



Possible origin of polymorphism for chromosome number in the assassin bug *Zelurus femoralis longispinis* (Reduviidae: Reduviinae)

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In this study, we analysed a population of *Zelurus femoralis longispinis* polymorphic for chromosomal number. The fundamental karyotype of this subspecies is $2n = 22 = 20A + XY$ (male), but individuals with $2n = 23 = 20A + XY + \text{extra chromosome}$ have been found at high frequency and collected at different time periods. We examined male meiotic behaviour, average length as percentage of the sex chromosomes, the content, distribution and composition of heterochromatin, and the number and location of ribosomal DNA in the two cytotypes found. The meiotic behaviour of the extra chromosome was highly regular and similar to that of sex chromosomes. The average length of the sex chromosomes in individuals not carrying the extra chromosome was significantly greater than in those carrying it. The results support a hypothesis that the extra chromosome might have originated by fragmentation of the original X chromosome into two unequal-sized chromosomes (X_1 and X_2), leading to an X_1X_2Y multiple system. Maintenance of the polymorphism with time appears to indicate that the new chromosomal variant is neutral or at least not detrimental, or that it could be selectively advantageous. This polymorphic population represents direct evidence of a multiple sex chromosome system originating through fragmentation of a single X in Reduviidae as well as in Heteroptera. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, 110, 757–764.

ADDITIONAL KEYWORDS: C- and fluorescent bands – chromosomal fragmentation – evolution – FISH – holokinetic chromosomes – multiple sex-chromosome system.

INTRODUCTION

About 1000 nominal species belonging to Reduviinae, distributed in 138 genera, have been described. This subfamily is a cosmopolitan group recognized in part by the presence of ocelli and the absence of a disc in the hemelytra cell (Henry, 2009). Most taxa in this subfamily are generalist predators, but have a preference for termites, bees and ants (Schaefer & Panizzi, 2000; Henry, 2009; Rimmel, Davison &

Tammaru, 2011). The subfamily Reduviinae has a polyphyletic origin; within this subfamily, the genus *Zelurus* Hahn would constitute of a monophyletic group together with the subfamilies Stenopodainae and Triatominae (Weirauch & Munro, 2009). Various species of *Zelurus* have been cited as predators of adults and nymphs of some triatomine species such as *Triatoma infestans* (Klug), *T. rubrovaria* (Blanchard), and *T. platensis* Neiva (Sosa, 1997). It has been observed that in the presence of many individuals of *Zelurus*, there are no hematophagous insects. In the subfamily Reduviinae, only four species have been cytogenetically studied (Payne, 1912; Jande, 1959a, b;

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Ueshima, 1979). The diploid autosomal number varies between 20 and 26, with both single (XY, male) and multiple sex-chromosome systems (X_nY , male).

In Heteroptera as well as in Reduviidae, it is generally agreed that multiple sex-chromosome systems are the result of fragmentation(s) of the X and/or Y chromosome(s) of a simple ancestral system. This hypothesis is supported by the holokinetic nature of these chromosomes, their achiasmatic behaviour and comparative cytogenetic studies (Ueshima, 1979; Manna, 1984; Poggio, Bressa & Papeschi, 2007). In addition, several researchers have described spontaneous mutants or polymorphic populations for the number of sex chromosomes (Heizer, 1950; Schrader & Hughes-Schrader, 1958; Papeschi, 1994; Papeschi, 1996). As exceptions to this hypothesis, three species of Coreidae (Pentatomomorpha) (Wilson, 1909) and one species of Cimicidae (Cimicomorpha) (Darlington, 1939; Slack, 1939; Ueshima, 1967) have multiple sex-chromosome systems originated by non-disjunction.

B chromosomes are considered selfish and parasitic elements that do not follow the Mendelian laws of inheritance and can originate from sex chromosomes, autosomes, or interspecific crosses. Most of these chromosomes possess a high transmission rate in meiosis and/or mitosis, which leads to an increase in their frequency and the establishment of polymorphisms in natural populations (Camacho, Sharbel & Beukeboom, 2000). In Heteroptera, B chromosomes are generally unstable, i.e. they are not present in all cells of the bearer individual, are positive heteropycnotic-like sex chromosomes during prophase I, do not recombine with other chromosomes of the complement, and divide equationally at anaphase I (Mikolajski, 1965, 1967; Papeschi, 1992; Mola & Papeschi, 1993; Grozeva & Nokkala, 2003). However, in some species of Nabidae (Cimicomorpha), B chromosomes are stable during meiosis and are associated with the X and Y sex chromosomes during metaphase II, and they could migrate indistinctly with either sex chromosome (Grozeva & Nokkala, 2003).

In the present study, we analysed a polymorphic population of *Zelurus femoralis longispinis* Lent & Wygodzinsky for the chromosomal number and observed two cytotypes ($2n = 22/23$). The meiotic behaviour, the content, distribution and composition of heterochromatin and the number and location of ribosomal DNA (rDNA) were analysed in the two cytotypes. In addition, the origin of the extra chromosome is discussed.

MATERIAL AND METHODS

We used 17 males of *Zelurus femoralis longispinis* (eight collected in 2008 and nine collected in 2011)

from Pampa del Indio (25°55'S, 56°58'W), Chaco province (Argentina). All the specimens analysed were brought alive to the laboratory.

Meiotic chromosome slides were made by the squash technique as described in Poggio, Bressa & Papeschi (2011), and chromosome slides for bandings and fluorescence *in situ* hybridisation (FISH) techniques were made by means of spreading as described in Traut (1976). The slides were then dehydrated in an ethanol series (70, 80, and 96%, 30 s each) and stored at -20 °C until use.

C-banding was performed according to Papeschi (1988) with slight modifications described in Poggio *et al.* (2011). Fluorescent bandings were made as described in Poggio *et al.* (2011). FISH with a biotinylated 18S rDNA probe was performed essentially following the procedure described in Sahara, Marec & Traut (1999) with several modifications described in Fuková, Nguyen & Marec (2005) and in Bressa *et al.* (2009).

Chromosome slides were observed in a Leica DMLB microscope equipped with a Leica DFC350 FX CCD camera and Leica IM50 software, version 4.0 (Leica Microsystems Imaging Solutions). Black-and-white images of chromosomes were recorded separately for each fluorescent dye. Images were pseudocoloured (light blue for DAPI, green for CMA₃, red for Cy3) and processed with an appropriate software.

The length of sex chromosomes was calculated with respect to the total length of chromosomal complement as a percentage, using photographs of cells at metaphase I and the computer software MicroMeasure version 3.3 (Reeves & Tear, 2000). Statistical analyses were performed using a *t*-test for samples with unequal variances and sample numbers.

RESULTS

CHROMOSOME COMPLEMENT AND MEIOSIS

Two cytotypes were observed in the 17 male specimens of the subspecies *Zelurus femoralis longispinis* studied: four of the eight specimens collected in 2008 and six of the nine specimens collected in 2011 showed $2n = 22 = 20A + XY$ (Fig. 1A, C), whereas the others individuals (four collected in 2008 and three collected in 2009) showed $2n = 23 = 20A + XY + \text{extra chromosome}$ (Fig. 1B, D). At spermatogonial prometaphase of both cytotypes, it was not possible to identify the sex chromosomes and the extra chromosome (Fig. 1A, B). Meiotic karyotypes of both cytotypes based on metaphase I bivalents were composed of ten autosomal bivalents (II) and two sex chromosomes of decreasing size (Fig. 1C, D), whereas

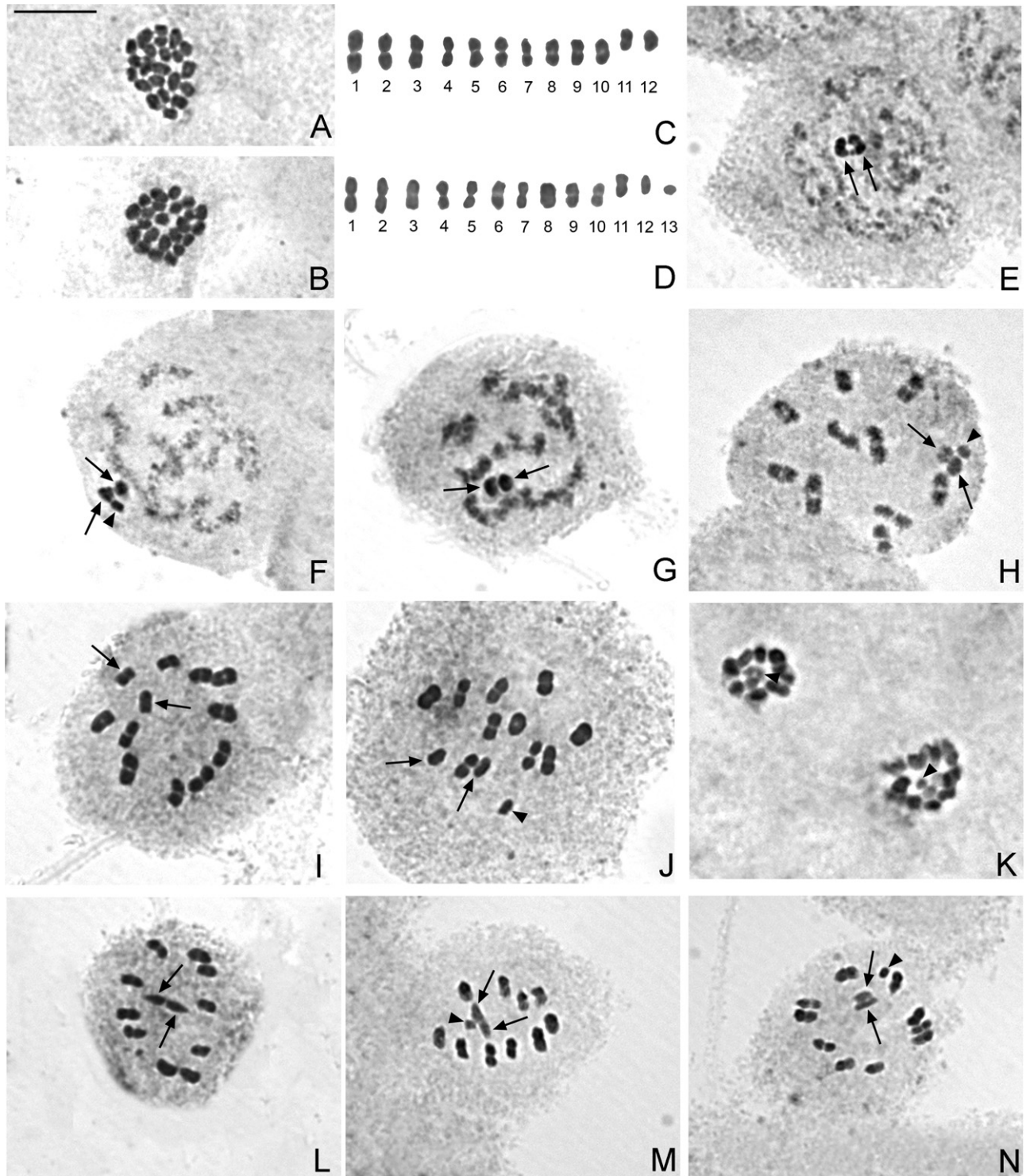


Figure 1. Male mitosis and meiosis in a polymorphic population of *Zelurus femoralis longispinis*. A, spermatogonial prometaphase ($2n = 22$). B, spermatogonial prometaphase ($2n = 23$). C, meiotic karyotype ($2n = 22$). D, meiotic karyotype ($2n = 23$). E, diffuse stage ($2n = 22$). F, diffuse stage ($2n = 23$). G, early diplotene ($2n = 22$). H, early diakinesis ($2n = 23$). I, diakinesis ($2n = 22$). J, metaphase I ($2n = 23$). K, telophase I ($2n = 23$). L, metaphase II ($2n = 22$). M–N, metaphase II ($2n = 23$). 1–10, autosomal bivalents; 11–12, X and Y sex chromosomes; 13, extra chromosome. Arrows: sex chromosomes. Arrowheads: extra chromosome. Chromosomes are stained with 2% iron acetic haematoxylin. Scale bar = 10 μm .

the extra chromosome is the smallest of the complement (Fig. 1D). All cells of all individuals, even of those individuals carrying the extra chromosome, showed a unique diploid number; moreover, the extra chromosome was present in all the cells examined, both at spermatogonial mitosis and at meiosis.

During the first meiotic division, at the diffuse stage, the autosomal bivalents (II) decondensed partially, whereas the sex univalents were positively heteropyknotic (Fig. 1E, F). In individuals with $2n = 23$, the extra chromosome was positively heteropyknotic and lay close to the sex chromosomes (arrowhead Fig. 1F). At early diplotene, bivalents began to condense again, although they could not be distinguished from each other (Fig. 1G). From diakinesis, ten II were recognized, and both sex univalents and the extra chromosome continued close to each other and became isopyknotic (Fig. 1H, I). At metaphase I, all the chromosomes acquired their maximum condensation. In particular, the II had one or two terminal chiasmata. The sex univalents and the extra chromosome were located apart from each other (Fig. 1J). At anaphase I, the II divided reductionally, whereas both X and Y and the extra chromosome segregated equationally (data not shown). As a result, telophase I nuclei showed 12 chromosomes in individuals without the extra chromosome and 13 chromosomes in those with it (Fig. 1K). At metaphase II, the autosomes formed a ring and the X and Y sex chromosomes came close together and associated to form a pseudo-bivalent, which lay at the centre of the ring (Fig. 1L–N). The extra chromosome was located in the centre of the ring, close to sex pseudo-bivalent in 94.7% of the 38 cells analysed (Fig. 1M) or it formed part of the autosomal ring in the remaining cells (Fig. 1N). At anaphase II, the autosomes divided equationally and the sex and extra chromosomes segregated reductionally (data not shown). As there were no cells in anaphase II and telophase II to determine the constitution of the resulting daughter cells, we may infer that there may be nuclei with $10A + X/10A + Y$ in individuals without the extra chromosome, and $10A + Y/10A + X + \text{extra chromosome}$ and $10A + X/10A + Y + \text{extra chromosome}$ in individuals with the extra chromosome.

To elucidate the possible origin of the diploid number polymorphism, we compared the average length in percentage of the sex chromosomes in individuals with and without the extra chromosome (23 and 22 chromosomes, respectively). The average total length of the sex chromosomes in individuals with $2n = 22$ ($12.66 \pm 0.71\%$) was significantly higher than those with $2n = 23$ ($11.15 \pm 0.85\%$); $T = 4.05 > CV_{(L=11)} = 2.2$.

CONTENT, DISTRIBUTION, AND COMPOSITION OF HETEROCHROMATIN, AND NUMBER AND LOCATION OF rDNA CLUSTERS

We found no differences in content, distribution and composition of heterochromatin or in the number and location of rDNA clusters between the two cytotypes (Fig. 2). We detected no C-heterochromatic regions in the ten autosomal pairs. One of the sex chromosomes was completely C positive, the other only had a terminal C-positive band and no C-positive bands were observed in the extra chromosome (Fig. 2A–C). The fluorescent banding showed that the C-positive sex chromosome was completely DAPI-positive (Fig. 2D, F) and CMA₃-negative (Fig. 2E, G), i.e. this chromosome is heterochromatic and rich in A + T. The C-positive band at one terminal region of the other sex chromosome was CMA₃-negative. The extra chromosome and all the autosomal pairs were stained homogeneously with DAPI (Fig. 2D, F) or CMA₃ (Fig. 2E, G). Thus, the C- and fluorescent banding patterns allowed us to differentiate the X and Y chromosomes and determine the arrangement of both sex chromosomes and the extra chromosome at metaphase II. In 85.4% of the cells analysed, the extra chromosome was orientated together with the sex chromosome that had the terminal C-positive band (Fig. 2B). By contrast, the extra chromosome was found in the middle of both sex chromosomes in 14.6% (41 cells analysed), i.e. without a determined position (Fig. 2C). FISH experiments revealed that rDNA clusters are placed at one terminal region of each sex chromosome. The extra chromosome presented no hybridization signals (Fig. 2H, I).

DISCUSSION

The population of *Zelurus femoralis longispinis* (Reduviinae) from Pampa del Indio (Chaco, Argentina) presented two different male karyotypes: $2n = 22 = 20A + XY$ and $2n = 23 = 20A + XY + \text{extra chromosome}$. Taking into account that four of the eight specimens collected in 2008 and six of the nine specimens collected in 2011 showed no extra chromosome and that the remainder showed the extra chromosome, we might conclude that this subspecies has a stable polymorphism for chromosome number.

Considering the cytogenetic features and the male meiotic behaviour of the specimens of *Z. femoralis longispinis* analysed, we can infer that this subspecies has a chromosome number and karyotype consistent with those previously described in the family Reduviidae: the autosomal bivalents are chiasmatic and divide reductionally at anaphase I and equationally at anaphase II, the sex chromosomes are positively heteropyknotic during early prophase I,

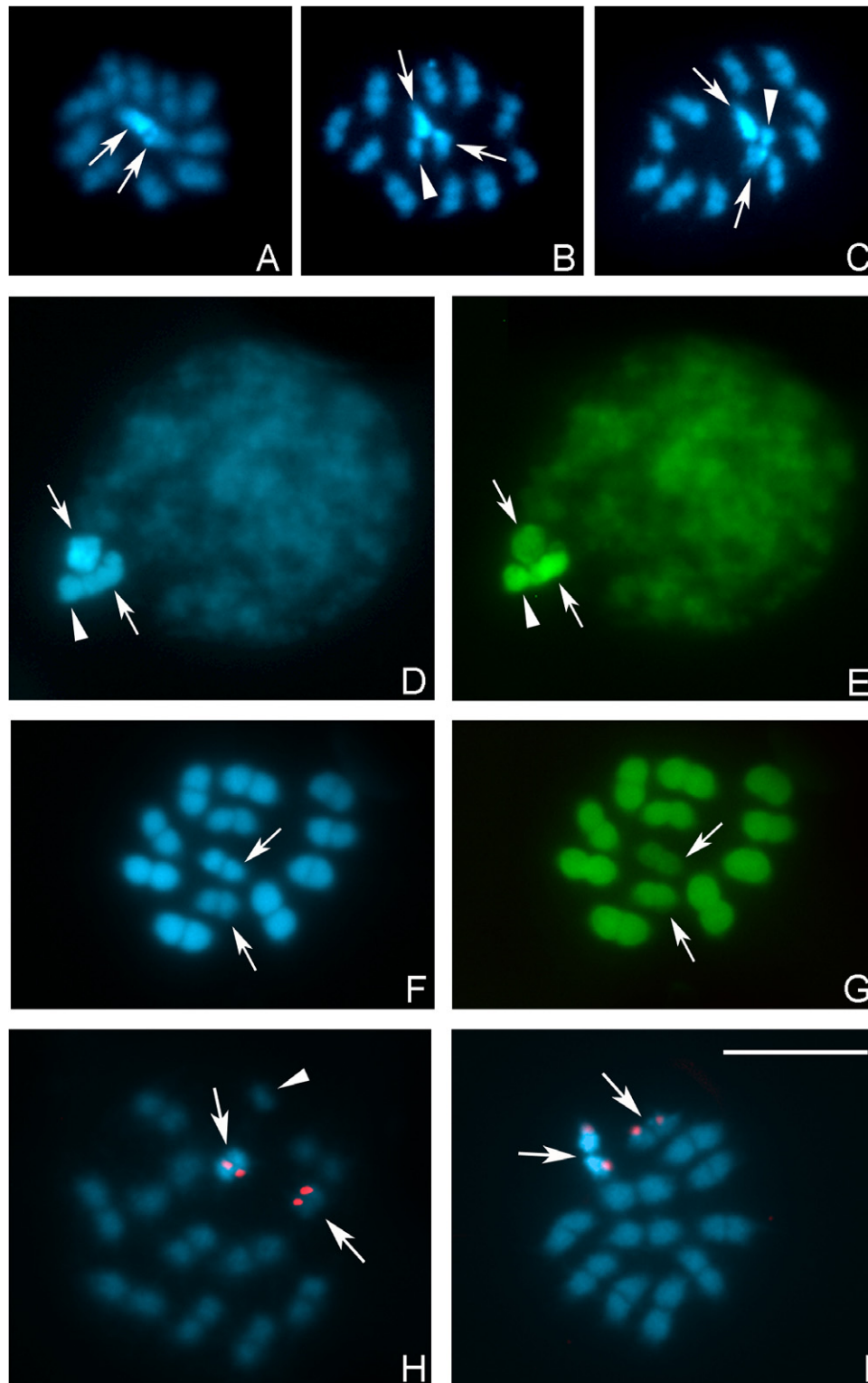


Figure 2. A–C, C-banding followed by staining with DAPI. D, F, DAPI banding. E, G, CMA₃ banding. H–I, FISH with rDNA 18S probe (hybridization signals in red) in a polymorphic population of *Zelurus femoralis longispinis*. Chromosomes were counterstained with DAPI (blue). A, metaphase II ($2n = 22$). B–C, metaphase II ($2n = 23$). D–E, diffuse stage ($2n = 23$). F–G, metaphase I ($2n = 22$). H, diakinesis ($2n = 23$). I, metaphase I ($2n = 22$). Arrows: sex chromosomes. Arrowheads: extra chromosome. Scale bar = 10 μ m.

asynaptic, achiasmatic, and divide equationally at anaphase I and reductionally at anaphase II (Ueshima, 1979; Manna, 1984; Poggio *et al.*, 2007; Panzera *et al.*, 2010). According to molecular phylogeny based on mitochondrial and nuclear ribosomal genes, the subfamily Reduviinae would have a polyphyletic origin, in which the genus *Zelurus* would form a monophyletic group with Triatominae and Stenopodainae (Weirauch & Munro, 2009). Cytogenetic data suggest the last two subfamilies are characterized by having a low autosomal diploid number, modal numbers ranging between 20 and 22 autosomes, and single (XY/XX) and multiple sex-chromosome systems (X_nY/X_nX_n) (Ueshima, 1979; Manna, 1984; Poggio *et al.*, 2007; Kaur, Kaur & Suman, 2009; Panzera *et al.*, 2010; Kuznetsova *et al.*, 2011). Besides, in light of the number of autosomes and the sex-chromosome system of *Z. femoralis longispinnis*, we may conclude that this subspecies shares the cytogenetic characteristics of its closest subfamilies. These lines of evidence support the placement proposed for the genus *Zelurus* in the phylogeny of Reduviidae (Weirauch & Munro, 2009).

In general, the Y chromosome is completely heterochromatic in Reduviidae (Panzera *et al.*, 1998; Poggio, 2007). Based on this background, we propose that the completely C-positive sex chromosome of *Z. femoralis longispinnis* would be the Y chromosome and that the chromosome with a terminal band would be the X chromosome. Moreover, rDNA-FISH revealed two rDNA clusters, which are located at the terminal position on the X and Y chromosomes, both in individuals with $2n = 22$ and in individuals with $2n = 23$. Neither nucleolus organizer region (NOR) is not associated with CMA₃ bright bands, indicating that the whole rDNA repeating unit is not rich in G + C base pairs. The same number and location of NORs have been determined in 12 triatomine species belonging to four different genera: *Rhodnius* Stål, *Psammolestes* Bergroth, *Eratyrus* Stål, and *Triatoma* Laporte (Morielle-Souza & Azeredo-Oliveira, 2007; Bardella *et al.*, 2010; Panzera *et al.*, 2012; Pita *et al.*, 2013). In only two of them, the rDNA clusters co-localized with a CMA₃-positive band; hence, the repeating unit of ribosomal genes is rich in G + C.

ORIGIN OF THE EXTRA CHROMOSOME

Seven of the 17 individuals analysed had an extra chromosome in all the cells observed. The extra chromosome was the smallest of the complement (Fig. 1D) and positively heteropyknotic at early meiotic prophase (Fig. 1F). At first meiotic division, this chromosome segregated equationally (Fig. 1K). At metaphase II, in most cases, the extra chromosome was associated with the sex chromosomes

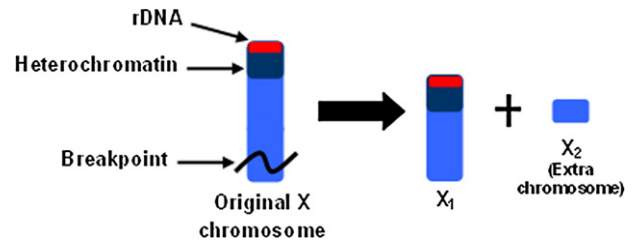


Figure 3. Possible location of breakpoints in the X chromosome and plausible origin of the extra chromosome in the polymorphic population of *Zelurus femoralis longispinnis*.

and orientated together with the X chromosome (Figs 1M, 2B).

To propose a probable origin of the extra chromosome, we compared the relative average length of the sex chromosomes in individuals with and without it. The results show that the average length in individuals without the extra chromosome ($2n = 22$) was significantly higher than that with this chromosome ($2n = 23$). This difference in the relative average length of the sex chromosomes between the two cytotypes suggests that the original X was fragmented into two unequal chromosomes, one larger (X_1) than the other (extra chromosome or X_2) (Fig. 3). By contrast, the analysis of content, distribution, and composition of constitutive heterochromatin as well as the location and number of rDNA clusters showed no differences between the two cytotypes. On the basis of these findings, we propose that the chromosome break might have been located near the terminal region of the original X chromosome, which lacks both a C-positive band and the NOR (Fig. 3). Given the presence of the extra chromosome in all the cells analysed of individuals who carry it, its achiasmatic and highly regular meiotic behaviour as sex chromosomes and its segregation together with the X chromosome in anaphase II, we infer that this extra chromosome would be a sex chromosome (X_2), but would not be considered as a B chromosome (Fig. 3). The present population of *Z. femoralis longispinnis* represents direct evidence of a multiple sex-chromosome system originating through fragmentation of the single X in Reduviidae as well as in Heteroptera.

Previously, sex-chromosome system polymorphisms have only been described in *Belostoma* sp. (Belostomatidae, Nepomorpha) and *Oechalia pacifica* (Stål) (Pentatomidae, Pentatomomorpha) by Papeschi (1996) and Heizer (1950), respectively. In these species, the multiple sex-chromosome systems have arisen from a simple XY/XX through X chromosome fragmentation, which was supported not only by comparison of the relative size of the sex chromosomes among individuals with single and multiple systems, but also by their chromosome behaviour. In most

multiple systems of Heteroptera, sex chromosomes associate by means of 'touch-and-go pairing' at metaphase II and generally arrange themselves with the X(s) facing the pole opposed to the Y(s) (double-plate arrangement). Nevertheless, when the multiple systems are not characteristic of the species, as in *O. pacifica* (Heizer, 1950), the three sex chromosomes (X_1X_2Y , male) can be associated in a chain at metaphase II (chain arrangement). In the polymorphic population of the sex chromosome of *Belostoma* sp., sex chromosomes show different types of association at metaphase II within each individual: (i) in double-plate, (ii) in a chain and (iii) in other transitional arrangements (Papeschi, 1996). Darlington (1939) suggested that the chain arrangement would be evolutionarily unstable and that there would be a trend towards the establishment of the double plate. In the present population of *Z. femoralis longispinis*, we always observed that the sex chromosomes were arranged in double plate at metaphase II. Hence, it could be considered that the multiple system of this population is stable. Keeping this chromosome behaviour in mind, it seems that a selective force towards a double-plate arrangement could be operating in the population under study.

Taking into account the meiotic configuration of the sex chromosomes, the frequency of the individuals that carry the multiple system (41%), and the presence of this cytotype $2n = 23 = 20A + X_1X_2Y$ with time (2008 and 2011), it can be assumed that the chromosome rearrangement could be neutral, or at least not detrimental, or even have some adaptive value. In summary, the population of *Z. femoralis longispinis* under study has a stable sex-chromosome system polymorphism, the origin of which would not be recent. We conclude that male individuals with $2n = 22 = 20A + XY$ and $2n = 23 = 20A + X_1X_2Y$ coexist in this population.

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