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Cytogenetic and morphologic approaches of hybrids from experimental crosses between *Triatoma lenti* Sherlock & Serafim, 1967 and *T. sherlocki* Papa et al., 2002 (Hemiptera: Reduviidae)

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ABSTRACT

The reproductive capacity between *Triatoma lenti* and *Triatoma sherlocki* was observed in order to verify the fertility and viability of the offspring. Cytogenetic, morphological and morphometric approaches were used to analyze the differences that were inherited. Experimental crosses were performed in both directions. The fertility rate of the eggs in crosses involving *T. sherlocki* females was 65% and 90% in F1 and F2 offspring, respectively. In reciprocal crosses, it was 7% and 25% in F1 and F2 offspring, respectively. The cytogenetic analyses of the male meiotic process of the hybrids were performed using lacto-acetic orcein, C-banding and Feulgen techniques. The male F1 offspring presented normal chromosome behavior, a finding that was similar to those reported in parental species. However, cytogenetic analysis of F2 offspring showed errors in chromosome pairing. This post-zygotic isolation, which prevents hybrids in nature, may represent the collapse of the hybrid. This phenomenon is due to a genetic dysregulation that occurs in the chromosomes of F1. The results were similar in the hybrids from both crosses. Morphological features, such as color and size of connexive and the presence of red–orange rings on the femora, were similar to *T. sherlocki*, while wins size was similar to *T. lenti* in F1 offspring. The eggshells showed characteristics that were similar to species of origin, whereas the median process of the pygophore resulted in intermediate characteristics in the F1 and a segregating pattern in F2 offspring. Geometric morphometric techniques used on the wings showed that both F1 and F2 offspring were similar to *T. lenti*. These studies on the reproductive capacity between *T. lenti* and *T. sherlocki* confirm that both species are evolutionarily closed; hence, they are included in the brasiliensis subcomplex. The extremely reduced fertility observed in the F2 hybrids confirmed the specific status of the species that were analyzed.

1. Introduction

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*, which is transmitted to mammalian hosts primarily through feces during the blood meal of insects of the Triatominae subfamily (Chagas, 1909; Lent and Wygodzinsky, 1979). It has been estimated that there are 7–8 million people infected by *T. cruzi* in Latin America, and that 25 million are exposed to infection (Moncayo and Silveira, 2009; Coura and Viñas, 2010; WHO, 2013).

Triatoma sherlocki was first studied by Cerqueira (1982), who performed experimental crosses between *T. sherlocki* and *Triatoma brasiliensis*, and between *Triatoma infestans* and *Triatoma lenti*. Experimental crosses with *T. lenti* and *T. infestans* has not resulted in hybrids, whereas fertile hybrids were obtained from the cross between *T. brasiliensis* and *T. sherlocki*. This result classifies the latter primarily as a subspecies of *T. brasiliensis*, designated *T. b. santinacensis* (Cerqueira, 1982).

Morphological analyses of the genital structures, pronotum and scutellum, suggest that *T. sherlocki* is related to *T. lenti*; however, peculiar characteristics, such as a reduced hemelytra, red–orange rings on the femora, allow for the description of *T. sherlocki* as a valid species (Papa et al., 2002).

According to Schofield and Galvão (2009), the brasiliensis subcomplex is comprised of nine species that are distributed throughout South America: *T. brasiliensis*, *Triatoma juazeirensis*, *T. lenti*,

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Triatoma melanica, *Triatoma melanocephala*, *Triatoma petrochii*, *T. sherlocki*, *Triatoma tibiamaculata* and *Triatoma vitticeps*. The parameters used to group these species were mainly morphological and geographical distribution. Through the use of cytogenetic data, Alevi et al. (2012a) proposed the exclusion of *T. melanocephala*, *T. tibiamaculata* and *T. vitticeps* from the brasiliensis subcomplex, and Alevi et al. (2012b, 2013a) confirmed the inclusion of *T. lenti*. Mendonça et al. (2009) used analyses of mitochondrial genes and Alevi et al. (2013a) used cytogenetic analysis to support the inclusion of the *T. sherlocki* in the brasiliensis subcomplex.

Experimental crosses between members of the brasiliensis subcomplex (as defined by Costa et al., 2013) revealed hybridization potential and reproductive compatibility under laboratory conditions (Costa et al., 2003; Almeida et al., 2012; Correia et al., 2013).

Human modifications to ecological landscapes may increase epidemiological risks, ease endemic disease emergence, and create new suitable environments for integration and mating between species, which may potentially result in natural hybrids (e.g., *T. infestans* with *Triatoma rubrovaria* (Salvatella et al., 1990).

Because triatomine hybridization (a) allows for the formulation of hypotheses on the origin and divergence of species, (b) may help understand the systematics of the group (Pérez et al., 2005), and (c) has enabled quantitative analyses of taxonomic relationships correlated with degrees of morphological similarities between species (Usinger et al., 1966), the present investigation sought to determine the reproductive compatibility between *T. sherlocki* and *T. lenti* and to use cytogenetic and morphologic approaches to analyze the characteristics of the hybrids from experimental crosses of these species.

2. Methods

2.1. Insects

The specimens used in these crosses were obtained from colonies established for at least six generations with Triatominae from non-overlapping areas. *T. sherlocki* (Fig. 1A) samples used in this study were taken from a colony generated from 26 specimens collected in a wild and rocky upland environment near the village of Santo Inácio in the district of Gentio do Ouro, in the state of Bahia, Brazil, in 2003. *T. lenti* (Fig. 1B) samples are from a colony generated from 23 adult specimens and 5 nymphs collected in a peridomestic environment in the rural village of Macaúbas, Bahia, Brazil, in 2009. Both colonies are kept at the Triatominae Insectarium of São Paulo State University (UNESP, Araraquara/SP, Brazil).

2.2. Experimental crosses

T. sherlocki and *T. lenti* were crossed in both directions: *T. lenti* females × *T. sherlocki* males and *T. sherlocki* females × *T. lenti* males. The insects were sexed as 5th instar nymphs, and males and females were kept separately until they reached the adult stage in order to cross adult virgins (Martínez-Ibarra et al., 2011). For the crosses, 3 couples from each set were placed in plastic jars (5 cm in diameter × 10 cm in height) and kept at room temperature.

The fertility rate and oviposition were calculated from each cross. The eggs were collected daily throughout the females' oviposition periods. Viable F1 offspring were maintained to the adult stage, according to Belisário et al. (2007).

To determine whether the F1 offspring were fertile, F1 × F1 crosses from the same couple were performed in order to obtain F2 offspring. As with the parental experimental crosses, the fertility rate and oviposition were calculated throughout the females' oviposition periods to ascertain the fertility of the F2 offspring.

2.3. Morphologic and morphometric analysis

The phenotypes of the offspring of each couple were described according to the major characteristics that species presented, such as a reduced hemelytra, red–orange rings on the femora and connexive (Papa et al., 2002).

Scanning Electron Microscopy (SEM) was used for the morphological analysis of 10 eggs, and 3 median processes of the

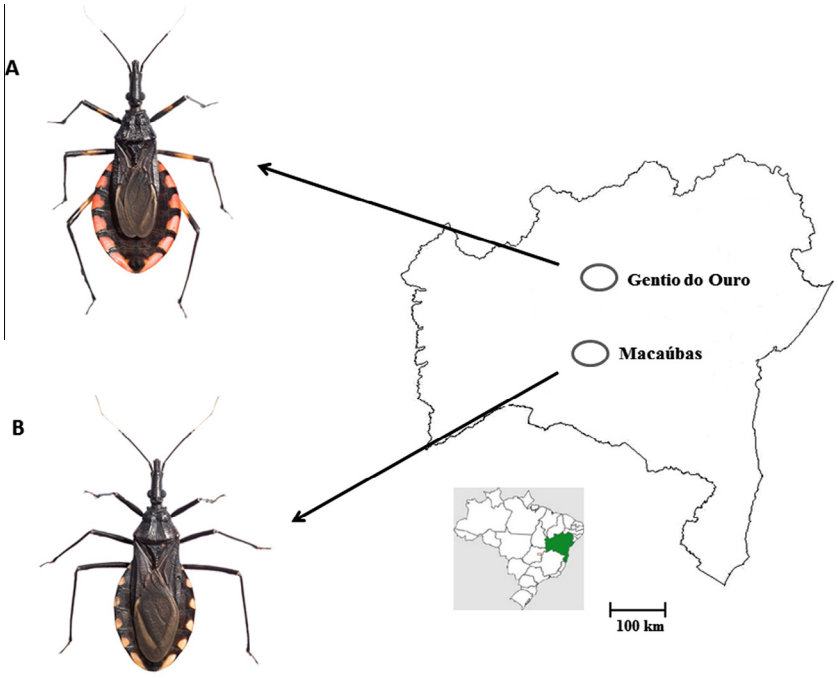

Fig. 1. Distribution of *T. sherlocki* and *T. lenti* in Bahia state, Brazil. *T. sherlocki* (A) is distributed around the region of Gentio do Ouro and *T. lenti* (B) is distributed around the region of Macaúbas.

pygophore of male genitalia were used to make comparisons among the parents and offspring. These images were examined under a Topcon SM-300 microscope at the UNESP Chemical Institute (Instituto de Química, UNESP, Araraquara, Sao Paulo, Brazil). The sample eggs and median processes of the pygophore were washed, dehydrated in an alcohol series, and oven-dried at 50 °C. Sputtering metallization was then performed for 2 min at 10 mA (Rosa et al., 2012).

Geometric morphometric techniques were applied to the wings to evaluate whether the morphotype exhibited by offspring presented any differences in shape compared to those of *T. lenti* and *T. sherlocki* (Almeida et al., 2012; Campos et al., 2011). Thirteen anatomical landmarks (Schachter-Broide et al., 2004) were collected at intersections between venations and processed by the same researcher using modules available from the CLIC (Collection of Landmarks for Identification and Characterization, http://www.mpl.ird.fr/morphometrics/clic/index.html) (Dujardin et al., 2010), and also using the COOWin software (Dujardin, 2004), and the methods described by Dujardin (2008). We identified a total of 11 type I landmarks (venation intersections) and 2 type II landmarks (Bookstein, 1990).

Analyses were computed as non uniform (partial warps) and uniform components, which describe regional and global deformations of the wing architecture (Bookstein, 1991). Prior to the Generalized Procrustes analysis, an isometric estimator of size variation (centroidsize) was calculated as the square root of the sum of the squared distances between the center of the configuration of the landmarks and each individual landmark (Bookstein, 1991). A factorial map was built to illustrate the variation, which resulted from the first and second principal components of the analysis, and which represented 95% of the shape.

2.4. Cytogenetic approaches

Seminiferous tubules of 10 F1 and F2 offspring adult males from the crosses between *T. sherlocki* females × *T. lenti* males were shredded, crushed and fixed in liquid nitrogen on glass slides using lacto-acetic orcein (De Vaio et al., 1985), C-banding (Sumner, 1972), and the Feulgen reaction (Mello and Vidal, 1978). To analyze the genomic affinity between the hybrids, the methodology presented by Techio et al. (2005) was used but with modifications, since the cells were analyzed during diakinesis, and also during metaphase I and II.

3. Results

3.1. Experimental crosses

The fertility rate of the eggs from the cross between *T. sherlocki* females and *T. lenti* males was 65%, while the reciprocal crosses resulted in 7% fertile eggs (Table 1). The rate of mortality before adulthood was 80% in the F1 offspring in both crosses. The fertility rate of the eggs from F1 offspring was greater in the cross between *T. sherlocki* females and *T. lenti* males than in crosses between *T. lenti* females and *T. sherlocki* males, 90% and 25%, respectively. In the crosses between *T. lenti* females and *T. sherlocki* males, only one couple obtained viable eggs.

The fertility of the F2 offspring was observed only from the cross between *T. sherlocki* females and *T. lenti* males. However, the percentage of viable eggs was 2%, and the rate of mortality before adulthood was 100%.

3.2. Morphological and morphometric analysis

Phenotypes of F1 offspring, such as size and color of the connexive, the presence or absence of red–orange rings on the femora, and the size of the hemelytra, were identical in both crossing directions (Fig. 2). The F1 offspring were found to have morphological characteristics (such as color and size of the connexive and the presence of red–orange rings on the femora) that were similar to those of *T. sherlocki*; however, the size of the wings was average (Fig. 2A, B). In

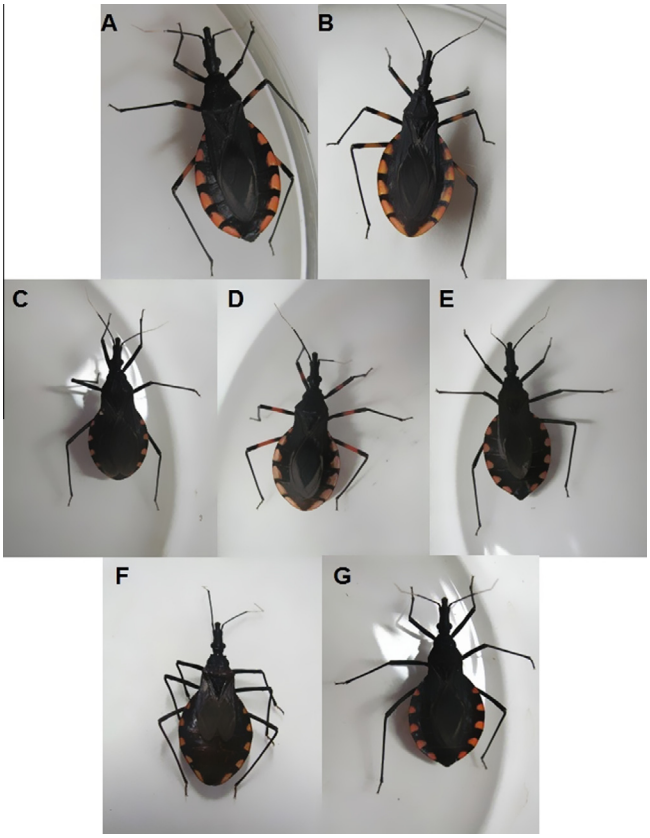

Fig. 2. Phenotypes of F1 and F2 offspring from crosses between *T. sherlocki* females and *T. lenti* males. (A–B) Female and male F1 offspring, respectively. (C–G) F2 offspring adults (C) similar to *T. lenti*; (D) connexive and red–orange rings on the femora similar to those of *T. sherlocki*, and size of hemelytra similar to those of *T. lenti*; (E–F) connexive and absence of red–orange rings on the femora similar to *T. lenti* and reduced hemelytra similar to those of *T. sherlocki*; (G) connexive and absence red–orange rings on the femora similar to *T. lenti* and color of connexive and reduced hemelytra similar to those of *T. sherlocki*.

Table 1
Crosses involving *T. sherlocki* × *T. lenti* showing the percentage of fertile eggs and rate mortality of the offspring until adulthood.

	T. sherlocki females × *T. lenti* males		*T. lenti* females × *T. sherlocki* males	
	Fertile eggs (%)	Mortality (%)	Fertile eggs (%)	Mortality (%)
F1	65	80	7	80
F2	90	90	25	40
F3	2*	100	–	–

* Only three nymphs emerged.

the F2 offspring, these same characteristics resulted in segregating patterns in adults (Fig. 2C–G).

Under Scanning Electron Microscopy (1000X), the exochorion was found to differ between *T. lenti* and *T. sherlocki* (Fig. 3A, C). In the F1 offspring, the characteristics of the exochorion were similar to those presented by the female of the original crossing (Fig. 3B, D).

The male genitalia, which was analyzed using the median process of the pygophore, revealed that the main difference between the two species was a slender point and a wider base in *T. sherlocki* (Fig. 4A) versus a rounded point and a narrower base in *T. lenti* (Fig. 4B). Average characteristics were observed in the F1 offspring, which differed from the parental species (Fig. 4C). In the F2 offspring, the median process of the pygophore was similar to that of *T. sherlocki* in that it revealed segregating characteristics (Fig. 4D). For this analysis, hybrids from cross between a *T. sherlocki* female and a *T. lenti* male were used.

In the case of the geometric morphometrics of the wings, the factorial map built with 40, 34, 43 and 15 specimens of *T. sherlocki*, *T. lenti*, F1 and F2, respectively, distinguished both species and hybrids as well-defined groups (Fig. 5). As for the shape variation components, the contribution of the first principal component (PC1) accounted for 34% of the total variation, whereas the second principal component (PC2) accounted for 14%.

3.3. Cytogenetic approaches

We analyzed the spermatogenesis of F1 offspring (Fig. 6) using the lacto-acetic orcein technique and F2 offspring (Fig. 9) using the Feulgen reaction. These analyses revealed striking differences in the meiotic chromosome behavior of the offspring. In the F1 offspring of both crosses (*T. sherlocki* females × *T. lenti* males and *T. lenti* females and *T. sherlocki* males), the same cytogenetic characteristics were observed (Figs. 6–8).

During the diffuse stage, a heteropyknotic corpuscle was found to be formed by the association between both sex chromosomes and some autosomes (Fig. 6A, arrow). During diplotene, chiasmata were visible in some of the 10 bivalents (Fig. 6B, asterisk). During metaphase I, the 10 autosomal bivalents and 2 sex chromosomes were clearly visible (Fig. 6C), while anaphase I was normal (Fig. 6D). The spermatids presented a heteropyknotic filament on their peripheries (Fig. 6E, F) and the spermatozoan was visible and (Fig. 6G).

C-banding revealed that the hybrids possessed the same diploid chromosome number and arrangement of constitutive heterochromatin as the parental species. During early meiotic prophase, we observed a large chromocenter made up of the association of both sex chromosomes plus two autosomal pairs (arrow), as well as multiple C-dots spread around the nucleus (Fig. 7A). During metaphase I, the 10 autosomal bivalents were found to have C-blocks at one or both chromosomal ends (Fig. 7B).

The Feulgen reaction was used to analyze 100 cells during diplotene (Fig. 8A), metaphase I (Fig. 8B), and metaphase II (Fig. 8C) in order to assess the compatibility between the genomic parental species. All cells showed 100% pairing. However, an analysis of 50 F2 offspring cells during diakinesis/metaphase I revealed that, in 90% of the cells, some errors in the pairing of autosomes occurred and resulted in monovalent chromosomes (Fig. 9A–D). Metaphase II was not visible in F2 offspring. The spermatids presented a heteropyknotic filament on their peripheries (Fig. 10A–C, arrows).

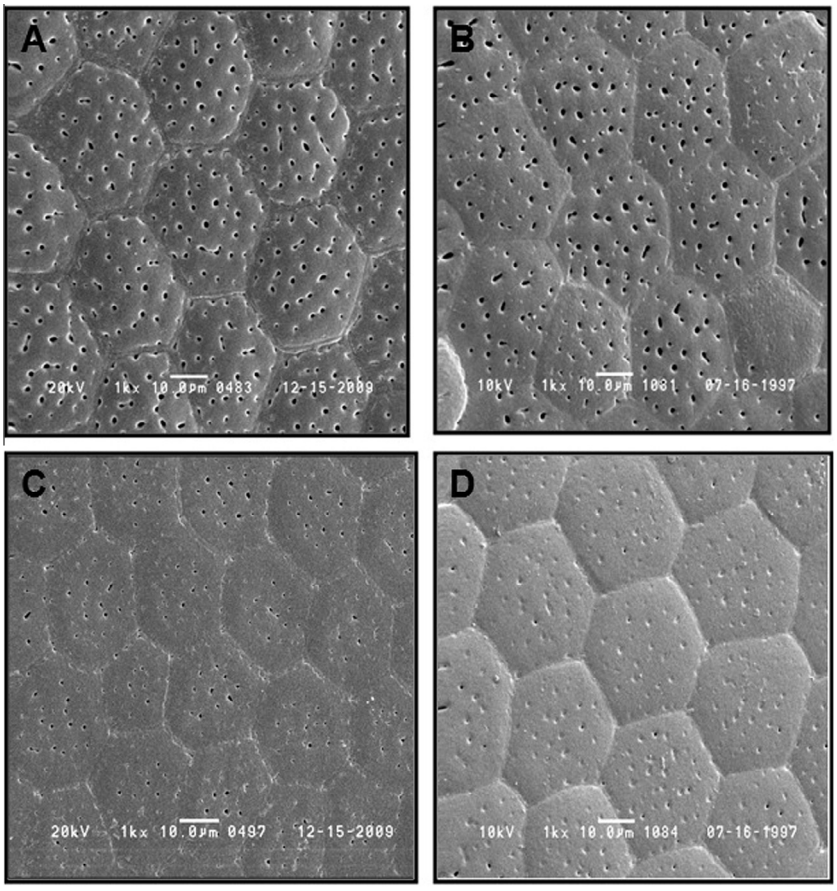

Fig. 3. Egg exochorion detail seen through SEM (1000X). (A) *Triatoma lenti*; (B) F1 offspring from *T. lenti* female × *T. sherlocki* male crossings; (C) *Triatoma sherlocki*; (D) F1 offspring from *T. sherlocki* female × *T. lenti* male crossings.

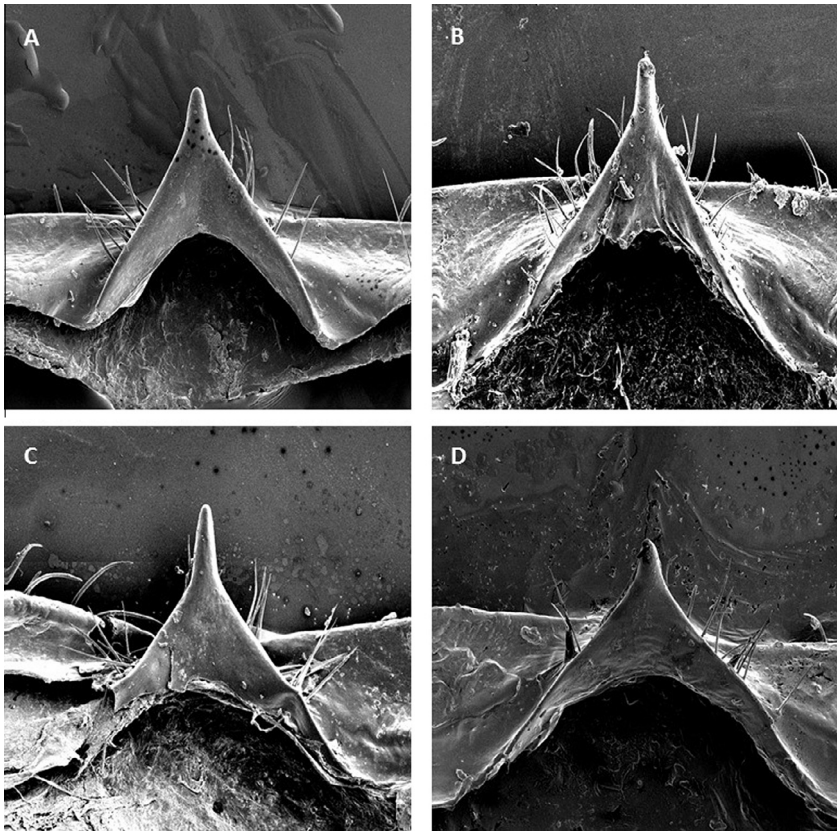

Fig. 4. Median process of the pygophore seen through SEM (100X). (A) *T. sherlocki*; (B) *T. lenti*; (C) F1 offspring; (D) F2 offspring (F1 and F2 offspring from *T. sherlocki* female × *T. lenti* male crossings).

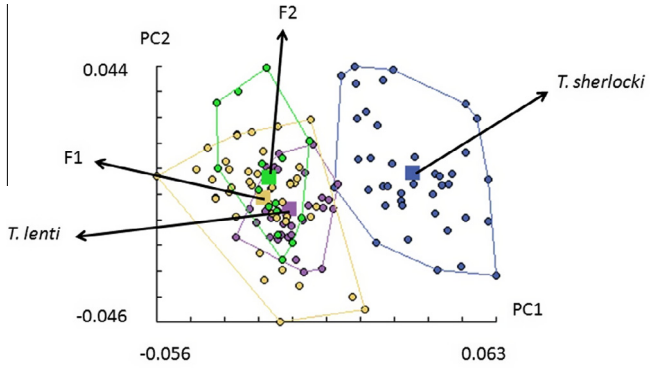

Fig. 5. Factorial map of wing shape. The factorial map of wing shape for *T. sherlocki*, *T. lenti*, and the F1 and F2 offspring.

4. Discussion

Morphological approaches provided important tools in the characterization, identification and description of the species of the brasiliensis subcomplex (Costa et al., 2006; Costa and Felix, 2007), as well as the characterization of hybrid forms (Almeida et al., 2012; Costa et al., 2003, 2013). Based on morphological observations, Costa et al. (2009) proposed that *T. b. macromelasoma* is a hybridization product between *T. b. brasiliensis* and *T. juazeirensis*.

Hybridization between closely related triatomine species is a well-known phenomenon that has been detected both in nature and in experimental laboratory cross-mating (Costa et al., 2003; Mas-Coma and Bargues, 2009; Pérez et al., 2005; Usinger et al., 1966). The subspecies *T. b. brasiliensis* and *T. b. macromelanosoma* and the species *T. juazeirensis* have presented genetic compatibility and generate fertile hybrids in the F1 and F2. However, the ability of hybridization may not be the only factor to be taken into account, since the species *T. melanica* presents genetic incompatibility with brasiliensis subcomplex members and has helped to produce an unviable hybrids (Costa et al., 2003).

Experimental crosses were made between *T. sherlocki* and the species of *T. brasiliensis* complex to confirm *T. sherlocki* as a member of the *T. brasiliensis* complex (Correia et al., 2013). In that study, all experimental combinations between *T. sherlocki* and members of the *T. brasiliensis* complex species produced viable eggs with variable rates of survival (52.3–73.5%), a result which confirms the inclusion of the species in the brasiliensis subcomplex.

Crossing experiments were previously performed by other researchers in order to check reproductive compatibility among the brasiliensis subcomplex members (according to Schofield and Galvão, 2009). In these other studies, *T. petrochii* and *T. lenti* failed to produce F1 viable offspring when they were crossed with *T. brasiliensis* (Espínola, 1971; Heitzmann-Fontenelle, 1984). In studies involving interspecific crosses between *T. lenti* and *T. sherlocki*, Cerqueira (1982) was unable to produce viable hybrids, a finding which contradicts the results presented in this study.

T. sherlocki and *T. lenti* are closely related geographically and ecologically. According to ecological niche modeling, *T. sherlocki* was found to be distributed in the city of Ipupiara (Almeida et al., 2009), a region known for the distribution of *T. lenti* (Sherlock and Serafim, 1967, 1972). Although the specimens used in the experiment were of different origins (*T. lenti* specimens were peridomiciliary and *T. sherlocki* specimens were wild), both species can be found in both ecotypes (Sherlock and Serafim, 1967, 1972; Almeida et al., 2009).

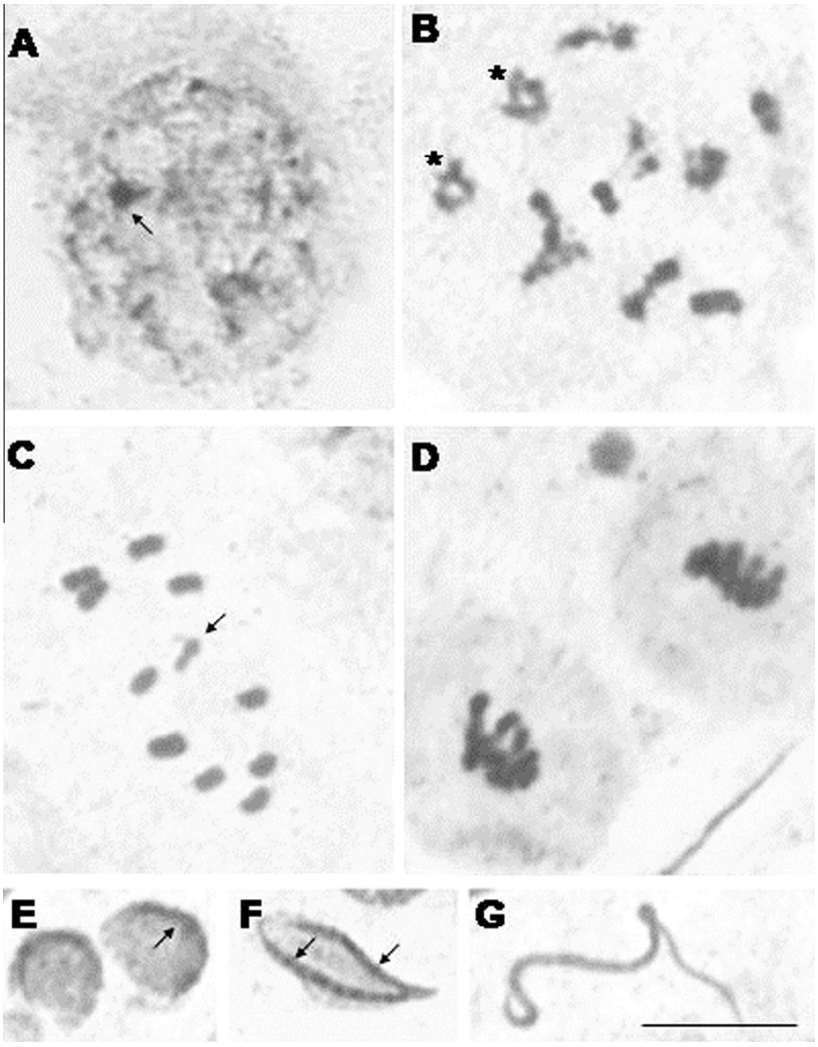

Fig. 6. Spermatogenesis of F1 hybrids stained with lacto-acetic orcein. (A–B) Prophase I. Note heteropyknotic corpuscle in the initial diffuse (A, arrow); during diplotene chiasmus was visible (B, asterisk). (C) Metaphase I. Note that it is possible to observe ten pairs of autosomes and the sex chromosomes, with a larger Y chromosome (arrow). (D) Anaphase. (E–F) Spermiogenesis. Note the heteropyknotic periphery in spermatids (arrows). (G) Spermatozoid. Bar: 10 um.

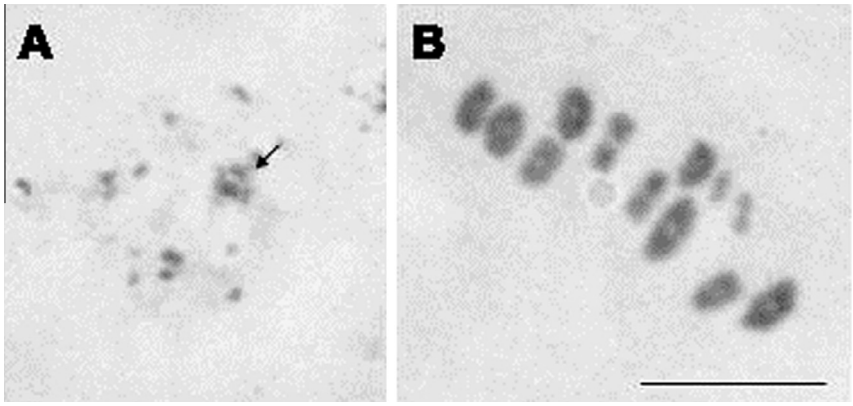

Fig. 7. Constitutive heterochromatin pattern in F1 hybrids stained with C-banding. (A–B) Constitutive heterochromatin pattern in hybrids. Note the chromosome configuration during early meiotic prophase (A), which is a large chromocenter made up of the association of both sex chromosomes plus two autosomal pairs (arrow), and multiple C-dots spread around the nucleus. During metaphase I (B), note the diploid chromosome number consists of 20 autosomes plus two sex chromosomes (XY in males and XX in females) and the amount of autosomal C-heterochromatin (25–32%). Bar: 10 um.

Because the ecological and geographic boundaries between *T. sherlocki* and *T. lenti* are not clear, the possible existence of natural hybrids would be expected. However, due to pre-and post-zygotic reproductive isolation, which has been described in this study, the formation of natural hybrids is unfeasible. According to Mas-Coma and Bargues (2009), *Triatoma delpontei* × *T. infestans* and *Triatoma*

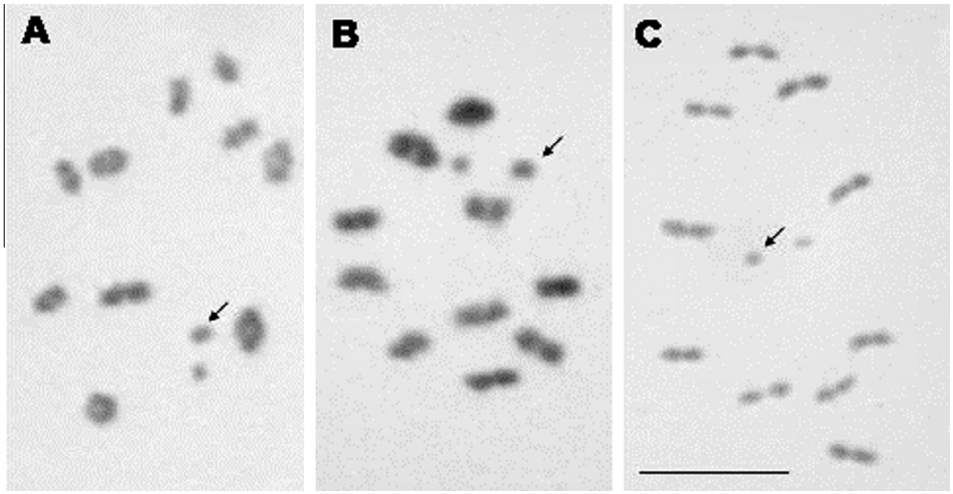

Fig. 8. Meiotic cells of F1 hybrids stained using the Feulgen reaction. (A) Diplotene. (B) Metaphase I. (C) Metaphase II. Note the sex chromosome Y (arrow). Bar: 10 um.

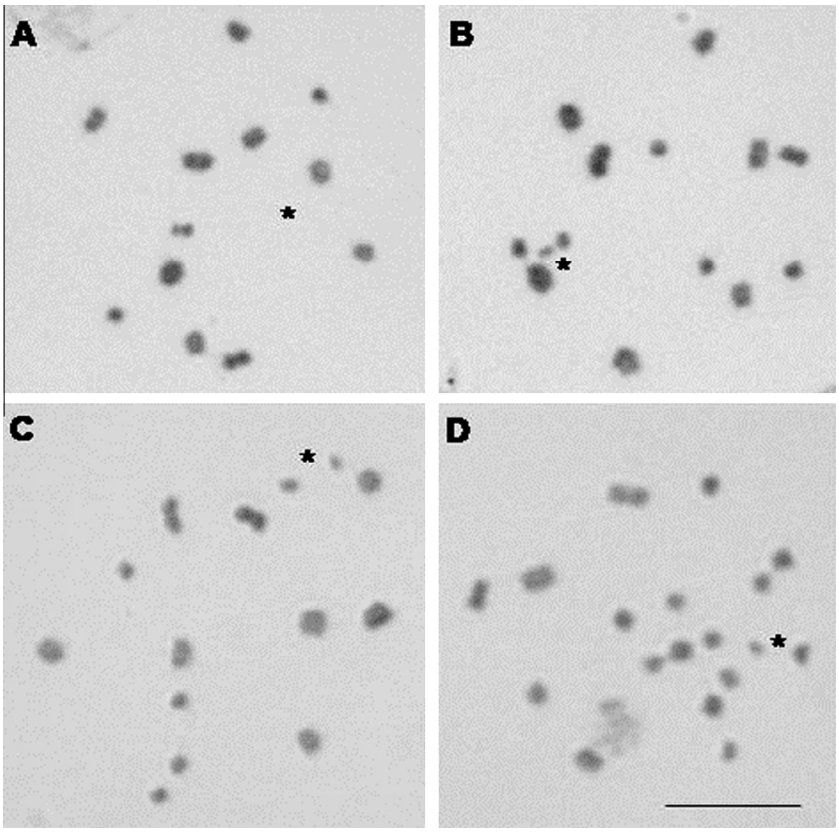

Fig. 9. Diakinesis of the F2 generation using the Feulgen reaction. (A–D) Diakinesis. Note the monovalent autosomes seen through the lack of pairing among homeologous and sex chromosomes (asterisk). Bar: 10 um.

platensis × *T. infestans* hybrids have been detected in natural populations, but it is still unclear if these represent accidental events or if they reflect the existence of large and stable hybrid populations.

Though *T. sherlocki* females × *T. lenti* males resulted in reproductive success, the reciprocal crosses did not show the same pattern. The crosses between *T. lenti* females and *T. sherlocki* males resulted in high mortality and low fertility of eggs in the F1 and F2 offspring. This difference in reproductive success between pairs of couples was also reported by Sasabe et al. (2007), who analyzed the genetic basis of interspecific differences in genital morphology of closely related carabid beetles.

Almeida et al. (2012) reported that laboratory-bred hybrids between *T. sherlocki* and *T. juazeirensis* possess average morphological traits. Average morphological traits were found in the male genitalia and in the median process of the pygophore in the F1 offspring resulting from the crosses between *T. sherlocki* and *T. lenti*. Furthermore, average forms have been observed in nature as crosses between *T. b. brasiliensis*, *T. b. macromelasoma*, and *T. juazeirensis* in the Brazilian state of Pernambuco (Costa et al., 2009).

Morphological features of the F1 offspring from both crossings, such as color and size of the connexion and the presence of red–orange rings on the femora were similar to those of *T. sherlocki*.

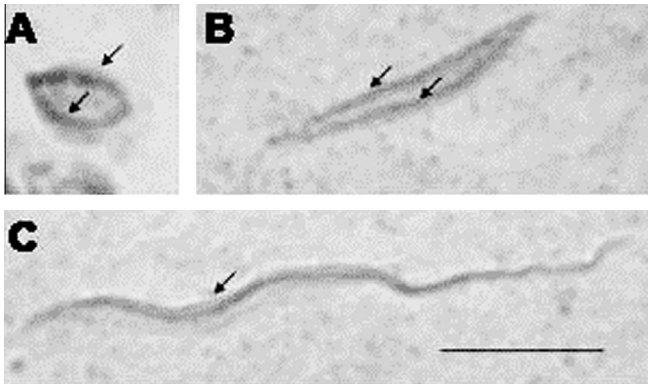

Fig. 10. Spermiogenesis of F2 generation. (A–C) Spermiogenesis. Note the heteropyknotic periphery in spermatids (arrows). Bar: 10 um.

Phenotypes of F1 offspring of crosses between *Meccus phyllosomus* and *Meccus pallidipennis* showed that all F1 specimens were morphologically similar to *M. pallidipennis* (Martínez-Ibarra et al., 2011).

Hybrid fertility and fitness are key parameters in determining the long-term outcome of the mixture of two species. The egg counts suggest that hybrid females are as fertile, at least as far as egg production, as parental populations, a finding which suggests little or no prezygotic isolation (Jiggins and Mallet, 2000; Barton and Cara, 2009). However, analyses of genotype frequencies of the different sexes and developmental stages strongly suggest the disappearance of hybrid females as they age, possibly because of increased mortality. A lack of hybrid fitness may thus lead to a postzygotic barrier that allows for the maintenance of reproductive isolation of parental genotypes (Wiwegweaw et al., 2009). The presence of recombinants between parental genotypes suggests that gene flow does occur between sibling species, but that selection processes act to maintain their distinctiveness.

The F1 offspring presented normal meiotic behavior. Pérez et al. (2005) also observed that, in hybrids resulting from the species *T. infestans* and *T. platensis*, meiotic division occurred normally. However, in experimental hybrids between *T. infestans* and *T. rubrovaria*, Pérez et al. (2005) reported sterility of F1 offspring in pairing failures among homeologous chromosomes, which led to the production of abnormal spermatids. An analysis of spermiogenesis revealed that the spermatids present the same characteristics outlined by Alevi et al. (2013b) in his description of *T. lenti*, including peripheral heteropyknotic filaments.

The parental species, *T. lenti* and *T. sherlocki*, present the same pattern of constitutive heterochromatin in the autosomes, as do all other species that are part of the brasiliensis subcomplex (Alevi et al. (2013a)). Alevi et al. (2013c) analyzed the heterochromatic pattern and confirmed the involvement of *T. lenti* in the brasiliensis subcomplex. Experimental crosses are considered to be a tool to evaluate the proximity found among species and, therefore, can be used to suggest whether a species is a member of a given group. However, we emphasize that, to confirm the true position of *T. lenti* in the complex, many other approaches must be used, especially since *T. melanica* presents genetic incompatibility with the triatomines of the complex and is still considered a member (Costa et al., 2003).

Pérez et al. (2005) analyzed hybrids resulting from a cross between evolutionarily related species and proposed that differences in the patterns of constitutive heterochromatin between homologous chromosomes in parental species are not a barrier that influences synaptic recombination. Thus, because the parents do not possess differences in heterochromatic patterns, F1 hybrids retained the same characteristics as *T. lenti* and *T. sherlocki*; namely, the location of the autosomal C-blocks (at one or both chromosomal ends in all autosomal pairs), the diploid chromosome number consisting of 20 autosomes plus two sex chromosomes (XY in males and XX in females) and the amount of autosomal C-heterochromatin (25–32%) (Panzera et al., 2010; Alevi et al., 2013a).

Like *T. lenti* (Alevi et al., 2013a) and *T. sherlocki* (Panzera et al., 2010), the F1 offspring were found to have diploid chromosome sets of $2n = 20A + XY$. Furthermore, we observed that homologous chromosomes presented 100% homeology. This result allows us to measure phylogenetic proximity between *T. lenti* and *T. sherlocki* since, according to Dewey (1982), the classical analysis of genomics which involves evaluating the behavior of chromosomes in metaphase I in interspecific hybrids, allows for the establishment of phylogenetic relationships in groups of different species, and can also be employed in the definition of taxonomic and evolutionary placements. Similar statements have been made by Riley (1966), who has affirmed that the two species have distinct genomes when their chromosomes are different in structure and gene content, so that no pairing occurs between one or more pairs of homeologous during meiosis of hybrids. This behavior leads to sterility, and, consequently, to the genetic isolation between species.

Thus, based only on the analysis of the F1 offspring derived from the crossing experiment, we propose an early homoploid speciation. Costa et al. (2009), performed morphological analyses and have suggested that *T. b. macromelanosoma* is a species derived from crosses between the species *T. b. brasiliensis* and *T. juazeirensis*. However, when we analyzed the F2 offspring, we found that other evolutionary barriers may be preventing hybridization.

The analysis of the F2 offspring revealed errors in chromosome pairing. These results are possibly related to one mechanism of post-zygotic isolation proposed by Dobzhansky (1970): the collapse of the hybrid. The collapse is a little-known phenomenon in which the F2 offspring or backcross has reduced viability or fertility, and was observed in our study in the amount of F3 offspring. We believe that this phenomenon is due to the genetic dysregulation that occurred in the chromosomes of F1. This imbalance is likely the result of crossing over that occurs in the homeologous chromosomes of the F1 offspring, which results in a lack of homology in euchromatic regions and prevents the normal pairing of chromosomes of some in F2 offspring.

Thus, in the F2 offspring, although some cells analyzed (10%) were normal (they presented 100% homeology between autosomes), most of the cells were abnormal and there was no homeology among autosomes. These factors resulted in monovalent autosomes. This phenomenon leads to the formation of unviable sperm. However, there were no significant changes in haploid cells during spermiogenesis, a finding which was also reported by Schreiber et al. (1975).

Studies the genetics and ecology of *T. sherlocki* and *T. lenti* are still in the initial stages, and there are few published works on these two species. It would therefore be reasonable to initiate quantitative trait locus (QTL) mapping, which provides fundamental information such as the number of loci involved in a given trait (morphological or behavioral), locations on chromosomes, and the magnitude of individual genetic effects. Thus, we believe that *T. lenti* is species of the *T. brasiliensis* complex, although new analysis are needed to confirm this initial finding. Furthermore, it can be seen that, although *T. sherlocki* and *T. lenti* species are evolutionarily and citotaxonomically closed, there is a post-zygotic reproductive barrier that reduces rates of fertility in the F2 offspring. These factors confirm the specific status of species analyzed.

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