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Research paper

Experimental crosses between *Mepraia gajardoi* and *M. spinolai* and hybrid chromosome analyses reveal the occurrence of several isolation mechanisms

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ABSTRACT

Hematophagous insects of the subfamily Triatominae include several species with a large variety of shapes, behavior and distribution. They have great epidemiological importance since most of them transmit the flagellated protozoan *Trypanosoma cruzi*, the etiologic agent of Chagas disease. In this subfamily several cases of species hybridization have been reported under experimental and natural conditions. *Mepraia* is a genus of Triatominae endemic in Chile, responsible for transmitting *T. cruzi* in the sylvatic cycle. This genus includes three species, *M. gajardoi*, *M. spinolai* and *M. parapatrica*; however, the differentiation of *M. parapatrica* as a separate species remains controversial considering the possible occurrence of introgression/hybridization processes in some populations of this putative species. *Mepraia* species show conspicuous wing polymorphism, and it has been proposed that the genes related to wings are linked to the Y chromosome, thus wingless males could not engender winged progeny. In order to determine the degree of reproductive isolation and to assess the wing phenotype in the offspring, we performed experimental crosses between the two most divergent *Mepraia* species (*M. gajardoi* and *M. spinolai*) together with chromosome analyses of hybrid progenies. Although fertile F_1 hybrids were obtained in only one direction of crossing, we verified the existence of different isolation mechanisms between parental species, including hybrid breakdown. The occurrence of winged males in the offspring of wingless parental males suggests that the wing character is not linked to the Y chromosome.

1. Introduction

The main criterion that defines a good biological species is reproductive isolation. However, there are many cases of species that hybridize under laboratory conditions and even in nature, violating the assumptions of the biological species concept (Mayr, 1942, 1996). Hematophagous insects of the subfamily Triatominae (Hemiptera: Reduviidae) include 150 species with a large variety of shapes, behavior and distribution (Galvão and Paula, 2014). They have great epidemiological importance since most of them transmit the flagellated protozoan *Trypanosoma cruzi*, the etiologic agent of Chagas disease (Lent and Wygodzinsky, 1979). According to Schofield and Galvão (2009), the genus *Mepraia*, endemic in Chile, belongs to the spinolai complex, which also includes two *Triatoma* species from Argentina: *Triatoma eratyrusiformis* and *T. breyeri*. These last two species are geographically separated from the *Mepraia* species by the Andes Mountains (Lent and Wygodzinsky, 1979). Phylogenetic analyses using mitochondrial fragments confirm a monophyletic clustering of the spinolai complex species (Campos et al., 2013a; Justi et al., 2014). The finding of shared mitochondrial genes between *Mepraia* and *T. eratyrusiformis* is strong evidence of the close relationship of these taxa (Campos-Soto et al., 2015). *Mepraia* species are endemic to semiarid and arid regions of Chile, distributed in coastal and interior valleys of the northern and central regions (Frías et al., 1998; Campos et al., 2013b), and are highly infected with *T. cruzi* (Campos-Soto et al., 2016). Their distribution in wild and peridomestic habitats, their opportunistic feeding behavior and human settlement in risky areas are features of high epidemiological significance that may turn *Mepraia* species into important *T. cruzi* vectors (Cattan et al., 2002; Toledo et al., 2013).

Currently three species are included in the genus: *M. spinolai*, *M. gajardoi* and *M. parapatrica*, based on differences in morphological characters and karyotypes (Frías et al., 1998; Frías and Atria, 1998; Jurberg et

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al., 2002; Frías-Lasserre, 2010). *M. gajardoi* is limited to coastal desert areas between 18° and 25° S, while *M. spinolai* is distributed in coastal and interior valleys between parallels 26° and 34° S. *M. parapatrica* is apparently restricted to coastal areas of the Antofagasta and Atacama Regions (24° to 26° S) at the distribution boundaries of the two previous species, and probably sympatric with *M. gajardoi* and *M. spinolai* in its northern and southern distribution, respectively (Frías-Lasserre, 2010). Nuclear and mitochondrial gene analyses support the specific status of *M. spinolai* and *M. gajardoi* (Calleros et al., 2010; Campos et al., 2013b). However, the differentiation as separated species from *M. parapatrica* remains controversial considering likely occurrence of introgression/hybridization processes in some populations of this putative species (Calleros et al., 2010; Campos et al., 2011).

Mepraia species show conspicuous wing polymorphism, unique in the Triatominae subfamily (Lent and Wygodzinsky, 1979; Schofield et al., 1998). Wing polymorphism occurs quite commonly in other Reduviidae subfamilies and frequently, although not exclusively, in desert species (Schofield et al., 1998). In insects, the presence or absence of wings seems to be controlled by a single locus with two alleles or a polygenic system, the last one was found in several hemipteran species (Roff, 1986). Females of the three *Mepraia* species are invariably wingless or micropterous, with hind wings reduced to minute pads. *M. spinolai* males have a wide variation in wing length; they may be wingless (micropterous) or winged, brachypterous (with wings that reach the abdominal end) or macropterous (with wings larger than the abdomen). *M. gajardoi* males are invariably winged (brachypterous), while *M. parapatrica* males are brachypterous or macropterous (Frías-Lasserre, 2010). Campos et al. (2011) reported a new wing phenotype (vestigial wings) in areas where the *M. parapatrica* has been reported. The study of Frías and Atria (1998) proposed that the genes related to wings are linked to the Y-chromosomes, thus wingless males could not produce winged progeny. However, another study argued that the sex chromosomes are not related to the wing polymorphism (Calleros et al., 2010). In this study we performed experimental crosses between the two most divergent *Mepraia* species (*M. gajardoi* and *M. spinolai*) and chromosomal analyses of hybrid progenies in order to answer the following questions: (i) Do *M. spinolai* and *M. gajardoi* exhibit reproductive isolation? if so (ii) What could be the mechanism involved?, and (iii) What are the wing phenotypes in the offspring?

2. Material and methods

2.1. Material analyzed and obtaining of parental individuals

All parental individuals were collected as fifth instar nymphs in Chile: *M. gajardoi* from two populations of the Arica and Parinacota Region (Caleta Vitor: 18° 45′45″S, 70° 20′34″W and Caleta Camarones: 19° 12′16″S, 70° 16′08″W), and *M. spinolai* from the Coquimbo Region (Las Chinchillas National Reserve: 31° 30′28″S, 71° 06′19″W). The localities are approximately 1450 km distant. The nymphs were maintained individually in 3.2 cm × 3.6 cm clear plastic containers inside a climatic chamber at 26 °C, 65–70% relative humidity and 14 h:10 h light:dark cycle. Each container was provided with a sandy bottom and a folded piece of paper as refuge. All insects were fed to engorgement every three weeks on laboratory mice (C_3H strain). All experiments were conducted with permission of the Ethics Committee of the Faculty of Science, University of Chile, and followed the recommendations for animal testing (Goldberg, 2010).

2.2. Breeding and hybridization

One day after molting to adult, virgin individuals were randomly assigned to mate with a partner of the same species or of the other species as follows: *M. gajardoi* male × *M. gajardoi* female (N = 2 couples), *M. spinolai* micropterous male × *M. gajardoi* female (N = 13 couples) and *M. gajardoi* male × *M. spinolai* female (N = 7 couples). For *M. spinolai* male × *M. spinolai* female crosses we used the data published by Frías et al. (1998) and Frías and Atria (1998). Each pair remained together in a 7 cm height 6 cm diameter plastic container with a meshed lid until one of the partners died. Containers were provided with a folded piece of paper as refuge. Laboratory conditions and pair feeding were as described above. Pair survivorship and sexual activity (e.g., males mounting or trying to mount females, Fig. 1) were recorded daily. Eggs were removed from parental containers daily, counted and placed in a new container. Hatching of first instar nymphs was recorded daily. Eggs were collected until female death; first nymph hatching was recorded until one month after female death. Table 1 summarizes the crosses performed, including F_1, first backcrosses and second backcrosses until adults were obtained (total time elapsed: 4 years). Egg fertility was calculated by dividing the number of first instar nymphs by the total number of eggs laid. Nymph mortality rate (%) was calculated by dividing the number of nymphs that did not reach adulthood by the total number of first instar nymphs.

2.3. Cytogenetic studies

Cytogenetic studies were restricted to adult specimens (males and females), since the nymphs do not present gonads with meiotic divisions. Chromosomal analyses involved parental, F_1 hybrids and first backcross individuals (2 matings), specified between parenthesis in Table 1. Testes and ovaries were removed from freshly killed adults, fixed in an ethanol–acetic acid mixture (3:1) and stored at −20 °C. Air-dried chromosome preparations were made by squashing gonads in 50% acetic acid, freezing them in liquid nitrogen and removing the coverslip with a razor blade. The C-banding technique was performed as described by Panzera et al. (1995). Slides were examined under a Nikon Eclipse 80i microscope and images were obtained with a DS-5Mc-U2 digital camera. Images were processed with the Adobe Photoshop® software. As far as possible, chromosome analyses were performed both in mitosis (gonial prometaphases) and meiosis (metaphases I, II or diplotene stages) to determine diploid chromosome number, distribution patterns of C-heterochromatin regions and meiotic behavior, including chromosome pairing and segregation during both meiotic divisions. In each male individual, we observed at least 50 cells in different stages. In females, chromosome analyses were restricted to gonial mitotic prometaphases because meiotic stages are not

Fig. 1. *Mepraia spinolai* male (lateral view) and *M. gajardoi* female (frontal view) copulating.

Table 1
Experimental crosses involving *Mepraia spinolai* and *M. gajardoi* species.

Mepraia species ♂		♀	No. of couples	No. eggs	No. nymphs 1st stage	Egg fertility (%)	Adult offspring	Mortality rate (%)
Control crosses								
gajardoi[a]	×	*gajardoi*	2 [2]	34	30	88.2	ND	ND
spinolai[a]	×	*spinolai*	38 [ND]	1952	956	48.9	ND	ND
spinolai wingless[b]	×	*spinolai*	21 [21]	ND	ND	ND	All males wingless	ND
spinolai winged[b]	×	*spinolai*	8 [8]	ND	ND	ND	Males wingless & winged	ND
Hybrids crosses								
spinolai	×	*gajardoi*[a]	4 [ND]	48	5	10.4	ND	ND
spinolai wingless	×	*gajardoi*	13 [2]	312	43	13.8	HYB = 25: 15 (3)♀, 10 (2)♂ winged	41.9
gajardoi	×	*spinolai*[a]	18 [ND]	539	32	5.9	ND	ND
gajardoi	×	*spinolai*	7 [0]	33	2	6.1	0	100.0
First backcrosses								
spinolai wingless	×	HYB	1 [1]	89	45	50.6	A = 19: 7 (3)♀, 8 (3)♂ winged, 4 (4)♂ wingless	57.8
HYB winged	×	*spinolai*	2 [ND]	83	17	20.5	B = 14: 6♀; 6♂ winged, 2 (2)♂ wingless	17.6
HYB winged	×	*gajardoi*	2 [ND]	178	43	24.2	C = 20: 11♀, 9♂ winged	53.5
gajardoi	×	HYB	5 [ND]	181	77	42.5	D = 25: 14♀, 1♂ winged	74.4
Second backcrosses								
A wingless	×	*spinolai*	1 [0]	0	0	–	0	–
A winged	×	*gajardoi*	1 [0]	0	0	–	0	–
spinolai wingless	×	A	1 [0]	27	10	37.0	0	100.0
gajardoi	×	A	1 [0]	9	0	0.0	0	–
B winged	×	*spinolai*	1 [0]	0	0	–	0	–
spinolai wingless	×	B	1 [1]	74	43	58.1	11 = 4♀, 2♂ winged, 5♂ wingless	74.4
gajardoi	×	B	1 [0]	15	0	0.0	0	–
C winged	×	*gajardoi*	1 [0]	13	0	0.0	0	–
gajardoi	×	C	1 [0]	32	0	0.0	0	–
gajardoi	×	D	2 [0]	37	0	0.0	0	–

Results obtained in this study and previous reports, including the number and sex of adult individuals obtained in experimental crosses. Egg fertility is defined as the percentage of hatching eggs to nymphs (first instar). The mortality rate (%) is calculated by dividing the number of nymphs that did not reach the adulthood on the total number of first instar nymphs. Between parentheses we included the number of individuals cytogenetically analyzed. ND: no data.
[a] Frías et al. (1998).
[b] Frías and Atria (1998).

usually observed in their ovaries. The female karyotype was determined by observing at least 10 mitotic cells.

3. Results

3.1. Experimental crosses (Table 1)

Experimental crosses between *M. spinolai* and *M. gajardoi* yielded adult hybrids only when *M. gajardoi* was the female progenitor. In the unsuccessful cross, i.e. *M. gajardoi* (male) with *M. spinolai* (female), copulation occurred but produced a very small number of eggs laid and hatched, and no adult progeny. In the successful crossing, i.e. *M. spinolai* (wingless male) with *M. gajardoi* (female), the rate of egg hatching of hybrids (egg fertility) was greatly reduced to 14% of those observed in the parental species (49–88%). In addition, less than 10% of the eggs laid reached adulthood (25/312), with a nymph mortality rate of almost 42%. The F_1 adult progeny (named HYB in Table 1) included both males and females, with the particularity that all males were winged (hybrid

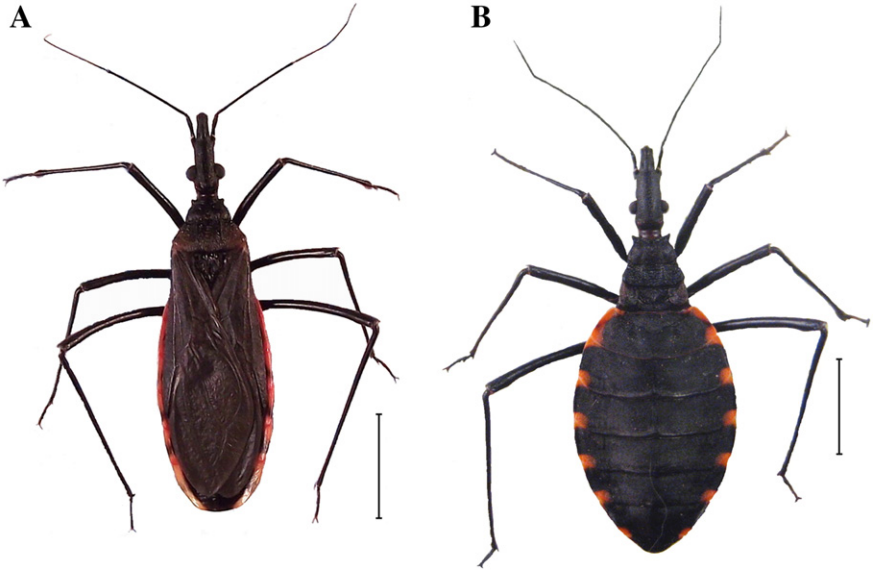

Fig. 2. Photographs of hybrids: (A) Male, (B) female. Scale bar: 5 mm.

specimens are shown in Fig. 2). These adult hybrids (males and females) were fertile when backcrossed with both parental species. Hatchability of eggs was highly variable among the first backcrosses (20–51%) as well as the mortality rates (18–74%). Progenies of the first backcrosses were mated with parental species and called second backcrosses, carrying out 10 of the 16 possible matings. Only one of the 10 second backcrosses resulted in adult offspring. In crosses using *M. spinolai* wingless males as progenitors, winged males were obtained.

3.2. Cytogenetic analyses

3.2.1. Karyotypes of parental individuals

Cytogenetic analyses of two males and one female of each of the three populations (two of *M. gajardoi* from Arica and Parinacota Region; *M spinolai* from Coquimbo Region) analyzed exhibited the same chromosome number and C-heterochromatin distribution as previously reported by several authors (Panzera et al., 1998, 2010; Pérez et al., 2004; Calleros et al., 2010). In both species, a diploid number of 23 and 24 chromosomes were observed in males and females, respectively (20 autosomes plus X_1X_2Y in males and $X_1X_1X_2X_2$ in females). The 10 autosomal pairs of *M. gajardoi* are euchromatic, with small C-dots only detected during early meiotic prophase. All autosomes of *M. spinolai* showed a C-heterochromatic region in one or two chromosome ends. A particular chromocenter during meiotic prophase characterizes and differentiates this species from all other triatomines. It is formed by the sex chromosomes surrounded by several autosomal heteropycnotic dots. Other heteropycnotic regions outside this chromocenter are observed. In *M. gajardoi*; only one chromocenter composed of the association of three sex chromosomes is observed during the first meiotic prophase. In both species the Y chromosome is almost entirely heterochromatic and medium-sized. The X_1 and X_2 are of similar size and the smallest of the complement; both are euchromatic in *M. gajardoi* and heterochromatic in *M. spinolai* (with terminal C-dots in one or both chromosome ends).

3.2.2. Hybrids (F_1): *M. spinolai* (♂) × *M. gajardoi* (♀) (Fig. 3)

We analyzed five individuals from the offspring, called HYB in Table 1. Gonial mitotic prometaphases of these hybrids showed the same chromosome number as those in the parental species, e.g., 23 chromosomes in males and 24 chromosomes in females (Fig. 3A). In one female we detected some gonial prometaphases having an extra small euchromatic chromosome (asterisk Fig. 3B). We can easily distinguish the autosomal complement of each parental species: ten autosomes with heterochromatic blocks in both chromosome ends coming from *M. spinolai* (arrows Fig. 3A–B), and ten autosomes without C-bands from *M. gajardoi* (arrowheads Fig. 3A–B). In females one X_1 and one X_2 chromosome were euchromatic (from *M. gajardoi*), while the other X_1 and X_2 chromosomes were heterochromatic (from *M. spinolai*) (Fig. 3A).

Meiotic behavior in male hybrids was entirely normal. The male meiotic prophase presented several chromocenters dispersed in the nuclei, very similar to *M. spinolai* but much smaller. One chromocenter is created by the association of autosomes and sex chromosomes (Fig. 3C). Normal pairing and recombination occurred between the two parental sets of homeologous chromosomes with different amounts of C-regions. For this reason, in first meiotic metaphase the 10 bivalents appear asymmetrical or heteromorphic for C-heterochromatin and show only one terminal or interstitial chiasma per bivalent (Fig. 3d). Typical for heteropteran sex chromosomes with inverted meiosis, the three sex chromosomes (X_1, X_2 and Y) appear as univalents (unpaired chromosomes with two chromatids each). Metaphases II were also completely normal with 10 half bivalents and the X_1, X_2 and Y chromatids forming a "pseudotrivalent", which is the product of equational segregation of sex univalents during the first meiotic division (Fig. 3e).

3.2.3. First backcrosses: *M. spinolai* wingless (♂) × hybrid F_1 (♀) (Fig. 4)

Ten individuals were analyzed, called "A" in Table 1. Gonial mitotic prometaphases of these hybrid males and females showed 23 and 24 chromosomes, respectively (Fig. 4A). We can easily distinguish three kinds of mitotic autosomes according to the distribution of C-heterochromatin: a) autosomes with C-regions in both chromosome ends from *M. spinolai* (arrows); b) autosomes without heterochromatin from *M. gajardoi* (arrowheads) and; c) autosomes with a C-region in only one chromosomal end as product of meiotic recombination in female hybrid progenitor (asterisk). Meiotic prophase presented more C-heterochromatic blocks than the hybrid F_1 individuals (compare Fig. 4B to Fig. 3C) due to the heterochromatic complement of the *M. spinolai* progenitor. All cells presented 10 bivalents in metaphase I as the product of normal pairing among homeologous autosomes. As a result of the chromosome pairing between autosomes with different amounts of C-heterochromatin, several bivalents showed asymmetrical distribution of C-regions (Figs. 4C, D). By contrast, the sex chromosomes exhibited normal and abnormal spatial arrangements in metaphase I. The three sex chromosomes may appear separately (normal behavior, Fig. 4C) or associated with each other, preferentially both X chromosomes (abnormal behavior, arrowhead Fig. 4D). Consequently, metaphases II exhibited different numbers of sex chromosomes, the autosomal number remaining constant. Most metaphases II (80% of the 75 cells analyzed) were normal, presenting ten half bivalents plus three sex chromosomes forming a typical pseudo-trivalent (Fig. 4E). Abnormal metaphases II exhibited different numbers of sex chromosomes without the typical arrangement, which is likely to lead to the generation of chromosomally unbalanced gametes: 10 half bivalents plus 3 X and 1 Y chromosome (Fig. 4F), or 2 Y plus 2 X chromosomes (Fig. 4G) or 2 X chromosomes without a Y chromosome (Fig. 4H). These male individuals, although most of their spermatids seemed to be genetically balanced, did not produce any offspring when crossed with either parental species (see A individuals in Table 1).

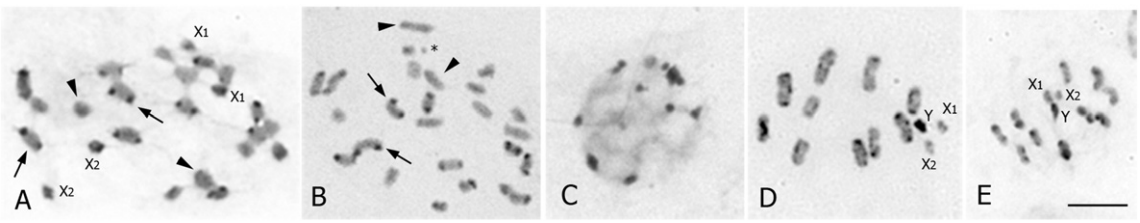

Fig. 3. Mitosis and male meiosis in experimental hybrids (F_1) between *M. spinolai* (♂) and *M. gajardoi* (♀) with C-banding. (A): Oogonial prometaphase with a diploid number of 24 chromosomes, 20 autosomes plus 4 sex chromosomes ($X_1X_1X_2X_2$). Ten autosomes have C-heterochromatic blocks in both chromosome ends (from *M. spinolai* - arrows) while the other ten autosomes are euchromatic without C-regions (from *M. gajardoi* - arrowheads). (B): Oogonial prometaphase showing an additional small euchromatic chromosome (asterisk). (C): Early meiotic prophase in male winged hybrid. Several C-positive chromocenters are dispersed in the nuclei; the largest is produced by the association of some autosomes with sex chromosomes. (D): Metaphase I. Normal cells with 10 bivalents. Each bivalent is formed by one homologue of *M. spinolai* and one of *M. gajardoi*. Due to their differences in C-blocks, the bivalents appear heteromorphic. (E): Metaphase II. This phase is entirely normal with ten half bivalents and a X_1X_2Y pseudo-trivalent. Scale bar: 10 μm.

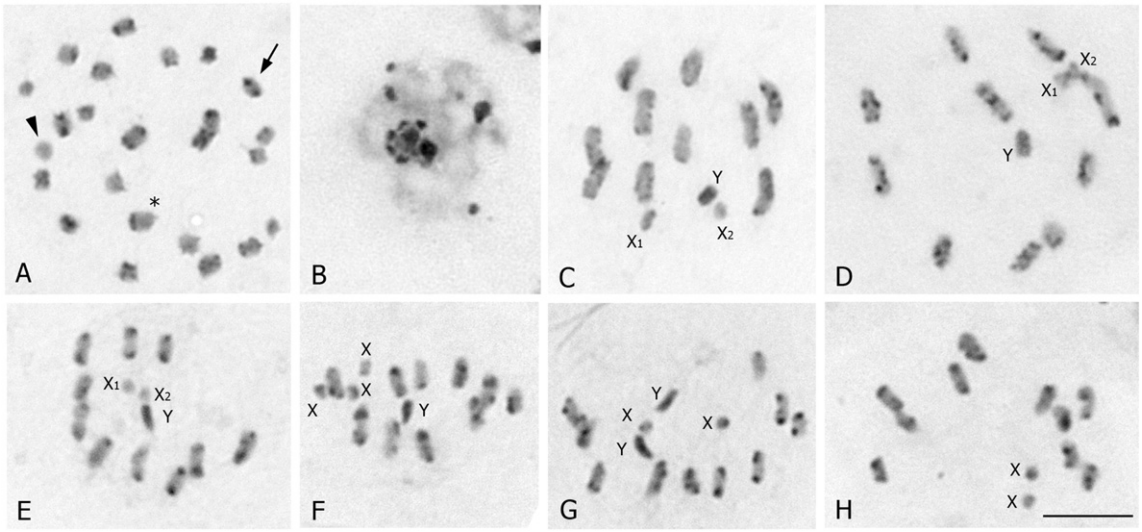

Fig. 4. Oogonial mitosis and male meiosis in experimental hybrids between *M. spinolai* wingless (♂) × hybrid F_1 (♀) by C-banding. (A): Oogonial prometaphase with a diploid number of 24 chromosomes, composed of 20 autosomes plus 4 sex chromosomes ($X_1X_1X_2X_2$). We can easily distinguish three kinds of autosomes: with C-regions in both chromosomal ends (arrows) from *M. spinolai*; without C-regions (arrowheads) from *M. gajardoi*, and autosomes with C-region in only one chromosome end as the product of meiotic recombination in the female hybrid progenitor (asterisk). (B): Early meiotic prophase in male hybrid (winged) showing more C-heterochromatic blocks than observed in the hybrid F_1 individuals. (C): First male meiotic metaphase. Normal cell with 10 bivalents plus clearly separate 3 sex chromosomes. (D): Abnormal metaphase I showing 10 bivalents plus 3 sex chromosomes, but X_1 and X_2 chromosomes are closely associated. (E): Metaphase II (normal) having a typical ring-shape configuration with 10 half bivalents and 3 sex chromosomes in the center (pseudo-trivalent- X_1X_2Y). (F): Metaphase II (abnormal) with 10 half bivalents plus one heterochromatic Y chromosome and three X chromosomes. (G): Metaphase II (abnormal) with 10 half bivalents plus two Y and two X chromosomes. (H): Metaphase II (abnormal) with 10 half bivalents plus two Xs but without Y chromosome. Scale bar: 10 μm.

3.2.4. First backcrosses: hybrid F_1 winged (♂) × M. spinolai (♀) (Fig. 5)

Two males (wingless) were analyzed, identified as "B" in Table 1. No mitotic prophases were observed. In the first meiotic division only a small fraction of metaphases I was normal (Fig. 5A). Most cells presented a high level of meiotic anomalies. Chromosomal pairing was altered; some autosomes synapsed and formed bivalents while others remained as univalents (arrowheads Fig. 5B-D). Trivalents and multiple associations (chains) of chromosomes were also observed (arrows Fig. 5B-C). The proportion of bivalents and univalents varied not only between the two analyzed individuals, but also between cells of the same specimen. These abnormalities produced metaphases I with variable numbers of autosomes and sex chromosomes. Second meiotic divisions, including metaphase II cells, were not observed. These individuals are completely infertile according to the cross-breeding results (see "B" individuals in Table 1).

4. Discussion

4.1. Hybrids in the subfamily Triatominae

There is an extensive and varied literature for the subfamily Triatominae on natural (Abalos, 1948; Martínez-Ibarra et al., 2005; Herrera-Aguilar et al., 2009) and experimental hybridization (Mazzotti and Osorio, 1941, 1942; Schreiber and Pellegrino, 1950; Perlowagora-Szumlewicz and Correia, 1972; Usinger et al., 1966; Heitzmann-Fontenelle, 1984; Almeida et al., 2012; Costa et al., 2003, 2016; Pérez et al., 2005; Martínez-Hernández et al., 2010; Correia et al., 2013; Martínez-Ibarra et al., 2015). The vast majority of these studies were conducted to determine the degree of reproductive isolation and compatibility existing among different species or morphologically distinct populations. This information was employed with different objectives, such as to decide the taxonomic status of the taxa involved (Ryckman, 1962; Espínola, 1971; Martínez-Ibarra et al., 2008; Martínez-Hernández et al., 2010; García et al., 2013; Mendonça et al., 2014), to clarify phylogenetic relationships (Usinger et al., 1966; Perlowagora-Szumlewicz and Correia, 1972; Belisário et al., 2007; Martínez-Ibarra et al., 2015) or to establish the role of natural hybridization in generating new genetic lineages (speciation process) (Costa et al., 2009, 2016).

Interspecific hybrid generation (adult F_1) is a fairly common phenomenon in triatomines, observed among several species of the genera *Meccus*, *Rhodnius* and *Triatoma* (Ryckman, 1962; Perlowagora-Szumlewicz and Correia, 1972; Schreiber et al., 1975; Carvalheiro and Barretto, 1976; Bello, 1978; Galíndez et al., 1994; Costa et al., 2003; Correia et al., 2013; Díaz et al., 2014; Martínez-Ibarra et al., 2015). The ease of obtaining inter-specific F_1

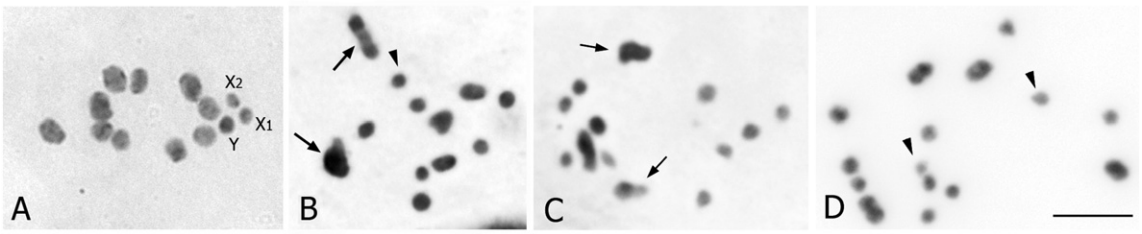

Fig. 5. Meiosis in experimental hybrids (2 wingless males) between Hybrid F_1 winged (♂) *M. spinolai* (♀) with C-banding. (A): First male meiotic metaphase (normal) showing 10 bivalents plus 3 sex chromosomes. (B, C, D): Metaphases I with several meiotic anomalies in chromosome pairing: autosomal univalents (arrowheads), bivalents (normal), trivalents or multiple chromosome associations (chains) (arrows). Scale bar: 10 μm.

adults reveals that several pre-zygotic isolation mechanisms can be easily avoided by experimental hybridization. Also, the morphology of the genital structures does not appear to provide an important pre-mating barrier that would prevent interspecific crosses in some triatomines, even between phylogenetically distant species such as *T. infestans* and *T. pseudomaculata* (Schreiber et al., 1974). However, the great majority of these hybrids are infertile, unable to produce viable offspring such as the crossings among *T. infestans*/*T. rubrovaria* (Schreiber and Pellegrino, 1950; Franca-Rodríguez et al., 1979; Scvortzoff et al., 1995; Pérez et al., 2005), *T. infestans*/*T. pseudomaculata* (Schreiber et al., 1974), *T. sinaloensis*/*T. protracta* (Usinger et al., 1966), *T. maculata*/*T. brasiliensis* (Perlowagora-Szumlewicz and Correia, 1972) and *T. maculata*/*T. pseudomaculata* (Belisário et al., 2007). However, fertile hybrids have been obtained between several Triatominae species, such as the crosses among several phyllosoma complex species (Martínez-Ibarra et al., 2008, 2009), brasiliensis complex species (Almeida et al., 2012; Costa et al., 2003, 2009; Correia et al., 2013; Mendonça et al., 2014), protracta complex species (*T. barberi*/*T. protracta*: Usinger et al., 1966; Ueshima, 1966) and infestans subcomplex species (Scvortzoff et al., 1995; Pérez et al., 2005).

Cross-breeding experiments were performed in *Mepraia* between *M. spinolai* and *M. gajardoi* (Frías et al., 1998). These authors reported data on egg fertility, but did not specify whether the hybrid generations reached the adult stage or the nymph mortality. This is the first report where adult hybrids are obtained and used for experimental crossbreeding with the parental species. Crosses between *M. spinolai* and *M. gajardoi* yielded adult hybrids when *M. gajardoi* was the female progenitor (Table 1). The reciprocal parental cross, i.e. *M. gajardoi* (male) with *M. spinolai* (female), did not produce adult progeny, with only two first instar nymphs obtained. Although copulation takes place, probably sperm transfer is nearly completely interrupted considering the very small number of eggs laid. This asymmetry in the reciprocal crosses was reported among some triatomine species, including both closely related species such as *T. delpontei*/*T. platensis* (Pérez et al., 2005) and *T. recurva*/*M. phyllosoma* (Martínez-Ibarra et al., 2015) and more distant species such as *T. maculata* with *T. brasiliensis* and *T. infestans* (Perlowagora-Szumlewicz and Correia, 1972).

In the successful crosses between *M. spinolai* (male) and *M. gajardoi* (female) hybrid progeny (HYB in Table 1) were obtained, including both fertile males and females in similar proportions. Despite the small number of individuals, in these hybrids Haldane's rule does not seem to be supported (Haldane, 1922). However, we also observed some reproductive alterations. Egg fertility rate in F_1 progeny was greatly reduced compared to those observed in both parental species; our results are very similar to those described by Frías et al. (1998) (Table 1). In addition, the proportions of eggs laid and first instar nymphs reaching adulthood were very small (10% and 42%, respectively). In all first backcrosses analyzed we obtained adult progeny of both sexes (A, B, C and D in Table 1), but egg fertility and nymph mortality were highly variable among them. Only one of the 10 second backcrosses resulted in adult offspring. These low values of egg fertility and viability of the nymphs are probably due to the joint action of several mechanisms of post-mating isolation acting with different intensity according to the cross performed. In the F_1 hybrids, and more intensely in the first backcrosses, high zygotic mortality (i.e., eggs are fertilized but zygotes do not develop) and partial hybrid inviability (i.e., nymphs are formed but with reduced viability) appear to be the predominant mechanisms. In the second backcrosses, partial gametic incompatibility (i.e., sperm transfer takes place but most of the eggs are not fertilized) seems to be the most important barrier. Perlowagora-Szumlewicz and Correia (1972) suggested for other triatomines that the primary component of hybrid sterility was a failure of the hybrid male to transfer sperm successfully during mating. In conclusion, the results obtained with experimental hybridization crosses suggest that the reproductive isolation between *M. spinolai* and *M. gajardoi* arises from the cumulative effect of different barriers to crossing.

4.2. Chromosomal analyses in Mepraia hybrid progenies

One of the main causes of infertility in interspecific hybrids is the production of genetically imbalanced gametes, mainly due to difficulties in chromosome pairing and/or irregular chromosome segregation during meiotic divisions. Interspecific hybrids formed between one species with autosomal C-heterochromatin regions (*T. infestans*) and another species without (such as *T. rubrovaria*) or a small amount of C-heterochromatin (such as *T. pseudomaculata*) showed lack of chromosome pairing and meiotic recombination between homeologous chromosomes (Schreiber and Pellegrino, 1950; Schreiber et al., 1974; Scvortzoff et al., 1995). Consequently, in the metaphase of the first meiotic division, several autosomal univalents are observed, which segregate irregularly during both meiotic divisions, producing genetically unbalanced gametes. Thus the fertility of these interspecific hybrids is drastically reduced to zero. As a result, Schreiber et al. (1974) proposed that the autosomal heterochromatin could prevent pairing between homeologous chromosomes, acting as a fertility barrier or isolation mechanism. However, Pérez et al. (2005) showed that male hybrids resulting from crosses between species with different C-heterochromatin quantities (*T. infestans*/*T. platensis*, or *T. platensis*/*T. delpontei*) are fertile. They have a regular meiosis, with normal meiotic pairing and genetic recombination, producing genetically balanced gametes.

In this study, the meiotic behavior in the interspecific F_1 hybrids between the two *Mepraia* species with (*M. spinolai*) and without (*M. gajardoi*) autosomal heterochromatin did not present irregular meiotic pairing between homeologous chromosomes and their segregation was normal in both meiotic divisions. We can conclude that at least in *Mepraia* F_1 hybrids, and similar to infestans subcomplex species, differential autosomal heterochromatin amounts do not seem to act as a fertility barrier or isolation mechanism.

Meiotic analysis of males resulting from the two first backcrosses analyzed showed striking differences between them and with that observed in F_1 hybrids. Male progeny of one of the first backcrosses analyzed (hybrid F_1 male with *M. spinolai* female), showed unbalanced chromosome numbers including both autosomes and sex chromosomes, generating non-viable gametes (Fig. 3). This is consistent with their incapacity to reproduce as detected in experimental crossbreeding (Table 1). On the contrary, in the other first backcrosses analyzed (*M. spinolai* male with hybrid F_1 female) most of the cells were normal and viable, and only a small fraction had disturbances in the sex chromosome number (Fig. 4). As a result these individuals were fertile, which is not consistent with the infertility shown when they were crossed with the parental species (Table 1). One possible explanation for this apparent incongruity would be the occurrence of the phenomenon called hybrid breakdown. This term is defined as inviability or sterility in the F_2 or later generations of interspecific crosses, even though the F_1 hybrids are viable and fully fertile (Johnson, 2010). In the parental species the alleles of different loci have been selected to form a "co-adapted" gene pool, including nuclear and organelle genes. In the F_1 hybrids meiotic division and recombination generates new allele combinations not presented in the parental species, which alter their original co-adaptation. The disruption of allele interaction at different loci increases in successive generations, and may be producing the inviability or sterility in F_2 or later hybrid generations.

In subspecies of *Mus musculus*, hybrid breakdown showing a reduced fertility/fecundity of F_2 is caused by disruption of the allele interactions of different loci involved in sperm head morphogenesis (Oka et al., 2004). Several years ago Perlowagora-Szumlewicz and Correia (1972) described that in different interspecific *Triatoma* crosses the sterility in hybrid males is due to their inability to transfer sperm to the female's spermatheca. Schreiber et al. (1975) speculated that the accessory glands of hybrid males are unable to produce essential secretions for sperm migration to the female receptacle. Something similar is probably occurring in *Mepraia* hybrids. Although spermatids are genetically balanced, the transformation processes of spermatids to spermatozoa

and/or their transference to females are not satisfactorily performed due to inadequate gene interactions, producing unviable sperm and consequently infertility of these hybrids.

4.3. Winged hybrids F_1

Frías and Atria (1998) proposed that genes related to wings are linked to the Y chromosome. According to these authors, the male wing polymorphism in *M. spinolai* involved a Y chromosome fragmentation. This rupture would originate two chromosomes, Y_1 and Y_2, with genes related to wings conserved in the Y_1 fragment. Male wingless individuals presented the Y_2 fragment without wing genes. This hypothesis predicts that wingless males could not engender winged progeny. However, in our study *M. spinolai* wingless males produced both wingless and winged males (see Table 1). This result suggests that in *Mepraia* species, wing character inheritance is not linked to the Y chromosome. Similar to other heteropteran and homopteran species, the inheritance of wings in *Mepraia* species probably involved a polygenic system, related with their capacity of reproduction (Roff, 1986).

5. Conclusion

This is the first report in the *Mepraia* genus where fertile adult hybrids were obtained and used for experimental crossbreeding with the parental species. Our results suggest that the reproductive isolation between *M. spinolai* and *M. gajardoi* arises from the cumulative effect of different crossing barriers. The presence of winged males in the offspring of wingless parental males suggests that the wing character inheritance is not linked to the Y chromosome.

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