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Karyotypes and Repetitive DNA Evolution in Six Species of the Genus *Mahanarva* (Auchenorrhyncha: Cercopidae)

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Key Words
Agricultural pests · Heterochromatin · Holocentric chromosomes · Repetitive DNAs · Telomeres

Abstract
Insects of the Cercopidae family are widely distributed and comprise 59 genera and 431 species in the New World. They are xylemophagous, causing losses in agricultural and pasture grasses, and are considered as emerging pests. Chromosomally, these insects have been studied by standard techniques, revealing variable diploid numbers and primarily X0 sex chromosome systems (males). We performed chromosome studies in 6 *Mahanarva* (Cercopidae) species using standard and differential chromosome staining as well as mapping of repetitive DNAs. Moreover, the relationship between the repetitive DNAs was analyzed at the interspecific level. A diploid chromosome number of 2n = 19,X0 was documented, with chromosomes gradually decreasing in size. Neutral or GC-rich regions were detected which varied depending on the species. Fluorescence in situ hybridization with a $(TTAGG)_n$ telomeric motif probe revealed terminal signals, matching those of the Cot DNAs obtained from each species, that were also restricted to the terminal regions of all chromosomes. Dot blot analysis with the Cot fraction from *M. quadripunctata* showed that at least part of the repetitive genome is shared among the 6 species. Our data highlight the conservation of chromosomal features and organization of repetitive DNAs in the genus *Mahanarva*, suggesting a low differentiation for chromosomes and repetitive DNAs in most of the 6 species studied.

Cercopidae belong to the hemipteran infraorder Cicadomorpha, suborder Auchenorrhyncha. These insects are distributed worldwide and represent the largest cercopoid family, with 431 species grouped in 59 genera in the New World [Carvalho and Webb, 2005; Cryan and Urban, 2012; Paladini and Cryan, 2012]. They are xylemophagous insects, and due to this habit, they cause losses in agricultural and pasture grasses, as pests of forage grasses, sugarcane, and occasionally other cultivated grass, such as rice [Bernardo et al., 2003; Peck and Thompson, 2008]. At least 11 genera of Cercopidae are considered emerging pests, including *Mahanarva,* with 39 species widely distributed in South and Central America [Paladini and Carvalho, 2007].

Chromosomal studies in Auchenorrhyncha have been performed in ~820 species, mostly using standard chro-

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mosome staining with description of diploid numbers, that range from 2n = 8 to 2n = 38 in females, and sex chromosomes, revealing predominance of XX♀/X0♂ [Boring, 1913; Halkka, 1964; Dey, 1991; Kuznetsova and Aguin-Pombo, 2015]. For Cercopidae, ~31 species were karyotyped showing variability for diploid number and sex chromosomes, e.g., 2n = 28, XY♂ in *Cosmoscarta* species *(C. dimidiata*, *C. septempunctata*, *C. decisa*, *C. egens*, and *C. fulviceps)*; 2n = 19, X0♂ in *Mahanarva fimbriolata*, *M. posticata,* and *Deois flavopicta*; and 2n = 15, X0♂ in *Notozulia entreriana* [Dey, 1991; Marin-Morales et al., 2002; Castanhole et al., 2010].

Repetitive DNAs are abundant in eukaryotic genomes, including in-tandem and dispersed elements, and could play an important role in genome and chromosome diversification [Cavalier-Smith, 1982; Charlesworth et al., 1994; Elder and Turner, 1995; Kidwell, 2002; Feschotte and Pritham, 2007]. These repetitive sequences represent excellent chromosomal markers and have been successfully used for the understanding of chromosomal evolution in distinct insect orders [e.g., Palomeque and Lorite, 2008; Nguyen et al., 2010; Anjos et al., 2015]. In species with holocentric chromosomes, repetitive DNA markers are even more important to understand evolution, due to the lack of other chromosomal features, such as the primary constriction, allowing the identification of specific chromosomes or chromosomal regions [Pita et al., 2014; Kuznetsova and Aguin-Pombo, 2015].

Among insects with holocentric chromosomes, the organization of heterochromatin has mostly been studied by conventional techniques, and studies using isolated repetitive fractions or specific repetitive DNAs have been undertaken in a few species [Kuznetsova et al., 2009; Bardella et al., 2014; Pita et al., 2014; Kuznetsova and Aguin-Pombo, 2015]. The telomeric repeat $(TTAGG)_n$ is considered ancestral for arthropods [Vítková et al., 2005], although in insects this motif was repeatedly lost [Frydrychová et al., 2004], including representatives of distinct orders, like Coleoptera, Hemiptera, Diptera [Frydrychová et al., 2004], and Hymenoptera [Gokhman et al., 2014].

Here, we performed chromosome studies in 6 species of *Mahanarva* applying standard staining and banding techniques, and FISH with the ancestral arthropod telomeric motif $(TTAGG)_n$. In addition, a repetitive DNA-enriched fraction (Cot-DNA) was obtained for each species, and the location of the pool of repetitive DNAs was established by FISH to the species of origin. The conservation of the repetitive DNA pools between the 6 species was tested by cross-hybridization using dot blot assays. The similarities and differences in the chromosomal markers were used to infer the evolutionary processes that led to chromosomal and repetitive DNA diversification in the species studied.

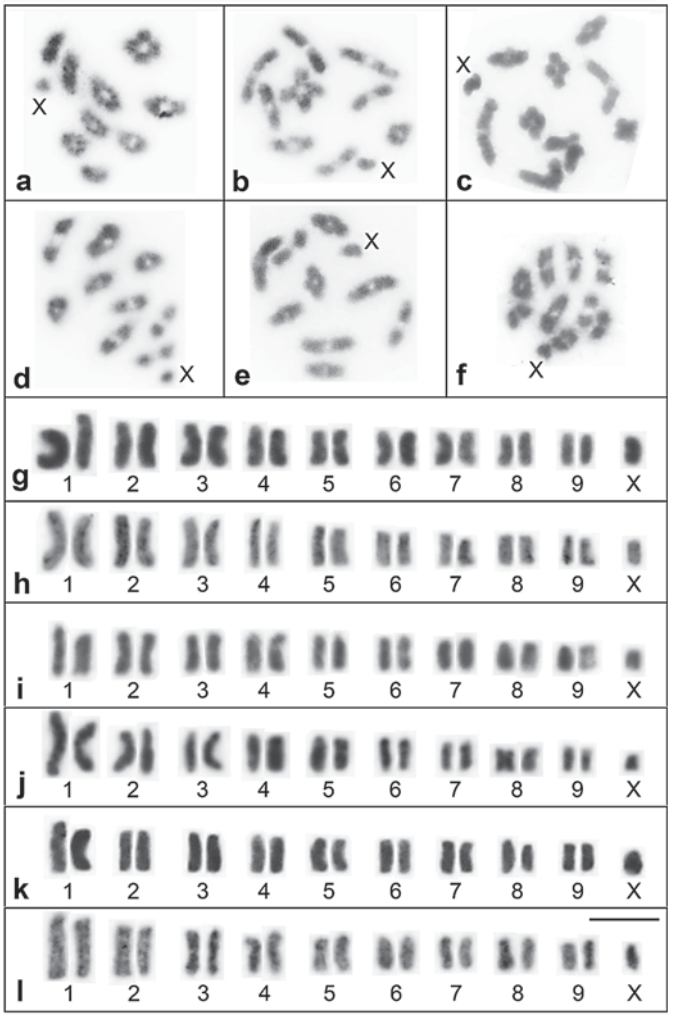

Fig. 1. Meiotic (**a–f**) and mitotic (**g–l**) chromosomes of 6 *Mahanarva* species stained with 5% Giemsa. **a**, **g** *M. fimbriolata*; **b**, **h** *M. liturata*; **c**, **i** *M. quadripunctata*; **d**, **j** *M. spectabilis*; **e**, **k** *M. tristis*; **f**, **l** *M. vittata*. **a–c**, **e** Diakinesis; **d**, **f** metaphase I; **g–l** arranged karyotypes. In **a–f**, the X chromosome is indicated. Bar = 5 μm.

Materials and Methods

Adult males of 6 *Mahanarva* species *(M. fimbriolata*, *M. liturata*, *M. quadripunctata*, *M. spectabilis*, *M. tristis*, and *M. vittata)* were sampled in natural areas of Rio Claro, São Paulo State, Brazil. Animals were anesthetized, the testes were dissected, left in distilled water for 5 min, and then fixed in modified Carnoy's solution (absolute ethanol:acetic acid, 3:1). Whole animals were stored in

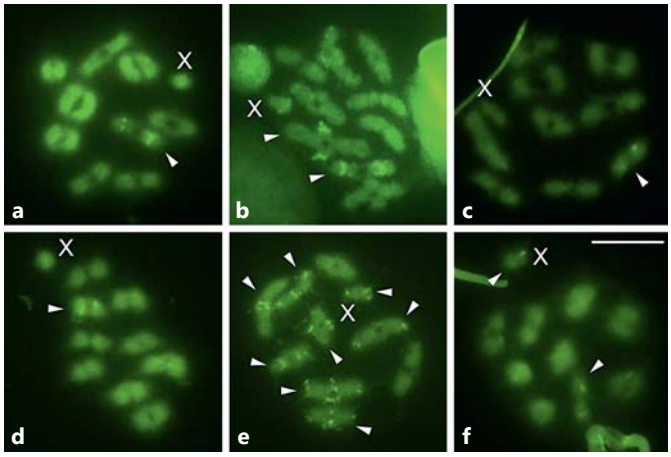

Fig. 2. Fluorochrome staining with CMA_3 in meiotic cells of 6 *Mahanarva* species. **a** *M. fimbriolata*; **b** *M. liturata*; **c** *M. quadripunctata*; **d** *M. spectabilis*; **e** *M. tristis*; **f** *M. vittata*. **b**, **e** Diakinesis; **a**, **c**, **d**, **f** metaphase I. The X chromosome is indicated, and chromosomes bearing positive signals are shown with arrowheads. Bar = 5 μm.

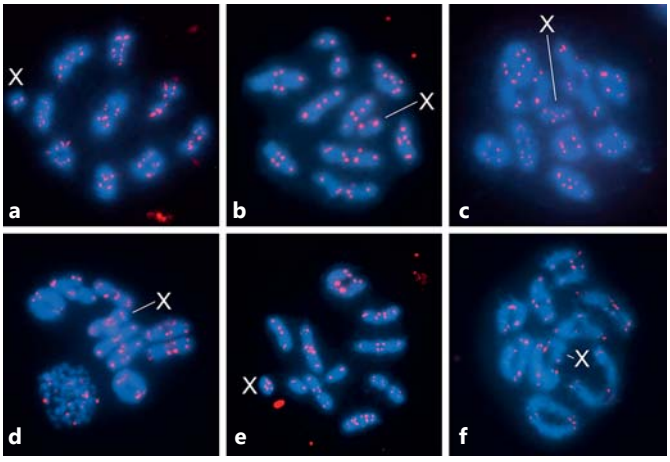

Fig. 3. FISH using the telomeric motif $(TTAGG)_n$ in meiotic chromosomes of 6 *Mahanarva* species. **a** *M. fimbriolata*; **b** *M. liturata*; **c** *M. quadripunctata*; **d** *M. spectabilis*; **e** *M. tristis*; **f** *M. vittata*. **a**, **b**, **d**, **e** Metaphase I; **c** diakinesis; **f** diplotene. The X chromosome is indicated in each cell. Bar = 5 μm.

100% ethanol for DNA extraction, following the protocol of Sambrook and Russell [2001], and for species identification. The testis follicles were macerated in a drop of 50% acetic acid, and the slides were dried using a hot plate at 40–45°C for chromosome preparation. The chromosomes were stained with 5% Giemsa, and fluorochrome staining with chromomycin A_3/distamycin A/DAPI (CMA_3/DA/DAPI) was performed as proposed by Schweizer et al. [1983].

The telomeric probe was obtained through PCR using the complementary primers $(TTAGG)_5$ and $(CCTAA)_5$ following the protocol of Ijdo et al. [1991]. Cot-DNA samples (DNA enriched for highly and moderately repetitive DNA sequences) were obtained according to the protocol described by Zwick et al. [1997] using the reannealing time of 25 min.

FISH was performed according to the protocol of Pinkel et al. [1986], with modifications described by Cabral-de-Mello et al. [2010]. The telomeric probe was labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany) through PCR, and Cot-DNA fractions were labeled with biotin-14-dATP through nick translation (Invitrogen, San Diego, Calif., USA). Probes labeled with digoxigenin-11-dUTP were detected using anti-digoxigenin rhodamine (Roche), and probes labeled with biotin-14-dATP were detected using Streptavidin Alexa Fluor 488 (Invitrogen). All preparations were counterstained with DAPI and mounted in Vectashield (Vector, Burlingame, Calif., USA). Chromosomes and signals were observed using an Olympus microscope BX61 equipped with a fluorescence lamp and appropriate filters. Photographs were recorded with a DP70 cooled digital camera. The images were merged and optimized for brightness and contrast with Adobe Photoshop CS2.

For dot blot analysis, the biotin-14-dATP-labeled Cot-DNA fraction of *M. quadripunctata* was used as probe. Approximately 100 ng of genomic DNA from each of the 6 *Mahanarva* species was applied to a Hybond N+ nylon membrane (GE Healthcare) and denatured with 0.5 M NaOH for 2 min. The membranes were then washed in 5× SSC for 1 min. Next, the membrane was dried for 90 min at 80°C. The hybridization was carried out overnight (16 h) at 37°C using 200 ng of denatured labeled probe diluted in ECL gold hybridization buffer hybridization solution (GE Healthcare), with the addition of bovine serum albumin (0.05% w/v) and NaCl (2.5 M). After hybridization, the membrane washes were performed as follows: 5× SSC for 5 min at 42°C; 3 times in primary buffer containing 6 M urea, 0.4% SDS (w/v), and 0.1× SSC for 10 min at 42°C each; and 20× SSC for 5 min at room temperature. Finally, the biotin chromogenic detection kit (K0661, Thermo Scientific) was used, following the manufacturer's recommendations for detection.

Results

All 6 *Mahanarva* species showed 2n = 19, X0♂ (fig. 1), with holocentric chromosomes decreasing gradually in size (fig. 1g–l). This pattern made the accurate identification of each pair difficult for meiotic cells, except for the X chromosome, which can be observed as a univalent during meiosis (fig. 1a–f). Fluorochrome staining was homogeneous for DAPI in all species (results not shown), but distinct patterns were detected for CMA_3 (fig. 2): interstitial blocks in 1 autosomal bivalent of *M. fimbriolata*, *M. quadripunctata,* and *M. spectabilis* (fig. 2a, c, d); 2 bivalents with GC-rich blocks, 1 located interstitially and the other terminally, in *M. liturata* (fig. 2b); an interstitial block in 1 autosomal bivalent and in 1 terminus of the X

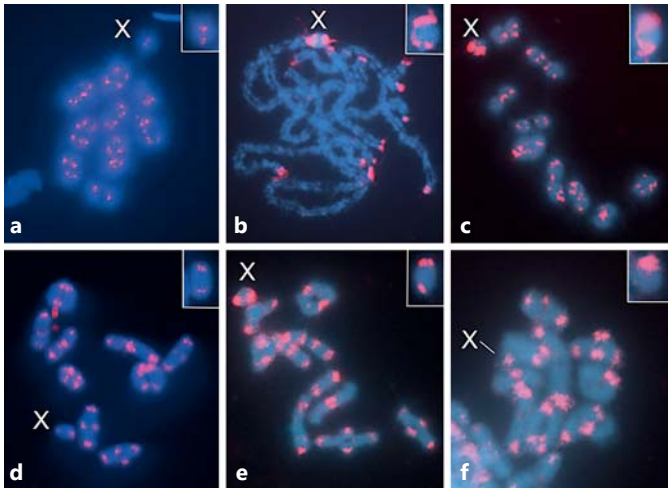

Fig. 4. FISH mapping of the Cot-DNA fraction obtained from each species (self-FISH) in meiotic cells. **a** *M. fimbriolata*; **b** *M. liturata*; **c** *M. quadripunctata*; **d** *M. spectabilis*; **e** *M. tristis*; **f** *M. vittata*. **a**, **c–f** Metaphase I; **b** pachytene. The X chromosome is indicated in each cell and is highlighted in the insets. Bar = 5 μm.

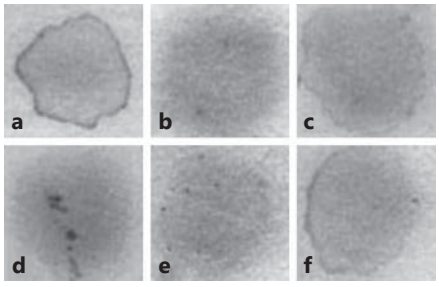

Fig. 5. Labeled Cot-DNA obtained from *M. quadripunctata* hybridized on a membrane with *Mahanarva* species unlabeled genomic DNAs. **a** *M. quadripunctata*; **b** *M. liturata*; **c** *M. spectabilis*; **d** *M. vittata*; **e** *M. tristis;* **f** *M. fimbriolata*.

chromosome in *M. vittata* (fig. 2f); and multiple sites, including 7 autosomal pairs and the X chromosome in *M. tristis*. For *M. tristis,* 2 autosomes presented signals in 1 terminus, 4 showed signals in both termini, 1 showed an interstitial signal, and the X harbored signals in both termini (fig. 2e).

The telomeric motif was restricted to the terminal regions of all chromosomes (fig. 3). These terminal regions were also strongly labeled with the Cot-DNA fraction obtained from each species, revealing stronger signals than those of the telomeric probes (fig. 4). A remarkable difference between the species for the location of Cot-DNA was the presence of only 1 terminal block in the X chromosome of *M. vittata* (fig. 4f), while the other species exhibited blocks in both X chromosome termini (fig. 4a–e). Finally, the dot blot using the Cot-DNA fraction obtained from *M. quadripunctata* as a probe revealed positive hybridization against the genomic DNA of the other *Mahanarva* species (fig. 5).

Discussion

Chromosomal studies in Cercopidae are scarce, and for *Mahanarva,* the karyotypes of *M. fimbriolata* and *M. posticata* were previously described [Marin-Morales et al., 2002]. Invariably, the 7 *Mahanarva* species studied until now share 2n = 19, X0♂, and this could represent the plesiomorphic character for the genus, which is also observed in other Cercopidae species, such as *D. flavopicta* [Castanhole et al., 2010], although other additional species should be karyotyped. Considering the ancestral diploid number for Cercopoidea, i.e., 2n = 26–28 [Kuznetsova and Aguin-Pombo, 2015], *Mahanarva* has a derived constitution that originated most parsimoniously through chromosomal fusions, leading to a reduction in 2n. Although extensive variability in diploid numbers has been reported in Auchenorrhyncha in high rank taxa such as tribes, families, and subfamilies [Kuznetsova and Aguin-Pombo, 2015], species within the same genus could present more stable karyotypes as noticed here for *Mahanarva*, and in *Cosmoscarta* [Dey, 1991] and *Alebra* [Kuznetsova et al., 2013].

The telomeric probe $(TTAGG)_n$ did not identify internal telomeric repeats that could be the result of chromosomal fusion suggested here for *Mahanarva*. The $(TTAGG)_n$ internal sequences could have been lost during chromosomal evolution, or may not have been identified due to low repeat numbers and classical FISH technique resolution limitations. The $(TTAGG)_n$ motif is canonical and considered ancestral for insects and arthropods as a whole [Sahara et al., 1999; Frydrychová et al., 2004], although it has been variably lost during the evolution of some orders, such as Diptera, Coleoptera, Hymenoptera, and Hemiptera [Sahara et al., 1999; Frydrychová et al., 2004; Gokhman et al., 2014]. Among hemipterans, the apomorphic heteropterans Cimicomorpha (families Miridae, Cimicidae, and Tingidae) and Pentatomomorpha (families Pyrrhocoridae and Pentatomidae) lost the $(TTAGG)_n$ ancestral motif [Frydrychová et al., 2004; Grozeva et al., 2011; Golub et al., 2015]. However, $(TTAGG)_n$ was not lost in other groups, including 4 aphid species [Monti et al., 2011], in the coccid *Planococcus li-*

lacinus [Mohan et al., 2011], in the suborder Coleorrhyncha [Kuznetsova et al., 2015], in the heteropteran infraorder Nepomorpha [Kuznetsova et al., 2012], and in Auchenorrhyncha representatives [Frydrychová et al., 2004; Maryańska-Nadachowska et al., 2012; Golub et al., 2014; Kuznetsova et al., 2015b]. For Auchenorrhyncha, $(TTAGG)_n$ is present in the families Aphrophoridae [Maryańska-Nadachowska et al., 2012], Cicadellidae [Kuznetsova et al., 2015b], Myerslopiidae [Golub et al., 2014], and Delphacidae [Frydrychová et al., 2004]. The data presented here for Cercopidae expand the knowledge of telomere types in this insect group, supporting the hypothesis that the ancestral arthropod telomere motif is conserved in Auchenorrhyncha.

The limited data for C-heterochromatin distribution in Auchenorrhyncha have shown accumulation of heterochromatin in one or both terminal/subterminal regions of the chromosomes [Kuznetsova et al., 2003, 2009, 2015b]. Here, the use of the anonymous Cot-DNA fraction that is enriched with highly repetitive DNA which is also abundant in heterochromatin, like satDNAs, revealed the accumulation of this genomic fraction mainly in terminal chromosome regions, a common location in *Mahanarva* as in other species of Auchenorrhyncha [Kuznetsova et al., 2009, 2015b] and as frequently reported in species with holocentric chromosomes [Spence et al., 1998; Bardella et al., 2014]. The observed difference in the size of Cot-DNA signals could suggest differential accumulation of repetitive DNAs between the species. Specifically, differences in the size of the X chromosome blocks were noticed, also indicating variability in the accumulation of repetitive DNAs. This pattern is more evident for *M. vittata*, in which the Cot-DNA hybridized in only 1 X terminus. The X chromosome of *M. vittata* could represent a variation related to the subgenus *Ipiranga* in which *M. vittata* is included, in comparison to the subgenus *Mahanarva* that includes the other species. These data also suggest that differential processes of expansion or contraction of repetitive DNAs act in the X chromosome of *Mahanarva* that led to its diversification among the 6 species.

Interestingly, the use of the Cot-DNA fraction from *M. quadripunctata* as a probe in dot blotting against genomic DNA from the 5 other *Mahanarva* species revealed conservation of at least part of the highly and moderately repetitive DNA among the species. It is known that highly repetitive DNAs, especially the satDNAs, are subject to the action of several molecular mechanisms causing variation and allowing rapid evolution, generating species-specific sequences [Dover, 1986; Charlesworth et al., 1994; López-Flores and Garrido-Ramos, 2012]. These molecular mechanisms could have less impact in the *Mahanarva* genomes, as evidenced by the conservation detected here, although we do not know the type of sequences conserved because the Cot-DNA is anonymous. Therefore, analysis of specific families of satDNAs should be undertaken. Among insects, conservation of a portion of the repetitive DNA pools was observed; for example, in terminal chromosomal regions of *Dichotomius* beetles [Cabral-de-Mello et al., 2011] through Cot-DNA interspecies FISH, and in Triatominae species using inter-specific GISH analysis [Pita et al., 2014].

Regarding repetitive DNA base pair composition, *Mahanarva* species appear to have a low specificity for AT or GC richness, except for *M. tristis*, with a predominance of GC blocks. Although few studies have addressed the heterochromatin base pair constitution in Auchenorrhyncha, the data show wide variation, evidencing heterogeneity for the constitutive heterochromatin composition of some species and homogeneity for others [Kuznetsova et al., 2003, 2009, 2015b]. The distinct patterns of GC-rich and neutral block distribution observed here reveal that the repetitive DNAs have diverged differently at the intragenomic level and between species, mainly between the species with 1 GC-rich block, i.e., *M. fimbriolata*, *M. quadripunctata*, and *M. spectabilis*, and the other species with 2 or multiple GC-rich blocks. However, some repetitive DNAs remained conserved, as demonstrated by Cot-DNA hybridization. Distinct families of repetitive DNAs differing in base pair composition could be compartmentalized in specific chromosomes among the 6 species, and are analyzed for 1 species of the genus using data from a sequenced genome [Anjos et al., in preparation]. A remarkable invariable feature among the 6 species is the presence of an interstitial GC-rich block in 1 medium-sized autosome pair, suggesting that this pair could be conserved, although other markers should be employed to confirm this statement. Finally, the data obtained from CMA_3 reinforce the differentiation of the X chromosome (that harbors a GC-rich block) of *M. vittata* in relation to the other species. This differentiation was also indicated by the results of Cot-DNA FISH, and could be a characteristic of the subgenus *Ipiranga*.

According to Melters et al. [2012], there are no consequences associated with chromosomal rearrangements arising from fission or fusion in holocentric chromosomes, because they can segregate perfectly after these rearrangements, encouraging rapid chromosomal evolution. However, in some other clades, the rate of karyotypic evolution is not increased [Gokhman and Kuz-

netsova, 2006; Panzera et al., 2012], as also observed here for *Mahanarva*. In contrast, there are some examples in which chromosomal diversity is noticed, such as *Myzus persicae* [Mandrioli et al., 2014] and Buthidae scorpions [Mattos et al., 2013]. Our data show that besides macrochromosomal stability, conservation for the pool of repetitive DNAs can be documented in *Mahanarva* species.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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