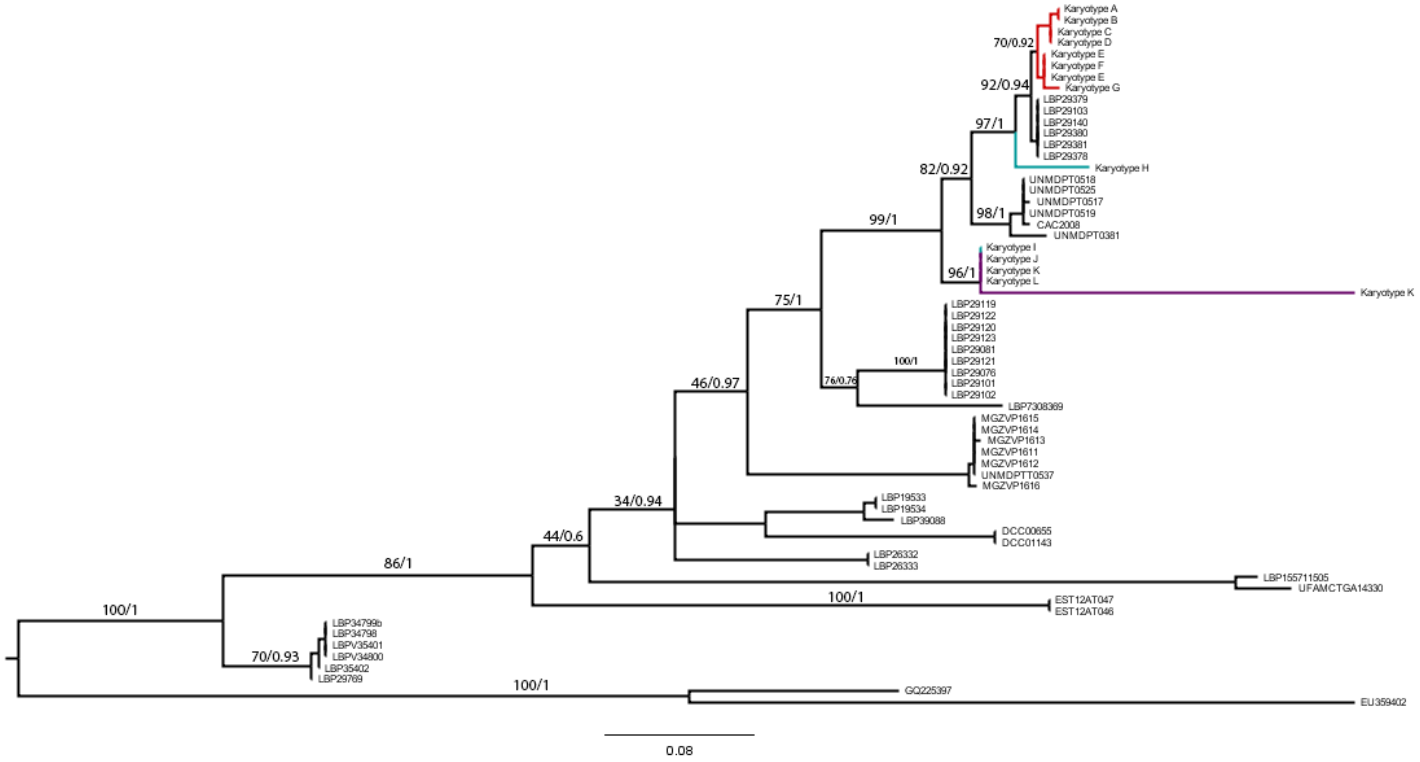


Figure 8. Maximum Likelihood consensus tree for the Rineloricaria species of the Iguaçu River performed using RAxML v. 8.2.4 software and the analysis first involved 100 ML searches, each starting from one randomized stepwise addition parsimony tree. Posterior probabilities are shown above the branches (ML/BI). Each color represents one of the three evolutionary lineages recovered. Codes: Karyotypes A-G representing clade 1 (in red); Karyotype H representing clade 2 (in blue); Karyotypes I-L representing clade 3 (in purple).

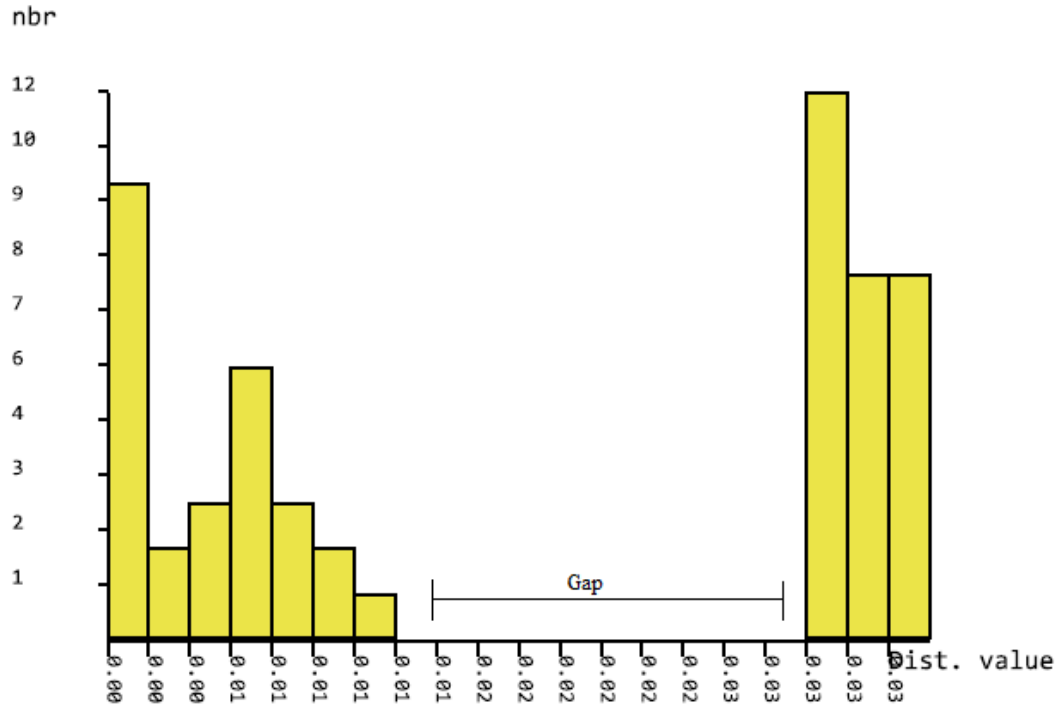
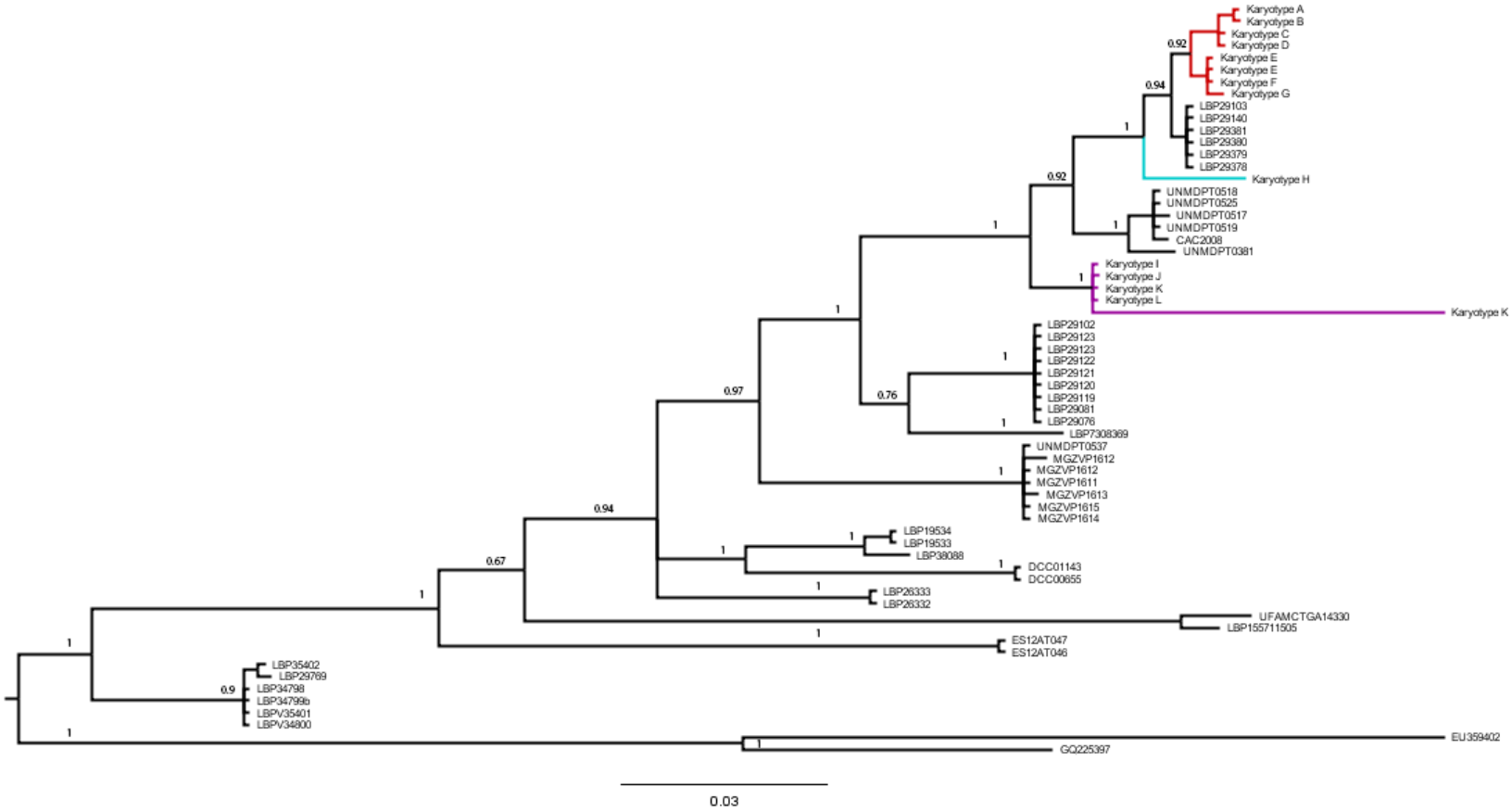


Figure 9. Histograms according ABGD method of intra-specific (left) and inter-specific (right) average distances between COI sequences both clade 1 and 3.



S.1. Bayesian consensus tree for the *Rineloricaria* species of the Iguaçu River produced Mr. Bayes 3.2.6 and obtained from 10 million generations. Posterior probabilities are shown above the branches. Each color represents one of the three evolutionary lineages recovered. Codes: Karyotypes A-G representing clade 1 (in red); Karyotype H representing clade 2 (in blue); Karyotypes I-L representing clade 3 (in purple).

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CAPITULO II

Distribution of DNA repetitive: a contribution to the understanding of the chromosomal rearrangements in three lineages of the *Rineloricaria* (Loricariidae, Loricariinae) from Iguaçu River, Paraná state, Brazil.

Este artigo será submetido a Zebrafish.

Distribution of DNA repetitive: a contribution to the understanding of the chromosomal rearrangements in three lineages of the *Rineloricaria* (Loricariidae, Loricariinae) from Iguaçu River, Paraná state, Brazil.

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Abstract

Repetitive DNA sequences represent an important tool in understanding evolutionary mechanisms, genomic structures and in the diversification of karyotypes. The genus *Rineloricaria* is an interesting group for the investigation of karyotype evolution, given its wide-ranging chromosomal variation and extensive chromosomal polymorphism (numerical and/or structural). The present study investigated the localization of the telomeric sequences and $(CA)_{15}$ and $(GA)_{15}$ microsatellite sequences in three lineages of the *Rineloricaria* from Iguaçu River, Paraná State, Brazil. The physical mapping of the telomeric sequences revealed the presence of interstitial telomeric sites (ITS) in the centromeric region in some metacentric chromosomes of the two lineages and both showed ITS localized in NOR pair. The $(CA)_{15}$ and $(GA)_{15}$ microsatellite probes hybridized preferentially in the subterminal and interstitial regions of most chromosome arms, and centromeric in most metacentric chromosomes, 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 18S rDNA sites. The distribution of telomeric and microsatellites and the locational relationship between both are discussed, and a possible evolutionary pathway is proposed for the wide karyotype variability and extensive polymorphism chromosomal in *Rineloricaria*.

Keywords: chromosomal mapping, telomeric probe, chromosomal polymorphism, karyotype variability, Loricariinae.

Introduction

DNA repetitive sequence, comprising satellite DNA, minisatellite and microsatellite repeats, integrate a significant portion of eukaryotic genomes, largely located at the heavily packed heterochromatic regions of the chromosomes (Charlesworth et al. 1994; Enukashvily and Ponomartsev 2013; Traldi et al, 2013). Jurka et al. (2003) ranked repetitive DNA into two major groups, the first group includes microsatellites, minisatellites, and satellites, and the second group composed by retroelements and DNA transposons. The accumulation and mobility of repetitive sequences in the genome promote chromosome differentiation with an important role in karyotype evolution, in addition, satellite DNAs rapidly diverge during evolution. Thereby, they has been important tools in studies of taxonomic and evolutionary problems (Wichman et al., 1991; Hamilton, 1992; Traldi et al, 2013; Pucci et al., 2014).

DNA sequences frequently involved in chromosomal rearrangements and, mainly short in tandem repeated sequences of telomeric DNA (TTAGGG)n, telomeres are substantial molecular tool for accurate replication and stability of chromosomal ends and can indicate the occurrence of possible chromosomal rearrangements . In some cases, traces of telomeric sequences (TTAGGGn) are found in interstitial sites, where is observed a high occurrence of chromosome rearrangements (Blackburn and Szostak 1984, Meyne et al. 1990; Ashley and Ward 1993; Meyne et al., 1990; Ruiz-Herrera et al., 2008; Slijepcevic et al., 1997). ITS are hotspots for chromosomal breakage and are naturally prone to breakage (Slijepcevic, 2016).

Tandem repetitive DNA sequences due to the variation in the number of repetitive units may exhibit a high degree of polymorphism and microsatellites (or short tandem repeats) are the most polymorphic and are formed of short sequences of one to six nucleotides repeated in tandem throughout the DNA (Tautz and Renz 1984), however the chromosomal mapping of microsatellite sequences has been little examined. Although they are often described as neutral markers, important functions of various biological phenomena have been attributed to microsatellites, in the organization of chromatin (Epplen et al., 1996), DNA replication (Li et al., 2002), recombination (Biet et al., 1999) and gene expression (Liu et al., 2001), among others. Thus, considering that microsatellites are the most dynamic genomic component, a better understanding of their chromosomal organization is important for improving knowledge regarding the role of repetitive DNA elements in the mechanisms of chromosomal evolution and heterochromatin composition.

In fishes, DNA sequences of multiple copies has been an important tool in understanding evolutionary mechanisms, genomic structures and karyotype differentiation (Barbosa, et al., 2017). Centric fusions in chromosomes are described in fish through studies involving sequences telomerics, for example, in the *Rineloricaria* (*Bleeker*, 1862) genus, *R. lima* (Rosa et al. 2012), *R. lanceolate* (Errero-Porto, et al., 2014) and *R. latirostris* and *R. pentamaculata* (Primo, et al., 2017). *Rineloricaria* present species with the great amount of inter and intrapopulational variations, both high morphological diversity and a wide variation of chromosomal number and formula, with diploid number reduction tendencies, ranging from 2n = 36 to 2n = 70 chromosomos. Centric fusions, inversions and translocations are considered the main rearrangements that leads reductions of the diploid number in this group and expressive numerical and/or structural polymorphisms (Giuliano-Caetano, 1998; Alves et al., 2003; Rodrigues e Almeida–Toledo, 2008; Rosa et al., 2012; Errero-Porto et al. 2014; Primo et al. 2017). However, the DNA sequences involved in chromosomal instability of Loricariidae group are still poorly understood (Barros, et al., 2017).

Cius et al (cap 1) through assistance of phylogenetic reconstructions and cytogenetic analysis characterized the presence of three "candidate species" among the *Rineloricaria* from the Iguaçu River located in União da Vitória (Paraná, Brazil). Differences in three distinct evolutionary lineages were recognized, based on their different diploid numbers, different karyotype formulae, heterochromatin patterns and multiple 5S rDNA sites patterns. Furthermore, two lineages revealed wide-ranging numerical and/or structural polymorphisms. Given the high chromosomal rearrangements diversity of the three lineages of *Rineloricaria* from Iguaçu River, and the efficiency of the analysis of repetitive sequences on chromosome studies, this study aimed at contributing to a better understanding of chromosome in three "candidate species" diversification, using the distribution of (CA)n (GA)n and (TTAGGG)n sequences in species of this genus.

Material and Methods

Specimens and Classical Cytogenetics

Specimens of *Rineloriicaria* collected from Iguaçu River and cytogenetics data analyzed initially by Cius et al (cap 1) are summarized in the Table 1. The protocols used in this study were submitted and reviewed by the Ethics Committee in Animal Experimentation (Protocol: 07/2011) of the State University of Ponta Grossa. Voucher specimens were deposited in the ichthyologic collection of the Limnology, Ichthyology and Aquaculture Research Center (Nupélia) at Maringá State University, Paraná, Brazil.

Fluorescence in situ hybridization (FISH)

Mitotic chromosomes were obtained from kidney cells following Bertollo et al 1978. For each FISH assay 30 cells were analyzed. The Fish with 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. This probe was generated by PCR (PCR DIG-Probe Synthesis Kit, Roche) in the absence of a DNA template, using $(TTAGGG)_5$ and $(CCCTAA)_5$ as primers (Ijdo et al. 1991).The telomeric (TTAGGG)n sequences were mapped by Fluorescence *in situ* Hybridization (FISH), following Pinkel et al. (1986).

The mapping 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 (2008).

The metaphases were photographed using an epifluorescence microscope and optimized for best contrast and brightness with Adobe Photoshop CS6 software. Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) or acrocentric (a) according to their arm ratios (Levan et al. 1964).

Table 1- Cytogenetic data of the specimens of three lineages of *Rineloricaria* obtained from the Iguaçu River.

Species	2n	Karyotypic formula	18S rDNA	5S rDNA
Clade 1				
Karyotype A	65	3m+62st/a	second st/a pair	8 and 13 pairs (four sites)
Karyotype B	65	4m+61st/a	second st/a pair	8 and 23 pairs (four sites)
Karyotype C	67	3m+64st/a	first st/a pair	8 and 13 pairs (four sites)
Karyotype D	66	3m+63st/a	first st/a pair	8 and 23 pairs (four sites)
Karyotype E	65	4m+61st/a	first st/a pair	8 and 23 pairs (four sites)
Karyotype F	67	2m+65st/a	first st/a pair	8 and 13 pairs (four sites)
Karyotype G	67	1m+66st/a	first st/a pair	8 and 23 pairs (four sites)
Clade 2				
Karyotype H	64	3m+61st/a	second st/a pair	8, 13 and 23 pairs (six sites)
Clade 3				
Karyotype I	66	2m+64st/a	second st/a pair	One homologue 8 and 23 pair (three sites)
Karyotype J1	66	3m+63st/a	first st/a pair	One homologue 8 and 23 pair
J2	65	4m+61st/a	second st/a pair	One homologue 8 and 23 pair
Karyotype K	65	3m+62st/a	second st/a pair	One homologue 8 and 23 pair
Karyotype L1	65	3m+62st/a	second st/a pair	One homologue 8 and 23 pair
L2	65	4m+61st/a	first st/a pair	One homologue 8 and 23 pair
L3	65	6m+59st/a	first st/a pair	One homologue 8 and 23 pair

Results

Chromosomal distribution of (TTAGGG)n sequence, (CA)n and (GA)n sequence

Based on the distribution of the chromosomal pairs bearing the 18S, 5S rDNA sites and heterochromatin blocks of the *Rineloricaria* lineages analyzed previously by Cius et al. (cap 1), was observed an interesting pattern, showing a considerable difference between species mainly in relation to 5S rDNA sites, were each clade showed different patterns (Table 1). The probe of the minisatellite sequence (TTAGGG)n was uniformly located in the telomeres of all chromosomes at the studied. In clade only Karyotype C (Fig. 1) showed ITS in metacentric and in NOR pair, and clade 2, did not showed interstitial signal (ITS) was found (data not shown). On the other hand, clade 3 showed interstitial signal (ITS) in three general karyotypes, beyond the karyotypes variations belonging to the karyotype L, in the other words, only karyotype J no interstitial signal (ITS) was found (data not shown). The karyotypes I and K (Fig 2) ITS was found in centromeric regions in two subtelocentric chromosomes corresponding a NOR pair. In

karyotype L1, in two karyotypes formulas we detect ITS (Fig 3), (table 1) showed a similar pattern of karyotypes I and K (ITS centromeric in NOR pair). However, a peculiar condition was found in the karyotype L3 (table 1), when we detect six interstitial signal (Fig 3), distributed in two metacentric chromosomes, subtelocentric pair and a NOR pair. All the karyotypes without ITS are in supplementary file (S1, S2 and S3).

Microsatellite sequences displayed a scattered distribution throughout most of the chromosomes in all species, without the evidence of preferential accumulations. The location of the oligonucleotide probes $(CA)_{15}$ and $(GA)_{15}$ microsatellites mostly coincided in the chromosomes, either co-located or adjacent to one another for all species and may help in investigating the heterochromatin nature (Fig 4 to Fig 10). The signal showed dispersed signal and with blocks among the chromosomes of the all chromosomes, however (GA) microsatellite coincided with heterochromatic regions for the most chromosomes in according to pattern of C-banding showed in our studies with *Rineloricaria.* However, both clade 1 and clade 3 $(CA)_{15}$ microsatellites showed some blocks in centromere regions in some metacentric chromosomes and clade 3 and showed blocks more discrete in relation to markers $(GA)_{15}$ microsatellites for the all karyotypes. Interestingly, we detected (GA) signals coinciding with the 18S rDNA sites in Karyotypes A, E, C, I, K, L1, L2 and L3.

Discussion

Numerous types of chromosome rearrangements were found in Loricariidae, in special fissions and centric fusions are responsible for the wide variation of diploid number (Artoni and Bertollo, 2001). In this family the karyotype diversification trends includes rearrangements as translocations, inversions and centric fusions that lead to a numerical reduction, from the ancestral diploid number 2n = 54 (Artoni and Bertollo, 2001). In addition, centric fusions in chromosomes are described in the Loricariidae family, in *Rineloricaria lima* (Rosa et al. 2012), *R. lanceolata* (Errero-Porto, et al., 2014) *Harttia carvalhoi* and *Harttia torrenticola* (Blanco et al., 2012). Cius et al. (in preparation) by analyzing mitochondrial cytochrome C oxidase subunit I (COI) and cytogenetic marks in *Rineloricaria* from the Iguaçu River suggested the presence of at least three lineages. Furthermore, revealed wide-ranging numerical and/or structural polymorphisms, in which karyotypes presented 2n=65–67 chromosomes with seven general karyotypes in clade 1 and 2n=65-66 with four general karyotype in clade 3, clade 2 showed only one karyotype with 2n=64. Cius et al (cap 1) suggested, for clade 1 and 3 (Table 1), events translocations and inversions, however, fusion-type rearrangements may be involved in this extensive polymorphism, in view of the clear reduction of the diploid number. Furthermore, clade 2 showed an expressive reduction about the diploid number when compared to the other clades. For this, studies involving telomeric sequences are fundamental, for the location of ITS is an important tool that helps to understand the evolutionary history of a group (Meyne et al. 1990) and it indicates the occurrence of possible chromosomal rearrangements (Ashley and Ward 1993), such as centric or tandem fusions and pericentric inversions (Slijepcevic 1998; Ocalewicz et al. 2013).

As proposed by Cius et al., (cap 1), the evolution of these three lineages has been marked by many chromosomal events and by crosses of individuals of the same species maintaining this extensive polymorphism. In clade 1, with the exception of the Karyotype C (Fig 1), although the studies involving DNA repetitive revealed ITS, we suggested the hypotheses that inversions and fusions are the principal factor to the reduction the diploid number. In the relation to origin of karyotype G (clade 1) we suggest that specimens with 2n = 68 may to represent the start point for the origin of this unique metacentric by fusion

events. In addition, the differences of karyotypes F (2m+65st/a) and C (3m+64st/a) (Table 1) with 2n=67, suggest pericentric inversions (Fig 11). As suggested by Bertollo and Artoni (2001) on the karyotype evolution in Loricariidae 2n=54 chromosomes as a primitive condition. Thus, Rosa et al., (2012) proposed that one lineage of *Rineloricaria* through centric fissions originated individuals with 2n = 70 chromosomes, as found in *R. lima*. Hence, we suggested a similar condition from the three species of *Rineloricaria* from Iguaçu River. In addition, hypothetical crossover between Karyotype C and Karyotype D resulting in individuals with 2n=68 chromosomes. In our collections, only one individual of this karyotype was found (Fig 12) and to exemplar did not preserved.

Studies involving telomeric probe in Karyotype D (2n=66 chromosomes, 3m+63 st/a clade 1) not revealed ITS, however based in *Rineloricaria* features two hypothesis was formulated: (1) the diploid number reduction occurred by fusion, using karyotype F (2m+65 st/a), as an example, fusion in two chromosomes subtelocentric/acrocentric resulted in one metacentric chromosome and, consequently the diploid number reduction, resulting in the 2n=66 chromosomes. (2) In addition to chromosomal rearrangements, the possible origin to Karyotype D could be crossover between specimens with different diploid number and karyotype formula. Cross hypothetical between Karyotype A and Karyotype C, individuals with 2n = 65 and 67 chromosomes, resulted in individual with 66 chromosomes and karyotype formula corresponding to Karyotype D. The Karyotypes A, B and E (2n=65, Tab 1) characterized by increase metacentric chromosomes number and decreases acrocentric chromosomes number and maintain the same diploid number, suggest types of rearrangements of translocations and inversions types. However, not ITS evidence was reported in these karyotypes.

Similar condition involving studies telomeric probe occurred in clade 2 (2n= 64), even in the presence of a significant diploid number reduction in relation to the other clades, no ITS were detected. Even so, hypothesis that principal tool about thus expressive diploid number reduction is consequence of fusions. Furthermore, as proposed by Nanda et al. (1995) in studies in *Mus musculus domesticusosses*, losses telomeric sites has been observed on chromosomes that underwent fusions. In fishes, Barbosa et al. (2017), using the telomeric probe in *Astyanax scabripinnis* there is no evidence of ITS, thus suggesting that possible rearrangements (centric fusions and/or paracentric inversions) able to lead to interstitial locations of ITS are not frequent, or even that interstitial telomeric sequences are rapidly eliminated from the genome of these fishes. Similar circumstance may be to explain the ITS absence in the karyotype H (clade 2).

As reported by Cius et al. (cap 1), Karyotypes J (2n=66 and 65) and Karyotype L (2n=65) reveals a mosaic condition, here, we realized FISH with DNA telomeric probe, only Karyotype L (L1 and L3) showed ITS in metacentric and subtelocentric chromosomes (Fig 8), suggested inversions pericentric events. We suggested that condition have originated due to a post-zygotic nondisjunction, however, even with ITS identification, it was not possible to explain the "starting point" for the variation of karyotypic formulas found in this specimens.

A few *Rineloricaria* species presented DNA sequences located in pericentromeric regions and between centromeres and the real telomeres suggested remnants of fusion or inversion/translocations events (Rosa et. al 2012; Errero-Porto 2014; Primo et al. 2017). Ruiz-Herrera et al. (2008) in studies in mammalian revised the knowledge on two types of ITS: 1) heterochromatic ITS (het-ITS) extended blocks of telomeric-like DNA mainly in centromeric or pericentromeric chromosomes, and 2) short ITSs (s-ITSs), that are stretches of limited numbers of telomeric hexamers distributed at internal positions and presumably present in all vertebrate species. In studies involving anuran species, a new category of ITS are found, euchromatic-ITS (euITS) which was allocated into: (1)

restricted euITS, restricted to few euchromatic regions with a random distribution pattern, and (2) dispersed eu-ITS, presented in many euchromatic regions (Schmid and Steinlein 2016). Recently, Primo et al. (2017) indicated het-ITS in *Rineloricaria latirostris*, and as suggested by Ruiz Herrera et al. (2008), there are considered as unstable regions, where fissions and inversions might occur during karyotype evolution. Thus, ITS found in metacentric Karyotypes C and L3 (present study) could be considered het-ITS. Beyond ITS in metacentric chromosomes, Karyotypes C, I, K, L1 and L3 showed ITS in NOR pair, which present a large amount of constitutive heterochromatin (Cius et al cap1) as showed in Figure 13. In some cases, these ITSs have been associated with the presence in these sites of repetitive sequences similar to those present in telomeres, or the (TTAGGG)n repeats are important component in the repetitive DNA of the heterochromatin itself (Meyne et al. 1990; Ocalewicz, 2013). Bruschi et al (2014) conducted studies in *Phyllomedusa*, were telomeric repeats were frequently found in association with heterochromatin regions, and the authors suggested that (TTAGGG)n repeats can be an important component of this heterochromatin and does not appear to be a remnant of structural chromosome rearrangements. Thus, in present study, we suggest that ITS in NOR pair does not involved structural chromosome rearrangements. In addition to sites of repetitive sequences similar to those present in the telomeres, the NOR pair also share sequences similar to $(GA)_{15}$ microsatellites (Fig 13).

The chromosomal mapping of the $(CA)_{15}$ and $(GA)_{15}$ microsatellite repeats showed pattern were well dispersed, but were accumulated primarily in the subterminal and centromeric regions, as found in study with $(GA)_{15}$ and $(A)_{30}$ microsatellites in *Rineloricaria latirotris* (Vanzela et al. 2002), in addition, in present study also some centromeric regions. In clade 1, there was a few distribution of $(CA)_{15}$ along the chromosomes, except for the Karyotype G that showed more evident markings (Fig 4), as well as in the karyotype I and J1 (clade 3). The distribution of microsatellite between the three clades did not present large difference. The interesting feature of $(GA)_{15}$ signals coinciding with the 18S rDNA sites in some Karyotypes (A, E, C, I, K, L1, L2 and L3) was found only present study. However, in others fishes the 5SrDNA sites coinciding with some microsatellites and this colocalization can stabilize DNA structures, acting as 'hot spots' for recombination (Merlo et al. 2010; Yano et al. 2014; Piscor and Parise-Maltempi 2016). In study conducted by Piscor and Parise-Maltempi (2016) in Astyanax, the authors suggested that dispersed pattern, as a found in present study, its evolution and distribution has been occur a free way of spreading and/or grouping. Modest blocks of heterochromatin were observed, mainly in the pericentromeric and centromeric chromosomal regions in all species (Cius, in preparation). As propose by Csink and Henikoff (1998) repetitive sequences can be showed in telomeric and centromeric regions that are rich in heterochromatin. The association of microsatellites with heterochromatinization regions suggests the involvement of this class of DNA in the amplification and differentiation of these chromosomes (Barbosa et al. 2017).

Conclusion

The expressive karyotype variability detected in three *Rineloricaria* lineages and extensive polymorphism numerical and structural chromosomal found in two lineages from Iguaçu River, suggested closely linked with the presence of repetitive elements, essentially involving telomeric sequences. The physical mapping of telomeric DNA supported the hypothesis that fusion and inversion chromosomal rearrangements were one of the main events to fascinating karyotypic variability, both inter and intrapopulational, found in these three lineages of *Rineloricaria*. Although the co-

localization of the two repetitive classes of DNA detected in the *Rineloricaria* genome reinforces the strong linkage between them in the eukaryotic genome, only $(GA)_{15}$ presented co-localization with region of 18S rDNA, raising questions about the constitution of the sequences corresponding to the NOR pair. In addition, the microsatellite pattern shared among the three lineages corroborate with the suggestion that recent divergence. In addition to these events, we suggested the maintenance of this polymorphism occur by crosses between individuals of the same lineage, with formation of viable gametes. Thus, the results of the present study provide new insights into the constitution and understanding of the rearrangements chromosomal of the three *Rineloricaria* lineages, probably in transition in the evolutionary process.

Acknowledgments

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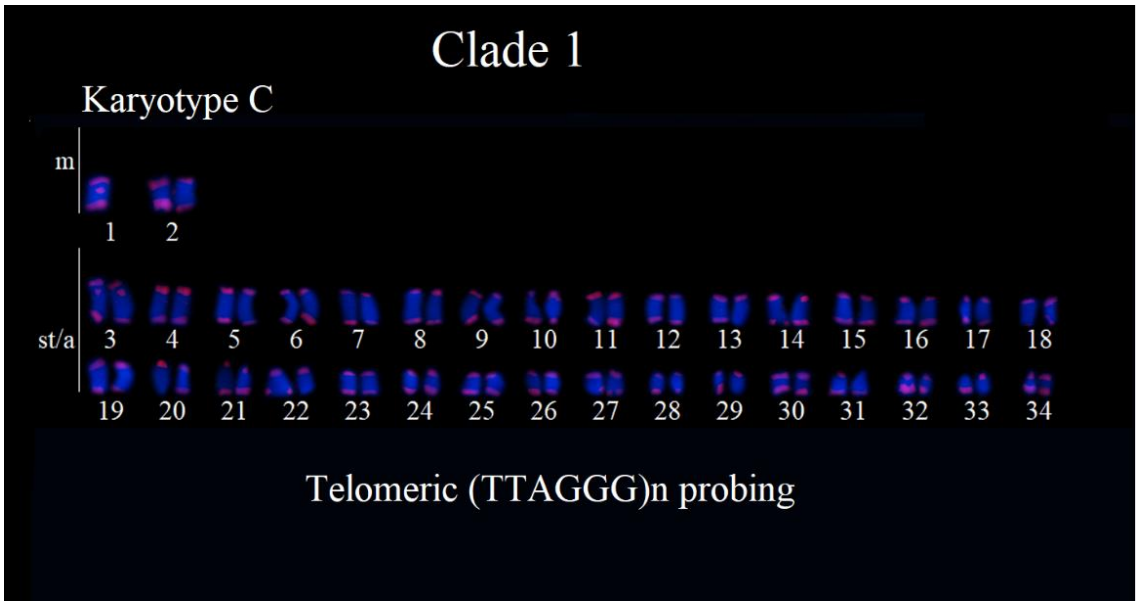


Fig. 1: *In situ* fluorescence hybridization using probe of (TTAGGG)*n* in karyotype C of clade 1 from Iguaçu River in southern South America. Chromosomes were counterstained with DAPI. The ITS are in NOR pair and metacentric chromosomes.

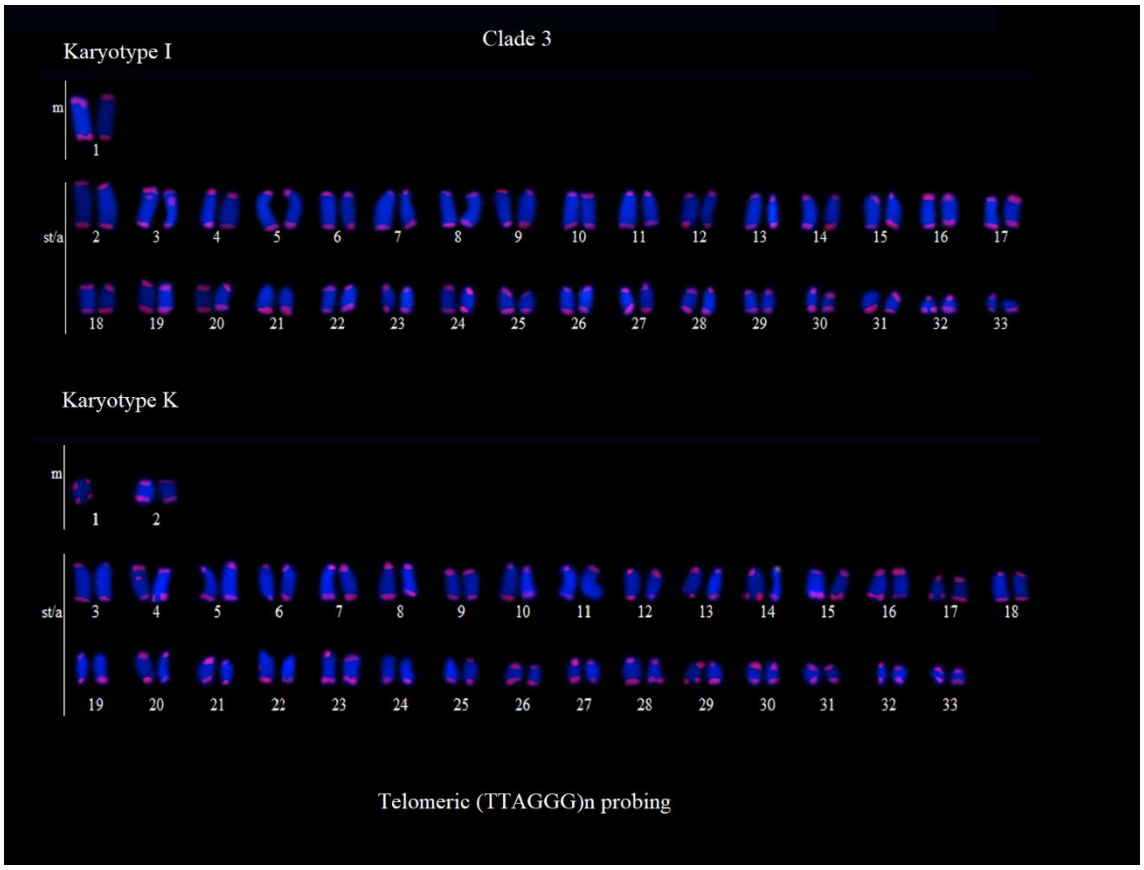


Fig. 2: *In situ* fluorescence hybridization using probe of (TTAGGG)*n* in Karyotypes (I and K) of clade 3 from Iguaçu River in southern South America. Chromosomes were counterstained with DAPI. The ITS are in NOR pair and metacentric chromosomes.

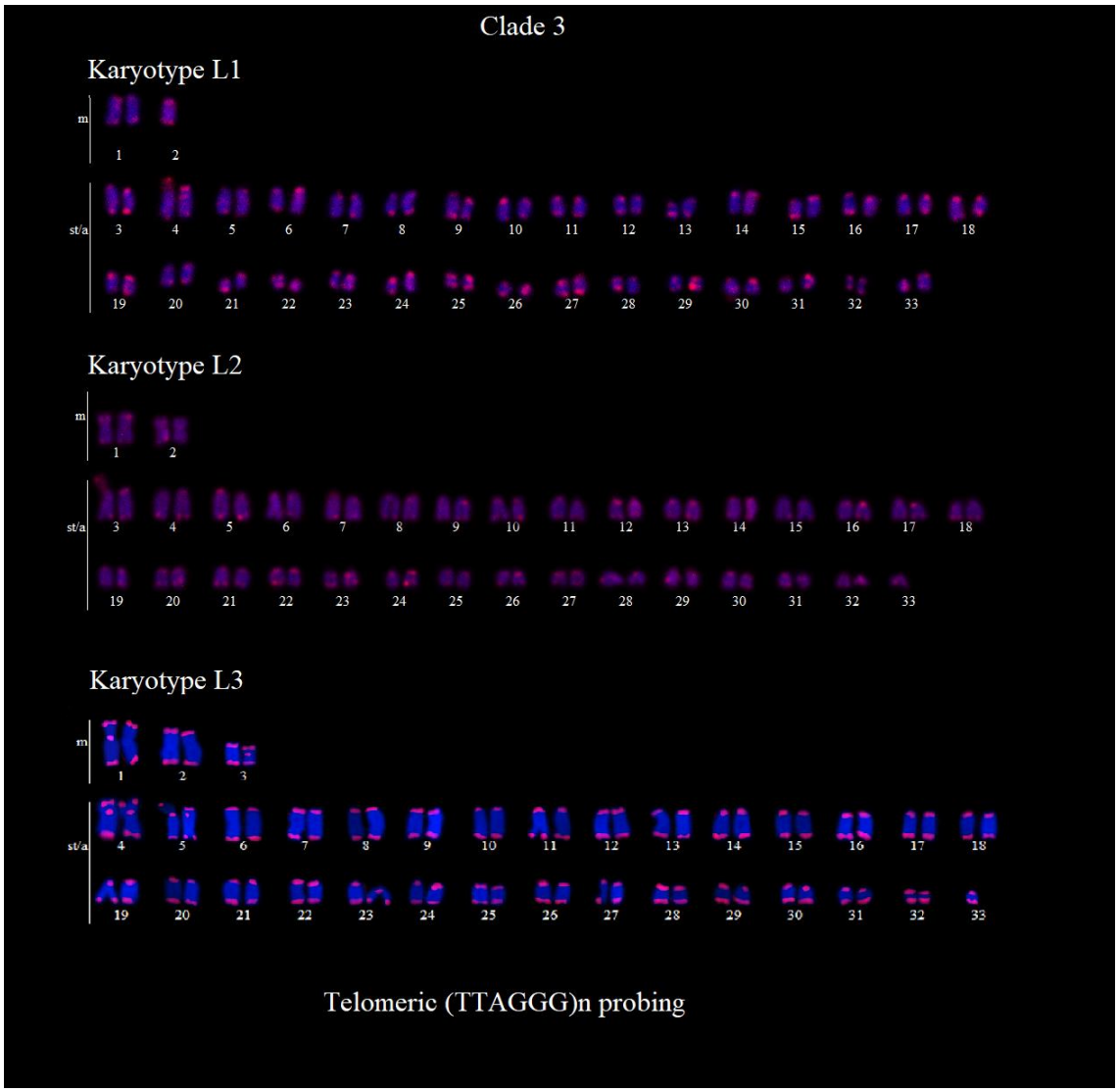


Fig. 3: *In situ* fluorescence hybridization using probe of (TTAGGG)*n* in Karyotypes (L1; L2 and L3) of clade 3 from Iguaçu River in southern South America. Chromosomes were counterstained with DAPI. The ITS are in NOR pair and metacentric chromosomes.

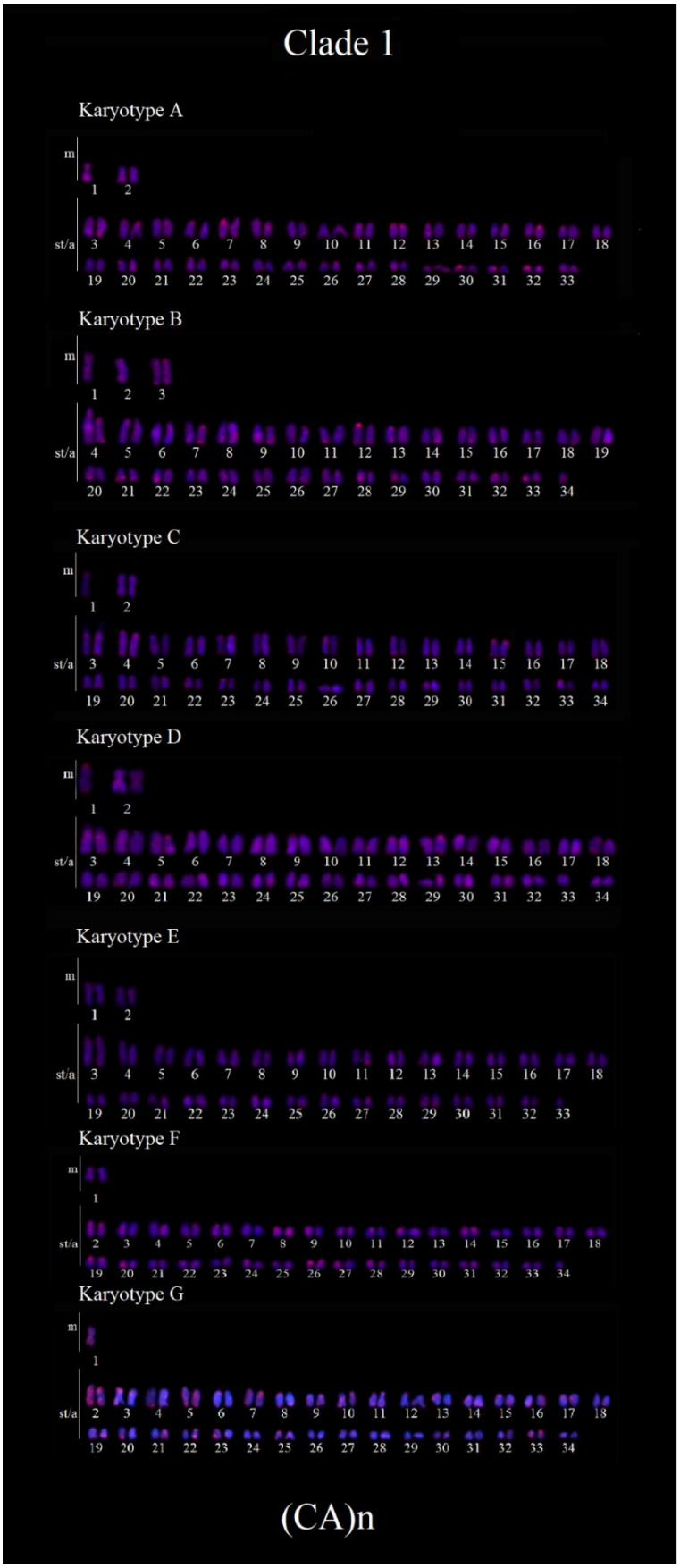


Fig. 4: *In situ* fluorescence hybridization using microsatellite $(CA)_{15}$ DNA probes (red signals) in karyotypes (A-G) of clade 1 from the Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.

Fig. 5: *In situ* fluorescence hybridization using microsatellite $(CA)_{15}$ DNA probes and $(GA)_{15}$ DNA probes (below) (red signals) in clade 2 with the only Karyotype H from the Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.

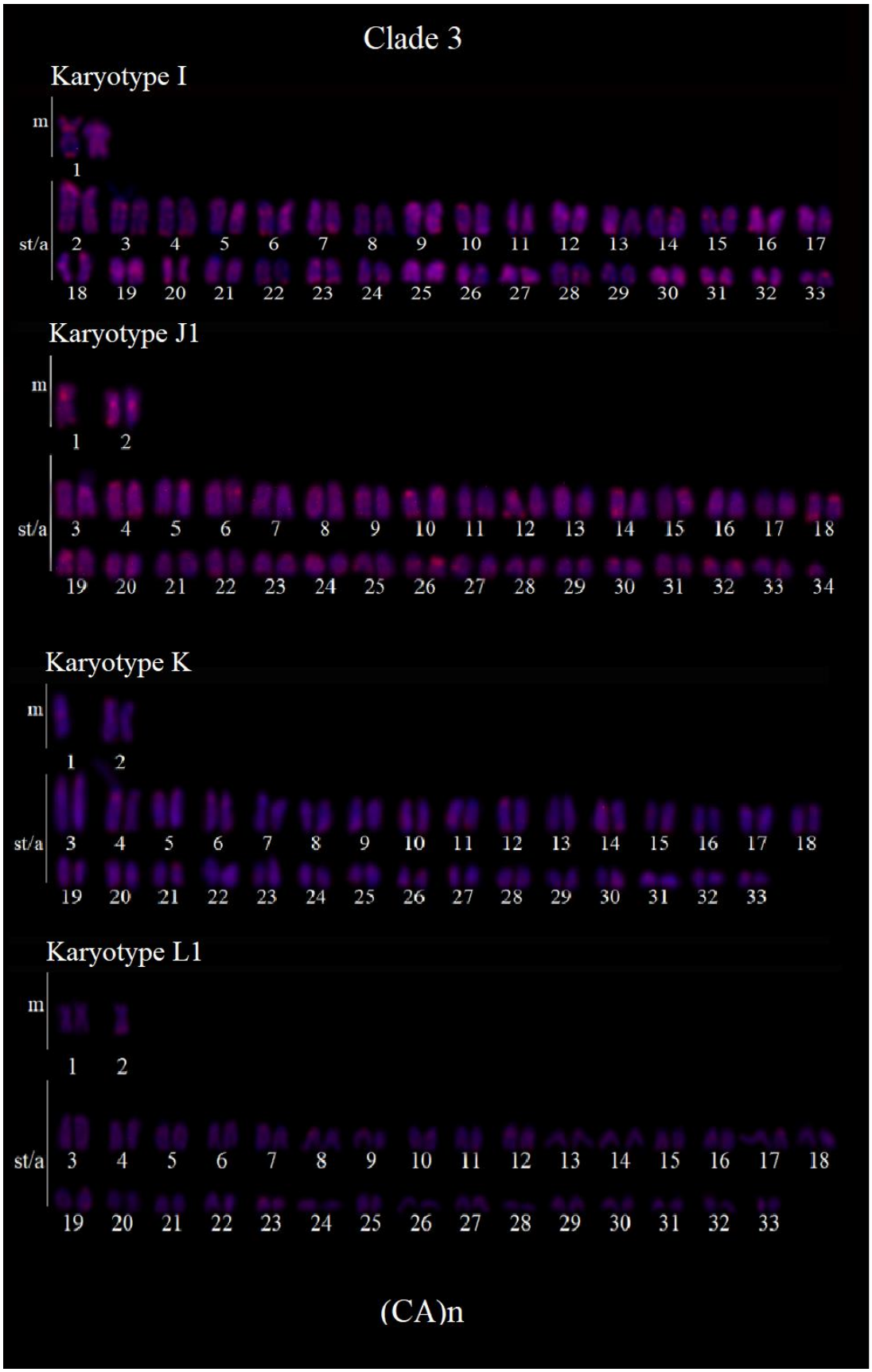


Fig. 6: *In situ* fluorescence hybridization using microsatellite $(CA)_{15}$ DNA probes (red signals) in Karyotypes (I-L1) of clade 3 from the Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.

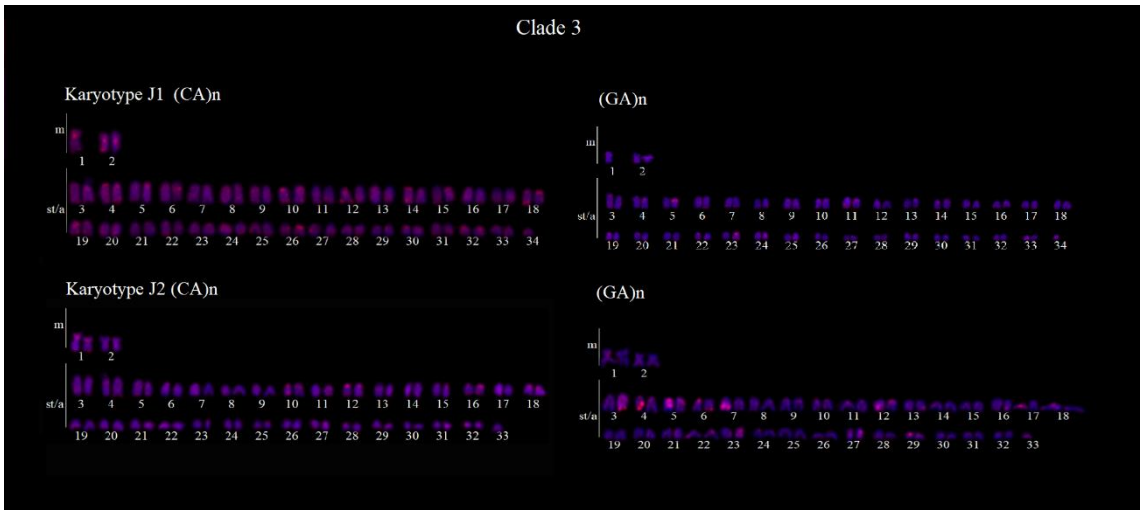


Fig. 7: *In situ* fluorescence hybridization using microsatellite $(CA)_{15}$ DNA probes (red signals) and $(GA)_{15}$ DNA probes (below) in Karyotypes (J1-J2) of clade 3 from the Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.

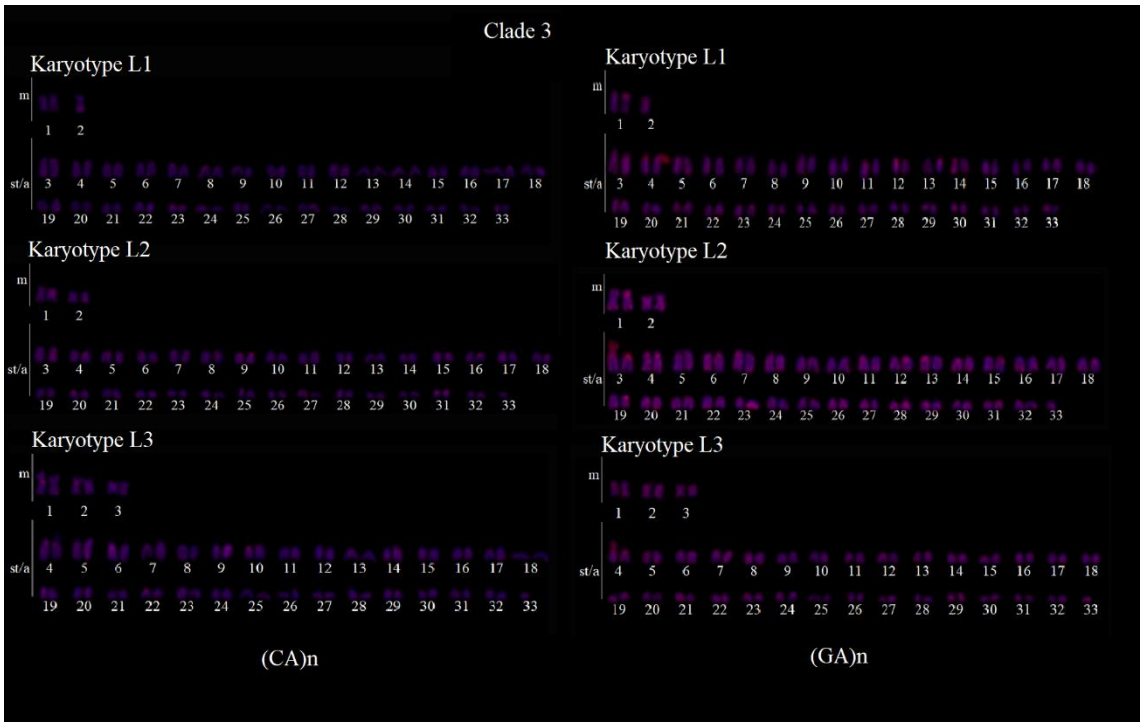


Fig. 8: *In situ* fluorescence hybridization using microsatellite $(CA)_{15}$ DNA probes and $(GA)_{15}$ DNA probes (below) (red signals) in Karyotypes (L1-L3) of clade 3 from the Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.

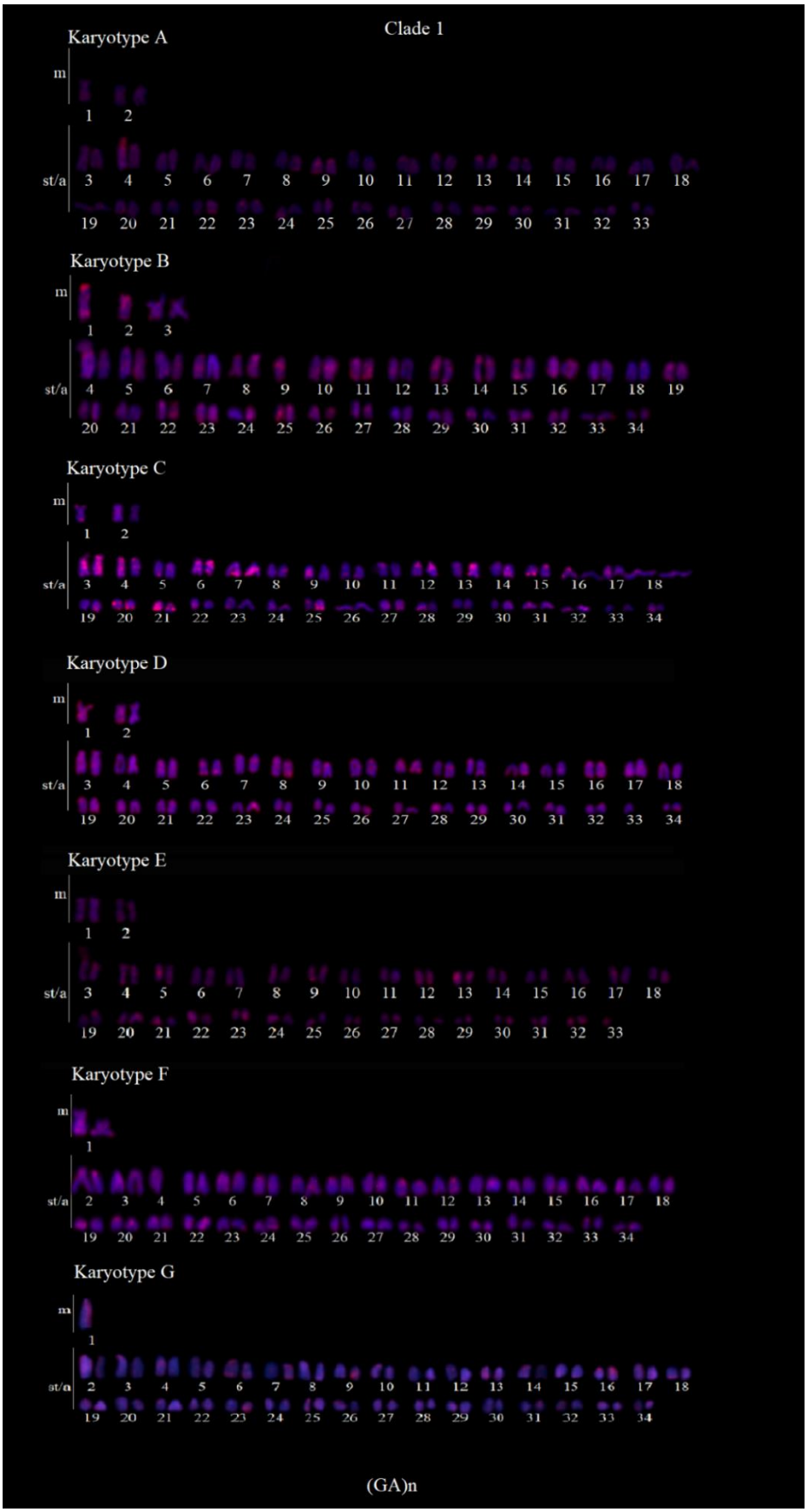


Fig. 9: *In situ* fluorescence hybridization using microsatellite $(GA)_{15}$ DNA probes (red signals) in Karyotypes (A-G) of clade 1 from the Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.

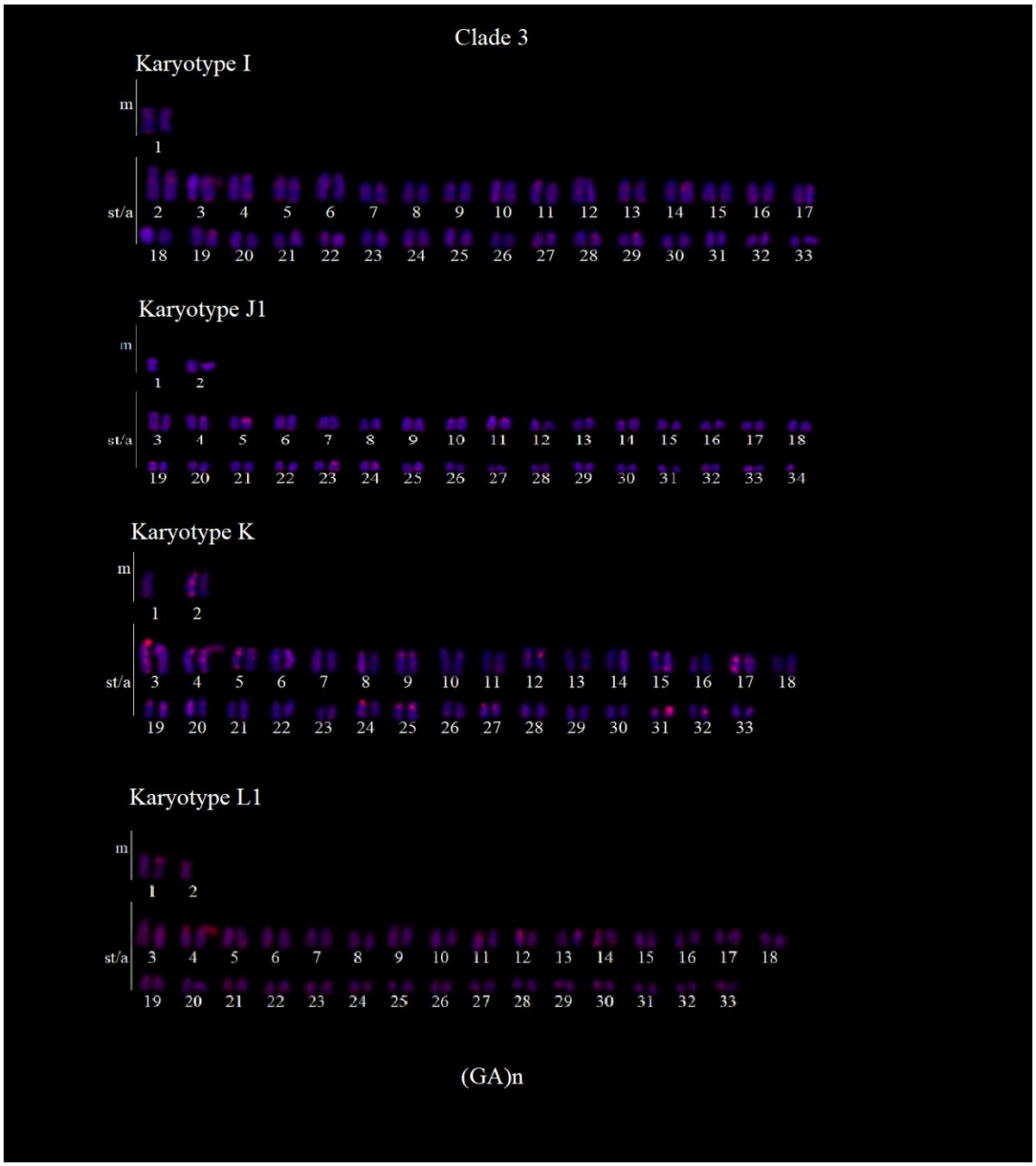


Fig. 10: *In situ* fluorescence hybridization using microsatellite $(GA)_{15}$ DNA probes (red signals) in Karyotypes (I-L1) of clade 3 from the Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.

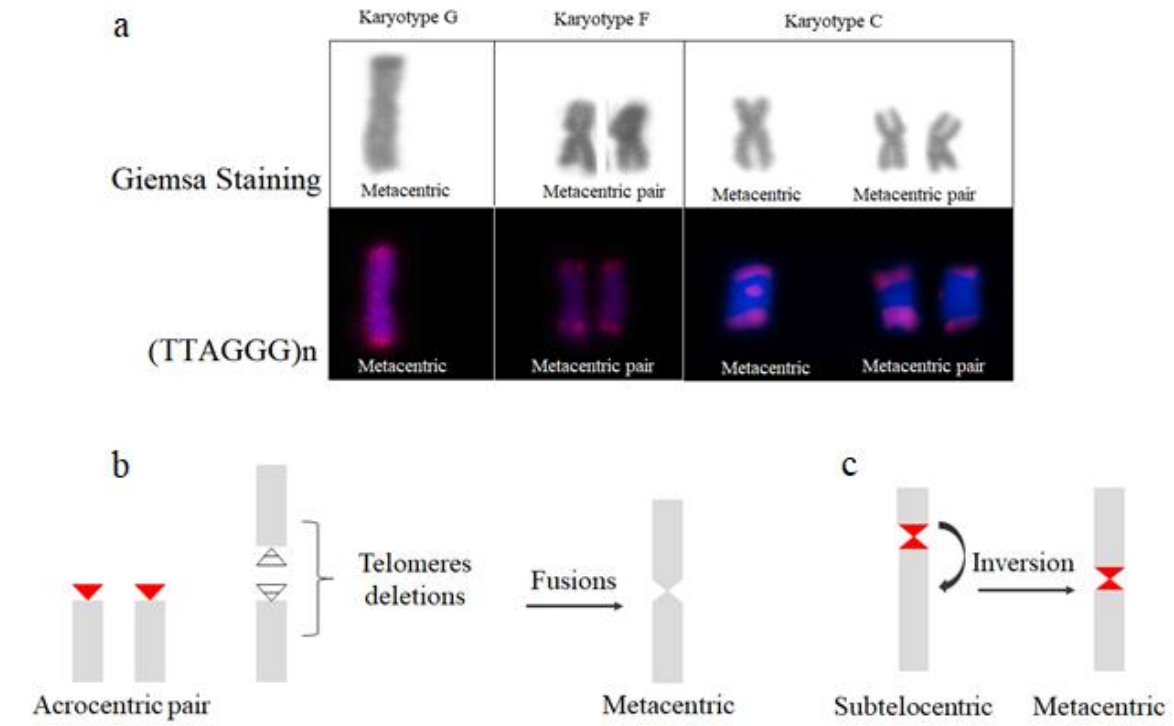


Fig. 11: In (a) metacentric chromosomes of *Rineloricaria* from Iguaçu River (Karyotypes G, F and C) in Giemsa staining and telomeric (TTAGGG)n probing (red color); in (b) fusion chromosomes origin metacentric chromosome belong Karyotype G without ITS vestiges; (c) inversion chromosome origin metacentric chromosome belong Karyotype C with ITS evidence (red color).

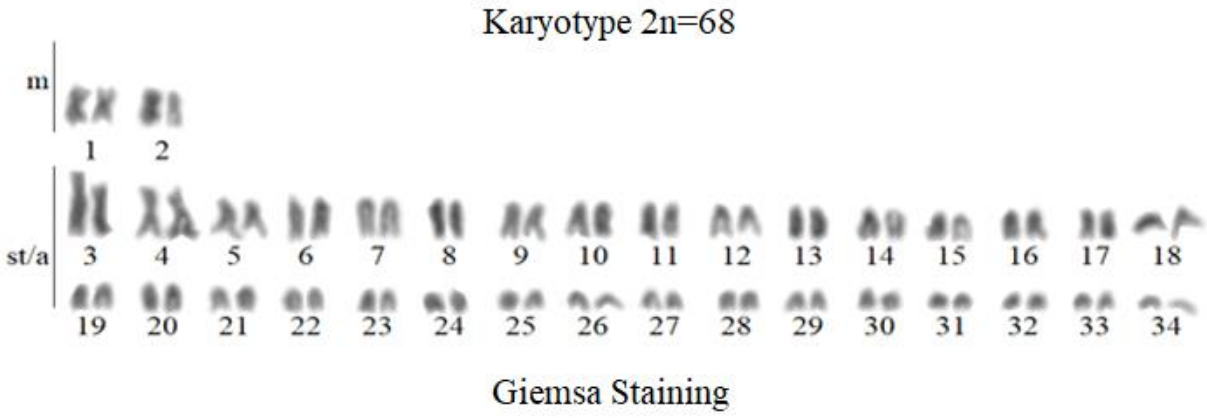


Fig. 12: Karyotype of *Rineloricaria* from Iguaçu River showed 2n=68 chromosomes with Giemsa staining.

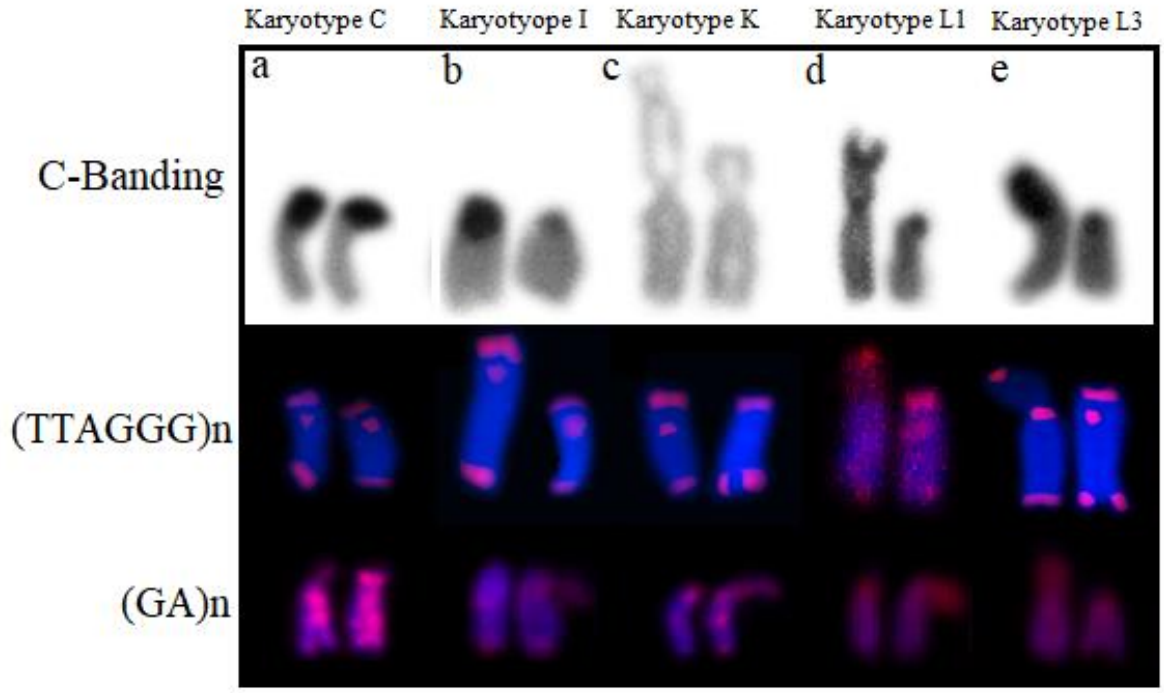
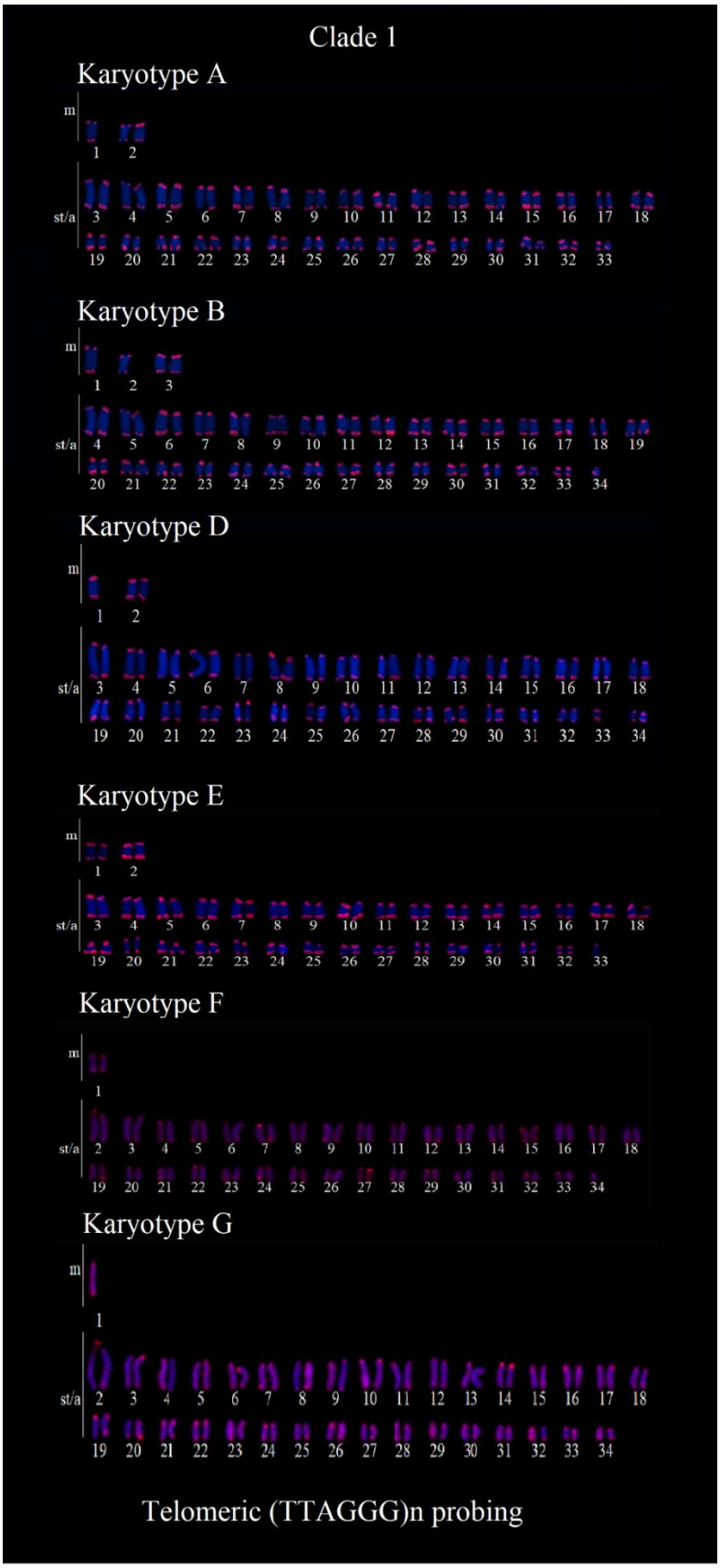
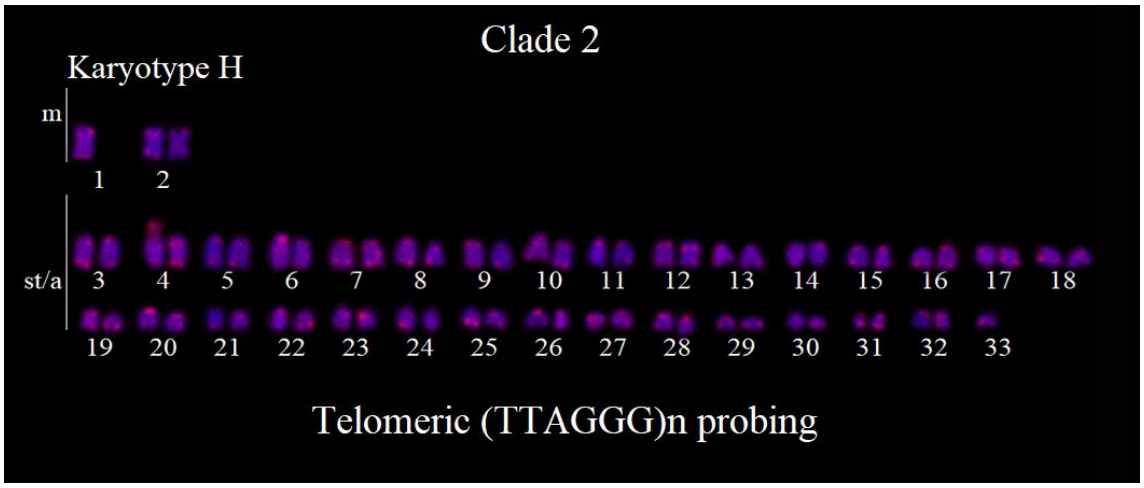


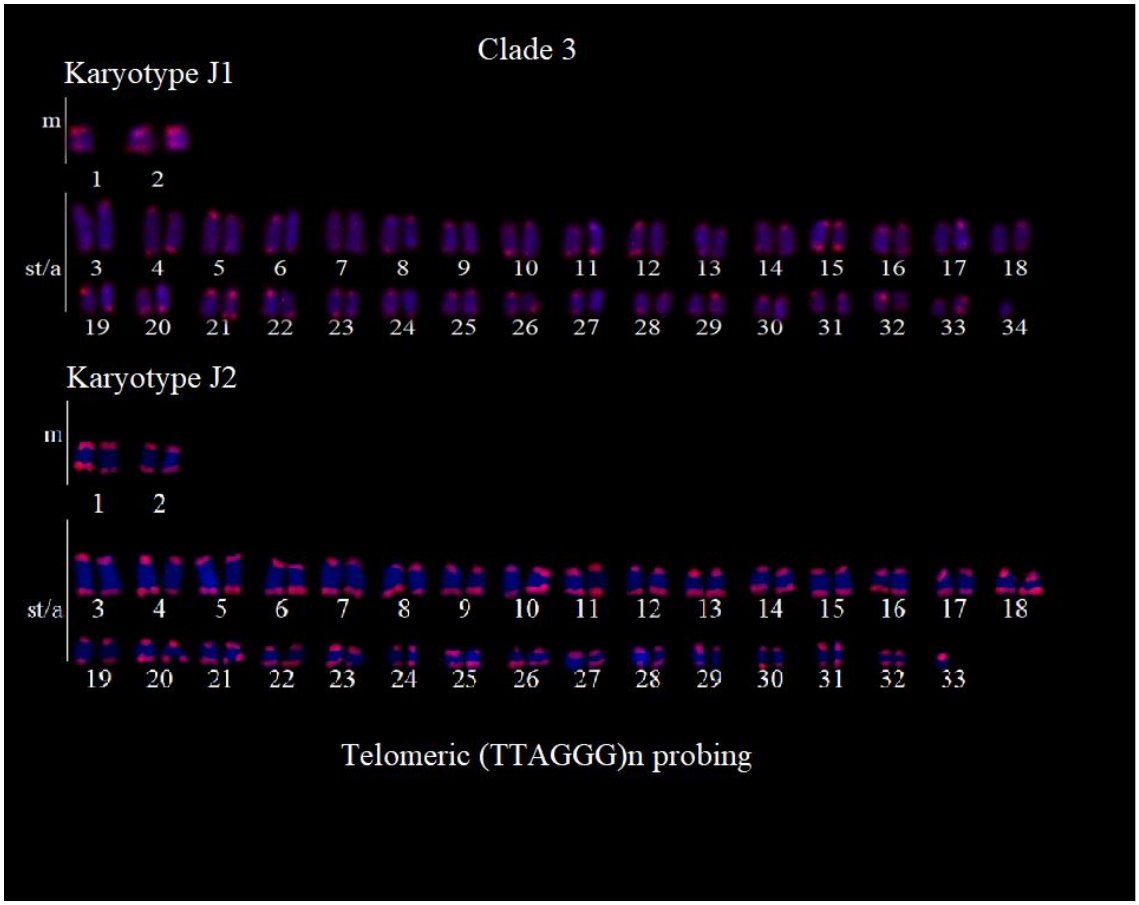
Fig. 13: In NOR pair with C-banding and telomeric (TTAGGG)n probing (red color) (below) of *Rineloricaria* from Iguaçu River; (a) Karyotype C; (b) Karyotype I; (c) Karyotype K; (d) Karyotype L1; (e) Karyotype L3.



S1: *In situ* fluorescence hybridization using probe of (TTAGGG)*n* in Karyotypes (A, B, D, E, F e G) of clade 1 from Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.



S2: *In situ* fluorescence hybridization using probe of (TTAGGG)*n* in Karyotypes H of clade 2 from Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.



S3: *In situ* fluorescence hybridization using probe of (TTAGGG)*n* in Karyotypes (J1 and J2) of clade 3 from Iguaçu River in southern South America. Chromosomes were counterstained with DAPI.

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