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Diversidade genética e citogenética de espécies de *Astyanax*, *Psalidodon* e *Oligosarcus* (Pices, Characidae) da bacia do rio Ivaí, PR

Maringá
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Tese apresentada ao programa de Pós-Graduação em Ciências Biológicas (área de concentração-Biologia Celular e Molecular), da Universidade Estadual de Maringá como requisito para a obtenção do grau de Doutor em Ciências Biológicas.

Orientadora: Profa. Dra. Ana Luíza de Brito Portela Castro

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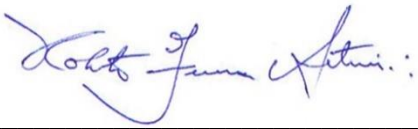

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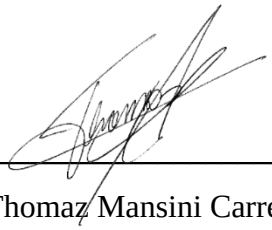

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BIOGRAFIA

Isabelle Pereira Mari Ribeiro, filha de Valdir Donizete Mari e Valmira Pereira Mari, nasceu em 22 de setembro de 1993 na cidade de Cianorte, Paraná.

Em 2007 concluiu o Ensino Fundamental, na Escola Estadual Professor Leo Kohler, e em 2010 o Ensino Médio no Colégio Estadual Helena Kolody, ambos no município de Terra Boa, Paraná.

Em 2011 ingressou no curso de Ciências Biológicas da Universidade Estadual de Maringá, o qual concluiu no ano de 2014 recebendo o título de licenciatura e bacharelado.

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A todos que, de alguma forma, contribuíram para a realização deste trabalho.

APRESENTAÇÃO

Esta tese é composta por três artigos científicos completos, os quais são apresentados abaixo.

Artigo um, será submetido ao periódico *Organisms Diversity & Evolution*:

Isabelle Pereira Mari-Ribeiro, Bárbara Scorsim, Leandro Ranucci Silva, Vania Aparecida, Alessandra Valéria de Oliveira and Ana Luiza de Brito Portela Castro. "Identification of *Astyanax* and *Psalidodon* species from the Ivaí river basin (upper Paraná river, PR) based on molecular and cytogenetic data."

Artigo dois, será submetido ao periódico *Genetics and Molecular Biology:*

Isabelle Pereira Mari-Ribeiro, Leandro Ranucci Silva, Marcos Otávio Ribeiro and Ana Luíza de Brito Portela Castro. "Karyotype variability among allopatric populations of the Neotropical fishes *Psalidodon fasciatus* and *Psalidodon aff. paranae* with repetitive sequences mapping."

Artigo três, foi submetido e aceito no periódico *Zebrafish*:

Isabelle Pereira Mari-Ribeiro, Bárbara Scorsim, Alessandra Valéria de Oliveira and Ana Luiza de Brito Portela Castro. "Cytogenetic and molecular characterization of *Oligosarcus pintoi* (Characidae): A new record of supernumerary chromosome in this species."

RESUMO GERAL

Characidae representa a quarta família mais diversa de peixes, e a mais rica entre os peixes neotropicais, reunindo 148 gêneros onde distribuem-se 1.149 espécies, as quais apresentam significativa importância ecológica e evolutiva. Dentre os gêneros que compõem Characidae, *Astyanax* Baird & Girard, 1854 se destaca por ser o mais especioso; seus representantes apresentam tamanhos entre 40mm e 200mm e morfologia conservada. Estudos filogenéticos classificam *Astyanax* como um grupo polifilético, para o qual recentemente foi sugerida uma nova classificação a partir da combinação de dados moleculares e morfológicos que realocou os "complexos de espécies" *Astyanax fasciatus* Cuvier, 1819 e *A. scabripinnis* Jenyns, 1842 em *Psalidodon* Eigenmann, 1911. *Oligosarcus* representa outro gênero de importância em Characidade, com espécies distribuídas em grande parte da região Neotropical. Estudos ictiofaunísticos sobre a região Neotropical concentram-se em ambientes fluviais de grande porte, enquanto pequenos rios e córregos ainda são pouco estudados; estes últimos comportam geralmente espécies de menor porte, que apresentam alto grau de endemismo e ocupam micro habitats específicos, tendo assim uma diversidade ainda considerada subestimada. A complexidade encontrada em alguns gêneros da família Characidae pode ser atribuída a presença de espécies morfologicamente semelhantes e em alguns casos delimitações taxonômicas pouco detalhadas, o que acaba tornando difícil a correta identificação das mesmas por métodos taxonômicos tradicionais. Nesse sentido, o uso de marcadores de DNA, associado à outras fontes de evidências como citogenética e morfologia têm sido cada vez mais explorados para real avaliação da diversidade em certos grupos. As espécies estudadas no presente trabalho foram coletadas em quatro diferentes tributários do rio Ivaí (bacia do alto rio Paraná): rio Keller, córrego Itiz, córrego Dezenove e rio dos Índios. A partir da associação das metodologias baseadas em marcadores moleculares de DNA mitocondrial *COI* (Citocromo Oxidase Subunidade I), *ATPase* (ATP sintase subunidade 6/8) e *ND2* (NADH desidrogenase subunidade 2) e citogenéticos (AgNOR, Banda C, 5S e 18S rDNA e microssatélites) foi possível a identificação de pelo menos cinco espécies para as localidades amostradas: *Astyanax lacustris*, *Psalidodon fasciatus*, *Psalidodon paranae*, *Psalidodon* aff. *paranae* e *Oligosarcus pintoi*. Os dados citogenéticos para as cinco espécies demonstraram variações relacionadas ao número de cromossomos, fórmula cariotípica, presença de cromossomo B distribuição da heterocromatina constitutiva, número e posição as regiões organizadoras de nucléolo e distribuição e quantidade de sequências repetitivas, as quais permitiram diferenciar as populações. Sendo assim, a utilização conjunta dos marcadores moleculares foi imprescindível para correta identificação das espécies, bem como para identificação de diferentes haplótipos. Os dados obtidos no presente trabalho contribuem para compreensão da diversidade de pequenos caracídeos presentes em rios e córregos da bacia do rio Ivaí, conhecimentos que fornecem subsídios para futuros projetos de conservação, bem como auxiliam na resolução de incertezas nestes grupos taxonomicamente complexos.

PALAVRAS-CHAVE: Taxonomia integrativa; DNA mitocondrial; rDNA; Microssatélites.

ABSTRACT

Characidae represents the fourth most diverse family of fish, and the richest among Neotropical fish, comprising 148 genera where 1,149 species are distributed, which have significant ecological and evolutionary importance. Among the genera that make up the Characidae, *Astyanax* Baird & Girard, 1854 stands out for being the most specious; its representatives have sizes between 40mm and 200mm and conserved morphology. Phylogenetic studies classify *Astyanax* as a polyphyletic group, for which a new classification was recently suggested from the combination of molecular and morphological data that relocated the "species complexes" *Astyanax fasciatus* Cuvier, 1819 and *A. scabripinnis* Jenyns, 1842 in *Psalidodon* Eigenmann , 1911. *Oligosarcus* represents another important genus in Characidade, with species distributed in most of the Neotropical region. Ichthyofaunistic studies on the Neotropical region are concentrated in large fluvial environments, while small rivers and streams are still poorly studied; the latter generally contain smaller species, which present a high degree of endemism and occupy specific microhabitats, thus having a diversity still considered underestimated. The complexity found in some genera of the Characidae family can be attributed to the presence of morphologically similar species and, in some cases, with poorly detailed taxonomic delimitation, which ends up making it difficult to correctly identify them by traditional taxonomic methods. In this sense, the use of DNA markers, associated with other sources of evidence such as cytogenetics and morphology, have been increasingly explored for real assessment of diversity in certain groups. The species studied in the present work were collected in four different tributaries of the Ivaí river (upper Paraná river basin): Keller river, Itiz stream, Dezenove stream and Índios stream. From the association of methodologies based on molecular markers of mitochondrial DNA *COI* (Cytochrome Oxidase Subunit I), *ATPase* (ATP synthase subunit 6/8) and *ND2* (NADH dehydrogenase subunit 2), and cytogenetic (AgNOR, Band C, 5S and 18S rDNA and microsatellites) it was possible to identify at least five species for the sampled locations: *Astyanax lacustris*, *Psalidodon fasciatus*, *Psalidodon paranae*, *Psalidodon* aff. *paranae* and *Oligosarcus pintoi*. Cytogenetic data for the five species showed variations related to the number of chromosomes, karyotypic formula, presence of chromosome B, distribution of the constitutive heterochromatin, number and position of the nucleolus organizing regions and distribution and quantity of repetitive sequences, which allowed to differentiate the populations. Thus, the joint use of molecular markers was essential for the correct identification of species, as well as for the identification of different haplotypes. The data obtained in this work contribute to understanding the diversity of small characids present in rivers and streams of the Ivaí river basin, knowledge that provides support for future conservation projects, as well as helping to resolve uncertainties in these taxonomically complex groups.

KEY WORDS: Integrative taxonomy; mitochondrial DNA; rDNA; Microsatellite.

SUMÁRIO

Identification of *Astyanax* and *Psalidodon* species from the Ivaí river basin (upper Paraná river, PR) based on molecular and cytogenetic data

Isabelle Pereira Mari-Ribeiro, Bárbara Scorsim, Leandro Ranucci Silva, Vania Aparecida, Alessandra Valéria de Oliveira and Ana Luiza de Brito Portela Castro

Abstract

Astyanax is one of the most specious fish groups in the Neotropical region, with a large number of cryptic species, which represents a challenge for their correct identification through traditional taxonomic methods. *Psalidodon* is a recently resurrected genus group of species that previously belonged to *Astyanax*, more specifically those with extensive chromosomal variation, of the *scabripinnis* and *fasciatu*s complexes. In the present study, the mitochondrial genes *COI*, *ATPase 6⁄8* and *ND2* were used in conjunction with chromosomal data to identify populations of *Astyanax* and *Psalidodon* from rivers and streams of the Ivaí river basin (Paraná Basin). The results demonstrated the effectiveness of the integrative use of molecular and cytogenetic techniques, with the identification of at least three species for the sampled sites: *Astyanax lacustris*, *Psalidodon paranae* and *Psalidodon fasciatus*, which showed inter and intrapopulation karyotype variations. The data obtained enrich the ichthyofaunistic knowledge of small rivers and streams, known to be less studied, in addition to contributing to future conservation projects in these areas.

Keywords: ATPase; ND2; Karyotype; Integrative taxonomy.

Introduction

Astyanax Baird & Girard, 1854 comprises about 170 valid species, and is the most species-rich genus in Characidade, composed of medium to large size individuals with conserved morphology (Oliveira et al., 2017; Alves et al., 2020; Terán et al., 2020; Fricke, 2021). Because its representatives share similar morphological characteristics, with the presence of many cryptic species, the identification and determination of phylogenetic relationships among them is difficult, being *Astyanax* a genus composed of several species complexes (Gavazzoni et al., 2018).

Recently, Terán et al. (2020) proposed a new classification for Characidae through the analysis of 520 morphological characters and 9 molecular markers from 608 taxa, of which 98 belonged to *Astyanax*. The results confirmed the non-monophily of the group in addition to moving *Astyanax* species to six other genera. Those whose cytogenetic variability is extensive intra- and interpopulationally and are considered "species complex" such as *A. fasciatus* (Pazza et al., 2006) and *A. scabripinnis* (Moreira-Filho and

Bertollo, 1991), were relocated in the resurrected genus *Psalidodon*, while coastal species were relocated in *Deuterodon* Eigenmann, 1907, and those belonging to the complex *A. bimaculatus* (Kavalco et al., 2011), along with the North American species, remained in the genus *Astyanax.* The genus *Psalidodon* Eigenmann, 1911 is currently composed for 33 species widely distributed throughout the Neotropical region (Fricke et al., 2021).

Large part of the studies on the ichthyofauna of the Neotropical region refer to large fluvial environments, however approximately 50% of the diversity comes from small rivers and streams (Viana et al., 2013). These environments usually harbor species with 15 cm or less in length, which have a high degree of endemism and occupy several specific microhabitats, however, they are less studied than species with larger size (Castro, 1999). The Ivaí basin is one of the main ones within the Paraná basin covering an area of about 35,845 km^2 (Araújo et al., 2011). The rivers and streams that make up the basin have very heterogeneous geomorphological and hydrological characteristics with the presence of rocky beds, steep banks, and several waterfalls which contribute to the presence of a restricted and peculiar ichthyofaunal (Maier et al. 2008; Viana et al. 2013).

The most recent survey carried out for the Ivaí basin recorded 118 fish species, with Siluriformes and Characiformes representing 83.9% of them, being Characidae one of the richest families (Frota et al., 2016). Among the genera that make up the family, *Astyanax* is represented in the basin with the specie *A. lacustris*; while *Psalidodon* with *P. bockmanni*, *Psalidodon* aff. *paranae*, and *Psalidodon* aff. *fasciatus* (Frota et al., 2016; Terán et al., 2021). In a review of species of the *A. bimaculatus* subgroup, Lucena and Soares (2016) recognized *Astyanax jacuhiensis*, *A. asuncionensis* and *A. altiparanae* as new junior synonyms of *A. lacustris*, which is considered a valid species.

Cytogenetic studies carried out for *Astyanax* from the Ivaí basin found mostly species belonging to the "*scabripinnis* complex" (now genus *Psalidodon*), with chromosome numbers ranging between 46, 48 and 50, and the presence of supernumerary chromosomes as a frequently characteristic (Fernandes and Martins-Santos, 2003, 2006; Barbosa et al., 2014; Nishiyama et al., 2016), what demonstrates the complexity of the group and the difficulty of identifying these species using isolated methods.

Therefore, in the present study, we report a cytogenetic and molecular diversity of *Astyanax* and *Psalidodon* from rivers and streams of the Ivaí basin, Paraná, using the

mitochontrial markers *COI*, *ATPase* 6/8 and *ND2* combined with cytogenetic data. Our findings reaffirm the importance of the integrative taxonomy for the correct identification of species, contributing to a better understanding of phylogenetic relationships in these groups.

Materials and Methods

Species, Collection sites and Ethical aspects

Specimens of *Astyanax* and *Psalidodon* were collected at four locations: Keller river (São Miguel do Cambuí, PR), Itiz stream (Marialva, PR), Nineteen stream (Paraíso do Norte, PR), and Índios river (Cianorte, PR), all tributaries of the Ívai River, in the upper Paraná river basin (Table 1). Specimen collection was authorized by the Brazilian Environment Ministry through its Biodiversity Information and Authorization System (SISBIO). All experimental procedures were approved and certified by the Ethics Committee on the Use of Animals (CEUA - State University of Maringá, Paraná, Brazil) through decision number 4640200717. Voucher specimens are in the cataloging process in the ichthyological collection of the Limnology, Ichthyology and Aquaculture Research Center (Nupélia) at the State University of Maringá, Paraná, Brazil (Uncatalogued NUP). The access to genetic heritage was authorized by the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado - SISBIO (nº ABABA91). Specimens were anesthetized by an overdose of clove oil (Inoue et al., 2005) and manipulated only once they became unresponsive to physical stimuli (approximately 1-2 min).

Table 1. Species, number and localities sampled in the Ivaí basin.

Locality	Cytogenetic analysis	Molecular Code	Geographical Coordinates
Keller river- São Miguel do Cambuí/ PR	15	KR3, KR5, KR6, KR7	23º37’8’S and 51º51’30’W
Itiz stream- Marialva/PR	30	IS1, IS2, IS5, IS7, IS17, IS20, IS22, IS32	23º29'06"S and 51º47'30"W
Nineteen stream- Paraíso do Norte/PR	14	C34	23º15’57’S and 52º34’17’W
Índios stream- Cianorte/PR	17	----	23º55’15”S and 52º40’12”W

Molecular analysis

Total genomic DNA extraction was performed from muscle samples and carried out using the Promega Wizard ®Genomics kit, following the manufacturer's instructions. After extraction, DNA was quantified using 1% agarose gel electrophoresis by comparison with lambda DNA concentration standard. Three mitochondrial regions were partially amplified: the Cytochrome Oxidase Subunit 1 (*COI*) gene using the primers L6448-F2 (5'-TCGACTAATCATAAAGATATCGGCAC-3') and H7152 (5'-CACCTCAGGGTGTCCGAARAAYCARAA-3') described by Ivanova et al. (2007); the NADH dehydrogenase 2 (*ND2*) gene using the primers ASN (5'-CGCGTTTAGCTGTTAACTAA -3') and B-L (5'-AAGCTTTCGGGCCCATACCC -3'); and the mitochondrial subunits 6 and 8 of the ATP synthase enzyme gene (*ATPase* 6/8) using the primers ATP8.2 (5'-AAAGCRTYRGCCTTTTAAGC -3') and CO3.2 (5'-GTTAGTGGTCAKGGGCTTGGR -3').

Polymerase chain reaction (PCR) consisted of Tris-KCl [Tris-HCl 20 mM (pH 8.4), KCl 50 mM], $MgCl_2$ (1.5 mM), primers (2.5 μM each), dNTPs (0.1 mM each), DNA Taq Polimerase (1 U) and template DNA in the concentration of 5ng/ul to make up a final volume of 25 μl. For *COI* the conditions comprised an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 1 min with a final elongation cycle at 72°C for 10 min. For *ND2* initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 1 min, 52°C for 1 min and 72°C for 1 min with a final elongation cycle at 72°C for 6 min. For *ATPase* initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 1 min with a final elongation cycle at 72°C for 5 min. The amplicons were verified on 1% agarose gel by comparison with a 100bp Ladder and purified using polyethylene glycol protocol following the protocol of Rosenthal et al. (1993). For the sequencing reaction, the Big Dye Terminator kit was used. The reactions and sequencing were performed at ACTGene Análises Moleculares Ltda (RS, Brazil) using the ABI-3500 automated sequencer.

The sequences obtained were edited and aligned using BioEdit (Hall, 1999) and MEGA 7.0 (Kumar et al., 2012) software, respectively. As a criterion for choosing the species, only those that occur in Ivaí River Basin were selected (Frota et al., 2016). Due to the large number of sequences available in GenBank, only the different haplotypes, from the Paraná River basin, identified by the DnaSP 6.0 software (Rozas et al., 2017), were considered in the analyses. The Kimura-2-parameter (K2P) (Kimura, 1980) distance

and neighbor-joining gene tree was obtained using MEGA 7.0 software. *Oligosarcus argenteus* (NC044969 and MN119396) was used as an outgroup. The program PopArt (Leigh and Bryant, 2015), was used to build haplotype networks by correlating the sequences obtained for each marker with the karyotype formulae found only for the specimens of the present study.

Cytogenetic analysis

The animals were injected with 1ml of Colchicine (0.1-0.2%) per 100g body weight for 40 min. Somatic metaphases were obtained from kidney cells by the air-drying technique according to Bertollo et al. (1978). The metaphases were analyzed and photographed under an epifluorescence photomicroscope (Olympus BX51). The images were captured using the software DP controller (Media Cybernetics). The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) according to Levan et al. 1964). The fundamental number (FN) was calculated according to the chromosomal arm numbers (the chromosomes m, sm, and st were considered to contain two arms and the a one arm only).

Results

The total sequences obtained in this study consisted of 12 *COI*, 10 *ATPase* and 10 *ND2* genes, with 481bp, 531bp and 839bp respectively, after alignment and editing. The specimen KR3 presented the lowest values of distance when compared to sequences of *Astyanax lacustris* with mean K2P of 0.021 for *COI*, 0.049 for *ATPase* and 0.005 for *ND2*. The specimens KR5, KR6, KR7, IS1, IS20 and IS22, presented the lowest values of distance with *Psalidodon paranae* with mean K2P ranging from 0.009 to 0.015 for *COI*; 0.012 to 0.015 for *ATPase*; and 0.009 to 0.013 for *ND2*. Whereas specimens IS2, IS5, IS7, IS17, IS32 and C34 presented the lowest distance with *Psalidodon fasciatus* with mean K2P values ranging from 0.007 to 0.018 for *COI*; 0.011 to 0.014 for *ATPase*; and 0.007 to 0.016 for *ND2* (S2, S3 and S4 Tables).

In the genetic tree reconstruct from *COI* sequences, a clear separation of *A. lacustris* is observed, forming a very distinct group. *P. fasciatus* also forms a more conspicuous group while the haplotypes referring to *P. paranae* and *P. bockmanni* appear belonging to the same group (Fig. 1).

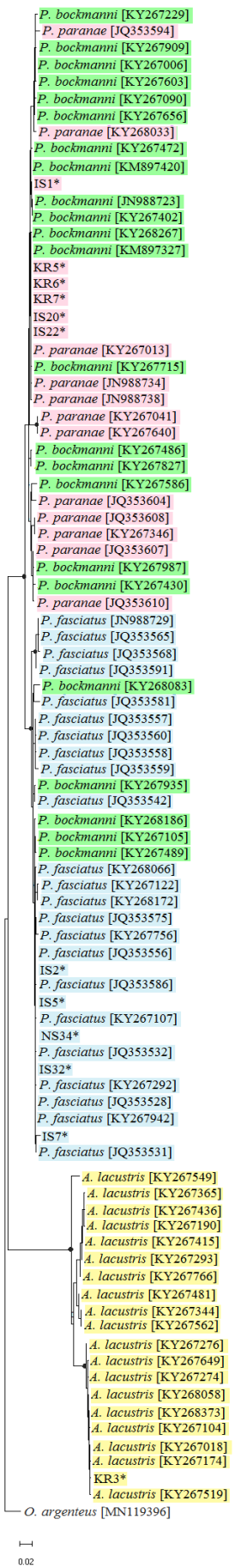


Figure 1. Neighbor joining gene tree constructed using *COI* gene sequences of *Astyanax* and *Psalidodon* species from GenBank and from the present study. Black dots on branches indicate support values above 95%. *Oligosarcus argenteus* was used as an outgroup.

The genetic tree recovered for *ATPase* was similar to that of *COI*. However, the mean K2P distances between species was greater than 2% (S2 Table), fact that did not prevent some of them from being superimposed on the tree (Fig. 2).

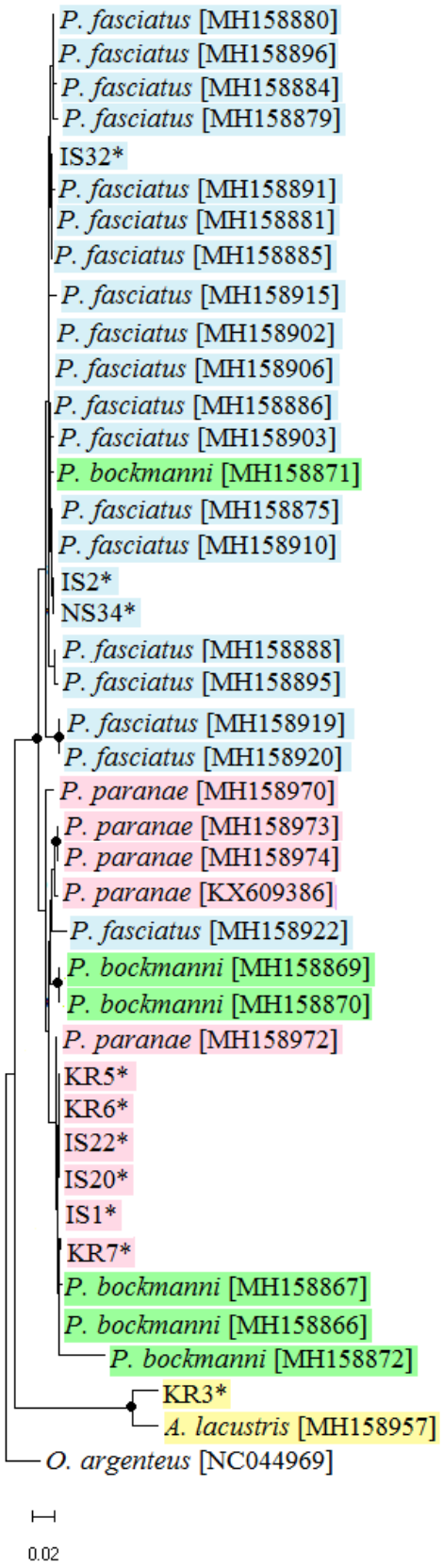


Figure 2. Neighbor joining gene tree constructed using *ATPase 6⁄8* gene sequences of *Astyanax* and *Psalidodon* species from GeneBank and from the present study. Black dots on branches indicate support values above 95%. *Oligosarcus argenteus* was used as an outgroup.

The genetic tree obtained for the *ND2* marker was construct with all sequences available in the databases (Fig. 3). In this, it is possible to clearly observe the separation of three groups with mean distance values between the species above 2% (S3 Table). The *P. bockmanni* specie could not be included in the analysis due to unavailability of sequences for this marker.

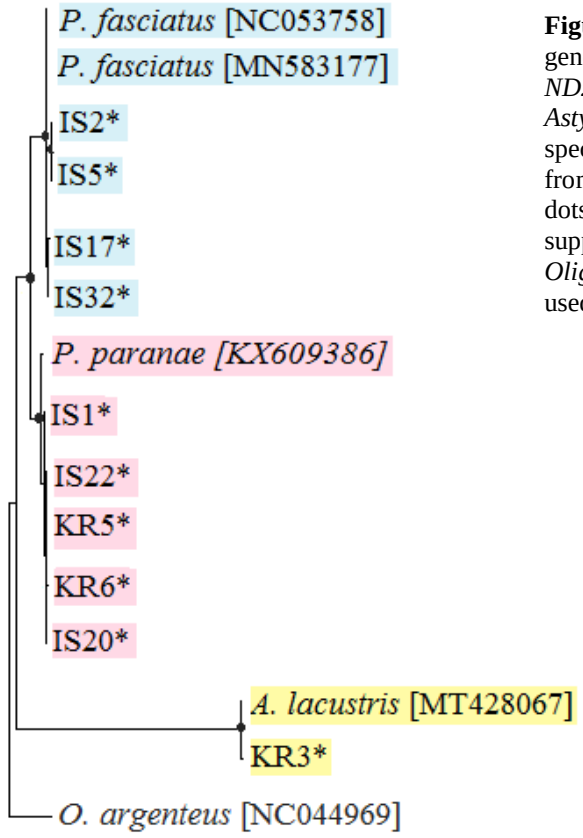


Figure 3. Neighbor joining gene tree constructed using *ND2* gene sequences of *Astyanax* and *Psalidodon* species from GeneBank and from the present study. Black dots on branches indicate support values above 95%. *Oligosarcus argenteus* was used as an outgroup.

In conjunction with the molecular analysis, the cytogenetic data are summarized in Table 2 and karyotypes represented in Figure 4. *A. lacustris* was recorded only in the Keller river, it had a high fundamental number as a result of the high number of sm chromosomes. *P. paranae* was recorded both in the Keller river and in the Itiz stream, however with inter and intrapopulation karyotype differences, as well as the presence of a B chromosome, a large metacentric with size equivalent to the first pair, for one of the

karyotypes. *P. fasciatus* was also recorded for two of the sampled locations: Itiz and Nineteen streams, where, among other characteristics, the difference in the diploid number can be highlighted. Finally, *Psalidodon* aff. *paranae* was registered for Índios river, however for this population was not possible to obtain DNA for genetic analysis.

Table 2. Cytogenetic data of the studied species of the different sampled locations.

Specie	Molecular code	Locality	2n	Kariotype Formulae	FN
A. lacustris	KR3	Keller river	50	6m+22sm+12st+12a	92
P. paranae	KR5, KR6, KR7	Keller river	50	6m+18m+12st+14a	86
P. paranae	IS1; IS20	Itiz stream	50	8m+18sm+12st+12a	88
P. paranae	IS22	Itiz stream	50+1	8m+18sm+10st+14a+1B	88
P. fasciatus	IS2, IS5. IS7, IS17, IS32	Itiz stream	48	8m+16sm+12st+12a	84
P. fasciatus	NS34	Nineteen stream	46	12m+14sm+16st+4a	88
Psalidodon aff. *paranae*	-	Índios river	48	8m+14sm+12st+14a	82

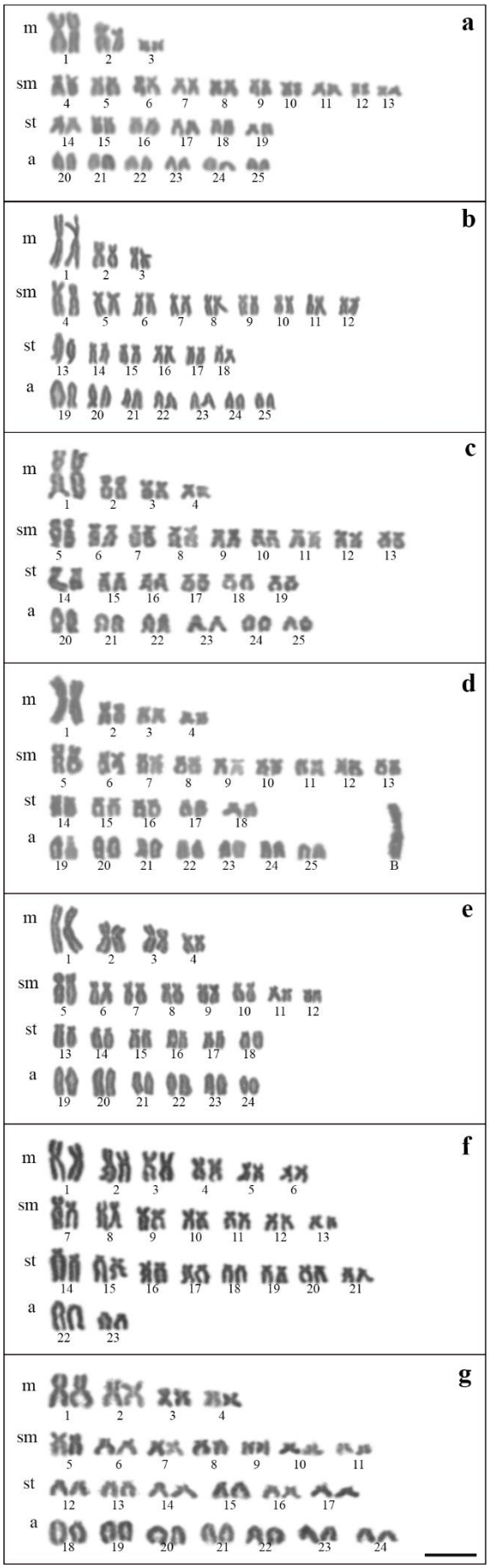
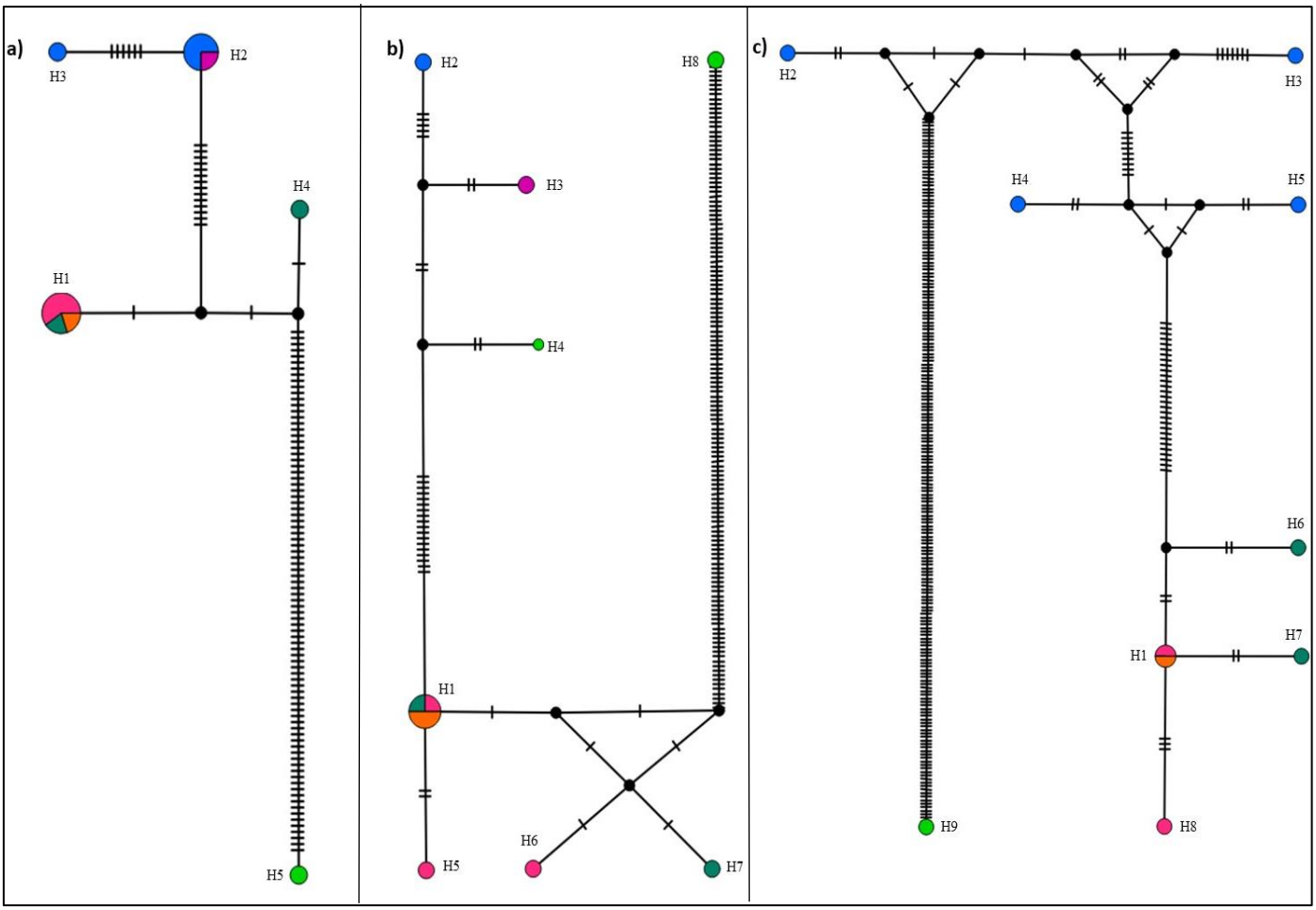


Figure 4. Karyotype of the *Astyanax* and *Psalidodon* populations sampled in different rivers of the Ivaí basin, stained with Giemsa. The colored bars represents each specie recovered in the molecular analysis. *A. lacustris* from Keller river **(a)**; *P. paranae* from Keller river **(b)** and from Itiz stream, karyotype 1 **(c)** and 2 **(d)**; *P. fasciatus* from Itiz stream **(e)** and from Nineteen stream **(f)**; *Psalidodon* aff. *paranae* from Índios river **(g)**. Bar= 10µm.

Associating the DNA sequences obtained for each marker with cytogenetic data, we can observe different numbers and types of haplotypes, the relationships of these haplotypes with the karyotypic formulas for each species are shown in Figure 5. Analyzing separately the haplotypes obtained for Keller river (Table 3) we found two haplotypes for *COI,* one corresponding to *A. lacustris* (KR3: H1) and the other corresponding to *P. paranae* specimens (KR5, KR6 and KR7: H5) (Fig. 5a). For the *ATPase* one haplotype was also recovered for *A. lacustris* (H8) while each of the *P. paranae* specimens presented an individual haplotype (KR5:H1; KR6: H5 and KR7: H6) (Fig. 5b). The distribution of haplotypes for *ND2* followed the same pattern seen for *ATPase* with one haplotype recovered for *A. lacustris* (KR3: H9) and two for *P. paranae* (KR5: H1 and KR6: H8). (Fig. 5c).

For Itiz stream (Table 3), *COI* recovered four haplotypes, two belonging to *P. paranae* (IS20 and IS22: H1; IS1: H4) and two belonging to *P. fasciatus* (IS2, IS5 and IS32: H2; IS7: H3) (Fig. 5a). *ATPase* marker also revealed two haplotypes for *P. paranae* (IS20 and IS22: H1; IS1: H7) and two for *P. fasciatus* (IS2: H2 and IS32: H4) however with different individuals in each of them (Fig. 5b). The *ND2* marker was the one that recovered the greatest number of haplotypes, generating unique haplotypes for each analyzed individual (Table 3); two for *P. paranae* without the B (IS1: H6 and IS20: H7), one for *P. paranae* with B (IS22: H1) and four for *P. fasciatus* (IS5:H2; IS2: H3, IS32: H4 and IS17: H5) (Fig. 5c).

Unique haplotype and karyotype formulae were found only for *A. lacustris* from Keller river (Fig. 5a-c). While unique karyotype formula with shared haplotype can be observed for *P. paranae* with B (orange) and *P. fasciatus* from Nineteen stream (purple).



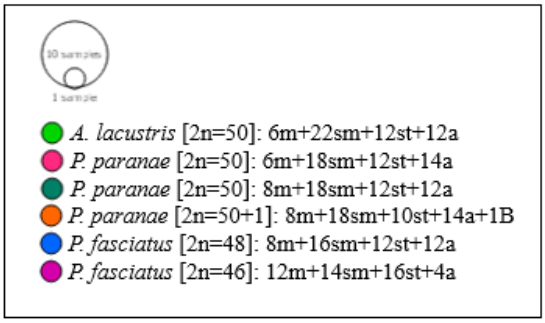


Figure 5. Haplotype network based on sequences of present study by the genes **(a)** *COI*, **(b)** *ATPase*, **(c)** *ND2*, colored according to the distribution of the karyotype formulae found in the cytogenetic analysis.

Table 3. Summary of the haplotypes obtained for each marker using the sequences of the specimens from the present study.

	Specimen	Haplotype			Identification
		COI	*ATPase*	*ND2*	
Keller river	KR3	H5	H8	H9	*A. lacustris*
	KR5	H1	H1	H1	*P. paranae*
	KR6	H1	H5	H8	*P. paranae*
	KR7	H1	H6	-	*P. paranae*
Itiz stream	IS1	H4	H7	H6	*P. paranae*
	IS2	H2	H2	H3	*P. fasciatus*
	IS5	H2	-	H2	*P. fasciatus*
	IS7	H3	-	-	*P. fasciatus*
	IS17	-	-	H5	*P. fasciatus*
	IS20	H1	H1	H7	*P. paranae*
	IS22	H1	H1	H1	*P. paranae*
	IS32	H2	H4	H4	*P. fasciatus*
Nineteen strem	NS34	H2	H3	-	*P. fasciatus*

Discussion

Species identification and characterization can be very complex since speciation is not uniform, and does not lead to changes in all aspects of an organism. Thus, the integrative taxonomy proposes the use of different characters becoming more reliable, minimizing the underestimation or overestimation of biodiversity (Gavazzoni et al., 2020). The use of multidisciplinary approaches has proven to be the most effective way to identify species and reconstruct phylogenies in species with recent diversification (Pazza et al., 2018; Terán et al., 2020).

Cytogenetics has proven to be a great tool for species characterization and for understanding evolutionary patterns through chromosomes studies in different groups (Piscor et al., 2019). This combined with DNA sequencing and morphological data have been called integrative taxonomy, which have helped to resolve taxonomic uncertainties. In this study, the identification of species such as *A. lacustris, P. paranae* and *P. fasciatus* using DNA markers enabled a comparative analysis of cytogenetic data with others representatives of the *Astyanax* and *Psalidodon* genera. However, different chromosomal characteristics such as 2n, karyotype formulae and fundamental number were observed among populations of the same species.

Analyzing separately the molecular markers used, the *COI* was the one that presented the lowest specificity, with lower distance values among the different species, especially between *P. paranae* and *P. bockmanni*, who present a mean KP2 distance of 0.002. *COI* has been a widely used marker, being successful in differentiate and identify both marine and freshwater fish species (Ward et al., 2005; Hubert et. al., 2008), however its already known that it is not as efficient for certain groups, which can be explained by the rate of evolutionary variation that is different between them (Pereira et al., 2013). In this way two fast-evolving genes (*ATPase* and *ND2*) were chosen to complement this analysis since *Astyanax* and *Psalidodon* seems to present a recent divergence as indicated by their evolutionary relationship and the presence of structured populations with distinct cytotypes (Kavalco et al., 2011; Pereira et al., 2013).

The mean K2P distances obtained for the *ATPase* marker showed higher values among the species than those obtained for the *COI* (except for *A. lacustris*), indicating that the specimens KR5, KR6, KR7, IS1; IS20 and IS22 belong to *P. paranae* since values were lower than 2%. *ATPase 6/8* has shown to be a promising marker in phylogeny and

phylogeographic analyses of diverse fish species; Pazza et al. (2018) analyzed sequences of this marker of 195 individuals from 16 nominal species of *Astyanax* that already had previously analyzed cytogenetic data, recovered four distinct clades. It is likely that this marker could be more informative if more DNA sequences are available in the database. The size of the fragment may also have influenced this aspect, since we obtained fragments of more than 900bp for the specimens of the present study; however, these had to be reduced at the time of the alignment with those from the database, generating sequences of 531bp. In the genetic tree recovered for this marker it is possible to observe species overlapping, as in the case of *P. bockmanni* grouped with *P. fasciatus* sequences; this fact may be related to the misidentification of specimens deposited in the database.

ND2 showed the greatest mean variation of K2P distance values among the different species and might be considered the best marker for ascertaining haplotype differences. Dowling et al. (2002) using *ND2* identified considerable variation in *A. mexicanus*, evidencing the existence of at least two genetically distinct lineages. Kavalco et al. (2011), grouping *ATPase* and *ND2* markers with cytogenetic data obtained phylogenetic trees that indicated the monophyly of *A. altiparanae* and *A.* aff. *bimaculatus*. Since *ND2* has been shown to be a good marker for *Astyanax* and *Psalidodon* species, a major limitation for its use is the small amount of sequences available in databases, demonstrating the importance of expanding the use of this marker for these species.

The importance of chromosomal rearrangements in the evolution of organisms has been discussed for a long time; it is already known that closely related species differ in their karyotypes, since chromosomal imbalances interfere in gametogenesis, decreasing gene flow, which can lead to reproductive isolation and consequently to speciation (Sumner, 2003). However, some organisms have karyotype plasticity and tolerate a certain degree of rearrangement in their chromosomes, therefore, these variations can result in speciation or remain as polymorphisms between populations, as observed for the populations under study (Pazza et al., 2018).

A. lacustris proved to be genetically very different and distant from the others species analyzed. Considering *A. altiparanae* and *A. lacustris* as synonyms (Lucena and Soares, 2016), the karyotypic data recorded for *A. altiparanae* (=*A. lacustris*) from different Brazilian hydrographic systems demonstrated a conserved diploid number (2n=50), however with an extensive structural chromosomal variability (Fernandes and Martins-Santos, 2004, Pazza and Kavalco, 2007; Pacheco et al., 2011). These

chromosomal differences arise between populations of *A. altiparanae* from the upper Paraná river basin have been attributed to the occurrence of chromosomal rearrangements, such as pericentric inversions during the karyotype evolution of the group (Fernandes e Martins-Santos, 2004; Pacheco et al., 2011).

P. paranae is part of the *P. scabripinnis* complex proposed by Moreira-Filho and Bertollo (1991) (formerly *Astyanax scabripinnis*), however, *P. paranae* can by itself be considered a complex of cryptic species that occur in the Upper Paraná River Basin since there is great variability in diploid numbers and cytotypes, with 2n=46, 48 and 50 (Alves et al., 2020). Analyzing different populations of *Astyanax*, whose specimens showed diagnostic characteristics of the "*scabripinnis* complex", Vicari et al. (2008) found three karyotypic forms, among which, one of them presented 2n=50, 8m+18sm+10st+14a; the specimens were collected in the proximities of the Castro region (PR), type locality for *A. paranae.* In our study, the karyotype formulas found for the populations of *P. paranae* from the Keller river and Itiz stream (table 2) present little variation in relation to that considered as representative of *P. paranae* (=*A. paranae*).

Cytogenetic studies in representatives of the *"scabripinnis* complex" were also carried out in the Ivaí river basin. Nishiyama et al. (2016) analyzing population of *Psalidodon* aff. *paranae* from the Itiz stream found 2n=50 and karyotype formula a little different from the one found in the present study, with lower number of st, but still with the presence of a large metracentric B chromosome. Fernandes and Martins-Santos (2005) and Castro et al. (2015) recorded the presence of different karyotype formulae for specimens of *P. scabripinnis* from the Tatupeba stream (Ivaí basin), with karyotype formulae varying especially in regard of the number of sm and a chromosomes, yet with a common feature, the presence of a large metacentric B chromosome.

Supernumerary chromosomes are frequent in the "*scabripinnis* complex"; they can be macrochromosomes like those observed in *P. scabrippinis* and *P. paranae* (Moreira-Filho and Bertollo, 1991), or microchromosomes like those observed in *P. bockmanni* (Daniel et al., 2012). Investigations into the origin of B chromosomes in a population of *A. paranae* from Tietê river basin (SP), by FISH mapping with B-specitifc probe generated from microdissection of a single chromosome arm of this element, support the hypothesis of the intraspecific origin of B chromosomes, that is an isochromosome (Silva et al. 2014).

P. fasciatus belong to the "*P. fasciatus* complex" initially proposed by Pazza et al. (2006) (formerly *Astyanax*), since it is composed of species that are also very similar morphologically, however, they present karyotype differences. The chromosomal numbers 2n=46 and 2n=48 are the most frequent, however, 2n=45 to 50 have already been described (Artoni et al., 2006; Pazza et al., 2006; Piscor et al., 2017). The karyotype formulae found for the Itiz and Nineteen streams populations differ somewhat from those already described for other populations of *P. fasciatus* although, a common feature is the presence of a larger number of chromosomes with two arms (submetacentric and subtelocentric chromosomes) resulting to higher fundamental numbers (Pazza et al., 2006; Medrado et al., 2008; Gavazonni et al., 2020).

Pazza et al. (2006) found a population in the Mogi-Guaçu river (São Paulo state) containing 2n=46 and karyotype formula of 12m+20sm+10st+4a, very similar to that found for the population of Nineteen stream (Fig. 5f). The same chromosome number was found by Gavazzoni et al. (2020) in a population of *P. fasciatus* from the Ijuí river (Rio Grande do Sul state), but with a karyotype formula equal to 8m+24sm+10st+4a. Medrado et al. (2008) found 2n=48 for *P. fasciatus* populations from Contas and Recôncavo Sul basins (State of Bahia), however with distinct karyotype formulae, highlighting the high number of sm chromosomes and low number of a. Ferreira-Neto et al. (2012), studying a population of *P. fasciatus* from Botucatu (SP) region, observed diploid numbers of 46, 48 and 50, as well as variations in the karyotype formulae, with demonstrated the complexity of this group.

From the joint analysis of DNA sequences and karyotype formulae it was possible to observe the presence of different haplotypes and distribution patterns of karyotype formulae. The presence of a high number of haplotypes for the same species recovered for the Paraná basin must also be highlighted. These findings can be related with the high mutation rates present in the mDNA of animals (Wilson et al., 1985; Stoeckle, 2003), some authors also suggests that small fish have a restricted dispersal capacity, which added to physical and chemical barriers leads to a decrease in the genetic flow, leading to the presence of specific genetic and cytogenetic variations in each population (Castro, 1999; Ward et al., 2009; Pereira et al., 2013).

The Neotropical region presents a high diversity of freshwater habitats, being the most diverse freshwater ichthyofaunal of the world (Rautemberg et al. 2021). The geology of the Ivaí basin presents sandy and clayey rocks that combined with the dense

drainage network and the tectonism allowed the formation of an uneven relief, with the presence of numerous waterfalls, these features contribute to the presence of a peculiar and restricted fish fauna, which together with the lack of studies in these environments result in underestimates of the diversity of fish in this basin (Araujo, 2011; Frota et al., 2016). It is already known that ichthyofaunistic studies are mostly concentrated in the main hydrographic basins, whereas minor and isolated river basins are still underrepresented. Thus, essential knowledge on patterns of genetic diversity of several species and populations and their relationship to the evolutionary history or environmental peculiarities of each basin remain unknown (Medrado et al., 2008).

From the analyses carried out in this study, it was possible to identify the presence of at least three species in the sampled locations: *A. lacustris*, *P. paranae* and *P. fasciatus*. *ATPase* and *ND2* proved to be efficient markers for the species studied, however, it is necessary to increase the number of studies mainly related to the *ND2*, since the number of available sequences is still small. Finally, this study expands the ichthyofaunistic knowledge of small rivers and streams, environments that are known to be less studied, providing subsidies for their future biodivesity.

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Supplementary Material

Table S1. GenBank sequences used in K2P distance calculations and construction of genetic trees.

Specie	Haplotype	Location	GenBank	Marker	Reference
Astyanax lacustris	4	Brazil: Botucatu, SP, Rio Tiete/Rio Parana, Rio Alambari	KY267018.1	*COI*	Rossini et al., 2016
Astyanax lacustris	8	Brazil: Nova Andradina, MS, Rio Parana, Rio Papagaio	KY267104.1	*COI*	Rossini et al., 2016
Astyanax lacustris	18	Brazil: Porto Rico, PR, Rio Parana, Corrego Caracu	KY267174.1	*COI*	Rossini et al., 2016
Astyanax lacustris	19	Brazil: Sao Paulo, Botucatu, SP, Rio Tiete, Rio Capivara"	KY267190.1	*COI*	Rossini et al., 2016
Astyanax lacustris	21	Brazil: Araras, SP, Rio Parana/La Plata Basin, Rio Araras	KY267274.1	*COI*	Rossini et al., 2016
Astyanax lacustris	22	Brazil: Avare, SP, Rio Paranapanema/Rio Parana, Rio Novo	KY267276.1	*COI*	Rossini et al., 2016
Astyanax lacustris	23	Brazil: Sao Paulo, Ipeuna/SP, Corrego Canta Galo	KY267293.1	*COI*	Rossini et al., 2016
Astyanax lacustris	26	Brazil: Colombia, SP, Rio Grande/Rio Parana, Corrego do Batata	KY267344.1	*COI*	Rossini et al., 2016
Astyanax lacustris	27	Brazil: Ivinhema, MS, Rio Parana, Rio sem nome	KY267365.1	*COI*	Rossini et al., 2016

Astyanax lacustris	28	Brazil: Conchas, SP, Rio Tiete, rio Conchas	KY267415.1	*COI*	Rossini et al., 2016
Astyanax lacustris	29	Brazil: Marapoama, SP, Rio Tiete, Ribeirao Cubatao	KY267436.1	*COI*	Rossini et al., 2016
Astyanax lacustris	31	Brazil: Colombia, SP, Rio Grande/Rio Parana, Rio Velho	KY267481.1	*COI*	Rossini et al., 2016
Astyanax lacustris	32	Brazil: Marapoama, SP, Rio Tiete, Ribeirao Cubatao	KY267519.1	*COI*	Rossini et al., 2016
Astyanax lacustris	34	Brazil: Cravinhos, SP, Rio Mogi-Guacu, Lagoa Margina rio Tamandua	KY267562.1	*COI*	Rossini et al., 2016
Astyanax lacustris	35	Brazil: Biritiba Mirim, SP, Rio Parana, Rio Tiete	KY267649.1	*COI*	Rossini et al., 2016
Astyanax lacustris	41	Brazil: Ipeuna, SP, Corrego Canta Galo	KY267766.1	*COI*	Rossini et al., 2016
Astyanax lacustris	44	Brazil: Diamante do Norte, PR, Rio Paranapanema, Corrego Fazenda Agua Mole	KY267898.1	*COI*	Rossini et al., 2016
Astyanax lacustris	49	Brazil: Marapoama, SP, Rio Tiete, Ribeirao Cubatao	KY268058.1	*COI*	Rossini et al., 2016
Astyanax lacustris	53	Brazil: Avare, SP, Rio Paranapanema/Rio Parana, Rio Novo	KY268273.1	*COI*	Rossini et al., 2016

Astyanax paranae (Psalidodon paranae)	1	Brazil: Parana, Upper Parana Basin	JN988734.1	*COI*	Pereira et al., 2013
Astyanax paranae (Psalidodon paranae)	2	Brazil: Parana, Upper Parana Basin	JN988738.1	*COI*	Pereira et al., 2013
Astyanax paranae (Psalidodon paranae)	4	Brazil: Minas Gerais, Upper Parana Basin	JQ353594.1	*COI*	Pereira (Umpublish)
Astyanax paranae (Psalidodon paranae)	7	Brazil: Parana, Upper Parana Basin	JQ353604.1	*COI*	Pereira (Umpublish)
Astyanax paranae (Psalidodon paranae)	8	Brazil: Sao Paulo, Upper Parana Basin	JQ353607.1	*COI*	Pereira (Umpublish)
Astyanax paranae (Psalidodon paranae)	9	Brazil: Sao Paulo, Upper Parana Basin	JQ353608.1	*COI*	Pereira (Umpublish)
Astyanax paranae (Psalidodon paranae)	10	Brazil: Sao Paulo, Upper Parana Basin	JQ353610.1	*COI*	Pereira (Umpublish)
Astyanax paranae (Psalidodon paranae)	15	Brazil: CarrancasMG, Rio Grande/La Plata Basin, Afluente corrego Beijinho	KY267013.1	*COI*	Rossini et al., 2016
Astyanax paranae (Psalidodon paranae)	16	Brazil: Nova Andradina, MS, Rio Parana, Rio São Bentinho	KY267041.1	*COI*	Rossini et al., 2016
Astyanax paranae (Psalidodon paranae)	17	Brazil: Sao Paulo, Botucatu, SP, Rio Tiete, Rio Capivara	KY267346.1	*COI*	Rossini et al., 2016
Astyanax paranae (Psalidodon paranae)	18	Brazil: Delfinopolis, MG, Rio Grande/La Plata Basin, Rio Claro	KY267640.1	*COI*	Rossini et al., 2016
Astyanax paranae (Psalidodon paranae)	20	Brazil: Capitolio, MG, Rio Grande/La Plata Basin, Rio Turvo	KY268033.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	1	Brazil: Sao Paulo, Upper Parana Basin	JN988723.1	*COI*	Pereira et al., 2013

Astyanax bockmanni (Psalidodon bockmanni)	3	Brazil: Parana, Upper Parana River Basin, Rio Tibagi, Londrina, Rio Taquara	KM897327.1	*COI*	Frantine-Silva et al., 2015
Astyanax bockmanni (Psalidodon bockmanni)	4	Brazil: Parana, Upper Parana River Basin, Rio Tibagi, Londrina, Rio Taquara	KM897420.1	*COI*	Frantine-Silva et al., 2015
Astyanax bockmanni (Psalidodon bockmanni)	5	Brazil: Delfinopolis, MG, Rio Grande/La Plata Basin, Rio Claro	KY267006.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	8	Brazil: Minas Gerais, Uberaba/MG, Rio Parana, Afluente rio Uberaba	KY267090.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	9	Brazil: Jataizinho, PR, Rio Parana, Ribeirao Agua da Floresta	KY267105.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	10	Brazil: Parana, Londrina/PR, La Plata Basin/Rio Tibagi, Ribeirao Cambezinho	KY267229.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	12	Brazil: Marapoama, SP, Rio Tiete, Ribeirao Cubatao	KY267402.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	14	Brazil: Sao Paulo, Bertioga/SP, Atlantico, Rio Itatinga	KY267430.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	16	Brazil: Goias, Apore/GO, Rio Parana, Riacho sem nome	KY267472.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	17	Brazil: CorumbaibaGO, Rio Paranaiba/La Plata Basin, Corrego Fazenda Balsamo	KY267486.1	*COI*	Rossini et al., 2016

Astyanax bockmanni (Psalidodon bockmanni)	18	Brazil: Jataizinho, PR, Rio Parana, Ribeirao Agua da Floresta	KY267489.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	19	Brazil: Parana, Jataizinho/PR, Rio Tibagi/Paranapanema, Riacho sem nome	KY267586.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	20	Brazil: Delfinopolis, MG, Rio Grande/La Plata Basin, Rio Claro	KY267603.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	21	Brazil: Delfinopolis, MG, Rio Grande/La Plata Basin, Rio Claro	KY267656.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	22	Brazil: Cabralia Paulista, SP, Rio Paranapanema, Riacho sem nome	KY267715.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	23	Brazil: Corumbaiba, GO, Rio Paranaiba/La Plata Basin, Corrego Fazenda Balsamo	KY267827.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	24	Brazil: Delfinopolis, MG, Rio Grande/La Plata Basin, Rio Claro	KY267909.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	25	Brazil: Goias, Chapadao do Ceu/GO, Rio Parana, Rio Formoso	KY267935.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	26	Brazil: Marapoama, SP, Rio Tiete, Ribeirao Cubatao	KY267987.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	28	Brazil: Parana, Sapopema/PR, Rio Paranapanema/Bacia do Prata, Corrego Lambari	KY268083.1	*COI*	Rossini et al., 2016

Astyanax bockmanni (Psalidodon bockmanni)	29	Brazil: Parana, Ibipora/PR, Rio Paranapanema, Rio Tibagi	KY268186.1	*COI*	Rossini et al., 2016
Astyanax bockmanni (Psalidodon bockmanni)	30	Brazil: Sao Paulo, Avare/SP, RioParanapanema/Rio Parana, Rio Novo	KY268267.1	*COI*	Rossini et al., 2016
Astyanax fasciatus (Psalidodon fasciatus)	1	Brazil: Goias, Upper Parana Basin	JN988729.1	*COI*	Pereira et al., 2013
Astyanax fasciatus (Psalidodon fasciatus)	2	Brazil: Sao Paulo, Upper Parana Basin	JQ353528.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	3	Brazil: Sao Paulo, Upper Parana Basin	JQ353530.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	4	Brazil: Sao Paulo, Upper Parana Basin	JQ353531.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	5	Brazil: Sao Paulo, Upper Parana Basin	JQ353532.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	6	Brazil: Goias, Upper Parana Basin	JQ353542.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	9	Brazil: Sao Paulo, Upper Parana Basin	JQ353556.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	10	Brazil: Parana, Upper Parana Basin	JQ353557.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	11	Brazil: Parana, Upper Parana Basin	JQ353558.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	12	Brazil: Parana, Upper Parana Basin	JQ353559.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	13	Brazil: Parana, Upper Parana Basin	JQ353560.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	15	Brazil: Goias, Upper Parana Basin	JQ353565.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	16	Brazil: Minas Gerais, Upper Parana Basin	JQ353566.1	*COI*	Pereira Unpublished

Astyanax fasciatus (Psalidodon fasciatus)	17	Brazil: Sao Paulo, Upper Parana Basin	JQ353575.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	19	Brazil: Sao Paulo, Upper Parana Basin	JQ353581.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	20	Brazil: Sao Paulo, Upper Parana Basin	JQ353586.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	21	Brazil: Goias, Upper Parana Basin	JQ353589.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	22	Brazil: Distrito Federal, Upper Parana Basin	JQ353591.1	*COI*	Pereira Unpublished
Astyanax fasciatus (Psalidodon fasciatus)	23	Brazil: Nova Andradina, MS, Rio Parana, Rio São Bentinho	KY267292.1	*COI*	Rossini et al., 2016
Astyanax fasciatus (Psalidodon fasciatus)	33	Brazil: Nova Andradina, MS, Rio Parana, Rio São Bentinho	KY267107.1	*COI*	Rossini et al., 2016
Astyanax fasciatus (Psalidodon fasciatus)	34	Brazil: Mato Grosso do Sul, Inocencio/MS, Rio Parana, Riacho sem nome	KY267122.1	*COI*	Rossini et al., 2016
Astyanax fasciatus (Psalidodon fasciatus)	42	Brazil: Sao Paulo, Avare/SP, Rio Paranapanema/Rio Parana, Rio Novo	KY267756.1	*COI*	Rossini et al., 2016
Astyanax fasciatus (Psalidodon fasciatus)	47	Brazil: Botucatu, SP, Rio Capivara (Fazenda Indiana)	KY267942.1	*COI*	Rossini et al., 2016
Astyanax fasciatus (Psalidodon fasciatus)	50	Brazil: Sao Paulo, Avare/SP, Rio Paranapanema/Rio Parana, Rio Novo	KY268066.1	*COI*	Rossini et al., 2016
Astyanax fasciatus (Psalidodon fasciatus)	51	Brazil: Mato Grosso do Sul, Inocencio/MS, Rio	KY268172.1	*COI*	Rossini et al., 2016

		Parana, Riacho sem nome			
Astyanax lacustris	1	Paraná River Basin	MH158957	*ATPase*	Pazza et al., 2018
Astyanax bockmanni (Psalidodon bockmanni)	1	Paraná River Basin	MH158866	*ATPase*	Pazza et al., 2018
Astyanax bockmanni (Psalidodon bockmanni)	2	Paraná River Basin	MH158867	*ATPase*	Pazza et al., 2018
Astyanax bockmanni (Psalidodon bockmanni)	3	Paraná River Basin	MH158869	*ATPase*	Pazza et al., 2018
Astyanax bockmanni (Psalidodon bockmanni)	4	Paraná River Basin	MH158870	*ATPase*	Pazza et al., 2018
Astyanax bockmanni (Psalidodon bockmanni)	5	Paraná River Basin	MH158871	*ATPase*	Pazza et al., 2018
Astyanax bockmanni (Psalidodon bockmanni)	6	Paraná River Basin	MH158872	*ATPase*	Pazza et al., 2018
Astyanax paranae (Psalidodon paranae)	1	Capivara river, Botucatu, São Paulo	KX609386	*ATPase*	Pazza et al., 2018
Astyanax paranae (Psalidodon paranae)	3	Paraná River Basin	MH158970	*ATPase*	Pazza et al., 2018
Astyanax paranae (Psalidodon paranae)	4	Paraná River Basin	MH158972	*ATPase*	Pazza et al., 2018
Astyanax paranae (Psalidodon paranae)	5	Paraná River Basin	MH158973	*ATPase*	Pazza et al., 2018
Astyanax paranae (Psalidodon paranae)	6	Paraná River Basin	MH158974	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	11	Paraná River Basin	MH158879.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	12	Paraná River Basin	MH158880.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	13	Paraná River Basin	MH158881.1	*ATPase*	Pazza et al., 2018

Astyanax fasciatus (Psalidodon fasciatus)	15	Paraná River Basin	MH158884.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	16	Paraná River Basin	MH158885.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	17	Paraná River Basin	MH158886.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	18	Paraná River Basin	MH158888.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	19	Paraná River Basin	MH158891.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	20	Paraná River Basin	MH158895.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	21	Paraná River Basin	MH158896.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	23	Paraná River Basin	MH158902.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	24	Paraná River Basin	MH158903.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	25	Paraná River Basin	MH158906.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	26	Paraná River Basin	MH158910.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	27	Paraná River Basin	MH158915.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	28	Paraná River Basin	MH158919.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	29	Paraná River Basin	MH158920.1	*ATPase*	Pazza et al., 2018
Astyanax fasciatus (Psalidodon fasciatus)	30	Paraná River Basin	MH158922.1	*ATPase*	Pazza et al., 2018
Astyanax lacustris	1	Brazil: Abaete river basin	MT428067	*ND2*	Pasa et al. Unpublished

Astyanax paranae (Psalidodon paranae)	1	Capivara river, Botucatu, São Paulo	KX609386	*ND2*	Silva et al. (2016)
Astyanax fasciatus (Psalidodon fasciatus)	1	Corrego das Araras stream (Mogi-Guaçu river basin	MN583177	*ND2*	Calegari et al. (2019)
Astyanax fasciatus (Psalidodon fasciatus)	2	Brazil: Bambui river basin	NC_053758	*ND2*	Pasa et al. Unpublished

Table S2. K2P distance values between *COI* gene sequences obtained in GenBank, grouped according to species, and *Astyanax* and *Psalidodon* sequences obtained in the present study.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. IS 1															
2. IS 2	0.030														
3. IS 5	0.030	0.000													
4. IS 7	0.041	0.010	0.010												
5. IS 20	0.006	0.028	0.028	0.038											
6. IS 22	0.006	0.028	0.028	0.038	0.000										
7. IS 32	0.030	0.000	0.000	0.010	0.028	0.028									
8. KR 3	0.177	0.181	0.181	0.195	0.180	0.180	0.181								
9. KR 5	0.006	0.028	0.028	0.038	0.000	0.000	0.028	0.180							
10. KR 6	0.006	0.028	0.028	0.038	0.000	0.000	0.028	0.180	0.000						
11. KR 7	0.006	0.028	0.028	0.038	0.000	0.000	0.028	0.180	0.000	0.000					
12. NS 34	0.030	0.000	0.000	0.010	0.028	0.028	0.000	0.181	0.028	0.028	0.028				
13. *Psalidodon paranae*	0.015	0.035	0.035	0.046	0.009	0.009	0.035	0.179	0.009	0.009	0.009	0.035			
14. *Psalidodon fasciatus*	0.033	0.007	0.007	0.018	0.030	0.030	0.007	0.185	0.030	0.030	0.030	0.007	0.038		
15. *Astyanax lacustris*	0.169	0.171	0.171	0.185	0.171	0.171	0.171	0.021	0.171	0.171	0.171	0.171	0.170	0.174	
16. *Psalidodon bockmanni*	0.015	0.028	0.028	0.039	0.012	0.012	0.028	0.179	0.012	0.012	0.012	0.028	0.020	0.031	0.170

Table S3. K2P distance values between *ATPase* gene sequences obtained from GenBank, grouped according to species, and *Astyanax* and *Psalidodon* sequences obtained in the present study.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. IS 1													
2. IS 2	0.035												
3. IS 20	0.002	0.033											
4. IS 22	0.002	0.033	0.000										
5. IS 32	0.031	0.008	0.029	0.029									
6. KR 3	0.153	0.178	0.156	0.156	0.176								
7. KR 5	0.002	0.033	0.000	0.000	0.029	0.156							
8. KR 6	0.002	0.033	0.000	0.000	0.029	0.156	0.000						
9. KR 7	0.002	0.037	0.004	0.004	0.033	0.156	0.004	0.004					
10. NS34	0.035	0.004	0.033	0.033	0.008	0.178	0.033	0.033	0.037				
11. *Psalidodon paranae*	0.013	0.035	0.012	0.012	0.031	0.166	0.012	0.012	0.015	0.035			
12. *Psalidodon fasciatus*	0.036	0.014	0.034	0.034	0.011	0.179	0.034	0.034	0.038	0.014	0.032		
13. *Astyanax lacustris*	0.169	0.173	0.171	0.171	0.171	0.049	0.171	0.171	0.171	0.173	0.175	0.172	
14. *Psalidodon bockmanni*	0.021	0.040	0.021	0.021	0.038	0.170	0.021	0.021	0.023	0.040	0.025	0.039	0.177

Table S4. K2P distance values between *ND2* gene sequences obtained in GenBank, grouped according to species, and *Astyanax* and *Psalidodon* sequences obtained in the present study.

	1	2	3	4	5	6	7	8	9	10	11	12
1. IS 1												
2. IS 2	0.049											
3. IS 5	0.043	0.009										
4. IS 17	0.039	0.023	0.016									
5. IS 20	0.005	0.053	0.047	0.043								
6. IS 22	0.003	0.051	0.046	0.040	0.002							
7. IS 32	0.039	0.021	0.015	0.005	0.043	0.040						
8. KR 3	0.298	0.305	0.298	0.305	0.300	0.296	0.302					
9. KR 5	0.003	0.051	0.044	0.040	0.002	0.000	0.040	0.296				
10. KR 6	0.007	0.055	0.048	0.044	0.005	0.003	0.044	0.302	0.003			
11. Psalidodon paranae	0.010	0.052	0.045	0.041	0.012	0.009	0.041	0.290	0.009	0.013		
12. Psalidodon fasciatus	0.036	0.016	0.010	0.008	0.040	0.038	0.007	0.299	0.038	0.042	0.039	
13. Astyanax lacustris	0.295	0.304	0.293	0.300	0.297	0.293	0.296	0.005	0.293	0.299	0.287	0.294

Karyotype variability among allopatric populations of the Neotropical fishes *Psalidodon fasciatus* and *Psalidodon paranae* (Pisces, Characidae) with repetitive sequences mapping

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Abstract

Fish represent the most diverse group of vertebrates; this wide variety of species may be linked with some intrinsic features of the genome of these organisms. In this way, karyotype and chromosomal characteristics from allopatric populations of *Psalidodon fasciatus, Psalidodon aff. paranae and Psalidodon paranae* were investigated using different staining techniques (C-bang and AgNOR banding) as well as fluorescent in situ hybridization (FISH) to detect 18S rDNA, 5S rDNA, and the microsatellites $(CGC)_{10}$ and $(GATA)_{8.}$ The populations of *P. fasciatus* showed chromosome numbers of 2n=46 and 48 with variations in their karyotype formulae. *Psalidodon aff. paranae* and *P. paranae* also differed in the number of chromosomes, the first with 2n=48 while the second with 2n=50. For all populations studied, variations in the number and position of NORs and distribution of the constitutive heterochromatin were observed. On the other hand, the 5S DNA sequences proved to be more conserve. The $(CGC)_{10}$ microsatellite coincided mostly with ribosomal DNA regions, while the $(GATA)_8$ sequences showed few markings with blocks conserved in at least one pair of acrocentric chromosomes. The data obtained allow a better understanding of the distribution of repetitive sequences in the chromosomal organization both among populations and among closely related species.

Key Words: Microsatellite; 5S rDNA, 18S rDNA.

Introduction

Psalidodon Eigenmann, 1911 is a recently resurrected genus composed of 33 species widely distributed throughout the Neotropical region (Alves *et al*., 2021; Fricke *et al.*, 2021). In a recent review based on morphological and molecular characters for the family Characidae, Terán *et al.* (2020) proposed a new classification for *Astyanax*, relocating species that previously belonged to the *A. scabripinnis* and *A. fasciatus* complexes to the genus *Psalidodon*. Species that make up these complexes share similar morphological characteristics, which makes identification by traditional taxonomic methods a challenge (Tenório *et al.*, 2013).

Cytogenetics studies in *P. scabripinnis* and *P. fasciatus* complexes indicates a great karyotype diversity, which is also seen in many species of the genus *Astyanax* (Morelli *et al.*, 1983; Moreira Filho and Bertollo, 1991; Pazza *et al.*, 2006; Pazza and

Kavalco, 2007 and others) The chromosomal data available of the "*scabripinnis* complex" proposed by Moreira-Filho and Bertollo (1991), show chromosome numbers ranging from 2n=46, 48 and 50. Similar fact is observed for the *"fasciatus* complex" (Pazza *et al.*, 2006) were a remarkable diversification of karyotypes is present with the occurrence of specific variants, and chromosome numbers ranging from 2n=45 to 50, with 2n=46 and 2n=48 being most frequent (Pazza *et al.*, 2006; Torres-Mariano and Morelli, 2006; Medrado *et al.*, 2008). In addition to the diversity of diploid number, the species belonging to these complexes are also diversified in terms of karyotype structure, presenting interpopulation chromosomal polymorphisms involving differences in karyotype formulae, presence of extra chromosomes (B chromosomes), natural triploid, as well as population differences between chromosomes markers of genetic segments or not (Machado *et al.*, 2012; Silva *et al.*, 2013; Pansonato-Alves *et al.*, 2013).

The presence of repetitive DNA sequences has proven to be a common feature in eukaryotes, and in many species, they represent a large portion of the genome (Charlesworth *et al.*, 1994). These sequences are generally classified into two main classes: the tandem repeats like the satelittes DNA's and multigene families; and the dispersed elements like the transposable elements (Mazuchelli and Martins, 2009; Vicari *et al.*, 2010; Cioffi and Bertollo, 2012). Studies have demonstrated the importance of these sequences in the evolution of eukaryotic genomes (Biémon and Vieira, 2006).

Therefore, this study presents a comparative chromosomal analysis among allopatric populations of *Psalidodon fasciatus* and *Psalidodon paranae* from tributaries of the Ivaí river basin (Paraná basin), using distinct staining methods (C-banding and AgNOR) and fluorescent in situ hybridization (FISH) with repetitive DNA probes (5S rDNA, 18S rDNA and microsatellite DNA $(CGC)_{10}$ and $(GATA)_8$). With this approach, we sought to chromosomally characterize the populations under study, evidencing the distribution of these repetitive sequences in chromosomes.

Material and Methods

Biological Samples and Ethical Aspects

Twenty-three specimens of *Psalidodon fasciatus* were collected in two localities: fourteen in Nineteen stream, Paraíso do Norte (23º15'57'S and 52º34'17'W) and nine in Itiz stream, Marialva (23º29'06"S and 51º47'30"W). Seventeen specimens of *Psalidodon*

aff. *paranae* were collected in Índios river, Cianorte (23º55'15"S and 52º40'12"W) and eleven specimens of *Psalidodon paranae* were collected in Keller river, São Miguel do Cambuí (23º37'8'S and 51º51'30'W) all tributaries of the Ivaí river (Paraná basin), Paraná state. Specimen collection was authorized by the Brazilian Environment Ministry through its Biodiversity Information and Authorization System (SISBIO). All experimental procedures were approved and certified by the Ethics Committee on the Use of Animals (CEUA) of the State University of Maringá (Paraná, Brazil), through decision number 4640200717. Voucher specimens are in the cataloging process in the ichthyological collection of the Limnology, Ichthyology and Aquaculture Research Center (Nupélia) at the State University of Maringá, Paraná, Brazil (Uncatalogued NUP). Specimens were anesthetized by an overdose of clove oil (Inoue et al., 2005) and manipulated only once they became unresponsive to physical stimuli (approximately 1-2 min), for the removal of tissue and organs for analysis.

Cytogenetic analysis

Mitotic chromosomes were obtained from kidney cells according to Bertollo *et al.* (1978). The Ag-NORs (active nucleolus organizer regions) were detected by the silver nitrate impregnation technique (Howell and Black, 1980) and the regions of heterochromatin were obtained by the C-banding method (Sumner, 1972). Preparations after C-banding were stained with propidium iodide, according to the method of Lui *et al.* (2012). The metaphases were analyzed and photographed under an epifluorescence photomicroscope (Olympus BX51). The images were captured using the software DP controller (Media Cybernetics).

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) and Kubat *et al.* (2008), with probes obtained from *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti, 1999) and *Prochilodus argenteus* Spix et Agassiz, 1829 (Hatanaka and Galetti, 2004). The 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-digoxigeninrhodamine for the 5S rDNA probe and avidin-FITC (fluorescein isothiocyanate) for the 18S rDNA probe. The chromosomes were counterstained with DAPI.

The chromosomal sites of the $(CGC)_{10}$ and $(GATA)_8$ microsatellites were mapping 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 also conduce according to the protocol of Kubat *et al.* (2008). All the metaphases, after hybridization, were photographed using an epifluorescence microscope and adjusted for best contrast and brightness using the Adobe Photoshop CS6 software.

Chromosomal morphology was established based on the arm ratio and arranged in order of decreasing size and classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) chromosomes (Levan *et al*., 1964)

Results

Variations in diploid number, karyotype formulae and fundamental numbers were observe among populations of *Psalidodon fasciatus*, *Psalidodon* aff. *paranae* and *Psalidodon paranae* of distinct localities revealing significant interpopulational differences (Table 1).

Table 1. Karyotype data of *Psalidodon* populations from Ivaí river basin, PR.

River/ Locality	n	Specie	2n	Karyotype formulae	Fundamental number
Nineteen stream-Paraíso do Norte/PR	14	*Psalidodon fasciatus*	46	12m+14sm+16st+4a	88
Itiz stream-Marialva/PR	9	*Psalidodon fasciatus*	48	8m+16sm+12st+12a	84
Índios river-Cianorte/PR	17	*Psalidodon* aff. *paranae*	48	8m+14sm+12st+14a	82
Keller river-São Miguel do Cambuí/ PR	11	*Psalidodon paranae*	50	6m+18sm+12st+14a	86

Psalidodon fasciatus (Nineteen and Itiz stream)

Specimens of *P. fasciatus* from Nineteen stream showed Ag-NORs located only in one of the homologues of three pairs of chromosomes; on the short arms of a submetacentric (No. 8) and a subtelocentric (No. 15) and on the long arm of a subtelocentric (No. 17) (Fig. 1a, in box). Specimens from the Itiz stream presented a simple Ag-NOR located in the short arm of the submetacentric pair 6 (Fig. 1c, in box). In the population from Nineteen stream FISH with the 18S rDNA probe showed signs of hybridization in the pairs 8, 15 and 17 coincident with Ag-NOR and additionally in pair 16 (Fig. 1a). FISH 18S were not obtained for the population of Itiz stream.

In both populations, the 5S rDNA probe hybridized in the pericentromeric region of the third pair of metacentric chromosomes (Fig. 1a and c). In *P. fasciatus* from Nineteen stream markings were also observed in only one of the homologues of the pair 15 (submetacentric) and 22 (acrocentric), evidencing a synteny with the 18S rDNA site in one of the chromosomes of the pair 15 (Fig. 1a). While for *P. fasciatus* from Itiz the markers appears on the short arm of the first pair of acrocentric chromosomes (No. 19) (Fig. 1c).

The mapping with microsatellite CGC_{10} probe showed mostly coincidences with both Ag-NOR/FISH 18S and FISH 5S as can be seen in the pairs 3, 8, 15 and 17 (Fig. 1b) and 19 (Fig. 1d).

Few heterochromatic blocks were observed in the karyotype of *P. fasciatus* from Nineteen stream, highlighting more strongly stained blocks in the telomeric regions of the long arms of the pairs 9, 10 (sm), 14 (st), 18 and 23 (Fig. 2a). In the population of Itiz stream, there was a greater amount of heterochromatin distributed in the pericentromeric and telometic regions; these last stand out in the long arms of pairs 19, 20 e 21 (acrocentric) (Fig. 2c).

The mapping chromosomal of the $GATA_8$ microsatellite in the population from Nineteen stream revealed telomeric sites in most chromosomes, however, an extensive blocks in the pericentromeric region of pair 4 (m) and in the short arms of pair 22 (a) were evidenced; in addition, blocks of this microsatellite are visualized in the interstitial regions of the short arms of pairs 2 e 3 (m) (Fig. 2b). In the population of Itiz stream, the $GATA_8$ microsatellite signals are restricted to pairs 6 (sm), close to the NOR sites and in the short arms of pair 19 (a) (Fig. 2d).

Psalidodon aff. *paranae* (Índios river)

The specimens from this population presented simple Ag-NOR located in the long arm of the first pair of acrocentric chromosomes (Fig. 1e, in box), which was confirmed by FISH with 18S rDNA (Fig. 1e). The 5S rDNA reveled pericentromeric markers on the third pair of metacentric chromosomes, and in the short arms in only one of the homologues of the acrocentric pairs 18 and 19, being the first syntenic with the 18S rDNA site (Fig. 1e). In this population, the microsatellite CGC_{10} markers were coincident with the 5S rDNA, with signals in the pairs 3 (m) and 18 (a), in addition to markings on pairs 2 (m) and 12 (st) (Fig. 1f).

The analysis of the heterochomatin revealed few C-band positives concentrated in the terminal region of the long arms of some sm, st and more evident in the acrocentric pairs 18, 19 and 20 (Fig. 2e). The mapping with $GATA_8$ microsatellite revealed few sites, being these restricted to the short arms of pair 18 (Fig. 2f).

Psalidodon paranae (Keller river)

Specimens from this population showed Ag-NORs located in the short arms of the chromosome pairs 6 and 10 (sm) (Fig. 1g, in box), which could not be confirmed, since in this population the FISH with 18S rDNA was not obtained. 5S rDNA probes hybridize in the pericentromeric region of the second pair of metacentric chromosomes and on the short arm of the first pair of acrocentrics. In this population the microsatellite CGC_{10} was not coincident with 5S rDNA sites, with markings localized in the pairs 6 (sm), 13 and 14 (st) (Fig. 1h).

C-band showed pericentromeric markings on most chromosomes as well as some terminal markings, as observed in pairs 4, 6, 8, 10 (sm); 19 and 20 (a) (Fig. 2g). The mapping chromosomal of the $GATA_8$ microsatellite revealed few sites, restricted to the short arms of pairs 7 (sm) and 20 (a) (Fig. 2h).

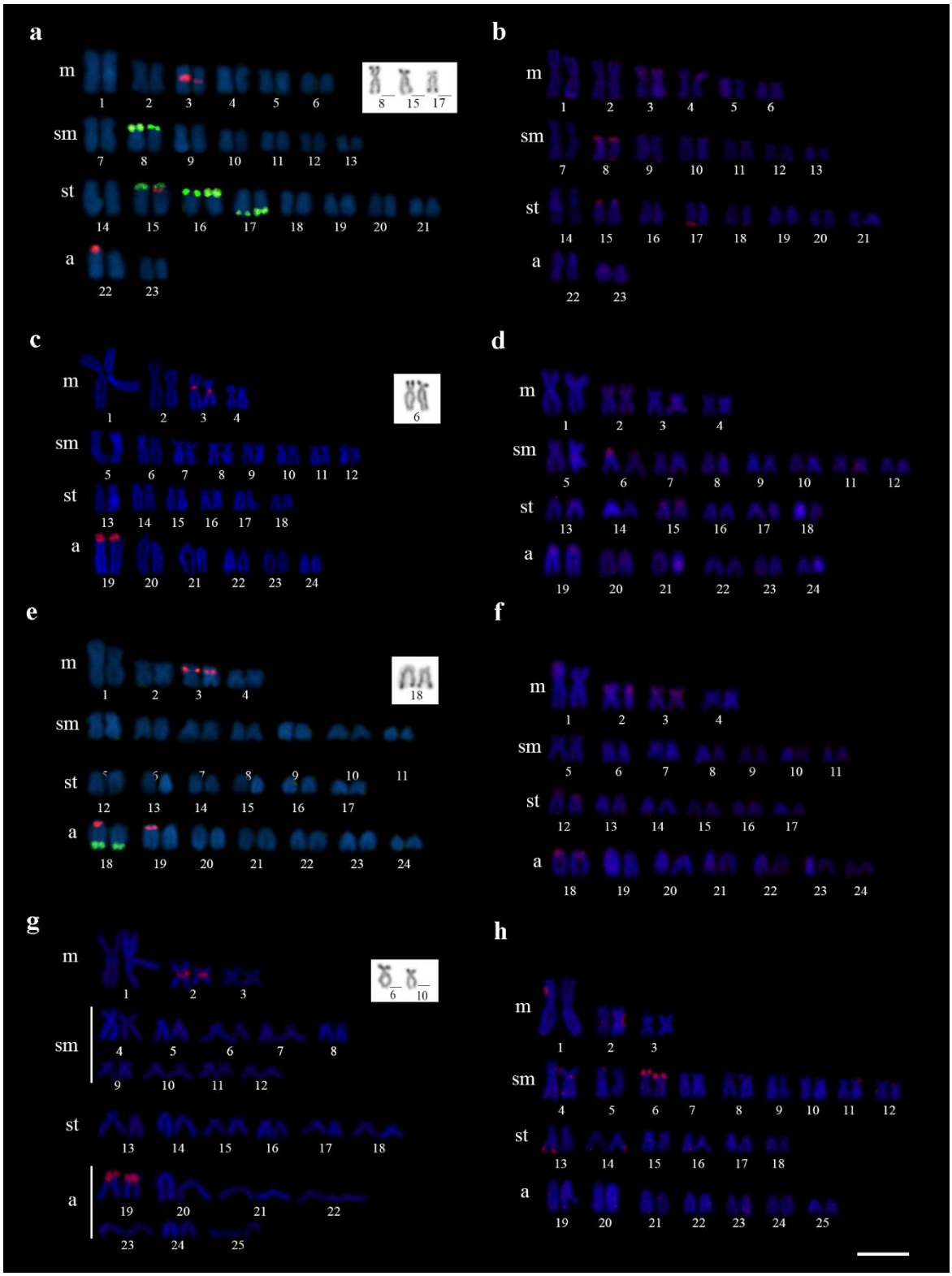

Figure 1. Karyotypes of *Psalidodon fasciatus* from Nineteen **(a** and **b)** and Itiz stream **(c** and **d)**, *Psalidodon* aff. *paranae* from Índios river **(e** and **f)** and *Psalidodon paranae* from Keller river **(g** and **h)** after FISH with 18S rDNA (green) and 5S rDNA (red) probes (**a**, **c**, **e** and **g**); and FISH with CGC_{10} microssatellite (**b**, **d**, **f** and **h**). In the boxes, NOR- bearing chromosomes. Bar= 10μm.

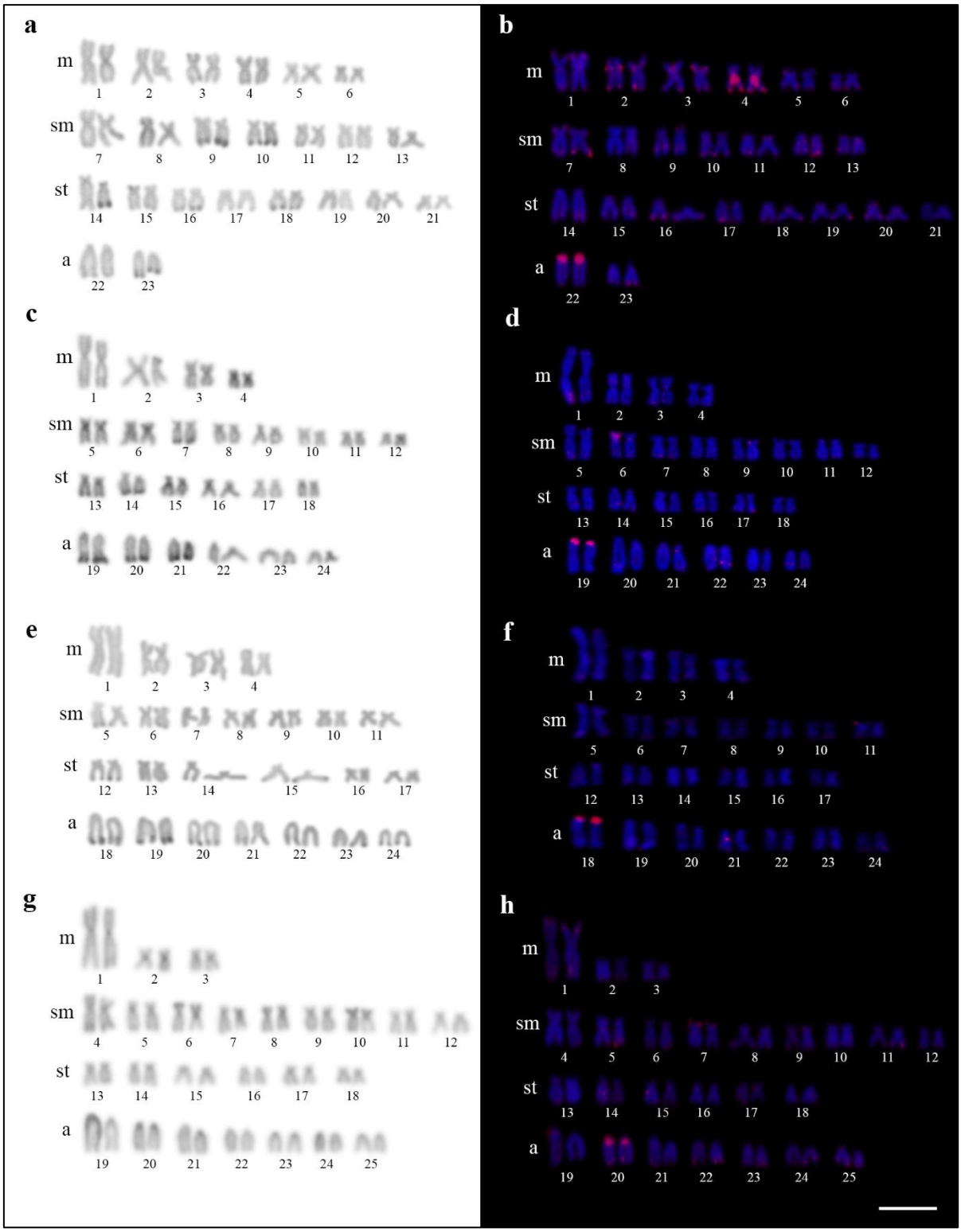

Figure 2. Karyotypes of *Psalidodon fasciatus* from Nineteen **(a** and **b)** and Itiz stream **(c** and **d)**, *Psalidodon* aff. *paranae* from Índios river **(e** and **f)** and *Psalidodon paranae* from Keller river **(g** and **h).** C-banding **(a**, **c**, **e** and **g)** and FISH with $GATA_8$ microssatellite **(b**, **d**, **f** and **h)**. Bar= 10μm.

Discussion

Variations related to the number and types of chromosomes, as well as the number and position of the nucleolus organizing regions and distribution of the constitutive heterochromatin are widely observed for the *P. fasciatus* and *P. scabripinnis* complexes (formerly *Astyanax*) (Morelli *et al.*, 1983; Moreira Filho and Bertollo, 1991; Pazza *et al.*, 2006; Artoni *et al.*, 2006; Ferreira-Neto *et al.*, 2012).

The diploid number found for the populations of Nineteen and Itiz stream, are the most commonly described for species of the "*P. fasciatus* complex", however the karyotype formulae differ somehow from other studied populations (Pazza *et al.*, 2006; Medrado *et al.*, 2008; Gavazonni *et al.*, 2020). A common feature is the presence of a larger number of chromosomes with two arms, and the small variation in size observed between the first and second pair of metacentric chromosomes, giving them a "ladder" appearance (Pazza *et al.*, 2008; Fernandes *et al.*, 2009). The low number of acrocentric chromosomes observed for Nineteen stream population has already been described for *P. fasciatus* populations from other basins, as for the São Francisco (Peres *et al.*, 2009), Mogi-Guaçu, Paranapanema (Pazza *et al.*, 2008; Fernandes *et al.*, 2009) and Uruguay (Gavazzoni *et al.*, 2020).

In *Psalidodon paranae* variations in diploid number and karyotype formulae are also present; being this specie part of the "*P. scabripinnis* complex" (Moreira Filho and Bertollo, 1991). The most common number of chromosomes for the complex is 2n=50 (Moreira-Filho *et al.*, 2004; Silva *et al.*, 2014; Alves *et al.*, 2020), however 2n=46 and 48 has been already described (Vicari *et al.*, 2008; Yano *et al.*, 2014). Fernandes and Martins-Santos (2003) analyzing *P. scabripinnis* specimens from Índios river found 2n=48 with the same number of metacentric and acrocentric chromosomes from those of present work; Vicari *et al.* (2008) analyzing population of the same complex of the Tibagi basin, observed 2n=50 and karyotype formulae very similar to that found for the Keller River population.

The polymorphisms verified for different populations of the same species can be explain due to chromosomal rearrangements that occur over time; some organisms have phenotypic plasticity and tolerate a certain degree of rearrangement in their chromosomes, as observed for the populations under study (Pazza *et al.*, 2018). However, the reduced dispersal capacity presented by small fish groups can facilitate the

geographical separation of populations, leading to a decrease in gene flow, process that after a long period can result in allopatric speciation (Castro, 1999; Ward *et al.*, 2009; Pereira *et al.*, 2013).

The presence of multiple NORs is a frequent characteristic for the *Astyanax* genus, to which *P. fasciatus* and *P. paranae* belonged. The location and number of these sequences varies according to the species, and may undergo intra-population variations, in the latter case this may be related to the expression pattern of these genes or even to the proximity of these regions during the interphase, which can facilitate their transfer when located in telomeric regions (Fernandes and Martins-Santos, 2006; Hashimoto and Porto-Foresti, 2010). On the other hand, the presence of simple NOR has already been described for *P. fasciatus* as seen by Peres *et al.* (2009) and for *P. paranae* as seen by Maistro *et al.* (2000) and Silva *et al.* (2013).

All populations from the present work showed 5S rDNA markings on the pericentromeric region of one pair of metacentric chromosomes, this being a frequent characteristic for *P. fasciatus* and *P. paranae* species (Ferro *et al.*, 2001; Almeida-Toledo *et al.*, 2002; Mantovani *et al.*, 2005; Vicari *et al.*, 2008; Ferreira-Neto *et al.*, 2012, among others). The presence of 5S DNA markings on non-homologous chromosomes, as well as the variation in number and position of these sequences observed for some species, has been attributed to translocation events mediated by transposable elements, as have already been demonstrated in *Erythinus erythrinus* (Cioffi *et al.*, 2010), cichlid species (Nakajima *et al.*, 2012) and *Ancistrus* populations (Prizon *et al.*, 2018).

The distribution of microsatellite $(CGC)_{10}$ varied somewhat among the populations studied, being more related with the regions of Ag-NOR⁄FISH 18S in *P. fasciatus* from Nineteen stream, while for *P. paranae* from Índios river it was coincident with the FISH 5S, providing an indication of the type of sequences present in these regions. According to Cioffi and Bertollo (2012), microsatellites are preferentially present in telomeres, centromeres and the sex chromosomes, where a large fraction of the repetitive DNA is located.

The distribution pattern of the constitutive heterochromatin varied both among the populations and the species studied. *P. fasciatus* from Nineteen stream presented few heterochromatic blocks, mostly located in the terminal regions of the long arms of some chromosomes. In the population of the Itiz stream, in addition to terminal markings,

pericentromeric blocks were observed. The presence of strongly stained blocks in terminal position of long arms was also described for *P. fasciatus* populations studied by Pazza *et al.* (2008) and Peres *et al.* (2009). *Psalidodon* aff. *paranae* have heterochromatic blocks more evident in terminal region of the long arms of some acrocentric chromosomes, while in *P. paranae* most of the markings are concentrated in the pericentromeric region, being its visualization possible in almost all chromosomes, in addition to some terminal markings. Vicari *et al.* (2008) and Yano *et al.* (2014) found the same scenario and for *A. paranae* populations from different localities.

The association of $(GATA)_{10}$ microsatellite sequences with heterochromatic regions was not a rule for the studied populations. Contrary to observed for species of the genus *Hypostumus* where the distribution of this microsatellite is very dispersed and variable (Traldi *et al.*, 2013), this presented punctual markings in the studied species; highlighting a marking on the short arm of a pair of acrocentric chromosomes in all populations. In addition to these, in the population of *P. fasciatus* from Nineteen stream it was possible to observe a strong pericentrometic marking on the fourth pair of metacentric chromosomes, which can be explained by the ability of microsatellites to originate variants with different repeat numbers (Cioffi and Bertollo, 2012).

The karyotype differences observed between the studied populations are the result of a continuous evolutionary process; repetitive sequences have been shown to be good evolutionary markers since they seem to escape the selective pressures that act on non-repetitive sequences, reinforcing the important role of the repetitive DNA to karyotypic diversification (Cioffi *et al.*, 2009; Prizon *et al.*, 2018).

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Cytogenetic and molecular characterization of *Oligosarcus pintoi* (Characidae): A new record of supernumerary chromosome in this species

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Abstract

The genus *Oligosarcus* currently comprises 24 valid species distributed in the major river basins of South America. In this group, nine species were cytogenetically investigated, and found to share a diploid number of 50 chromosomes. Despite the conservation of the diploid number, variations in the karyotypic formula, number and position of the nucleolar organizer regions, and longitudinal bands have been described between both species and populations. In this study, we present cytogenetic and molecular data from *Oligosarcus pintoi* specimens from the Keller River, a tributary of the Ivaí River (Upper Paraná basin), using DNA barcoding and cytogenetic markers (C-band, silver-stained nucleolar organizer regions, and fluorescence in situ hybridization of 18S and 5S rDNA). The genetic inferences reached after analyzing the *Cytochrome c oxidase subunit I* gene allowed us to confirm the identity of the individuals with 2n=50 chromosomes. However, one specimen contained a medium subtelocentric supernumerary chromosome (2n=51). This is the second record of additional chromosomes in *O. pintoi*, thereby confirming the existence of a

supernumerary chromosome in allopatric populations of this species, fact that demonstrates an evolutionary path that is divergent from other populations and/or species of *Oligosarcus* analyzed so far, contributing to the karyotypic diversification of the group.

Keywords: Supernumerary chromosome, *COI*, 5S rDNA, 18S rDNA.

Introduction

The Ivaí River basin is one of the most important tributaries of the Paraná River, with an ichthyofauna comprising an estimated 118 species classified into 8 orders and 29 families. These are predominantly small to medium-sized species of the orders Characiformes and Siluriformes.[1] The Characidae family is one of the most diverse among Neotropical fish, comprising more than 1150 species,[2] and in the Ivaí River basin it accounts for approximately 37% of the species, among them are two species of *Oligosarcus* (Günther, 1864), i.e., *Oligosarcus pintoi* Campos, 1945 and *Oligosarcus paranensis* Menezes & Géry, 1983.[1]

Oligosarcus is mainly distributed in the major river basins to the south of the 14th parallel, in South America.[3,4] Representatives of this genus are characterized as predators of small fish and insects; they have an elongated body and a mouth armed with canine and conical teeth and are popularly known as dogfish.[5] Phylogenetic relationships within *Oligosarcus* have been widely discussed. Based on the morphological and molecular characters, Mirande[2] recovered *Oligosarcus* as a monophyletic group, proposing a new tribe, Gymnocharacini, from the subfamily Stethaprioninae, which belongs to a clade that includes the *Astyanax* Baird & Girard, 1854, *Hypessobrycon* Durbin, 1908, *Hasenmania* Ellis, 1911 and *Gymnocharacinus* Steindachner, 1903 species.

Taxonomic descriptions of the species have been based only on morphological characteristics for a long time; however, it is already known that this can lead to misidentification cases in cryptic species or in those that have some phenotypic plasticity or only genotypic variations.[6] Thus, the use of molecular tools such as DNA barcoding has been instrumental in the resolution of taxonomic uncertainties and delimitation of species in more complex groups of fish.[7,8]

Cytogenetic studies in *Oligasarcus* have been conducted in nine species with an increase in information in the last decade (Table 1). The data showed stability in the diploid number (2n=50), but intra- and inter-specific variations in their karyotype formula, fundamental number, number and position of nucleolus organizing regions (NORs), and the distribution of heterochromatin by the C band have been reported. Despite the stability in the diploid number, Falcão *et al*.[9] registered two specimens of *O. pintoi* (cited as *Paroligosarcus pintoi*) from the Mogi-Guaçu River in the state of São Paulo with 2n=51, indicating the possible occurrence of supernumerary chromosomes.

Supernumerary or accessory chromosomes were first observed in insects by Wilson,[10] and since then they have been described in many species of animals, plants, and fungi.[11] They appear in addition to complement A, which is their most likely origin, but they follow their own evolutionary pathway.[12] Among the Neotropical fish with studied karyotypes, approximately 1,000 species, at least 4% of them have supernumerary chromosomes.[13] Characiformes is the

order with the highest number of species carrying these additional chromosomes (31), were these additional elements show considerable variability in number, size, and morphology.[14]

Thus, considering intraspecific karyotypic divergences between allopatric populations of *O. pintoi*, an integrative analysis of cytogenetic and molecular data is presented here with a new record of supernumerary chromosomes for a population from Keller River, whose data allowed a better characterization of this species.

Material and Methods

Biological Samples

Specimens of *O. pintoi* were collected from the Keller River, located near the town of São Miguel do Cambuí (23º37'8'S and 51º51'30'W), a tributary of the Ivaí River. Specimen collection was authorized by the Brazilian Environment Ministry through its Biodiversity Information and Authorization System. All experimental procedures were approved and certified by the Ethics Committee on the Use of Animals (CEUA, State University of Maringá, Paraná, Brazil), decision number 4640200717. Voucher specimens were cataloged in the ichthyological collection of the Limnology, Ichthyology and Aquaculture Research Center (Nupélia) at the State University of Maringá, Paraná, Brazil (NUP 23220). Access to the genetic heritage was authorized by the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (register number ABABA91). For tissue sampling, specimens were anesthetized by an overdose of clove oil[15] and manipulated only after they became unresponsive to physical stimuli (approximately 1-2 min).

Molecular analysis

DNA was extracted from the tissue samples using the Promega Wizard ®Genomics kit, following the manufacturer's instructions. The *Cytochrome c oxidase subunit I* (*COI)* gene was partially amplified using the primers L6448-F2 (5′ TCGACTAATCATAAAGATATCGGCAC-3′) and H7152 (5′-CACCTCAGGGTGTCCGAARAAYCARAA-3′) described by Ivanova *et al*.[16]

DNA fragments were amplified using polymerase chain reaction (25 μL reactions consisting of Tris-KCl [Tris-HCl 20 mM (pH 8.4), KCl 50 mM], $MgCl_2$ (1.5 mM), primers (2.5 mM each), dNTPs (0.1 mM each), DNA Taq Polymerase (1 U), and template DNA at a concentration of 10 ng/μL). The conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 1 min and 30 s, 72 °C for 1 min and 30 s, and a final elongation cycle at 72 °C for 10 min.

Samples for sequencing were prepared using BigDye™ Terminator 3.1. Cycle sequencing kit. Sequencing was performed following the manufacturer's instructions, and the products were sent to the Complexo de Centrais de Apoio à Pesquisa of the Universidade Estadual de Maringá for automated sequencing using an ABI3500 Applied Biosystems sequencer.

The sequences obtained were edited and aligned using BioEdit 7.2[17] and MEGA 7.0[18] software, respectively. The Kimura-2-parameter (K2P) distance was calculated between sequences of *Oligosarcus* present in GenBank (except *Oligosarcus* sp.) and those obtained in this study. Due to the substantial number of available sequences, one sequence of each haplotype, as identified by the DnaSP 6.0 software[19] was included in the analyses. The K2P distance calculation was performed between groups (according to species) and pairwise using MEGA 7.0[18] (Supplementary Material 1).

A haplotype network was constructed using the median-joining method in PopArt software[20] with all sequences available in GenBank for *Oligosarcus* (except *Oligosarcus* sp.) and those obtained in the present study (OK285066 and OK285067), totaling 95 sequences.

Cytogenetic analysis

Mitotic chromosomes were obtained from kidney cells according to Bertollo *et al*.[21] The silver-stained NORs (AgNORs) were revealed using the silver nitrate impregnation technique.[22] The heterochromatin regions were determined by the C-banding technique[23] and stained with propidium iodide according to Lui *et al*.[24] Physical mapping of the 5S rDNA and 18S rDNA sequences was carried out by fluorescence in situ hybridization (FISH) according to Kubat *et al*.[25] with probes obtained from *Leporinus elongatus* Valenciennes, 1850[26] and *Prochilodus argenteus* Agassiz, 1829[27]. The probes were labeled by nick translation using digoxigenin-11-dUTP. The signals were detected using conjugated antidigoxygenin-rhodamine, and the chromosomes were counterstained with DAPI.

The metaphases were analyzed and photographed under an epifluorescence photomicroscope (Olympus BX51). The images were captured using the DP Controller (Media Cybernetics) software. Karyotypes were organized according to their arm ratios following Levan *et al*.[28]

Table 1. Review of cytogenetic data in *Oligosarcus*.

Specie	Location	Hydrographic Basin	2n	Karyotype formula	FN	C-band	AgNOR	18S rDNA	5S rDNA	Ref.
Oligosarcus argenteus	Casca River-MG	São Francisco Basin	50	6m+14sm+18st+12a	88	–	1 pair sm; 1pair st	–	1 pair st	3
	Coimbra-MG	São Francisco Basin	50	6m+14sm+18st+12a	88	–	–	1 pair m; 1pair sm; 2pairs st	1 pair st	
	Ponte Nova-MG	São Francisco Basin	50	6m+12sm+20st+12a	88	–	–	1 pair sm; 4 pairs st	1 pair st	
	Viçosa-MG	São Francisco Basin	50	6m+14sm+16st+14a	86	Bitelomeric and centromeric blocks	1 pair sm; 1pair st	4 pairs st	1 pair st	
Oligosarcus hepsetus	Ipiranga River-SP	Paraná Basin	50	2m+26sm+4st+18a	82	–	–	–	–	43
	Juquiá River-SP	Paraná Basin	50	2m+26sm+4st+18a	82	–	–	–	–	
	Jacui Stream-SP	Paraná Basin	50	6m+10sm+16st+18a	82	NOR associed blocks	1 pair a	2 pairs a	1 pair m/sm; 1 pair a	44
	Paraitinga River-SP	Paraná Basin	50	6m+10sm+16st+18a	82					
	Ribeirão Grande Stream-SP	Paraná Basin	50	6m+12sm+14st+18a	82	Pericentromeric and some telomeric	1 pair a + 1 chrom a	2 pairs a	–	45
	Santo Antônio Stream-SP	Paraná Basin	50	4m+12sm+16st+18a	82	Pericentromeric and some bitelomeric	1 chrom sm + 1 pair a	1 pair sm; 2pairs a	–	
	Paraíba do Sul River-SP	Paraná Basin	50	2m+16sm+16st+16a	84	Telomeric and centromeric smal blocks	1 pair a	1 pair a + 1 chrom a	1 pair a	38
Oligosarucs jenynsii	Doce River Valley-MG	São Francisco Basin	50	6m+22sm+6st+16a	84	–	–	–	–	43
	Ipiranga River-SP	Paraná Basin	50	6m+22sm+6st+16a	84	–	–	–	–	
	Uruguay River-SC	Uruguay Basin	50	2m+24sm+10st+14a	86	Telomeric and centromeric smal blocks	1 pair a	1 pair a	1 pair a	38
Oligosarcus longirostris	Iguaçu River-PR	Paraná Basin	50	4m+10sm+16st+20a	80	–	1 pair a	–	–	37
	Iguaçu River-PR	Paraná Basin	50	2m+20sm+10st+18a	82	–	2 pairs a	–	–	34
Oligosarcus macrolepis	Turvo River-MG	São Francisco Basin	50	8m+20sm+6st+16a	84	–	–	–	–	43
Oligosarcus paranensis	Tunas River-PR	Paraná Basin	50	4m+10sm+16st+20a	80	Large amaout and telomeric smal blocks	1 pair a	–	–	37

Oligosarcus cf. paranensis	Três Bocas Stream-PR	Paraná Basin	50	8m+18sm+10st+14a	86	Terminal and pericentromeric blocks	2 to 8 chroms	7 chroms	1 pair	42
	Quexada River-PR	Paraná Basin	50	6m+10sm+16st+18a	82		2 to 6 chroms	9 chroms	1 pair	
	Keller River-PR	Paraná Basin	50	2m+26sm+8st+14a	86	–	1 st pair	–	–	34
	Mourão River-PR	Paraná Basin	50	2m+26sm+8st+14a	86	–	2 pairs st; 1 pair a	–	–	
Oligosarcus pintoi	Mogi-Guaçu River-SP	Paraná Basin	50	4m+20sm+10st+16a	84	–	–	–	–	43
	Tunas River-PR	Paraná Basin	50	4m+10sm+16st+20a	80	NOR associed blocks	1 pair a +1 to 2 chroms	–	–	37
	Mogi-Guaçu River-SP	Paraná Basin	50	2m+20sm+12st+16a	84	Terminal and pericentromeric blocks	1 pair a	–	–	38
	Keller River-PR	Paraná Basin	50+1	4m+12sm+14st+20a+1*	82	Pericentromeric and some telomeric blocs	1 pair st; 1 pair a	1 pair st; 1 pair a	1 pair sm; 1 pair st; 1 pair a	PS
Oligosarcus solitatius	Aguapé Lake-MG	São Francisco Basin	50	4m+14sm+16st+16a	84	–	–	1 pair	1 pair	3
	Almecéga Lake-MG	São Francisco Basin	50	4m+14sm+14st+18a	82	Centromeric and telomeric blocks	–	–	–	
	Cure Like-MG	São Francisco Basin	50	4m+14sm+20st+12a	88	–	1 pair st	–	–	
	Hortência Lake-MG	São Francisco Basin	50	4m+14sm+18st+14a	86	–	–	–	–	
	Juiz de Fora Lake-MG	São Francisco Basin	50	4m+14sm+20st+12a	88	–	–	–	–	
	Lingüiça Lake-MG	São Francisco Basin	50	4m+14sm+16st+16a	84	–	1 pair st	8 chroms	2 chroms	
	Tiririca Lake-MG	São Francisco Basin	50	4m+14sm+20st+12a	88		–	–	–	
Oligosarucs sp.	Das Velhas River-MG	São Francisco Basin	50	6m+14sm+18st+12a	88	–	2 pairs sm	–	–	3

Abbreviations: 2n= diploid number; m= metacentric; sm= submetacentric; st= subtelocentric; a= acrocentric; FN= fundamental number; AgNOR = silver stained nucleolar organizer regions; chroms = chromosome; PR= state of Paraná; SP= state of São Paulo; MG= state of Minas Gerais. *Supernumerary chromosome. PS: Present Study

Results

Molecular analysis

A total of 95 sequences were obtained for *Oligosarcus*, including two sequences with 458 base pairs of partial fragments of the *COI* gene amplified from specimens collected from the Keller River and 93 *COI* sequences available in GenBank (Supplementary Material 2). The sequences obtained in this study were identical to the *O. pintoi* sequences available in the database and shared a unique haplotype. The partial sequences that were newly generated in this study were deposited in GenBank (accession numbers: OK285066 and OK285067).

Thirty-seven single haplotypes were obtained and used in the analysis that presented with 50 polymorphic sites featuring 57 mutations and 49 parsimony informative sites. The haplotype (h) and nucleotide diversity (π) indices were 0.9545 and 0.03790, respectively. The haplotype network (Fig. 1) was generated based on the classification of Wendt *et al*.[29] through Bayesian inference that revealed two groups of *Oligosarcus*, i.e., the continental and coastal, the first comprising ten species, including *O. pintoi*, and the second comprising seven species.

Different haplotypes for the same species were identified, as observed for *O. paranensis*, *O. brevioris*, *O. hepsetus*, *O. jenynsii*, and *O. jacuiensis.* The highest number of haplotypes were observed in *O. jenynsii* and *O. hepsetus* that presented with seven haplotypes each. Haplotype sharing also occurred between distinct species. H1 and H25 were shared between *O. jenynsii* and *O. hepsetus* (coastal and continental groups), and H24 was shared between *O. solitarius* and *O. argenteus* (coastal group).

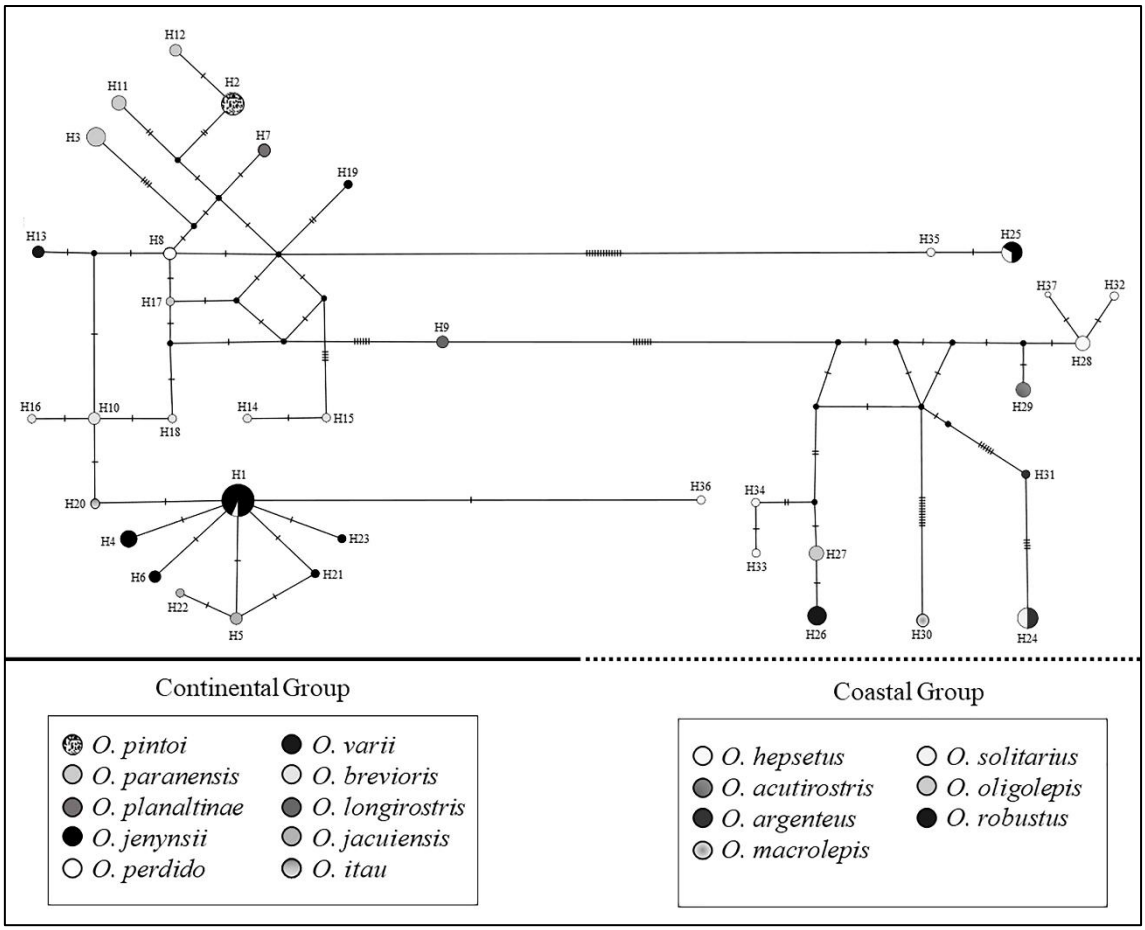

Figure 1. Haplotype network based on *COI* gene sequences of *Oligosarcus* species from GenBank of the present study. **H1; H25:** *O. jenynsii* and *O. hepsetus*; **H2:** *O. pintoi*; **H3; H11; H12; H17:** *O. paranensis*; **H4; H6; H19; H21; H23:** *O. jenynsii*; **H5; H22:** *O. jacuiensis*; **H7:** *O. planaltinae*; **H8:** *O. perdido*; **H9:** *O. longirostris*; **H10; H14- H16; H18:** *O. brevioris*; **H13:** *O. varii*; **H20:** *O. itau*; **H24:** *O. solitarius* and *O. argenteus*; **H26:** *O. robustus*; **H27:** *O. oligolepis*; **H28**; **H32-H37:** *O. hepsetus*; **H29:** *O. acutirostris*; **H30:** *O. macrolepis*; **H31:** *O. argenteus*.

The mean K2P distances between the specimens in the present study and those available in the database (Table 2) ranged from 0.0% (*O. pintoi*) to 5.5% (O. *macrolepis*). When analyzing all the species, the K2P distances ranged from 0.8% to 6.7%. The species genetically closest to *O. pintoi* were *O. perdido* (1.0%) and *O. paranensis* (1.1%), while *O. acutirostris* (5.2%) and *O. macrolepis* (5.5%) were the most distant. The intraspecific distance values ranged from 0.0% to 2.9%. *O. paranensis*, *O. jenynsii*, and *O. hepsetus* showed intraspecific values above 2%, whereas *O. brevioris* and *O. jacuiensis* showed intraspecific values of less than 2%.

Table 2. K2P interespecific and intraspecific genetic distances of the partial fragment of *COI* gene of *Oligosarcus* from the GenBank and present study, grouped according species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	Intraspecific
1. *O. pintoi*																		0.000
2. *O. perdido*	0.010																	0.000
3. *O. paranensis*	0.011	0.010																0.011
4. *O. planaltinae*	0.012	0.008	0.015															0.000
5. *O. itau*	0.012	0.008	0.019	0.019														n/c
6. *O. brevioris*	0.014	0.008	0.020	0.016	0.009													0.007
7. *O. varii*	0.015	0.008	0.017	0.018	0.007	0.012												0.000
8. *O. jacuiensis*	0.019	0.011	0.023	0.021	0.007	0.015	0.012											0.001
9. *O. jenynsii*	0.021	0.014	0.024	0.022	0.012	0.018	0.017	0.016										0.013
10. *O. longirostris*	0.022	0.018	0.024	0.024	0.016	0.018	0.021	0.021	0.024									0.000
11. *O. oligolepis*	0.049	0.039	0.049	0.042	0.043	0.043	0.043	0.049	0.046	0.028								0.001
12. *O. solitarius*	0.050	0.041	0.048	0.047	0.043	0.046	0.044	0.048	0.048	0.038	0.036							0.000
13. *O. hepsetus*	0.050	0.046	0.054	0.052	0.046	0.047	0.051	0.049	0.050	0.031	0.016	0.043						0.009
14. *O. robustus*	0.050	0.041	0.047	0.044	0.046	0.045	0.048	0.048	0.047	0.031	0.003	0.039	0.017					0.000
15. *O. argenteus*	0.051	0.043	0.048	0,046	0.044	0.046	0.046	0.046	0.047	0.036	0.026	0.010	0.036	0.031				n/c
16. *O. acutirostris*	0.052	0.056	0.061	0.067	0.055	0.059	0.061	0.058	0.058	0.037	0.021	0.049	0.013	0.020	0.041			0.000
17. *O. macrolepis*	0.055	0.049	0.054	0.053	0.048	0.048	0.053	0.050	0.053	0.033	0.033	0.032	0.034	0.034	0.033	0.039		0.000

Cytogenetic analysis

Oligosarcus pintoi specimens showed a diploid number of 50 chromosomes with a karyotype formula equal to 4m+12sm+14st+20a and fundamental number equal to 82. However, one specimen revealed metaphases with 2n=51 chromosomes; the extra element was a medium subtelocentric chromosome present in 43% of all analyzed metaphases (Fig. 2a).

The heterochromatin revealed by C-banding was evidenced as conspicuous blocks located in the pericentromeric region and the terminal region mainly on the acrocentric chromosomes and had coincident marking with the intercalary region of the ribosomal sites. Meanwhile, the B chromosome presents a large heterochromatic block that extends from the centromere throughout the short arm (Fig. 2b).

AgNORs are located in two pairs of chromosomes, i.e., in the short arm of pair 9 (submetacentric) and in the long arm of pair 17 (acrocentric) (Fig. 2a, in box). FISH with an 18S rDNA probe confirmed the presence of ribosomal sites coincident with AgNOR (Fig. 2c). The 5S rDNA probe hybridized with three pairs of chromosomes, i.e., in the terminal region of the short arm of pair 5 (submetacentric), in the pericentromeric region of pair 10 (subtelocentric), and in the telomeric region of pair 17 (acrocentric), thereby demonstrating the synteny between the 18S and 5S sites in this last pair (Fig. 2d). None of these markers were observed on the supernumerary chromosome (Fig. 2a-d).

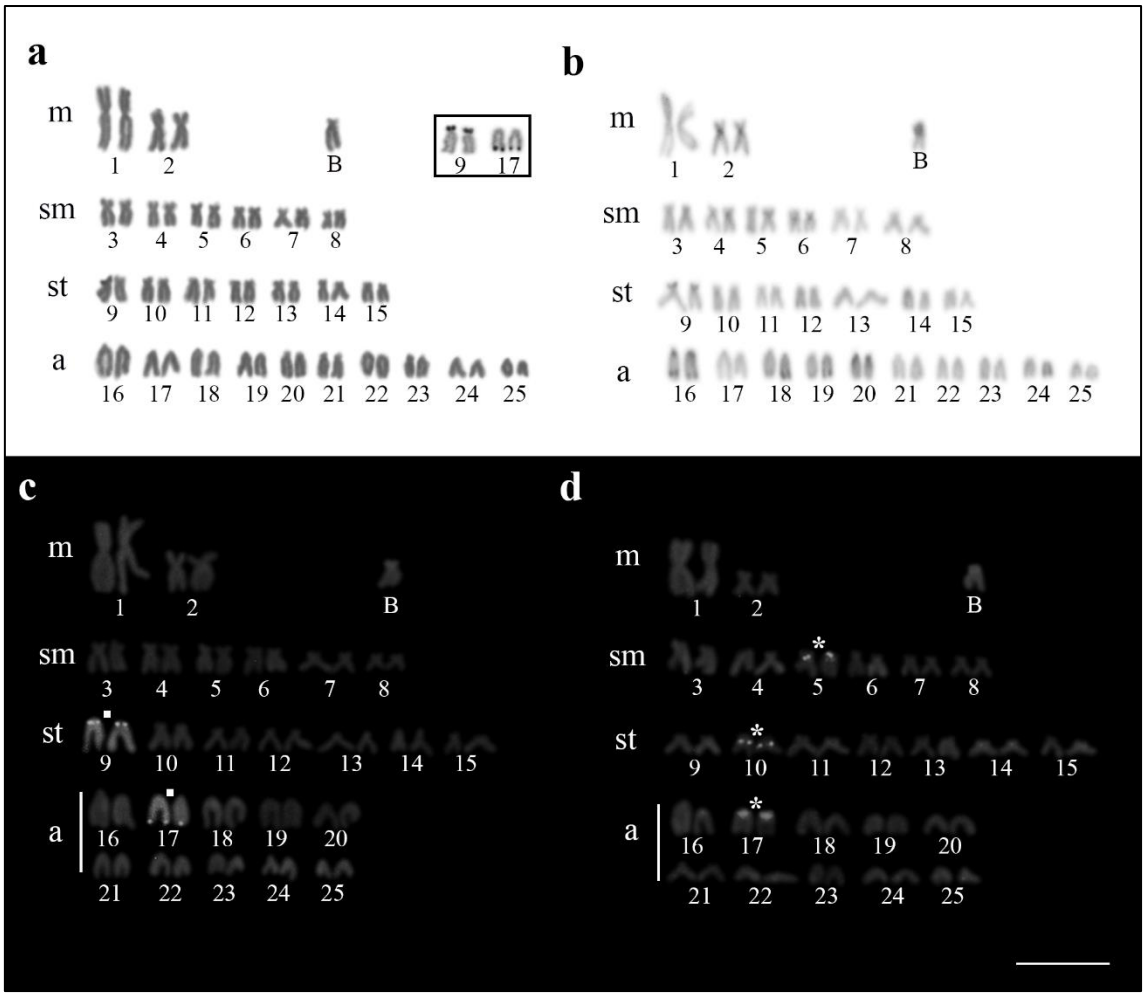

Figure 2. Karyotypes of *Oligosarcus pintoi* from Keller River **(a)** stained with Giemsa, in box the Ag-NOR pairs; **(b)** C-banded; **(c)** FISH with 18S rDNA probe (square) and **(d)** FISH with 5S rDNA probe (asterisk). FISH= fluorescence in situ hybridization. B= supernumerary chromosome. Bar= 10µm.

Discussion

COI gene sequences are currently used for the identification of fish species and population studies.[30,31]. This marker enabled the identification of the specimens studied here as *O. pintoi*, grouping them with other *O. pintoi* sequences from the databases that shared the same haplotype. Considering all analyzed sequences, it was possible to observe a separation of coastal (*O. hepsetus*, *O. acutirostris*, O. *argenteus*, *O. macrolepis*, *O. solitarius*, *O. oligolepis*, and *O. robustus)* and continental (*O. pintoi*, *O. paranensis*, *O. planaltinae*, *O. jenynsii*, *O. perdido*, *O. varii*, *O. brevioris*, *O. longirostris*, *O. jacuiensis*, and *O. itau*) species, according to Wendt *et al*.[29]

Using the classical approach of applying the threshold value of 2% *COI* divergence proposed by Hebert *et al*.,[30] pairs of sequences with >2% divergence were considered different species; however, in this study, low genetic distance was observed between *O. pintoi* and other *Oligosarcus* species, particularly in the large continental group. This has already been observed among species of other genera, such as *Astyanax*, which could suggest a relatively recent separation or a very close relationship between them.[32] Pereira *et al*.[33] observed low interspecific

genetic distance values for some Neotropical fish groups, including *Oligosarcus*, and suggested that applying the threshold value of 2% *COI* divergence can hide the real diversity of some genera. Therefore, these values must be used in conjunction with other data, such as those obtained through cytogenetic and morphological techniques, for effective species identification.

Considering the intraspecific percentage, two species (*O. jenynsii* and *O. hepsetus*) showed values >2%. This can be attributed to the presence of two haplotypes (H1 and H25) shared between this species, which may be due to misidentifications, since the species are quite distinct and belong to different groups (continental and coastal groups). The same occurred for *O. solitarius* and *O. argenteus* (coastal group), which shared the H24 haplotype.

Oligosarcus pintoi presented a low mean K2P distance when compared to *O. perdido* and, in particular, *O. paranensis* species (1% and 1.1%, respectively). The proximity between *O. pintoi* and *O. paranensis*, which occur in the same basin, was also observed by Pereira *et al*.[31] and Wendt *et al*.[6], where the species were recovered in the same clade in both Bayesian and neighbor-joining inferences. According to Wendt *et al*.[6] *O. pintoi* diverged in sympatry from a common ancestor with *O. paranensis*, considering that both have distinct morphologies of mouth and teeth.

Despite the low genetic distance values present, cytogenetic data supports the separation of these two species; a population of *Oligosarcus* cf. *paranensis* from the Keller River studied by Martinez *et al*.[34] has a different karyotype formula from the one found for the specimens in the present study, mainly regarding the number of subtelocentric and acrocentric chromosomes (Table 1). Differences were also observed in the number of chromosomes carrying NOR (Table 1). Considering the integration of cytogenetic and molecular data in the present work, as well as distribution data and a relatively recent separation of species within the genus, we can conclude that the specimens studied here belong to the species *O. pintoi*.

Following the classification of Wendt *et al*.[29], cytogenetic data were obtained for four species from the coastal group (*O. argenteus, O. hepsetus, O. solitarius,* and *O. macrolepis*) and five species from the continental group (*O. jenynsii, O. paranensis, Oligosarcus cf. paranensis, O. loginrostris, and O. pintoi*) (Table 1). Although all analyzed species share a conservative diploid value of 50 chromosomes, the karyotype formulas vary inter- and intra-specifically. However, the fundamental number ranges for species from the coastal and continental groups were 82-88 and 80-86, respectively. The intraspecific variation in the karyotype formula seems smaller among the species of the coastal group, for example, in *O. argenteus* and *O. hepsetus*. In the continental group, divergent karyotypes were observed between populations of *O. paranensis* (NF=80-86) and *O. pintoi* (NF=80-84). In addition, the occurrence of a supernumerary chromosome in the populations of *O. pintoi* from the Mogi-Guaçú[9] and Keller rivers (present study) demonstrates an evolutionary path divergent in relation to the other populations analyzed so far.

In some continental species, different haplotypes showed significant differences. For example, *O. paranensis*, which presented four different haplotypes in the upper Paraná River basin, with intraspecific distance values of 1.1%. Some authors have suggested that small fish have a restricted dispersal capacity which adds to physical and chemical barriers leading to a decrease in the genetic flow, and further, leading to the presence of specific genetic variations in each population.[33,35,36] These results seem to indicate alternative regional evolutionary scenarios for *Oligosarcus* in the upper Paraná River basin.

The presence of extra chromosomes is not a common feature of *Oligosarcus*. The first and only supernumerary chromosome mention was published by Falcão *et al*.[9] and it found a large metacentric chromosome in two specimens of *Paroligosarcus pintoi*, (also known as *Oligosarcus pintoi*) from the Mogi-Guaçu River, with a frequency of 10.5% among the specimens, while in the present study the frequency of this chromosome was 43% of analyzed metaphases. One of the most striking features of supernumerary chromosomes is that they are not necessarily found in all individuals of a species and can be restricted to specific cells, i.e., they have inter- and intra-individual variability.[11,12]

The distribution of constitutive heterochromatin shows light variation between species; it is more evident in the pericentromeric regions and in some chromosomes at the telomeres (Table 1). In the *O. pintoi* populations studied so far, small heterochromatic blocks are located mostly in the pericentromeric regions, as well as NOR-associated blocks.[37,38] According to Souza *et al*.[39] this localization of heterochromatin facilitated the exchange among other non-homologous chromosomes with posterior amplification, playing an important role in the chromosomal evolution of these fish.

Fully heterochromatic supernumerary chromosomes are more frequently reported in fish, but these elements can present different degrees of heterochromatization and consequent genetic deactivation of these chromosomes.[40,41] The typical heterochromatic nature of these elements reveals the presence of repetitive DNA; in many cases, this amount exceeds that observed in the genome of origin suggesting massive and rapid amplification of these regions.[12]

The number and location of the AgNORs varied intra- and inter-specifically within the genus *Oligosarcus* (Table 1). The population of *O. pintoi* from the Tunas River showed multiple NORs (pair 21 and variations between chromosomes 2, 8, and 22), while that from the Mogi-Guaçu River showed a signal only at pair 18; however, FISH with 18S rDNA probe revealed another pair of NOR-bearing chromosomes for this last population.[37,38]

Multiple 18 rDNA sites are present in almost every species studied (Table 1), but exceptions were found in *O. jenynsii* from the Uruguay River (Santa Catarina state),[38] *O. longirostris* from the Iguaçu River (Paraná state),[37] and *O. solitarius* from Aguapé Lake (Minas Gerais state).[3] The telomeric distribution of these sequences facilitates genetic material exchange during interphase, making this an efficient population marker.[3] However, a lack of or low

variation in the position of the 5S rDNA sequences has been observed for different populations of the same species within the genus, suggesting that this genomic region is very stable, as reported for other characins.[3,42] In *O. pintoi* from the Mogi-Guaçu River, three acrocentric chromosomes carried 5S DNA sequences including the two NOR-bearing acrocentric ones;[38] this synteny between 5S and 18S DNA sequences can also be observed in the acrocentric chromosomes of the specimens of the present study.

The new occurrence of a supernumerary chromosome in *O. pintoi*, even with different morphologies, confirms the previous data obtained by Falcão *et al*.[9] indicating a peculiar characteristic for this species suggesting that it was fixed in some populations and is not a sporadic event. This fact demonstrates a divergent evolutionary path from other populations and/or species of *Oligosarcus* studied so far, playing an important role in the karyotypic evolution of the group. In summary, the data presented here show that *Oligosarcus* is an interesting genus for integrative analysis involving cytogenetic and molecular tools, which may support phylogenetic relationships in this group.

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Disclousure Statament

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