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Genetic variation, population structure, and phylogenetic relationships of *Triatoma rubida* and *T. recurva* (Hemiptera: Reduviidae: Triatominae) from the Sonoran Desert, insect vectors of the Chagas' disease parasite *Trypanosoma cruzi*

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Abstract

Nucleotide and amino acid sequence data from the mitochondrial cytochrome *b* (*Cyt*b) and cytochrome *c* oxidase subunit I (*COI*) gene segments were used to gain insights into the population biology and phylogenetic relationships of two species of hematophagous kissing bugs (Hemiptera: Reduviidae: Triatominae) from the Sonoran Desert of northwestern Mexico and southern Arizona, USA, *Triatoma rubida* (Uhler, 1894) and *T. recurva* (Stål, 1868), both of which are vectors of the protozoan parasite *Trypanosoma cruzi* responsible for Chagas' disease. Analysis of molecular variance of gene sequences indicated significant structure among populations of both species from widely separated geographic localities. Phylogenetic analyses of gene and amino acid sequences employing both Bayesian and parsimony methods showed that *T. recurva* clustered within the *phyllosoma* complex of *Triatoma* species from central and southern Mexico with high statistical support, and that it was closely related to *T. longipennis. Triatoma dimidiata* also was shown to be closely related to the *phyllosoma* complex, as was *T. sanguisuga* which has historically been assigned to the *lecticularia* complex. Analyses of gene sequences were unable to confidently resolve relationships of *T. rubida*, although weak support for a *T. nitida* + *T. rubida* clade was seen under certain conditions. A provisional calibration of a mitochondrial DNA molecular clock for *T. rubida*, based on geological dates for the vicariant separation of the Baja California peninsula from mainland Mexico, suggested that pairwise sequence divergences for the *Cyt*b and *COI* genes were 1.1–1.8% and 0.6–1.0% per million years, respectively. Two highly supported sympatric lineages of *T. rubida uhleri* from southern Arizona, which are hypothesized to have diverged approximately 550,000–900,000 years ago, were detected in the *Cyt*b gene trees.

Keywords: Kissing bugs; Reduviidae; Triatomine relationships; Population genetics; Genetic divergence; Molecular clock calibration; Cytochrome *c* oxidase subunit I; Cytochrome *b*

1. Introduction

Reduviid bugs of the subfamily Triatominae are important vectors of the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease, or American trypanosomiasis, the most important parasitic infection in Latin America (Miles et al., 2003). Currently there is no vaccine available against the parasite (Monteiro et al., 2001), although recent sequencing of the complete genome of *T. cruzi* (El-Sayed et al., 2005) holds promise for the development of specific treatments in the future. The only effective control measure presently

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available is to eliminate the domestic triatomine vectors with insecticides.

Accurate information on population structure and dispersal potential of triatomine species is essential for optimum vector control. Not surprisingly, the majority of research in this area has focused on the most important vectors in southern Mexico and Central and South America, especially *Triatoma infestans*, *T. brasiliensis*, *T. dimidiata* (tribe Triatomini) and *Rhodnius* spp. (tribe Rhodniini). However, in Mexico alone there are at least 24 species of *Triatoma*, most of which are vectors of *Trypanosoma cruzi*, including *Triatoma rubida* (Uhler, 1894) and *Triatoma recurva* (Stål, 1868) (Lent and Wygodzinsky, 1979).

Triatoma rubida occurs over a wide geographic area of northwestern Mexico (Baja California peninsula, and the states of Nayarit, Sinaloa, and Sonora) and the southwestern United States (Arizona, California, New Mexico, and Texas), and is also reported from the state of Veracruz, Mexico (Lent and Wygodzinsky, 1979). *Triatoma recurva* also is an inhabitant of northwestern Mexico (Chihuahua, Nayarit, Sinaloa, and Sonora) and Arizona (Lent and Wygodzinsky, 1979), thus the two species are sympatric throughout a large portion of their range. No information is available on dispersal capabilities of either species. In addition, phylogenetic relationships of *T. recurva* to other members of the Triatomini have not been examined with molecular methods. In the two mtDNA studies that included *T. rubida*, analyses of 16S rDNA sequences suggested that *T. rubida* was closely related to *T. nitida* (Hypša et al., 2002), whereas combined analyses of 12S and 16S rDNA genes suggested a close relationship with *T. protracta* (Sainz et al., 2004), but both groupings were poorly supported.

Populations of *T. rubida* have been subdivided into several geographically distinct subspecies (Table 1) based mainly on differences in the pattern and color of the light markings along the connexival margin of the body (Lent and Wygodzinsky, 1979). Throughout their range, both *T. rubida* and *T. recurva* occupy sylvatic habitats, with *T. rubida* also being found peridomestically. Recent observations, however, indicate that both species are becoming more closely associated with domestic habitats, especially *T. rubida* in the city of Guaymas, Sonora, Mexico where new housing construction is invading previously sylvatic habitats of both species (Paredes et al., 2001). Paredes et al. (2001) documented an infestation rate of 68% for *T. rubida* in houses in Guaymas, and concluded that it should now be considered a domestic species. *Triatoma recurva* was reported to be less abundant, and was found only peridomestically (Paredes et al., 2001), but our recent observations indicate that this species is also invading houses in Guaymas. Chagas' disease is presently not a major health problem in northern Mexico and the southern U.S., but the increase in domestic infestations in Sonora gives rise to concern because *Trypanosoma cruzi* infection rates in both *Triatoma rubida* and *T. recurva* have been reported to exceed 90% in the Guaymas area (Paredes et al., 2001), underlining their potential epidemiological significance.

Table 1
Subspecies designations and geographic distribution of *Triatoma rubida*[a]

Subspecies	Geographic distribution
T. rubida rubida (Uhler)	Cape region, Baja California Sur, Mexico
T. rubida cochimiensis Ryckman	Central Baja California peninsula, Mexico
T. rubida jaegeri Ryckman	Pond Island (= Isla Estanque), Gulf of California, Mexico
T. rubida sonoriana Usinger	Mainland Mexico (Sonora, Sinaloa, Nayarit)
T. rubida uhleri Usinger	Southwestern USA, Veracruz, Mexico

[a] After Usinger (1944), Ryckman (1967), and Lent and Wygodzinsky (1979).

Previous studies have shown that mitochondrial DNA (mtDNA) sequence analysis is an effective tool for assessing intraspecific population genetics in triatomines, and for inferring relationships among closely related and morphologically similar species (García et al., 2003; Monteiro et al., 2003, 2004). Nucleotide sequence analysis of mtDNA genes has also been used to infer phylogenetic relationships among the more widely diverged triatomine taxa (Lyman et al., 1999; García et al., 2001; Hypša et al., 2002; Sainz et al., 2004). Although molecular studies provide support for the divergence between the tribes Triatomini and Rhodniini, in some cases relationships within the Triatomini are poorly resolved. For the most part, molecular studies have supported the morphologically based grouping of *Triatoma* species into various species complexes (Lent and Wygodzinsky, 1979; Schofield, 1988), but they have also suggested that some assignments to these complexes will require re-evaluation (García et al., 2001; Sainz et al., 2004).

Here we used both nucleotide and amino acid sequence data obtained from segments of the mitochondrial cytochrome *b* (*Cyt*b) and cytochrome *c* oxidase subunit I (*COI*) genes to (1) provide a phylogenetic hypothesis for relationships of *T. rubida* and *T. recurva* to other members of the genus *Triatoma* and its various species complexes within the tribe Triatomini, and (2) to obtain information on population structure and the extent of dispersal and gene flow in populations of both species from northwestern Mexico and southern Arizona. We also provide a provisional calibration of a mtDNA molecular clock for *T. rubida*.

2. Materials and methods

2.1. Collection of bugs

Individuals of *T. rubida* and *T. recurva* were obtained from domestic, peridomestic and sylvatic habitats in Mexico and southern Arizona, USA (Fig. 1). For *T. rubida*, we obtained representatives of three of the five described subspecies (Table 1). Specimens of *T. rubida sonoriana* were collected from Guaymas and San Carlos (~20 km W of Guaymas), Sonora. Individuals of *T. rubida cochimiensis*

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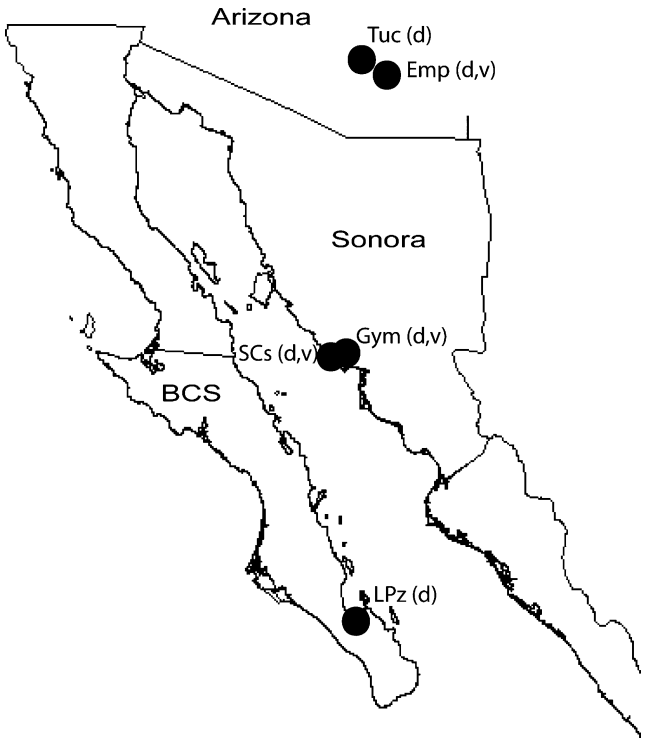

Fig. 1. Map showing collecting localities for *Triatoma rubida* (d) and *T. recurva* (v) in southeastern Arizona, USA and northwestern Mexico. Abbreviations: BCS, Baja California Sur; LPz, La Paz; Gym, Guaymas; SCs, San Carlos; Tuc, Tucson (including the Santa Catalina Mountains); Emp, Empire Mountains.

were from La Paz, Baja California Sur. *Triatoma rubida uhleri* from southern Arizona were collected in Tucson, the Santa Catalina Mountains (near Tucson), and the Empire Mountains (~50 km SE of Tucson). Specimens of *T. recurva* were collected at Guaymas and San Carlos, Sonora, and from the Empire Mountains, Arizona.

New *Cyt*b and *COI* sequences also were obtained for six species from the *phyllosoma* complex (*T. phyllosoma*, *T. longipennis*, *T. pallidipennis*, *T. mexicana*, *T. mazzottii*, and *T. picturata*) from central and southern Mexico [although a different genus has been suggested for species of the *phyllosoma* complex (Carcavallo et al., 2000), here they are assigned to the genus *Triatoma*]. Sequence data for additional triatomine species were taken from GenBank. A list of the species, collection localities, and GenBank accession numbers, including those of the new sequences, is given in Table 2.

2.2. DNA extraction, gene amplification, sequencing, and alignment

Total genomic DNA was extracted from thoracic muscle or leg muscle using either the DNAzol® (Molecular Research Center, Inc., Cincinnati, Ohio) or the DNeasy™ (QIAGEN Inc., Valencia, California) protocol with proteinase K digestion. The polymerase chain reaction (PCR) was used to amplify a 682 bp segment of the *Cyt*b gene using the primers 7432F (5′-GGACGWGGWATTTATT ATGGATC-3′) and 7433R (5′-GCWCCAATTCARGTT ARTAA-3′) (Monteiro et al., 2003). A 636 bp segment of the *COI* gene was also amplified using the primers LCO1490f (5′-GGTCAACAAATCATAAAGATATTG G-3′) and HCO2198r (5′-TAAACTTCAGGGTGACCAA AAAATCA-3′) (Folmer et al., 1994). PCR was performed on a Perkin-Elmer Thermal Cycler 480 in a reaction mixture containing 1 µl template DNA, 5 µl 10× PCR buffer (0.1 M Tris–HCl, 0.5 M KCl, and 0.015 M $MgCl_2$, pH 8.3), 5 µl of 2.5 mM dNTP, 2 µl of each 10 µM primer, 5 µl 50 mM $MgCl_2$, and 1.5–2.5 U *Taq* DNA polymerase (Takara Shuzo Co., Shiga, Japan or Fisher Scientific, Fair Lawn, NJ) and brought up to 50 µl with water. After an initial denaturation at 94 °C for 3 min, PCR conditions were 30 cycles of 94 °C for 1 min of denaturation, 45 °C for 1 min of annealing, and 72 °C for 1 min of extension, followed by a final extension of 10 min. PCR also was conducted on an Eppendorf Mastercycler under similar conditions in a final volume of 25 µl. Verification of successful amplification was assessed by agarose gel electrophoresis.

Sequencing reactions were performed on an Applied Biosystems (Foster City, CA) ABI 3700 DNA sequencer using the PCR primers. For most samples, sequencing was conducted in both forward and reverse directions. Alignments were performed in ClustalX 1.81 (Thompson et al., 1997) with manual adjustment as required. The first base in the amplified *COI* and *Cyt*b gene segments corresponds to position 1429 and 10572, respectively, in the complete mitochondrial genome of *T. dimidiata* (GenBank Accession No. AF301594; Dotson and Beard, 2001).

2.3. Data analyses

Aligned DNA sequences were imported into MEGA version 3.1 (Kumar et al., 2004) for analysis of base composition and determination of genetic distances using Kimura's (1980) 2-parameter (K2P) method. Maximum parsimony (MP) analysis implemented in MEGA using the CNI heuristic search option and 110 random addition of sequences was used to examine both intra- and interspecific relationships within the Triatominae. Relative support for tree topology was obtained by bootstrapping (Felsenstein, 1985) using 1000 iterations of the data matrix.

Phylogenetic relationships within the Triatominae also were examined by Bayesian methods implemented in MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001). Clade support was estimated utilizing a Markov chain Monte Carlo (MCMC) algorithm. Parameters in MrBayes were set to one million generations and 4000 trees sampled (sampled every 250th generation), using the general time-reversible (GTR) model of DNA substitution (nst = "6"; rates = "invgamma") and the default random tree option to begin the analysis. Log-likelihood values from four simultaneous MCMC chains (three hot and one cold) stabilized at about 20,000 generations, resulting in the first 80 trees being discarded from the analysis (burnin = 80).

Table 2
Taxa, collection localities and GenBank accession numbers for species of Triatominae

Taxon	Locality (if known)	*COI*	*Cyt*b	Reference[a]
Tribe Triatomini				
Triatoma rubida uhleri	Arizona, USA			
Clade I		DQ198801	DQ198808	This study
Clade II			DQ198809	This study
T. rubida sonoriana	Sonora, Mexico	DQ198800	DQ198810	This study
T. rubida cochimiensis	La Paz, BCS, Mexico	DQ198802	DQ198811	This study
T. nitida			AF045723	[2]
T. protracta			AF045727	[2]
T. barberi			AY130137	
T. phyllosoma	Oaxaca, Mexico	DQ198806	DQ198818	This study
T. pallidipennis (a)	Morelos, Mexico		DQ198814	This study
T. pallidipennis (b)	?		AF045724	[2]
T. longipennis	Zacatecas, Mexico	DQ198804	DQ198815	This study
T. mexicana	Guanajuato, Mexico	DQ198807		This study
T. mazzottii	Oaxaca, Mexico	DQ198805	DQ198816	This study
T. picturata	Jalisco, Mexico		DQ198817	This study
T. dimidiata	Guatemala	AF301594	AF301594	[1]
T. recurva (a)	Sonora, Mexico	DQ198803	DQ198812	This study
T. recurva (b)	Arizona, USA		DQ198813	This study
T. sanguisuga	Georgia, USA		AF045725	[2]
T. infestans (a)		AF021199		[3]
T. infestans (b)			AF045721	[2]
T. rubrovaria		AF021206		[3]
T. guasayana		AF021193		[3]
T. sordida (a)		AF021213		[3]
T. sordida (b)	Cochabamba, Bolivia		AF045730	[2]
T. brasiliensis (a)		AF021184		[3]
T. brasiliensis (b)	Brazil		AY494161	[5]
T. platensis		AF021202		[3]
T. vitticeps		AF021219		[3]
T. maculata		AF449139		[6]
T. circummaculata		AF021191		[3]
Dipetalogaster maxima	Mexico		AF045728	[2]
Panstrongylus megistus (a)		AF021182		[3]
Panstrongylus megistus (b)			AF045722	[2]
Tribe Rhodniini				
Rhodnius prolixus (a)		AF449138		[6]
Rhodnius prolixus (b)	Honduras		AF421339	[4]

[a] Key to references: [1], Dotson and Beard (2001); [2], Lyman et al. (1999); [3], García and Powell (1998); [4], Monteiro et al. (2003); [5], Monteiro et al. (2004); [6], Gaunt and Miles (2002).

Analysis of molecular variance (AMOVA) was used to test for population structure in *T. rubida* and *T. recurva* using ARLEQUIN version 2.000 (Schneider et al., 2000). The calculation of significance (5% level) of pairwise comparisons (hierarchical analysis) of the F_{ST} analogue Φ_{ST} among collection localities was based on 1000 permutations of the data matrix. Calculation of haplotype diversity, nucleotide diversity and neutrality tests [Tajima's (1989) *D*] were performed in DnaSP version 3.51 (Rozas and Rozas, 1999).

3. Results

3.1. Sequence analysis

Because of inconsistent amplifications of the *COI* gene in *T. recurva* (see below), results are presented for separate analyses of the *Cyt*b and *COI* gene segments, as well as for the combined analyses (1318 bp) when feasible. As expected for protein coding genes, no insertions, deletions or stop codons were present in any of the sequences. There was a pronounced bias against G for both *Cyt*b and *COI* gene fragments (mean G composition: 13.2% ($N = 37$) and 17.2% ($N = 28$), respectively), especially at the third codon position (4.6 and 2.2% G, respectively). These results support the conclusion that the sequences represent mtDNA and are not nuclear pseudogenes.

The *Cyt*b gene in *T. rubida* ($N = 23$) contained 85 variable sites, 75 of which were at the third codon position. Six of the substitutions were non-synonymous. The translated gene consisted of 227 amino acid residues. Five of the amino acid changes were unique to *T. rubida cochimiensis* from La Paz. The *COI* gene in *T. rubida* ($N = 21$) contained 46 variable sites, 34 of which were at the third position. The translated gene contained 211 amino acid residues. Only two of the substitutions were non-synonymous, but one

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Table 3
Summary of genetic diversity indices of the *Cyt*b (682 bp) and *COI* (636 bp) gene segments in *Triatoma rubida* and *T. recurva*

	N^a	K	h (±SD)	π (±SD)	Tajima's D
T. rubida					
*Cyt*b	22	11	0.913 (±0.035)	0.03276 (±0.00773)	−0.21798 (n.s.)
COI	21	12	0.900 (±0.048)	0.01618 (±0.00474)	−0.52314 (n.s.)
T. recurva[b]					
*Cyt*b	8	5	0.857 (±0.108)	0.01126 (±0.00175)	1.32719 (n.s.)

[a] Abbreviations: N, number of sequences; K, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; n.s., not significant ($P > 0.10$).
[b] Values for *COI* in *T. recurva* not given as only two sequences were obtained.

was unique to *T. rubida cochimiensis*. In *T. recurva*, the *Cyt*b fragment contained 15 variable sites, 12 of which were at the third codon position. Two of the variable sites resulted in amino acid substitutions. The *COI* gene was successfully amplified in only two individuals of *T. recurva* (from San Carlos and Guaymas, Sonora), and the sequences were similar (three variable sites). Genetic diversity indices for both genes in *T. rubida*, and for *Cyt*b in *T. recurva*, are summarized in Table 3. None of the Tajima's D tests for neutrality were significant. For both species, no haplotypes for either gene were shared among the main geographic regions (Baja California Sur, Sonora, and Arizona).

3.2. Population analyses of Triatoma rubida and T. recurva

Because AMOVA results of each gene revealed no evidence of population structure in *T. rubida* among the three southern Arizona collecting localities, data from these localities were grouped for population analyses. Similarly, data for *T. rubida* from the two collecting localities in Sonora also were grouped.

AMOVA results for separate analyses of *Cyt*b and *COI* sequences in *T. rubida* showed that most (>85%) of the genetic variation occurred among the three populations sampled (Table 4). Global Φ_{ST} values were high (>0.86), indicating significant population genetic structure. All pairwise comparisons of Φ_{ST} among the three geographic areas (La Paz, Sonora, and southern Arizona) were significant for both genes (not shown), indicating low gene flow among populations from these regions. Similar results were obtained when data from the two genes were combined ($N = 20$). The estimated number of migrants per generation (Nm) between the La Paz and Sonora populations was very low ($Nm = 0.06$–0.07 in separate and combined analyses). AMOVA results for the *Cyt*b gene in *T. recurva* also indicated significant population structure among populations from Sonora and southern Arizona (Table 4).

Table 4
AMOVA results for *Triatoma rubida* and *T. recurva* based on separate analyses of the *Cyt*b (682 bp) and *COI* (636 bp) gene segments

Species	Gene	Source of variation	*df*	Percentage of variation	Φ_{ST}
T. rubida	*Cyt*b	Among populations	2	87.05	0.870 ($P < 0.0001$)
		Within populations	20	12.95	
	COI	Among populations	2	85.56	0.856 ($P < 0.0001$)
		Within populations	18	14.44	
T. recurva	*Cyt*b	Among populations	1	91.67	0.917 ($P = 0.018$)
		Within populations	6	8.33	

MP analysis of *Cyt*b gene sequences from individuals of *T. rubida* is shown in Fig. 2. Bayesian analysis recovered a similar topology (not shown). With the exception of a single specimen from Guaymas [individual T9 which differed from the other Sonoran samples by an average K2P genetic distance (d) of ~2.8%], the populations from the three geographic areas clustered into three well-supported groups. Average genetic distances between the population from La Paz and the other populations were ~9.0%. A single *Cyt*b haplotype was found in the four individuals from La Paz. Average K2P distance between the Guaymas and Arizona populations of *T. rubida* was ~2.8%. An unexpected result of the MP and Bayesian analyses was the clustering of southern Arizona individuals into two distinct clades (clades I and II), each consisting of seven individuals. There was no apparent geographic partitioning among the two closely related ($d = 1.0\%$) clades, which showed diagnostic base substitutions at five sites, all third position transitions. For one Tucson collecting site, individuals from both clades were collected within the same house. Relative rate tests (Tajima, 1993) indicated no significant difference in the rate of evolution between the two clades. The overall topology and similar bootstrap values seen in the *Cyt*b MP tree, including the resolution of clades I and II in Arizona, was also recovered in a combined MP analysis (length = 330; CI = 0.912; RI = 0.914; $N = 20$; not shown).

Results from MP analysis of *T. rubida* using *COI* sequences (length = 121; CI = 0.934; RI = 0.916; $N = 21$) were generally concordant with those from the *Cyt*b gene, showing a highly supported (99%) La Paz clade that was distinct ($d \sim 5.0\%$) from the two other populations (not shown). Although the topologies of the *COI* and *Cyt*b trees were similar, the *COI* tree alone failed to resolve clades I and II in the southern Arizona population. In addition, the separate clustering of Arizona and Sonora populations, although still evident, was poorly supported in the *COI* tree, and there was little genetic differentiation between the two populations ($d = 0.8\%$).

Bayesian analysis of *Cyt*b sequences from *T. recurva* (Fig. 3) showed that individuals from Sonora ($N = 4$) and Arizona ($N = 4$) each clustered into two distinct and reasonably well-supported groups ($d = 1.9\%$; Table 5). Fig. 3 also shows results obtained from analysis of new sequences (except for *T. dimidiata*) from the *phyllosoma* complex. *Triatoma recurva* unambiguously clustered within the *phyllosoma* complex, and was most closely related ($d \sim 11.0\%$; Table 5) to *T. longipennis*. Except for the position of *T. phyllosoma*, the same topology was recovered in the MP tree (length = 384; CI = 0.672; RI = 0.674; not shown).

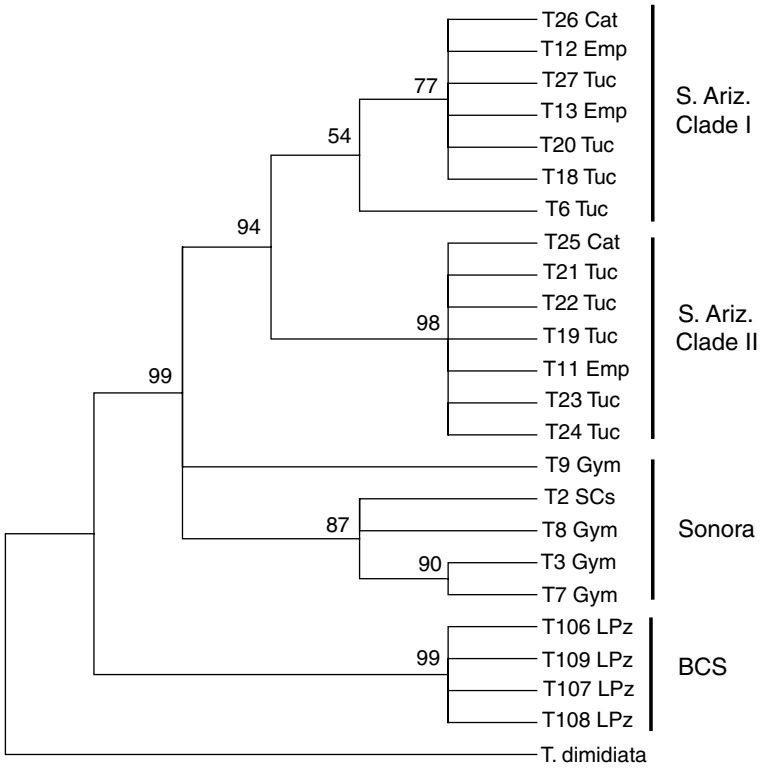

Fig. 2. Most parsimonious tree (length = 179; CI = 0.910; RI = 0.942) obtained for individuals of *Triatoma rubida* ($N = 23$) from northwestern Mexico and southeastern Arizona based on analysis of a 682 bp segment of the *Cyt*b gene (73 parsimony informative sites). *Triatoma dimidiata* was used as an outgroup. All codon positions were used in the analysis. Bootstrap support values are shown on branches; nodes with <50% support were collapsed. Key to localities: Tuc, Tucson AZ; Cat, Santa Catalina Mountains, AZ; Emp, Empire Mountains, AZ; Gym, Guaymas, Sonora; SCs, San Carlos, Sonora; LPz, La Paz, Baja California Sur (BCS).

3.3. Phylogenetic relationships

To generate phylogenetic hypotheses for the genus *Triatoma* based on *Cyt*b and *COI* sequences, additional taxa from different species complexes available in the literature (Table 2) were incorporated into the data matrix and analyzed by both Bayesian and parsimony methods. This required trimming the new sequences for *T. rubida*, *T. recurva*, and species of the *phyllosoma* complex to 399 bp (*Cyt*b) and 588 bp (*COI*). The split between members of the South American *infestans* complex and the remaining species of Triatomini is estimated at 19.5–38.3 million years ago (mya) (Bargues et al., 2000), suggesting that third codon position transition substitutions are probably saturated in the relatively fast evolving *Cyt*b and *COI* genes. When we plotted the number of transitions and transversions at each codon position against Tamura and Nei (1993) genetic distances using the computer program DAMBE (Xia and Xie, 2001), third position transitions were indeed saturated for both genes as predicted (not shown). Phylogenetic relationships shown in Figs. 4 and 5, therefore, were obtained after deleting third codon positions.

The Bayesian *Cyt*b tree, using *Rhodnius prolixus* as an outgroup, is shown in Fig. 4. A comparison of Figs. 3 and 4 shows that trimming the *Cyt*b sequences by >40% (from 682 to 399 bp), in addition to deleting all third codon positions, had little effect on tree topology within the *phyllosoma* complex. Fig. 4 again showed that *T. longipennis* was the most closely related taxon to *T. recurva*. Interestingly, *T. sanguisuga* from the *lecticularia* complex clustered into a highly supported clade that included the *phyllosoma* complex. Similar results were seen in the MP tree after deleting third codon positions (length = 105; CI = 0.476; RI = 0.684; not shown). However, the MP tree also showed a weakly supported (54%) clade consisting of *T. nitida* + *T. rubida*. The *T. nitida* + *T. rubida* clade (68% support) was also resolved in a Bayesian tree in which all codon positions were included (not shown). Fig. 4 shows that the three representatives from the *infestans* complex (*T. infestans*, *T. brasiliensis*, and *T. sordida*) + *Panstrongylus megistus* formed a clade that was separate from the remaining species which were primarily from Central and North America. The clustering of *P. megistus* with the *infestans* complex, however, was not seen in the Bayesian tree when all codon positions were included.

The results of Bayesian analyses of triatomine *COI* sequences were generally concordant with the *Cyt*b tree, again showing that members of the *phyllosoma* complex, including *T. mexicana* for which *Cyt*b data were not available, formed a highly supported clade (Fig. 5). With the exception of *T. vitticeps* and *T. maculata*, the species of the *infestans* complex + *T. circummaculata* formed a

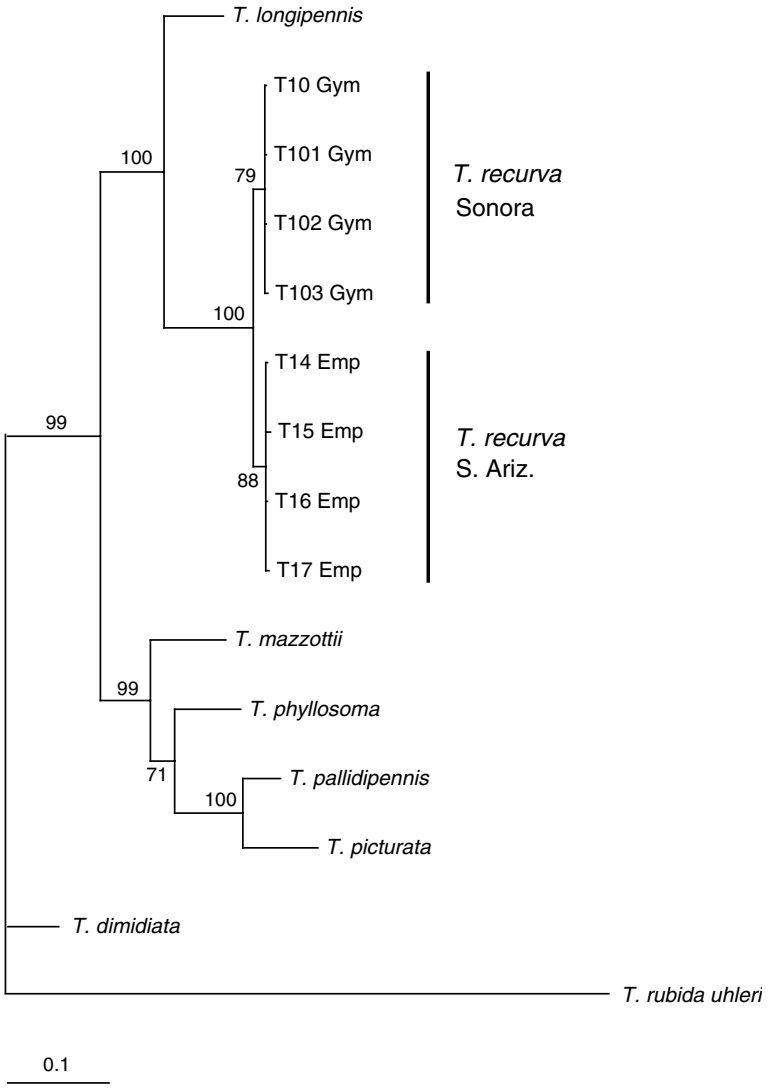

Fig. 3. Fifty-percent majority rule consensus tree showing relationships of *Triatoma recurva* ($N = 8$) from Sonora and Arizona, together with members of the *phyllosoma* complex from central and southern Mexico, based on Bayesian analysis of a 682 bp segment of the *Cyt*b gene. Data for *T. dimidiata* were taken from GenBank (Table 2). *T. rubida uhleri* was used as an outgroup. All codon positions were used in the analysis. Abbreviations are the same as in Fig. 2. Clade credibility values are shown on branches. Scale indicates expected substitutions per site.

Table 5
Average K2P genetic distances (*d*) for the *Cyt*b gene (682 bp) among populations of *Triatoma recurva* from Sonora, Mexico (a) and southern Arizona (b), and species from the *phyllosoma* complex

Species	1	2	3	4	5	6	7	8
1. *T. recurva* (a)	—							
2. *T. recurva* (b)	0.019	—						
3. *T. dimidiata*	0.154	0.148	—					
4. *T. pallidipennis*	0.164	0.162	0.155	—				
5. *T. longipennis*	0.106	0.112	0.147	0.152	—			
6. *T. mazzottii*	0.155	0.149	0.145	0.120	0.133	—		
7. *T. picturata*	0.161	0.161	0.164	0.082	0.150	0.127	—	
8. *T. phyllosoma*	0.138	0.138	0.135	0.113	0.127	0.106	0.131	—

highly supported lineage. The *COI* tree again showed that *T. longipennis* was the sister taxon to *T. recurva*. The subspecies of *T. rubida* formed a highly supported clade, with *T. rubida cochimiensis* from La Paz occupying a basal position within the *rubida* lineage in agreement with the MP *Cyt*b tree (Fig. 2).

Amino acid composition at variable positions in the trimmed *Cyt*b and *COI* protein segments in the Triatominae is shown in Table 6. The *Cyt*b protein segment consisted of 133 amino acid residues and contained 33 variable sites. The *COI* segment was much less informative, with only 12 variable positions out of a total of 195 amino acid residues. Species of the *phyllosoma* complex, including *T. dimidiata* and *T. recurva*, contained five unique *Cyt*b substitutions, and two unique *COI* substitutions, that separated them from the other triatomines examined. All of the unique *Cyt*b amino acid substitutions in the *phyllosoma* complex also were present in *T. sanguisuga* (Table 6). Bayesian and MP phylogenetic trees based on *Cyt*b protein sequences also resolved a clade consisting of the *phyllosoma* complex + *T. sanguisuga* with 100 and 99% support, respectively, again show-

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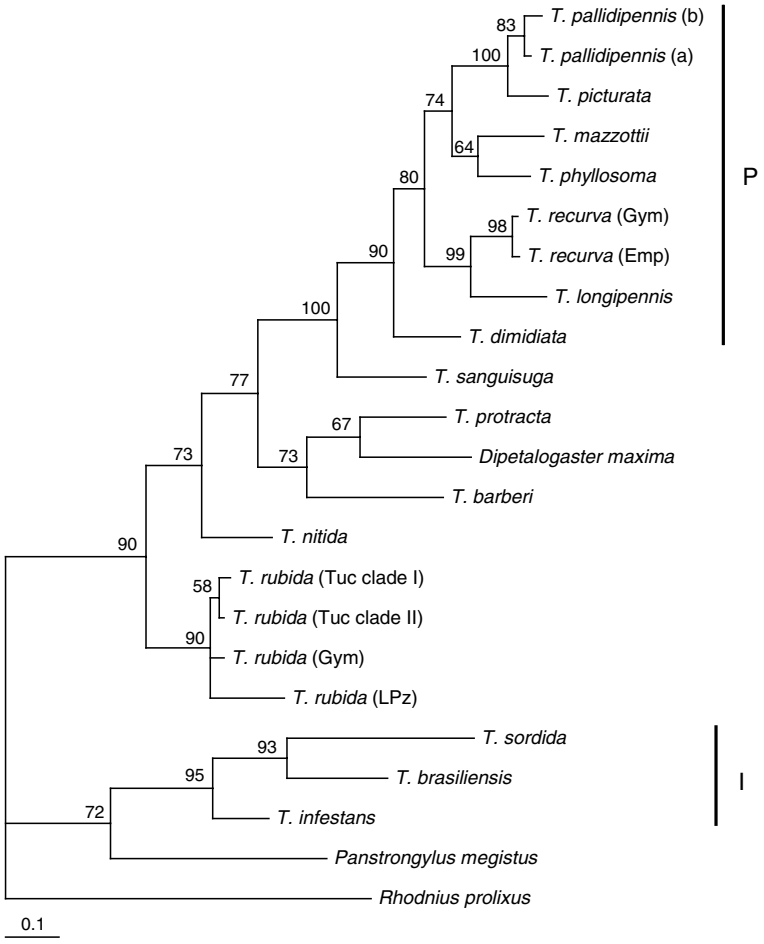

Fig. 4. Fifty-percent majority rule consensus tree showing relationships among *Triatoma* spp. (Triatomini) based on Bayesian analysis of a 399 bp segment of the *Cyt*b gene using first and second codon positions only. The tree was rooted with *Rhodnius prolixus* (Rhodniini). Numbers on branches are clade credibility values. Two sequences for *T. pallidipennis* are included: (a) is a new sequence from an individual from Morelos, Mexico; (b) is GenBank sequence AF045724. Abbreviations: P, *phyllosoma* complex; I, *infestans* complex; others abbreviations as in Fig. 2. Scale represents expected substitutions per site.

ing their close relationship and supporting the results from the *Cyt*b gene tree (Fig. 4). The protein trees, however, did not provide good resolution of triatomine affinities, with the exception of a well-supported clade composed of the *T. rubida* subspecies, and therefore are not shown.

Table 6 shows two *Cyt*b amino acid substitutions between *T. rubida cochimiensis* and the other subspecies of *T. rubida*. In the complete *Cyt*b protein segment of 227 amino acids, a total of five amino acid differences separated the two groups. In addition, analysis of the complete segment revealed nine additional amino acid substitutions in the *phyllosoma* complex, including *T. dimidata*, that were not present in the subspecies of *T. rubida* or in *Rhodnius prolixus*. Because sequences for the complete *Cyt*b segment were not available for the other triatomines examined here, it is uncertain whether these additional differences are unique to the *phyllosoma* complex, and whether they also will be found in *T. sanguisuga*, but the results are consistent with the view that *T. dimidiata* is a member of the *phyllosoma* complex.

4. Discussion

4.1. Population structure and taxonomic status of Triatoma rubida and T. recurva

The applicability of *Cyt*b sequence data to infer population divergence and structure in *Triatoma* spp. has previously been demonstrated in *T. brasiliensis* from Brazil, where different chromatic forms characteristic of discrete populations generally showed large divergences ($d > 7.5\%$) suggesting the existence of a species complex (Monteiro et al., 2004). The large genetic distances ($d \sim 9\%$) between *T. rubida* from La Paz, BCS and the populations from Sonora and southern Arizona, together with chromatic differences (Ryckman, 1967; Lent and Wygodzinsky, 1979), suggest that *T. rubida cochimiensis* could be considered a valid species. The level of divergence between *T. rubida cochimiensis* and the other populations exceeds the value of $d \sim 8\%$ that has been reported to separate several closely related species of *Triatoma* (Monteiro et al., 2004), and is similar to the average divergences between *T. nitida* and *T. rubida sonori-*

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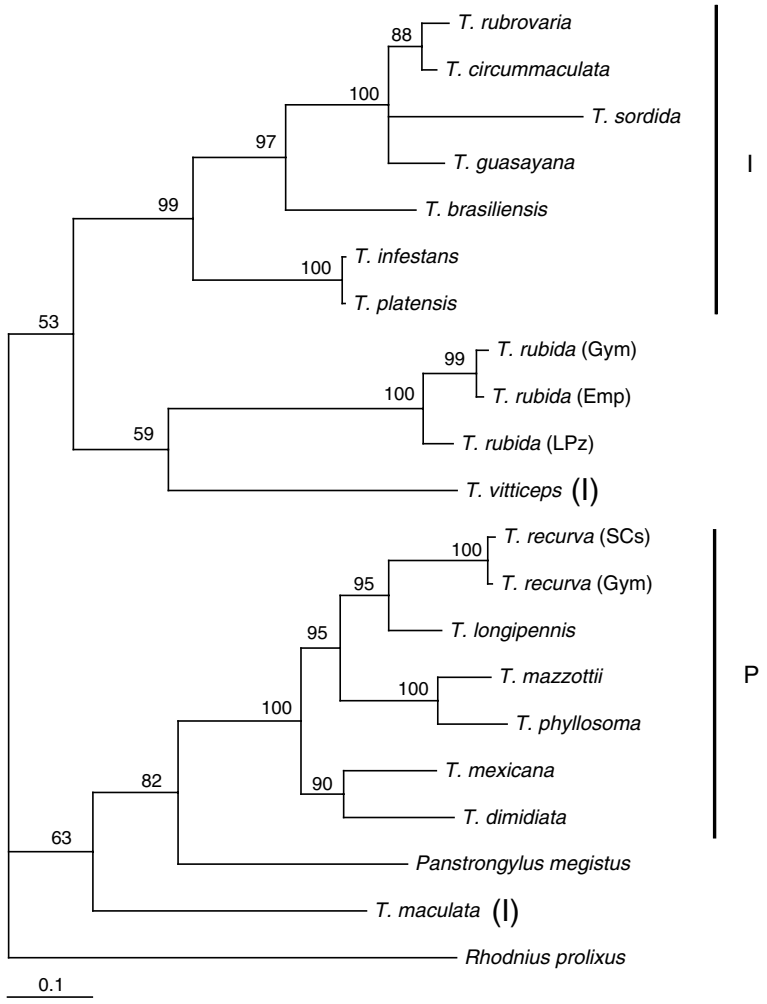

Fig. 5. Fifty-percent majority rule consensus tree showing relationships among *Triatoma* spp. (Triatomini) based on Bayesian analysis of 588 bp segment of the *COI* gene using first and second codon positions only. The tree was rooted with *Rhodnius prolixus* (Rhodniini). Numbers on branches are clade credibility values. Abbreviations as in Figs. 2 and 4. Scale represents expected substitutions per site.

ana/uhleri ($d \sim 10$–11%) and between *T. longipennis* and *T. recurva* ($d \sim 11\%$). We would caution, however, that a more thorough study of morphological differences among populations of *T. rubida* should be undertaken before any taxonomic changes are considered. The low gene flow and modest genetic divergence between populations of *T. rubida* from Sonora and Arizona ($d \sim 2.8\%$) support their subspecific designations (Ryckman, 1967).

Although only limited molecular data are available for *T. recurva* ($N = 8$), AMOVA (Table 4) showed significant population structure and limited gene flow between the central Sonora and southern Arizona populations of this species.

4.2. Relationships of T. recurva and the phyllosoma complex

One of the major findings of the present study was that *T. recurva*, which had not been placed previously in any species complex of *Triatoma* (Schofield, 1988), consistently clustered with high support within the *phyllosoma* complex in phylogenetic analyses of both *Cyt*b and *COI* gene segments, and that it was closely related to *T. longipennis* (Figs. 3–5). *Triatoma mexicana* and *T. dimidata*, which were tentatively placed in the *phyllosoma* complex (Lent and Wygodzinsky, 1979; Schofield, 1988), also clustered within the complex. Overall, seven of the 10 valid species that had been assigned previously to the *phyllosoma* complex (*T. phyllosoma*, *T. longipennis*, *T. pallidipennis*, *T. mazzottii*, *T. picturata*, *T. mexicana*, *T. brailovskyi*, *T. bassolsae*, *T. bolivari*, and *T. dimidiata*) (Lent and Wygodzinsky, 1979; Carcavallo et al., 1987; Schofield, 1988; Alejandre-Aguilar et al., 1999; Martínez et al., 2005) were analyzed here. All seven species are principal vectors of *Trypanosoma cruzi* in Mexico.

Analyses of *Cyt*b and *COI* protein sequences were concordant with results from nucleotide sequences and they provided additional support for species assignments to the *phyllosoma* complex. All members of the complex, including *Tritatoma recurva* and *T. dimidiata*, shared five unique amino acid substitutions (out of a total of 133 residues) that, except for *T. sanguisuga*, were not found in the other triatomine species analyzed (Table 6). In addition, both

Table 6
Variable amino acid positions in triatomine *Cyt*b and *COI* gene segments

Species	*Cyt*b Position 1	15	18	113	114	134	135	144	145	146	147	148	150	158	161	167	168	170	177	179	181	185	188	189	191	193	194	196	198	1102	1103	1104	1106
T. rubida uhleri/sonoriana	I	L	I	N	E	F	A	I	A	A	M	V	V	T	S	N	S	F	P	F	I	M	S	L	L	F	F	M	N	P	R	M	G
T. rubida cochimiensis	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.
T. nitida	.	.	.	.	D	.	.	.	.	.	.	.	I	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.
T. phyllosoma	.	M	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	S	A	P	I	M
T. pallidipennis (a)	.	M	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	S	A	P	I	M
T. pallidipennis (b)	Y	M	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	S	A	P	I	M
T. longipennis	.	M	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	S	A	P	I	M
T. mazzottii	.	M	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	S	A	P	I	M
T. picturata	.	M	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	L	L	S	A	P	I	M
T. dimidiata	.	M	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	S	A	P	I	M
T. recurva	.	M	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	S	A	P	I	M
T. sanguisuga	.	M	.	.	D	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	S	A	P	I	M
T. protracta	.	.	V	.	.	.	.	.	.	.	L	.	I	.	.	Q	G	.	.	.	.	L	.	M	.	.	.	.	.	.	.	I	.
T. barberi	?	?	?	?	?	?	.	V	.	.	L	.	I	S	N	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	I	.
T. infestans	.	.	.	.	.	.	.	.	.	.	L	T	I	.	.	T	.	.	.	Y	.	.	.	I	.	.	.	L	.	.	.	I	.
T. brasiliensis	.	.	.	.	.	.	.	.	.	.	L	T	.	.	N	T	.	.	.	.	T	.	.	I	.	L	.	.	.	.	.	I	.
T. sordida	.	.	.	.	.	Y	.	.	.	T	L	T	I	.	.	T	.	.	S	.	.	.	.	I	.	L	.	L	.	.	.	I	.
Dipetalogaster maxima	.	.	.	.	.	.	.	.	.	.	L	.	I	.	.	.	.	.	.	.	.	.	I	.	.	.	.	L	.	.	.	I	.
Panstrongylus megistus	.	.	.	M	.	.	.	.	.	.	L	.	I	.	.	T	.	.	.	.	.	.	.	I	.	L	.	.	.	.	.	I	.
Rhodnius prolixus	.	.	V	.	.	.	.	.	T	.	L	T	I	.	.	K	.	L	.	.	.	.	I	I	M	L	.	L	.	.	.	I	.

Species	*COI* Position 144	145	152	163	174	176	182	204	213	242	255	267
T. rubida uhleri/sonoriana	L	S	V	I	I	S	M	I	L	A	R	D
T. rubida cochimiensis	.	.	.	.	.	.	.	.	.	.	.	.
T. phyllosoma	I	.	.	V	.	A	V	.	M	.	.	.
T. longipennis	I	.	.	V	.	A	V	.	M	.	.	.
T. mazzottii	I	.	.	V	.	A	V	.	M	.	.	.
T. mexicana	I	.	I	.	.	A	V	.	M	.	.	.
T. dimidiata	I	.	.	V	.	A	V	.	M	.	.	.
T. recurva	I	.	.	V	.	A	V	.	M	.	.	.
T. infestans	.	.	I	.	V	A	.	V	.	E	.	.
T. platensis	.	.	I	.	V	A	.	V	.	E	.	.
T. brasiliensis	.	.	.	.	.	A	.	.	.	E	.	.
T. sordida	.	.	I	.	.	A	.	.	.	E	Q	E
T. rubrovaria	.	.	.	.	.	A	.	.	.	E	.	.
T. vitticeps	.	.	I	.	.	A	.	.	.	E	.	.
T. guasayana	.	.	.	.	.	A	.	.	.	E	.	.
T. maculata	.	.	.	.	.	A	.	.	.	S	.	.
T. circummaculata	?	?	.	.	.	A	.	.	.	E	.	.
Panstrongylus megistus	.	.	.	.	.	A	.	.	.	T	.	.
Rhodnius prolixus	I	P	I	V	.	.	T	.	.	.	T	E

Shaded areas indicate unique amino acid substitutions in the *phyllosoma* complex + *T. sanguisuga*.

T. recurva and *T. dimidata* shared two unique amino acid substitutions in the *COI* gene segment (out of a total of 195 residues) with the *phyllosoma* complex (Table 6). The close relationship between *T. sanguisuga* and the *phyllosoma* complex suggested by protein sequence analysis (Table 6) was surprising given that this small species from the USA has historically been placed in the *lecticularia* complex (Schofield, 1988). The Bayesian *Cyt*b tree also clustered *T. sanguisuga* at the basal position of the *phyllosoma* complex clade with 100% support (Fig. 4), a topology which is in agreement with previous the findings (Lyman et al., 1999; Hypša et al., 2002). These results suggest that the apparent close relationship between *T. lecticularia* and *T. sanguisuga* needs to be reevaluated.

Although considered valid species in the present study, the taxonomic status of members of the *phyllosoma* complex has been the subject of debate for many years. As reviewed by Marcilla et al. (2001), the different morphotypes of this group were originally designated as subspecies of *T. phyllosoma* (Usinger, 1944), but then were elevated to full species by Lent and Wygodzinsky (1979). Based on sequence analysis of ~470–480 bp of the nuclear rDNA second internal transcribed spacer (ITS-2), Marcilla et al. (2001) suggested that subspecific status was more appropriate for members of the *phyllosoma* complex, and that *T. dimidata*, although closely related to this group, as suggested previously (Lyman et al., 1999; García et al., 2001), belonged to a separate lineage. Marcilla et al. (2001) also

found no ITS-2 sequence differences between *T. longipennis* and *T. picturata*.

Average K2P genetic distances for the *Cyt*b gene among members of the *phyllosoma* complex (*T. dimidiata* and *T. recurva* included) were relatively large ($d = 8.2$–16.4%; Table 5) suggesting that they represent valid species. The same conclusion was reached by Sainz et al. (2004) in their study on triatomine 12S and 16S rDNA sequences. In contrast to ITS-2 analyses (Marcilla et al., 2001), the *Cyt*b data showed a clear split ($d = 15\%$) between *T. longipennis* and *T. picturata* (Table 5). Also, phylogenetic analyses of nucleotide and corresponding protein sequences from both mitochondrial genes, together with results from other mtDNA studies (Lyman et al., 1999; García et al., 2001; Hypša et al., 2002; Sainz et al., 2004), support the inclusion of *T. dimidiata* within the *phyllsoma* complex. It should be pointed out, however, that differences in ITS-2 sequences among populations of *T. dimidiata* from Yucatan, Mexico and those from other areas suggest the existence of more than one species in Mexico (Marcilla et al., 2001).

4.3. Phylogenetic relationships of the Triatomini

Bayesian and parsimony analyses of *Cyt*b and *COI* nucleotide sequences generally supported the major findings from previous molecular phylogenetic studies of the Triatominae, especially the divergence of the South American *infestans* complex from the northern species (Lyman et al., 1999; García et al., 2001; Hypša et al., 2002; Sainz et al., 2004) and provided additional information on relationships within the Triatomini. But in contrast to the strongly supported relationships found for *T. recurva*, the molecular data did not provide a clear resolution of the species complex affinities of *T. rubida*. The Bayesian *Cyt*b tree (third positions deleted; Fig. 4) showed that the different geographic populations (subspecies) of *T. rubida* resolved as a highly supported clade that was sister to a clade containing the remaining species of Triatomini from Central and North America. The Bayesian *COI* tree (third positions deleted; Fig. 5), however, showed the *T. rubida* clade clustering with *T. vitticeps* from the *infestans* complex, but with weak support. The weakly supported clustering of *T. rubida* with *T. nitida* in the both the MP tree (third positions deleted) and the Bayesian tree using all codon positions is in agreement with the findings of Hypša et al. (2002) using nuclear 18S rDNA sequences. Schofield (1988) included *T. nitida* in the *protracta* complex, but with reservation, a grouping followed by later workers (Lyman et al., 1999; Peterson et al., 2002). Fig. 4, however, suggests that *T. rubida* is not closely related to the *protracta* complex species, *T. barberi* and *T. protracta*. Analysis of 16S rDNA sequences clustered *T. rubida* and *T. nitida* with *T. protracta*, but with poor support (Hypša et al., 2002). Similar results were found by Sainz et al. (2004) who were able to resolve a *T rubida* + *T. protracta* clade from analysis of 12S + 16S rDNA sequences, but again with poor support. It is clear that more work will be required before the affinities of *T. rubida* within the Triatomini can be confidently determined.

Although the mtDNA protein sequences were informative and provided insights into the makeup of the *phyllosoma* complex, generally they provided little new information concerning relationships of the other species complexes. For example, we found no unique amino acid substitutions that characterized the *infestans* complex for either gene, although COI amino acid site No. 142 was characterized by an A to E substitution in all species of the complex, except *T. maculata* (Table 6). The same substitution was seen in *T. circummaculata* which, although previously placed in the *circummaculata* complex (Schofield, 1988), has been shown to cluster with high support within the *infestans* complex (García et al., 2001; Sainz et al., 2004; also see Fig. 5). Also noteworthy is that *T. infestans* and *T. platensis*, which are known to be very closely related from nucleotide data, share two unique I to V amino acid substitutions at COI sites No. 41 and 104 (Table 6).

4.4. Molecular clock calibration for mtDNA in T. rubida

By applying geological estimates for dates of separation of the Baja California peninsula from mainland Mexico during the formation of the Gulf of California, and by assuming that this vicariant event resulted in geographic isolation and restricted gene flow in a panmictic population of *T. rubida* which ultimately led to the divergence that we see today between the geographically isolated *T. rubida cochimiensis* (Baja peninsula) and *T. rubida sonoriana* (Sonora), we can obtain a rough calibration of a molecular clock for estimating ages of population divergences in this species. Geological data suggest that the Gulf of California began to form roughly 5–8 million years ago, during the late Miocene–early Pliocene (Holt et al., 2000; Riddle et al., 2000; Oskin and Stock, 2003). Pairwise K2P sequence divergence between *T. rubida cochimiensis* and *T. rubida sonoriana* is about 9% for the *Cyt*b gene. This is equivalent to a 1.1–1.8% pairwise sequence divergence per million years, lower than the 2.3% divergence for mtDNA generally applied to mtDNA in insects, including the Triatominae (Brower, 1994; Monteiro et al., 2003), but in close agreement with results obtained with sand flies (Psychodidae: *Phlebotomus* spp.) (Esseghir et al., 1997). Also, a molecular clock applied to nuclear 18S rDNA dates the split of the ancestors of the *infestans–phyllosoma* complex to about 23 mya (Bargues et al., 2000). The average *Cyt*b divergence that we found between members of these two complexes ($d = 26.1\%$) yields a divergence rate of 1.1% per million years, consistent with our calibration. Our *Cyt*b molecular clock calibration suggests that the two sympatric lineages of *T. rubida uhleri* from southern Arizona (clades I and II) probably diverged between 550,000 and 900,000 years ago. A mid-Pleistocene vicariant separation which restricted gene flow among isolated populations, followed by secondary contact, is a plausible explanation for the formation of the *Cyt*b subclades seen in *T. rubida uhleri*.

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The average *COI* divergence between *T. rubida cochimiensis* and *T. rubida sonoriana* ($d \sim 5\%$) yields a pairwise divergence rate of about 0.6–1.0% divergence per million years, suggesting that the *COI* gene is evolving more slowly than *Cyt*b in this species. The postulated slower rate of evolution of the *COI* gene would explain the absence of a clear genetic signature of clades I and II in *T. rubida uhleri* seen with the faster evolving *Cyt*b gene. Also, our results suggest that applying a molecular clock calibration of 2.3% sequence divergence per million years for the *Cyt*b and *COI* genes overestimates divergence rates in *T. rubida*, and possibly other triatomines as well, and will therefore yield underestimates of dates of population separations.

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