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Research paper

Uncovering the molecular organization of unusual highly scattered 5S rDNA: The case of *Chariesterus armatus* (Heteroptera)

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ABSTRACT

One cluster of 5S rDNA per haploid genome is the most common pattern among Heteroptera. However, in *Chariesterus armatus*, highly scattered signals were noticed. We isolated and characterized the entire 5S rDNA unit of *C. armatus* aiming to a deeper knowledge of molecular organization of the 5S rDNA among Heteroptera and to understand possible causes and consequences of 5S rDNA chromosomal spreading. For a comparative analysis, we performed the same approach in *Holymenia histrio* with 5S rDNA restricted to one bivalent. Multiple 5S rDNA variants were observed in both species, though they were more variable in *C. armatus*, with some of variants corresponding to pseudogenes. These pseudogenes suggest birth-and-death mechanism, though homogenization was also observed (concerted evolution), indicating evolution through mixed model. Association between transposable elements and 5S rDNA was not observed, suggesting spreading of 5S rDNA through other mechanisms, like ectopic recombination. Scattered organization is a rare example for 5S rDNA, and such organization in *C. armatus* genome could have led to the high diversification of sequences favoring their pseudogenization.

1. Introduction

Ribosomal genes are repeated and organized in two distinct clusters in eukaryotic genomes (45S rDNA and 5S rDNA) located in a single locus or on multiple chromosomes. The major ribosomal DNA (rDNA) consists of the 18S, 5.8S and 28S rRNA genes and spacers, while the minor ribosomal DNA is composed of the 5S rRNA gene and gene spacers (Long and David, 1980). The 5S rDNA transcriptional unit consists of a 120-bp that is evolutionarily conserved, even between phylogenetically distant organisms, and a non-transcribed spacer (NTS) that is subject to sequence modification, exhibiting variations in size and/or nucleotide sequence (Denis and Wegnez, 1978; Long and David, 1980; Wasko et al., 2001; Rebordinos et al., 2013). Variability within NTS sequences is caused by nucleotide insertions-deletions or substitutions, as well as by the presence of microsatellites and transposable elements (TEs), contributing for NTS evolution within and between taxa (Pasolini et al., 2006; da Silva et al., 2011; Merlo et al., 2010; Merlo et al., 2013; Bueno et al., 2016).

Concerted evolution results in repeat homogenization, while birth-and-death promotes sequence diversification (Nei and Rooney, 2005; Rooney and Ward, 2005). Mixing patterns involving concerted evolution and birth-and-death have been proposed for 5S rDNA evolution in some groups such as freshwater stingrays (Pinhal et al., 2011), mollusks (Freire et al., 2010; Vizoso et al., 2011), echinoderms (Caradonna et al., 2007) and grasshoppers (Bueno et al., 2016). Furthermore, the 5S rDNA association with other sequences, as well as loci number and chromosomal location, could influence the evolution of 5S rDNA (Cross and Rebordinos, 2005; Manchado et al., 2006; Vierna et al., 2011; Bueno et al., 2016). The high diversification of the 5S rDNA unit has caused the emergence of variants within genomes, like those primarily reported in fish (Martins and Galetti, 2001; Wasko et al., 2001). Extreme genome variability for this sequence was reported in fishes reaching up to ten different variants (Campo et al., 2009; Merlo et al., 2013; Barman et al., 2016). Similar cases have been reported in invertebrates with up to five variants per genome (Vierna et al., 2009; Freire et al., 2010; Perina et al., 2011; Vizoso et al., 2011; Bueno et al., 2016).

Evolutionary processes previously mentioned involved in the diversification of rDNA arrays are well known in some organisms. However in Heteroptera, the processes underlying molecular organization and evolution of rDNAs are poorly known. In this group, the rDNAs were mostly studied via FISH (Fluorescent in situ hybridization).

Abbreviations: bp, base pair; ICR, internal control region; IE, intermediate element; NTS, Non Transcribed Spacer; PCR, polymerase chain reaction; rDNA, ribosomal DNA; rRNA, ribosomal RNA; SINE, Short Interspersed Nuclear Element; snDNA, small nuclear DNA; TE, transposable element

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The presence of one chromosomal loci for 18S rDNA is common hallmark, though multiple chromosomal loci were also reported (Panzera et al., 2012; Bardella et al., 2013, 2016). 5S rDNA mapping has been restricted to 15 species of Pentatomidae and Coreidae, and higher loci number and chromosomal location variability than the found for 18S rRNA cluster was reported (Bardella et al., 2016). An extreme distribution of 5S rDNA was observed in *Chariesterus armatus* (Coreidae), which has clusters spreading over almost all chromosomes, except in the m-chromosomes. This contrasts abruptly with respect to the other fourteen species studied, which present two or four 5S rDNA clusters, with discrete localization (Bardella et al., 2016). Thus, to improve the knowledge of molecular organization and evolution of the 5S rDNA in Heteroptera and to understand possible causes and consequences of 5S rDNA chromosomal spreading in *C. armatus* (2n = 22 + 2 m + X0) we isolated and characterized the 5S rDNA + NTS from its genome. For comparative analysis, the 5S rDNA + NTS from another Coreidae, *Holhymenia histrio* (2n = 24 + 2 m + X0), a species with its 5S rDNA cluster restricted to one bivalent (the most common pattern and putatively ancestral), was also isolated and characterized.

2. Results

As previously reported by Bardella et al. (2016), chromosomal mapping of the 5S rRNA gene revealed one pair of clusters (one bivalent) in *H. histrio* (Fig. 1a–c), while the signal was spread throughout all the chromosomes except the m-chromosome in *C. armatus* (Fig. 1d–f). These results were confirmed in five distinct individuals for each species.

Using divergent PCR primers to amplify the 5S + NTS sequence, we observed five and three conspicuous bands in agarose gels for *H. histrio* and *C. armatus*, respectively. After molecular cloning we recovered 15 sequences from *H. histrio* and 39 sequences from *C. armatus*. The sequenced clones ranged from 666 bp to 1168 bp for *H. histrio* and from 170 bp to 256 bp for *C. armatus*. The gene regions were compared to the 5S rDNA from *Drosophila mojavensis* (XR_047820.1) to confirm 5S rDNA isolation.

Comparison of the 5S + NTS units from both species revealed some differences in size and nucleotide composition. Based on these

Table 1
Principal sequence characteristics of the 5S rDNA variants in two species from Heteroptera.

	Region	Size (bp)	n	ICR	Potentially functional gene	Poly-T
H. histrio						
Hh-1	Entire unit	666	1		Yes	
	5S rDNA	117	1	Yes		
	NTS	549	1			Yes
Hh-2	Entire unit	703	3		Yes	
	5S rDNA	117	3	Yes		
	NTS	586	3			Short
Hh-3	Entire unit	734–735	5		Yes	
	5S rDNA	118	5	Yes		
	NTS	616–617	5			Yes
Hh-4	Entire unit	849	3		Yes	
	5S rDNA	117	3	Yes		
	NTS	732	3			Yes
Hh-5	Entire unit	1168	3		Yes	
	5S rDNA	117	3	Yes		
	NTS	1051	3			Yes
C. armatus						
Ca-1	Entire unit	170	7		No	
	5S rDNA	112	7	No		
	NTS	58	7			No
Ca-2	Entire unit	179	1		No	
	5S rDNA	117	1	No		
	NTS	62	1			No
Ca-3	Entire unit	186–187	4		No	
	5S rDNA	128	4	Yes		
	NTS	58–59	4			No
Ca-4	Entire unit	192	1		Yes	
	5S rDNA	114	1	Yes		
	NTS	78	1			Short
Ca-5	Entire unit	246–247	25		No	
	5S rDNA	125	25	Yes		
	NTS	121–122	25			No
Ca-6	Entire unit	256	1		No	
	5S rDNA	112	1	No		
	NTS	144	1			No

n: number of sequences; ICR: Internal Control Regions.

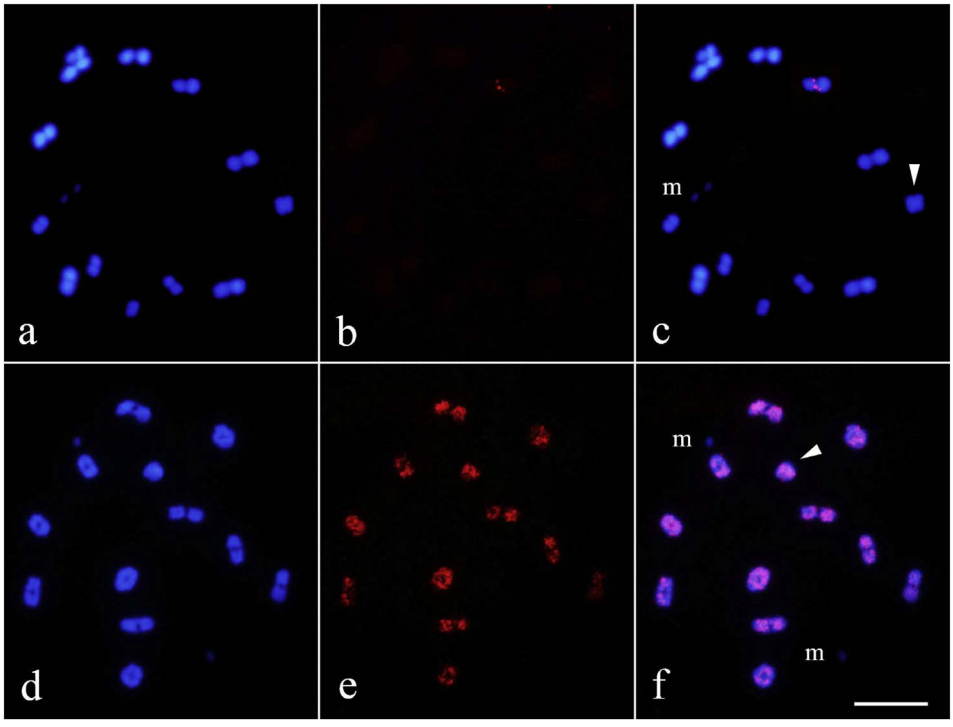

Fig. 1. 5S rDNA gene unit mapping through FISH in (a–c) *Holhymenia histrio* and (d–e) *Chariesterus armatus*. Note the remarkable differences in the hybridization signals with (a–c) discrete bands and (d–f) scattered signals. Arrowheads indicate the sex chromosome. The m-chromosomes are also indicated. (a and d) DAPI, (b and e) signal, and (c and f) merge. Scale bar = 5 μm.

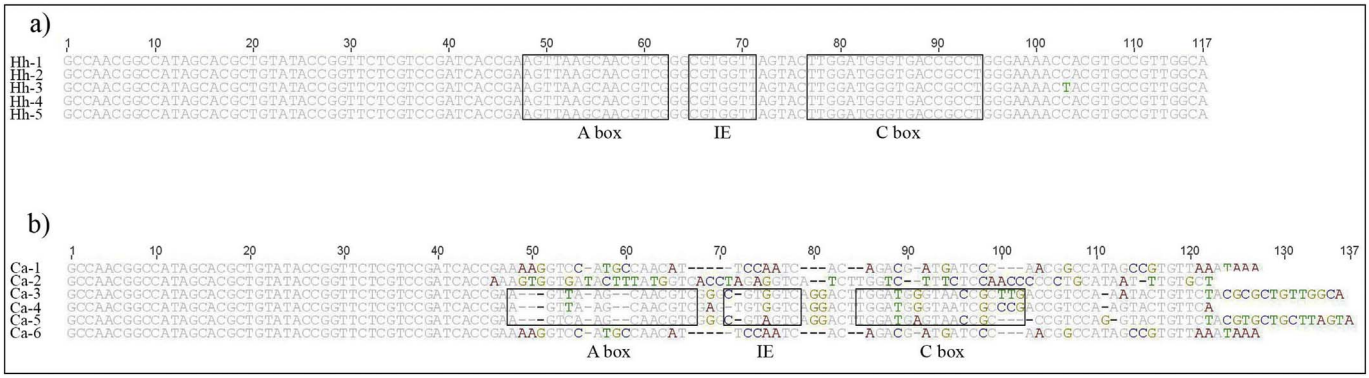

Fig. 2. Gene unit alignments of the 5S rDNA from different consensus sequence variants from (a) *Holhymenia histrio* and (b) *Charieserus armatus*. Note the low nucleotide variation (only one mutation) in (a) and observe the difference in the size and nucleotide sequence between the sequence variants for (b). Similar nucleotides between the sequence variants are in gray and the nucleotide variations are in colors (blue - cytosine, red - adenine, yellow - guanine, and light green - thymine). Regulatory elements are highlighted in the boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

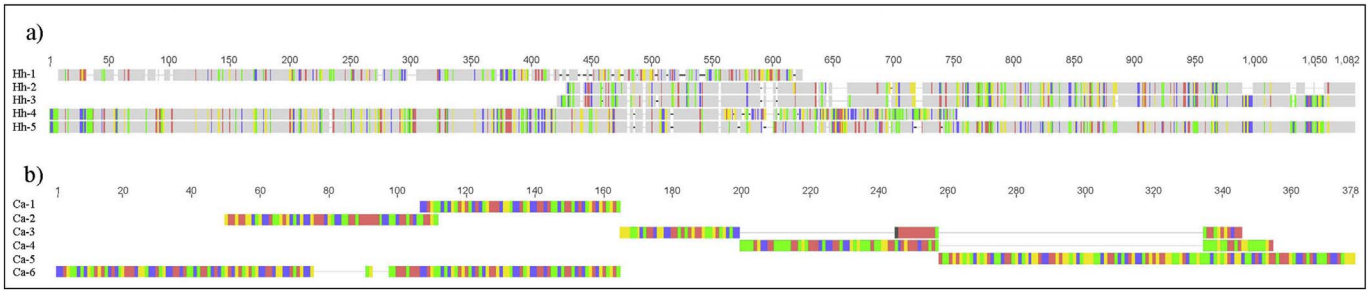

Fig. 3. 5S rDNA NTS region alignment of 5S rDNAs from (a) *Holhymenia histrio* and (b) *Charieserus armatus* consensus sequences. Note the extensive differences in (b) *C. armatus*. Similar nucleotides between the variants are in gray and the nucleotide variations are in colors (blue - cytosine, red - adenine, yellow - guanine, and light green - thymine). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differences, the sequences were classified in 11 distinct sequence variants (Table 1). Among them, five were present in the *H. histrio* genome, named Hh-1 to Hh-5. These sequences were highly similar in the 5S rRNA gene coding region (Fig. 2a) but showed differences in the NTS region (Fig. 3a). Ca-1 through Ca-6 variants were observed in *C. armatus*. These sequences were more variable than those from *H. histrio* in both the 5S rRNA coding region (Fig. 2b) and the NTS (Fig. 3b). Sequences of the 5S rRNA gene coding regions can be organized in three groups according to similarity: first group sequences Ca-3, Ca4 and Ca-5; second group sequences Ca-1 and Ca-6; third group sequence Ca-2 (Fig. 2b). The NTS regions were remarkably different in size and composition in all sequences caused by nucleotide insertions-deletions or substitutions (Fig. 3b). Comparatively the NTS region was larger in *H. histrio* than in *C. armatus*, mean sizes 707 bp and 87 bp, respectively (Table 1).

The variability of the sequences was calculated considering only the putative coding region of 5S rDNA. Only this analysis was performed because the NTS region from *C. armatus* is highly different, preventing comparisons between the two species. The nucleotide diversity for *H. histrio* was 0.00410 and only contained one mutation (substitution). For *C. armatus*, the nucleotide diversity was 0, 17, 361; this sequence contained 39 mutations including deletion-insertions and substitutions. Regarding the presence of regulatory elements, a box A, intermediate element (IE), and box C were identified in eight of the 5S rRNAs recovered (Hh-1 to Hh-5 and Ca-3 to Ca-5) (Fig. 2a–b). The poly-T at the end of coding region was observed only in the five *H. histrio* sequence variants (Hh-1 to Hh-5). In *C. armatus*, a short poly-T sequence (4 bases) was observed in the Ca-4 sequence. Using the RepBase query (http://www.girinst.org/repbase/), no significant similarity was observed with other repetitive elements in NTS regions, such as TEs. Moreover, no microsatellites were observed as are frequently reported in NTS regions in other species (Pasolini et al., 2006; Campo et al., 2009; Úbeda-Manzanaro et al., 2010; Merlo et al., 2013).

3. Discussion

The presence of one bivalent harboring the 5S rDNA is common in Heteroptera (Bardella et al., 2016), as in other eukaryotes (see for example Roa and Guerra, 2015; Cabral-de-Mello et al., 2011a). However, multiple loci number for 5S rDNA were reported in some taxa (see Cabral-de-Mello et al., 2011b; Nakajima et al., 2012; Roa and Guerra, 2015), including the extreme case in the plant *Tulipa fosteriana* with 71 clusters (Mizuochi et al., 2007). In *C. armatus*, the highly scattered hybridization signals of 5S rDNA, forming multiples blocks over *C. armatus* chromosomes, suggest putative duplication episodes for this gene that have acquired genome mobility. This contrasts with *T. fosteriana* that showed discrete blocks (Mizuochi et al., 2007) as commonly seen in other Heteroptera (Bardella et al., 2016) and other animal groups (Insua et al., 2001; Martins and Galetti, 2001; Martins et al., 2002; da Silva et al., 2011; Rodrigues et al., 2012; Merlo et al., 2013; Bueno et al., 2016; Liu et al., 2017). The scattered chromosomal pattern of 5S rDNA seen in *C. armatus* is unusual, however, it has been reported for other multigene families, such as U2 snDNA in the fish *Amphuchthys cryptocentrus* and *Porichtys plectrodon* (Úbeda-Manzanaro et al., 2010). In *Synbranchus marmoratus* H3 and H4 histone genes were also scattered (Utsunomia et al., 2014). These data suggest that diverse molecular mechanisms are shaping the chromosomal organization of multigene families in distinct genomes.

Even though the 5S rDNA is scattered throughout all chromosomes and in distinct regions (interstitial or terminal), it did not colonize the m-chromosome or it was not amplified in these chromosomes preventing FISH signals detection. Despite the limited knowledge about m-chromosome composition (Nokkala, 1986; Suja et al., 2000) it is known that these chromosomes can exchange sequences with other

chromosomes. For example, Bardella et al. (2013) observed 18S rDNA clusters in m-chromosomes from *Leptoglossus gonagra*. The factors that inhibited 5S rDNA dispersion to the m-chromosomes remain unknown and deserve further investigations.

The highly divergent chromosomal organization of the 5S rDNA between *H. histrio* and *C. armatus* caused distinct patterns of sequence diversification that are reflected in their evolutionary histories. As consequence of its scattered organization, which makes the process of sequence homogenization difficult, the 5S rDNA coding region was much more variable in *C. armatus* than in *H. histrio*. Similar patterns were also observed for NTS regions, which are almost completely different between them, reinforcing the occurrence of a less effective homogenization process in *C. armatus* compared with *H. histrio*. In other species, it has been suggested that the organization of 5S rDNA clusters in distinct genome environments (i.e., distinct chromosomes) influences the level of sequence homogenization or diversification (Pinhal et al., 2011). Sequences in the same chromosome region tend to experiment more effective episodes of homogenization due to gene conversion, unequal crossing over and selection, than those located in different chromosomes (Nei and Rooney, 2005).

In some species, different variants of 5S rDNA – variable in size and composition – occupy different chromosomal loci (see Martins and Galetti, 2001; Martins et al., 2002; Rodrigues et al., 2012; Merlo et al., 2013; Bueno et al., 2016; Liu et al., 2017). 5S rDNA variants were noticed for example in grasshoppers (Bueno et al., 2016), crustaceans (Perina et al., 2011), mollusks (Vierna et al., 2009; Vizoso et al., 2011), amphibians (Rodrigues et al., 2012; Liu et al., 2017), freshwater stingrays (Pinhal et al., 2011) and fish (Martins and Galetti, 2001). Interestingly, in *H. histrio*, which has one cluster per haploid genome, more than two variants of 5S rDNAs were recognized based on the NTS regions; this is similar to the fish *D. sargus*, which has multiple 5S rDNA variants but exhibits restriction of the clusters to only two chromosomes (Merlo et al., 2013). This indicates that the mutational rate causing variability for NTS region could be higher in Coreidae heteropterans or that the homogenization mechanisms, which results in sequence similarity, are lower in comparison to other organisms. This should be confirmed by analyzing other species with single 5S rDNA clusters.

Remarkably, the size of NTS sequences was highly divergent between the two species, with longer sequences in *H. histrio* than *C. armatus*. NTS size variation has been reported in other species, including for example *Pollicipes pollicipes* (Perina et al., 2011) and *Amolops mantzorum* (Liu et al., 2017). Small sized NTS like observed in *C. armatus* is common in animals (Perina et al., 2011; Rodrigues et al., 2012; Bueno et al., 2016; Liu et al., 2017). Martins and Galetti (2001) suggested NTS of about 80 bp as the minimum size for organization in the genome. The small size of NTS in *C. armatus* could be resultant from process of pseudogenization influenced by the scattered organization for 5S rDNA units. We cannot completely rule out that the dispersion of 5S rDNA units were favored by the small NTS size. However, the absence of scattered chromosomal 5S rDNA units in other species with small NTS suggests that small NTS size do not influence its spreading (Bueno et al., 2016; Liu et al., 2017).

The presence of the internal control regions (ICRs) in the gene coding region and the poly-T tail at the start of the NTS in *H. histrio* suggests that the sequences recovered have the capacity to be transcribed. On the contrary in *C. armatus*, some regulatory elements are absent and multiple mutations are present, suggesting that pseudogenes originated after birth-and-death process, which has been proposed in other species (Perina et al., 2011; Vizoso et al., 2011). Although the birth-and-death mechanism is effectively acting on *C. armatus* 5S rDNA repeats, homogenization also occurred as the result of concerted evolution. The latter evolutionary mechanism is also effective in driving the homogenization of the 5S rDNA coding region in *H. histrio*. Mixed model involving both birth-and-death and concerted evolution might explains the patterns observed here. Similar results have been observed in fish (Úbeda-Manzanaro et al., 2010; Pinhal et al., 2011; Merlo et al., 2013), amphibians (Liu et al., 2017), grasshoppers (Bueno et al., 2016) and mollusks (Freire et al., 2010). We speculate that a correlation exists between the presence of pseudogenes and 5S rDNA chromosomal distribution in *C. armatus*. Further evidence for a potential link between pseudogenization and rDNA distribution is reinforced by the absence of pseudogenes (at least recovered here) in *H. histrio*, with only one 5S rDNA cluster.

In other species several TEs or considerable parts of them were observed within NTS regions. For example, SINEs were noticed in fish and grasshoppers (Nakajima et al., 2012; Merlo et al., 2012, 2013; da Silva et al., 2011, 2016; Bueno et al., 2016). Because no putative TEs were identified by our analysis, the dispersion of 5S rDNA might occur via other mechanisms, such as ectopic recombination. An alternative scenario might result from insertion of TEs within regions flanking the 5S + NTS followed by intragenomic dispersion of 5S rDNA arrays via capture by TEs. However, further experimental evidences are needed to test this possibility.

The genome plasticity of 5S rDNA sequences at the chromosomal and molecular level is known, including the unique or multiple clusters and their association with TEs (da Silva et al., 2011, 2016; Nakajima et al., 2012; Merlo et al., 2013; Bueno et al., 2016), other multigene families (Eirín-López et al., 2004; Cabral-de-Mello et al., 2011b; Vierna et al., 2011), as well their potential to generate satDNA (see for example Gatto et al., 2016). The presence of scattered 5S rDNA signals in *C. armatus* chromosomes is uncommon. In general, 5S rDNA displays a clustered organization even in species with multiple FISH signals, although molecular studies have reported dispersed copies for this gene (Drouin and de Sá, 1995; Nakajima et al., 2012). It is evident that this particular organization led to higher diversification among 5S rDNA copies in *C. armatus* genome when compared with the most common placement observed in Coreidae, which is observed in *H. histrio*.

4. Materials and methods

Adult males of *H. histrio* and *C. armatus* (Coreidae) were collected in Rio Claro, São Paulo, Brazil. The testes were removed from anesthetized insects and fixed in modified Carnoy's solution (absolute ethanol:acetic acid, 3:1) followed by subsequent storage in a freezer at − 20 °C. The pronotum and legs were stored in 100% ethanol in the freezer at − 20 °C for the extraction of genomic DNA according to Sambrook and Russell (2001). Slides were prepared using a portion of the testis, which was macerated in 50% acetic acid and then dried on a hot plate at 45–50 °C.

The 5S rDNA + NTS sequences were obtained by polymerase chain reaction (PCR) from *H. histrio* and *C. armatus* genomic DNA using divergent primers described by Bueno et al. (2016), 5S–NTS-F 5′ TACC GGTTCTCGTCCGATCAC and 5S–NTS-R 5′ TACAGCGTGCTATGGCCG TTG. PCR was performed using final concentrations of 1 × PCR Rxn Buffer, 2 mM $MgCl_2$, 250 μM dNTPs, 0.5 μM of each primer, 0.5 U of *Taq* Platinum DNA Polymerase (Invitrogen, San Diego, CA, USA), and 50–100 ng template DNA. The components were subjected to the following cycling conditions: (1) an initial denaturation at 94 °C for 5 min; (2) 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 80 s; and (3) a final extension at 72 °C for 5 min. The PCR products were separated on a 1% agarose gel, and the DNA bands were purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., The Epigenetics Company, USA) according to the manufacturer's recommendations. Purified PCR products were ligated into pGEM-T plasmids (Promega, Madison, WI, USA), which were then inserted into DH5α *Escherichia coli* competent cells. Positive clones were sequenced using an ABI Prism 3100 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA) with a Dynamic Terminator Cycle Sequencing Kit (Applied Biosystems).

The quality of the sequences was determined using the Geneious 4.8.5 software (Drummond et al., 2009). When more than one sequence

for a variant was retrieved, a consensus sequences was assembled. Each sequence and consensus sequence were subjected to BLAST (Altschul et al., 1990) searches on NCBI (http://www.ncbi.nlm.nih.gov/blast) and to Repbase (http://www.girinst.org/censor/index.php). Consensus sequences were deposited into NCBI database under accession numbers MF804485-MF804495. For DNA sequence analyses, basic sequence statistics were computed with the DnaSP v.5.10.01 program (Librado and Rozas, 2009).

FISH was performed according to Pinkel et al. (1986), with modifications from Cabral-de-Mello et al. (2010). The probe corresponded to 5S rRNA gene isolated from *Anisoscelis foliaceus* (Coreidae) using the Sca5SF 5′-AAC GAC CAT ACC ACG CTGAA-3′ and Sca5SR 5′-AAG CGG TCC CCC ATC TAA GT-3′ primers (Cabral-de-Mello et al., 2010) and labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany). The same probe was used by Bardella et al. (2016) and corresponded to 92 bp of the 120 bp coding region of 5S rDNA. The probe was detected using anti-digoxigenin-rhodamine (Roche). Chromosomes were counterstained using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and the slides were mounted using Vectashield (Vector, Burlingame, CA, USA). The results were observed using an Olympus microscope BX61 equipped with a fluorescence lamp and appropriate filters. Photographs were recorded using a DP70 cooled digital camera. The images were merged and optimized for brightness and contrast using the Adobe Photoshop CS2 software.

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