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High genetic diversity in a single population of *Triatoma sanguisuga* (LeConte, 1855) inferred from two mitochondrial markers: *Cytochrome* b and 16S ribosomal DNA

N. de la Rua [a,b], L. Stevens [b], P.L. Dorn [a,*]

[a] Loyola University New Orleans, New Orleans, LA 70118, USA
[b] University of Vermont, Burlington, VT, USA

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ABSTRACT

Chagas disease remains endemic across much of Latin America, but is largely enzootic – restricted to wild mammals and triatomine vectors in the United States. Within the United States, there are ten recognized species of triatomines and 18 mammals reported naturally infected with *Trypanosoma cruzi*, the causative agent of Chagas disease. However, only six cases of autochthonous vector-borne transmission of *T. cruzi* to humans have been reported in the United States. As a follow-up to the sixth reported case, triatomine insects were collected from the index case site, in a rural area of New Orleans, LA, USA. During the summer months of 2006 and 2007, 344 *Triatoma sanguisuga* were collected and showed a *T. cruzi* infection prevalence of 56%. A subset of these insects was analyzed to infer intraspecific genetic variation from a 606 bp fragment of *cytochrome* b ($n = 54$) and a 340 bp fragment of 16S ribosomal DNA ($n = 17$). From the 54 samples, 37 *cyt*b haplotypes ($H_d = 0.978$) were observed and 14.7% of positions were polymorphic. Phylogenetic analysis divides the population into two distinct groups with an average pairwise genetic distance of ~5%. The 16S rDNA sequences revealed 6 haplotypes among 17 specimens ($H_d = 0.713$) with 1.2% of the positions exhibiting polymorphisms. 16S polymorphism data support the concept of two groups within this single population. The genetic distance of Group 1 from Group 2 suggests that Group1 could represent a sub-species as this level of divergence is similar to that observed among other triatomine subspecies.

1. Introduction

Chagas disease, which causes the greatest negative economic impact on Latin America of any parasitic disease, remains endemic in this region with 8–9 million people infected with the causative agent, *Trypanosoma cruzi* (Hotez et al., 2008). In 2004, there were a reported 11,000 annual deaths, and an annual loss of 430,000 disability adjusted life years (W.H.O., 2008). The parasite is transmitted primarily by triatomine insects (Hemiptera: Reduviidae) as parasite-laden feces are deposited on host skin during or following engorgement with a blood meal. *T. cruzi* can also be transmitted congenitally, by blood transfusion, organ transplantation, oral transmission, and laboratory accident.

Chagas disease is not considered endemic in the United States; only six human cases of autochthonous vector-borne transmission have been reported (Dorn et al., 2007; Herwaldt et al., 2000). However, Chagas disease is gaining attention in the United States due to the presence of an estimated 300,000 *T. cruzi* infected immigrants from endemic areas (Bern and Montgomery, 2009). So while the risk of local, vector-borne transmission is considered low, Chagas disease is present in the United States. Screening of the United States blood supply began in 2007 and to date >1300 blood units have been confirmed positive for antibodies to *T. cruzi* (AABB, 2011).

Although autochthonous vector-borne transmission of *T. cruzi* to humans is rare in the United States, a robust sylvan cycle appears to be maintained by a close association of the triatomine insect with mammalian hosts acting as reservoirs for *T. cruzi*. Eighteen species of mammals have been reported naturally infected with the parasite in the United States (John and Hoppe, 1986). There are ten species of *Triatoma* found in 27 states across the southern ~2/3 of the United States (Usinger, 1944) and seven of the ten have been found naturally infected with *T. cruzi* (Davis et al., 1943; Kofoid and Whitaker, 1936; Sullivan et al., 1949; Wood, 1941a,b, 1958).

As a follow-up study to our description of the sixth human autochthonous transmission of *T. cruzi* (Dorn et al., 2007), triatomine insects were collected from the location of the index case over a two-

* Corresponding author at: Department of Biological Sciences, Loyola University New Orleans, 6363 St. Charles Ave., New Orleans, LA 70118, USA.
Tel.: +1 504 865 3672.
E-mail address: dorn@loyno.edu (P.L. Dorn).

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year period in a rural area of New Orleans, Louisiana. All insects collected from the location were identified as *Triatoma sanguisuga*. Traditionally, *T. sanguisuga* has been classified as a sylvatic species (Lent and Wygodzinsky, 1979) with a very broad geographic range spanning 23 states across the southern United States. However, in addition to sylvan environments, it has been regularly found in close association with houses, including urban settings, although without colonizing homes (Lent and Wygodzinsky, 1979). *T. sanguisuga* in homes was reported as early as 1868 (Walsh and Riley, 1868) and, indeed, this vector species has been implicated in two of the six documented cases of human autochthonous transmission in the United States (Dorn et al., 2007; Ochs et al., 1996).

Previous studies have addressed the vector capacity, *T. cruzi* infection prevalence, and mammalian-triatomine interactions of *T. sanguisuga* (Hays, 1965; Kjos et al., 2008; Pippin, 1970). However, to the best of our knowledge, no population genetic studies have been reported for this vector species. The purpose of this study is to describe the genetic diversity and population structure of a *T. sanguisuga* population associated with the location of the most recently reported transmission of *T. cruzi* to humans in the United States. Mitochondrial *cytochrome* b (*cyt*b) DNA was chosen as the molecular marker for its utility for resolving within population relationships and the availability of a wealth of comparative data from other triatomine studies (Mas-Coma and Bargues, 2009). 16S mitochondrial ribosomal DNA was also amplified in a subset of specimens in order to observe if the diversity and phylogenetic relationships observed from the *cyt*b sequence data might be reflected in a much more highly conserved marker.

2. Materials and methods

2.1. Specimen collection and sample design

Trained personnel collected all insect specimens, in 2006 sporadically and in 2007 systematically, two people for one hour weekly (2-person-hours) during the months of April–November. All specimens were collected from inside and in the immediate vicinity of the home and other buildings at the site of the sixth United States reported human case of locally acquired *T. cruzi* infection, which occurred in a rural area of New Orleans, LA, USA. All triatomines collected were identified as *T. sanguisuga* using the morphological key of Lent and Wygodzinsky (Lent and Wygodzinsky, 1979).

A subset of 54 *T. sanguisuga* was selected to assess the genetic diversity of this population. The sample set was designed to include approximately 30 individuals from each year and roughly equal numbers of males and females (Table 1). In addition, two specimens collected from St. Gabriel, Louisiana (~112 km from New Orleans) were included in the study.

2.2. DNA extraction, T. cruzi detection, PCR amplification, and DNA sequencing

Triatomine specimens were stored in 5% glycerol/95% ethanol at −80 °C. To assess *T. cruzi* infection, DNA was extracted from the last two segments of the abdomen by boiling for 15 min in 1X PCR buffer or using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. For the triatomine DNA sequence studies, DNA was isolated from 3 to 5 legs of each specimen using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. All samples were stored at −80 °C until PCR amplification. The infection prevalence of *T. cruzi* was determined by amplifying a 276 bp fragment of minicircle DNA (Dorn et al., 2007). PCR products were subjected to electrophoresis on a 1.8% agarose gel and visualized by UV transillumination after staining with ethidium bromide. A positive control of 5 μl of *T. cruzi* parasites boiled in 1× PCR buffer and a negative control without the DNA template were included with every PCR.

A 682 bp fragment of the mitochondrial *cytochrome* b gene was amplified in all 56 specimens (Table 1) according to previously published methods (Monteiro et al., 2003; Pfeiler et al., 2006). PCR products were purified for sequencing using Qiagen's PCR Clean Up kit (Qiagen, Valencia, CA). Both forward and reverse PCR products were commercially sequenced (Eton Bioscience, Inc., San Diego, CA), aligned using ClustalW (Larkin et al., 2007), edited and trimmed (MacVector v. 11.1.0, Cary, NC and) to 606 bp.

A 386 bp fragment of the mitochondrial 16S rDNA sequence was amplified in a subset of 17 specimens according the to the method described by Lyman et al. (1999). PCR amplifications were verified by UV transillumination of 5 μl of each amplicon following agarose gel electrophoresis. PCR products were purified, sequenced, edited, and trimmed to 340 bp as described above.

Table 1
Collection information.

Location	Year of collection	*n*	Males	Females
New Orleans, Louisiana[a]				
	2006	27	12	15
	2007	27	16	11
Total		54	51.9%	48.1%
St. Gabriel, Louisiana				
	2007	2	Unknown	Unknown

[a] Site of sixth autochthonous case of human transmission.

2.3. Data analysis

Genetic analysis was performed using DNAsp v. 5.10 (Librado and Rozas, 2009) including generation of the polymorphism table, haplotype analysis, and polymorphism statistics. Haplotypes were named based on the convention proposed for triatomines (Bargues et al., 2006). Haplotype diversity was calculated as: $H_d = (n/n-1)(1-\sum_{i=1}^{k} p_i^2)$ where p_i represents the frequency of each of k haplotypes in a sample size of n (Nei, 1987). Nucleotide diversity, $\hat{\pi}$ was calculated as the average number of nucleotide differences per site between two sequences: $\hat{\pi} = (n/n-1)(\sum_{i=1}^{k}\sum_{j=1}^{k} p_i p_j \pi_{ij})$ where π_{ij} is the proportion of nucleotides that differ between the ith and jth sequences (Nei, 1987). Analysis of molecular variance (AMOVA) tested for differences between specimens collected 2006 and 2007 at the index case site using Arlequin ver. 3.11 (Excoffier et al., 2005).

2.4. Phylogenetic analysis

Two methods of inferring evolutionary relationship were investigated; distance based Neighbor-Joining (Saitou and Nei, 1987) and character based Maximum Parsimony (Eck and Dayhoff, 1966) and phylogenetic trees were generated using MEGA 4 (Kumar et al., 2008). For the Neighbor-Joining analysis and genetic distance estimations, the best fit model of nucleotide substitution was determined using Akaikie Information Criterion in jModel Test (Posada, 2008). Genetic distances were determined by the Tamura–Nei model (Tamura and Nei, 1993). Maximum Parsimony trees were generated using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000). The statistical significance of the nodes of both types of trees was tested by bootstrap analysis (1000 replicates) (Felsenstein, 1985). A Median Joining Network analysis (Bandelt, 1994) was generated using Network (fluxus-engineering.com) using the default settings and assumptions. Both star contraction pre-processing and maximum parsimony post processing was conducted in order to reduce the complexity of the network and purge non-parsimonious nodes.