



Research paper

Phenotypic variability confirmed by nuclear ribosomal DNA suggests a possible natural hybrid zone of *Triatoma brasiliensis* species complex



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ABSTRACT

Triatoma brasiliensis macromelasoma occurs in Pernambuco state, Brazil, which is situated between the distribution areas of *Triatoma brasiliensis brasiliensis* (north) and *Triatoma juazeirensis* (south). *T. b. macromelasoma* displays greater variations in its chromatic phenotype than either *T. b. brasiliensis* or *T. juazeirensis*, and patterns reminiscent of one or the other. Experimental crosses from each of these members of the *T. brasiliensis* species complex generated fertile offspring suggesting that viable hybrids could be present in nature, despite their significant genetic distances. Considering the geographical position of occurrence of the *T. b. macromelasoma* (in Pernambuco) it was proposed to be an area capable of supporting natural hybridization between *T. b. brasiliensis* and *T. juazeirensis*. Since phenotypic variability is expected, this study investigated the existence of intermediate chromatic phenotypes for *T. b. macromelasoma* in various locations in areas between the *T. b. brasiliensis* and *T. juazeirensis* occurrences. Thirteen different color patterns were for the first time characterized and nine of those displayed intermediate phenotypes. Molecular analysis performed using ribosomal DNA intergenic region, grouped all within the *T. brasiliensis* complex. The intermediate chromatic phenotypes, molecular analysis and experimental crosses all support the distinction of a zone of hybridization that gave rise to the *T. b. macromelasoma* through homoploid evolution.

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1. Introduction

The origin and significance of phenotypic variations within groups of insects is not well understood despite numerous references in the literature. In the Triatominae subfamily, several species have been described with wide variations including: *Triatoma rubrovaria* (Blanchard, 1843), *Triatoma pseudomaculata* Corrêa and Espínola,

1964, *Panstrongylus geniculatus* Latreille, 1811 and *Triatoma dimidiata* (Latreille, 1811). These variations in appearance can underscore multiple changes in the taxonomic status (see Lent and Wygodzinsky, 1979, Almeida et al., 2002). The case of *Triatoma infestans melanosoma* Martínez, Olmedo and Carcavallo, 1987, is illustrative. It was initially considered to belong to one species (Lent and Wygodzinsky, 1979), but was later validated as a separate species, *Triatoma melanosoma*, based only on color patterns (Lent et al., 1994). In the argumentation, the genital structures were not retained since *Triatoma infestans* (Klug, 1834) is known to exhibit intraspecific variations (Pires et al., 1995). Subsequently, an analysis of mitochondrial DNA sequences using the Cyt B gene showed no differences between them suggesting that *T. melanosoma* was indeed a dark morphotype of *T. infestans* (Monteiro et al., 1999). Additionally, geometric morphometrics analyses synonymized *T. melanosoma*, confirming that it was a dark morphotype of *T. infestans*, since size and shape

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variations were of the same order as geographic variation in *T. infestans*. This finding was in complete agreement with the molecular analyses previously carried out (Gumiel et al., 2003).

The lack of research on the distribution and characterization of the chromatic variations of *T. b. brasiliensis* Neiva, 1911, as mentioned by Lent and Wygodzinsky (1979), has led to multidisciplinary approaches that examined geographic distributional patterns, morphological, biological, ecological and molecular biology aspects and geometric morphometrics (Costa et al. 1997a,b, 1998, 2002, 2003a, b, 2009, 2014; Monteiro et al., 2004; Neiva-Lima et al., 2012, Correia et al., 2013). Those approaches revealed that the distinct chromatic variations mentioned by Lent and Wygodzinsky (1979) were indeed members of the *T. brasiliensis* species complex which is currently composed of three species and two subspecies. Here we summarize the taxonomic updates of the five members of the group according to Costa and Felix (2007) and Costa et al. (2006, 2013): *T. b. brasiliensis* Neiva 1911, *T. b. macromelasoma* Galvão, 1956, *Triatoma melanica* Neiva & Lent, 1941, *T. juazeirensis* Costa and Felix, 2007 and *T. sherlocki* Papa et al., 2002.

The use of multidisciplinary approaches has improved the classification of the Triatominae group and also of the insects in general. Classifications independent of morphology provided an opportunity to study the phenotypic variability and polymorphisms within a species of insects and to examine the evolutionary process (Gumiel et al., 2003; Pacheco et al., 2003, 2007; Adams et al., 2009; Costa et al., 2013). As a result, it was suggested that *T. b. macromelasoma*, arose through homoploid hybridization. This hypothesis was proposed based on color patterns and morphological traits such as egg size and wing shape (Costa et al., 2009). The hypothesis was further supported by the study of distribution patterns and ecological modeling (Costa et al., 2002, 2014). While the available molecular data (Costa et al., 1997b; Monteiro et al., 2004) was not conclusive, it did not refute the possibility of hybrid speciation. This scenario suggests that *T. b. macromelasoma* was the product of crosses between *T. b. brasiliensis* and *T. juazeirensis* within sites located in the state of Pernambuco (PE), which covers an area between the distributions of *T. b. brasiliensis* (in the north) and *T. juazeirensis* (in the south) and encompasses the natural range of *T. b. macromelasoma* (Costa et al., 2009, 2014).

Given the importance of members of the *T. brasiliensis* complex in the occurrence of Chagas disease (Silveira and Vinhaes, 1999; Costa et al., 2003a; Almeida et al., 2008), ribosomal DNA (rDNA) characterization and verification of phenotypic variability are important aspects for the precise identification of the species vectors bringing support for the more accurate measures for disease control. Furthermore, domiciles in the state of PE exhibit a high incidence of infestation by several species of triatomines (Costa et al., 2003a) making the state of PE an area strategically located to better understand the phenotypic variability of the members of the *T. brasiliensis* complex. Here we present the analysis of field collections of triatomines from various locations within the state of PE using a multidisciplinary approach. Despite the relative genetic distances between the proposed parental species of *T. b. macromelasoma*, the results of Costa et al. (2009) are consistent with the observed laboratory hybridization of *T. b. brasiliensis* and *T. juazeirensis* (Costa et al., 2003b) and suggest the possibility that *T. b. macromelasoma* evolved through homoploid hybridization.

The present paper investigates the existence of intermediate chromatic phenotypes for *T. b. macromelasoma* collected from natural and artificial ecotopes in various locations between the distributions of *T. b. brasiliensis* and *T. juazeirensis* and examines their relationships with genetic variation, based on the complete rDNA region including ITS-1, 5.8S and ITS-2.

2. Materials and methods

2.1. Collection of specimens

Collections were carried out in fifteen counties that were chosen according to the records of domicile infestations in the state of PE

from the Secretary of Health Surveillance (SVS). Collection points were distributed within three geographical areas: of “Sertão do Araripe”, “Sertão do São Francisco” and “Agreste”. Fig. 1 shows the geographical spots where bugs were collected, along with the number of phenotypes and number of specimens collected at each collection point. Collections were performed individually during the day with the aid of forceps and each captured triatomine was placed into small vials containing filter paper. In some instances, Pirisa (5 mL/500 mL water) was utilized to dislodge insects from inaccessible spaces. Home environments, peridomestic spaces and sylvatic areas were searched using the same procedures described by Costa et al. (1998). Part of the collected specimens were identified and deposited in the Entomological Collection of Instituto Oswaldo Cruz, in the section Lima-Neiva & Costa of Triatominae collection and the remaining were kept in alcohol for additional studies.

2.2. Identification and characterization

Only adult insects were examined alive on a stereoscopic microscope (Zeiss Stemi SV6) and the specimens were categorized according to the standards proposed by Lent and Wygodzinsky (1979), Galvão (1956), and Costa et al. (2013). All observed patterns were photographed and included into a Table 1 of color variations based on the general color of the insect, as well as the bands on the prothorax and legs, independently from the location of collection. Latter, the patterns of coloration were linked to the sites of collection (Supplementary Fig. 1).

2.3. Phenetic analyses

The chromatic variation was converted into a presence-absence table using characters related to the colors and their observed patterns. This table allowed for a quantitative estimation of phenetic distances between color patterns, as derived from the following similarity indexes: the Jaccard, the Hamann, the Rogers–Tanimoto and the Sokal–Michener indexes. Binary distances were then tested for their correlation with corresponding evolutionary distances (Tajima and Nei, 1984) using a Mantel test (10,000 iterations). A descriptive test of hybridization hypothesis was undertaken by examining the chromatic phenotypes in relation to various locations between the areas of distribution of *T. b. brasiliensis* and *T. juazeirensis*.

2.4. rDNA sequence analyses

The complete sequences of ITS-1, 5.8S and ITS-2 comprising the whole intergenic region of rDNA of 28 specimens representing all members and different chromatic patterns of the *T. brasiliensis* complex were analyzed (Table 2). Two legs for representative specimens of the main phenotypes were used and processed individually for DNA extraction using methods outlined before (Bargues et al., 2006). This intergenic region was amplified using primers Lim1657 and 28T, designed in conserved positions of 18S and 28S rRNA genes, previously described (Bargues and Mas-Coma, 1997; Marcilla et al., 2001; Bargues et al., 2006). Amplification procedures and thermal cycler conditions were carried out in a Mastercycler EP gradient (Eppendorf, Hamburg, Germany), by 30 cycles of 30 s at 94 °C, 30 s at 50–55 °C and 1 min at 72 °C, preceded by 30 s at 94 °C and followed by 7 min at 72 °C. Ten µl of each PCR product were checked by staining with ethidium bromide on 1% Nusieve® GTG agarose (FMC) gel electrophoresis, using the Molecular Weight Marker VI (Boehringer Mannheim) at 0.1 µg DNA/µl as control. Primers and nucleotides were removed from PCR products by purification using Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) according to the manufacturer's protocol and resuspended in 50 µl of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm. The sequencing of the complete intergenic region was performed on both strands by the dideoxy chain-termination method. It was carried out with the Taq dye-terminator

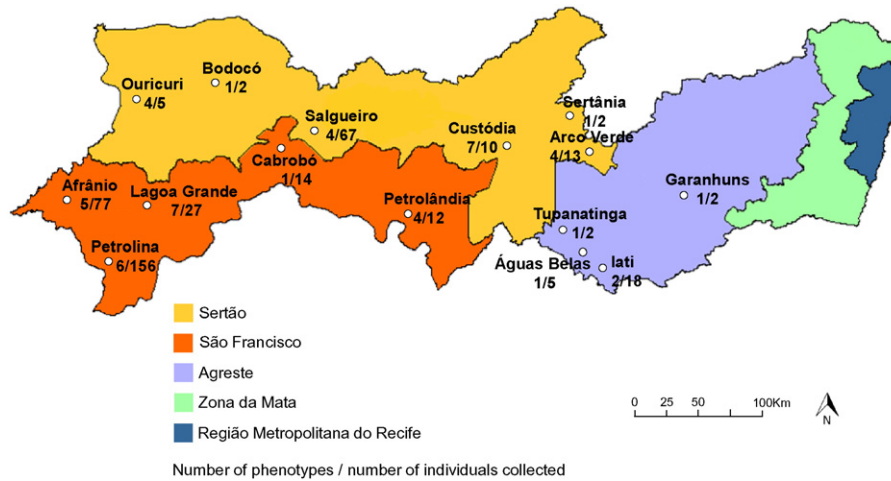


Fig. 1. Map of the state of Pernambuco, Brazil. The state is divided into the general geographical areas that represent different ecotopes and the specific locations where collections were performed. Source: www.pernambuco.gov.br. Number of phenotypes/number of specimens collected in each locality is reported in parentheses.

chemistry kit for the sequencers ABI 3130 and ABI 3700 (Applied Biosystems, CA, USA), using PCR primers. Given the importance of the recent discovery of a pseudogenic 5.8S + ITS-2 sequence, designed as “ps(5.8S + ITS-2)”, widely distributed in triatomines of North, Central and South America (Bargues et al., 2014), special efforts were made to ensure that no double signal was present in the chromatograms, in the way to confirm that variable positions in the intergenic region were not due to an underlying paralogous sequence. The haplotype (H) terminology used for both ITS follows the nomenclature for composite haplotyping (CH) previously proposed (Bargues et al., 2006; Mas-Coma and Bargues, 2009). Accordingly, ITS-2 haplotypes are noted by numbers and ITS-1 haplotypes are noted by capital letters.

2.5. Sequence alignment, length and AT-content and similarity/identity matrix

Sequences were aligned with CLUSTAL W2 (Larkin et al., 2007), using default parameters, including corresponding penalties for gaps in pairwise and multiple alignments. The NCBI BLAST (Basic Local Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used for sequence comparison against the GenBank database. Length and AT-content of each one of the markers including in the complete intergenic rDNA region was determined using MEGA 5.2 program (Tamura et al., 2011). Sequence divergence within and among different ITS-1 and ITS-2 sequence types were measured using total character differences, including all possible changes, transitions (ts), transversions (tv) and insertions/deletions (indels) as character states in PAUP 4.0b10 (Swofford, 2002). The MatGAT (Matrix Global Alignment Tool) software has been used to calculate identity/similarity between every pair of sequences using the Myers and Miller global alignment algorithm (Myers and Miller, 1988).

2.6. Phylogenetic and network analyses

A phylogenetic network estimation using median-joining or MJ network algorithm using default parameters (equal character weight = 10, transitions/transversions weight = 1:1, and connection cost as a criterion) was constructed, for the ITS-1 and ITS-2 combined haplotypes, using Network 6.4.1.1 (<http://www.fluxus-engineering.com/>). Hypothetical median vectors were added to the network for shortest connection between the data set. Phylogenetic trees were inferred using the Maximum Likelihood (ML) and distance-based (Neighbor Joining) (NJ) methods implemented in PAUP 4. ML parameters and the evolutionary model best fitting our dataset were determined using Akaike information criteria (AIC) (Akaike, 1974; Posada and Buckley, 2004),

implemented in jModeltest version 0.1.1 (Posada, 2008). The evolutionary distances were computed using the Tajima–Nei method (Tajima and Nei, 1984). Starting branch lengths were obtained using the least-squares method with ML distances. To provide an assessment of the reliability of the nodes in trees, statistical support was evaluated with 1000 bootstrap pseudoreplicates, using NJ search, with fast stepwise pseudoreplicates (random sequence addition replication) and heuristic search with nearest-neighbor-interchange (NNI) approach.

3. Results

3.1. Collected specimens

A total of 412 triatomine specimens were collected with 286 from the area “Sertão do São Francisco”, 99 from the region of “Sertão do Araripe” e and 27 from the “Agreste” (Fig. 1).

3.2. Chromatic phenotypes for *T. b. macromelasoma* and members of the *T. brasiliensis* complex collected in PE

The triatomine specimens displayed numerous color variations, especially in the thorax and legs. The general color of the body, along with the color and specific patterns on the prothorax and legs, were used to categorize the specimens into thirteen color patterns independently from their collection site. Each of the thirteen patterns was assigned names, which refer to the color characteristics and the species of the *T. brasiliensis* complex it most closely resembles. Two of the chromatic patterns are similar to those of *T. juazeirensis* and *T. b. macromelasoma*, nine patterns were consistent with patterns intermediate to members of the *T. brasiliensis* complex and two patterns showed no similarities with members of the *T. brasiliensis* complex or an intermediate pattern. A description of each color pattern is presented in Table 1.

Fig. 2a shows representative images of examples for *T. b. brasiliensis*, *T. b. macromelasoma* and *T. juazeirensis*, members of the *T. brasiliensis* complex. Examples of ten of the determined thirteen color patterns are shown in Fig. 2b. The predominant feature observed for *T. b. brasiliensis* was the presence of a ring on the appendages, which was used to determine the categories that were intermediate. The intermediates of *T. b. macromelasoma* used the shape of the longitudinal stripe on the pronotum as an indicator. For *T. juazeirensis*, the general dark color pattern was consistent with intermediate patterns.

After the determination of the various color patterns, the collection of triatomines was tabulated based on collection site and the chromatic phenotype (Supplementary Fig. 1). From the data, the total number of

Table 1

Different phenotypes of *Triatoma brasiliensis* collected in Pernambuco state, Brazil. Descriptions are based on the general color of the insect, as well as the bands on the prothorax and legs.

Color pattern	Description
Juaz	General color pattern of black with a black pronotum and black appendages; as described by Costa and Felix (2007)
Mac/juaz	General color pattern of black with a black pronotum and appendages marked with clear spots that do not form rings; or General color pattern of black with a pronotum marked with narrow yellow bands reaching the anterior lobe, but not past the posterior margin of the posterior lobe. The appendages are black;
Mac	General color pattern of a dark yellow with a pronotum marked with narrow bands that extend to the anterior lobe, but not a part of the posterior margin of the posterior lobe. The appendages have pale spots that do not form distinct rings;
Mac/bn	General color pattern of brown with a pronotum marked with narrow yellow bands reaching the anterior lobe, but not past the posterior margin of the posterior lobe. The appendages have clear spots that do not form rings; or General color pattern of brown with a brown pronotum and appendages marked with clear spots that do not form rings;
Bras/mac	General color pattern of dark yellow with a pronotum marked with yellow longitudinal bands extending outward from the median carinae in the posterior margin of the posterior lobe to the closed region of the anterior lobe. The appendages have pale spots that do not form distinct rings; or General color pattern of dark yellow with a pronotum marked with narrow yellow longitudinal bands reaching the anterior lobe, but not parting the posterior margin. The appendages have bright spots that form a sharp ring;
Dark bras	General color pattern of black with a pronotum marked with yellow longitudinal bands extending from the carinae at the posterior margin of the posterior lobe until the closed anterior lobe. Appendages have a sharp ring in the middle;
Brown	General color pattern of brown with a light brown pronotum and light brown appendages;
Juaz mcfp	General color pattern of black with a pronotum displaying small pale spots on the prothorax. The appendages are black;
Light brown	General color pattern of light brown with a light brown pronotum and light brown appendages;
Bn cc	General color pattern of brown with a brown pronotum, but a carinae without color. The appendages are brown;
Bras/bn	General color pattern of brown with a pronotum marked with yellow longitudinal bands extending from the carinae at the posterior margin of the posterior lobe until the closed anterior lobe. Appendages have a sharp ring in the middle;
Mac/lt.	General color pattern of light brown with a pronotum marked with narrow yellow bands reaching the anterior lobe, but not past the posterior margin of the posterior lobe. The appendages are marked with clear spots that do not form rings;
bn	General color pattern of black with a pronotum marked with yellow longitudinal bands extending from the median carinae of the posterior margin of the posterior lobe until the closed region of the anterior lobe. The appendages are black.

Juaz.: form similar to *T. juazeirensis*; mac/juaz.: form intermediate between *T. b. macromelasoma* and *T. juazeirensis*; mac.: form similar to *T. b. macromelasoma*; mac/bn.: form intermediate between the *T. macromelasoma* and the Brown form; bras/mac.: form intermediate between *T. b. brasiliensis* and *T. macromelasoma*; dark/bras.: Intermediate form of dark *T. b. brasiliensis*; bn.: Brown; juaz mcfp.: form similar to *T. juazeirensis* with thin pale spots on prothorax; lt. bn.: Light Brown; bn.cc.: Brown with a clear carinae; bras/bn.: Intermediate form between *T. b. brasiliensis* and the Brown form; mac/lt.bn.: form intermediate between *T. macromelasoma* with Light Brown; bras/juaz.: form intermediate between *T. b. brasiliensis* and *T. juazeirensis*.

triatomines for each chromatic phenotype was plotted based on the geographical region of its collection (Supplementary Fig. 2). The greatest number of triatomines was collected within the São Francisco region consisting predominantly of the *mac/juaz* phenotype followed in decreasing order by the *mac*, *juaz* and *mac/bn* chromatic patterns. The region of Sertão Araripe yielded a greater number of *mac/bn*. The least number of specimens were collected in the Agreste region consisting of only the dark bras and bras/mac phenotypes.

The distribution of triatomines within each area was not uniform. In the São Francisco regions, greater one half of the collections occurred around the Petrolina metropolitan area and together with the Afranio

area accounted for 81% of the samples collected. Of those, 55% displayed chromatic characteristics reminiscent of *T. juazeirensis*, which is consistent with their proximity to the northern boundary of the distribution of *T. juazeirensis*. In the north of Pernambuco, the preponderance of collection came from around the Salgueiro metropolitan area and 88% were observed with patterns consisting of a brown phenotype. All of the triatomines collected in the Agreste region displayed chromatic patterns similar to *T. b. brasiliensis*.

3.3. Ribosomal DNA sequence analyses

3.3.1. Complete intergenic rDNA sequence

The complete intergenic region revealed the existence of 16 combined haplotypes (CH) (Table 2). Their length and AT content ranged between 1468 and 1475 bp (mean, 1471.61 bp) and 65.10% and 65.36% (65.23%), respectively. The alignment of the 16 CH generated a 1475 bp dataset which contained 33 variable positions (2.23%) of which 7 were singleton sites (0.47%), 15 parsimony informative positions (1.17%) and 11 indels (0.74%) (Table 3). The 5.8S gene had a length of 155 bp and an AT content of 41.94% and was identical in all specimens analyzed. A similarity/identity matrix was generated for the calculation of the similarity and identity between every pair of sequences in the data set including 16 CH. Maximum similarities/identities (99.9%) were obtained between CH of *T. b. brasiliensis* and *T. b. macromelasoma* while the less similarity were obtained between *T. melanica* and *T. b. brasiliensis*-3A (98.8%); *T. melanica* and *T. juazeirensis* (98.8%) and *T. b. brasiliensis* vs *T. melanica*, *T. sherlocki* or *T. juazeirensis* (average, 98.6%) (Supplementary Table 1).

3.4. First internal transcribed spacer ITS-1

A total of 14 different ITS-1 haplotypes were detected among the samples analyzed and codified as ITS1-A to ITS1-N. Length and AT content ranged between 836 and 837 bp (836.9 bp) and 61.89%–62.20% (62.04%), respectively. Nine of them correspond to *T. b. brasiliensis* and *T. b. macromelasoma* (haplotypes ITS1-A to ITS1-I) (Tables 2, 3). Seven polymorphic sites (0.84%) appear in the alignment of *T. b. brasiliensis* (ITS1-A,B,C) and *T. b. macromelasoma* (ITS1-A,D,E,F,G,H,I) haplotypes. These nine haplotypes representing both subspecies, show a range of 1–4 (average of 2.1) nucleotide differences in their respective pairwise sequence comparisons. Haplotype ITS1-J was detected in samples of *T. melanica*; haplotypes ITS1-K and ITS1-L in samples of *T. sherlocki* and haplotypes ITS1-M and ITS1-N in samples of *T. juazeirensis* (Tables 2, 3). Haplotype ITS1-J differ in only one mutation in comparison with haplotypes ITS1-K and ITS1-L (positions 229 and 808, respectively of their respective alignment – Table 3). Supplementary Table A and Supplementary Table B show pairwise distances between all sequenced specimens of *T. brasiliensis* members by using combined (ITS-1 + ITS-2) haplotypes.

In the 837 bp-long alignment including the 14 ITS-1 haplotypes representing the members of the *T. brasiliensis* complex analyzed, 22 variable positions appeared (2.63%) of which, 21 were substitutions (2.51%), including 15 parsimony-informative positions and 6 singleton sites, and one (0.12%) gapped position (Table 3).

Sequence analysis comparison allows the distinction of three ITS-1 haplogroups corresponding to *T. b. brasiliensis* + *T. b. macromelasoma*; *T. melanica* + *T. sherlocki*; and *T. juazeirensis*. Average number of absolute differences and p-distances between these haplogroups ranged between 7.61 and 12.16 (mean, 9.49) and 0.009 and 0.015 (0.011), respectively. Total number of absolute differences ranged between 1 to 13 (average 5.76) between all members of this complex.

The presence of a very large minisatellite of 193 bp long was detected tandemly repeated in all samples of the *T. brasiliensis* complex. This minisatellite area is of 386 bp long including two repeats of the minisatellite, located respectively between positions 213 and 405 and 406 and 598, of the alignment including all ITS-1 haplotypes described. The two repetitions of this long minisatellite, are not identical in all

Table 2
Geographic localities of the *T. brasiliensis* species complex analyzed, haplotypes obtained for rDNA ITS-1 and ITS-2 markers, and corresponding GenBank accession numbers.

Species	State	Municipality	Coordinates	Color pattern*	ITS-1 haplotype	Long (bp)	AT (%)	ITS-2 haplotype	Long (bp)	AT (%)	Combined haplotype	Long (bp)	AT (%)	ITS-1, 5.8S, ITS-2 GenBank Acc. No.
<i>T. brasiliensis</i> (related)	Pernambuco	Salgadinho	07° 56' 09" S 35° 37' 58" W	Bras/mac	ITS1-A	837	61.89	ITS2-2	478	78.24	CH2A	1470	65.10	KJ125137
<i>T. brasiliensis</i> (related)	Pernambuco	Salgadinho	07° 56' 09" S 35° 37' 58" W	Mac/br	ITS1-A	837	61.89	ITS2-1	480	78.33	CH1A	1472	65.15	KJ125136
<i>T. brasiliensis</i> (related)	Pernambuco	Garanhuns	08° 53' 25" S 36° 29' 34" W	Mac/juaz	ITS1-C	837	62.01	ITS2-2	478	78.24	CH2C	1470	65.17	KJ125140
<i>T. brasiliensis</i> (related)	Pernambuco	Garanhuns	08° 53' 25" S 36° 29' 34" O	Bras/dark	ITS1-C	837	62.01	ITS2-2	478	78.24	CH2C	1470	65.17	KJ125140
<i>T. brasiliensis</i> (related)	Pernambuco	Garanhuns	08° 53' 25" S 36° 29' 34" w	Bras/dark	ITS1-B	837	62.01	ITS2-1	480	78.33	CH1B	1472	65.22	KJ125139
<i>T. b. brasiliensis</i>	Ceará	Tapera	03° 57' 51" S 38° 21' 00" W	Typical	ITS1-A	837	61.89	ITS2-3	482	78.42	CH3A	1474	65.20	KJ125138
<i>T. b. brasiliensis</i>	Ceará	Tapera	03° 57' 51" S 38° 21' 00" W	Typical	ITS1-A	837	61.89	ITS2-3	482	78.42	CH3A	1474	65.20	KJ125138
<i>T. b. brasiliensis</i>	Ceará	Tapera	03° 57' 51" S 38° 21' 00" W	Typical	ITS1-A	837	61.89	ITS2-3	482	78.42	CH3A	1474	65.20	KJ125138
<i>T. b. macromelasoma</i>	Pernambuco	Petrolina/reared in lab.	09° 23' 55" S 40° 30' 03" W	Typical	ITS1-D	837	62.01	ITS2-1	480	78.33	CH1D	1472	65.22	KJ125141
<i>T. b. macromelasoma</i>	Pernambuco	Petrolina/reared in lab.	09° 23' 55" S 40° 30' 03" W	Typical	ITS1-E	837	62.13	ITS2-1	480	78.33	CH1E	1472	65.29	KJ125142
<i>T. b. macromelasoma</i>	Pernambuco	Petrolina/reared in lab.	09° 23' 55" S 40° 30' 03" W	Typical	ITS1-G	837	62.0	ITS2-1	480	78.33	CH1G	1472	65.22	KJ125144
<i>T. b. macromelasoma</i>	Pernambuco	Petrolina/reared in lab.	09° 23' 55" S 40° 30' 03" W	Typical	ITS1-F	837	62.01	ITS2-1	480	78.33	CH1F	1472	65.22	KJ125143
<i>T. b. macromelasoma</i>	Pernambuco	Petrolina/reared in lab.	09° 23' 55" S 40° 30' 03" W	Typical	ITS1-G	837	62.01	ITS2-1	480	78.33	CH1G	1472	65.22	KJ125144
<i>T. b. macromelasoma</i>	Pernambuco	Petrolina/reared in lab.	09° 23' 55" S 40° 30' 03" W	Typical	ITS1-E	837	62.1	ITS2-1	480	78.33	CH1E	1472	65.29	KJ125142
<i>T. b. macromelasoma</i>	Pernambuco	Petrolina/reared in lab.	09° 23' 55" S 40° 30' 03" w	Typical	ITS1-H	837	62.01	ITS2-1	480	78.33	CH1H	1472	65.22	KJ125145
<i>T. b. macromelasoma</i>	Pernambuco	Petrolina/reared in lab.	09° 23' 55" S 40° 30' 03" W	Typical	ITS1-I	837	62.13	ITS2-1	480	78.33	CH1I	1472	65.29	KJ125146
<i>T. b. macromelasoma</i>	Pernambuco	Petrolina/reared in lab.	09° 23' 55" S 40° 30' 03" W	Typical	ITS1-I	837	62.13	ITS2-1	480	78.33	CH1I	1472	65.29	KJ125146
<i>T. b. macromelasoma</i>	Pernambuco	Salgadinho/reared in lab.	09° 24' 50" S 40° 30' 10" W	Typical	ITS1-A	837	61.89	ITS2-1	480	78.33	CH1A	1472	65.15	KJ125136
<i>T. melanica</i>	Bahia	Urandi/reared in lab.	14° 46' 15" S 42° 39' 18" W	Typical	ITS1-J	837	62.01	ITS2-4	480	78.33	CH4J	1472	65.22	KJ125147
<i>T. melanica</i>	Bahia	Urandi/reared in lab.	14° 46' 15" S 42° 39' 18" W	Typical	ITS1-J	837	62.01	ITS2-4	480	78.33	CH4J	1472	65.22	KJ125147
<i>T. melanica</i>	Bahia	Urandi	14° 55' 34" S 42° 49' 09" W	Typical	ITS1-J	837	62.01	ITS2-4	480	78.33	CH4J	1472	65.22	KJ125147
<i>T. sherlocki</i>	Bahia	Gentio do Ouro/reared in lab.	11° 25' 44" S 42° 30' 21" W	Typical	ITS1-K	837	62.13	ITS2-5	480	78.54	CH5K	1472	65.36	KJ125149
<i>T. sherlocki</i>	Bahia	Gentio do Ouro/reared in lab.	11° 25' 44" S 42° 30' 21" W	Typical	ITS1-K	837	62.13	ITS2-5	480	78.54	CH5K	1472	65.36	KJ125149
<i>T. sherlocki</i>	Bahia	Gentio do Ouro/reared in lab.	11° 25' 44" S 42° 30' 21" W	Typical	ITS1-L	837	62.01	ITS2-6	483	78.47	CH6L	1475	65.29	KJ125148
<i>T. juazeirensis</i>	Bahia	Juazeiro	09° 24' 50" S 40° 30' 10" W	Typical	ITS1-M	836	62.20	ITS2-8	477	78.35	CH8M	1468	65.26	KJ125150
<i>T. juazeirensis</i>	Bahia	Juazeiro/reared in lab.	09° 24' 50" S 40° 30' 10" W	Typical	ITS1-N	836	62.08	ITS2-7	479	78.29	CH7N	1470	65.24	KJ125151
<i>T. juazeirensis</i>	Bahia	Juazeiro/reared in lab.	09° 24' 50" S 40° 30' 10" w	Typical	ITS1-M	836	62.20	ITS2-8	477	78.35	CH8M	1468	65.26	KJ125150
<i>T. juazeirensis</i>	Bahia	Juazeiro/reared in lab.	09° 24' 50" S 40° 30' 10" W	Typical	ITS1-M	836	62.20	ITS2-8	477	78.35	CH8M	1468	65.26	KJ125150

* According to the taxonomic key for all members of the complex (Costa et al., 2013).

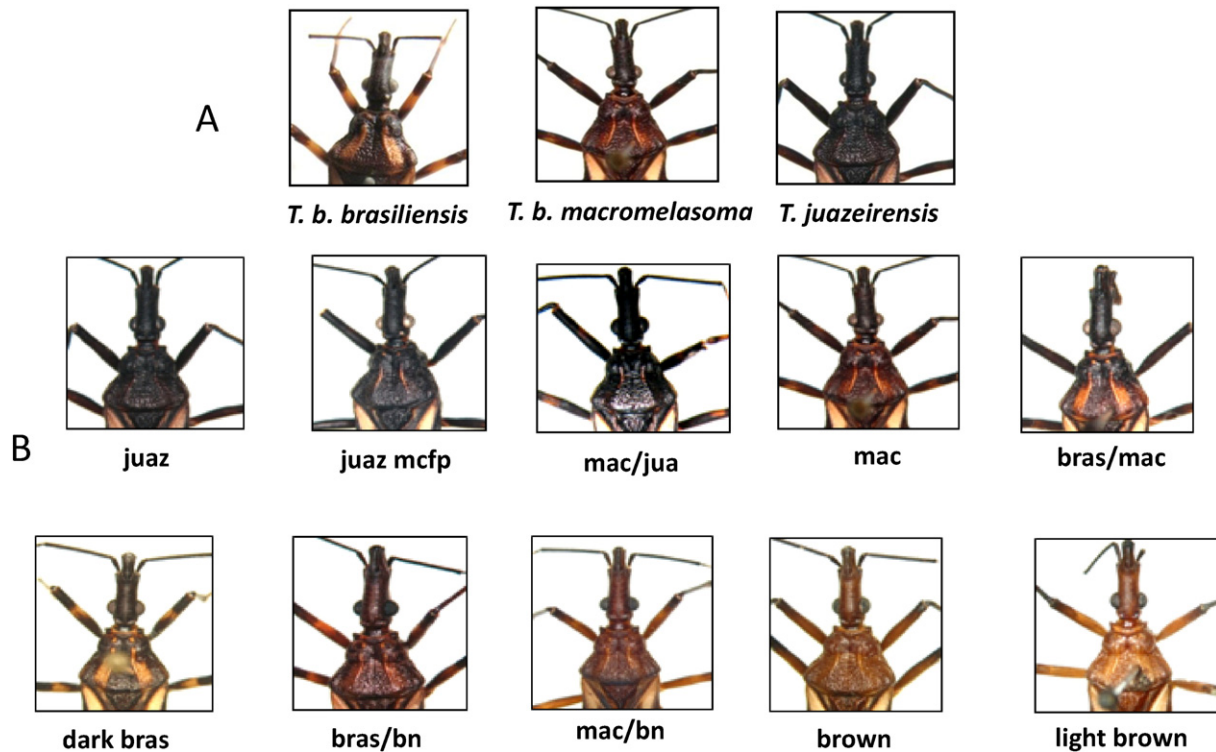


Fig. 2. Chromatic patterns presented by the *Triatoma brasiliensis* complex. 2A: the members of the *Triatoma brasiliensis* complex collected in Pernambuco state (*T. b. brasiliensis*, *T. b. macromelasoma*, *T. juazeirensis*); 2B: distinct chromatic phenotypes encountered in the state of Pernambuco.

ITS-1 haplotypes, providing a total of 9 variable positions of which, 7 were parsimony informative and 2 singleton sites.

3.5. Second internal transcribed spacer ITS-2

A total of 8 different ITS-2 haplotypes were detected among the samples analyzed and codified as ITS2-1 to ITS2-8. Length and AT content ranged between 477 and 485 bp (479.8 bp) and 78.20%–78.54% (78.35%), respectively. The most frequent haplotype coexisting with ITS1-A in the same specimens was selected for H1. These eight ITS-2 haplotypes were compared with the only complete ITS-2 sequence of *T. brasiliensis* available in the GenBank (AJ293591; [Marcilla et al., 2001](#)) from Ceará, Brazil, showing close similarity with haplotypes of *T. b. brasiliensis*/*T. b. macromelasoma*. The presence of a tandemly repeat minisatellite (TCTGC) in positions 380–384 in the ITS-2 alignment allows the distinction between populations from Ceará and all other states evaluated.

Haplotypes ITS2-1 to ITS2-3 were detected in samples of *T. b. brasiliensis* and *T. b. macromelasoma*, being the most abundant the haplotype ITS2-1, present in the majority of specimens analyzed. Haplotype ITS2-4 was detected in samples of *T. melanica*, haplotypes ITS2-5 and ITS2-6 in samples of *T. sherlocki* and haplotypes ITS2-7 and ITS2-8, in samples of *T. juazeirensis* (Tables 2, 3).

The 488 bp-long alignment including the nine ITS-2 haplotypes (*T. brasiliensis* complex ITS2-1 to ITS2-8 and *T. brasiliensis* AJ293591) generate the presence of a total of 16 variable positions (3.27%) of which, 14 were indels (2.87%), only 2 were mutations (0.41%) and no parsimony informative position was detected. This very low degree of genetic differences, being in the majority only gapped positions, generate a very high similarity of the ITS-2 sequences between all the members of the *T. brasiliensis* complex (Supplementary Table 1). Total number of absolute differences ranged between 0 to 2 (average 0.44) between all members of this complex.

As in the case of the ITS-1, three ITS-2 haplogroups corresponding to *T. b. brasiliensis* + *T. b. macromelasoma*; *T. melanica* + *T. sherlocki*; and

T. juazeirensis have been considered and evaluated. Average number of absolute differences and p-distances between these haplogroups ranged between 0.250 and 0.583 (mean, 0.389) and 0.0005 and 0.0012 (0.0008), respectively.

3.6. Phylogenetic and network analyses

The ITS-1 and ITS-2 combined haplotype network constructed using MJ network algorithm shows the existence of three discrete lineages: (i) a big group with a high diversity of haplotypes (11 CH), including samples of *T. b. brasiliensis* and *T. b. macromelasoma* from the states of Pernambuco and Ceará, suggesting a gene flow or a close relationship between these two subspecies; (ii) a second group including haplotypes of *T. melanica* and *T. sherlocki*, both from Bahia; and (iii) one small group including the only two haplotypes of *T. juazeirensis* from Juazeiro in Bahia (Fig. 3). Only one haplotype (1A) was present in two members simultaneously (*T. b. brasiliensis* and *T. b. macromelasoma*). Pernambuco and Ceará depict the highest number of haplotypes, but concentrated in only one of the lineages in the network, while Bahia depicts haplotypes in two distant lineages. This genetic diversity found in the first lineage, supports the consideration of haplotype 1A as the ancestral one, because of its central location in the network, and its relationship with other derived haplotypes. Moreover, haplotype ITS-1A is present in a significant proportion of individuals coming from a wide geographic distribution, including several regions (Table 3).

Phylogenetic analyses were constructed using a total of 26 CH of ITS-1 + 5.8S + ITS-2 sequences including: 16 CH provided by the members of *T. brasiliensis* complex, together with the complete intergenic region of: *Triatoma sordida* (AJ576063), *T. infestans* (AJ576051), *T. platensis* (AJ576061), *T. delpontei* (AJ576057) ([Bargues et al., 2006](#)); *T. rubrovaria* (AJ557258) ([Pacheco et al., 2007](#)); and *Panstrongylus megistus* (HF678458), *P. herreri* (AM949584, AJ306550); *P. geniculatus* (AM949585, AJ306543) ([Marcilla et al., 2001](#); [Mas-Coma and Bargues, 2009](#)). The sequences of *Mepraia spinolai* FN396516 and *Triatoma eratyrusiformis* FN396537 ([Calleros et al., 2010](#)) were used as outgroups.

Table 3

Polymorphic sites allowing differences between complete intergenic rDNA haplotypes of species and populations of *T. brasiliensis* complex analyzed from Brazil.

ITS1+ITS2 Haplotype	Members of <i>T. brasiliensis</i> Complex	Color Pattern	Variable Positions
			11111111 111
			1111222234 4556666777 7800023333 333
			5678256801 2664569226 6055564445 556
			7306921468 2032745451 7826707897 898
1-A	<i>T.b.brasiliensis</i> , <i>T.b.macromelanosoma</i>	mac/br; typical (Tbm)	CCATC C AGGC CGGACACCAG ATTAA- T T- -TC
2-A	<i>T.b.brasiliensis</i>	bras/mac
3-A	<i>T.b.brasiliensis</i>	typical
1-B	<i>T.b.brasiliensis</i>	bras/darkA
2-C	<i>T.b.brasiliensis</i>	mac/juaz; bras/dark A
1-D	<i>T.b.macromelanosoma</i>	typical	... T
1-E	<i>T.b.macromelanosoma</i>	typical T ..A
1-F	<i>T.b.macromelanosoma</i>	typical	... A
1-G	<i>T.b.macromelanosoma</i>	typical T
1-H	<i>T.b.macromelanosoma</i>	typical A
1-I	<i>T.b.macromelanosoma</i>	typical	... A
4-J	<i>T.melanica</i>	typical	T... GAA ..T...G... GA...T...A T-
5-K	<i>T.sherlocki</i>	typical	T... T.GAA ..T...G... G...T...A T..
6-L	<i>T.sherlocki</i>	typical	T... GAA ..T...G... G...T... -A
8-M	<i>T.juazeirensis</i>	typical	TTCA..... -..AAT.
7-N	<i>T.juazeirensis</i>	typical	TTCA..... -..AT.

Shaded areas correspond to minisatellite area including a tandemly repeat minisatellite of 193 bp long. Color pattern according to the taxonomic keys for all members of the complex (Costa et al., 2013 submitted). Position = numbers (to be read in vertical) refer to variable positions obtained in the ITS-1 alignment made with MEGA 5.2. Identical = .; Indel = -.

The ML model best fitting this data set was HKY85 + I + G using the ti/tv ratio of 1.39, base frequencies for A, C, G, and T of 0.3550, 0.1333, 0.1614 and 0.3503, respectively, proportion of invariable sites = 0.156, and the gamma distribution = 3.23. The resulting phylogeny (-Ln =

7119.1284) was evaluated using least squares method with ML distances, showing high bootstrap values (> 70 for at least one criterion) supporting the reliability of the nodes corresponding to *T. brasiliensis* complex, infestans complex and rubrovaria subcomplex (Fig. 4A). Inside

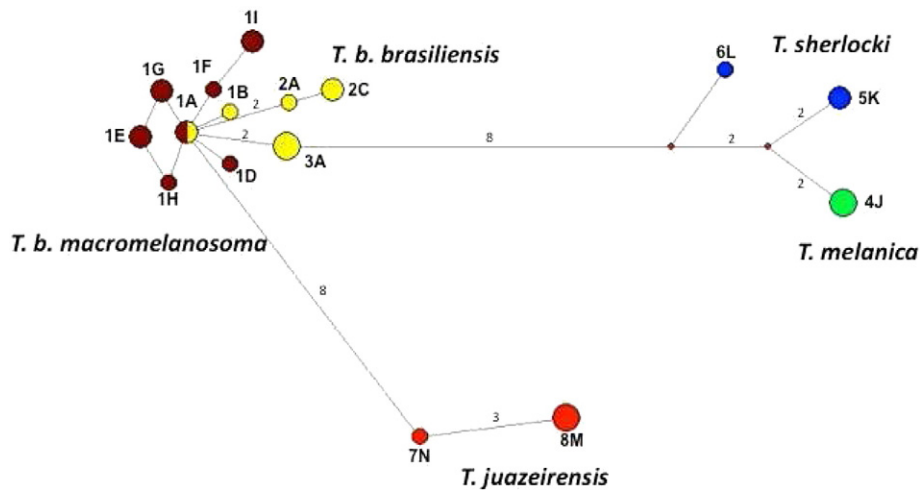


Fig. 3. Median network analysis of the *T. brasiliensis* complex combined haplotypes based on rDNA ITS-1 and ITS-2 sequences. The area of each haplotype is proportional to the total sample. Small red-filled circles represent suggested intermediate haplotypes not present in the sample. Combined haplotype codes and corresponding geographical localities listed in Fig. 4. Yellow = *T. b. brasiliensis*; brown = *T. b. macromelanosoma*; Green = *T. melanica*; Blue = *T. sherlocki*; Red = *T. juazeirensis*. Mutational steps between haplotypes are represented by a line. More than one mutational step is represented by numbers.

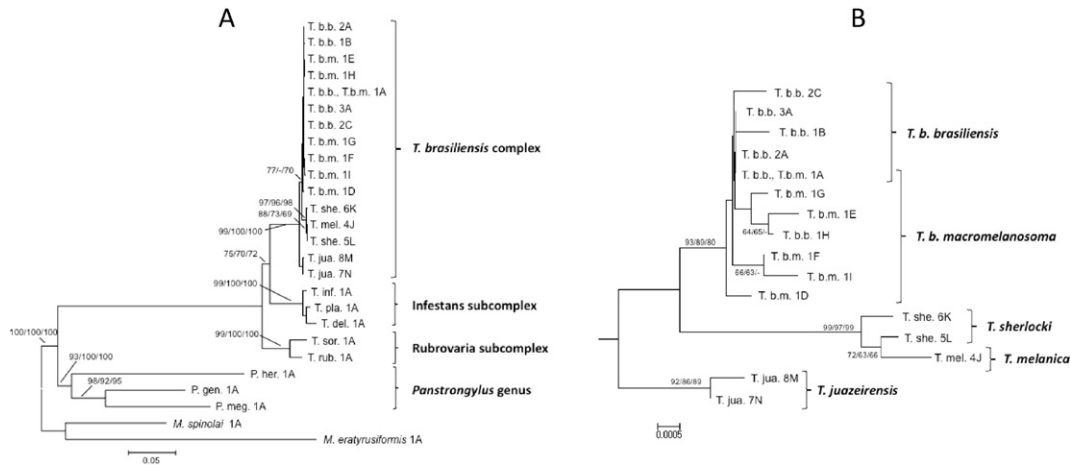


Fig. 4. A. Phylogenetic ML tree based on HKY85 + I + G model including complete ITS-1, 5.8S and ITS-2 sequences of 16 combined haplotypes of *T. brasiliensis* complex, together with other ten South American species of genus *Triatoma*, *Panstrongylus* and *Mepraia*, representatives of other complex and subcomplex whose same complete rDNA intergenic region is available in GenBank database. Molecular evolution model was HKY85 + I + G. *Mepraia* species used as outgroup. Scale bars (number of substitutions per sequence position): 0.05. B. Phylogenetic ML tree based on HKY85 + I + G model ($-\ln = 2431.7462$; ti/tv ratio = 1.552; p-inv. = 0.577; gamma shape = 0.73) including complete ITS-1, 5.8S and ITS-2 sequences of *T. brasiliensis* complex. Node support values with 1000 bootstrap replicates are those that were >60% based on a/b/c: a: NJ reconstruction using Tajima Nei distances; b: ML reconstruction using ML distances and 1000 fast stepwise replicates; c: ML reconstruction using heuristic search and NNI approach. Molecular evolution model was HKY85 + I + G. Infestans subcomplex members (*T. infestans*, *T. platensis* and *T. delponteii*) used as outgroup (not shown). Scale bar (number of substitutions per sequence position): 0.0005.

the monophyletic clade comprising *T. brasiliensis* complex, tree subclades appear grouping *T. b. brasiliensis* and *T. b. macromelasoma*; *T. sherlocki* and *T. melanica*; and *T. juazeirensis*. The tree obtained using only species of the infestans complex as outgroup (Fig. 4B) shows that *T. juazeirensis* appears as sister species of the clade grouping all *T. b. brasiliensis*, *T. b. macromelasoma*, *T. sherlocki* and *T. melanica* phenotypes and haplotypes analyzed. The topology of the clades was similar to that of the groupings obtained with the MJ network (Fig. 3).

3.7. Relationships between genetic and phenetic distances

The Mantel tests performed between the genetic (Tajima–Nei) and the phenetic (Jaccard, Hamann, Rogers–Tanimoto or Sokal–Michener) matrices of distances were significant (P ranging from 0.001 to 0.005). The highest level of correlation was obtained with the Jaccard's phenetic distances ($r = 0.38$, $r_2 = 0.62$).

4. Discussion

Pernambuco state, Brazil is situated between the distributional areas of *T. b. brasiliensis* (north) and *T. juazeirensis* (south), where it is occupied by *T. b. macromelasoma* (Costa et al., 2014). Based on (i) the greater chromatic variation of *T. b. macromelasoma*, (ii) morphometric geometrics (iii) and also considering the geographical position of occurrence of this subspecies in Pernambuco, Costa et al. (2009) proposed that this area might represent a zone of natural hybridization between *T. b. brasiliensis* and *T. juazeirensis*. Our results showed that *T. b. macromelasoma* displays greater chromatic variations within *T. brasiliensis* species complex, exhibiting intermediate chromatic phenotypes between the *T. b. brasiliensis* and *T. juazeirensis*. We conducted phylogenetic analyses using ribosomal DNA intergenic region under a Maximum Likelihood framework that grouped these phenotypes within the *T. brasiliensis* complex. Together with previous findings (Costa et al., 2009), results herein obtained supported the idea of a hybridization zone in Pernambuco state that gave rise to the *T. b. macromelasoma* through homoploid evolution.

Laboratory crosses between *T. b. brasiliensis*, *T. juazeirensis* and *T. macromelasoma* generated fertile hybrid offspring (Costa et al., 2003b). This, combined with multiple morphological parameters and their geographical distributions, made possible the first thoughts

concerning the hybridization between *T. b. brasiliensis* and *T. juazeirensis* as a mode to originate *T. b. macromelasoma* (Costa et al., 2009). This was the first time that hybridization was suggested to be a possible speciation mechanism in Triatominae. According to Galvão (1956), Petrolina, in the state of PE, represents the type locality and the original distribution of *T. b. macromelasoma*. In addition, its northern border correlates with the southernmost distribution of *T. b. brasiliensis* and its southern border with the northern most distribution of *T. juazeirensis*.

Coloration is one of the most variable characteristics and exists widely in natural populations of diverse insects (Gray and McKinnon, 2006). Specimens of the *T. brasiliensis* complex, which include *T. b. brasiliensis*, *T. b. macromelasoma*, *T. melanica*, *T. sherlocki* and *T. juazeirensis*, may be distinguished by their color patterns and genetic profiles (Monteiro et al., 2004; Costa et al., 2013). Variations, such as those observed in the *T. brasiliensis* complex, can be indicators of genetic drift and, within its geographic distribution, the highest phenotypic variability was observed in the state of PE (Monteiro et al., 2004). Areas of hybridization are expected to permit a greater combination of alleles that would consequently generate a greater variability in chromatic phenotypes in relation to other areas of the species (Rieseberg et al., 1999).

Thirteen distinct phenotypes of the *T. brasiliensis* complex were here defined according to the most evident chromatic traits for the 412 specimens collected in the PE before correlating to the capture sites. The greatest diversity in coloration was observed in the areas of Sertão do São Francisco and Sertão do Araripe where 11 and 12 phenotypes were collected, respectively. In the Agreste region, only 2 different phenotypes were identified. Even though the number of collected specimens cannot be considered as the expression of the absolute percentage for each phenotype, some indicative differences were observed in the three studied mesoregions of PE.

Nearly one third of the specimens collected in the São Francisco region had a color pattern related to *T. b. macromelasoma*, almost 30% presented a phenotype of *mac/juaz* and 12.59% presented a phenotype of *juaz*. The patterns suggest an influence of the *T. juazeirensis* chromatic phenotype, whose distribution borders the southern edge and is separated by São Francisco river. In the region of Sertão Araripe, the *T. b. macromelasoma* phenotype was reduced to 14.1% of the total and no specimens with a *juaz* phenotype were identified. The most common phenotype was *mac/bn* and the number of *T. b. brasiliensis* chromatic

phenotypes was the greatest in specimens captured in this area. The third most collected phenotype was defined as brown (*bn*), which suggests a transgressive segregated phenotype since none of the parental characteristics were observed in these specimens (Rieseberg et al., 1999). In the Agreste region, the influence of the *T. b. brasiliensis* appeared most evident since both phenotypes were characterized as *dark bras* and *bras/mac*. The Sertão Araripe and Agreste regions both border the distribution of *T. b. brasiliensis*.

4.1. Nuclear ribosomal DNA analyses

It is the first time that a genetic analysis of the members of *T. brasiliensis* complex has been performed based on sequences of the complete ITS-1, 5.8S and ITS-2 rDNA intergenic region. Fourteen and eight haplotypes have been detected for each ITS-1 and ITS-2 spacers, respectively, and a total of sixteen combined haplotypes (ITS-1 + ITS-2) for specimens representing 3 Brazilian states, 7 geographical localities and 9 color patterns.

The whole intergenic rDNA region provides a data set with an average length and AT content of 1471.7 bp and 65.23%, respectively. This region was considerably longer than the 1377 bp described for *T. rubrovaria* (Pacheco et al., 2003, 2007), the 1375 bp of the *infestans* subcomplex (Bargues et al., 2006) and the 1371 bp in the genus *Mepraia* (Calleros et al., 2010). The total of 16 combined haplotypes described for this intergenic region provides a genetic variability of 2.23% in the members of the *T. brasiliensis* complex analyzed, allowing for species and population differentiation. This genetic variability is similar to those obtained in the *infestans* subcomplex, suggesting evolving divergence processes presently ongoing within the species in question (Piccinali et al., 2011).

The ITS-1 in *T. brasiliensis* complex proved to be markedly longer than ITS-2, and its bias in AT composition is lower than in ITS-2, as observed in other Triatominae (Bargues et al., 2006, 2008; Pacheco et al., 2007; Calleros et al., 2010).

Total variability obtained (considering total character differences = indels and mutations) was 2.63% and 3.27% for the ITS-1 and ITS-2, respectively. When only true mutations are considered, the percentages of genetic variability of both markers differed considerably, being of 2.51% and 0.41% for ITS-1 and ITS-2, respectively. These results are in agreement with the molecular clock dating proposed for triatomines, according to which ITS-1 evolved 1.12–2.60 times faster than ITS-2 (Bargues et al., 2000, 2002, 2006). Without considering indels because of their weight lower than that of mutations, the number of transitions (ts) and transversions (tv) enables for a better species and population differentiation in the ITS-1 than in the ITS-2 in *T. brasiliensis* complex. It is known in other organisms that the coexistence of many indels with very few mutations usually indicates active evolutionary divergence processes (Bargues et al., 2001).

Only two mutations (tv) between ITS-2 sequences of all members of the *T. brasiliensis* complex have been detected. This reduced variability in the ITS-2 is in agreement with the chromosome homogeneity observed between the four chromatic members of *T. brasiliensis* complex (Panzera et al., 2000). A low variability in rDNA ITS-2 sequences has also been described in the phyllosoma complex (Marcilla et al., 2001; Bargues et al., 2002; Martínez et al., 2006; Martínez-Hernández et al., 2010). For those Mexican species it was concluded that a subspecies status would fit best (Bargues et al., 2002), as such a low number of differences are typical in organisms able to hybridize (Mas-Coma and Bargues, 2009).

The pairwise comparisons performed in members of the *T. brasiliensis* complex, considering total number of absolute differences, ranged from 1 to 13 (average 5.76) and 0 to 2 (average 0.44) for ITS-1 and ITS-2, respectively. These values were in agreement with the information capacity of the different DNA markers, related to the speed of their evolutionary rates (Bargues et al., 2006; Mas-Coma and Bargues, 2009).

The presence of a minisatellite of 193 bp long tandemly repeated in the ITS-1 is reported for first time in members of *T. brasiliensis* complex. It does not contain enough information for molecular species or subspecies distinction, but was able to differentiate *T. melanica* and *T. sherlocki* from the other unities of the complex. This minisatellite demonstrates expected changes during evolution. Each members had the same number of repeats, but could differ in the mutations detected in repeat 1 (5) and in repeat 2 (4). These observations suggest that the replication of the repeats occurred before the *T. brasiliensis* complex separated into the current species grouping with the individual mutations arising afterwards.

This long minisatellite repeat had not been reported previously in any other *Triatoma* species. It differs from those of 10 and 15 bp-long minisatellites tandemly repeated in the *infestans* subcomplex members, and able to distinguish between sylvatic and domestic populations of *T. infestans* (Bargues et al., 2006). In *T. rubrovaria* from Argentina, Uruguay and Brazil, the ITS-1 includes another minisatellite of 31 bp tandemly repeated two times which does not however discriminate the different populations of *T. rubrovaria* (Pacheco et al., 2003, 2007). The total variability of the ITS-2 provided by members of *T. brasiliensis* complex analyzed is relatively high (3.27%) when compared to the small genetic diversity of 1.83% obtained in the phyllosoma complex (including *Triatoma bassolsae*, *Triatoma longipennis*, *Triatoma mazzotti*, *Triatoma picturata*, *Triatoma pallidipennis* and *Triatoma phyllosoma*) and lower than that of 4.58% in the *infestans* subcomplex (including *Triatoma infestans*, *Triatoma platensis* and *Triatoma delpontei*) or that of 5.62% in *Triatoma dimidiata* sl. (including all subspecies and excluding the species *Triatoma* sp. aff. *Dimidiata*) (Bargues et al., 2006, 2008).

In the case of the ITS-1, the genetic diversity of 2.63% obtained in the *T. brasiliensis* complex is very low when compared with the 10.87% detected in the *infestans* species subcomplex, but when only true mutations are considered (and not indels generated by microsatellites), results are very similar for both groups of species, being of 2.51% and 2.29% for *Triatoma brasiliensis* complex and *infestans* subcomplex, respectively.

Both rDNA markers suggest that there are not enough genetic criteria to support different species inside this group. However, the detection of three haplogroups using the ITS-1 and the ITS-2 suggest that the *T. brasiliensis* group is composed by three genetically distinct forms referred to (i) *T.b. brasiliensis/T. b. macromelasoma*; (ii) *T. melanica* and *T. sherlocki*, and (iii) *T. juazeirensis*, that present a total genetic divergence of 2.63% and 3.27%, an interhaplogroup p-distance average values of 0.011 and 0.0008, for ITS-1 and ITS-2, respectively, and a pattern of percentage number of differences compatible with the existence of a species complex in a clear process of speciation. The reproductive capacity between these unities could be the cause that prevented complete speciation of these taxa, as reported in the case of *T. dimidiata* complex of species and subspecies (Bargues et al., 2008). However, as each member of *T. brasiliensis* species complex is fit in a particular environmental condition (Costa et al., 2014), it might be avoiding interbreeding events.

The genetic closeness obtained between *T. sherlocki* and *T. melanica* using the complete intergenic region of rDNA is coincident with the results obtained using two mtDNA markers (Cyt b and 16S). Molecular phylogenetic analyses suggest that *T. sherlocki* is a sister species of *T. melanica*, as supported by robust credibility rates of 98% (Mendonça et al., 2009; Gardim et al., 2014).

The grouping by analysis of ribosomal DNA sequences of *T. b. macromelasoma* with *T. b. brasiliensis* confirms that both are close evolutionary unities, which was evidenced by low genetic distances among taxa. Phylogenetic results provided higher resolution for the distinction of *T. b. brasiliensis* + *T. b. macromelasoma*, *T. melanica*, *T. sherlocki* and *T. juazeirensis* as independent evolutionary unities. The position of *T. melanica* and *T. juazeirensis* in the ML tree further suggests geographic separation between species as an important factor increasing genetic

differences, in agreement with results with Cyt B sequences (Monteiro et al., 2004).

In general, a good correlation between phenotype and genotype have been observed, although previous studies reported some phenotypes intermediate between *bra* and *mac*, as well as some specimens presenting *mac* and *jua* phenotypes that clustered with *bra* in the DNA sequence analyses (Costa et al., 2009). Our results clearly agree with the results obtained with a fragment of the *Cytb* gene of the mtDNA (Monteiro et al., 2004), in the sense that rDNA genetic distances also separate members of the *T. brasiliensis* complex, especially the “typical” color patterns (Costa et al., 2013) as for *T. b. brasiliensis* + *T. b. macromelasoma*, *T. melanica* + *T. sherlocki* and *T. juazeirensis*.

The high degree of chromatic variations, along with the correlations of genetic and phenetic distances through Mantel test, supported to the idea of a natural hybridization zone for the speciation of *T. b. macromelasoma* in Pernambuco state. However, these evidences are not conclusive, because ITS 1 and ITS 2 sequences may have not the resolution to point out the hybrid genetic profile evidenced by the intermediate phenotypes between the *T. brasiliensis* and *T. juazeirensis*. The observed fertile experimental hybrids between *T. b. brasiliensis* and *T. juazeirensis* demonstrate the potential for viability of these offspring in nature (Costa et al., 2003b). For other taxa, as members of the Phyllosoma complex in the Yucatan Peninsula in Mexico, viable lab-hybrids could have been produced (Martinez-Ibarra et al., 2009) and also indicated areas of natural hybridization events (Herrera-Aguilar et al., 2009).

Other aspects of triatomine biology that lend support to hybridization leading to speciation are the evidence of rapid evolution and the model of ecological niches. It is known that ecological traits are crucial to permit a species to maintain its distinctive traits with different species of triatomine having population dynamics that peak at different times during the year (Lucena, 1960). It has been proposed that the differentiation of *T. b. macromelasoma* was achieved due to a distinctive ecology compared to the other members of the complex (Costa et al., 2002, 2014). In this scenario, despite the apparent overlap in distributions, *T. b. macromelasoma* could be in an ecological niche isolated from the proposed parental species in a way that its differentiation can be maintained (Costa et al., 2009).

The state of PE is under strong environmental impact from increases in agricultural activity and human population growth. This can create greater pressure and increasing the chances of crosses between *T. b. brasiliensis*, *T. juazeirensis* and *T. b. macromelasoma* is through new mechanisms of transport and altering the natural habitats. Another similar scenario for *T. juazeirensis* and *T. sherlocki* must be further investigated because their distributional boundaries are unknown (Almeida et al., 2012) in an area now getting impacted by mining activities (Almeida et al., 2009). In addition to that, the *T. cruzi* susceptibility of hybrid specimens is very poorly understood, some previous results on the *T. dimidiata* were presented by Herrera-Aguilar et al. (2009), although recent clues in this sense have been provided for members of *T. brasiliensis* species complex (Costa et al., 2015).

In this study, 13 phenotypes of the *T. brasiliensis* complex were for the first time recorded in several sites of the PE state, compatible with the hypothesis of the existence of a natural hybrid zone for members of that complex, specifically: *T. b. macromelasoma*, *T. b. brasiliensis*, and *T. juazeirensis* are supposed to be involved. Also, an important fact is that all the recorded phenotypes were kept under the same laboratory conditions (data not published yet) and the heredity, homogeneity and stability of the different color patterns were confirmed. Finally, an important question arises related to whether an epigenetic phenomenon (Rieseberg et al., 1999; Liu and Wendel, 2003; Rieseberg and Willis, 2007) could also be involved in the morphological diversity of the *T. brasiliensis* complex is still to be assessed. This research is crucial to better understand the speciation process of the Triatominae group and brings relevant questions that relates also to the vectorial capacity. Arguments for an evolutionary significance of phenotypic variability

were discussed by Dujardin et al. (2009) and epigenetics phenomenon was suggested for the Triatominae group and, results in the present study are also supportive of that phenomenon.

The successful campaign against *T. infestans*, previously the predominant cause of *T. cruzi* infections in humans, has elevated the importance of members of the *T. brasiliensis* complex due to their capacity to maintain and transmit *T. cruzi*. Improving the knowledge of each member of the *T. brasiliensis* complex, including their characterization and originating process, are important aspects for monitoring Chagas disease vector transmission.

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