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ORIGINAL PAPER

Molecular evidence of intraspecific variability in different habitat-related populations of *Triatoma dimidiata* (Hemiptera: Reduviidae) from Costa Rica

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Abstract Intraspecific genetic variation among *Triatoma dimidiata* (Hemiptera: Reduviidae) from seven Costa Rican populations and from different domestic, peridomestic, and sylvatic ecotopes were analyzed. The complete nucleotide sequence of the nuclear ribosomal DNA internal transcribed spacer (ITS-2) and partial sequences of the cytochrome B (Cyt b) gene and the large ribosomal subunit RNA (16S) of mitochondrial DNA (mtDNA) were analyzed and compared. All ITS-2 sequences analyzed were identical and correspond to the haplotype T.dim-H1, the most common haplotype in Central American populations. Sequences of mtDNA revealed a 10.17% of polymorphism in Cyt b and 2.39% in 16S, suggesting that the Cyt b fragment is a useful marker to describe the genetic structure of populations, even at habitat-related level. The analyses of the 18 new combined *T. dimidiata* haplotypes (Cytb/16S/ITS-2) showed that the two main geographical locations and populations studied are genetically structured showing different haplotype profiling. Only one combined haplotype was shared in the studied areas (Cytb.d/16S.a). Seven haplotypes exclusive for domestic/peridomestic populations, five for sylvatic, and six shared haplotypes for both habitat-related ecotopes are described. Although the relationship between the habitat and the haplotype profiling is less clear, there are different patterns of haplotype distribution in each geographic area between the two habitat-related ecotopes studied (domestic/peridomestic and sylvatic), some of them reflected in the phylogenetic relationships analyzed. The intraspecific variability detected may underlie the known plasticity of *T. dimidiata*, an important vector for Chagas disease transmission, suggesting that this species must be continuously monitored.

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Introduction

The Triatominae (Hemiptera: Reduviidae) represents a subfamily of hematophagous insects which are vectors of *Trypanosoma cruzi*, the agent of Chagas disease. The disease is a substantial public health problem in Latin America, affecting approximately 16–18 million people throughout this region, with 100 million more estimated to be at risk (WHO 1995, 2002). Nevertheless, these figures have suffered a marked decrease within the last 15 years, due to the efforts of the control initiatives in the different regions (Moncayo and Ortiz 2006; Schofield et al. 2006).

Control efforts in Central America include *Rhodnius prolixus* as a target for eradication because it is considered to be an introduced and exclusively domestic species (Dujardin et al. 1998; Zeledón 2004). This is in marked contrast with *Triatoma dimidiata*, a vector adapted to domestic and peridomestic habitats which has a large sylvatic reservoir that provides bugs to recolonize houses following pesticide application, thereby making monitoring and vigilance necessary (Dujardin et al. 1996; Ponce 1999; Zeledón et al. 2001; Cordón-Rosales 2002; Dumonteil et al. 2004). A

better understanding of the genetic structure of *T. dimidiata* populations is required for the design of more effective control strategies. Genetic analysis of subpopulations can reveal bug migration to houses either from residual peridomestic or from wild foci.

Validated molecular markers are needed for the determination of population structure and gene flow occurrence among subpopulations (Dotson and Beard 2001). Sequencing of nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) appears as the method furnishing more valuable information concerning the two objectives of triatomine systematics and vector characteristics up to the present. Both rDNA and mtDNA include neutral markers whose usefulness has already been emphasized in Chagas disease vectors (Monteiro et al. 2001; Bargues et al. 2002). The broad usefulness of these markers explains why the number of triatomine studies using them published in recent years has increased so pronouncedly (Mas-Coma and Bargues 2009). The sequences of the internal transcribed spacer 2 (ITS-2) of the rDNA have been proven to be a useful tool in the analysis of species, subspecies, hybrids, and populations and for inferring phylogenetic relationships in Triatominae (Bargues et al. 2000, 2002, 2006, 2008; Marcilla et al. 2001). Molecular analysis of mtDNA has also been widely used for phylogenies, population studies, and species relationships in Triatominae (Mas-Coma and Bargues 2009). The higher variability of mtDNA makes it a valuable tool for distinguishing differences among similar species and among populations of the same species from different geographic locations (García and Powell 1998; Lyman et al. 1999; Monteiro et al. 1999, 2000; García et al. 2001, 2003; Sainz et al. 2004; Giordano et al. 2005; Pfeiler et al. 2006; Fitzpatrick et al. 2008; Segura et al. 2009). In the case of *T. dimidiata*, the entire mitochondrial genome has been sequenced. It exhibits a relatively simple genetic structure, a straightforward form of transmission with no recombination, and a rapid rate of change that is up to ten times faster than nuclear DNA substitutions (Dotson and Beard 2001).

A study of the ITS-2 of rDNA in populations of *T. dimidiata* from several countries throughout its geographical dispersion showed that there are four well-defined groups that must be considered under the categories of species and subspecies (Bargues et al. 2008). Additionally, these groups exhibit different characteristics that affect the control strategies against them. Central American populations constitute a group that has been classified as the subspecies *Triatoma dimidiata dimidiata.* Its main characteristic is the capacity to occupy sylvatic, peridomestic, and domestic habitats and, therefore, it represents a serious challenge to the control programs as the extra-domiciliary populations act as reservoirs to repopulate the treated houses (Bargues et al. 2008; Dorn et al. 2009).

Due to the epidemiological importance of *T. dimidiata* as a vector of Chagas disease, the present work aims to genetically characterize the population structure of specimens from different regions and habitats in Costa Rica in the way to evaluate its spreading capacity and human influence on its population structure. Combined analysis of the complete sequence of the ITS-2 of the rDNA and partial sequences of the 16S gene and the Cyt b gene of mtDNA are used to investigate the haplotype profiling, genetic variation, and phylogenetic relationships of *T. dimidiata* populations from Costa Rica and other Mesoamerican countries.

Materials and methods

Triatomine specimens A total of 58 adults of *T. dimidiata* were collected in seven geographic locations of Costa Rica, including 19 specimens from Guanacaste province and 39 from the Central Valley region. The bugs were collected from two forests in the northwest province of Guanacaste, characterized by a dry tropical forest at low elevations, and from five communities located in the Central Valley region, the most populated area of Costa Rica, where the vegetation is mostly secondary and is characterized by a humid tropical forest. Both regions are separated by about 200 km in the Costa Rican territory. The insects were classified into three groups concerning their habitat as domestic (six specimens), peridomestic (21 specimens), and sylvatic (31 specimens). After being brought to the laboratory, triatomines were given unique ID codes. All the specimens were used for sequencing, genetic variation, and phylogenetic analysis (Table 1 and Fig. 1).

Amplification and sequencing of DNA for triatomine haplotyping Leg muscle tissue of each specimen was used and processed individually for DNA extraction using the Wizard Genomic Purification Kit (Promega Corporation, Madison, WI, USA), following the manufacturer's protocol and stored at −20°C until used. rDNA and mtDNA markers were amplified by standard polymerase chain reaction (PCR) using previously described primers for Cyt b (Monteiro et al. 2003), 16S (Lyman et al. 1999), and ITS-2 (Marcilla et al. 2001). The mitochondrial fragments were amplified using a total volume of 50 μl, containing 2 μl of DNA template; amplifications were generated in a Gene Amp 2400 (PE Applied Biosystems, Foster City, CA, USA) by 30 cycles of 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C, and followed by 7 min at 72°C for Cyt b; and by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and followed by 7 min at 72°C for 16S. The complete ITS-2 rDNA was PCR amplified using 4–6 μl of genomic DNA for each 50 μl reaction; amplifications were generated in a Peltier thermal cycler (MJ Research, Watertown, MA, USA) by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, preceded by 30 s at 94°C and followed by 7 min at 72°C. PCR products

Table 1 Geographic locations and habitats of the *Triatoma dimidiata* analyzed, haplotypes obtained for Cyt b, 16S, and ITS-2 markers, and GenBank accession numbers of the sequences

Geographic location	Habitat	Cyt b nucleotide haplotype	Cyt b aminoacid haplotype	Cyt b GenBank accession no.	16S Nucleotide haplotype	16S GenBank accession no.	ITS-2 Nucleotide haplotype	ITS-2 GenBank accession no.
Vuelta de Jorco, San José (3)	Peridomestic	T.dim-Cytb.h	T.dim-Cytb-AA.I	FN641811	T.dim-16S.c	FN641821	T.dim-H1	AM286693
Vuelta de Jorco, San José (2)	Peridomestic	T.dim-Cytb.k	T.dim-Cytb-AA.I	FN641814	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Vuelta de Jorco, San José (4)	Peridomestic	T.dim-Cytb.g	T.dim-Cytb-AA.II	FN641810	T.dim-16S.d	FN641822	T.dim-H1	AM286693
Vuelta de Jorco, San José	Peridomestic	T.dim-Cytb.l	T.dim-Cytb-AA.I	FN641815	T.dim-16S.c	FN641821	T.dim-H1	AM286693
Vuelta de Jorco, San José (2)	Sylvatic	T.dim-Cytb.a	T.dim-Cytb-AA.I	FN641804	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Santa Ana, San José	Sylvatic	T.dim-Cytb.c	T.dim-Cytb-AA.I	FN641806	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Santa Ana, San José (2)	Sylvatic	T.dim-Cytb.c	T.dim-Cytb-AA.I	FN641806	T.dim-16S.b	FN641820	T.dim-H1	AM286693
Santo Domingo, Heredia (4)	Peridomestic	T.dim-Cytb.c	T.dim-Cytb-AA.I	FN641806	T.dim-16S.b	FN641820	T.dim-H1	AM286693
Santo Domingo, Heredia	Peridomestic	T.dim-Cytb.n	T.dim-Cytb-AA.I	FN641817	T.dim-16S.c	FN641821	T.dim-H1	AM286693
Getsemaní, Heredia (2)	Domestic	T.dim-Cytb.j	T.dim-Cytb-AA.I	FN641813	T.dim-16S.b	FN641820	T.dim-H1	AM286693
Getsemaní, Heredia (3)	Domestic	T.dim-Cytb.e	T.dim-Cytb-AA.I	FN641808	T.dim-16S.b	FN641820	T.dim-H1	AM286693
Getsemaní, Heredia	Domestic	T.dim-Cytb.l	T.dim-Cytb-AA.I	FN641815	T.dim-16S.c	FN641821	T.dim-H1	AM286693
Getsemaní, Heredia	Peridomestic	T.dim-Cytb.a	T.dim-Cytb-AA.I	FN641804	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Getsemaní, Heredia	Peridomestic	T.dim-Cytb.e	T.dim-Cytb-AA.I	FN641808	T.dim-16S.b	FN641820	T.dim-H1	AM286693
Getsemaní, Heredia	Peridomestic	T.dim-Cytb.d	T.dim-Cytb-AA.I	FN641807	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Getsemaní, Heredia	Peridomestic	T.dim-Cytb.c	T.dim-Cytb-AA.I	FN641806	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Getsemaní, Heredia (3)	Sylvatic	T.dim-Cytb.d	T.dim-Cytb-AA.I	FN641807	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Getsemaní, Heredia	Sylvatic	T.dim-Cytb.a	T.dim-Cytb-AA.I	FN641804	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Lagunilla, Heredia (5)	Sylvatic	T.dim-Cytb.a	T.dim-Cytb-AA.I	FN641804	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Mayorga, Guanacaste	Peridomestic	T.dim-Cytb.b	T.dim-Cytb-AA.I	FN641805	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Mayorga, Guanacaste	Peridomestic	T.dim-Cytb.i	T.dim-Cytb-AA.III	FN641812	T.dim-16S.e	FN641823	T.dim-H1	AM286693
Mayorga, Guanacaste (8)	Sylvatic	T.dim-Cytb.b	T.dim-Cytb-AA.I	FN641805	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Mayorga, Guanacaste (2)	Sylvatic	T.dim-Cytb.f	T.dim-Cytb-AA.I	FN641809	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Mayorga, Guanacaste	Sylvatic	T.dim-Cytb.d	T.dim-Cytb-AA.I	FN641807	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Mayorga, Guanacaste	Sylvatic	T.dim-Cytb.i	T.dim-Cytb-AA.III	FN641812	T.dim-16S.e	FN641823	T.dim-H1	AM286693
Santa Rosa, Guanacaste	Sylvatic	T.dim-Cytb.i	T.dim-Cytb-AA.III	FN641812	T.dim-16S.c	FN641821	T.dim-H1	AM286693
Santa Rosa, Guanacaste	Sylvatic	T.dim-Cytb.o	T.dim-Cytb-AA.IV	FN641818	T.dim-16S.e	FN641823	T.dim-H1	AM286693
Santa Rosa, Guanacaste	Sylvatic	T.dim-Cytb.m	T.dim-Cytb-AA.I	FN641816	T.dim-16S.a	FN641819	T.dim-H1	AM286693
Santa Rosa, Guanacaste	Sylvatic	T.dim-Cytb.f	T.dim-Cytb-AA.I	FN641809	T.dim-16S.f	FN641824	T.dim-H1	AM286693
Santa Rosa, Guanacaste	Sylvatic	T.dim-Cytb.f	T.dim-Cytb-AA.I	FN641809	T.dim-16S.a	FN641819	T.dim-H1	AM286693

The numbers in brackets indicate the number of individuals analyzed with the same results

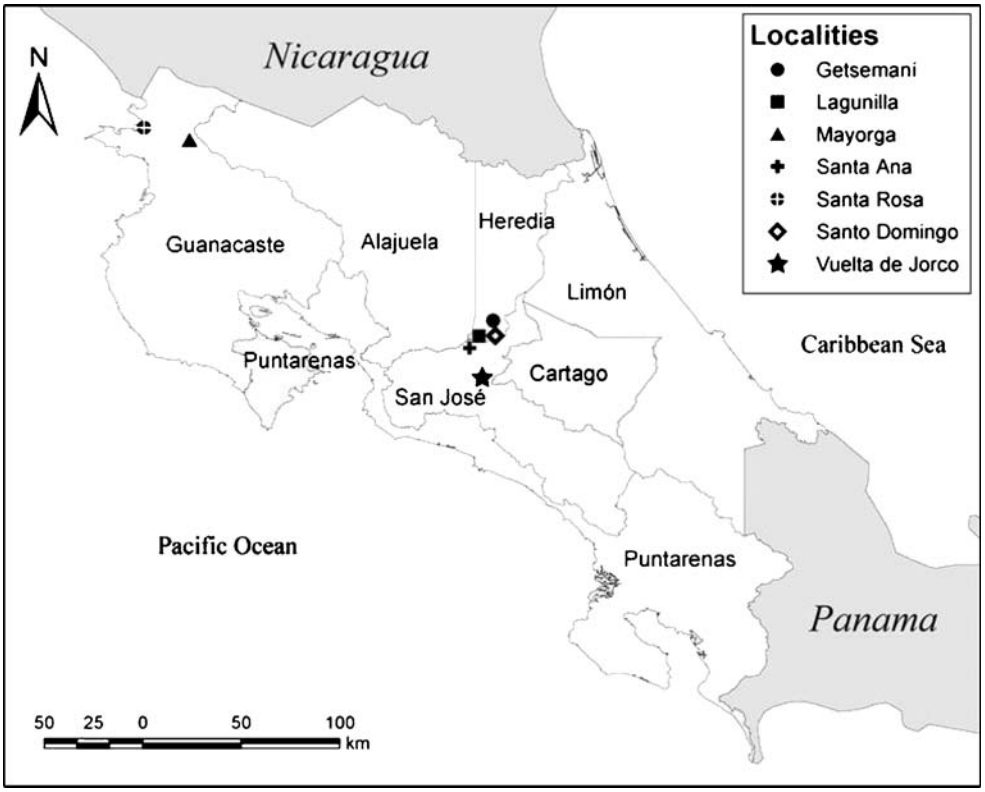

Fig. 1 Map of Costa Rica, indicating the seven studied geographical localities in the Guanacaste Province and Central Valley region

were purified with Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA), according to the manufacturer's protocol, and resuspended in 50 µl of 10 mM TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6). In both cases, amplification products were analyzed in 1% agarose gel, and purified products were sequenced by the dideoxy chain-termination method, using the same amplification PCR primers.

Haplotype terminology The haplotype terminology used in the present paper follows the nomenclature for composite haplotyping recently proposed by Bargues et al. (2006). Accordingly, ITS-2 haplotypes are noted by numbers, and mt fragments are preceded by the name of the gen and followed by a letter. Mitochondrial haplotypes here described are considered as preliminary, becuase only fragments of the genes are analyzed.

Sequence alignments Sequences were aligned using CLUSTAL-W version 1.8 (Thompson et al. 1994) and MEGA 4.0 (Tamura et al. 2007), and assembly was made with the Staden Package (Staden et al. 2001). Genetic distances were measured using parameters provided by PAUP v.4.0b10 (Swofford 2002).

Sequence comparisons The following sequences from GenBank-EMBL have been used for comparison analyses:

- rDNA ITS-2: *T dimidiata* 31 haplotypes (T.dim-H1 to T.dim-H31, GenBank accession numbers AM286693 to AM286723; Bargues et al. 2008); *T. dimidiata* eight haplotypes T.dim-H32 to T.dim-H39 (FJ197146 to FJ197153; Dorn et al. 2009).
- Cyt b: *T. dimidiata* Guatemala (Dotson and Beard 2001); *T. dimidiata* clone TdcGUA1 Guatemala (AY062157), *T. dimidiata* clone TdcTHON1 Honduras (AY062152), *T. dimidiata* clone TdcTHON4 Honduras (AY062156), *T. dimidiata* clone TdcTHON6 Honduras (AY062155), *T. dimidiata* clone TdcTHON5 Honduras (AY062154), *T. dimidiata* clone TdcTHON7 Honduras (AY062153), *T. dimidiata* clone TdcVMEX1 Mexico (AY062150); *T. dimidiata* clone TdcYUC1 Yucatan, Mexico (AY062162) (Harris and Beard 2003); *T. dimidiata* isolate BzToSP33, Belize (FJ197154), *T. dimidiata* isolate BzToST32 Belize (FJ197155), *T. dimidiata* isolate BzCaCC34 Belize (FJ197156) (Dorn et al. 2009).
- 16S: *T. dimidiata* Guatemala (Dotson and Beard 2001); *T. dimidiata* clone TdlGUA1 Guatemala (AY062140), *T. dimidiata* clone TdlTHON1 Honduras (AY062135), *T. dimidiata* clone TdlVMEX1 Mexico (AY062133), *T. dimidiata* clone TdlYUC1 Yucatan, Mexico (AY062145) (Harris and Beard 2003).

Phylogenetic inference Phylogenies were inferred from DNA sequences using distance analysis estimates with PAUP 4b10 software (Swofford 2002). Total character differences and mean character differences adjusted for

missing data were determined by pair-wise comparisons. When more than one individual from a population yielded the same sequence, only a single haplotype was included in the analysis. PAUP 4.0b10 and MEGA4 software (Tamura et al. 2007) were used to generate a Neighbor-joining tree (Saitou and Nei 1987) based on Kimura 2-parameter distance matrix (Kimura 1980). Statistical support for clades in the phylogenetic tree was assessed by the bootstrap method (Felsenstein 1985) with 1,000 replications. Cyt b and 16S sequences retrieved from GenBank were included in the analysis, and the sequences from Yucatan, Mexico, were used as outgroup.

Results

Cytochrome b gene partial sequence analysis A total of 15 haplotypes (T.dim-Cytb.a to T.dim-Cytb.o) were found in the analysis of the cytochrome b sequence fragments of *T. dimidiata* from Costa Rica. The most frequent haplotypes were T.dim-Cytb.a, T.dim-Cytb.b, and T.dim-Cytb.c, which were found mainly in the sylvatic ecotopes and sporadically in peridomestic ecotopes. The rest of the haplotypes exhibited a low frequency and were spread in both studied habitats. Regarding their geographical distribution, only one haplotype (T.dim-Cytb.d) was found in both areas of study, nine haplotypes were exclusive from the Central Valley (T.dim-Cytb.a, T.dim-Cytb.c, T.dim-Cytb.e, T.dim-Cytb.g, T.dim-Cytb.h, T.dim-Cytb.j, T.dim-Cytb.k, T.dim-Cytb.l, and T.dim-Cytb.n), and four were from Guanacaste (T.dim-Cytb.b, T.dim-Cytb.f, T.dim-Cytb.i, and T.dim-Cytb.m; Table 1). The length of the studied fragment was 600 bp, and its AT content was 63.7% on average. A total of 61 variable positions were detected in the 600-bp-long alignment (variability of 10.17%), of which, 16 were autapomorphies, and 45 were parsimony informative sites. Of the mutations, 88.9% occurred in the third position of the codon, being 66.7% of them transitions between C and T. The number of non-synonymous mutations calculated pair by pair varied from 0 to 3, with an average of 1.02. When the sequences extracted from GenBank were included in the analyses, the number of variable positions rose to 131 (21.83% of variability), with 66 autapomorphies and 65 parsimony informative sites. This increment of variability was mainly due to the Mexican sequences, especially to the sequence from Yucatan. When converted to amino acid sequences, only four haplotypes remained within the Costa Rican populations. The most frequent amino acidic haplotype was T.dim-Cytb-AA.I, which was identical in 12 of the 15 nucleotide haplotypes described and was spread through all the studied locations. The other three amino acid haplotypes (T.dim-Cytb-AA.II, T.dim-Cytb-AA.III, and T.dim-Cytb-AA.IV) were the product of individual nucleotide haplotypes, which were found only in one location or province (Table 1).

For an analysis of populations relationships, a comparison between these 15 Cyt b haplotypes from Costa Rica was analyzed in a pair-wise distance matrix performed with PAUP 4.0b10 including *T. dimidiata* populations from other Mesoamerican countries (Table 2).

The new 15 haplotype sequences (T.dim-Cytb.a to T.dim-Cytb.o) have been deposited in the EMBL, GenBank, and DDBJ databases, where they are available under the following accession numbers: FN641804 to FN641819.

16S mitochondrial rRNA gene partial sequence analysis The analysis of the 16S rRNA sequences revealed the existence of six different haplotypes within the Costa Rican populations analyzed (T.dim-16S.a to T.dim-16S.f). The most frequent haplotype was T.dim-16S.a, followed by T.dim-16S.b and T.dim-16S.c. However, none of them turned to be specific from a habitat, appearing indistinctly in peridomestic and sylvatic ecotopes. In relation to the geographical distribution, two haplotypes were restricted to the Central Valley (T.dim-16S.b and T.dim-16S.d); the other two to Guanacaste (T.dim-16S.e and T.dim-16S.f) and the two remaining were shared in both areas (T.dim-16S.a and T.dim-16S.c). The length of the analyzed fragment was 376 bp, and its AT content was 71.1% on average. Only nine variable positions were found in the 376-bp-long alignment (variability of 2.39%) including six autapomorphies and three parsimony informative sites. Eighty-three percent of the mutations were transitions, all of them between A and G. When the sequences extracted from GenBank were added to the analysis, the number of variable positions increased to 25 (6.65% of variability), including 18 autapomorphies and seven parsimony informative sites. As occurred with Cyt b, the Mexican sequences were mostly accountable for the increased variability.

For an analysis of populations relationships, a comparison between these six 16S rRNA sequences and those of *T. dimidiata* from other Mesoamerican was made with a pair-wise 16S rRNA distance matrix performed with PAUP 4.0b10 (Table 3).

The six haplotype sequences (T.dim-16S.a to T.dim-16S.f) have been deposited in the EMBL, GenBank, and DDBJ databases, where they are available under the following accession numbers: FN641819 to FN641824.

ITS-2 sequence analysis All the specimens analyzed presented the same ITS-2 sequence, corresponding to the T.dim-H1 haplotype (AM286693) described by Bargues et al. (2008), which is the most frequent variant in the Central American populations of *T. dimidiata*. No intra or interpopulational differences were detected.

Molecular combined analysis A total of 18 new combined (Cytb/16S/ITS-2) haplotypes were described for the total of

Table 2 Total character differences (above diagonal) and mean character differences adjusted for missing data (below diagonal), as determined using the PAUP program, in pair-wise comparisons between the Cytb sequences of the *Triatoma dimidiata* populations studied

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	–	0.01000	0.05500	0.05667	0.06000	0.03667	0.03833	0.04167	0.04167	0.04167	0.05500	0.05500	0.04667	0.04833
2	6	–	0.05500	0.05333	0.05667	0.03333	0.03500	0.04167	0.03833	0.04167	0.05167	0.05167	0.04333	0.04500
3	33	33	–	0.02000	0.02333	0.04667	0.04833	0.04833	0.04500	0.04167	0.06167	0.06167	0.06000	0.06500
4	34	32	12	–	0.01667	0.03667	0.03833	0.04167	0.04000	0.03833	0.05667	0.05667	0.05500	0.06000
5	36	34	14	10	–	0.04000	0.04167	0.04500	0.03667	0.03500	0.05667	0.05667	0.05500	0.06000
6	22	20	28	22	24	–	0.00167	0.00833	0.02333	0.02167	0.04000	0.04000	0.03500	0.04333
7	23	21	29	23	25	1	–	0.01000	0.02500	0.02333	0.04167	0.04167	0.03667	0.04500
8	25	25	29	25	27	5	6	–	0.02833	0.02333	0.04833	0.04833	0.04333	0.05167
9	25	23	27	24	22	14	15	17	–	0.00500	0.04667	0.04667	0.04167	0.04333
10	25	25	25	23	21	13	14	14	3	–	0.05167	0.05167	0.04667	0.04833
11	33	31	37	34	34	24	25	29	28	31	–	0.00000	0.01500	0.03000
12	33	31	37	34	34	24	25	29	28	31	0	–	0.01500	0.03000
13	28	26	36	33	33	21	22	26	25	28	9	9	–	0.01833
14	29	27	39	36	36	26	27	31	26	29	18	18	11	–
15	30	28	40	37	37	27	28	32	27	30	19	19	12	1
16	29	27	37	34	34	24	25	29	24	27	16	16	9	2
17	30	28	38	35	35	25	26	30	25	28	17	17	10	3
18	31	29	39	36	36	26	27	31	26	29	16	16	9	4
19	29	27	37	34	34	26	27	31	26	29	18	18	11	4
20	30	28	38	35	35	27	28	32	27	30	19	19	12	5
21	33	31	37	34	34	28	29	33	24	27	18	18	13	6
22	24	24	34	33	33	25	26	28	25	26	21	21	12	15
23	23	23	33	32	32	24	25	27	24	25	20	20	13	14
24	25	25	33	32	32	22	23	23	24	25	20	20	13	16
25	44	42	50	46	46	46	47	47	45	47	45	45	46	47
26	75	71	83	83	80	71	70	74	70	69	72	72	73	74
27	78	74	86	86	81	74	75	77	73	72	75	75	76	75

1, *2*: *Triatoma dimidiata* Belize (FJ197154 and FJ197155); *3–5*: *T. dimidiata* Costa Rica (haplotypes Tdim-Cytb.g, Tdim-Cytb.o, and Tdim-Cytb.i); *6–10*: *T. dimidiata* Honduras (AY062152, AY062154, AY062156, AY062153, and AY062155); *11–12*: *T. dimidiata* Guatemala (AF30159 and AY06215); *13–24*: *T. dimidiata* Costa Rica (haplotypes Tdim-Cytb.b, Tdim-Cytb.a, Tdim-Cytb.d, Tdim-Cytb.c, Tdim-Cytb.e, Tdim-Cytb.j, Tdim-Cytb.f, Tdim-Cytb.k, Tdim-Cytb.m, Tdim-Cytb.h, Tdim-Cytb.n, and Tdim-Cytb.l); *25*: *T. dimidiata* Mexico (AY062150); *26*: *T. dimidiata* Belize (FJ197156); *27*: *T. dimidiata* Mexico, Yucatan (AY06216)

	15	16	17	18	19	20	21	22	23	24	25	26	27
1	0.05000	0.04833	0.05000	0.05167	0.04833	0.05000	0.05500	0.04000	0.03833	0.04167	0.07333	0.12500	0.13000
2	0.04667	0.04500	0.04667	0.04833	0.04500	0.04667	0.05167	0.04000	0.03833	0.04167	0.07000	0.11833	0.12333
3	0.06667	0.06167	0.06333	0.06500	0.06167	0.06333	0.06167	0.05667	0.05500	0.05500	0.08333	0.13833	0.14333
4	0.06167	0.05667	0.05833	0.06000	0.05667	0.05833	0.05667	0.05500	0.05333	0.05333	0.07667	0.13833	0.14333
5	0.06167	0.05667	0.05833	0.06000	0.05667	0.05833	0.05667	0.05500	0.05333	0.05333	0.07667	0.13333	0.13500
6	0.04500	0.04000	0.04167	0.04333	0.04333	0.04500	0.04667	0.04167	0.04000	0.03667	0.07667	0.11833	0.12333
7	0.04667	0.04167	0.04333	0.04500	0.04500	0.04667	0.04833	0.04333	0.04167	0.03833	0.07833	0.11667	0.12500
8	0.05333	0.04833	0.05000	0.05167	0.05167	0.05333	0.05500	0.04667	0.04500	0.03833	0.07833	0.12333	0.12833
9	0.04500	0.04000	0.04167	0.04333	0.04333	0.04500	0.04000	0.04167	0.04000	0.04000	0.07500	0.11667	0.12167
10	0.05000	0.04500	0.04667	0.04833	0.04833	0.05000	0.04500	0.04333	0.04167	0.04167	0.07833	0.11500	0.12000
11	0.03167	0.02667	0.02833	0.02667	0.03000	0.03167	0.03000	0.03500	0.03333	0.03333	0.07500	0.12000	0.12500
12	0.03167	0.02667	0.02833	0.02667	0.03000	0.03167	0.03000	0.03500	0.03333	0.03333	0.07500	0.12000	0.12500
13	0.02000	0.01500	0.01667	0.01500	0.01833	0.02000	0.02167	0.02000	0.02167	0.02167	0.07667	0.12167	0.12667
14	0.00167	0.00333	0.00500	0.00667	0.00667	0.00833	0.01000	0.02500	0.02333	0.02667	0.07833	0.12333	0.12500
15	–	0.00500	0.00667	0.00833	0.00833	0.01000	0.01167	0.02667	0.02500	0.02833	0.07833	0.12333	0.12500
16	3	–	0.00167	0.00333	0.00333	0.00500	0.00667	0.02167	0.02000	0.02333	0.07833	0.12667	0.12833
17	4	1	–	0.00167	0.00500	0.00667	0.00833	0.02333	0.02167	0.02500	0.07667	0.12833	0.13000
18	5	2	1	–	0.00667	0.00833	0.01000	0.02500	0.02333	0.02667	0.07833	0.13000	0.13167
19	5	2	3	4	–	0.00167	0.01000	0.02167	0.02000	0.02333	0.07833	0.12333	0.12500
20	6	3	4	5	1	–	0.01167	0.02333	0.02167	0.02500	0.08000	0.12500	0.12667
21	7	4	5	6	6	7	–	0.02833	0.02667	0.03000	0.08167	0.13000	0.13167
22	16	13	14	15	13	14	17	–	0.00167	0.00833	0.07333	0.12833	0.13333
23	15	12	13	14	12	13	16	1	–	0.00667	0.07500	0.12667	0.13167
24	17	14	15	16	14	15	18	5	4	–	0.07167	0.12333	0.12833
25	47	47	46	47	47	48	49	44	45	43	–	0.12500	0.13000
26	74	76	77	78	74	75	78	77	76	74	75	–	0.01333
27	75	77	78	79	75	76	79	80	79	77	78	8	–

Table 3 Total character differences (above diagonal) and mean character differences adjusted for missing data (below diagonal), as determined using the PAUP program, in pair-wise comparisons between the 16S sequences of the *Triatoma dimidiata* populations studied

	1	2	3	4	5	6	7	8	9	10	11
1	–	0.00266	0.00532	0.01330	0.01330	0.00266	0.00798	0.00798	0.00532	0.01330	0.03723
2	1	–	0.00798	0.01596	0.01596	0.00532	0.01064	0.01064	0.00798	0.01064	0.03989
3	2	3	–	0.01330	0.01330	0.00266	0.01330	0.01330	0.00532	0.01064	0.03723
4	5	6	5	–	0.01064	0.01064	0.02128	0.02128	0.01330	0.02128	0.04787
5	5	6	5	4	–	0.01064	0.02128	0.02128	0.01330	0.02128	0.04787
6	1	2	1	4	4	–	0.01064	0.01064	0.00266	0.01064	0.03989
7	3	4	5	8	8	4	–	0.00000	0.01330	0.02128	0.03989
8	3	4	5	8	8	4	0	–	0.01330	0.02128	0.03989
9	2	3	2	5	5	1	5	5	–	0.01330	0.04255
10	5	4	4	8	8	4	8	8	5	–	0.04255
11	14	15	14	18	18	15	15	15	16	16	–

1–6: *T. dimidiata* Costa Rica (haplotypes Tdim-16S.a, Tdim-16S.b, Tdim-16S.c, Tdim-16S.d, Tdim-16S.e, and Tdim-16S.f), *7–8*: *T. dimidiata* Guatemala (AF301594 and AY062140); *9*: *T. dimidiata* Honduras (AY062135); *10*: *T. dimidiata* Mexico (AY062133); *11*: *T. dimidiata* Mexico, Yucatan (AY062145)

T. dimidiata populations and ecotopes studied. All of these haplotypes present the same ITS-2 haplotype (T.dim-H1). For this reason, an analysis of geographical and habitat-related distribution was carried out combining only both mitochondrial sequences. The comparison was performed separating the samples by its geographical origin: Guanacaste and Central Valley, and by its type of habitat: sylvatic and domestic/peridomestic (these two habitats were grouped since their relationship with human behavior). Of the 18 combined haplotypes, seven were exclusive for domestic/peridomestic populations and five for sylvatic ones. Shared haplotypes between both habitat-related ecotopes were six. In the same words, 13 combined haplotypes were described for domestic/peridomestic populations and 11 for sylvatic ones, and only six, between them, are shared haplotypes in D/P/S. This represents a genetic structuration for habitat-related ecotopes of *T. dimidiata* populations studied. Only one combined haplotype was present in both study areas and in both habitats (Cytb.d/16S.a). Haplotype exclusive/shared and presence/absence by regions and ecotopes were detailed in Fig. 2.

Phylogenetic analysis The neighbor-joining tree constructed combining Cyt b and 16S partial sequences (Fig. 3) do not show a clear geographical differentiation within the Costa Rican populations studied. Both sample groups, corresponding to Guanacaste and Central Valley regions, are present in the different clades obtained. The Costa Rican sequences are distributed in two main branches of the tree together with the other Mesoamerican sequences included in the analysis. The upper branch clusters 14 haplotypes (nine from Central Valley, four from Guanacaste, and one shared) from Costa Rica with the sequences from Guatemala, while the lower branch clusters the remaining four Costa Rican haplotypes (three from Guanacaste and one from Central Valley) with the sequence from Honduras. A subdivision in the base of the upper branch separates a group of three haplotypes found exclusively in the domestic/peridomestic

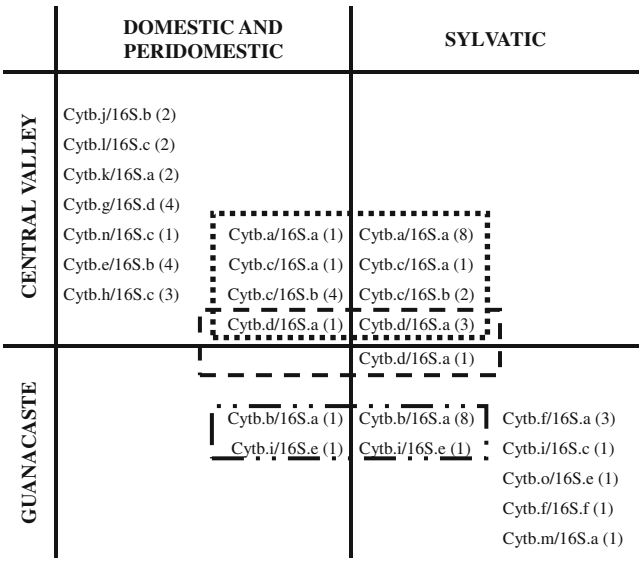

Fig. 2 Distribution of Cyt b/16S combined haplotypes on each study zone (Central Valley and Guanacaste) and each type of habitat (domestic/peridomestic and sylvatic). The *dashed line* groups the haplotype shared between both study areas. The *dotted line* groups the shared haplotypes between both habitats in Central Valley. The *dash-dot-dot line* groups the shared haplotypes between both habitats in Guanacaste. Non-grouped haplotypes are exclusive from the area and habitat where are represented. *Numbers in brackets* indicate the number of individuals with the combined haplotype in each region and habitat

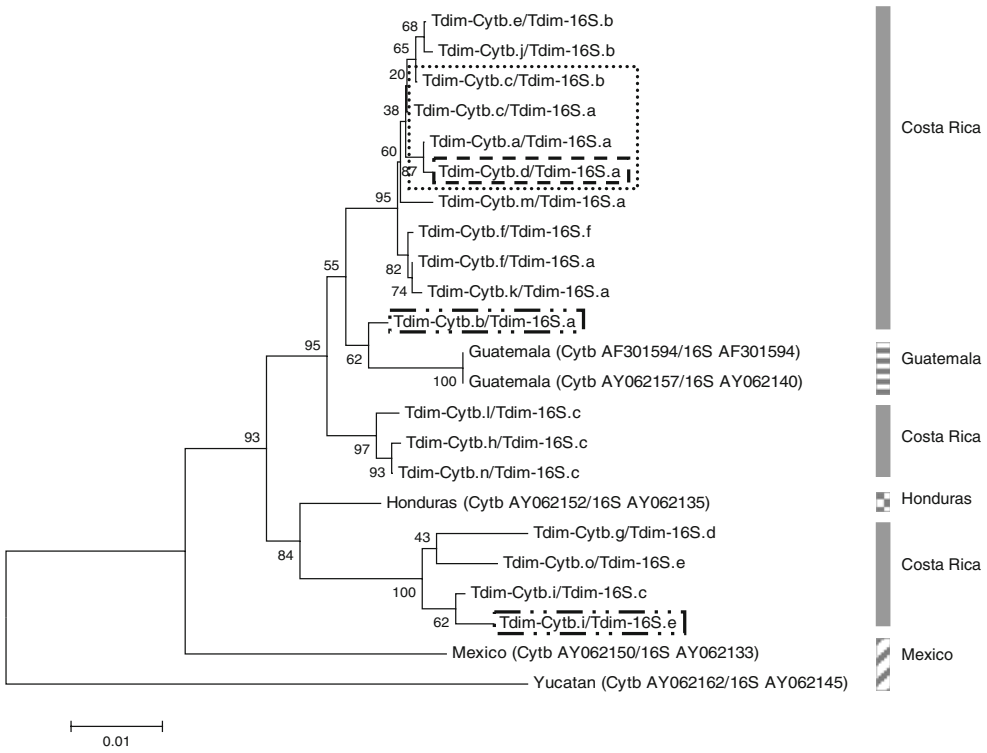

Fig. 3 Neighbor-joining tree based on Kimura 2-parameter model of Cyt b and 16S partial sequences. The *scale bar* indicates the number of substitutions per sequence position. *Numbers* represent the percentage of 1,000 bootstrap replicates. For line significance, see legend of Fig. 2

habitat of the Central Valley region. All the haplotypes present in sylvatic ecotopes of Central Valley cluster together.

In a tree constructed only with the Cyt b fragment, the sequences from Honduras (AY062156, AY062155, AY062154, and AY062153) and Belize (FJ197154, FJ197155, and FJ197156) were included in the analysis. The Costa Rican sequences presented the same distribution as in the combined tree, clustering in the same two branches. The new Honduran sequences and two sequences from Belize (FJ197154 and FJ197155) clustered in the lower branch of Mesoamerican sequences, while the remaining sequence from Belize clustered with the Yucatan one (tree not shown).

Discussion

The high level of polymorphism (10.17%) at the Cyt b fragment of mtDNA suggests that this marker is a useful tool at the level of population genetic structure. Although very little is known about this gene in *T. dimidiata*, some studies have already been performed with other species and genera, as *Triatoma brasiliensis*, which showed a level of polymorphism of 19.4% (Monteiro et al. 2004); *Rhodnius robustus* and *R. prolixus*, which showed 16.7% of variable sites (Monteiro et al. 2003); and species complexes among tribes Rhodniini and Triatomini, which showed a 48% of polymorphism (Lyman et al. 1999). In this study, the Cyt b sequence analysis supported the existence of intraspecific variability among *T. dimidiata* populations and domestic/peridomestic and sylvatic habitat-related ecotopes from Costa Rica.

Lyman et al. (1999) examined the region of the rDNA 16S mitochondrial gene from 18 reduviid species and reported a 43% of polymorphism that included base substitutions, insertions, and deletions, demonstrating the utility of this gene for taxonomic analysis of different species of triatomine bugs, but they did not assess intraspecific sequence variation. Segura et al. (2009) carried out an intraspecific study of *Triatoma infestans* from Argentina based on this marker and found a low variability rate, with a predominant haplotype present in the 76.9% of the individuals, due to a low mutation rate in this gene. Our study showed only a 2.39% of intraspecific variation at the level of the 16S rRNA gene for *T. dimidiata* populations and ecotopes studied. This marker was able to provide two restricted haplotypes for the Central Valley (T.dim-16S.b and T.dim-16S.d), other two for Guanacaste (T.dim-16S.e and T.dim-16S.f), and the two remaining were shared haplotypes for both geographical regions. The most abundant 16S haplotype was T.dim-16S.a, which was present in more than half of the samples analyzed (53.45%). However, none of them turned to be specific from a habitat, appearing indistinctly in peridomestic and sylvatic ecotopes. These features suggest that this region is a poor molecular marker for intrapopulational analyses in *T. dimidiata*, and probably it is a suitable marker for

distinguishing genetically more distant taxa. Our study revealed significant differences between the Cyt b and 16S regions, with 10.17% polymorphism in Cyt b as compared to 2.39% in 16S. This observation is consistent with the results of Lyman et al. (1999), who used both genes for interspecific analysis and concluded that the Cyt b region appears to have evolved faster than the examined region of 16S.

The study of the ITS-2, a region which has proven to be an excellent marker to discriminate between species, subspecies, and population groups (Bargues et al. 2000), revealed the presence of one unique haplotype in the analyzed samples, the haplotype T.dim-H1 (Bargues et al. 2008). The lack of intrapopulational variability in this marker is explained by the concerted evolution that rDNA follows, which with sufficient time, effectively homogenizes the many copies of nuclear rDNA among both homologous and non-homologous chromosomes containing rDNA clusters within a genome (Hillis and Dixon 1991; Liao 1999). Although this pronouncedly simplifies interpopulation studies within a concrete species, it also represents a limitation for genetic intrapopulation analyses (Mas-Coma and Bargues 2009).

The analyses of the combined mitochondrial preliminary haplotypes showed that the two main geographical locations and populations studied are genetically structured showing different haplotype profiling. Only one combined haplotype was present in both study areas (Cytb.d/16S.a), suggesting very little contact between them most probably due to geographical barriers.

At intrapopulational level and related to domestic/peridomestic and sylvatic ecotopes, we found that for the same geographical location, no evident differences appear between the ecotopes. In the case of Central Valley region, all combined haplotypes detected in sylvatic ecotopes are also present in domestic/peridomestic habitats, as well as in the Guanacaste region, where the only two haplotypes detected in domestic/peridomestic habitats are also present in sylvatic ones. However, our study demonstrates the existence of genetic variability between domestic/peridomestic and sylvatic *T. dimidiata* from Costa Rica analyzed based on the different haplotype profiling they show, allowing the distinction between the two main geographical areas studied in this country. A detailed distribution of the new 18 combined haplotypes (Cytb/16S/ITS-2) allows us to describe seven haplotypes exclusive for domestic/peridomestic populations, five for sylvatic and six shared haplotypes for both habitat-related ecotopes.

The phylogenetic analysis did not show a clear relationship between the geographical precedence of the samples or between their habitat and its position on the tree. However, there is predominance of Central Valley sequences in the upper main branch, with interesting associations within it, as a subdivision clustering three haplotypes exclusive from the peridomestic and domestic habitat, and all the haplotypes present in the sylvatic habitat of this region clustering together. There is also predominance of sequences from Guanacaste in the lower main branch, where only one Central Valley haplotype is present. These results prove the intraspecific variability in the studied populations is also related with the geographical and habitat-related precedence of the samples. The intraspecific variability detected may underlie the known plasticity of *T. dimidiata*, an important vector for Chagas disease transmission, suggesting that this species must be continuously monitored.

Regarding the vectorial control in this country, it must be highlighted that Costa Rican populations are identified as the subspecies *T. dimidiata dimidiata*, which is the most common variant in Mesoamerica and the origin of the group of species and subspecies known as *T. dimidiata sensu lato* (Bargues et al. 2008). This subspecies is characterized by its capacity of occupying all kind of habitats, which permits the re-infestation of the houses by peridomestic and wild insect populations shortly after the fumigation, compromising the effectiveness of control campaigns. In Costa Rica, a high degree of recolonization by wild adults has been reported (Zeledón and Rojas 2006), and chromosomal variation on *T. dimidiata* has shown the existence of gene flow among domestic, peridomestic, and sylvatic populations in Central America (Panzera et al. 2006). In this situation, other strategies, alternatives to the fumigation with insecticides, as environmental management, must be taken into account to improve the control (Zeledón and Rojas 2006; Zeledón et al. 2008).

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