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High Dynamics of rDNA Cluster Location in Kissing Bug Holocentric Chromosomes (Triatominae, Heteroptera)

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Key Words
Chagas disease vectors · FISH · Hemiptera · Holocentric chromosomes · rDNA variability · Triatominae

Abstract
In this paper, we determine by fluorescent in situ hybridization the variability in the chromosomal location of 45S rDNA clusters in 38 species belonging to 7 genera of the Triatominae subfamily, using a triatomine-specific 18S rDNA probe. Our results show a striking variability at the inter- and intraspecific level, never reported so far in holocentric chromosomes, revealing the extraordinary genomic dynamics that occurred during the evolution in this group of insects. Our results also demonstrate that the chromosomal position of rDNA clusters is an important marker to disclose chromosomal differentiation in species karyotypically homogenous in their chromosome number.

Triatominae is a subfamily of Reduviidae (Hemiptera, Heteroptera), generally defined by their blood-sucking habit [Lent and Wygodzinsky, 1979]. Most triatomines are vectors of Chagas disease or American trypanosomiasis, recognized as the most serious human parasitic disease of Latin America in terms of its social and economic impact, with 15 million people infected and 12,500 deaths per year [World Health Organization, 2006].

There are currently 140 triatomine species, classified into 5 tribes and 15–19 genera [Galvão et al., 2003; Schofield and Galvão, 2009]. Karyotypic information is currently available for 80 species from 9 genera [Panzera et al., 2010]. The triatomines present a very uniform chromosome complement; most species have 20 autosomes, with the exception of only 3 species. The number of sex chromosomes is more variable; 3 sex chromosome systems can be found in males (XY, X_1X_2Y and $X_1X_2X_3Y$) (table 1) [Panzera et al., 2010]. As observed in all heteropteran species, the triatomines have holocentric chromosomes characterized by the presence of a diffuse or nonlocalized centromere [Hughes-Schrader and Schrader, 1961].

The lack of a primary constriction and the relatively small chromosomes of similar size are factors that cause difficulties in studying karyotype evolution in Heteroptera. In some cases, C-banding and silver staining of nucleolus organizer regions have been successfully used for longitudinal differentiation in holocentric chromosomes [Kuznetsova et al., 2011].

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Table 1. Available data on the chromosomal location of 45S rDNA through FISH and relevant cytogenetic traits in Triatominae species

Species	Male diploid number (2n)	Chromosomal location of 45S rDNA	Autosomal C-bands	X with C-bands	Heterologous association	Geographic origin[a]	Reference
Genus *Rhodnius*							
R. prolixus (fig. 1a)	20A+XY	X chromosome	no	no	no	Insectary CDC (USA). Origin: Colombia Colombia, Casanare. S Guatemala, Las Palmas. D	this paper
R. domesticus (fig. 1b)	20A+XY	both sex chromosomes; small signal in Y chromosome	no	no	no	Brazil, Santa Catarina. S	this paper
R. pallescens	20A+XY	both sex chromosomes; small signal in Y chromosome	C-dots in 1 or both ends of almost all autosomes	no	no	• ND • Colombia, Norcasia, Caldas. S	• Morielle-Souza and Azeredo-Oliveira [2007] • this paper
R. colombiensis	20A+XY	X chromosome	C-dots in 5–6 autosomal bivalents	no	no	Colombia, Tolima, Coyaima, Totarco. S	this paper
Genus *Psammolestes*							
P. tertius (fig. 1c)	20A+XY	both sex chromosomes; small signal in Y chromosome	no	no	no	Insectary Araraquara, Brazil. Origin: Ceará, Spirito Santo, Brazil	this paper
Genus *Dipetalogaster*							
D. maxima (fig. 1d)	20A+XY	X chromosome	no	no	no	Mexico, Baja California, La Paz. S	this paper
Genus *Eratyrus*							
E. cuspidatus (fig. 1e)	20A+X_1X_2Y	1 X chromosome (X_1) and Y chromosome	no	no	no	Colombia, Sucre, San Onofre. S	this paper
Genus *Panstrongylus*							
P. megistus (fig. 1f)	18A+X_1X_2Y	• 1 autosomal pair and 1 sex chromosome • the largest autosomal pair	no	no	no	• ND • Insectary Fiocruz (Rio de Janeiro, Brazil). Origin: Minas Gerais, Brazil	• Morielle-Souza and Azeredo-Oliveira [2007] • this paper
P. lignarius	20A+X_1X_2Y	largest autosomal pair	C-blocks in 1 or both ends of almost all autosomes	yes	yes	Peru, San Martín, Mallobamba. S	this paper
P. chinai (fig. 1g)	20A+X_1X_2Y	largest autosomal pair	C-blocks in both ends of all autosomes	yes	yes	Peru, Lambayeque, Zaña. S	this paper
Genus *Mepraia*							
M. spinolai (fig. 1h)	20A+X_1X_2Y	1 X chromosome	C-blocks in 1 or both ends of all autosomes	yes	yes	Chile, Region III, Copiapó, Inca Oro. P	this paper
M. gajardoi (fig. 1i)	20A+X_1X_2Y	1 X chromosome	C-dots in some autosomes	no	no	Chile, Region XV, Arica/Parinacota, Caleta Vitor. S	this paper
Genus *Triatoma*							
Group Rubrofasciata							
Complex Phyllosoma							
Subcomplex *Dimidiata*							
T. dimidiata maculipennis *T. dimidiata dimidiata* *T. dimidiata capitata* (fig. 2a)	20A+X_1X_2Y	a large autosomal pair	C-dots in 1 or both ends of almost all autosomes	no	no	Mexico, Oaxaca, Nopala. P Guatemala, Jutiapa, Carrizal. D Colombia, Magdalena. S	this paper

Table 1 (continued)

Species	Male diploid number (2n)	Chromosomal location of 45S rDNA	Autosomal C-bands	X with C-bands	Heterologous association	Geographic origin[a]	Reference
Subcomplex *Phyllosoma*							
T. pallidipennis	20A+X_1X_2Y	a large autosomal pair	no	no	no	Mexico, Morelos, Cuernavaca. S	this paper
T. phyllosoma	20A+X_1X_2Y	a large autosomal pair	no	no	no	Mexico, Oaxaca, Santo Domingo. P	this paper
T. mazzottii	20A+X_1X_2Y	a large autosomal pair	no	no	no	Mexico, Oaxaca, Puerto Escondido. S	this paper
Subcomplex *Flavida*							
T. flavida	20A+X_1X_2Y	a large autosomal pair	no	no	no	Cuba, Peninsula Guanahacabibes Pinar del Río, Caimanera cave. S	this paper
Complex Protracta							
T. nitida (fig. 2b)	18A+X_1X_2Y	1 autosomal pair (euchromatic)	C-blocks in the 2 largest heterochromatic pairs	no	no	Guatemala, Quiché, San Andrés Saycabajá. P	this paper
T. protracta (fig. 2c)	20A+X_1X_2Y	• only 1 chromosome of an autosomal pair • a large autosomal pair	C-blocks in both ends of all autosomes	yes	yes	• Insectary Araraquara (SP, Brazil). Origin: ND • Insectary Fiocruz (RJ, Brazil). Origin: Monte Diablo, Calif., USA	• Severi-Aguiar and Azeredo-Oliveira [2005] • this paper
Complex Lecticularia							
T. lecticularia (fig. 2d)	20A+XY	1 autosomal pair	no	no	no	Insectary Fiocruz (RJ, Brazil). Origin: Valkiria, Okla., USA	this paper
Group Dispar							
Complex Dispar							
T. carrioni (fig. 2e)	20A+XY	X chromosome	C-dots in the 2 largest autosomal pairs	no	no	Perú, Piura, Ayacuiba. S	this paper
T. boliviana	20A+XY	X chromosome	no	no	no	Bolivia, La Paz, Muñecas, Vilaque. S	this paper
Group Infestans							
Complex Infestans							
Subcomplex *Rubrovaria*							
T. carcavalloi (fig. 2f)	20A+XY	largest autosomal pair	no	no	no	Insectary Fiocruz (RJ, Brazil). Origin: Rio Grande do Sul, Brazil	this paper
T. rubrovaria	20A+XY	largest autosomal pair	no	no	no	• Insectary Araraquara (SP) and Fiocruz (RJ), Brazil. Origin: ND • Uruguay, Artigas. S	• Bardella et al. [2010] • this paper
Subcomplex *Brasiliensis*							
T. sherlocki	20A+XY	largest autosomal pair	C-blocks in both ends of all autosomes	no	yes	Insectary Fiocruz (RJ, Brazil). Origin: Brazil, Bahia	this paper
T. brasiliensis	20A+XY	largest autosomal pair	C-blocks in both ends of all autosomes	no	yes	• Insectary Araraquara (SP) and Fiocruz (RJ), Brazil. Origin: ND • Brazil, Pernambuco, Terra Nova, P	• Bardella et al. [2010] • this paper
Subcomplex *matogrossensis*							
T. mattogrossensis (fig. 2g)	20A+XY	both sex chromosomes; small signal in Y chromosome	no	no	no	• Insectary Araraquara (SP) and Fiocruz (RJ, Brazil). Origin: ND • Insectary Fiocruz (RJ, Brazil), Origin: Serra das Arenas, M.G. do Sul, Brazil	• Bardella et al. [2010] • this paper
T. vandae	20A+XY	both sex chromosomes; small signal in Y chromosome	no	no	no	Insectary Fiocruz (RJ, Brazil). Origin: Rondonopolis, Matto Grosso, Brazil	this paper

Table 1 (continued)

Species	Male diploid number (2n)	Chromosomal location of 45S rDNA	Autosomal C-bands	X with C-bands	Heterologous association	Geographic origin[a]	Reference
Subcomplex *Sordida*							
T. sordida (fig. 2h)	20A+XY	X chromosome	C-blocks in only 1 end of almost all autosomal pairs	yes	yes	Brazil, Matto Grosso, São Jose do Povo, P	this paper
T. garciabesi	20A+XY	X chromosome	no	no	no	Argentina, Salta, S	this paper
Subcomplex *Maculata*							
T. maculata (fig. 2i)	20A+XY	both sex chromosomes; small signal in Y chromosome	C-dots in 1 or both ends of almost all autosomal pairs	no	no	Brazil, Roraima, Boa Vista	this paper
T. pseudomaculata (fig. 2j)	20A+XY	a large autosomal pair	C-dots in 1 or both ends of 3 or 4 autosomal pairs	no	yes	Brazil, Ceará, Sobral, P	this paper
T. wygodzinsky	20A+XY	largest autosomal pair	no	no	no	Brazil, São Paulo, São João da Boa Vista, S	this paper
Subcomplex *Infestans*							
T. delpontei (fig. 2k)	20A+XY	a large autosomal pair and X chromosome	C-blocks in only 1 end of all autosomes	yes	yes	Insectary Córdoba (Arg.). Origin: La Merced, Rivadavia, Salta, Argentina. S	this paper
T. platensis	20A+XY	X chromosome	C-blocks in 1 or both ends of the 3 largest autosomal pairs	yes	yes	• Insectary Araraquara (SP, Brazil). Origin: ND • Insectary Córdoba (Arg.). Origin: San Marcos Sierras, Cruz del Eje, Córdoba, Argentina. S • Uruguay, Paysandú. S	• Severi-Aguiar and Azeredo-Oliveira [2005] • this paper • this paper
T. infestans Andean group (fig. 3a, b)	20A+XY	a large autosomal pair	C-blocks in 1 or both ends of almost all autosomes	yes	yes	• Bolivia, Potosí, Sud Chichas, La Deseada. S • Bolivia, Chuquisaca, Sucre, Oropeza, Carapari. D	• this paper • this paper
T. infestans Non-Andean group (fig. 3c, d)	20A+XY	• 1 hybridization signal (not determined) • X chromosome	C-blocks in 1 or both ends of the 3 largest autosomal pairs	no	yes	• ND • Argentina, Catamarca, Palo Blanco. P • Brazil, Paraiba, Monteiro. D • Uruguay, Rivera. P	• Morielle-Souza and Azeredo-Oliveira [2007] • this paper • this paper • this paper
T. infestans melanosoma	20A+XY	X chromosome	same as above	no	yes	Insectary Araraquara (Sao Paulo, Brazil) and Fiocruz (RJ, Brazil). Origin: ND	Bardella et al. [2010]
Species without group assigned							
T. tibiamaculata	20A+X_1X_2Y	• 1 autosomal pair • a large autosomal pair	terminal C-dots in some autosomal pairs	no	no	• Insectary Araraquara (SP, Brazil). Origin: ND • Insectary Fiocruz (RJ, Brazil). Origin: Belem, Pará, Brazil	• Severi-Aguiar and Azeredo-Oliveira [2005] • this paper
T. vitticeps (fig. 2l)	20A+$X_1X_2X_3Y$	• 2 X chromosomes • the smallest X chromosome (X_3) and Y chromosome	no	yes	no	• Insectary Araraquara (SP, Brazil). Origin: ND • Insectary Fiocruz (RJ, Brazil). Origin: Rio de Janeiro, Brazil	• Severi-Aguiar et al. [2006] • this paper

Species are grouped according to the classification of Galvão et al. [2003] and Schofield and Galvão [2009]. [a] ND = Not determined; P = peridomiciliary; D = domiciliary; S = sylvatic.

Within Triatominae species, C-heterochromatin is the main source of karyotype differentiation both at the interspecific and intraspecific level [Panzera et al., 2010]. C-heterochromatin variability in autosomes and sex chromosomes involves changes in quantity, size, composition, location and behavior of C-blocks during cell divisions. These analyses have been very useful to differentiate several cryptic species [Panzera et al., 1997, 2006] and to detect population variability in species from different genera, such as *Triatoma infestans* [Panzera et al., 1992, 2004], *Panstrongylus geniculatus* [Pérez et al., 2002] and *Rhodnius pallescens* [Gómez-Palacio et al., 2008]. The role of the C-heterochromatin in chromosomal evolution is still unclear, thus creating a need for other cytogenetic markers to reveal the occurrence of chromosomal changes in the genomes with holocentric chromosomes. Using fluorescent in situ hybridization (FISH) with a heterologous ribosomal probe from *Drosophila*, varying 45S ribosomal cluster location has been reported in triatomine species, supporting its utility as chromosomal marker [Severi-Aguiar and Azeredo-Oliveira, 2005; Severi-Aguiar et al., 2006; Morielle-Souza and Azeredo-Oliveira, 2007; Bardella et al., 2010].

The aim of this paper was to determine the degree of variability in the chromosomal location of the 45S rDNA clusters in 38 species belonging to 7 genera of the Triatominae subfamily. To perform the FISH assay, we generated a triatomine-specific 18S rDNA probe. Our findings show a striking variability at inter- and intraspecific levels, revealing the extraordinary genomic dynamics that occurred during the evolution of this group of insects.

Materials and Methods

Interspecific Studies

We studied 38 species from 7 genera included in the Rhodniini (*Psammolestes* and *Rhodnius* genera) and Triatomini tribes (*Dipetalogaster*, *Eratyrus*, *Mepraia*, *Panstrongylus* and *Triatoma* genera) (table 1) by FISH. For each species, at least 2 male individuals were analyzed. As far as possible we used individuals collected from natural populations (table 1).

The species analyzed here comprised the 3 sex systems described in males for Triatominae: XY, X_1X_2Y and $X_1X_2X_3Y$. For the *Triatoma* genus, which involves 80 of the 140 described species, we studied the 3 main clades or groups: the Infestans group (from south and east of the Amazon region), the Rubrofasciata group (species from north of the Amazon, including Central and North America and Old World species) and the Dispar group (west of the Amazon region, only including the Dispar complex). At least 2 species pertaining to 12 of the 13 *Triatoma* groups (complexes or subcomplexes) were studied [Schofield and Galvão, 2009] (table 1).

Intraspecific Studies

We analyzed different populations from the 3 main vector species of Chagas disease: *Rhodnius prolixus, Triatoma dimidiata and T. infestans*. For each species, 12 individuals and at least 3 individuals from each geographical origin were studied. In *R. prolixus*, we studied field specimens from Colombia (sylvatic) and Guatemala (domestic) and insectary individuals (Centers for Disease Control and Prevention of Atlanta, Ga., USA). This last material is being used for the entire genome sequencing by The Genome Center at Washington University (http://genome.wustl.edu/genomes/view/rhodnius_prolixus). In *T. dimidiata* we analyzed specimens from 3 subspecies identified by molecular markers [Bargues et al., 2008], all of them showing the cytotype 1 described by Panzera et al. [2006]. In *T. infestans* we studied several populations from both previously described chromosomal groups, named Andean and non-Andean, which have substantial differences in C-heterochromatin content and genome size [Panzera et al., 2004, 2007, 2010].

DNA Isolation, PCR Amplification and Probe Generation

Genomic DNA was isolated from adult *T. infestans* from Uruguay (non-Andean chromosomal group) with standard procedures. The cephalothoraxes were homogenized and solubilized in a lysis buffer containing 50 μg/ml proteinase K and RNAse A (100 μg/ml). Following phenol:chloroform (1:1) extractions, the DNA was precipitated with ethanol and resuspended in H_2O. The 18S rRNA gene of *T. infestans* (accession number Y18750) was used to design new primers (forward: 5′-GTC GGT GTA ACT GGC ATG T-3′ and reverse: 5′-GTG TCG TCG GTA GCA TTG A-3′). An 807-bp fragment was amplified by PCR, which was performed in 50 μl using 1 ng genomic DNA, 1× buffer, 5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas Life Sciences Inc., Hanover, Md., USA), 0.3 μM primers (each), and 1 U/μl Taq polymerase (Fermentas). The amplification conditions employed were 30 s at 94°C, 40 s at 48°C and 1 min at 72°C. The PCR product was cloned into a GeneJet vector (Fermentas) and sequenced bidirectionally with vector primers using an ABI Prism 377-Perkin Elmer automated sequencer. Plasmid DNA (1 μg) was labeled by the Nick Translation System (Invitrogen, Carlsbad, Calif., USA) using Cy3-dUTP (GE Healthcare, Life Sciences, Oreg., USA).

Fluorescence in situ Hybridization

FISH was carried out using squashed gonad preparations, previously fixed in 3:1 ethanol:acetic acid solution. The chromosome preparation was pre-treated with 100 μg/ml RNAse A and 0.01 mg/ml pepsin, and post-fixed in 3.7% formaldehyde. The slides were denatured at 75°C for 6 min in a hybridization solution which contained the DNA probe (50 ng per slide), 50% formamide, 2× SSC and 10% dextran sulfate and were hybridized over night at 37°C. Slides were then washed twice in 2× SSC for 5 min at 42°C and twice in 0.1× SSC for 5 min at room temperature. The slides were mounted with Vectashield H-100 (Vector Laboratories, Inc., Burlingame, Calif., USA) containing 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI).

Microscopy and Imaging

Slides were analyzed under a Nikon Eclipse 80i epifluorescence microscope. Images were obtained with a Nikon DS-5Mc-U2 digital cooled camera using Nikon Nis Elements 3.1 Advanced

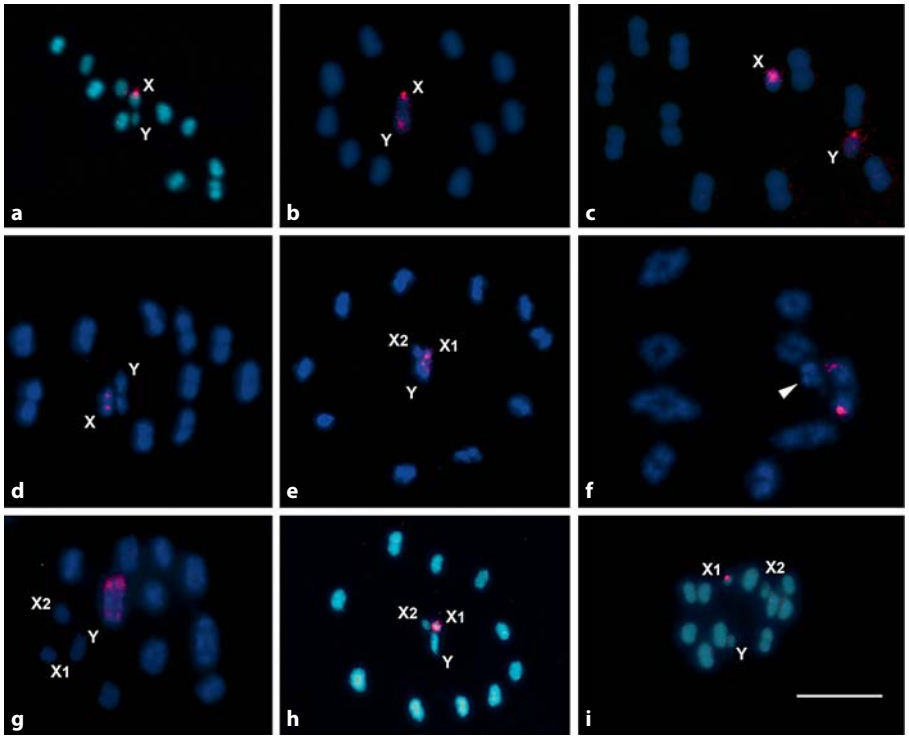

Fig. 1. Interspecific variability in the chromosomal location of 45S rDNA in species from 6 genera of Triatominae, excluding *Triatoma*. The rDNA signals are located in 1 sex chromosome (**a**, **d**, **h**, **i**), in 2 sex chromosomes (**b**, **c**, **e**) or in 1 autosomal pair (**f**, **g**). **a** *Rhodnius prolixus* (2n = 20A+XY), MII; **b** *R. domesticus* (2n = 20A+XY), MII; **c** *Psammolestes tertius* (2n = 20A+XY), MI; **d** *Dipetalogaster maxima* (2n = 20A+XY), MI; **e** *Eratyrus cuspidatus* (2n = 20A+X_1X_2Y), MII; **f** *Panstrongylus megistus* (2n = 18A+X_1X_2Y), diplotene. The arrowhead indicates the associated sex chromosomes. **g** *P. chinai* (2n = 20A+X_1X_2Y), MI; **h** *Mepraia spinolai* (2n = 20A+X_1X_2Y), MII; **i** *M. gajardoi* (2n = 20A+X_1X_2Y), MI. Bar represents 10 μm.

Research software, and processed with Adobe Photoshop® software. For each species at least 30 meiotic metaphases I (MI) or II (MII) or diplotene stages were studied.

Results

The 45S rDNA cluster has 1 or 2 chromosome loci per haploid genome, showing 4 location patterns: in the X chromosome (11 species), in the X and Y chromosomes (8 species), in 1 autosomal pair (19 species), or in the X chromosome plus 1 autosomal pair (1 species) (table 1, figs. 1–3). In all cases, the hybridization signals are located in a terminal or subterminal chromosome position. Table 1 summarizes our results and includes previous reports of rDNA cluster location by FISH and other relevant cytogenetic traits of the Triatominae, which will be considered in the results and discussion.

Genus Rhodnius

We analyzed 4 species: 2 without autosomal C-heterochromatin *(R. prolixus, R. domesticus)* and 2 with autosomal C-heterochromatin *(R. pallescens, R. colombiensis)* (table 1). In *R. prolixus* and *R. colombiensis*, the rDNA cluster was located on the X chromosome (fig. 1a). In *R. domesticus* and *R. pallescens* the hybridization signals were observed in both sex chromosomes (X and Y) (fig. 1b).

Genus Psammolestes

The only species studied of this genus, *P. tertius,* presented the rDNA clusters on the X and Y chromosomes (fig. 1c).

Genus Dipetalogaster

D. maxima is the only species belonging to this genus. The rDNA clusters were localized on the X chromosome (fig. 1d).

Genus Eratyrus

The only species analyzed, *E. cuspidatus*, has no autosomal C-heterochromatin (table 1) and carried the rDNA in 1 euchromatic X chromosome (X_1) and the Y chromosome (heterochromatic). The hybridization signals presented the same intensities on both sex chromosomes (fig. 1e).

Genus Panstrongylus

The 3 species analyzed showed the same rDNA location: in the largest autosomal pair. In *P. lignarius* and *P. chinai* the clusters were located on 1 C-heterochromatic

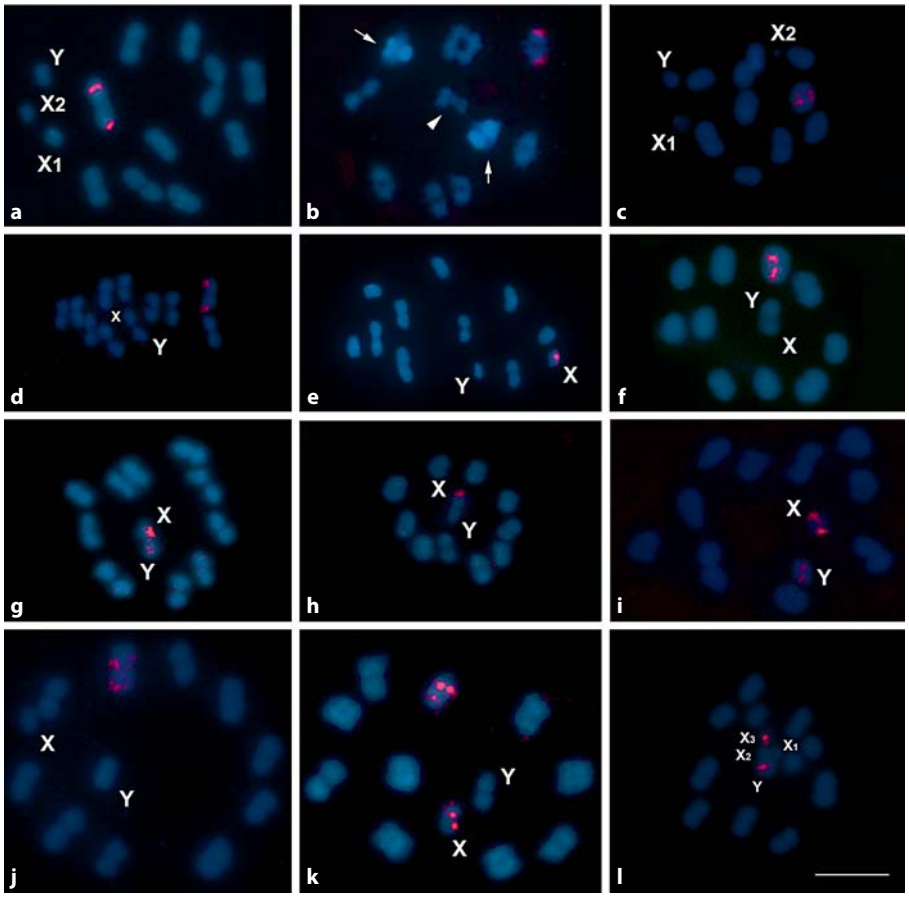

Fig. 2. Interspecific variability in the chromosomal location of 45S rDNA in triatomine species of the genus *Triatoma*. The rDNA signals are located in 1 sex chromosome (**e**, **h**), in 2 sex chromosomes (**g**, **i**, **l**), in 1 autosomal pair (**a**–**d**, **f**, **j**), or in 1 autosomal pair and X chromosome (**k**). **a** *T. dimidiata* (2n = 20A+X_1X_2Y), MI; **b** *T. nitida* (2n = 18A+X_1X_2Y), diplotene. Two heterochromatic bivalents are indicated by arrows. The association of 3 sex chromosomes is indicated by the arrowhead. **c** *T. protracta* (2n = 20A+X_1X_2Y), MI; **d** *T. lecticularia* (2n = 20A+XY), MII; **e** *T. carrioni* (2n = 20A+XY), MII; **f** *T. carcavalloi* (2n = 20A+XY), MI; **g** *T. matogrossensis* (2n = 20A+XY), MII; **h** *T. sordida* (2n = 20A+XY), MII; **i** *T. maculata* (2n = 20A+XY), MI; **j** *T. pseudomaculata* (2n = 20A+XY), MI; **k** *T. delpontei* (2n = 20A+XY), MI; **l** *T. vitticeps* (2n = 20A+$X_1X_2X_3Y$), MII. Bar represents 10 μm.

autosome (table 1, fig. 1g), while in *P. megistus* it was on a euchromatic autosome (table 1, fig. 1f).

Genus Mepraia

In both species *(M. spinolai* and *M. gajardoi)* the rDNA cluster was observed in one of the 2 X chromosomes. This X chromosome had C-heterochromatin in *M. spinolai* but not in *M. gajardoi* (table 1, fig. 1h, l, respectively).

Genus Triatoma

In this genus we analyzed 24 species belonging to 3 main clades or groups, and 2 unassigned species. This genus presented the 4 chromosomal location patterns of 45S rDNA described above (table 1, figs. 2, 3).

Group Rubrofasciata. The 8 species analyzed presented the hybridization signal in 1 autosomal pair, despite different cytogenetic features (table 1). The species analyzed have C-heterochromatic blocks (fig. 2c) or not (fig. 2a, b, d), a multiple sex system (X_1X_2Y) (fig. 2a–c) or an XY sex system (fig. 2d) and different autosomal numbers (18 or 20 chromosomes).

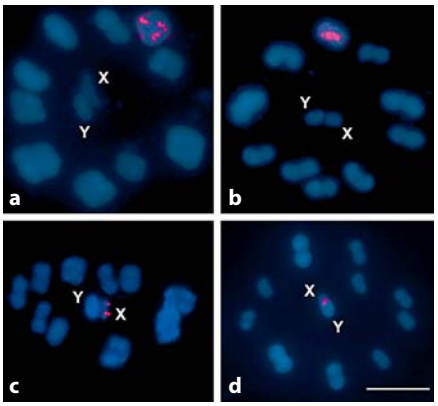

Fig. 3. Intraspecific variability in the chromosome location of the 45S rDNA in *Triatoma infestans* (2n = 20A+XY). **a**, **b** Andean group, MI and MII, respectively. The rDNA signals are located in 1 autosomal pair. **c**, **d** Non-Andean group, MI and MII, respectively. The rDNA signals are located in 1 sex chromosome (X chromosome). Bar represents 10 μm.

Group Dispar. Here, we described the chromosome complement of *T. boliviana* and *T. carrioni* for the first time. Both species present a diploid chromosome number of 22 chromosomes (2n = 20A+XY in males, XX in females) and the 45S rDNA cluster was located on the euchromatic X chromosome (table 1, fig. 2e).

Group Infestans. We studied 16 species from the 5 subcomplexes included in this group (table 1).

Subcomplexes Rubrovaria and Brasiliensis. The 4 species analyzed (2 for each subcomplex) showed the same rDNA location: in the largest autosomal pair (fig. 2f).

Subcomplex Matogrossensis. In the 2 species analyzed, rDNA signals were located on both sex chromosomes (X and Y). However, the intensity of the hybridization signals was different, the one on the Y chromosome being less intense (fig. 2g).

Subcomplex Sordida. The 2 species analyzed showed the same rDNA location, i.e. in the X chromosome (fig. 2h).

Subcomplex Maculata. In the 3 species analyzed, we found 2 different chromosome locations for the 45S rDNA cluster. In *T. maculata*, 45S rDNA was located in both sex chromosomes (fig. 2i), while in *T. pseudomaculata* and *T. wygodzinsky* it was observed in 1 autosomal pair (fig. 2j).

Subcomplex Infestans. We studied the 3 species of this subcomplex: *T. delpontei*, *T. platensis* and *T. infestans*. In *T. delpontei*, hybridization signals were located on an autosomal pair and on the X chromosome (fig. 2k). In *T. platensis*, the rDNA clusters were located in the X chromosome. In the 2 chromosomal groups of *T. infestans* (Andean and non-Andean) previously identified by Panzera et al. [2004], we found a striking intraspecific variability. The Andean group showed the rRNA genes located in 1 autosomal pair (fig. 3a, b) and the non-Andean group showed the signal on the X chromosome (fig. 3c, d).

Unassigned Species. T. tibiamaculata showed the signal on the largest autosomal pair, while in *T. vitticeps* it was located on the smallest X chromosome (X_3) and in the largest sex chromosome identified as Y (fig. 2l).

Discussion

Variability of rDNA Location in Heteroptera

Reports on the number and location of rDNA clusters in Heteroptera using silver staining techniques and FISH with rDNA probes are very scarce and include no more than 33 species distributed in 8 families [for review see Papeschi and Bressa, 2006a, b; Grozeva et al., 2010, 2011]. This is the first report attempting to address and study the variability of the 45S rDNA cluster location in a great number of species included in a single subfamily of Heteroptera.

We observed 1 or 2 loci of the rDNA cluster per haploid genome located either in the sex chromosomes and/or in 1 autosome (table 1). The low number of loci, their chromosomal position (terminal or subterminal) and their distribution in sex chromosomes or autosomal pairs are in agreement with other reports in Heteroptera [for review see Papeschi and Bressa, 2006a, b; Grozeva et al., 2011; Poggio et al., 2011]. The distribution patterns of the rDNA clusters reveal an extensive variability among the 38 analyzed species, never reported so far. The results described herein, as well as some results previously reported [for review see Panzera et al., 2010], indicate that the Triatominae is one of the chromosomally most diverse subfamilies within the Heteroptera suborder, in spite of their homogeneity in chromosome number.

Comparative Results in Triatominae rDNA Location

We analyzed a new sample of the 10 species previously described through FISH [Severi-Aguiar and Azeredo-Oliveira, 2005; Severi-Aguiar et al., 2006; Morielle-Souza and Azeredo-Oliveira, 2007; Bardella et al., 2010], most of them from natural populations (table 1). For 3 species, our results were different from the previously reported data (table 1). In *T. vitticeps* ($2n = 20A+X_1X_2X_3Y$), Severi-Aguiar et al. [2006] described the hybridization signals on 2 X chromosomes. Based on the chromosome segregation during both meiotic divisions, we established that the rDNA is located in 1 X chromosome and in the Y chromosome (fig. 2l). In *T. protracta*, Severi-Aguiar and Azeredo-Oliveira [2005] described a heterozygous pattern in 1 autosomal pair. We found the rDNA signals on both autosomal homologues (homozygous pattern) (fig. 2c). In *P. megistus*, Morielle-Souza and Azeredo-Oliveira [2007] described the hybridization signals on the sex chromosome. Our results indicate that the 45S rDNA is located in an autosomal pair (fig. 1f). These discrepancies may derive from the different geographical origins of the individuals analyzed, changes in the FISH conditions and/or the use of different rDNA probes (i.e. heterologous *Drosophila* probe vs. homologous *Triatoma* probe).

Correlation between rDNA Location and Chromosomal Traits

In Reduviidae, a correlation between rDNA chromosomal location and sex systems [Poggio et al., 2011] has

been suggested. However, our data indicates that in triatomines, the position of rDNA in autosomes or sex chromosomes does not appear to be correlated with the sex chromosome mechanism (table 1). Species with the same sex mechanism, even from the same genus, presented different location patterns; for example *T. carrioni* and *T. carcavalloi* (fig. 2e, f, respectively). Furthermore, species with different sex mechanisms presented the rDNA cluster in the same location; for example *T. protracta* and *T. pseudomaculata* (fig. 2c, j, respectively).

In a wide range of organisms, both among closely related species and even within a given species, variations in the position and number of rDNA clusters are associated with C-heterochromatin changes [Andronico et al., 1985; Hirai et al., 1996; Criniti et al., 2005; Roy et al., 2005; Raskina et al., 2008; Gomes de Oliveira et al., 2010]. Our results show that in most triatomine species the rDNA is not associated with C-heterochromatin regions (table 1). In closely related species within the *Panstrongylus* genus, the rDNA signal is located in the second largest autosome pair, regardless of the presence or absence of C-heterochromatin (fig. 1g, f, respectively). In *T. nitida*, despite the fact that it has 2 almost entirely heterochromatic autosomal pairs, the rDNA signal is located in a euchromatic autosomal pair (table 1, fig. 2b). As observed for the autosomes, the rDNA cluster may be located either on the C-heterochromatic (fig. 1h, 2h) or euchromatic X chromosomes (figs. 1a, b, d, i, 2e, 3c, d).

We cannot rule out a relationship between rDNA clusters and repetitive sequences not revealed by C-banding. Repetitive sequences are thought to play an important role in ribosomal cluster rearrangements. In several species, including Hemiptera, rDNA clusters have been related with the presence of macrosatellite and telomere-like repeats [Mandrioli et al., 1999a, b; Bizarro et al., 2000].

Evolutionary and Taxonomic Aspects

Within different groups of insects, including species with holocentric chromosomes, interspecific comparisons of the rDNA location have been frequently used to trace evolutionary pathways [Hirai et al., 1996; Roy et al., 2005; Cabrero and Camacho, 2008; Almeida et al., 2010; Nguyen et al., 2010]. However, among Heteroptera this type of analysis has not been successfully implemented, taking into account the few species that have been studied. The only previous study about sex chromosome evolution involving the ribosomal genes in Heteroptera was performed in 3 species of the genus *Dysdercus* [Bressa et al., 2009].

Despite the extensive variation found in the rDNA position within the Triatomine subfamily, in several closely related species, such as the Rubrofasciata group of the genus *Triatoma* and the *Panstrongylus* species, the 45S ribosomal cluster is observed in the same chromosomal location (table 1). This would reflect that in these groups of species this location is an evolutionarily conserved genetic trait. However, the interspecific variations within the genus *Rhodnius* (fig. 1a, b) and the subcomplex Infestans (figs. 2k, 3) clearly show that the rDNA cluster position can vary in a short period of time, even among closely related species.

The subcomplex Infestans comprises 3 closely related species: *T. platensis, T. delpontei* and *T. infestans*. These species share a recent common ancestor [Ueshima et al., 1966] and present an identical chromosome number [Panzera et al., 1995]. However, the 45S rDNA cluster is observed in different locations, in the X chromosome in *T. platensis* and in 1 X and 1 autosomal pair in *T. delpontei* (fig. 2k). Moreover, *T. infestans* presents a polymorphism that depends on the geographic origin of the individuals (see intraspecific variation below) (fig. 3). This striking ribosomal gene variation revealed that these closely related species have undergone major chromosomal rearrangements in a relatively short period of time when compared to other triatomine groups. This indicates that different triatomine groups have dissimilar chromosomal differentiation rates, as suggested for the C-heterochromatin variation [Panzera et al., 2010].

Intraspecific Variability of rDNA Location

Population studies were performed in the 3 main Chagas disease vectors (table 1). No intraspecific variation was revealed in *R. prolixus* and *T. dimidiata* populations. In *T. infestans*, we detected striking differences in rDNA location between both previously described chromosomal groups (Andean and non-Andean) (fig. 3). These groups can hybridize and produce viable progeny, and they are distinguishable by C-banding, flow cytometry [Panzera et al., 2004] and molecular markers [Bargues et al., 2006; Quisberth et al., 2011]. The variation in rDNA cluster location reveals that the differentiation process between both chromosomal groups of *T. infestans* involved significant genomic reorganization of essential coding sequences.

Although intraspecific variation of rDNA cluster location is relatively common in plants [Pedrosa et al., 2006], its occurrence in animals is exceptionally rare and it has only been reported in certain species of amphibians [Andronico et al., 1985] and fishes [Castro et al., 2001]. In

insects, there is only 1 description in the grasshopper species *Eyprepocnemis plorans* [Cabrero et al., 2003a, b]. The intraspecific variation in *E. plorans* involved an increase in the number of chromosomes which harbor a ribosomal cluster, resulting in populations with a variable number of rDNA-bearing chromosomes (from 4 to 8 chromosomes) [Cabrero et al., 2003a]. On the contrary, in *T. infestans* populations the rDNA cluster changes its location between autosomes and sex chromosomes (fig. 3). As far as we know, the complete transference of rDNA between different chromosome types (autosomes vs. sex chromosomes) has never been reported in any species. In conclusion, this is not only the first report of population polymorphism detected in Heteroptera and in holocentric chromosomes, but it is also the description of a novel type of intraspecific reorganization of rDNA clusters.

Mechanisms of rDNA Variation

The capability to relocate, expand and contract the number of copies is a well recognized property of rDNA. Changes in the location of rDNA loci could be the consequence of structural chromosome rearrangements that imply modifications in chromosome number, such as reported in plants [Hall and Parker, 1995] and certain groups of insects [Hirai et al., 1996; Bressa et al., 2009]. However, in Triatominae, given that the number of autosomes remains almost unchanged (77 of the 80 described species have 20 autosomes), it is possible to rule out this type of chromosomal rearrangements as the primary mechanism of variation in the location of rDNA loci.

The diversity in the rDNA position and its occurrence in more than one chromosome suggest that transposition [Schubert, 1984; Schubert and Wobus, 1985] and/or ectopic recombination [Hanson et al., 1996] are the mechanisms responsible for Triatominae rDNA changes. Certainly the occurrence of these mechanisms is largely controlled by chromosome proximity patterns in the nucleus. In triatomines, heterologous associations among heterosomes (Xs/Y sex chromosomes), as well as non-homologous autosomes, are very common phenomena and could favor the observed rDNA variation.

Sex chromosomes are achiasmatic but remain intimately associated during mitotic interphase and the first meiotic prophase. This heterologous association among sex chromosomes during cell divisions is particularly regular and it is present in all triatomine species [Panzera et al., 2010]. The large number of triatomine species with rDNA loci in 2 sex chromosomes (see table 1), may reflect the existence of chromosomal exchanges between X and Y sex chromosomes during this association.

Other triatomine species with autosomal heterochromatin present different kinds of chromosomal associations among non-homologous chromosomes, i.e. sex chromosomes with autosomes and/or autosomes with autosomes, giving rise to different types of chromocenters [for review see Panzera et al., 2010]. The existence of these heterologous associations should greatly facilitate the occurrence of ectopic recombination and transposition, and as a consequence, rDNA variation. Those groups of species with heterologous associations show greater variation in the chromosome position of the ribosomal loci, such as the Infestans subcomplex (table 1).

Conclusions

Our findings reveal 4 features of 45S rDNA loci not previously observed in Heteroptera: (1) high level of rDNA location variability within a chromosomal homogenous group (Triatominae subfamily), (2) four chromosomal patterns of rDNA location within a single genus *(Triatoma)*, (3) simultaneous presence of 45S rDNA clusters in 1 sex chromosome and 1 autosomal pair (*T. delpontei*, fig. 2k), and (4) detection of intraspecific variation in the rDNA cluster location (*T. infestans*, fig. 3). This conspicuous diversity shows that the genomes of the Triatominae species are very dynamic and have undergone several chromosomal reorganizations. Our results support the hypothesis that the chromosomal position of rDNA clusters is an important marker to evidence chromosomal differentiation in species karyotypically homogenous in their chromosome number.

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