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Short communication

Multiple mitochondrial genes of some sylvatic Brazilian *Triatoma*: Non-monophyly of the *T. brasiliensis* subcomplex and the need for a generic revision in the Triatomini

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ABSTRACT

Multiple fragments of mitochondrial DNA genes (cytochrome *b*, cytochrome oxidase I, and 16S rDNA) were used to evaluate the phylogenetic relationships among *Triatoma melanocephala*, *Triatoma tibiamaculata*, *Triatoma vitticeps*, and other members of *Triatoma brasiliensis* subcomplex under a Bayesian framework and maximum parsimony criterion. With the addition of new sequences of *T. tibiamaculata* and *T. vitticeps*, *Triatoma juazeirensis*, *Triatoma melanica* and the newly sequenced *T. melanocephala,* the three first sylvatic species, *T. melanocephala*, *T. tibiamaculata* and *T. vitticeps*, were strongly recovered into a clade separate from the other with the remaining *Triatoma* species from South America, such as the members of *T. brasiliensis* subcomplex. *Panstrongylus megistus* was recovered as a sister to *T. tibiamaculata*, whereas *T. vitticeps* was a sister to *T. melanocephala.* This study revealed the non-monophyly of the *T. brasiliensis* subcomplex, and the polyphyly of *Triatoma* was reinforced by the placement of these three sylvatic species with *Dipetalogaster*, *Meccus, Mepraia*, and *Panstrongylus*. The results herein shown highlight the need of generic revision in Triatomini.

1. Introduction

Chagas disease affects 7–8 million people worldwide, mostly in Latin America, yet no vaccine has been developed. It is caused by a protozoan parasite, *Trypanosoma cruzi,* transmitted to humans mainly by blood-sucking bugs, the triatomines. Vector transmission is still considered the main mode of infection in Brazil. Therefore, most efforts against Chagas disease focus on the interruption of its natural transmission by controlling domiciliary vector populations with pyrethroid insecticides (Vinhaes and Dias, 2000; WHO, 2013). However, the epidemiological importance of these Chagas disease vectors is continually changing. *Triatoma sherlocki*, for example, was described in 2002 as a sylvatic species, and was recently found invading and colonizing human domiciles in Bahia State (Papa et al., 2002; Almeida et al., 2012); and several other species can be mentioned in this context (e.g., Costa, 1999; Costa and Lorenzo, 2009; Schofield and Galvão, 2009).

These changes in vector importance from an epidemiological standpoint are in part a reflection of an increase of studies on triatomine diversity, especially in Brazil. After the first reported human cases of the disease by Chagas (1909), studies on vector species reservoirs and description of new triatomine species started to increase. Currently, the subfamily Triatominae consists of 147 species and 18 genera, of which sixty-five species occur in Brazil (Galvão et al., 2003; Poinar, 2005; Costa et al., 2006; Galvão and Ângulo, 2006; Bérenger and Blanchet, 2007; Costa and Felix, 2007; Martínez et al., 2007; Sandoval et al., 2007; Jurberg et al., 2009, 2013; Rosa et al., 2012, Gonçalves et al., 2013; Alevi et al., 2013a). More recently, phylogenetic approaches have been used to understand evolutionary relationships among these species (Marcilla et al., 2001, 2002; Bargues et al., 2008, 2010; Costa et al., 2013; Costa and Lorenzo, 2009; Schofield and Galvão, 2009; Gurgel-Gonçalves et al., 2012; Rosa et al., 2012; Gardim et al., 2013), but most of these studies lack from good taxa or character sampling to produce a robust phylogeny. A robust phylogenetic hypothesis can be used as the basis for an informative and

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predictive classification for the group and highlight sylvatic species with potential to become epidemiologically important.

Members of *Triatoma* are grouped into complexes and subcomplexes based on morphological similarities, geographic distribution, epidemiological importance, phylogenetic relationships, and other features. At the moment, there is no consensus about the features that define complexes (Usinger et al., 1966; Lent and Wygodzinsky, 1979; Costa and Lorenzo, 2009; Rosa et al., 2012). In an attempt to consider most classifications proposed based on different rationales, Schofield and Galvão (2009) grouped together *Triatoma melanocephala*, with *Triatoma petrochiae*, *Triatoma lenti*, *Triatoma brasiliensis*, *Triatoma juazeirensis*, *Triatoma melanica*, and *T. sherlocki* in the *T. brasiliensis* subcomplex of the *Triatoma infestans* complex. They also tentatively placed *Triatoma tibiamaculata* and *Triatoma vitticeps* in this group based on morphological similarities, but point out that based on 16S rDNA sequences and cytogenetics they were not found to be closely related to members of the *T. brasiliensis* subcomplex. To date, only the last four (*T. brasiliensis*, *T. juazeirensis*, *T. melanica*, and *T. sherlocki*) were recovered as a monophyletic group based on the analysis of the mitochondrial genes cytochrome *b* (Cytb) and 16S rDNA (16S) (Monteiro et al., 2004; Mendonça et al., 2009). Given that DNA samples for sylvatic species are difficult to be obtained, species complexes and subcomplexes definition has been chiefly based on morphological and ecological features; and for this reason, Cytb, cytochrome oxidase I (COI), and 16S sequences of mitochondrial DNA were obtained and used to evaluate the phylogenetic relationships among *T. melanocephala*, *T. tibiamaculata*, *T. vitticeps*, and other triatomines, with focus on members of *T. brasiliensis* subcomplex.

2. Material and methods

Insects for DNA extraction were randomly obtained from colonies (CTA) maintained at the Triatominae Insectarium of the Department of Biological Sciences, School of Pharmaceutical Sciences, São Paulo State University (Araraquara, Brazil). Details about the origin of insects can be found in Table 1. Multiple specimens were used for gene sequencing of *T. tibiamaculata* ($N = 3$), *T. vitticeps* ($N = 3$) and specimens of three geographic occurrence points of *T. melanocephala* distribution ($N = 3$ for each), due to the reasonable geographic distance among sites (22–80 km), herein called 1, 2, and 3. In addition, we deposited new sequences for *T. juazeirensis* and *T. melanica* (COI and 16S) extracted from a single specimen for each species (see Table 2).

The species set choice for the analysis was based on (i) a broad taxon sampling representing species of different genera of Triatomini, previously recovered as related to the species of interest (members of *T. brasiliensis* subcomplex) and (ii) availability of sequences for the genes studied for these species in GenBank. Therefore, after an explorative search, not all species chosen had all three markers available for the analysis.

Total DNA extraction was performed according to the protocol described by Sambrook and Russell (Gaunt and Miles, 2002). From the extracted DNA, 16S and COI fragments were amplified as described by Mello (1982), and for Cytb as by Hypsa and collaborators (2002), and purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Life Sciences). Purified products were subjected to a sequencing reaction using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and were analyzed in the ABI PRISM® 377 DNA Sequencer (Applied Biosystems). Additional sequences of Cytb and 16S and COI deposited at GenBank were added to the analysis, including several representative species of South and North American species complexes and subcomplexes. *Rhodnius prolixus* were used to root the resulting phylogenies (Table 2).

Sequences were edited with BioEdit 7.0.5 and aligned with ClustalW (Larkin et al., 2007). Nucleotide data for Cytb and COI were transformed into aminoacid sequences to check the alignment. Phylogenetic analyses of concatenate 16S, Cytb, and COI sequences were run under a Bayesian framework in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; 2 independent runs, 4 chains, and 1 M gens) and under a maximum parsimony criterion in PAUP* 4.0b10 (Swofford, 1998; *hsearch*, 1000 random addition replicates with TBR branch swap, gaps treated as "?"). The following evolutionary models were chosen for the three partitions using the Akaike Information Criterion in MrModeltest (Nylander, 2004): HKY + G for 16S rDNA; GTR + I + G for Cytb; and for COI was used HKY + I + G. Clade support was estimated by Bayesian posterior probabilities (BPP) and 1,000 parsimony bootstrap pseudoreplicates (BP).

3. Results

Sixteen new sequences were obtained (five for 16S, five for Cytb, and six for COI) and aligned with others available at GenBank (Table 2) and cropped according to the shorter sequences. The alignment constructed for the phylogenetic analysis included 256 bp of 16S (95 variable sites, including 79 parsimony informative), 313 bp of Cytb (141 variable sites, including 128 parsimony informative), and 202 bp of COI (87 variable sites, including 76 parsimony informative), totalizing 771 bp evaluated. However, sequences deposited in the GenBank were much longer, with variable length.

A single haplotype for each gene was found for *T. tibiamaculata* and *T. vitticeps*. Each population of *T. melanocephala* also exhibited a single haplotype for each gene. Interspecific pairwise divergences (uncorrected p-distances) between *T. melanocephala* specimens varied in 16S sequences from 0.8% (between 1 vs. 3) to 1.2% (2 vs. 3 and 1), in Cytb sequences from 0.3% (between 1 vs. 3) to 4.1% (2 vs. 1), and in COI sequences from 4.8% (between 1 vs 2) to 10.8% (3 vs. 2). Given that 1 and 2, 2 and 3, and 1 and 3 are localities distant by 73.9 km, 80.4 km, and 22.0 km, the variation found

Table 1
Number and data of colonies studied.

Species	Number (CTA)[a]	Origin	Initiated	Coordinates
T. juazeirensis	207	Juazeiro - BA	01/12/2007	-9 42′ 63.43637″, -40 50′ 31.55027″
T. melanica	206	Urandi - BA	29/07/2008	-14 76′ 40.25299″, -42 64′ 99.30749″
T. melanocephala	221 (1)[b]	Bom Jesus da Serra – BA	26/04/2010	-14 22′ 04.46160″, -40 30′ 52.55281″
	219 (2)[b]	Jequié – BA	08/10/2009	-13 51′ 03.75834″, -40 04′ 52.22281″
	220 (3)[b]	Poções – BA	17/11/2009	-14 31′ 01.94880″, -40 22′ 43.37040″
T. tibiamaculata	195	Mogi-Guaçu – SP	17/06/1986	-22 22′ 14.51637″, -46 56′ 16.73298″
T. vitticeps	198	Guarapari - ES	01/11/1979	-20 39′ 01.41478″, -40 30′ 25.29000″

[a] CTA - Colony number registration.
[b] 1, 2 and 3 - different collection spots of *T. melanocephala*.