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Evolutionary Relationships of the *Triatoma matogrossensis* Subcomplex, the Endemic *Triatoma* in Central-Western Brazil, Based on Mitochondrial DNA Sequences

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Abstract. The phylogenetic relationships among species of *Triatoma matogrossensis* subcomplex (*T. baratai*, *T. guazu*, *T. matogrossensis*, *T. sordida*, *T. vandae*, and *T. williami*) was addressed by using fragments of cytochrome oxidase I (COI), 16S rDNA (16S), and cytochrome *b* (Cytb) through Bayesian and parsimony analyses. We did not recover a monophyletic *T. matogrossensis* subcomplex, and their members were found clustered in three strongly supported clades, as follows: i) *T. jurbergi* + *T. matogrossensis* + *T. vandae* + *T. garciabesi* + *T. sordida*; ii) with *T. guasayana* as the sister group of clade (i); and iii) *T. williami* + *T. guazu*, however not closely related to the clade formed by the previously mentioned species. The other two endemic species from Central-Western Brazil, *T. baratai* and *T. costalimai*, were not recovered with strong clade support as related to other members of this subcomplex. Results call for a further revision in the classification of the subcomplexes within the genus *Triatoma*.

INTRODUCTION

Carlos Chagas[1] described *Trypanosoma cruzi*, the causative agent of American trypanosomiasis, in 1909 and noted that this protozoan was transmitted by triatomines. After the first reported human cases of the disease, studies on the vector species reservoirs and description of new triatominae species started to increase. Galvão and others[2] included 19 genera and 137 species into the subfamily Triatominae, but currently it consists of 145 valid species belonging to 18 genera, of which 65 species occur in Brazil.[3–11]

Some *Triatoma* species are grouped into complexes, and subcomplexes based on morphological similarities, geographical distribution, epidemiological importance, phylogenetic relationships, and others. At the moment, there is no consensus about the features that define complexes.[11–14] Thirteen *Triatoma* species have been found in Central-Western Brazil, but only seven are considered endemic: *T. baratai*, *T. costalimai*, *T. deanorum*, *T. jurbergi, T. matogrossensis, T. vandae*, and *T. williami*.[2,13,15,16] Based on morphological similarities, Carcavallo and others,[17] grouped these species into the *T. oliveirai* complex together with *T. klugi* and *T. oliveirai*, which are found in Southern Brazil. We must stress that *T. oliveirai*, *T. baratai*, and *T. deanorum* are rare species, difficult to collect[18]; and for this reason studies about them are scarce on all grounds. The epidemiological importance related to Chagas disease vectors is continually changing. *Triatoma sherlocki*, for example, was described in 2002 as a sylvatic species,[19] and was recently found invading and colonizing human domiciles in Bahia State[20,21]; it highlights the importance of understanding the phylogenetic relationships of sylvatic species. Several other species can be mentioned in this context.[14]

The latest classification scheme, proposed by Schofield and Galvão,[13] summarizing results from different analyses, placed members previously considered belonging to the *T. oliveirai* complex into two subcomplexes (within the *T. infestans* complex): i) *T. baratai*, *T. costalimai*, *T. deaneorum*, *T. guazu*, *T. jurbergi*, *T. matogrossensis*, *T. vandae*, and *T. williami*, forming the *T. matogrossensis* subcomplex; and ii) *T. oliveirai* and *T. klugi*, included in the *T. rubrovaria* subcomplex, along with four other species from the Southern region of Brazil: *T. carcavalloi*, *T. circummaculata*, *T. limai*, and *T. rubrovaria.* Of additional interest to this work, *Triatoma sordida* was placed in the *T. sordida* subcomplex, which included *T. garciabesi*, *T. guasayana*, and *T. patagonica*.

Triatominae species that overlap in geographic distribution are sometimes subjected to the same ecological pressures, and it remains a question whether the evident morphological similarities among them are a result of ecological convergences or of their phylogenetic relationships. On the other hand, distinct ecological forces might also promote morphological differentiation among sympatric phylogenetically related species.[22] In sylvatic species, for which samples are difficult to be obtained, species complex definition has been chiefly based on morphological and ecological features; and for this reason, cytochrome b (Cytb), cytochrome oxidase I (COI), and 16S rDNA (16S) sequences of mitochondrial DNA were used to evaluate the phylogenetic relationships among seven *Triatoma* species occurring in Central-Western Brazil.

MATERIAL AND METHODS

Multiple specimens of seven sylvatic species of *Triatoma* were used for gene sequencing: *T. baratai* (N = 4), *T. costalimai* (N = 6), *T. guazu* (N = 6), *T. matogrossensis* (N = 5), *T. vandae* (N = 4), *T. williami* (N = 6), and *T. sordida* (N = 5) (Table 1, Figure 1). Except for *T. sordida*, all of the previously mentioned species belong to the *T. matogrossensis* subcomplex. However, *T. sordida* was included in the analysis because of its epidemiological importance and because some studies have shown this species as related to *T. matogrossensis*.[12,23,24] According to Forattini[25] and Galvão and others,[2] Central-Western Brazil is the center of dispersion of these seven species. The specimens studied

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TABLE 1
Colony number (CTA - from the Triatominae Insectarium, São Paulo State University), origin and start date of colonies studied

Species	Colony number	Initiated	Origin	Coordinates
Triatoma baratai	CTA 247	08/2007	Nioaque, Mato Grosso do Sul (MS)	55.83°W 21.13°S
Triatoma costalimai	CTA 191	03/1996	Mambaí, Goiás (GO)	46.11°W 14.48°S
Triatoma guazu	CTA 232	07/2001	Barra do Garça, Mato Grosso (MT)	52.25°W 15.89°S
Triatoma matogrossensis	CTA 248	05/2007	Rio Verde de Mato Grosso, Mato Grosso do Sul (MS)	54.84°W 18.91°S
Triatoma sordida	CTA 028	08/1982	Brasilândia, Mato Grosso do Sul (MS)	52.03°W 21.25°S
Triatoma vandae	CTA 231	07/2001	Itiquira, Mato Grosso (MT)	54.15°W 17.20°S
Triatoma williami	CTA 184	12/1995	Barra do Garça, Mato Grosso (MT)	52.25°W 15.89°S

were randomly obtained from colonies maintained at the Triatominae Insectarium of the Department of Biological Sciences, School of Pharmaceutical Sciences, São Paulo State University (Araraquara, Brazil). Because sequences of the mitochondrial genes studied herein of *T. vandae* and *T. baratai* were obtained for the first time, representative species of other groups of Latin America triatomines, such as the *T. brasiliensis* species complex and *T. rubrovaria* subcomplex, were also included for comparison.

The origin of the colonies of *T. matogrossensis* subcomplex maintained at the insectarium and their published distributional records were used in the construction of a map of the known geographic distribution (Figure 2).[2,12,13,16–18,23–32] In Brazil, *T. sordida* overlaps the geographic distribution of all species herein studied (also occurring in the states of Bahia, Goiás, Mato Grosso, Mato Grosso do Sul, Maranhão, Minas Gerais, Paraná, Pernambuco, Piauí, Rio Grande do Sul, Santa Catarina, and São Paulo). This species is also found in the Chaco eco-region of other countries: Argentina, Bolivia, Paraguay, and Uruguay.[2,25,33] Regarding the members of *T. matogrossensis* subcomplex, only *T. deaneorum* has not been included in this phylogenetic study because of its rarity, which has been also addressed by others authors.[18].

Genomic DNA extraction was performed according to the protocol described by Sambrook and Russell.[34] From the extracted DNA, 16S and COI fragments were amplified as described by Sainz and others,[23] and for Cytb as by Lyman and others,[35] and purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Piscataway, NJ). Purified products were subjected to a sequencing reaction using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) 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. *Rhodnius prolixus* was used as an outgroup (Table 2).[12,20,23,24,35–43]

Sequences were edited with BioEdit 7.0.5 and aligned with ClustalW.[44] Nucleotide data for Cytb and COI were transformed into amino acid sequences to check the alignment. Separate and combined phylogenetic analyses of 16S, Cytb, and COI sequences were run under a Bayesian framework in MrBayes 3.1 (2 independent runs, 4 chains, and 1 M gens)[45] and the maximum parsimony criterion in PAUP (*hsearch*, 1,000 random addition replicates with TBR branch swap, gaps treated as "?").[46] The following evolutionary models were chosen for the three partitions in the mixed-model Bayesian analysis using the Akaike information criterion in MrModeltest[47,48]: HKY+I+G for 16S rDNA; HKY+I+G for Cytb; for COI was used GTR+I+G. Clade support was estimated by Bayesian posterior probabilities

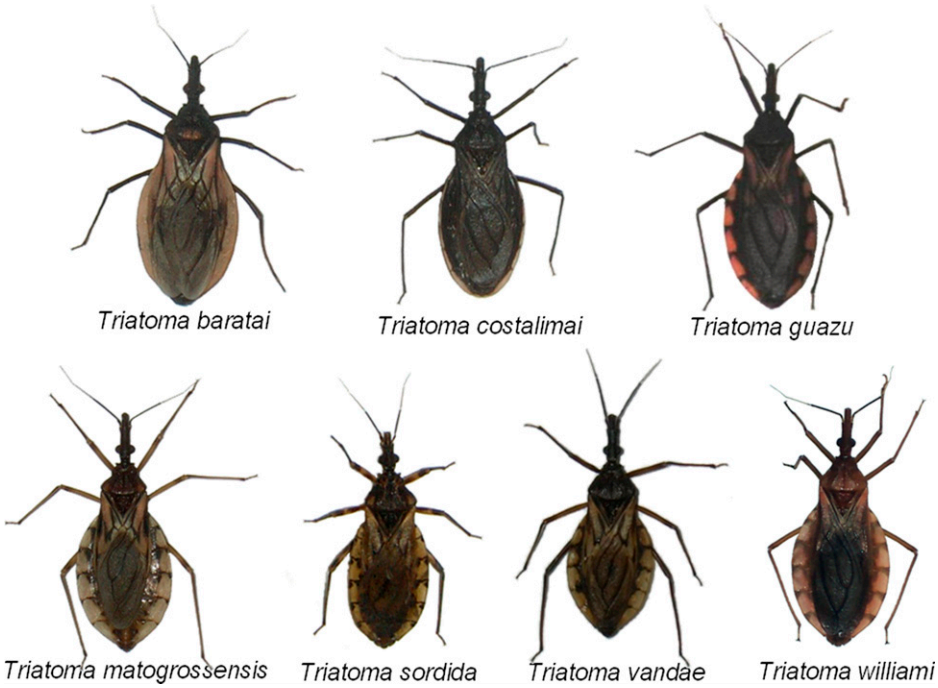

FIGURE 1. Dorsal view of specimens from the colonies studied: *T. baratai*, *T. costalimai*, *T. guazu*, *T. matogrossensis*, *T. sordida*, *T. vandae*, and *T. williami*. Pictures of Sueli Gardim.

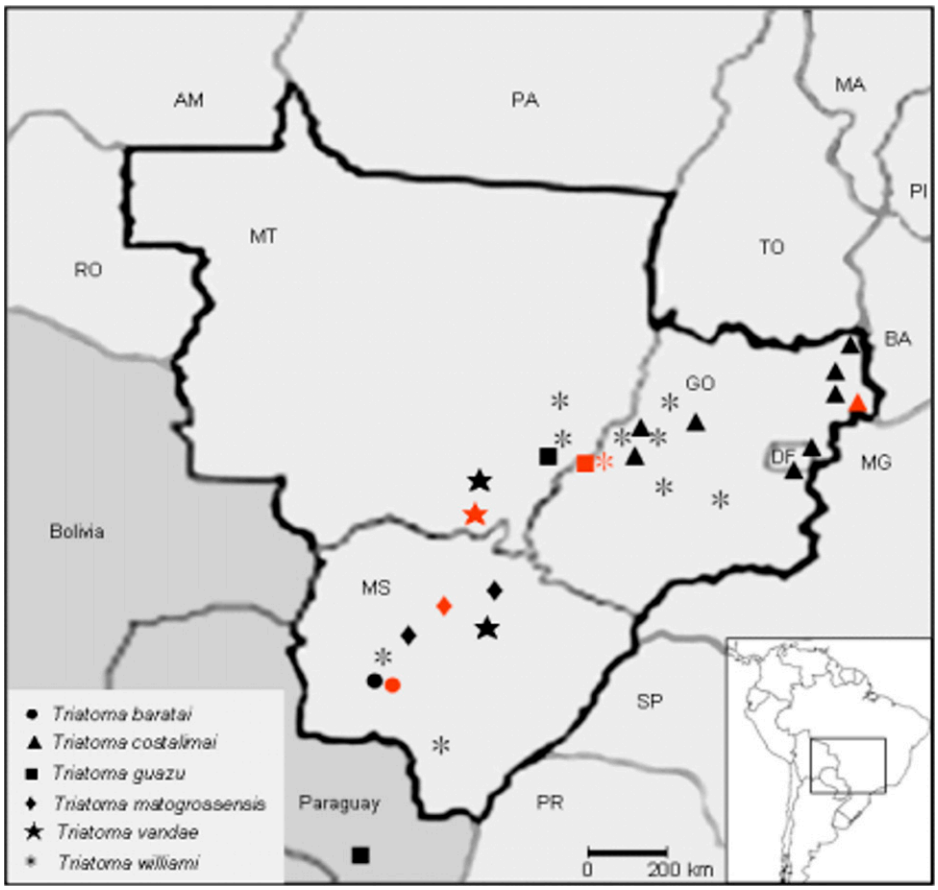

FIGURE 2. The known geographic distribution of species of the *T. matogrossensis* subcomplex in the Central-Western Region of Brazil.[2,13–16,22–28] In red spots where the specimens herein studied originated. For details, see Table 1. The geographic distribution of *T. sordida* overlaps all spots for the members of *T. matogrossensis* subcomplex, and the Chaco eco-region of Argentina, Bolivia, Paraguay, and Uruguay.[2,25,33]

TABLE 2

Accession codes from GeneBank sequences of *Triatoma* and outgroup species used in this study

Subcomplex	Species	16S	Cytb	COI
T. brasiliensis	*T. brasiliensis*	EU827222	AY336524	AF021186
	T. juazeirensis (haplotype F)	–	AY494168	–
	T. melanica (haplotype H)	–	AY336527	–
	T. sherlocki	EU489057	EU489058	**KC608987**
T. infestans	*T. infestans*	EU143699	AY702023	AF021199
T. maculata	*T. maculata*	EU827231	**KC608977**	AF449139
	T. pseudomaculata	EU827225	**KC608979**	**KC608986**
T. matogrossensis	*T. baratai*	**KC571991**	**KC608974**	–
	T. costalimai	**KC571993**	**KC608975**	**KC608983**
	T. guazu	**KC571994**	**KC608976**	**KC608984**
	T. jurbergi	AY035456	–	–
	T. matogrossensis	**KC571995**	**KC608978**	**KC608985**
	T. vandae	**KC571997**	–	**KC608989**
	T. williami	**KC571998**	**KC608981**	**KC608990**
T. rubrovaria	*T. carcavalloi*	**KC571992**	–	**KC608982**
	T. circummaculata	AF021188	–	AF021191
	T. klugi	AY035463	*	–
	T. rubrovaria	AF021203	GQ398003*	AF021204
T. sordida	*T. garciabesi*	AY185835	–	–
	T. guasayana	AF021194	*	AF021193
	T. patogonica	AY035464	–	–
	T. sordida	**KC571996**	**KC608980**	**KC608988**
outgroup	*P. megistus*	AF045701	AF045722	AF021179
	R. prolixus	AF045707	EF011726	AF449138

(Rocha and others, 2009, unpublished data with asterisks).[20,23–43] Sequences obtained in this study are in bold.
(–) Not available.

(BPP) and 1,000 pseudoreplicates of non-parametric parsimony bootstrap (PB).

RESULTS

Twenty-five new sequences were obtained (8 for 16S, 8 for Cytb, and 9 for COI; Table 2). These sequences were aligned with other sequences available at GenBank and cropped according to the shorter sequences. The alignment constructed for the phylogenetic analysis included 256 basepairs (bp) of 16S (83 variable sites, including 70 parsimony informative), 313 bp of Cytb (109 variable sites, including 84 parsimony informative), and 202 bp of COI (75 variable sites, including 58 parsimony informative), totalizing 771 bp evaluated.

For the *Triatoma* species a single haplotype for each gene was found, except for two Cytb haplotypes for *T. matogrossensis* and *T. baratai*, and three haplotypes of Cytb for *T. guazu*. In cases of two haplotypes, *p*-distances (all < 0.6%) were within the expected for intraspecific variation and after pilot phylogenic reconstructions, they were always clustered together. Therefore, a single haplotype was chosen as representative. Two of the sequenced Cytb haplotypes of *T. guazu* showed stop codons, and most probably represent pseudogenes, therefore were excluded from the analysis (see discussion below). *Triatoma costalimai* showed a large deletion of position 182 to 220 of Cytb sequence referring to a string of 13 amino acids. If this fragment is the homologous mitochondrial copy, apparently this deletion is not deleterious because it was confirmed in six adult specimens. However, because of the absence of stop codons, this sequence was maintained in the analysis, although there is a possibility it might be a pseudogene like as in *T. guazu*.

Interestingly, finding intraspecific variation in only these three species is consistent with the date of the start of the colony. These younger colonies have been maintained from 5 to 11 years, whereas most other species have been maintained from 15 to 30 years and are possibly highly inbred. *Triatoma baratai* and *T. vandae* did not have any sequences of 16S deposited in GenBank, and also *T. baratai*, *T. costalimai*, *T. guazu*, *T. matogrossensis*, *T. vandae,* and *T. williami* of Cytb.

Interspecific pairwise divergence (uncorrected *p*-distances) within *Triatoma* herein focused varied in 16S sequences from 0.4% (between *T. pseudomaculata–T. maculata*) to 8.7% (*T. garciabesi–T. brasiliensis* and *T. sordida–T. infestans*), in Cytb sequences from 0.7% (*T. pseudomaculata–T. maculata*) to 21.3% (*T. sordida–T. sherlocki*), and in COI sequences from 1.5% (*T. williami–T. guazu*) to 22.9% (*T. sordida–T. infestans*) (Supplemental files).

Figure 3 shows the contribution of each gene for the phylogenetic reconstruction of members of *T. matogrossensis* subcomplex. In the analysis of 16S (Figure 3A), a clade including *T. jurbergi*, *T. vandae*, *T. sordida*, *T. garciabesi*, and *T. matogrossensis* was recovered with high support (PB = 97% and BPP = 99%). However, for this gene, *T. guasayana* was not included in this group, being placed in *T. rubrovaria* subcomplex. The analysis of COI (Figure 3C) gene by itself recovered *T. sordida* and *T. matogrossensis* as closely related (PB = 91% and BPP = 81%), a relationship also recovered by the Cytb analysis (Figure 3B), but in this case, *T. sordida* is found to be sister to *T. guasayana* + *T. matogrossensis* (PB = 98% and BPP = 100%). Except for the analyses based on the 16S gene, all others recovered *T. williami* and *T. guazu* as sister species with high clade support (All PB ≥ 95% and BPP ≥ 99%; Figures 3B and C and Figure 4).

Both Bayesian and parsimony analyses resulted in a similar topology (Figure 4) for the analyses of the combined genes, with a markedly higher branch support for certain clades, as compared with separate analyses (Figure 3). Focusing on the Central-Western species, three strongly supported clades were recovered as follows: i) one including the species *T. jurbergi*, *T. matogrossensis*, *T. vandae*, *T. garciabesi*, and *T. sordida* (PB = 83% and BPP = 100%); ii) one with *T. guasayana* as sister to this previously mentioned clade (PB = 59% and BPP = 100%); and iii) another with *T. williami* + *T. guazu* (PB = 99% and BPP = 100%). Based on the results of the combined analysis presented herein, it is not possible to say whether clade (iii) is related to the other previously mentioned Central-Western species. However, *T. williami* and *T. guazu* were recovered (without strong support) as related to each other and, also to the remaining Central-Western species, *T. baratai* and *T. costalimai*, in both 16S and Cytb separate analyses.

According to our results, the *T. matogrossensis* subcomplex as currently defined by Schofield and Galvão[13] was not found to be a monophyletic unit, because other species, currently placed in the *T. sordida* subcomplex, including one of the main Chagas disease vectors in Brazil, *T. sordida*, were recovered as strongly related to some species from this group, especially *T. matogrossensis*, *T. jurbergi*, and *T. vandae*.

DISCUSSION

Traditionally, the phylogeny of newly described or strictly sylvatic triatomine species has been inferred based on morphological comparisons.[16,49,50] In this study, the phylogenetic relationships among *Triatoma* species (*T. baratai*, *T. costalimai*, *T. guazu*, *T. matogrossensis*, *T. sordida*, *T. vandae*, and *T. williami*) occurring in Central-Western Brazil were analyzed using molecular information (mtDNA fragments of 16S ribosomal RNA, Cytb, and COI) through Bayesian and parsimony analysis. Both analyses provided satisfactory information for species, which had never been studied, addressing the phylogenetic reconstruction of these groups of triatomines found in Central-Western Brazil. Overall, not a single gene was particularly great in recovering these relationships, showing low clade support in most clades, however the combined analysis showed higher clade support for the clades of interest.

The use of insects from colonies for several kinds of approaches remains controversial because of genetic drift and loss of genetic variability[51]; an interesting observation of this study is the apparent homogeneity of mtDNA from bugs maintained in colonies for several generations. Garcia and Powell[36] sequenced 16S (AF021212) and COI (AF021213) from *T. sordida* from the same colony used herein when it was 14 years old. In this study, 16 years later, the same haplotypes obtained by these authors were also found for both genes. Considering that no selective pressure appears to be affecting and driving mtDNA variation, we concluded that mtDNA is a suitable source of information for exploring phylogenetic relationships, even

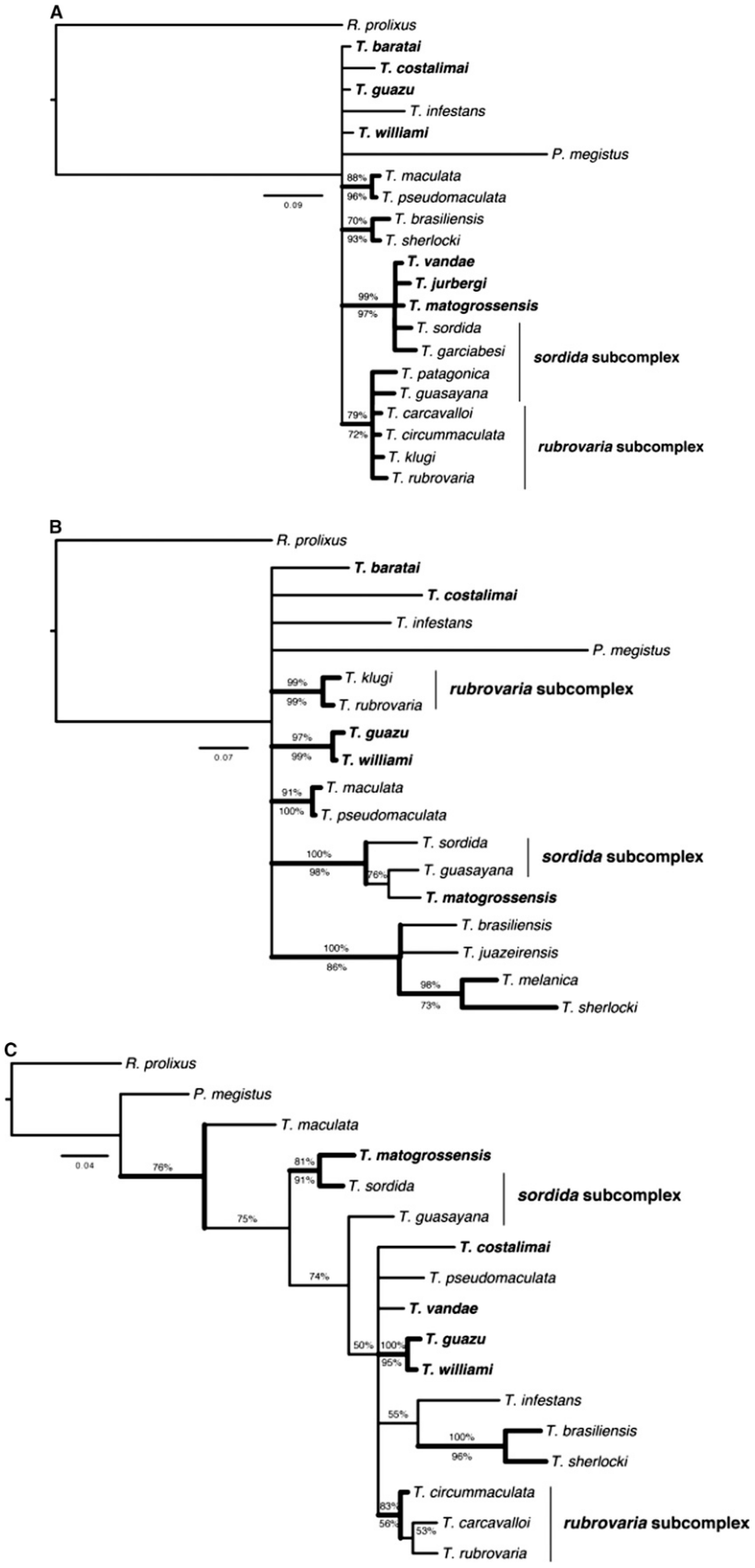

FIGURE 3. Bayesian inference consensus for each molecular marker of *Triatoma* species: (**A**) 16S (HKY+I+G), (**B**) Cytb (HKY+I+G), and (**C**) COI (GTR+I+G). Thick clades represent those also recovered by parsimony. Percentages above the nodes indicate Bayesian posterior probabilities (BPP), whereas those below indicate parsimony bootstrap percentages (PB). Taxa in bold are current members of the *T. matogrossensis* subcomplex.

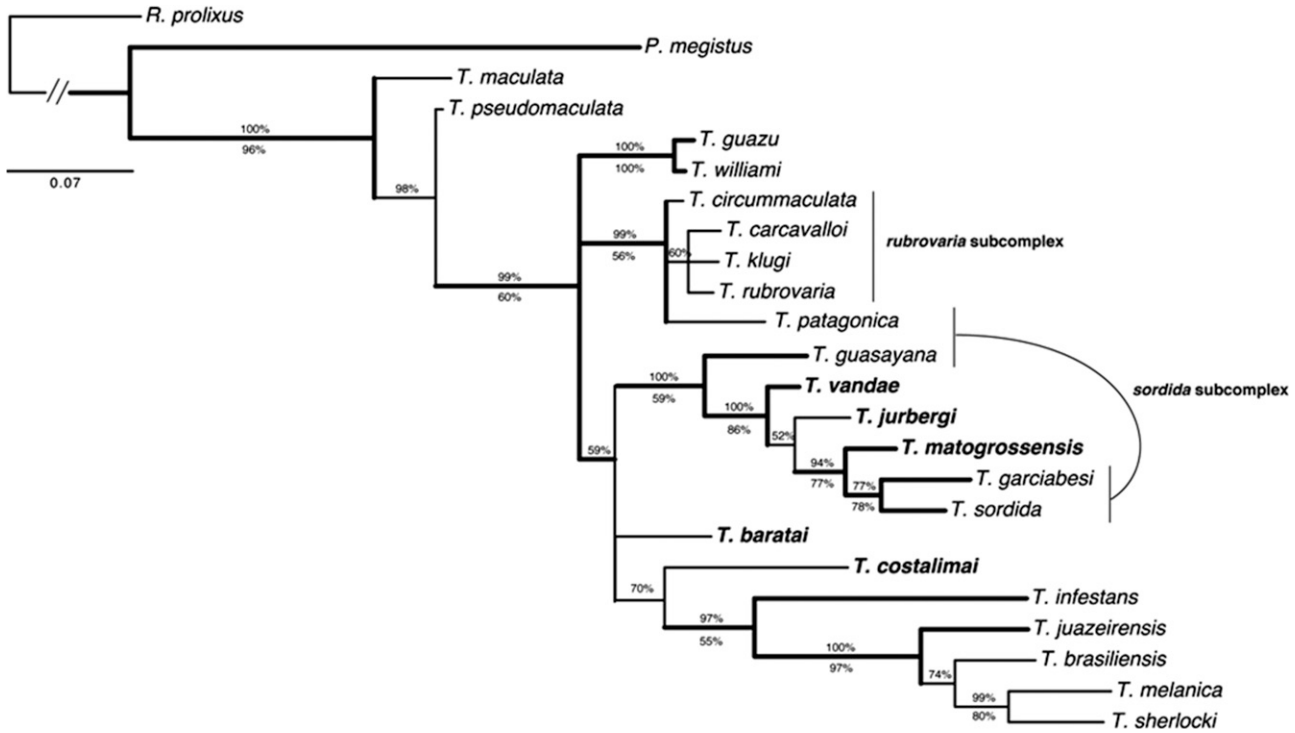

FIGURE 4. Bayesian inference consensus of the combined analysis of sequences of *Triatoma* species occurring in Central-Western Brazil. Molecular evolution models for each partition were HKY+I+G for 16S, GTR+I+G for COI, and HKY+I+G for Cytb. Thick clades represent those also recovered by parsimony. Percentages above the nodes indicate Bayesian posterior probabilities (BPP), whereas those below indicate parsimony bootstrap percentages (PB). Taxa in bold are current members of the *T. matogrossensis* subcomplex.

when obtained from individuals maintained in laboratory conditions over decades.

Sequences of 16S were overall more informative for the combined analysis between species, whereas Cytb and COI were possibly more useful for recovering relationships among more closely related species, because of its faster rate of evolution (in average 3.3 times faster), which can be observed by comparing the pairwise divergences.

Aside from the poor resolution obtained by the combined analyses, at least for separate analyses of the molecular markers, this study does not recover a strict monophyletic group for the seven aforementioned species placed in the *T. matogrossensis* subcomplex, mostly because members of the *T. sordida* subcomplex, such as *T. guasayana*, *T. garciabesi*, and *T. sordida*, tend to be recovered as related to them. This proximity found among members of the *T. sordida* subcomplex to members of the *T. matogrossensis* subcomplex is in agreement with previously published analyses, although this was previously recovered only with 16S and 12S sequences.[12,13,23,24] With the herein newly added Cytb and COI sequences of species belonging to the *T. matogrossensis* subcomplex, the need for a future revision for both *T. matogrossensis* and *T. sordida* subcomplexes to better define the limits of the current classification is reinforced.

The placement of *T. guasayana* in our analyses disagrees in part with previous published analyses. In none of the analyses conducted herein, *T. guasayana* was recovered as sister to *T. sordida*, which was expected because of isoenzyme electrophoresis genetic distances, morphological and ecological similarities, and overlapping distribution in northern Argentina and part of the Bolivian Chaco and Paraguay.[52] Furthermore, it also disagrees with Sainz and others,[23] Garcia and others,[24] and Almeida and others,[53] which reconstructed the phylogeny of selected *Triatoma* species based on fragments of the mtDNA genes: 12S +16S + COI and placed this species as more related to members of the *T. rubrovaria* subcomplex. In fact, by using only 16S, *T. guasayana* was clustered with members of the *T. rubrovaria* subcomplex; however, by using the COI and Cytb, *T. guasayana* (both obtained in this study) was clustered with members of *T. sordida* and *T. matogrossensis* subcomplexes (Figure 3).

The position of *T. costalimai* was also incongruent in each of the separate analyses, but usually it was recovered as related to a member of the *T. matogrossensis* subcomplex. This is not a surprising result considering that Hypsa and others[12] based on a parsimony analysis of 16S sequences and Sainz and others[23] based on a parsimony analysis of 16S + 12S, found this species as the sister species to all other Central-Western Brazil species. A maximum likelihood reanalysis of Hypsa and others[12] data set by Schofield and Galvão,[13] however placed this species at an even more distant position, relating it to a clade composed of members of some other subcomplexes like *T. brasiliensis*, *T. maculata*, and *T. rubrovaria*, in addition to *T. matogrossensis* and *T. sordida*.

The other two recovered clades of *Triatoma* from Central-Western Brazil actually corroborated morphometric and isoenzymatic phylogenetic analyses by Noireau and others[16] focusing on the called "*T. oliveirai* complex" (actually *T. matogrossensis* subcomplex, according to Carcavallo and others[54] and Schofield and Galvão[13]). Both distance analyses of morphological measurements and 20 isoenzyme loci support a close relationship between *T. vandae* and *T. jurbergi*

as a sister clade of *T. matogrossensis*, whereas *T. guazu* and *T. williami* with a significant distance from *T. klugi*. These relationships are in complete agreement with the present analysis. In fact, *T. guazu* and *T. williami* showed only 1.2% sequence divergence of 16S, representing the lowest found herein, concerning the sylvatic species from Central-Western Brazil. The close phylogenetic relationship between these two species had already been addressed by Noireau and others[16] and De Paula and others,[55] and was confirmed herein with high node support for the Bayesian analysis (BPP = 100). Low values of *p*-distances associated with an overlapping geographic distribution have also been found by Almeida and others[53] for species occurring in Southern Brazil, which might be a result of recent speciation events.

Triatoma sordida is currently one of the most important Chagas disease vectors in some states of Brazil (e.g., Minas Gerais).[56,57] According to Forattini,[25] its dispersion center was the Central-Western Region of Brazil and it stands out from the others species because it is a predominant species in Mato Grosso do Sul, Goiás and other states in Brazil and it forms large colonies in human dwellings, especially in the peridomicile.[58,59] This distribution was confirmed by Gurgel-Gonçalves and others.[33] The epidemiologic scenario related to new sylvatic vectors that have been invading and colonizing homes are continually increasing.[14,53,60] Therefore, the close relationship between *T. sordida*, *T. jurbergi*, *T. matogrossensis, T. vandae*, and *T. garciabesi* calls the attention for the epidemiologic potential of these species; as such *T. guasayana*, has already been found invading and colonizing homes.[52]

Two of the Cytb sequences for *T. guazu* obtained, but not used in this analysis, most likely represent pseudogenes, possibly a nuclear copy (*numts*) that can be very common in insect genomes[61] and not uncommon to amplify using direct polymerase chain reaction (PCR) methods.[62] The assumption that the recovered sequences were paralogous copies, was a result of being impossible to properly align both haplotypes of Cytb to other sequences. When aligned based on nucleotide similarity, it required three indel events, two insertion events of a single nucleotide and a downstream deletion event of two nucleotides, and it shifted the open reading frame in the area between these events, resulting in stop codons being observed in all sequences. Previously, amplification of pseudogenes in Triatominae was reported for ND1, ND2, and COI[63] and is herein reported for Cytb. Considering that the density of *numts* in insect genomes is apparently lineage-specific and, as far as it is known, there is little information in Hemiptera,[61,63] future sequencing by direct PCR of Cytb for phylogenetic reconstruction should be done with caution.

CONCLUSION

Considering species from Central-Western Brazil, which had some genes sequenced for the first time, *T. baratai* and *T. vandae* were analyzed phylogenetically based on DNA sequences. The analyses confirmed the relationship of both *T. vandae* and *T. baratai* as members of the *T. matogrossensis* subcomplex, however the monophyly of this subcomplex was not recovered because of the close relationship of some members to members of the *T. sordida* subcomplex. This added phylogenetic evidence calls for a re-evaluation of the current classification of triatomines. This re-evaluation should be based on additional multiple molecular markers, especially nuclear genes, as mitochondrial protein-coding genes used so far are apparently too variable, and possibly saturated, to recover these relationships with strong support.

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Note: Supplemental table appears at www.ajtmh.org.

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