

The Chagas Vector, *Triatoma dimidiata* (Hemiptera:Reduviidae), is Panmictic within and Among Adjacent Villages in Guatemala

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ABSTRACT *Trypanosoma cruzi*, the hemoflagellate parasite and cause of Chagas disease in Latin America, is carried by Triatomine vectors, principally *Triatoma dimidiata* and *Rhodnius prolixus* in Central America. To assist control efforts and to understand the epidemiology of the disease in Guatemala, the population genetics of *T. dimidiata* was analyzed among three houses within a village and two adjacent villages in Guatemala. Eleven Randomly Amplified Polymorphic DNA-polymerase chain reaction (RAPD-PCR) primers were screened and three used to amplify bands, 29 of which were scored, from *T. dimidiata* DNA of ≈ 50 bugs per house from three houses within a village and from 66 and 33 bugs, respectively, from adjacent villages. Results show very small genetic distances among the three *T. dimidiata* subpopulations from the houses ($D = 0.013\text{--}0.022$) and the two villages ($D = 0.0199$). The amount of differentiation among houses (fixation index, F_{ST}) was also very small, $F_{ST} = 0.025$ among the houses and the two villages $F_{ST} = 0.019$. These fixation indices give an average number of mating migrants per generation (N_m) of 9.7 (among houses) and 12 (among villages). Average heterozygosity (H) appears to be high, ranging from $H = 0.299\text{--}0.325$ among the houses and $H = 0.273$ among the villages. The low genetic distance and fixation indices, and high heterozygosity suggest that the subpopulations in the houses and in the adjacent villages are not reproductively isolated but are in fact, one large panmictic population. Therefore the geographic coverage necessary for effective control must include, at least, the area encompassing adjacent villages.

KEY WORDS *Triatoma dimidiata*, population genetics, Chagas, Guatemala, vector control

CHAGAS DISEASE REMAINS a serious problem in Latin America: ≈ 12 million people are infected with the causative parasite, *Trypanosoma cruzi*, and ≈ 120 million people, or 25% of the population of Latin America, live in conditions that put them at risk for infection (Moncayo 1999). The *T. cruzi* parasite is transmitted by Triatomine bugs and is a leading cause of heart disease in Latin America. The most important Chagas vectors are: *Triatoma infestans* in the Southern Cone countries, *Triatoma dimidiata* and *Rhodnius prolixus* in Central America (Tabaru et al. 1999) and all three species in the Andean countries. An intensive effort to eliminate transmission of *T. cruzi* was begun by the Southern Cone Countries in June of 1991 and Andean and Central American Countries in 1997 with the help of the World Health Organization. Transmission is interrupted by spraying houses with residual insecticides and screening blood donations followed by discarding infected units (Moncayo 1999). Elimination of

transmission by the domestic vector *T. infestans* has met with remarkable success with transmission interrupted for Uruguay, Chile, and most of the most endemic states of Brazil and Argentina. However, long-term monitoring and vigilance is necessary because it is possible that sylvan populations will repopulate areas "cleansed" of domestic vectors. Control efforts are moving ahead in Central America and *R. prolixus* is a target for eradication because it is apparently exclusively domestic in Central America and following pesticide application does not return. This is in marked contrast to *T. dimidiata*, where the large sylvan reservoir provides bugs to recolonize houses following pesticide application (Cordon-Rosales et al. 1999).

The Southern Cone initiative has been aided by extensive work on the natural history and the genetics of *T. infestans*. In contrast, very little research has been done on *T. dimidiata*, and what has been done has been limited to studies of distribution, risk factors for infestation of houses, and susceptibility to particular insecticides. *T. dimidiata* is found from northern South America (Colombia, Venezuela, Ecuador, and Peru), throughout all the countries of Central America and into Southern Mexico (Schofield and Dujardin 1997).

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In the Yucatan, Mexico, *T. dimidiata* was found in 61% of the houses examined and 16% of the captured bugs were infected with *T. cruzi* (Guzman-Marin et al. 1991). In Costa Rica and Belize, *T. dimidiata* is the only Triatomine species that transmits Chagas (Guzman-Marin et al. 1991) and in Costa Rica the presence of a dirt floor and roof tiles were both significantly associated with a higher risk of infestation (Starr et al. 1991). Studies in three communities in Nicaragua showed that 3.8–48.3% of the houses were infested with *T. dimidiata* and 33–60% of the bugs captured in infested houses were infected with *T. cruzi* (Palma-Guzman et al. 1996). Extensive work has described entomological indices and insecticide sensitivity of *T. dimidiata* as part of a national survey and control effort of Chagas disease in Guatemala (Tabaru et al. 1998, 1999). *T. dimidiata* is clearly the most significant vector for *T. cruzi* in Guatemala based on its widespread distribution (16/22 provinces) and adaptation to both sylvan and domestic habitats. The highest risk of transmission of Chagas disease occurs in the Southeastern regions of Guatemala and $\approx 330,000$ people are considered to live in conditions that place them at high risk of infection with *T. cruzi* (Tabaru et al. 1999). Approximately 39% of *T. dimidiata* captured in Guatemala are infected with *T. cruzi* (Dorn et al. 1999). In summary, *T. dimidiata* is a major Chagas vector in Mexico, Central and Northern South America and presents significant challenges to control efforts. For effective control efforts, in addition to knowledge of the life history of the vectors, habitat preference, etc., it is important to know something of the genetic structure of the vector populations. Knowledge of genetic structure will show if there are distinct subpopulations of *T. dimidiata* that might differ in vector competence or sensitivity to insecticides. Analysis of gene flow among subpopulations will indicate if there is migration among subpopulations and the geographic coverage necessary for effective control. Identification of distinct subpopulations will reveal the source (residual bugs or new infestation) of bugs that reappear following spraying.

Randomly Amplified Polymorphic DNA (RAPD-PCR) analysis, because it samples the genome, generally reveals more genetic variability than is evident with isoenzyme analysis and is useful for distinguishing several species of Triatomine bugs (Garcia et al. 1998). RAPD-PCR analysis is independent of the sex of the organism, lifecycle stage and environmental factors and requires only a small amount of template DNA. Multiple bands may be amplified with only a few PCR reactions, and many bands can be simultaneously visualized on large polyacrylamide gels which also facilitates analyzing large numbers of individuals (Tabachnick and Black 1997). RAPD-PCR analysis was able to clearly distinguish three populations of *T. brasiliensis* in Brazil and showed that they were genetically isolated by distance (Borges et al. 2000). In addition, RAPD-PCR data showed that domestic *R. prolixus* and sylvan *R. colombiensis* were not interbreeding thus *R. colombiensis* was not a significant risk for domestic transmission of *T. cruzi* in a region in

Colombia (Jaramillo et al. 2001). In the current study, RAPD-PCR analysis was used to investigate the genetic structure of *T. dimidiata* in two villages in Guatemala, Central America. Subpopulations of *T. dimidiata* found in three houses in a single village or in several houses in two adjacent villages were analyzed to determine if the subpopulations were reproductively isolated, had limited gene flow or came from one, large panmictic population.

Materials and Methods

Specimen Collection. For the comparison of house populations within a village, 34–53 (average 44.3) *T. dimidiata* were collected from three houses in the rural community of Aguazarca, Santa Maria, Ixhúatan, Santa Rosa, Guatemala, which is 1,140 m above sea level at 14° 09' 23" N, latitude and 90° 14' 28" W, longitude. For comparison between two rural villages, 66 additional bugs were collected from 10 houses in Aguazarca and 33 from eight houses in the village of El Cuje, Pueblo Nuevo Viñas, Santa Rosa, Guatemala which is 1,250 m above sea level at 14° 14' 52" N latitude and 90° 28' 12" W longitude. The bugs were collected between 1998 and 2000 inside houses, before application of pesticides. Aguazarca and El Cuje are rural communities containing houses at least 50 m from each other set among forest and agricultural crops on rolling hills. There are no major topographical barriers between the two communities (mountains, large rivers, etc.). Distinct groups of individuals, e.g., bugs from a single house or bugs from a single village are referred to as subpopulations.

The live bugs were placed in vials with folded paper and transported to the laboratory at the University of San Carlos Guatemala where the lifecycle stage or sex (for adults) and infection status with *T. cruzi* (assessed by microscopic examination of the intestines and rectum) was recorded (to be reported elsewhere). The bug legs were removed and placed in 1.5 μ l microcentrifuge tubes containing 95% alcohol and stored at -20°C . The bug legs were selected as the site for DNA isolations because it provided DNA free of contaminants that could have been acquired from blood meals or from gut symbionts.

DNA Isolation. Bug legs were then brought to Loyola University New Orleans and stored at -80°C until the DNA was isolated using the method originally from Coen et al. 1982 with modifications as described in Black and Munstermann 1996 and below. Briefly, bug legs from individual bugs were separately ground using a Kontes pestle in 100 μ l grind buffer (0.1 M NaCl, 0.2 M sucrose, 50 mM EDTA, 100 mM Tris-HCl [pH 8.0–9.0], 0.05% SDS). (Contaminated pestles were soaked in one M HCl, washed with soap, rinsed with de-ionized water and autoclaved before reusing.) The debris was removed by centrifuging the lysate briefly at $14,000 \times g$. The homogenate was incubated at 65°C for 15–30 min. Fourteen microliters of 8M potassium acetate was added for a final concentration of one M potassium acetate and the solution incubated for 15 min on ice to precipitate the SDS. The sample

was centrifuged at $14,000 \times g$ at 4°C for 10 min and the supernatant transferred to a cold 1.5-ml microfuge tube. Two hundred microliters of 100% ethanol was added to precipitate the DNA. The sample was then incubated on ice for at least 10 min and centrifuged for 20 min at $14,000 \times g$ at 4°C . The resultant pellet was washed with 70% ethanol, allowed to dry and then resuspended in $50 \mu\text{l}$ sterile TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, pH 8.0) containing 1U RNAase (Sigma-Aldrich Co., St. Louis, MO). The samples were then stored at -80°C until amplification.

Random Amplification of Polymorphic DNA. The RAPD-PCR protocol was modified from (Garcia et al. 1998). Reaction mixtures of $40 \mu\text{l}$ contained 1X PCR buffer (Applied Biosystems, Foster City, CA) 10 mM Tris-HCl (pH 8.3, 50 mM KCl), 3 mM MgCl_2 and 0.01% gelatin, 40 pmol of selected decameric primer, and 200 μM of each dNTP. Primers used included: H3: 5'-CATCCCCCTG-3', L1: 5'-CGGCCCTG-3', and L5: 5'-AAGAGCCCGT-3' (Garcia et al. 1998). To the reaction mixture $2 \mu\text{l}$ of DNA template and $2 \mu\text{l}$ *Taq* DNA polymerase was added for a final volume of $40 \mu\text{l}$. The DNA was then amplified using a PTC-100 Thermal cycler (MJ Research- Watertown, MA) with the following cycle conditions: 80°C for 10 min, 94°C for 1 min, 30°C for 2 min, 72°C for 1 min (two cycles); 94°C for 30 s, 40°C for 2 min, 72°C for 1 min (31 cycles); 95°C for 30 s, 40°C for 2 min, 72°C for 5 min, (one cycle), hold at 4°C . The *Taq* was added once the thermocycler had reached 80°C . Negative controls were included in all runs and included all components listed above except DNA template, which was replaced by $2 \mu\text{l}$ sterile water.

Randomly Amplified Polymorphic DNA was electrophoresed on 1.5% agarose gel containing 50 ng/ml ethidium bromide in 0.5X TBE buffer (44.5 mM Tris-base, 44.5 mM boric acid, 1 mM EDTA, pH 8.0). The gel was photographed under UV light. Samples that amplified were electrophoresed on 3.3% polyacrylamide gels in 1X TBE buffer and silver stained (Tabachnick and Black 1997).

The gel was digitized by scanning and molecular weights of the individual bands determined using Quantity One Program (BIO-RAD; Hercules, CA) by comparison to the migration of molecular weight markers, $\phi\text{X174HaeIII}$ (Promega Madison, WI). Band sizes that were consistent throughout many samples for a given primer in the gel (DNA fragments that migrated the same distance) were recorded and lanes containing this band ($\pm 0.5\%$ size difference) were scored as positive. The few bands whose appearance could be considered equivocal were not scored. These data were entered into an Excel spreadsheet (Microsoft, Redmond, WA), and analyzed using RAPDIST, RAPDFST (Tabachnick and Black 1997), RAPDBIOS (Apostol et al. 1996), and BIOSYS-2 (Swofford DL 1997). RAPDIST was used to compute the genetic distance among populations. The diploid option was selected along with the Lynch and Mulligan correction for small sample sizes (Lynch and Milligan 1994) and Nei's genetic distance was calculated (Nei 1972).

The F_{ST} shows the overall differentiation among subpopulations from the three houses and among subpopulations from the two villages. A fixation index = 0 indicates no genetic divergence whereas a value = 1 indicates fixation of alternate alleles in different subpopulations. The effective migration rate (N_m , number of mating migrants per generation) was calculated directly from the fixation index using RAPDFST (Tabachnick and Black 1997) from the formula originally derived in Wright 1931: $F_{\text{ST}} = 1 / (4N_m + 1)$.

Results

Initially, the 11 primers shown to amplify *Triatoma* DNA (Garcia et al. 1998) were used on *T. dimidiata* and the three primers: H3, L1, and L5, that showed multiple, strong, distinct bands were selected. These primers were then used in RAPD-PCR reactions on ≈ 20 individuals, with duplicates run every five samples, from each of the three houses and electrophoresed on one large polyacrylamide gel. Twenty-nine bands that were strong, reproducible and isolated from other bands were selected and scored for all individuals.

To determine local patterns of gene flow among subpopulations of *T. dimidiata* among three houses and within the entire village, Nei's genetic distance, D , was calculated. A small genetic distance was found among the populations from nearby houses ($D = 0.0219$ among houses separated by 0.08 km; $D = 0.0127$ for houses separated by 0.12 km; and $D = 0.0186$ for houses separated by 0.16 km). Individual bugs from each of the three houses were not clustered but dispersed throughout a dendrogram generated using unweighted pair-group method with arithmetic average on data from RAPDIST (data not shown). The genetic distance among the populations found in the two villages, 26.7 km apart, was also small ($D = 0.0199$) and quite similar to levels of genetic distance among the populations in the same village.

The fixation index (F_{ST}) shows a low degree of subdivision of *T. dimidiata* into subpopulations. Both the fixation indices among the populations from one village and between the two villages are quite low. The fixation index among the nearby houses all from a single village is: $F_{\text{ST}} = 0.025 \pm 0.038$, which translates into an effective migration rate of 9.7 mating migrants per generation. The F_{ST} among the two villages is slightly lower, $F_{\text{ST}} = 0.019 \pm 0.033$, for an effective migration rate of 12.7 mating migrants per generation.

Fairly high levels of genetic variability in the subpopulations were evident by the high average expected heterozygosity as well as very high polymorphism of the RAPD-PCR markers (Table 1).

Discussion

Genetic Distance. Although almost no work has been done on the population genetics of *T. dimidiata*, a significant amount of work has been done on the population genetics of the related South American vector, *T. infestans*, and on *R. prolixus* mostly using

Table 1. Mean expected heterozygosity averaged over all loci and percent of polymorphic loci^a

Subpopulation	Mean heterozygosity per locus	Percentage of polymorphic loci ^a
House 2	0.299+/-0.022	100
House 1	0.318+/-0.022	96.6
House 3	0.326+/-0.017	100
Village AG	0.301+/-0.200	96.6
Village EC	0.273+/-0.029	79.3

^a Locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95.

isoenzymes. Studies in Bolivia indicated that *T. infestans* showed very low levels of genetic variability among populations within ≈ 20 km (genetic distance $D = 0.001-0.004$ for nearby domestic and/or sylvan populations) (Dujardin et al. 1987), although there is evidence for isolation by distance (Dujardin et al. 1998). *T. infestans* from laboratory colonies from bugs originally from Argentina, Bolivia and Brazil showed slightly more diversity, $D = 0.001-0.011$ (Garcia et al. 1995) and three laboratory colonies of *R. prolixus* showed considerably more genetic distance among subpopulations, $D = 0.0646$ (Harry et al. 1992), however, these differences could have arisen by genetic drift. Values of genetic distance for the three subpopulations of *T. dimidiata* in Aguazarca and among Aguazarca and El Cuje are intermediate between those of *T. infestans* and *R. prolixus*. There is no correlation with genetic and geographic distance among *T. dimidiata* subpopulations, at least for these fairly small distances, thus, no evidence of isolation by distance. This is consistent with the lack of topographical barriers separating the populations.

Population Subdivision. The fixation index among all three subpopulations within and among villages was extremely low showing a low degree of population subdivision. This level was comparable to that seen in nine laboratory colonies of *T. infestans* originating from Argentina, Bolivia and Brazil (range, 0.0048–0.0874, average 0.0413, average migration rate $Nm = 11.35$) (Garcia et al. 1995). F_{ST} values for both *T. dimidiata* and *T. infestans* are considerably lower than that found for *R. prolixus*, domestic and sylvan from Colombia (average 0.598) (Lopez and Moreno 1995). Interestingly, a large study in Bolivia using isoenzymes found low genetic distance between geographic regions and much data supportive of the isolation by distance model, however also found significant F statistics because of a deficiency of heterozygotes in about one-fourth of the villages as well as strong deviations from Hardy-Weinberg equilibrium (Breniere et al. 1998). It will be interesting to test the *T. dimidiata* populations with another method where the levels of heterozygosity can be directly determined rather than calculated, such as microsatellite analysis, over a large geographic area to determine whether or not particular villages in Guatemala have reproductively isolated subpopulations. The F_{ST} values for the *T. dimidiata* subpopulations are also considerably

lower than those obtained using RAPD-PCR data for domestic *R. prolixus* and sylvan *R. colombiensis* in Colombia ($F_{ST} = 0.24$) (Jaramillo et al. 2001).

Genetic Variability. High levels of genetic variability in the subpopulations were evident by a high mean expected heterozygosity per locus in all three house subpopulations (0.299–0.326) and village subpopulations (0.273–0.301), and a high percentage of polymorphic loci as is seen in RAPD-PCR data (79.3–100%). The low genetic distance and F_{ST} , and high heterozygosity suggest that the subpopulations in the houses are not reproductively isolated but are in fact, one large panmictic population. Thus control efforts, to be effective, must at least encompass the area including adjacent villages and may require housing improvements that would eliminate the vector habitat, because migrating bugs or bugs carried in wood or personal belongings could recolonize sprayed houses.

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