Chromosome Painting in Triatomine Insects Reveals Shared Sequences Between X Chromosomes and Autosomes

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Abstract

In order to provide a broad picture on the origin and evolution of holocentric X chromosomes in heteropteran species, we prepared a sex chromosome painting probe by microdissection of the X_1 and X_2 chromosomes from a kissing bug Mepraia spinolai (Hemiptera: Reduviidae: Triatominae). Fluorescence in situ hybridization on four species of the Triatomini having different amounts of autosomal heterochromatin and sex chromosome systems show that the Xs probe hybridizes on the euchromatin, located both on autosomes and X chromosomes. The heterochromatic Y chromosome and autosomal heterochromatic regions always appear free of hybridization signals. The hybridization results of the Xs probe on Rhodnius prolixus (Rhodniini) is completely different to that observed in Triatomini species. The hybridization signals are small and scattered on all euchromatin, without specific regions including the X chromosome. These results are in accordance with previous data obtained by genomic in situ hybridization and fluorescent banding, suggesting a clear differentiation in the repeat sequence composition of both sex chromosomes between Triatomini and Rhodniini tribes. These results also support that each sex chromosome in Triatomini has evolved independently from different autosomal pairs of a common ancestor, as described in other insect orders.

Key words: fluorescence in situ hybridization, holocentric chromosome, kissing bug, microdissection X chromosome, sex chromosome evolution

Chromosomal painting analysis using whole chromosome-specific probes has contributed to the knowledge on the origin and evolution of sex chromosomes in a great diversity of organisms such as mammals (Cortez et al. 2014), fish (Cioffi et al. 2011a,b), reptilia (Matsubara et al. 2014), platyhelminthes (Hirai et al. 2012), and plants (Kejnovsky and Vyskot 2010). However, the application of chromosome painting for comparative chromosome studies in insects, excepting Diptera, is very limited due to the difficulties in obtaining probes. In fact, just a few studies with chromosome painting probes have already been conducted in this animal group, including studies focused on both autosomes and sex chromosomes (Traut et al. 1999; Rutten et al. 2004; Bressa et al. 2009; Teruel et al. 2009a,b; Martins et al. 2013; Yoshido et al. 2013; Menezes-de-Carvalho et al. 2015).

Insects of the Heteroptera order (true bugs) represent an attractive group to study sex chromosome evolution due to holocentric nature of their chromosomes and their particular segregation during cell divisions. Holocentric chromosomes, also called chromosomes with diffuse or nonlocalized centromeres, are characterized by lack of a primary constriction (Hughes-Schrader and Schrader, 1961). In Triatominae subfamily, three male sex systems are recorded: XY, X_1X_2Y, and $X_1X_2X_3Y$, the first one considered to be ancestral (Ueshima 1966). Sex chromosomes are very well differentiated from autosomes by their behavior during meiotic divisions. As a rule, heteropteran sex chromosomes are considered asynaptic, achiasmatic, and behave as univalents during the first meiotic division (Solari 1979). From first meiotic prophase until diakinesis, the sex chromosomes are grouped together forming a positive heteropycnotic body. At first metaphase, the sex chromosomes appear clearly separated but lying side by side, without any visible physical connection between them. During anaphase I, the two sister chromatids of each sex chromosome segregate to opposite poles resulting in their equal division. In metaphase II, the sex chromosomes appear associated end-to-end to form a pseudobivalent (pseudotri or tetravalent according to the sex system), which is located in the center of the ring formed by the autosomes. In anaphase II, these chromatids segregate to opposite poles resulting in two gametes with different sex

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chromosomes. So, in this group, sex chromosomes exhibit an equational division in first meiotic division and reductional division in meiosis II. This reversion in the order of segregation of sex chromosomes is called postreductional segregation or inverted meiosis (Hughes-Schrader and Schrader 1961; Ueshima 1979). The meiotic association between sex chromosomes does not involve recombination or association by homologous regions.

In different insect groups such as Diptera, Lepidoptera, and Orthoptera distinct types of DNAs have been identified on sex chromosomes, such as different repetitive DNA sequences and transposable elements (Kaiser and Bachtrog 2010). However, in Heteroptera, knowledge about the molecular composition and evolution of sex chromosomes is very limited. Recently, the sequencing of the R. prolixus genome has allowed the identification of nine Y-linked genes (Mesquita et al. 2015). Of the nearly 90 Triatominae species cytogenetically studied so far, the Y chromosome is entirely C-heterochromatic (Panzera et al. 2010). However, at the molecular level, the Y chromosome shows striking differences between the two main tribes of the subfamily, Triatomini and Rhodniini, which comprise almost 90% of the 150 recognized species (Galvão and Paula 2014). In Triatomini, the Y chromosome is mainly composed of A þ T rich repeated DNA sequences (Bardella et al. 2014, 2016). Genomic in situ hybridization (GISH) studies reveal that these repetitive sequences are highly conserved and probably represent an ancestral character of this tribe (Pita et al. 2014). In Rhodniini, the heterochromatic Y chromosome is constituted by other types of DNA sequences that are not revealed by GISH or fluorescence analyses (Pita et al. 2014, Bardella et al. 2016; respectively).

Unlike the Y chromosome, the X chromosomes of Triatominae presented an extensive variability with C and fluorescence bandings. In most species, they are euchromatic, but in others, the X chromosomes revealed heterochromatic regions (Panzera et al. 2010). Several of these heterochromatic regions are CMA positive (G þ C rich) and are associated with ribosomal clusters (Bardella et al. 2016). Currently, there are no data on sequence composition of the X chromosomes in this group, nor in the Heteroptera order.

In order to provide a preliminary study on the origin and evolution of X chromosomes, chromosome painting analyses were performed in several triatomine species. We obtained a sex chromosome painting probe by microdissection of the X_1 and X_2 chromosomes from Mepraia spinolai meiotic nuclei, followed by polymerase chain reaction (PCR) amplification and labeling. We have tested the probe by fluorescence in situ hybridization (FISH) on meiotic preparations of the same species. For comparative chromosome studies, we applied this probe on male preparations of other triatomine species with different sex mechanisms and amounts of C-heterochromatin (Panzera et al. 1995, 2010, 2014; Calleros et al. 2010).

Materials and Methods

Biological Material

Table 1 summarizes the geographic origin and cytogenetic data of the material here analyzed. Microdissection was performed on two individuals of M. spinolai (Chile, Til Til). Hybridizations were performed on at least two individuals of each species, whose geographical origin is specified in Table 1.

Chromosome Preparations, C-Banding, and Microdissection of X Sex Chromosomes

Cytological preparations (squashes) from microdissection, hybridizations, and C-banding were obtaining from testes removed from alive adult insects and fixed in an ethanol–glacial acetic acid mixture (3:1) and finally stored at −20 °C. Microdissection of sex chromosomes was performed from M. spinolai individuals (2n ¼ 20 autosomes plus X_1X_2Y sex chromosomes) without pooling chromosomes. C-banding was performed as described by Panzera et al. (1995).

Microdissection was performed using an inverted microscope (Zeiss Axiovert 200, Göttingen, Germany) with a sterile glass needle attached to a micromanipulator (Eppendorf Transfer Man NK2, Hamburg, Germany). Forty chromatids of each X chromosome (total ¼ 80 chromatids) obtained from metaphase I or II cells were microdissected. Before degenerate oligonucleotide-primed PCR (DOP-PCR) amplification, the microdissected chromosomes were pretreated by using a precycling incubation of 15 cycles at 30°C/1 min and 50°C/1 min. For the DOP-PCR amplification, 30 ml of a PCR reagent mix was added to give a final concentration of 200 mM dNTPs, 2.5 mM $MgCl_2$, 100 pg/ml BSA, 2 U Biotaq DNA polymerase, and 1 mM DOP primer (50-CCG ACT GCA GNN NNN NAT GTG G).

Degenerate oligonucleotide-primed PCR was carried out with an initial denaturation at 94°C for 5 min, followed by eight cycles at 94°C/1 min, 45°C/1 min, and 72°C/3 min, and finally 28 cycles at 94°C/1 min, 56°C/1 min, and 72°C/3 min, with a final extension

Table 1. Geographic origin and chromosomal traits of the species here analyzed.

Species	Male diploid number (2n)	Autosomal C-banding	Y chromos.	X chromos.	Geographic origin
Mepraia spinolai	20A þ X_1X_2Y	10 II with blocks in 2 ends	Cþ, DAPIþ, CMA-	X_1 & X_2 with dots in both ends (Cþ & CMAþ)	Chile, Metropolitan Region, Til Til, S.
Triatoma infestans Non-Andean Group	20A þ XY	3–4 II with blocks in 1 or 2 ends; 6–7 II euchromatic	Cþ, DAPIþ, CMA-	Euchromatic with DAPI dot in 1 end	Argentina, Chaco, Tres Estacas, P.
T. infestans Andean Group	20A þ XY	8–10 II with blocks in 1 or 2 ends; 0–2 II euchromatic	Cþ, DAPIþ, CMA-	Cþ dot & DAPIþ in both ends	Bolivia, Potosí, Palquiza, S.
T. delpontei	20A þ XY	10 II with block in only 1 end	Cþ, DAPIþ, CMA-	Cþ dot & DAPIþ	Bolivia, Santa Cruz, Tita, S.
T. dimidiata	20A þ X_1X_2Y	10 II with dots in 2 ends	Cþ, DAPIþ, CMA-	Euchromatic	Guatemala, Jutiapa, Carrizal, D.
Rhodnius prolixus	20A þ XY	No, all II euchromatic	Cþ, DAPI-, CMA-	Euchromatic	Guatemala, Quezaltenango, D. Insectary CDC (USA)

Chromosome data from Calleros et al. (2010), Panzera et al. (1995, 2010, 2014), and Bardella et al. (2016).

A, autosomes; II, bivalents; P, peridomiciliary; D, domiciliary; S, sylvatic; DAPI, 4',6-diamino-2-fenilindol; CMA, chromomycin A_3.

at 72°C for 7 min. A second labeling DOP-PCR amplification was made with 5 µl of the first PCR product as template in a reaction mixture (30 µl volume) containing of 200 µM dNTPs, 2.5 mM $MgCl_2$, 4 µM DOP primer, 2 U Taq DNA polymerase (Roche), and 20 µM Biotin-16-dUTP (Roche). The amplification was carried out with an initial denaturation at 95°C for 5 min, followed by five cycles at 94°C/30 s, 30°C/30 s, and 72°C/1.5 min, and finally 35 cycles at 94°C/30 s, 62°C/30 s, and 72°C/1.5 min, with a final extension at 72°C for 7 min.

Squashed preparations for FISH were dehydrated in an increasing series of ice-cold ethanol and then air-dried. Chromosomes were denaturized in 70% deionized formamide and 2×SSC at 70°C for 3.5 min, then incubated in 2×SSC at room temperature for 1 min and dehydrated in an ethanol series. Approximately 200 ng of probe (chromosome paint) was coprecipitated with 5 µg of denatured salmon sperm DNA and was finally dissolved in 10 µl of hybridization solution containing 50% deionized formamide, 10% dextran sulfate, and 2×SSC. After denaturation (6 min at 73°C), the probe was dropped onto each slide and spread over the hybridization area with a 22- by 22-mm glass cover slip. Hybridization was conducted at 37 °C overnight in a moist chamber. Fluorescence immunological detection was performed using the avidin-Fluorescein/anti-avidin-biotin system with two amplification rounds. The slides were counterstained with DAPI and mounted in an antifade solution (Vectashield from Vector laboratories). Finally, images were captured using a fluorescence microscope (Olympus BX51) equipped with a CCD camera (Olympus DP70). Hybridization pattern for each species was determined by the chromosomal analyses of at least two individuals.

Results

Control Hybridization of the Xs Probe (Self-Hybridization)

The sex chromosome-painting probe derived from microdissected X_1 and X_2 chromosomes of *M. spinolai* was first hybridized on meiotic cells from male individuals of *M. spinolai* with the same geographical origin of the microdissected X chromosomes in order to verify the probe specificity (Fig. 1a-b). The Xs probe displayed strong hybridization signals on both X chromosomes; however, it did not paint the Y chromosome evenly. Also most of the chromatin in all autosomes (10 pairs) presents strong hybridization signals, excepting their terminal regions (arrowheads Fig. 1a-b). This hybridization pattern is exactly the opposite of that observed with C-banding: the Y chromosome is entirely C-heterochromatic, while the 10 autosomal pairs exhibit terminal C-blocks (Table 1, Fig. 1c). In conclusion, in our control species the Xs probe hybridizes with the euchromatin of both X chromosomes and on the euchromatic regions of all 10 autosomal pairs, but not with the heterochromatic regions of autosomes and Y chromosome.

Interspecific X Probe Hybridization

In the analyzed species from the genus *Triatoma*, the Xs probe hybridized with the euchromatic regions. The Xs probe on *T. infestans* (non-Andean group) male chromosomes displays strong labeling on the X chromosome and in the seven minor autosomal bivalents while the heterochromatic Y chromosome is free of labeling (Fig. 1d). The three largest autosomal bivalents show a nonuniform hybridization with regions without hybridization signals (arrowheads Fig. 1d), which correspond with the large C-heterochromatic regions presented in these chromosomes (Table 1, Fig. 1e). The pattern of hybridization in *T. infestans* (Andean group) is similar to that observed in the non-Andean group, but the level of hybridization is less uniform and less strong (Fig. 1f). As in the non-Andean group, the heterochromatic regions of the autosomal bivalents lack hybridization signals, especially visible in the largest bivalents (arrowheads Fig. 1f).

In *T. delpontei* the Y chromosome appears without hybridization. Unlike the results in the other X chromosomes, in this species this chromosome shows scattered signals (Fig. 1g), probably due to possessing heterochromatic regions (Table 1). The 10 autosomal bivalents have strong signals located in the middle regions that correspond to one of the chromosomal ends (arrowheads Fig. 1g). The other chromosome ends are heterochromatic (Fig. 1h) and lack hybridization signals (Fig. 1g).

In *T. dimidiata*, the 10 autosomal bivalents and both X chromosomes (X_1 and X_2) show strong hybridizations signals (Fig. 1i). Only the heterochromatic Y chromosome does not display labeling. Finally, in *R. prolixus* (Rhodniini), all autosomal bivalents as well as the heterochromatic Y chromosome and the euchromatic X chromosome show scattered hybridization signals (Fig. 1j).

Discussion

Knowledge of the sequences that compose the X chromosomes in organisms with holocentric chromosomes is very limited. In parthenogenetic aphids with X0/XX system, the X chromosome presents levels of genetic diversity, allelic richness, and recombination rates similar as autosomes (Jaquiéry et al. 2012). In species with heteromorphic sex chromosomes, several studies support the accumulation of different sequences between both sex chromosomes. In lepidopteran species with a ZZ/ZW system, the overall sequence composition of Z chromosome (equivalent of X chromosome) is much like that of autosomes than the W chromosome, showing extensive conserved synteny in their gene content (Sahara et al. 2012). In Heteroptera, only one report using an X probe has been published, involving *Dysdercus* species (Pentatomomorpha) with X0 and neo-X neo-Y sex systems (Bressa et al. 2009). These authors showed the existence of conserved regions among X chromosomes that are not present in the neo-Y chromosome.

The results obtained here provide new information about the origin of the sex chromosomes in triatomines. The DOP-PCR of microdissected chromosomes preferentially amplifies anonymous repeat DNA sequences (Houben et al. 2001). The hybridization of *M. spinolai* X_1-X_2 probe on the same species chromosomes (self-hybridization) and other three Triatomini species shows the existence of shared repeat DNA sequences between the X chromosomes and the autosomes. Since hybridization signals are located in euchromatic regions it is very likely that they recognize interspersed repeat sequences, common between autosomes and X chromosomes. This hybridization pattern is completely reverse to that observed with C-banding that point that these sequences are not present in the Y chromosome heterochromatin neither in the autosomal heterochromatic ends, which appear free of labeling (Fig. 1a-i). Our results are similar to the obtained in two grasshopper species *Locusta migratoria* and *Eyprepocnemis plorans* (Teruel et al. 2009a, 2009b). In both species, with localized centromere, chromosome painting using an X chromosome probe results in the presence of dot-like painting pattern over euchromatic autosomal regions. The authors suggested that this hybridization pattern is due to the presence of some repetitive elements interspersed in euchromatin that are shared between the X chromosome and the autosomes.

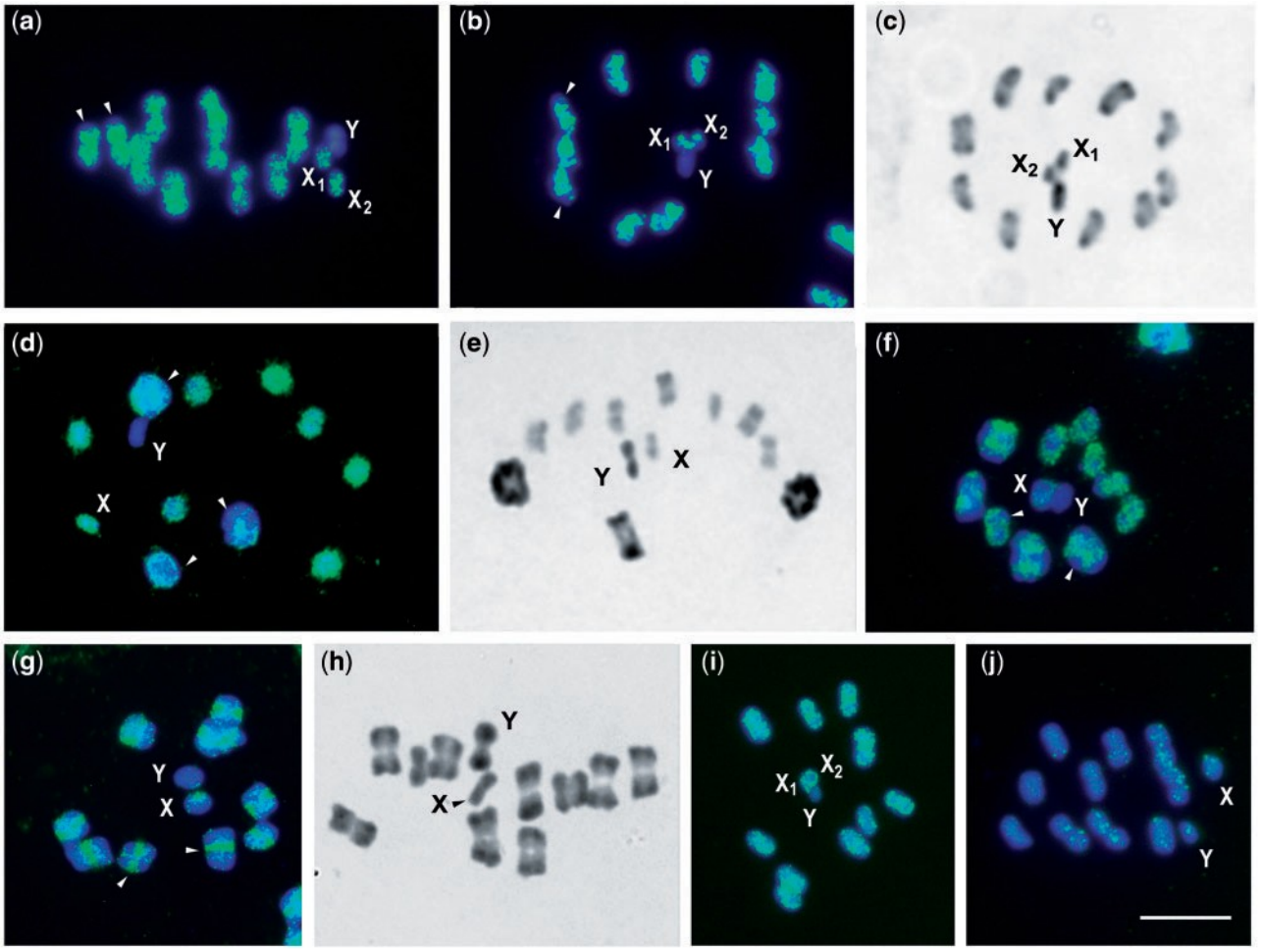

Fig. 1. Hybridization results using an Xs probe obtained by the microdissection of the X_1 and X_2 chromosomes of M. spinolai on male meiotic chromosomes from different triatomine species. (a) Metaphase I (MI) and (b) Metaphase II (MII) of M. spinolai. Both X chromosomes and all autosomes (10 pairs) show strong hybridization signals (in green), except on their terminal regions, which do not have labeling (arrowheads). The Y chromosome is completely unlabeled. (c) C-banding in MII of M. spinolai showing the heterochromatic Y chromosome and the presence of small and terminal heterochromatic regions in all autosomes. (d) Triatoma infestans (non-Andean group). MI. Hybridization signals involve almost completely the seven smaller bivalents and the X chromosome. The three largest autosomal bivalents have regions lacking hybridization signals (arrowheads) while the Y chromosome is completely unlabeled. (e) C-banding in T. infestans (non-Andean group). MI showing the heterochromatic nature of the Y chromosome and the euchromatic X chromosome. Only the three biggest bivalents show prominent heterochromatic regions, the remaining autosomes being entirely euchromatic. (f) T. infestans (Andean group). MI. The 10 bivalents and the X chromosome present hybridization signals. The heterochromatic Y chromosome is free of labeling as well as the heterochromatic regions of the autosomes (arrowheads). (g) T. delpontei. MI. Hybridization signals are located in the middle regions of 10 bivalents that correspond to one of the chromosomal ends (arrowheads). The X chromosome shows label in a small region while the Y chromosome does not show hybridization signals. (h) C-banding in T. delpontei. MI shows the presence of large heterochromatic blocks in the terminal regions of all autosomes as well the heterochromatic Y chromosome. (i) T. dimidiata. MII. All autosomes and both X chromosomes appear almost entirely labeled while the Y chromosome is completely unlabeled. (j) Rhodnius prolixus. MI. Autosomes and sex chromosomes show small and scattered hybridization signals. Scale bar ¼ 10 **1**m.

Previous GISH and fluorescent banding analyses (Pita et al. 2014, Bardella et al. 2016) and the results presented here support that the X and Y chromosomes in Triatomini species presented substantial differences in their chromatin constitution. Bardella et al. (2016) showed that C-heterochromatic Y chromosome is DAPI^{þ}, preferentially formed by A þ T rich repeated sequences, being extremely conserved among all Triatomini species (Pita et al. 2014). By contrast the X chromosome would consist of dispersed repeated sequences located in euchromatin, and similar to those observed in euchromatic autosomal regions (this paper; Fig. 1a, b, d, f). Occasionally in some species, the X chromosomes may have heterochromatic regions which may be similar to those observed in the autosomes, as in T. delpontei (Fig. 1g).

In several insects orders such as Coleoptera, Diptera, and Lepidoptera, different authors suggest that each sex chromosome (X and Y) have evolved independently from different pairs of autosomes from a common ancestor (Carvalho 2002, Kaiser and Bachtrog 2010, Pease and Hahn 2012, Yoshido et al. 2013). For example in Drosophila melanogaster, the Y chromosome does not share any genes with the X chromosome, except for Ste-Su (Ste) and rDNA gene clusters (Carvalho 2002). In Triatominae, the XY sex chromosomes contain different types of repeat sequences, as revealed by GISH studies (Pita et al. 2014), fluorescent banding (Bardella et al. 2016), and this paper. All these results strongly support that heteromorphic XY sex chromosomes in Triatomini species derived from different autosomes of a common ancestor, as has been described in other insect groups.

The results of the Xs probe on R. prolixus (Rhodniini) are completely different to that observed in Triatomini species (Fig. 1j). The hybridizations signals are small and scattered on all euchromatin,

without specific regions including the X and Y chromosomes. The Xs probe results joint with fluorescent banding data indicate that the X and Y chromosomes in Triatomini and Rhodniini are very different in each tribe. In Rhodniini, the C-heterochromatic Y chromosome has not fluorescence signals and probably it is constituted by other types of repeated sequences (Bardella et al. 2016). The results obtained with chromosome painting show that a similar phenomenon occurs with the X chromosomes. The M. spinolai Xs probe resulted in a strong hybridization on the euchromatin of the four Triatomini species here analyzed (Fig. 1a–f), suggesting that the X chromosome sequences are widely conserved among them. However, this Xs probe, obtained from a Triatomini species, does not show hybridization on the R. prolixus X chromosome (Fig. 1j).

The high chromatin differentiation between X and Y chromosomes in Triatomini and Rhodniini could help to elucidate the controversial evolutionary origin of the Triatominae subfamily. Phylogenetic trees based on nuclear and mitochondrial sequences support Triatominae as monophyletic (Hypša et al. 2002, Justi et al. 2016), polyphyletic (Schofield and Galvão 2009), or paraphyletic group (Hwang and Weirauch 2012). If Triatominae is monophyletic, the sex chromosomes could have diverged after separation between both tribes about 40 million years ago (Justi et al. 2016). Conversely, if Triatominae is polyphyletic, they might have been derived from different ancestors with dissimilar sex chromosomes. The molecular characterization of specific sequences on sex chromosomes and their comparison with putative ancestors or sister species could clarify the origin of the Triatominae subfamily.

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