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Cytomolecular investigations using repetitive DNA probes contribute to the identification and characterization of *Characidium* sp. aff. *C. vidali* (Teleostei: Characiformes)

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Characidium sp. aff. *C. vidali* is a species found in coastal streams in southeastern Brazil, which has karyotypic explanatory elements as the occurrence of microstructural variations, keeping the chromosomal macrostructure of the genus. The objective of this study was to apply cytomolecular tools in the chromosomes of *Characidium* sp. aff. *C. vidali* to identify characteristics in their karyotype contributing to cytogenetic definition of this species, adding information about the evolution of the chromosomal structure of the group. The species showed 2n = 50 chromosomes and from 1 to 4 additional B microchromosomes. FISH technique showed histone H3 and H4 genes in the short arm of pair 10, and microsatellites $(CA)_{15}$, $(CG)_{15}$, $(GA)_{15}$ and $(TTA)_{10}$ clustered in the subtelomeric portions of all A chromosomes, with total accumulation by supernumerary. The telomeric probe marked terminal regions of all chromosomes, in addition to the interstitial portion of four pairs, called ITS sites, with these markings being duplicated in two pairs, hence the double-ITS classification. C-banding revealed that supernumerary chromosomes are completely heterochromatic, that ITS sites are C-banding positive, but double-ITS sites are C-banding negative. So, throughout the evolution to *Characidium*, genomic events are occurring and restructuring chromosomes in populations.

Keywords: Fish cytogenetics, Crenuchidae, Repetitive DNA, Molecular markers.

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Characidium sp. aff. *C. vidali* é uma espécie encontrada em riachos costeiros do sudeste do Brasil, que apresenta elementos cariotípicos elucidativos quanto à ocorrência de variações microestruturais, conservando a macroestrutura cromossômica do gênero. O objetivo deste estudo foi aplicar ferramentas citomoleculares para identificar características no cariótipo de *Characidium* sp. aff. *C. vidali*, que contribuam para a definição citogenética desta espécie, agregando informações quanto à evolução da estruturação cromossômica do grupo. A espécie apresentou 2n = 50 cromossomos, além de 1 a 4 microcromossomos B por célula. A FISH mostrou os genes de histona H3 e H4 sintênicos no braço curto do par 10, e os microssatélites $(CA)_{15}$, $(CG)_{15}$, $(GA)_{15}$ e $(TTA)_{10}$ clusterizados nas porções subteloméricas de todos os cromossomos do complemento A, com grande acúmulo nos supranumerários. A sonda telomérica identificou marcações terminais em todos os cromossomos, além de quatro pares marcados intersticialmente, chamados de sítios ITS, e dois pares com duas marcações intersticiais, chamados de double-ITS. O bandamento C revelou que os cromossomos supranumerários são completamente heterocromáticos, que os sítios ITS são banda C positivos, mas os sítios double-ITS são banda C negativos. Então, ao longo da evolução de *Characidium*, eventos genômicos estão ocorrendo e reestruturando cromossomos nas populações.

Palavras-chave: Citogenética de peixes, Crenuchidae, DNA repetitivo, Marcadores moleculares.

INTRODUCTION

Characidium Reinhardt, 1867, is considered the most species rich genus within the family Crenuchidae, with 65 valid species (Buckup, Van der Sleen, 2017). These fishes are widely distributed in the Neotropical region, from eastern Panama to northeastern Argentina (Buckup, 2003), with some ecological characteristics that restrict their habitat (Caramaschi, 1986). They generally are small fish, that lives in isolated populations at the head of streams (Maistro *et al*., 1998), which in most cases are environments of high altitudes and intense water flow, so cases of endemism and allopatric speciation are common for these fish (Buckup, 2003, 2011). Therefore, it is probable that such factors directly impact the evolution of the karyotype structure of *Characidium* species, mostly in the microstructure of their chromosomes.

From the cytogenetic point of view, *Characidium* is considered a genus with conserved karyotypes, since most species have diploid number of 2n = 50 chromosomes (metacentrics and submetacentrics). However, some studies identified the occurrence of structural variation in the karyotypes of some species and populations, such as occurrence of heteromorphic ZZ/ZW sex chromosomes (Maistro *et al*., 1998, 2004; Centofante *et al*., 2001, 2003; Noleto *et al*., 2009; Pansonato-Alves *et al*., 2014; Serrano *et al*., 2017, 2019a,b), presence of supernumerary chromosomes (Maistro *et al*., 1998; Pansonato-Alves *et al*., 2010, 2011a,b, 2014; Serrano *et al*., 2017, 2019a,b), distribution of repetitive DNA sequences (Vicari *et al*., 2008; Machado *et al*., 2011; Pansonato-Alves

et al., 2011a, 2014; Scacchetti *et al*., 2015a,b; Serrano *et al*., 2017, 2019a,b; Pucci *et al*., 2018) and occurrence of natural triploidy (Centofante *et al*., 2001; Pansonato-Alves *et al*., 2011b). These particularities make the genus *Characidium* an interesting group for cytogenetic studies because rearrangements that occur in chromosome microstructure certainly can set the biological characteristics of the species and populations.

The eukaryote genome is composed of a large number of repetitive sequences, and the physical mapping as well as the knowledge of the molecular organization of these sequences has contributed significantly to a better understanding of the structural differences that occur in the karyotypes of many fish species (Cioffi, Bertollo, 2012; López-Flores, Garrido-Ramos, 2012). The accumulation of these repetitive elements in specific regions of the genome can generate breaks, inversions, deletions and amplifications in the chromosomes (Lim, Simmons, 1994; Dimitri *et al*., 1997; Raskina *et al*., 2008), which could reflect directly on the micro and/or macro structure of karyotype of a species or population.

There is a group of *Characidium* known to inhabit coastal drainages in Southeastern region of Brazil, and the species *Characidium* sp. aff. *C. vidali*, although not yet formerly described, has already been found in the Macaé River, São João River and Paraíba do Sul River (Leitão, Buckup, 2014). Using cytomolecular tools, Scacchetti *et al*. (2015c) initiated investigations on the karyotype of a population of this species, verifying its importance in studies on the evolution of sex chromosomes within the genus *Characidium*, and in the species differentiation process, since *Characidium* sp. aff. *C. vidali* was found in sympatry with *Characidium vidali* Travassos, 1967. An interesting feature observed was the occurrence of supernumerary chromosomes present only in *Characidium* sp. aff. *C. vidali*, making the specie more interesting for cytogenetic studies related to the origin of the extra genomic elements.

Cytomolecular markers were used in the present study to investigate the genome of *Characidium* sp. aff. *C. vidali* aiming at the cytogenetic characterization of the species, contributing to a better understanding of the karyotype diversification process in this fish group. The mapping of repetitive DNA sequences permitted to identify characteristics of the karyotype structure throughout the evolutionary history of the species. It is considered that the information obtained can help to the construction of a cytogenetic map for the species of the genus *Characidium*, establishing better knowledge about the genomic structuring of the species, as well as the mechanisms involved in the chromosomal diversification process and speciation.

MATERIAL AND METHODS

Twenty-three specimens of *Characidium* sp. aff. *C. vidali* (10 females and 13 males) from the Bananeiras Stream, São João River basin, municipality of Silva Jardim, state of Rio de Janeiro (22º28'51.8"S 42º23'39"W) were analyzed. The individuals were deposited in fish collection of Laboratório de Biologia e Genética de Peixes, Universidade Estadual Paulista "Julio de Mesquita Filho" (UNESP), Botucatu, São Paulo, Brazil, LBP 28520.

Cell suspensions with mitotic chromosomes were obtained from anterior portion of the kidney, according to Foresti *et al*. (1981). The heterochromatic regions were identified by C-banding (Sumner, 2003), and the chromosomes were classified following Levan *et al*. (1964).

Genomic DNA from *Characidium* sp. aff. *C. vidali* was extracted using the Kit Wizard Genomic DNA Purification (PROMEGA), from liver fragments preserved in ethanol. Telomeric probes (TTAGGG), histone H3 and histone H4, were obtained by PCR (*Polymerase Chain Reaction*) and the primers used are in accordance with Ijdo *et al*. (1991), Colgan *et al*. (1998), and Pineau *et al*. (2005), respectively. The marking of the three probes were by PCR, with histone H3 and telomeric being marked with Biotin-16-dUTP (Roche), while histone H4 was marked with Digoxigenin-11-dUTP (Roche). In the present study, oligonucleotide probes with microsatellite sequences were also used $(CA)_{15}$, $(GA)_{15}$, $(CG)_{15}$ and $(TTA)_{10}$, all already marked directly with Carboxy-tetramethylrhodamine N-succinimidyl ester (TAMRA), by Sigma (Kubat *et al*., 2008).

The physical mapping of the probes was performed under high stringency conditions, on slides of males and females of *Characidium* sp. aff. *C. vidali*, according to Pinkel *et al*. (1986). Signals were detected using anti-digoxigenin–Rhodamine for digoxigenin-11-dUTP and avidin–FITC (Sigma Aldrich, St Louis, MO, USA) for biotin-16-dUTP. Chromosomes were counterstained with DAPI (Vector Laboratories, Burlingame, Calif, USA), and the images analyzed with an epifluorescence microscope (Olympus BX61) were captured using the software Image Pro Plus 6.0 (Media Cybernetics, Rockville, Md. USA).

RESULTS

All specimens of *Characidium* sp. aff. *C. vidali* analyzed showed a basic diploid number of 2n = 50 chromosomes, composed of 32m + 18sm (Figs. 1A–D). All individuals analyzed presented micro B chromosomes mitotically unstable and eventually some difference in size, varying from one to four per cell in the individuals. Differences between the karyotypes of males and females were found only with the chromosomes of the second pair identified as sex-linked, being represented by two metacentrics of the same size (ZZ) in males (Figs. 1A, C) and by one metacentric (Z) and one submetacentric (W) in females, both medium-sized (Figs. 1B, D). Fig. 1 also shows an example of the species under study.

C-banding revealed heterochromatic blocks accumulated in a centromeric portion of all the chromosomes of the analyzed karyotypes, in addition some marked regions were observed in the terminal region in some pairs. In addition, a conspicuous block of constitutive heterochromatin was observed in the pericentromeric region of the long arm of the Z chromosome, while the W chromosome was completely heterochromatic. All supernumeraries present in the species were also considered to be completely heterochromatic (Figs. 1C, D, boxes).

The sequences for histone H3 (green) and H4 (red) genes, as revealed by the FISH technique were found clustered in synteny in the pericentromeric region of the short arm in a median sized chromosome pair (Fig. 2). The physical mapping of the four microsatellites motifs $(CA)_{15}$, $(CG)_{15}$, $(GA)_{15}$ and $(TTA)_{15}$ showed a preferential accumulation of these sequences in the subtelomeric regions of the A chromosomes, without differences between the karyotype of males and females (Fig. 3). In addition, the B chromosomes presented all tested microsatellites (Fig. 3).

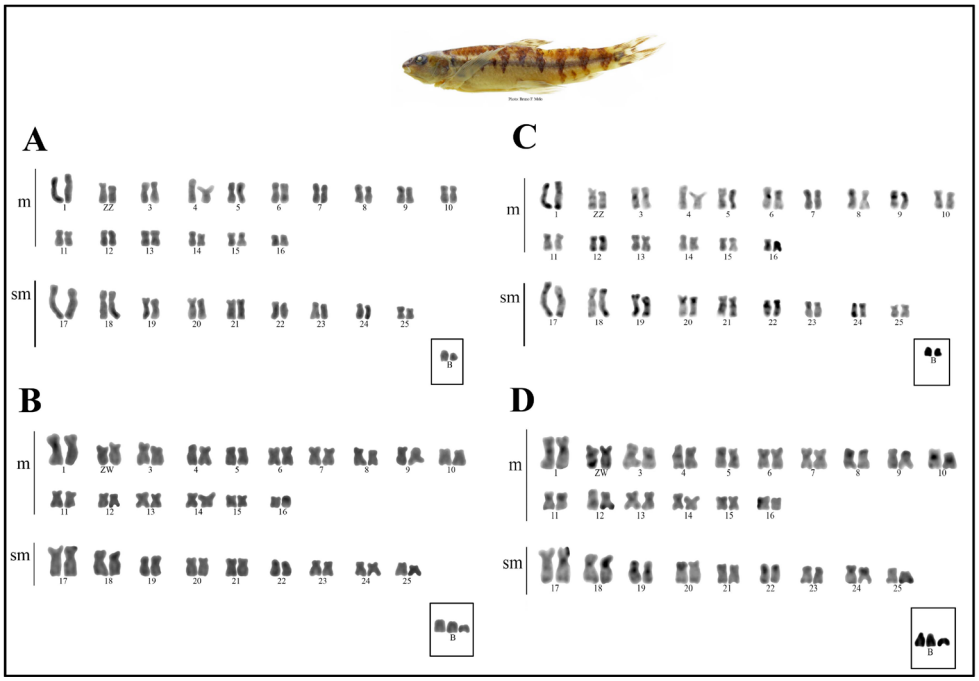

FIGURE 1 | *Characidium* sp. aff. *C. vidali* karyotypes arranged from mitotic metaphases after to conventional Giemsa staining and C-banding. **A.** and **C.** male karyotypes. **B.** and **D.** female karyotypes. B chromosomes are in the boxes. In evidence a preserved specimen under study. Photo of *Characidium* sp. aff. *C. vidali* by Bruno F. Melo.

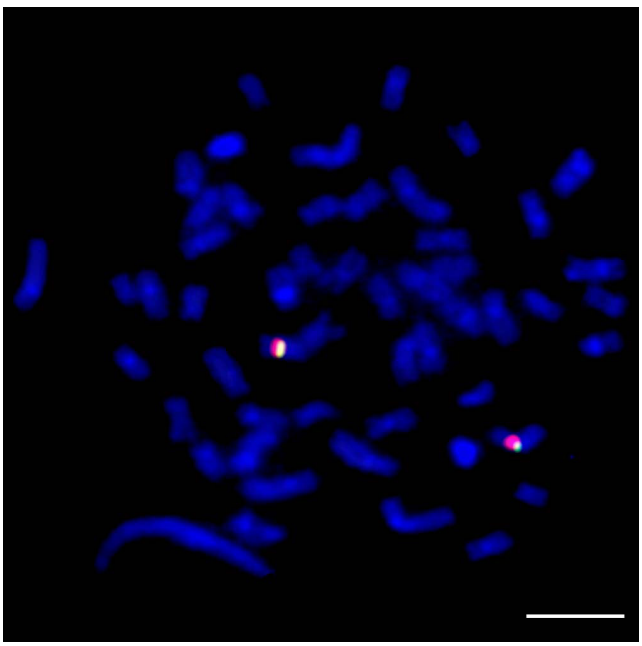

FIGURE 2 | Metaphase of *Characidium* sp. aff. *C. vidali* after FISH with histone H3 (green) and H4 (red) probe. Synteny marked in par 10. Scale bar = 10 µm.

Regarding the markings with telomeric probe TTAGGG, the chromosomes of *Characidium* sp. aff. *C. vidali* showed signals of hybridization in the terminal portions of all chromosomes, including the supernumeraries (Fig. 4A). Additionally, ITS (Interstitial Telomeric Sites) sites in pericentromeric regions of four chromosomal pairs and double-ITS sites, in interstitial portions of the short arm of two chromosome pairs were also observed (Fig. 4A). The sequential use of FISH and C-banding showed that ITS regions were coincident with constitutive heterochromatic blocks, while the double-ITS marks were C-band negative (Fig. 4B). B chromosomes showed telomeric marks only in the terminal portion and they were fully heterochromatic as revealed by C banding (Figs. 4A–B).

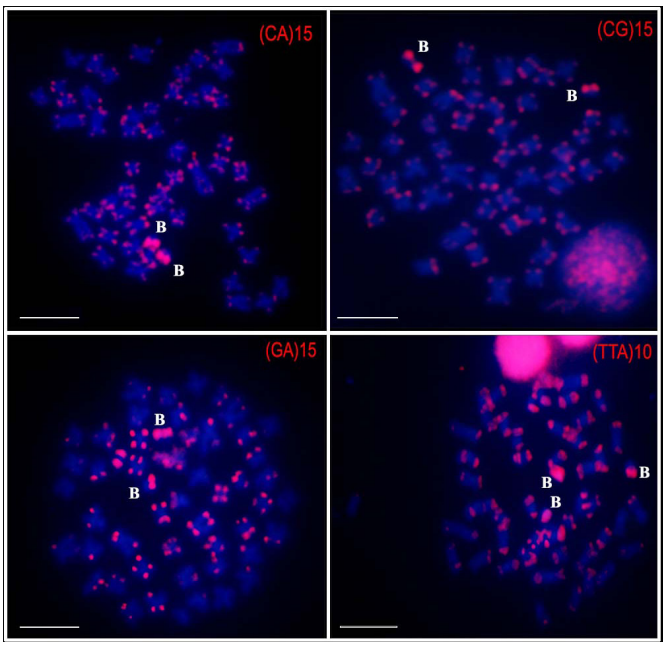

FIGURE 3 | Metaphase plates of *Characidium* sp. aff. *C. vidali* after fluorescent *in situ* hybridization (FISH) with four microsatellite motifs. B chromosomes present in the species are indicated. Scale bar = 10 µm.

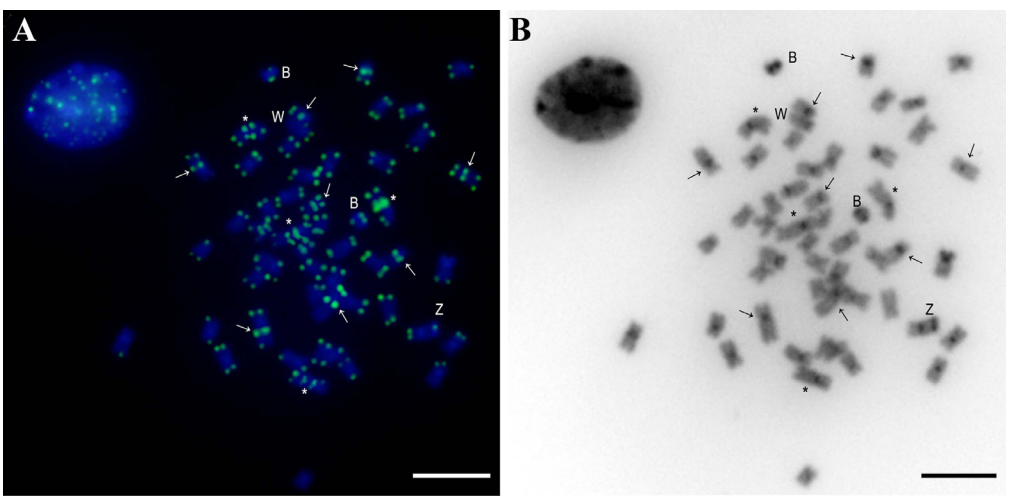

FIGURE 4 | Metaphase of *Characidium* sp. aff. *C. vidali* after fluorescent *in situ* hybridization (FISH); **A.** With a telomeric probe (TTAGGG) n. **B.** After sequential C-banding. The arrows indicate Interstitial Telomeric Sites (ITS), and asterisks highlight double-ITS marks; B = B-chromosomes; Z, W = sex chromosomes. Scale bars = 10 µm.

DISCUSSION

Characidum are an ideal group for karyotype studies, due to the structural variation in karyotype in populations. In this study, it is the first time that the markers H3, H4, telomeric sequences and motifs CA, CG, TTA, GA are described for *Characidum* sp. aff. *C. vidali* that will reinforce the classic cytogenetic data already described and increase the knowledge about the composition of these sequences in the karyotypes of this species.

The results observed in the karyotype analysis of *Characidium* sp. aff. *C. vidali*, corroborate the findings of Scacchetti *et al*. (2015c) concerning the diploid number, presence of heteromorphic chromosomes linked to sex and distribution pattern of constitutive heterochromatin. The identification of B microchromosomes in all the cells analyzed, varying only in quantity, was also reported by the authors, drawing attention to the chromosomal structure of the species, which in both works maintained the diploid number of 50 chromosomes. In addition, the present work suggests the occurrence of evident characteristics in the genomic elements that are acting and reorganizing the karyotype microstructure during differentiation of the species and the evolutionary processes of this group of fish.

The physical mapping with H3 and H4 histone probes showed that in *Characidium* sp. aff. *C. vidali* these genes are colocalized in the short arm of the pair 10. This particularity was also observed by Pucci *et al*. (2018) in *Characidium zebra* Eigenmann, 1909, and *Characidium gomesi* Travassos, 1956, both species collected in the Paiol Grande stream, while in *C. gomesi* from the São João River the two clusters were syntenic but located in the short arms of the chromosomes in pair 5. Additionally, Serrano *et al*. (2017) mapped the H3 histone gene in *Characidium alipioi* Travassos, 1955, verifying that the gene was located in the long arm of the chromosomes also in the par 10.

The histone genes H1, H2A, H2B, H3 and H4 form a complex multigenic family, encoding basic and essential proteins (Eirín-López *et al*., 2009). The organization of these sequences has been the subject of many studies in fish. In some fish groups, the location of these genes are considered conserved, as the histone H1 in *Astyanax* Baird & Girard, 1854 (Hashimoto *et al*., 2011) and *Pseudoplatystoma* Bleeker, 1862 (Hashimoto *et al*., 2013), histone H3 in *Hypostomus* Lacepède, 1803 (Pansonato-Alves *et al*., 2013) and histone H1, H3, and H4 in *Psalidodon bockmanni* (Vari & Castro, 2007) (Silva *et al*., 2013). In *Characidium*, the H3 and H4 histone genes appear to be frequently colocalized and so far, in most cases, clustered in the chromosomes of par 10; therefore, the variations observed by Serrano *et al*. (2017) and Pucci *et al*. (2018) would be consequence of structural rearrangements occurred in the karyotypes of the species. These findings increase the evidence that *Characidium* is a group of fish that frequently undergoes significant microstructural changes in its karyotype, although conserving the chromosomal macrostructure in most species and populations. The mapping of repetitive sequences particularly contributes to reveal this particularity.

The four microsatellite sequences mapped in this study showed an interesting similar pattern of distribution on the karyotype of *Characidium* sp. aff. *C. vidali*, with preferential accumulation of the sequences in the subtelomeric portions of the chromosomes. This distribution model observed in several species of the genus *Characidium* (Scacchetti *et al*., 2015b; Serrano *et al*., 2017) is also common among other Characiformes (Cioffi *et al*., 2010, 2012; Poltronieri *et al*., 2014; Terencio *et al*., 2013), representing

a usual pattern among the components of this order. In most eukaryotic organisms, the microsatellites distribution does not occur randomly. As being highly repetitive sequences, they are usually found accumulated in the heterochromatic regions of the genome where the exchange rate is reduced (Lohe *et al*., 1993; Cuadrado, Jouve, 2011; Pathak, Ali, 2012). The four microsatellites motifs analyzed in the present work showed a preferential accumulation near the subtelomeric regions in almost all chromosomes of the A complement, without differences between the sex of individuals. However, B chromosomes were almost completely positive for C-banding and conspicuous marks characterized the cumulative presence of the four motifs along this element.

The accumulation of repetitive sequences in genome, can change the structures of chromosomes, and mapping these regions in DNA can provide information about origin, structure organization, and functions of specific chromosomes (Ruiz-Ruano *et al*., 2015). In this sense, many studies have attempted to investigate the location of microsatellite sequences on sex-linked chromosomes to understand about the participation of these repetitive regions in the chromosome differentiation process (Kubat *et al*., 2008; Poltronieri *et al*., 2013; Ziemniczak *et al*., 2014; Scacchetti *et al*., 2015b,c). In the case of *Characidium* sp. aff. *C. vidali* the accumulation of the four motifs highlighted the B chromosomes of the species. It can be supposed that the accumulation could occur due to the low rate of recombination of these chromosomes, and the fact that they are completely heterochromatic (Lohe *et al*., 1993; Cuadrado, Jouve, 2011). It is interesting to note that the same four motifs were mapped in *Characidium gomesi* (Scacchetti *et al*., 2015b), as well as $(CA)_{15}$ and $(GA)_{15}$ sequences in *C. alipioi* (Serrano *et al*., 2017), and although the two species also carry heterochromatic B chromosomes, no microsatellite clusters were observed in their supernumerary element. This indicates that the B chromosomes of the three species differ in their structural composition as revealed by their specific degree of heterochromatic enrichment, and probably in *Characidium* sp. aff. *C. vidali* the four motifs have accumulated after the establishment of the supernumerary chromosome in the karyotype of the population.

The distribution of microsatellites in the genome of a population can be attributed to different events such as uneven crossing-over, ectopic recombination, disturbs in DNA repair or slippage, and action of transposable elements (Dover, 1993; McMurray, 1995; Hancock, 1996; Ruiz-Ruano *et al*., 2015). In *Characidium,* we believe that the spread of microsatellites occurred by ectopic recombination, as pointed out by Pucci *et al*. (2018) when mapped transposable elements in some species of the genus and observed a low accumulation of these sequences in the genomes. In addition, the similar pattern of microsatellite allocated in the subterminal regions of the chromosomes may be further evidence that reinforces the macrostructural homogeneity of the karyotypes in this group of fish.

In the vertebrate genome, the identification of telomeric sequences also increase knowledge about the chromosomal structure of the species. Such sequences are made up of six nucleotides TTAGGG that repeat *in tandem* (Meyne *et al*., 1989; Guerra *et al*., 2004), and the present study showed the physical mapping of these sites in *Characidium* sp. aff. *C. vidali*. All chromosomes of the species showed the telomeric region marked, including the supernumerary elements. As telomeric sequences confer integrity and protect DNA from damage (Zakian, 1995; de Lange 2005; Palm, de Lange, 2008; Luke, Lingner, 2009; Chan, Chang, 2010; Feuerhahn *et al*., 2010; O'Sullivan, Karlseder, 2010),

these sequences play an important role also in the stability of the extra elements in the genome and could explain the presence of B chromosomes in the carrier individuals.

In *Characidium* sp. aff. *C. vidali* telomeric probes hybridized with the terminal portion of all chromosomes in the karyotype, including the supernumerary elements. Additionally, interstitial telomeric sequence (ITS) signals are highlighted in several regions of many chromosomes. These marks were previously found in other species of the genus varying only in number of labeled homologous chromosomes (Scacchetti *et al*., 2015a). C-banding revealed that interstitial sites accumulate constitutive heterochromatin, and this could mean that ITSs behave as satellite components, turning possible their spread to more internal chromosomal regions (Pagnozzi *et al*., 2000, 2003; Metcalfe *et al*., 2004; Scacchetti *et al*., 2015a). This could explain the variation in the number of interstitial sites in the species here studied and in other six species of *Characidium* analized by Scacchetti *et al*. (2015a). An interesting point observed was the occurrence of chromosomes bearing double-ITS marks, not evidenced by C-banding, in addition to single-ITS marks C-banding positive. One can consider the hypothesis of the possible occurrence of a position effect acting in the determination of the negative heterochromatic pattern of regions with double ITS that, when changing from the pericentromeric position to a region along the short arm of the chromosomes would promote changes in these segments previously heterochromatic, modifying their structural behavior. Alternatively, even due to the occurrence of changes in the composition and structure of the sequences in these segments, modifying and distending the heterochromatic components in these blocks, turning them euchromatic.

Many studies try to identify and understand the dispersion process of telomeric sequences in the internal portion of the chromosomes of several organisms (Meyne *et al*., 1989; Pagnozzi *et al*., 2000, 2003; Bolzán, Bianchi, 2006; Lin, Yan, 2008; Ruiz-Herrera *et al*., 2008; Cioffi *et al*., 2010; Scacchetti *et al*., 2015a). In general, these sites are interpreted as remnants of chromosomal rearrangements, which can modify the karyotype structure of the taxon (Holmquist, Dancis, 1979; Hastie, Allshire, 1989; Meyne *et al*., 1989; Pagnozzi *et al*., 2003; Cioffi *et al*., 2010). However, there are cases where variation in number and structure of the chromosomes are evident but ITS are not observed, indicating that the sequences (TTAGGG)n may have been eliminated or suffered structural modification throughout the evolution of the genome (Ocalewicz *et al*., 2013). In this context, the presence of interstitial telomeric marks in *Characidium* cannot be interpreted as absolute evidence of structural chromosomal rearrangements, since the genus is known by its conserved karyotype macrostructure. Scacchetti *et al*. (2015a) proposed that species with ITS belong to a small group of phylogenetically related *Characidium* species and the spread of this mark within the genomes would be a consequence of ectopic transposition and/or events of recombination. The physical mapping of telomeric sequences here described were similar to those presented by Scacchetti *et al*. (2015a), evidencing a possible relationship between the species (MLMO, work in progress).

The results obtained revealed that *Characidium* sp. aff. *C. vidali* shares the common and conserved diploid number of the genus, and corroborates the hypothesis that the ecological characteristics of the species with restrict habits to headwaters of small streams favors the occurrence of endemism and speciation by allopatry, a particularly common situation for these fish. It is clear that such factors directly affect the evolution

of the karyotype structure of *Characidium* populations, mostly in the composition of the microstructure of their chromosomes. The mapping of the sequences performed here provide an identification of the species by means of cytomolecular tools, in addition to aggregating information about the evolution of repetitive portions of the genome within *Characidium*. The findings also reveal that, despite presenting a conserved chromosomal macrostructure, different micro alterations can occur in the genome of the species and provide an important and significative mechanism of diversification and, consequently, for speciation in this group of fish.

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ETHICAL STATEMENT

The procedures were carried out in accordance with the National Council for Control of Animal Experimentation (CONCEA) and Ethics Committee on Use of Animals (CEUA) (protocol 971) of the BIOSCIENCE INSTITUTE/UNESP.

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