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Chromosomal Spreading of Microsatellites and $(TTAGGG)_n$ Sequences in the *Characidium zebra* and *C. gomesi* Genomes (Characiformes: Crenuchidae)

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Chromosome differentiation · Heterochromatin · Repetitive DNA · Sex chromosomes

Abstract
Sex chromosome evolution involves the accumulation of repeat sequences such as multigenic families, noncoding repetitive DNA (satellite, minisatellite, and microsatellite), and mobile elements such as transposons and retrotransposons. Most species of *Characidium* exhibit heteromorphic ZZ/ZW sex chromosomes; the W is characterized by an intense accumulation of repetitive DNA including dispersed satellite DNA sequences and transposable elements. The aim of this study was to analyze the distribution pattern of 18 different tandem repeats, including $(GATA)_n$ and $(TTAGGG)_n$, in the genomes of *C. zebra* and *C. gomesi*, especially in the *C. gomesi* W chromosome. In the *C. gomesi* W chromosome, weak signals were seen for $(CAA)_{10}$, $(CAC)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAC)_{10}$, and $(CA)_{15}$ probes. $(GA)_{15}$ and $(TA)_{15}$ hybridized to the autosomes but not to the W chromosome. The $(GATA)_n$ probe hybridized to the short arms of the W chromosome as well as the $(CG)_{15}$ probe. The $(GATA)_n$ repeat is known to be a protein-binding motif. GATA-binding proteins are necessary for the decondensation of heterochromatic regions that hold coding genes, especially in some heteromorphic sex chromosomes that may keep genes related to oocyte development. The $(TAA)_{10}$ repeat is accumulated in the entire W chromosome, and this microsatellite accumulation is probably involved in the sex chromosome differentiation process and crossover suppression in *C. gomesi*. These additional data on the W chromosome DNA composition help to explain the evolution of sex chromosomes in *Characidium*.

The accumulation of repetitive DNAs is one of the key features of genome and sex chromosome differentiation. These sequences have been used to solve problems related to the evolutionary history of chromosomes [Cross et al., 2006; Traldi et al., 2013]. Sex chromosome evolution is also characterized by chromosomal rearrangements, heterochromatinization, and repeat sequence accumulation [Ross and Peichel, 2008; Poltronieri et al., 2013; Pucci et al., 2014; Ziemniczak et al., 2014]. These sequences can be classified as multigenic families, noncoding tandemly re-

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peated DNA (satellite, minisatellite, and microsatellite), and mobile elements such as transposons and retrotransposons [Hardman, 1986; Sumner, 2003].

Minisatellites are tandemly arranged repeats that may have 6–24 bp [Hardman, 1986]. One of these sequences is located at the terminal end of every chromosome and structures the telomeres. The telomeric DNA sequences confer stability to chromosomes and prevent end-to-end fusions and DNA degradation [de Lange, 2002; Ocalewicz, 2013]. A well-studied telomeric DNA sequence is $(TTAGGG)_n$ that is conserved in most vertebrates and can provide important information about the evolutionary status of the karyotype. Thus, chromosome mapping of interstitial telomeric sequences (ITSs) may help detect chromosomal fusion and inversion events to provide insight into karyotype evolution in vertebrate species [Meyne et al., 1990; Abuín et al., 1996; Rosa et al., 2012; Ocalewicz, 2013].

Microsatellites or simple sequence repeats (SSRs) are tandemly repeated DNA sequences composed of 1–6 bp long units and show several patterns of localization in coding and noncoding regions in eukaryotes. The SSRs are involved in chromosomal structure and even in gene expression control. They show an abundance of taxon-specificity and vary in degree of repetition [Tóth et al., 2000]. In addition, microsatellite sequences have been described to move through the genome in association with transposable elements [Akagi et al., 2001; Coates et al., 2010].

Characidium species show a large amount of DNA repeats in the sex chromosomes [Machado et al., 2011; Pansonato-Alves et al., 2011; Pucci et al., 2014; Scacchetti et al., 2015a–c]. This genus belongs to the Crenuchidae family which is composed of 71 valid species [Eschmeyer et al., 2015]. All *Characidium* species hitherto cytogenetically studied have a diploid chromosome number of 2n = 50 [for a review, see Pucci et al., 2014; Scacchetti et al., 2015c].

In the genus *Characidium*, some species present a differentiated ZZ/ZW sex chromosome system, others like *C. zebra*, *C. lagosantense*, *C. tenue*, *C. xavante*, and *C. stigmosum* do not have any heteromorphic sex chromosomes. The absence of differentiated sex chromosomes can be considered as a plesiomorphic feature [Vicari et al., 2008; Pazian et al., 2013; Scacchetti et al., 2015b]. This suggests that sex chromosome differentiation is a more recent evolutionary event in the *Characidium* genus and is reinforced by the absence of sex chromosomes in the *Crenuchus* outgroup [Pazian et al., 2013]. In *Characidium* species with differentiated sex chromosomes, the Z and W sex chromosomes vary in size and morphology as well as the degree of heterochromatinization and presence/absence of rDNA. These differences in the ZW sex chromosomes in *Characidium* may have accumulated after vicariance events that prevent gene flow among species with a differentiated proto sex chromosome pair. These variations may lead to mechanisms of reproductive isolation and meiotic barriers among sympatric and syntopic species [Pucci et al., 2014].

The karyotype evolution of *Characidium* includes the differentiation of ZW chromosomes, which involves intense heterochromatinization and accumulation of repetitive DNA in the W sex chromosome [Centofante et al., 2001; Pansonato-Alves et al., 2010, 2011, 2014; Pucci et al., 2014]. Comparative chromosome mapping of repetitive sequences as microsatellites can be a useful tool to gain insights into genome evolution. Thus, the main aim of this paper was to study the molecular composition of the W chromosome by analyzing the location pattern of 18 different tandem repeats including the well-studied $(GATA)_n$ repeats and the minisatellite telomere repeats over the genomes of *C. zebra* and *C. gomesi*.

Materials and Methods

Sampling and Chromosome Preparation

Two *Characidium* species were cytogenetically studied: *C. zebra* Eigenmann, 1909 (Paiol Grande Stream, São Bento do Sapucaí, SP) and *C. gomesi* Travassos, 1956 (São João River, Carambeí, PR). The specimens (15 *C. zebra*; 17 *C. gomesi*) were then deposited in the following ichthyology museums: Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia), Universidade Estadual de Maringá, and Museu Nacional, Rio de Janeiro, Brazil, voucher numbers (NUP 14577–14580; MNRJ 29183). Chromosomes were prepared for analysis using the air-drying procedure [Bertollo et al., 1978]. Constitutive heterochromatin was detected by the method of Sumner [1972].

Probe Preparation

Microsatellite $(GATA)_n$ and minisatellite $(TTAGGG)_n$ sequence probes were synthetized by polymerase chain reaction (PCR) in the absence of DNA templates according to Ijdo et al. [1991] using $(GATA)_7$/$(TATC)_7$ and $(TTAGGG)_5$/$(CCCTAA)_5$ primers, respectively. The PCR reaction mix contained 2.5 mM $MgCl_2$, 400 μM dNTPs, 100 μM of each primer, and 2 U of Platinum®*Taq* DNA Polymerase (Invitrogen). The PCR cycle conditions were set at 5 min 94°C; 9 cycles of 1 min at 94°C, 30 s at 55°C, 1 min at 72°C; 29 cycles of 1 min at 94°C, 30 s at 60°C, 90 s at 72°C; with final elongation for 5 min at 72°C. The purified sequences were then labeled with digoxigenin-11-dUTP by nick translation using the DIG Nick Translation Mix (Roche Applied Science).

The other microsatellites included mono-, di-, tri-, and tetranucleotides: $(A)_{30}$, $(C)_{30}$, $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CG)_{15}$, $(CGG)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAC)_{10}$, $(GACA)_4$, $(GAG)_{10}$, $(TA)_{15}$, and $(TAA)_{10}$. These oligonucleotides were labeled directly with biotin at the 5′ end during synthesis (Sigma-Aldrich) and were used as probes.

The *C. gomesi* W-specific probe was made by microdissecting *C. gomesi* W chromosomes (the specimens were from the Quebra Perna River population). The microdissected W chromosomes were transferred to a microtube and used for PCR amplification employing a Whole Genome Amplification kit (WGA4; Sigma-Aldrich) according to the manufacturer's instructions. The W chromosome amplification product was purified with a GenEluteTM PCR Clean-Up kit (Sigma-Aldrich). Thereafter, the purified product was used as a template for reamplification, employing the Genome Plex® WGA3 kit (Sigma-Aldrich). In the WGA3 amplification reaction, the nucleotide digoxigenin-11-dUTP was incorporated with a 7:3 ratio of dTTP:digoxigenin-11-dUTP (Roche Applied Science) according to Pucci et al. [2014]. The 18S rDNA probe (GenBank accession numbers AY449632 and AY449633), isolated from the genomic DNA of *Prochilodus argenteus* Spix & Agassiz, 1829 [Hatanaka and Galetti, 2004], was labeled with biotin through nick translation using biotin-16-dUTP (Nick Translation Biotin; Roche Applied Science).

Fluorescence in situ Hybridization

Chromosome spreads were prepared from the *Characidium* species and were studied by fluorescence in situ hybridization (FISH) using microsatellite and minisatellite probes (around 50 metaphase spreads were analyzed). FISH was performed under high stringency (2.5 ng/μl of each probe, 50% formamide, 2× SSC, 10% dextran sulfate, pH 7.0–7.2, at 37°C overnight) following the general procedure described by Pinkel et al. [1986]. Slides were then washed at room temperature, twice for 5 min each in 2× SSC and once for 1 min in 1× PBS. Signal detection was performed using an anti-streptavidin antibody conjugated to Alexa Fluor 488 (Molecular Probes) and an anti-digoxigenin antibody conjugated to rhodamine (Roche Applied Science). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 0.2 μg/ml) in the mounting medium Vectashield (Vector) and observed under the epifluorescence microscope Zeiss Axio Imager A2 coupled with the ZEISS pro 2011 software in Zeiss AxioCam MRm camera of monochromatic capture with CCD sensor and 1.4 megapixels resolution (Carl Zeiss®).

Results

Karyotypes, 18S rDNA Sites and Localization of the C. gomesi W-Specific Probe

Both species studied had a diploid chromosome number of 2n = 50 (figs. 1 and 2) and had the karyotypes previously published [Centofante et al., 2001; Vicari et al., 2008]. The fundamental number (FN) was 100 in both species. There were no karyological differences between *C. zebra* males and females. All chromosome pairs in the *C. zebra* karyotype are homomorphic (fig. 1a, b) and have scattered signals of the *C. gomesi* W-specific probe (fig. 1c). FISH with the 18S rDNA probe revealed the presence of this sequence in the subterminal region of the long arm of pair 23 (fig. 1c).

C. gomesi presents heteromorphic ZZ/ZW sex chromosomes (fig. 2a, b). The Z chromosome is a large metacentric chromosome, and the W sex chromosome is easily recognizable. It is the only subtelocentric and completely heterochromatic element (fig. 2a, b) among the metacentric and submetacentric chromosomes of the complement. In *C. gomesi*, the W-specific probe used in this study marked the entire W chromosome and the pericentromeric region of the Z chromosome (fig. 2c). The 18S rDNA probe hybridized to chromosome pairs 10 and 17 in *C. gomesi* (fig. 2c).

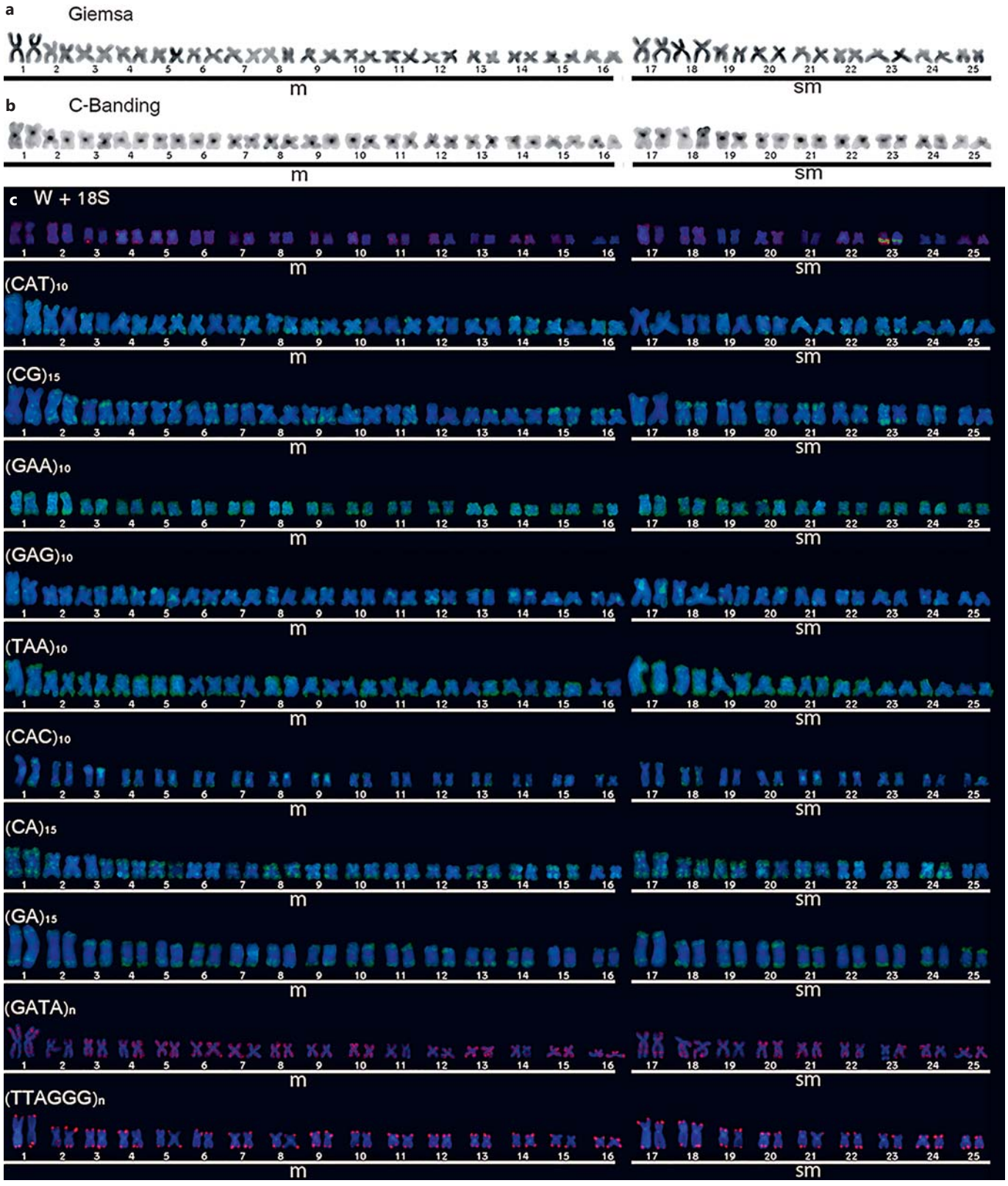
Microsatellite Distribution in C. zebra and C. gomesi Chromosomes

FISH with the microsatellite probes revealed different patterns on chromosomes. In *C. zebra*, only 10 of the 18 hybridized probes showed results. FISH analyses with $(CAT)_{10}$, $(CG)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$, and $(TAA)_{10}$ microsatellite probes revealed scattered signals on all chromosomes (fig. 1). Beside dispersed hybridization signals of the $(CAC)_{10}$ probe, significant signal blocks were seen in different positions in euchromatic regions of some chromosomes (fig. 1). Signals from $(CA)_{15}$, $(GA)_{15}$, and $(GATA)_n$ probes were clustered in the subterminal region of most chromosomes (fig. 1). The FISH results with telomere probes did not show any ITS in *C. zebra* (fig. 1). In *C. gomesi*, only 12 of the 18 probes hybridized to different chromosomes. FISH analyses with $(CAA)_{10}$, $(CAC)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAC)_{10}$, and $(TAA)_{10}$ probes revealed dispersed signals throughout the autosomes with weak blocks in the subterminal region of some of them (fig. 2). The FISH experiments using probes for $(CA)_{15}$, $(CG)_{15}$, $(GA)_{15}$, $(TA)_{15}$, and $(GATA)_n$ showed huge blocks in the subterminal region in most of the autosomes (fig. 2). FISH with telomere probes revealed no ITSs in *C. gomesi* (fig. 2).

Fig. 1. Karyotypes of female specimens of *C. zebra* subjected to Giemsa staining (**a**), C-banding (**b**), and FISH with different probes (**c**): W chromosome probe (red signals) and 18S rDNA (green signals), microsatellite probes $(CAT)_{10}$, $(CG)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$, $(TAA)_{10}$, $(CAC)_{10}$, $(CA)_{15}$, $(GA)_{15}$, $(GATA)_n$, and the $(TTAGGG)_n$ telomere repeat.

Fig. 2. Karyotypes of female specimens of *C. gomesi* subjected to Giemsa staining (**a**), C-banding (**b**), and FISH with different probes (**c**): W chromosome probe (red signals) and 18S rDNA (green signals), microsatellite probes $(CAA)_{10}$, $(CAC)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAC)_{10}$, $(TAA)_{10}$, $(CA)_{15}$, $(CG)_{15}$, $(GA)_{15}$, $(TA)_{15}$, $(GATA)_n$, and the $(TTAGGG)_n$ telomere repeat.

(For figures see next pages.)



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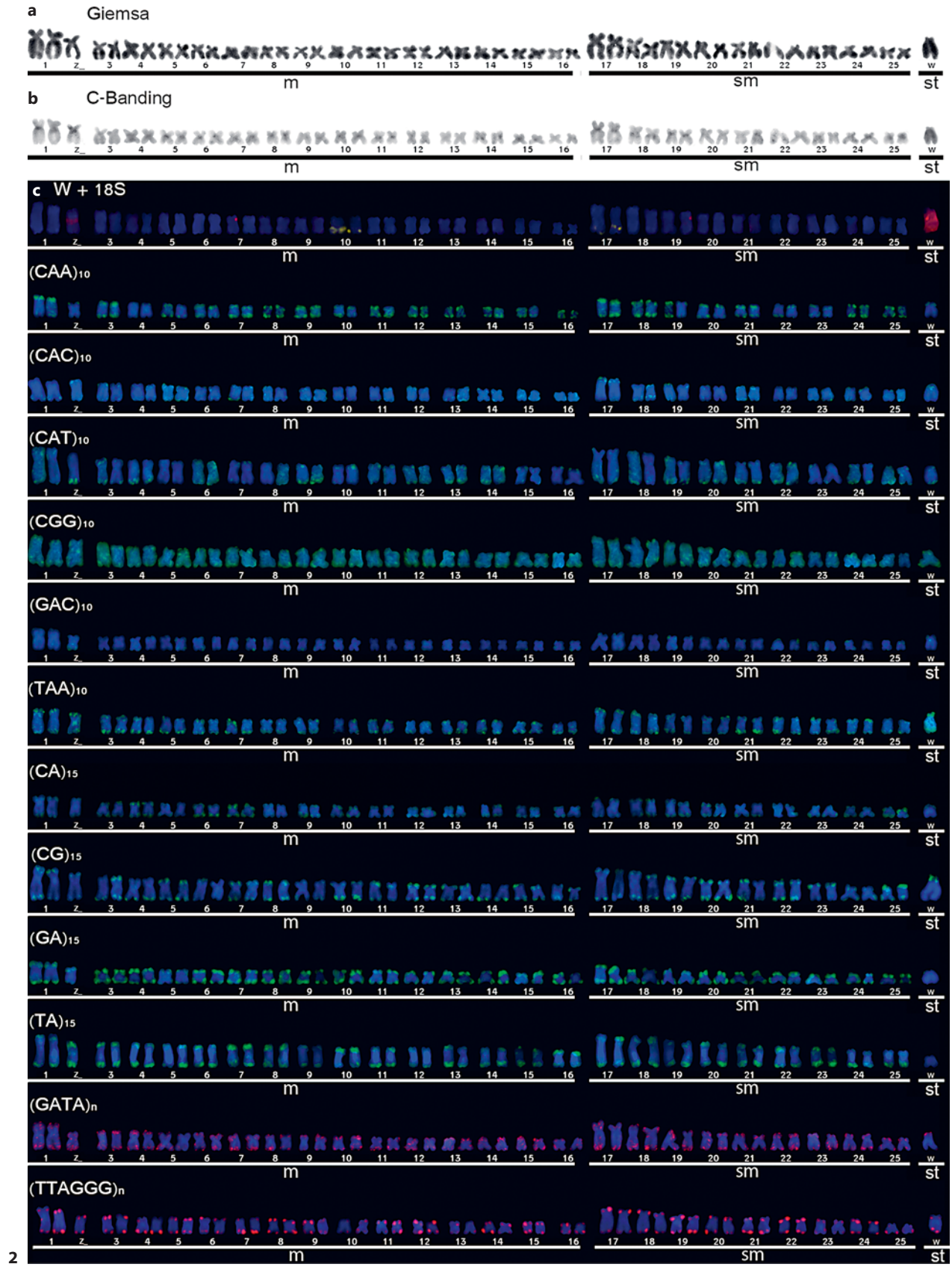

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Regarding the sex chromosomes, weak signals were seen with $(CAA)_{10}$, $(CAC)_{10}$, $(GAC)_{10}$, $(CA)_{15}$, $(CG)_{15}$, $(GA)_{15}$, and $(GATA)_n$ in the Z chromosome (fig. 2). Signals bunched out in the terminal region of the long arm of the Z chromosome in FISH with $(CAT)_{10}$ and in the terminal regions of both arms in FISH with $(TA)_{15}$ (fig. 2). Only FISH with $(CGG)_{10}$ and $(TAA)_{10}$ probes showed signals over the entire Z sex chromosome (fig. 2). In the W chromosome, weak signals were obtained by $(CAA)_{10}$, $(CAC)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAC)_{10}$, and $(CA)_{15}$ probes (fig. 2). Signals bunched in the short arm of the W chromosome in FISH with $(CG)_{15}$ and $(GATA)_n$ probes (fig. 2). Only FISH with the $(TAA)_{10}$ probe resulted in signals over the entire W sex chromosome (fig. 2). The $(GA)_{15}$ and $(TA)_{15}$ probes hybridized to the autosomes and did not hybridize to the W chromosome. ITSs were detected neither in the Z nor the W chromosomes (fig. 2).

Discussion

Localization of Microsatellites in the C. zebra and C. gomesi Genomes

The evolution of the genome, sex chromosomes, and B chromosomes is associated with a specific accumulation of repeat DNA sequences including microsatellites [Milani and Cabral-de-Mello, 2014]. Herein, *C. zebra* and *C. gomesi* showed different patterns of microsatellite distribution. In *C. zebra*, FISH with the triplex $(CAC)_{10}$ indicates that this repeat may have a functional role in the euchromatic region. More dispersed signals were seen with $(CAT)_{10}$, $(CG)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$, and $(TAA)_{10}$ probes in *C. zebra* and with $(CAA)_{10}$, $(CAC)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAC)_{10}$, and $(TAA)_{10}$ probes in *C. gomesi*. The dispersed pattern of microsatellites in the *Characidium* chromosomes may be due to the association with mobile elements. In fact, transposable elements may contain microsatellites and contribute to the spreading of these sequences. For instance, in *Abracris flavolineata*, *Mariner*-like transposable elements are thought to be associated with microsatellites [Milani and Cabral-de-Mello, 2014] and are involved in the movement of these repeats. Moreover, the transposable elements are also considered hotbeds for new microsatellites because they are involved in the 'birth process' during the lifecycle of these repeats [Kelkar et al., 2011; Grandi and An, 2013]. Other plausible mechanisms of the microsatellite dispersion in the *Characidium* chromosomes may be ectopic recombination, unequal crossing over, and replication slippage [Kelkar et al., 2011].

The dinucleotides $(CA)_{15}$ and $(GA)_{15}$ accumulated more intensely in the subterminal regions of the chromosomes in both *C. zebra* and *C. gomesi*. These regions can be hotspots for the accumulation of SSRs and might be associated with the structural formation of the telomere and participate in chromosome rearrangements as proposed for *Semaprochilodus* [Terencio et al., 2013]. The same subterminal pattern was seen for $(CG)_{15}$ and $(TA)_{15}$ probes, but only in *C. gomesi*. Scacchetti et al. [2015b] reported similar results in the chromosome mapping of $(CG)_{15}$ in *C. gomesi*, *C. lanei*, *C. pterostictum*, and *C. vidali*; these microsatellites were partially eliminated in *C. timbuiense* and *C.* cf. *zebra*.

Distribution Pattern of Microsatellites and Evolution of the W Chromosome

The early stages in the evolution of sex chromosomes comprise the acquisition of a sex determination function by one chromosome of the proto sex chromosome pair. Chromosomal rearrangements such as paracentric/pericentric inversions can prevent the recombination between these chromosomes and avoid DNA sequence exchange. Next, heterochromatinization and accumulation of repetitive DNA (mobile elements, satellite and microsatellite DNAs) in one of the homologues increases the recombination suppression. These facts make the sex chromosomes cytologically distinguishable/heteromorphic and participate in the genetic erosion of the sex-specific chromosome [Steinemann and Steinemann, 2005; Palacios-Gimenez et al., 2013; Ziemniczak et al., 2014].

The SSR $(CG)_{15}$ accumulated in the short arm of the W chromosome of *C. gomesi*. An accumulation is also seen in the karyotype of *C. zebra* and is associated with the NOR site in pair 23. This result corroborates the hypothesis of the origin of the ZZ/ZW sex chromosome system by a translocation that involved the NOR-bearing chromosome pair 23 and the metacentric pair 2 [Machado et al., 2011; Pansonato-Alves et al., 2014; Pucci et al., 2014].

Here, FISH experiments using the $(GATA)_n$ probe revealed the same distribution pattern in autosomes of *C. zebra* and *C. gomesi*. In both species wide blocks in the terminal sites on almost all autosomes were labeled. The GATA sequence has been described in snakes and highlighted as a satellite fraction in female DNA [Singh et al., 1981]. Later, it was discovered that the GATA sequence is a motif associated with transcription factors [Trainor et al., 1996].

These simple repeats are generally described as a motif for the sex- and tissue-specific GATA-binding protein called BBP (Bkm-binding protein). It is hypothesized that this BBP is involved in the decondensation of heterochro-

matic regions in heteromorphic sex chromosomes where there are genes related to oocyte development [Singh et al., 1994]. *Bombyx mori* has a BmBBP, a GATA-binding protein, expressed predominantly in the pupal ovary [Priyadarshini et al., 2003]. Thus, the localization of the $(GATA)_n$ sequence in the sex chromosomes of *C. gomesi* can be associated with W chromatin modifications or transcriptional regulatory events.

The W chromosome in *C. gomesi* is completely heterochromatic and is thought to be inactive. However, the presence of the GATA motif in the short arm of the W chromosome may indicate the existence of coding, W-linked genes in this region. The GATA motif and its associated proteins are involved in the decondensation of the genic regions and possibly play a role in the decondensation of the W chromosome as well. In mice, $(GATA)_n$ repeats were evidenced in Y chromosomes and in the proximal region of chromosome pair 17. This suggests that autosomes could be involved in the male germ cell differentiation [Kiel-Metzger and Erickson, 1984].

Other patterns of microsatellite distribution were seen in *C. gomesi* where FISH experiments using the probes $(CGG)_{10}$ and $(GAC)_{10}$ revealed small marks in the W, and $(TAA)_{10}$ repeats were strongly evident over the entire chromosome. The $(TAA)_{10}$ data in *C. gomesi* confirms the chromosome localization of the complementary $(TTA)_{10}$ in the W sex chromosome of other *C. gomesi* populations [Scacchetti et al., 2015b]. The $(CGG)_{10}$ and $(GAC)_{10}$ probes did not show hybridization signals in the *C. zebra* karyotype indicating that these microsatellites do not represent a plesiomorphic trait. In addition, the $(TAA)_{10}$ sequence is widely distributed in the *C. zebra* karyotype. However, the FISH experiments with this microsatellite in *C. gomesi* revealed strong signals on the W chromosome and scattered signals on the Z chromosome.

This distribution pattern is not related to the rDNA site translocation movement as corroborated by the $(CG)_{15}$ repeat; $(TAA)_{10}$ showed no interaction with NOR sites in *C. zebra*. However, the $(TAA)_{10}$ accumulation contributed in the W chromosome molecular degeneration event. The association of this microsatellite with the sex chromosome is probably a consequence of an interaction with some transposable element or satellite DNA.

Localization of $(TTAGGG)_n$ Sites

In vertebrates, the telomeres are composed of tandem repeat sequences of $(TTAGGG)_n$. In studies of karyotype evolution, chromosomal mapping of telomere sequence probes is usually performed to localize ITSs that indicate chromosome rearrangements such as fusions and/or inversions [Lui et al., 2013; Scacchetti et al., 2015a]. Here, rearrangements such as fusions and inversions involving the telomeric region were not the main cause of the W chromosome evolution. The absence of interstitial $(TTAGGG)_n$ hybridization signals in this sex chromosome suggests the occurrence of other rearrangements such as paracentric/pericentric inversions in the evolutionary process of this genus. Scacchetti et al. [2015a] described the distribution of ITSs in the chromosomes of 9 *Characidium* species, where *C. vidali*, *C. zebra*, and *C. gomesi* also presented no ITSs. However, all the other analyzed species, *C. pterosticum* (2 different populations), *C. schubarti*, *C. serrano*, *C. timbuiense*, *C. lanei*, and *C. lauroi* presented ITSs, even more than 1 ITS block in the same chromosome except in the sex chromosomes. It has been proposed that the presence of ITSs is a derived condition in this genus, which probably occurred by transposition-mediated events and ectopic recombination [Scacchetti et al., 2015a]. Thus, the telomeric sequence shows an intense transposition dynamic in the autosomes of *Characidium* and is stable at the terminal regions of the sex chromosomes.

New insights into the sex chromosome differentiation of *Characidium* were gained by mapping microsatellites and $(TTAGGG)_n$ sequences in *C. zebra* and *C. gomesi* genomes. The $(CG)_{15}$ and $(GATA)_n$ probes revealed blocks only in the short arm of the W chromosome. The short arm of the W chromosome keeps homology with the terminal region of the Z chromosome in relation to the $(CG)_{15}$, $(GATA)_n$, and $(TAA)_{10}$ sequences. The homology is also present with the centromeric region in relation to the $(GATA)_n$ and $(TAA)_{10}$ sequences. The W chromosome of *C. gomesi* particularly accumulated $(TAA)_{10}$ repeats. In addition, the $(TAA)_{10}$ microsatellite accumulation in the W chromosome is probably involved in the chromosome differentiation process and crossover suppression in *C. gomesi* sex chromosome evolution.

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Statement of Ethics

All procedures with the animals were performed in accordance with the Ethical Committee on Animal Use (CEUA 11/2013) of the Universidade Estadual de Ponta Grossa and current Brazilian legislation.

Disclosure Statement

The authors have no conflicts of interest to declare.

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