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Combined phylogenetic and morphometric information to delimit and unify the *Triatoma brasiliensis* species complex and the Brasiliensis subcomplex

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Abstract

"*Triatoma brasiliensis* species complex" was defined as a monophyletic group of the species: *T. brasiliensis*, *T. juazeirensis*, *T. melanica*, and *T. sherlocki*. An alternative grouping scheme proposed the concept of "Brasiliensis subcomplex" which included the former species together with *T. melanocephala*, *T. petrocchiae*, *T. lenti*, *T. tibiamaculata*, and *T. vitticeps*. To evaluate the relationship among these taxa we combined the results obtained with four mitochondrial genes (12S, 16S, COI and Cytb, adding to 1811 bp) and geometric morphometric analysis of wings and heads. *Panstrongylus megistus* was included in the analysis as it was previously found related to *T. tibiamaculata, T. melanocephala* and *T. vitticeps.* The results of both molecular and morphometric approaches clearly grouped the species analyzed into two monophyletic units, supported by both genetic and wing variability. The first one (G1) comprises the four species originally included in the *T. brasiliensis* species complex plus *T. lenti* and *T. petrocchiae*. The second group (G2) was

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Appendix A. Supplementary data

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composed by *T. melanocephala*, *T. tibiamaculata* and *T. vitticeps*, and remarkably, *P. megistus* if considering wing variability and phylogenetic results. Nevertheless, geometric morphometrics of heads provided a quantitative measurement that discriminates *Panstrongylus* from the *Triatoma* species based on the position of the antennal insertion relative to eyes, as it is used as the generic distinctive character. The discrepancy among approaches questions the validity of this character to define *Panstrongylus* genus. Independently of the chosen group definition —"*T. brasiliensis* species complex" or "Brasiliensis subcomplex"—we propose to delimit it to species of G1 that are all associated with the Caatinga biome in the Brazilian Northeast. G2 are the ones associated with the Atlantic Forest biome.

Keywords

Triatominae; Phenotype; Genotype

1. Introduction

Classifying organisms by delimiting taxonomic units is a complex and dynamic research endeavor. Scientific classification has given rise to modern systematics through the incorporation of integrative approaches based on an array of new techniques, statistical models, theories and new information from genes, genomes and ecological attributes (Dayrat, 2005). Consequently, systematics has suggested major rearrangements of biological classification by reshaping taxa phylogenies and redefining species boundaries, which in turn leads to an increase in numbers of known taxonomic units, especially in megadiverse regions. In this context, the determination of "species complexes" is particularly useful. Distinctly from other formal ranks ruled by the International Code of Zoological Nomenclature (ICZN, 1999), grouping species into complexes is more permissive; but for this reason, is also a more contentious matter. Yet, the concept is useful to approach large taxonomic systems or whole systematic research assemblages, often used to ponder diverse organisms of interest to economic activities or human health (e.g., White, 1985; Perring, 2001).

Trypanosoma cruzi, the causing agent of Chagas disease, is transmitted by triatomine vectors (Reduviidae: Triatominae) also known as kissing bugs. The genus *Triatoma* includes the largest number of species within the Triatominae. The validity of subgenera within *Triatoma* has been discussed, but was never fully resolved (Carcavallo et al., 2000); thus to consider related species, authors have proposed the use of "species complex", "species subcomplex", and "species group". Grouping species of *Triatoma* begun as early as the first attempt of understanding the phylogenetic relationships within the genus: Usinger (1944) grouped species based only on morphology and under a phylogenetic perspective in his revision of North and Central American species, presenting an identification key for some *Triatoma* species of North America. Lent and Wygodzinsky (1979) followed Usinger's (1944) model, but proposed a more detailed scheme, which comprised all *Triatoma* species known at the time, in which they considered features of immature stages. Following the same line, Dujardin et al. (2002) presented a classification adapted from Lent and Wygodzinsky (1979), with a hierarchical assembly of groups > subgroups > complexes >

subcomplexes > species. Afterwards, it was followed by several authors (e.g. Almeida et al., 2009a; Justi et al., 2014, 2016; Justi and Galvão 2017).

Lucena (1970) provided the first attempt to group species that should compose the *T. brasiliensis* species complex, considering *T. lenti* and *T. petrocchiae* as members. Later, Costa and collaborators initiated in 1997 a large set of studies to understand the relationships among *T. brasiliensis* s.l. that occurred across the whole semiarid region of the Brazilian Northeastern region. The authors included a comprehensive array of information, considering aspects on species biology, ecology, phylogenetics, among others (e.g., Costa et al., 1997a, 1997b, 1998, 2013, 2002; Monteiro et al., 2004). Based on the obtained results, the "*Triatoma brasiliensis* species complex" was proposed as a monophyletic group including some *T. brasiliensis* chromatic forms, previously called "brasiliensis", "juazeiro" and "melanica" populations (Costa and Felix 2007; Costa et al., 2009, 2013). Members of the complex present distinct epidemiologic importance, as analyzed by Costa et al. (2003a) and Almeida et al. (2009b). The outcome was an exhaustive taxonomic revision of *T. brasiliensis* s.l. "populations", from which some were raised to specific status as *T. juazeirensis* (Costa and Felix 2007) and *T. melanica* (Costa et al., 2006). Later, *T. sherlocki* was included (Mendonça et al., 2009) and considered all part of the *Triatoma brasiliensis* species complex (Costa et al., 2013). An alternative classification scheme proposed by Schofield and Galvão (2009), intended to give continuity to the proposal of Usinger (1944) and Lent and Wygodzinsky (1979) re-arranging species to create a lower hierarchical level, called "subcomplexes". In this sense, the authors created the "Brasiliensis subcomplex" of the "Infestans complex" that included the four species of the *T. brasiliensis* species complex of Mendonça et al. (2009) and Costa et al. (2013), some rare species, such as *T. lenti*, *T. petrocchiae* and *T. melanocephala*, in addition to *T. vitticeps* and *T. tibiamaculata*. However, Schofield and Galvão (2009) highlighted the uncertain position for the two last species, based on previous cytogenetic signals.

Cytogenetic (Alevi et al., 2012, 2013, 2014a,b) and phylogenetic (Gardim et al., 2014) evidence later determined that *T. tibiamaculata, T. melanocephala*, and *T. vitticeps* were not closely related to the species of the Brasiliensis subcomplex. Instead, these three species were related to lineages including other genera of Triatomini, such as *Panstrongylus*. These studies reflected the limitation of classification based exclusively in qualitative morphology, emphasizing the need of evaluating the degree of specific relatedness using combined approaches. Specifically, in this work we intended to investigate phylogenetic relationships of the rare species *T. lenti* and *T. petrocchiae* in relation to other species of the *T. brasiliensis* species complex and Brasiliensis subcomplex. Additionally, we aimed to evaluate the morphological relationships of *P. megistus* with the analyzed species, by using two distinct structures: the wings and the heads.

2. Materials and methods

2.1. Terminology and taxon sampling

We will hereafter refer to "*T. brasiliensis* complex" as the group defined by Mendonça et al. (2009) and Costa et al. (2013): *T. brasiliensis*, *T. juazeirensis*, *T. sherlocki*, and *T. melanica*; and to "Brasiliensis subcomplex" to the group defined by Schofield and Galvão (2009),

comprising the species aforementioned, in addition to *T. lenti*, *T. petrocchiae*, *T. melanocephala*, *T. tibiamaculata*, and *T. vitticeps*, This study focused on the newly sequenced species, *T. lenti* and *T. petrocchiae*, referred to as "candidate" members. *Panstrongylus megistus* does not belong to any abovementioned groups, but was included because molecular phylogenetics (Gardim et al., 2014) suggested it is closely related to *T. melanocephala*, *T. tibiamaculata*, and *T. vitticeps*, in fact it clustered as a sister to *T. tibiamaculata*. For the phylogenetic reconstruction, all species of the *T. brasiliensis* complex and Brasiliensis subcomplex were sampled. Overall, taxon sampling focused on the monophyletic clade of South American *Triatoma* (Gardim et al., 2014; Justi et al., 2014; Justi et al., 2016; Justi and Galvão, 2017; Justi and Galvão 2017) by gathering sequences deposited at GenBank (Table 1). *Rhodnius prolixus* was used as outgroup.

2.2. DNA extraction, amplification, and sequencing

Specimens from the species analyzed were collected in rocky outcrops, while *P. megistus* was found in animal burrows. *T. vitticeps* and *T. tibiamaculata* specimens were insects that invaded homes, as the natural ecotope of these species is not fully known and (Lent and Wygodzinsky 1979; Ribeiro et al., 2015). Details about their geographic origin are presented in Table 2. DNA was extracted from three individuals of each species using two legs or each, according to the protocol described by Sambrook and Russell (2001). DNA amplification was carried out specifically for each target as published elsewhere (Garcia and Powell, 1998; Lyman et al., 1999; and Monteiro et al., 2003). Because specific mutations prevented amplification of the *cytb* locus in *T. petrocchiae* a new reverse primer was designed for this species (cybTprR: GCTC-CRATTCATGTTARRAG) that successfully amplified the target region using the 7432F primer (Monteiro et al., 2003). Products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Life Sciences), subjected to a sequencing reaction using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), and analyzed in the ABI PRISM ® 377 DNA Sequencer (Applied Biosystems). Sequences were edited with BioEdit 7.0.5 and aligned with ClustalW (Larkin et al., 2007). Nucleotide data for Cytb and COI were translated into aminoacid sequences to verify the alignment, as recommended by Mas-Coma and Bargues (2009) and Bargues et al., (2014) to avoid interpreting pseudogenes in the analysis. The sequences were manually checked for quality and the DNA fragments were cropped to the reliable sizes of 305 bp for 12S rDNA, 547 bp for 16S rDNA, 398 bp for cytochrome oxidase subunit I (COI), and 561 bp for cytochrome *b* (Cytb) genes. New sequences here obtained were deposited at GenBank (Table 1).

2.3. Phylogenetic analyses

Phylogenetic inference was based on a concatenated matrix of the four markers, 12S, 16S, COI, and Cytb sequences (26 taxa and 1811 characters) under three methods: (i) maximum parsimony (MP) criterion run in PAUP* 4.0a145 (Swofford, 2002) with 1000 random addition replicates and Tree Bisection Reconnection (TBR) branch swap, (ii) maximum likelihood criterion run in Garli 2.01 (Zwickl, 2006) with 100 search replicates and enforcing termination when no significant topological change occurred after 20,000 generations, and (iii) Bayesian inference (BI) run in MrBayes 3.2 (Ronquist et al., 2012) with two independent runs of 4 MCMC chains for 5 M generations, sampling every 1000

trees. For both parametric approaches, each partition was independently modeled according to the following evolutionary models selected with the Akaike Information Criterion in MrModeltest (Nylander, 2004): GTR+I+G for cytb, COI; and 16S, and HKY+G for 12S. In the Bayesian analysis, the convergence of independent runs was assessed by values of standard deviation of split frequencies <0.05 at the last generation and visually inspecting combined sampled distributions of parameters using Tracer v. 1.5 (Rambaut and Drummond, 2009), as well as adequate mixing of sampled parameters assessed by effective sampling size (ESS) values > 200. Clade support was estimated by Bayesian posterior probabilities (BPP) and bootstrap frequencies for parsimony (PB) and maximum likelihood (MLB), calculated over 1000 and 500 matrix pseudoreplicates, respectively.

2.4. Morphometric analyses

For morphometric analyses we used 30 F1 individuals (15 males and 15 females), for each species of the Brasiliensis subcomplex, except for the unavailable *T. melanica.* Capture location details shown in Table 2, adapted of Batista et al. (2012). To explore patterns of morphological variation, 9 landmarks of heads (Fig. 1) and 10 landmarks of right wings (Fig. 2) were used. For both structures, pictures were taken under identical conditions with the camera positioned 13 cm from the photographic plane. We used a mil-limetered paper as background to indicate the scale and to align insects (for head pictures). Landmarks were recorded with TpsDig2 software (Rohlf, 2010). Then the files with the raw landmark coordinates were transferred to IMP software (Sheets, 2004) to generate shape variables. A procrustes superimposition was carried out to eliminate all information related with size, position and orientation (Rohlf 2010). Shape matrix were projected into a Euclidian space to generate a set of partial warps scores (Bookstein 1991).

Exploratory analysis of head and wing shape was carried with a Principal Component Analysis (PCA). Shape difference of heads and wings between species was tested with two factor (species and sex) procrustes ANOVA and a MANOVA. Pairwise permutations tests (10,000 iterations) between pair of species used procrustes and Mahalanobis distances. The degree of shape differentiation between species was assessed through procrustes and Mahalanobis distance as well as cross-validated correct classification percentages of a pairwise discriminant analysis. Shape similarity was visualized by unrooted neighbor joining trees obtained from procrustes and Mahalanobis distance matrices. Procrustes distances matrices represent shape differences in shape space whereas Mahalanobis are distances between group centroids normalized by the within group variances. To examine the separation between two species we applied cross-validation and discriminant function, following Dunne and Stone (1993) and Lachenbruch (1967) algorithms. All analyses were run as implemented in MorphoJ (Klingenberg 2011).

3. Results

3.1. Phylogenetic reconstruction

Molecular analysis results based on the three methods, consistently recovered two groups: the first (G1), a monophyletic clade including the species found in the Caatinga biome, comprising *T. petrocchiae* + *T. juazeirensis* + *T. brasiliensis* + *T. sherlocki* + *T. lenti* + *T.*

melanica; and the second group (G2) including species from the Atlantic Forest biome: *T. melanocephala* + *T. vitticeps* + *T. tibiamaculata* + *P. megistus*. However G2 was not recovered in the parsimony analysis. All phylogenetic methods (Fig. 3) strongly supported *T. lenti* as a sister species to *T. melanica* (MLB = 99, BPP = 100, PB = 100) and *T. petrocchiae* related to the *T. brasiliensis* species complex, including *T. lenti* (MLB = 75, BPP = 100, PB = 62). Finally, results also recovered G1 closely related to remaining South American *Triatoma* included in the analysis, except of *T. maculata* (MLB = 83, BPP = 99, PB = 58).

3.2. Morphometry

3.2.1. Statistical differences—Wing and head shape variations among species were all statistically significant (P <0.01). Wings showed sexual dimorphism in shape and, as expected, females wings were larger than males' ($p < 0.05$) whereas heads were not dimorphic. Results of the Principal Component (PC) Analysis for wings and heads showed distinct patterns. Whereas wings showed a continuum of variations of PC 1 and 2 (47.8% and 12.6% of variance respectively) with *P. megistus* at one extreme of PC1, heads variation showed three distinct groups on the same projection (83.2% and 8.5% of variance for PC1 and 2). The first cluster on the positive values of PC1 was solely composed of *P. megistus.* The second cluster, towards null values, included *T. melanocephala*, *T. tibiamaculata*, and *T. viticeps.* The third cluster at the negative values of PC1 included *T. brasiliensis*, *T. juazerensis*, *T. lenti*, *T. petrocchiae*, and *T. sherlocki.* PC2 showed a marked difference of *P. megistus* females.

3.2.2. Morphometric species differentiation: procrustes distances (PD) and Mahalanobis (MD)—Pairwise distance matrices represent the true distance between groups unlike bidimensional projection (e.g. PCAs, CVAs) that only capture part of the distance. With many taxa neighbor-joining algorithm provide a graphical representation of the distances between groups. The four morphometric distance matrices (heads and wings/ Mahalanobis and procrustes distances) were congruent with the phylogenetic analyses in clustering G1 and G2 (Fig. 4). All NJ trees of shape distances were also congruent in showing that *P. megistus* is phenetically closer to *T. melanocephala* in accordance with the phylogenetic tree. Phenograms also showed that head shape is more conservative than wing shape and that in all cases head shape of *P. megistus* is highly divergent. *Panstrongylus megistus* exhibited much higher values even within G2 (all MD ≥10.73, P ≥0.11) than within G1 (all MD ≤7.29, PD ≤0.07) (Fig. 4, Supplementary Table 1). The analysis of values for wings, showed that some intergeneric distances within groups (e.g., *P. megistus*-*T. tibiamaculata*, MD = 6.22, PD = 0.047) were lower than intrageneric ones also within group (e.g., within G1 for *T. sherlocki*-*T.petrocchiae*, MD = 7.38, PD = 0.05). Within G1 and G2, neither head nor wing shape were congruent with the topology of the phylogenetic tree. The topology of phenograms from head shapes were congruent with the grouping ((*T. petrocchiae*, *T. sherlocki*), *T. lenti*). The other phenetic relationships varied depending on the distance matrix used, suggesting the need of further analyses of intraspecific patterns of shape variation.

3.2.3. Statistical differences between species pairs—All procrustes and Mahalanobis pairwise distances among species were significant (P < 0.01). Most pairwise

species comparisons were correctly assigned (100% probability), but some exceptions were observed: probably due the close relationship among G1 species, there were some misclassifications within the pair *T. sherlocki*-*T. juazeirensis*, which exhibited the lowest overall correct classifications percentages (86.2–96.6%), but only for wings. The pair *T. lenti*-*T. sherlocki* also exhibited lower (89.6–96.6%) correct classification. A few incorrect classifications between G1-G2, were also observed, as for *T. juazeirensis*-*T. melanocephala*, of which one wing of each other species were misclassified for cross validation (96.5–96.6%), and one sample of *T. lenti* was misclassified as *T. melanocephala* on this same parameter and structure. The remaining misclassifications between G1-G2 were observed for *T. vitticeps*: for cross validation analysis of heads: one sample of *T. brasiliensis* was misclassified as *T. vitticeps*, and one sample of this last species was misclassified as *T. juazeirensis*, generating 96.7% of correct assignment for both. One *T. vitticeps* was misclassified as *T. lenti* for cross validation of wings. Within G2, one or two samples for the comparison between *T. melanocephala*-*T. viticeps* were misclassified for cross validation, generating 96.7% and 93% of correct assignment for heads. Also, one *T. melanocephala* was assigned to *T. vitticeps* for cross validation of wings (97%) (Supplementary Table 2).

3.2.4. Shape changes—Shape changes for the heads in the first principal component (Fig. 5.A) clearly show that the main distinction between G1 and G2 is related with the position of the maximum curvature of eyes, making the heads of species from G1 thinner. Notably, the distances between the eyes and the antennal insertion (landmarks 2–3 and 7–8) were markedly differentiated between these groups. For wings (Fig. 5.B), the main difference is related to distances between two points: the first is the intersection between cubitus and postcubitus and the second are the intersections between media and cubitus (m–cu), involving landmarks 3, 4 and 5.

4. Discussion

For triatomines, the terms “species complex”, “species subcomplex”, and “species group” are used to group species with similar morphological, ecological, cytogenetic, or even geographic distribution patterns. In this work, we aimed to evaluate the bases for grouping species of the *T. brasiliensis* complex as defined by Mendonça et al. (2009) and Costa et al. (2013): *T. brasiliensis*, *T. juazeirensis*, *T. sherlocki* and *T. melanica*, considering as “candidate” members the ones included by Schofield and Galvão (2009): *T. lenti* and *T. petrocchiae* as well as the Brasiliensis subcomplex (Schofield and Galvão, 2009) which included all aforementioned species with the addition of *T. melanocephala*, *T. vitticeps* and *T. tibiamaculata*. Both morphological and molecular evidence analyzed here suggest that the species herein studied conform two distinct groups, namely G1 and G2. The first included species of the *T. brasiliensis* species complex in addition to candidate members *T. lenti* and *T petrocchiae*, which conform a monophyletic clade composed of species entirely confined to the Caatinga Biome. The second (G2) was composed by *T. vitticeps*, *T. melanocephala*, and *T. tibiamaculata*, which were recovered as related to *P. megistus.* Hence all methods suggest the inclusion of *T. lenti* and *T. petrocchiae* within the *T. brasiliensis* complex, while suggesting the exclusion of *T. melanocephala*, *T. vitticeps*, and *T. tibiamaculata* from the Brasiliensis subcomplex based on its closer relationship to lineages of other genera in South

American Triatomini, as *Panstrongylus*. Indeed, the inclusion of the later three species was questioned in a recent study based on phylogenetic reconstruction (Gardim et al., 2014). Cross validation and discriminant function showed a few incorrect classifications between G1 and G2, which may explain why species of these groups were included together in the Brasiliensis subcomplex by Schofield and Galvão (2009).

All species included in G2 are from the Atlantic Forest biome — except for *P. megistus*, a species believed to have been introduced in the Caatinga but later eliminated by control measures, as it had remained confined to domiciliary structures in this biome (Dias et al., 2000). As the Atlantic Forest biome extends to other states outside Bahia, the distribution of G2 species are expected to be broaden to other states. A detailed study on the geographic (and potential) distribution of *T. brasiliensis* complex species using ecological niche modeling, reported *T. brasiliensis*, *T. juazeirensis*, *T. sherlocki*, and *T. melanica* as allopatric or parapatric (Costa et al., 2014). Galvão et al. (2003) reported the occurrence of *T. petrocchiae* in some spots of the Caatinga biome of states of Bahia, Paraíba, Pernambuco and Rio Grande do Norte, although it probably occurs throughout the Brazilian Caatinga, as occasional findings were reported in Paraíba, Rio Grande do Norte and Ceará, in the same rocky habitats with *T. brasiliensis* (Almeida et al., 2016; Caranha et al., 2011). Up to date, *T. lenti* exhibits a restricted geographic distribution as it has only been recorded in Bahia State (BA) in the municipality of Macaúbas (Mendonça et al., 2014). A limited geographic distribution had also been recorded for *T. sherlocki*, only in the municipality of Gentio do Ouro (BA), and *T. melanica* recorded for Urandi (BA), both in the Caatinga Biome (Bahia State), but their distribution might extend to some parts of Cerrado biome in Minas Gerais (Costa et al., 2014; Souza et al., 2015). Indeed, Bahia state seems to have been the area of original diversification for members of G1 except for *T. brasiliensis* and *T. petrocchiae* that present broader distribution. It must be stressed that little is known about the geographic distribution of the rare *T. melanocephala* and *T. lenti*. Galvão et al. (2003) mentioned the occurrence of *T. melanocephala* in the states of Bahia, Paraíba, Pernambuco, Rio Grande do Norte and Sergipe, without details about the municipality nor the biome. The potential geographic distribution for the remaining vectors herein considered are discussed in Gurgel-Gonç alves et al. (2012). Moreover, a new putative member for the *T. brasiliensis* complex (*T. bahiensis*) has been recently suggested (Mendonça et al., 2016), but was not approached here due to its recent description released as this work was almost completed.

All applied phylogenetic methods strongly recovered *T. lenti* as a sister species to *T. melanica* and *T. petrocchiae* in a monophyletic clade that also contained the remaining species of the *T. brasiliensis* species complex, thus strongly supporting the inclusion of both species in this complex. *Triatoma petrocchiae* however, is more distant to other members of the complex. This fact is reflected on its inability to hybridize with other members of the complex. All experimental crossings combinations among species of the *T. brasiliensis* species complex (*T. brasiliensis*, *T. juazeirensis*, *T. melanica*, *T. sherlocki* and *T. lenti*) produced viable hybrids (Costa et al., 2003b; Almeida et al., 2012; Correia et al., 2013; Mendonça et al., 2014), whereas *T. petrocchiae* and *T. brasiliensis* s.l. failed to produce hybrids under laboratory conditions (Espínola, 1971). Interestingly, *T. petrocchiae* is the only species with a sympatric distribution with two members of *T. brasiliensis* species complex: *T. brasiliensis* and *T. juazeirensis* (Costa et al., 2014; Caranha et al., 2011), and

intermediate forms between members of *T. brasiliensis* species complex and *T. petrocchiae* were never found in nature, which reinforces the laboratory evidence of reproductive incompatibility (Espínola 1971). In contrast, intermediate forms between *T. brasiliensis* and *T. juazeirensis* were recently characterized throughout morphological and ribosomal gene analysis, suggesting the occurrence of a natural hybrid zone placed in the semi-arid areas in Pernambuco state (Costa et al., 2016).

Overall, confronting morphometrics with phylogenetic data led to congruent results, except for the pronounced higher morphometric distances of *P. megistus* obtained from heads, which was not followed by wing morphometrics nor genetic distances. Indeed, the head structural differentiation derived landmarks related to the generic distinctive characteristic: according to Lent and Wygodzinsky (1979), *Panstrongylus* differs of *Eratyrus*, *Paratriatoma*, *Dipetalogaster*, and *Triatoma* by exhibiting heads larger in width and with antenniferous tubercles inserted extremely close to eyes. We suggest that although useful to distinguish *P. megistus*, this taxonomic character may not be the reliable to obtain phylogenetic inferences of *Panstrongylus*, because it does not correlate with the genetic and morphometric distances for wings. Indeed, Gardim et al. (2014) have already alerted the need of a generic revision in Triatomini, mainly regarding this inconsistency to cluster *Panstrongylus* isolated to *Triatoma*.

Combining morphometric and molecular approaches has provided important clues about species complexes delimitation (Almeida et al., 2009a; Márquez et al., 2011). For the *T. brasiliensis* species complex, either morphological (Costa et al., 1997a; Costa et al., 2009) or molecular (Costa et al., 1997b; Hypsa et al., 2002; Monteiro et al., 2004; Almeida et al., 2008; Mendonça et al., 2009; Guerra et al., 2016) approaches have been used independently. By using both approaches in this study we provide strong support to include *T. lenti* and *T. petrocchiae* in the *T. brasiliensis* species complex. It remains however a question whether the similarity between *T. petrocchiae* and *T. brasiliensis* is a result of retention of ancestral characters or convergence. This last supposition is reinforced by the finding of *T. brasiliensis* and *T. petrocchiae* over the same rock in some semi-arid spots of Paraiba and Rio Grande do Norte (Almeida et al., 2016). The influence of geological changes on the diversification of Neotropical triatomines might provide insights to explain the diversification history among the studied species (Justi et al., 2016), although the use of other markers (e.g. Single Nuclear Polymorphisms) would be required to elucidate it.

4.1. Epidemiological implications

Triatoma brasiliensis is beyond doubt the most domiciliated native species in Brazil and currently the most important Chagas disease vector in semiarid zones (Costa et al., 2003a). Moreover, its involvement in recent Chagas disease outbreaks has been suggested (Lilioso et al., 2017). The constraint presented by classical morphology can be exemplified by the *T. petrocchiae* and *T. lenti*, as well as the most differentiated species, *T. sherlocki. Triatoma petrocchiae* is so similar to *T. brasiliensis* that it has been considered in the past as just a chromatic variation of *T. brasiliensis* (Lucena 1970). Instead, *T. sherlocki* is distinguishable even for the laymen, as the local people call it “procoto vemelho” (=red bug, see Almeida et al., 2009b). Despite this morphological differentiation of *T. sherlocki*, all approaches applied

here were congruent to suggest it is properly placed within the *T. brasiliensis* species complex. As *T. petrocchiae* with *T. brasiliensis* overlap in geographic distribution, their superficial similarity may confound those involved in vector control campaigns, and if *T. petrocchiae* bugs are entering in domiciles they may be recorded as *T. brasiliensis*. To complicate more this possible scenario, there is no objective morphological way to recognize nymphs. Therefore, for future studies we recommend developing a key to differentiate nymphs and adults for all members of *T. brasiliensis* species complex (species of G1). Finally, as *T. petrocchiae* is related to members of the *T. brasiliensis* species complex — species of recognized vector capacity (Folly-Ramos et al., 2016)—we recommend strengthening its surveillance and improving the knowledge on its capacity to get infected and to transmit *T. cruzi*.

Classifying species into groups of species is an important means to retrieve taxonomic information and therefore it should not be based on a single type of evidence, such as morphological resemblance, but take into consideration species phylogeny, cytogenetic evidence, and morphometry. Lately, inappropriate grouping for species that involve *T. brasiliensis*—the most important Chagas disease vector in semi-arid zones of Brazil —has led to continuous confusion, caused by a variety of prevailing opinions. Neither morphological nor eco-geographic characters alone have been sufficient to propose natural groups. In this study, we combined morphological characters from two distinct structures and four mitochondrial genes and came to the conclusion that independently of the species group name ("*T. brasiliensis* species complex" or "Brasiliensis subcomplex") it should be composed of *T. brasiliensis, T. juazeirensis, T. melanica, T. sherlocki, T. lenti*, and *T. petrocchiae*, and probably *T. bahiensis* (not included in our study). Moreover, we recognized that the main head characters used to differentiate *Panstrongylus* and *Triatoma* is not consistent with the genetic information neither with wings morphological variation, and we reinforce previous concerns about the need of a taxonomic revision in Triatomini.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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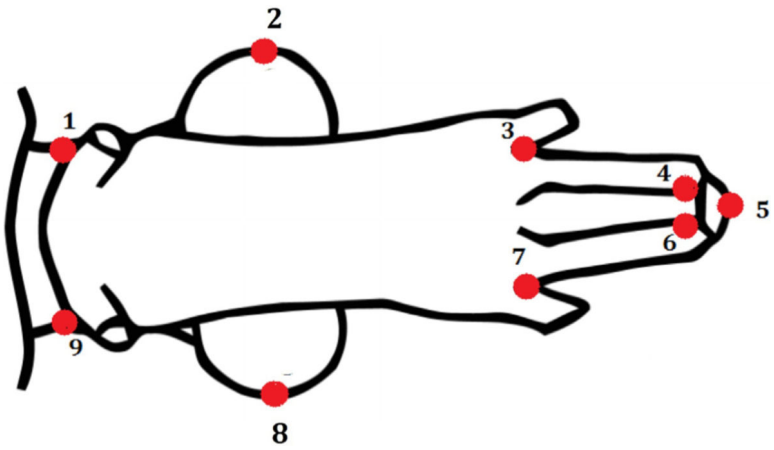

Fig. 1.

Illustration adapted from Lent and Wygodzinsky (1979), showing landmarks on the heads. 1. Meeting point between neck and head at the left side; 2. Maximum curvature of the left eye; 3. Left point for the antenniferous tubercle insertion; 4. Closest left point between gena and anteclypeus; 5. Median anterior point of the anteclypeus; 6. Closest right point between gena and anteclypeus; 7. Right point for the antenniferous tubercle insertion; 8. Maximum curvature of the right eye; 9. Meeting point between neck and head at the right side.

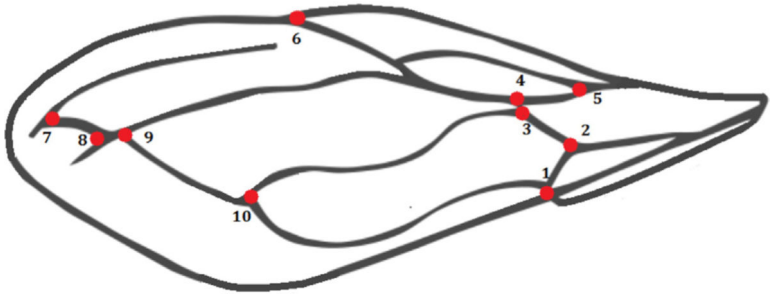

Fig. 2.

Landmarks on the right wings. Corium portion: 1. Intersection of Pcu and Pcu + first anal vein; 2. Intersection of Cu and Cu–postocubitus (Cu–Pcu); 3. Intersection of Cu and M–Cu; 4. Intersection of media and cubitus (M–Cu); 5. Bifurcation of the radius (R) and median (M) veins; 6. Membrane portion on radius vein; 7. First intersection of R + M and Pcu (postocubitus); 8 Second intersection of R + M and Pcu (postocubitus); 9. Intersection of M and extension of Cu–Pcu veins; 10. Intersection of Pcu and Cu.

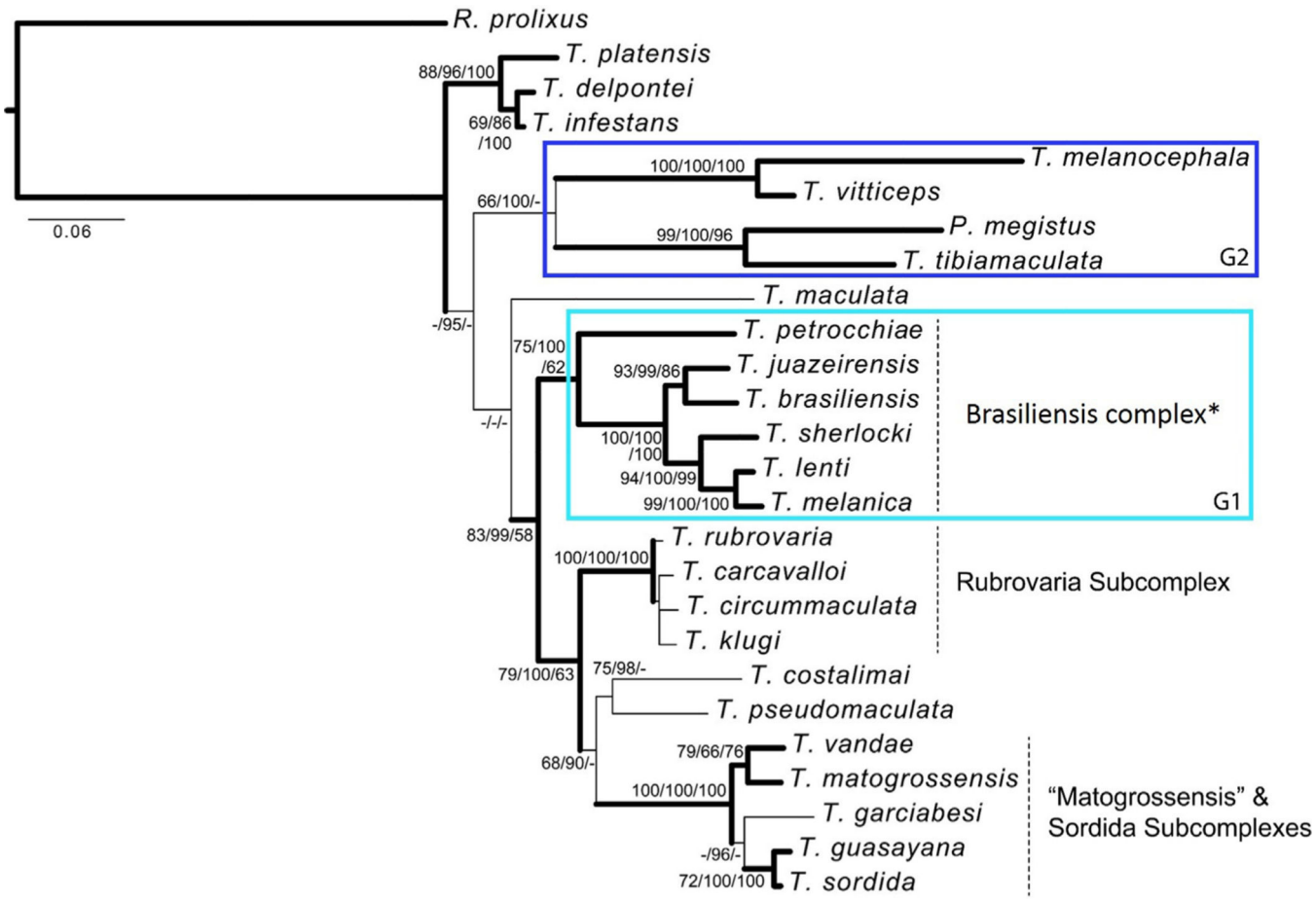

Fig. 3.

Maximum likelihood tree (-lnL = 10360.4245) of the combined analysis of 1811 bp of 16S, COI, CytB, and 12S sequences of South American triatomines. Molecular evolution models for each partition were GTR+I+G, except HKY+G for 12S. Thick clades represent those also recovered by at least one other phylogenetic method. Clade supports are: Maximum Likelihood bootstrap/Bayesian Posterior Probability/parsimony bootstrap. Monophyletic groups of species herein focused are represented by G1 (light blue) and G2 (dark blue).

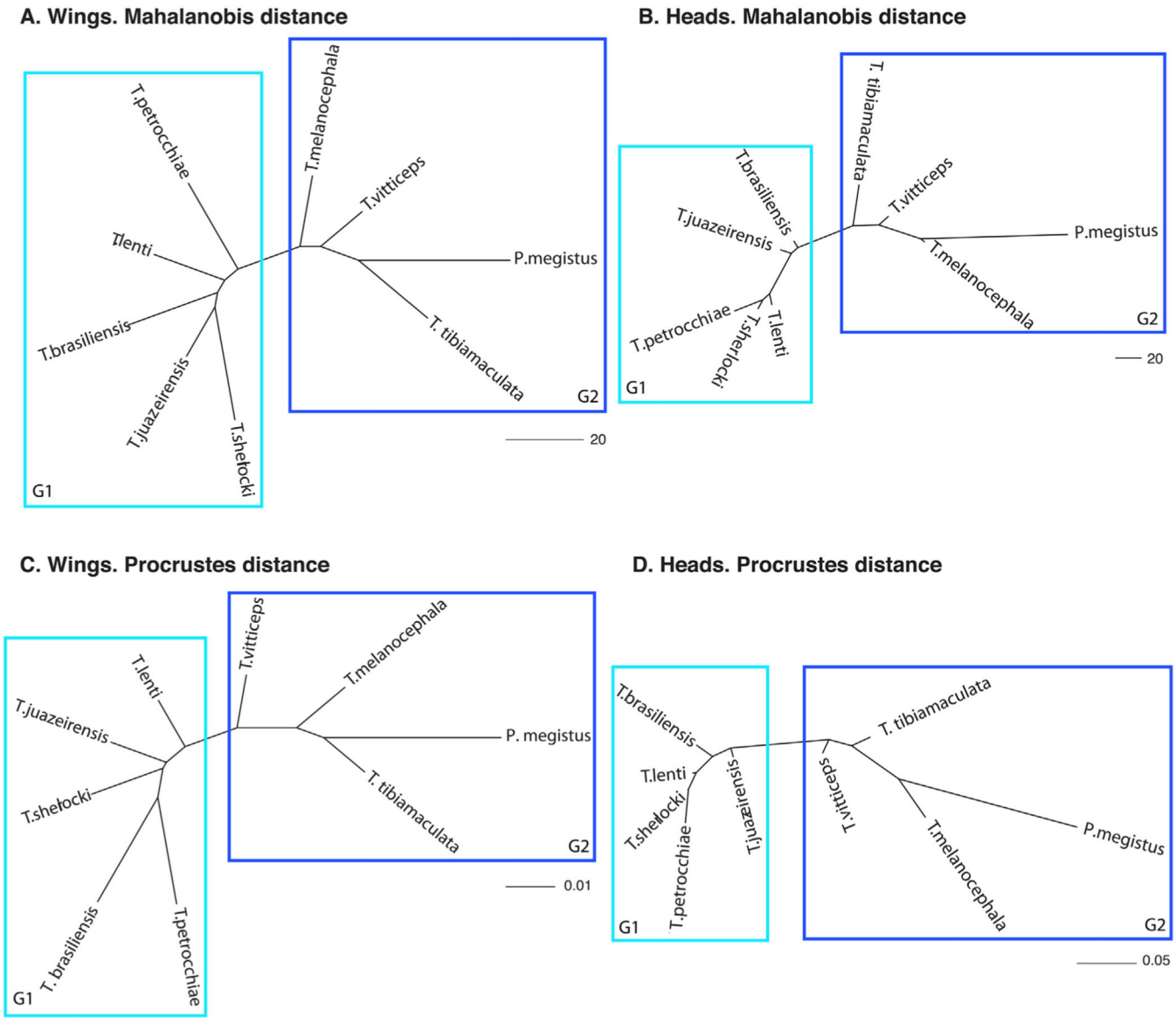

Fig. 4.

Interspecific shape differences. Unrooted neighbor-joining trees from pairwise Mahalanobis (A, B) and procrustes (C, D) distance matrices for wings (A, C) and heads (B, D) geometric morphometric datasets. Monophyletic groups of species herein focused are represented by G1 (light blue) and G2 (dark blue).

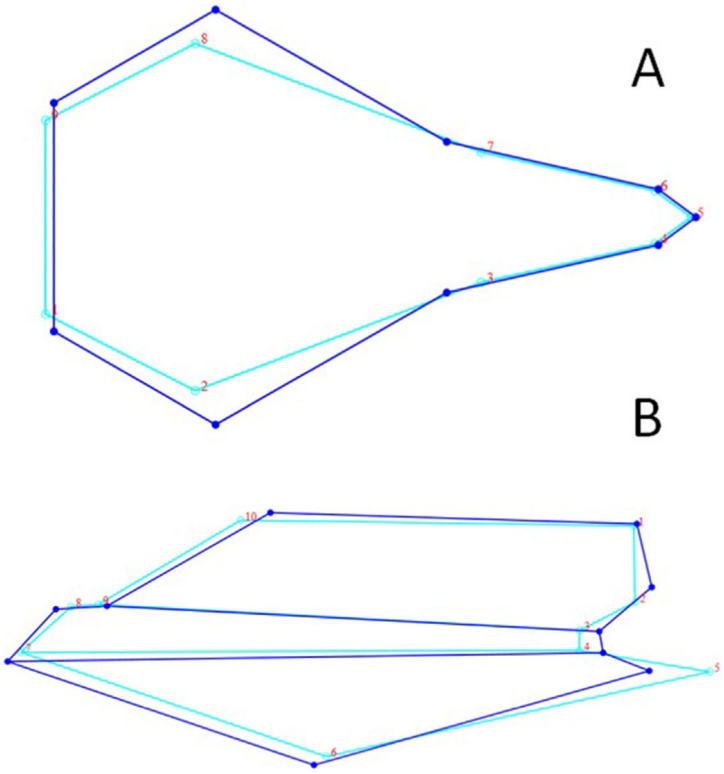

Fig. 5.

Shape changes for the first component analysis of heads (A) and wings (B). Light blue consensus is related to Group 1 (*Triatoma brasiliensis*, *T. juazeirensis*, *T. lenti*, *T. petrocchiae*, and *T. sherlocki*) whereas the dark blue for Group 2 (*T. tibiamaculata*, *T. vitticeps*, and *Panstrongylus megistus*).

Table 1

Taxon and gene sampling with accession codes from GeneBank sequences used in this study.

Subcomplex	Species	12S	16S	COI	Cytb
Triatoma brasiliensis	*T. brasiliensis*	AF021187	EU827222	AF021186	FJ623064
	T. juazeirensis	–	KC249026	AF826892	AY494169 *
	T. lenti	–	KY576788 **	KY576791 **	KY576789 **
	T. melanica	–	KC249461	KC249041	AY336527 *
	T. melanocephala	–	KF769452	AF826893	KF826898
	T. petrocchiae	KY654072 **	KY654073 **	KY654074 **	KY654075 **
	T. sherlocki	–	EU489057	KC608987	EU489058
	T. tibiamaculata	AY185829	AY185843	KC249390	KC249297
	T. vitticeps	AF021217	KC249088	AF021219	KC249303
T. infestans	*T. delpontei*	AF021197	AF324520	FJ439768	HQ333241
	T. infestans	AF021197	EU143699	AF021199	AY702023
T. maculata	*T. maculata*	AF324512	EU827231	AF449139	KC608977
	T. pseudomaculata	AY185827	EU827225	KC608986	KC608979
T. matogrossensis	*T. guazu*	–	KC571994	KC608984	KC608976
	T. matogrossensis	–	KC571995	KC608985	KC608978
	T. vandae	–	KC571997	KC608989	KC249298
	T. williami	–	KC571998	KC608990	KC608981
T. rubrovaria	*T. circummaculata*	AF021190	AF021188	AF021191	–
	T. carcavalloi	–	KC248991	KC249322	KC249244
	T. rubrovaria	AF021207	AF021203	AF021204	KC249281
T. sordida	*T. garciabesi*	AY185821	AY185835	EF451041	KC249249
	T. sordida	AF021208	KC571996	KC608988	KC608980
Panstrongylus megistus	*P. megistus*	AF021180	KC248970	AF021179	AF045722
Outgroup	*Rhodnius prolixus*	AF324508	AF324519	AF449138	EF011726

(−) Not available.

*Haplotypes named as *T. brasiliensis* in GenBank, because they were deposited before being raised to *T. juazeirensis* or *T. melanica*.

**sequenced obtained in this study.

Table 2

Geographic origin, sex, and number of specimens used for morphometric analyses.

Species	Locality, State/Biome	Geographic coordinates	
Panstrongylus megistus	Ortiqueira, Paraná/Atlantic Forest	24° 12′ 46.9“S	50° 55′ 35.8“W
Triatoma brasiliensis	Pedra do Sino, Rio Grande do Norte/Caatinga	6° 16′ 23.7“S	36° 29′ 44.2“W
T. juazeirensis	Curaç a, Bahia/Caatinga	8° 59′ 33.3“S	39° 54′ 17.6“W
T. lenti	Macaúbas, Bahia/Caatinga	13° 01′ 08.3“S	42° 41′ 10.4“W
T. melanocephala	Jequié, Bahia/Atlantic Forest	13° 52′ 08.5“S	40° 04′ 48.0“W
T. petrocchiae	Caicó, Rio Grande do Norte/Caatinga	6° 27′ 51.8“S	37° 04′ 19.2“W
T. sherlocki	Santo Inacio, Bahia/Caatinga	11° 06′ 41.9“S	42° 43′ 20.2“W
T. tibiamaculata	Mogi-Guaç u, São Paulo/Atlantic Forest	22° 23′ 04.8“S	46° 55′ 42.6“W
T. vitticeps	Guarapari, Espirito Santo/Atlantic Forest	20° 40′ 49.7“S	40° 30′ 33.7“W