Published in final edited form as:

Acta Trop. 2013 December ; 128(3): . doi:10.1016/j.actatropica.2013.09.003.

Reproductive isolation revealed in preliminary crossbreeding experiments using field collected *Triatoma dimidiata* (Hemiptera: Reduviidae) from three ITS-2 defined groups

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Abstract

Triatoma dimidiata, a Chagas disease vector distributed in Mexico, Central America, Colombia, Venezuela, Peru and Ecuador, has been studied using genetic markers and four groups have been defined by ITS-2 sequences: 1A, 1B, 2 and 3. To gather evidence on the divergence and reproductive isolation among *T. dimidiata* ITS-2 groups, we carried out 15 crossbreeding experiments with field-collected sylvan and domestic *T. dimidiata* from Guatemala where three groups are found: 1A, 2 and 3. Reciprocal crosses between individuals from groups 1A and 2, and a cross between group 2 individuals from different habitats, produced an average 129.78±42.29 eggs with hatching success ranging from 31.6% to 90.1%. The offspring of these crosses reached the adult stage, and crosses between F1 insects produced eggs. These results suggest that there are no pre- or post-zygotic reproductive barriers between groups 1A and 2, or within group 2. Crosses between group 3 females and males from groups 1A or 2 produced on average 85.67±30.26 eggs and none of them hatched. These results support the existence of pre-zygotic barriers between *T. dimidiata* group 3 and groups 1A and 2. The group 3 individuals were collected in sylvatic environments in Yaxha, Peten, Guatemala. Previously, distinct chromosomal characteristics (cytotype 3) were described in individuals from this population. Based on this evidence we suggest that this population is divergent at the species level from other *T. dimidiata* populations.

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Keywords

Triatoma dimidiata; Chagas disease; ITS–2; crossbreeding; reproductive isolation

1.1. Introduction

The subfamily Triatominae (Hemiptera–Reduviidae) is of medical importance given that most species are vectors of *Trypanosoma cruzi*, the parasite that causes Chagas disease. Taxonomic classification of triatomine bugs has been based primarily on external morphological characteristics (Lent and Wygodzinsky, 1979). However, the results of different genetic techniques and crossbreeding experiments have cast doubt upon the status of many taxa (Mas Coma and Bargues, 2009). Cryptic species (morphologically similar but genetically distinct) and especially many subspecies (morphological variants) have been described in triatomines (Dujardin et al., 2009).

Triatoma dimidiata is distributed from Northern Mexico through most of Central America to Colombia, Venezuela, Peru and Ecuador. It is the most important vector of Chagas disease in Central America and it can be found in domestic, peridomestic, and sylvan habitats (Dorn et al., 2007). *Triatoma dimidiata* is characterized by its phenotypic plasticity; it is highly variable in morphology, behavior, and chromatic pattern (Lent and Jurberg, 1985; Schofield, 2000; Bustamante et al., 2004; Dorn et al., 2007). Since the description of *T. dimidiata* in 1811, its phenotypically variable populations have been recognized as three distinct species or subspecies (Usinger, 1941; 1944, respectively). Based on an extensive study of chromatic variation in individuals from different countries, Lent and Wygodzinsky (1979) synonymized the subspecies and recognized *T. dimidiata* as a polytypic species.

Since 2001, several genetic and phenetic approaches have supported *T. dimidiata* as a species complex including sibling species and subspecies (Marcilla et al., 2001; Panzera et al., 2006; Dorn et al., 2007, 2009; Bargues et al., 2008; Calderón–Fernández et al., 2011). Sequencing of the nuclear internal transcribed spacer 2 (ITS–2) suggested the existence of four ITS–2 groups, including at least two different species. Populations from the Yucatan peninsula in Mexico, extending to the State of Chiapas (Mexico), Yaxha (Petén–Guatemala), Yoro (Honduras), and Belize are highly divergent in ITS–2 sequence from the rest and were designated as a new species named *T. sp. aff. dimidiata* or group 3 (Bargues et al., 2008). The remaining *T. dimidiata* populations show a level of nucleotide differences that would be consistent with the three subspecies described by Usinger (1944), i.e., (1) *T. dimidiata maculipennis* or group 2: southern Mexico, Guatemala, and Belize; (2) *T. dimidiata dimidiata* or group 1A: Guatemala, Honduras, El Salvador, Nicaragua, and Costa Rica; (3) and *T. dimidiata capitata* or group 1B: Panama and Colombia. Several authors show that the Yucatan peninsula (including Mexico, Belize, and northern Guatemala) and Honduras is a region where the group 2 and the putative new species (group 3) coexist in sympatry (Bargues et al., 2008; Tamay–Segovia et al., 2008; Dorn et al., 2009). Another study using cyt b and ND4 markers supports that *T. dimidiata* is a complex of “sibling species or near sibling-taxa” composed of four groups and *Triatoma hegneri* (Monteiro et al., 2013): group I (corresponding to ITS–2 1A and 1B), group II, group III, and group IV from Belize, with all groups being assigned specific status. Information from all of the aforementioned studies suggest that northern Guatemala is an important area for *T. dimidiata* from an evolutionary standpoint given that three ITS–2 groups (1A, 2 and 3) occur there, and more research is needed on potential hybridization among groups within the country.

Crossbreeding experiments offer a powerful tool for analyzing phenomena that may cause population divergence and speciation (i.e., the formation of isolating mechanisms), and hence can help in clarifying their taxonomy (Arnold, 1997). Experimental crosses have been done to determine isolating mechanisms that limit gene flow between species and to elucidate the taxonomic status among putative cryptic species or morphological subspecies in several triatomine groups, such as in *Brasiliensis* (Costa et al., 2003; 2009), *Phyllosoma* (Martínez-Ibarra et al., 2009; 2011), and in *Infestans* subcomplexes (Pérez et al., 2005).

In this study, we carried out preliminary crossbreeding experiments using field-collected individuals of *T. dimidiata* from domestic and sylvatic habitats from five provinces of Guatemala where three ITS-2 groups were previously reported. The results provide additional evidence of the divergence and reproductive isolation among particular *T. dimidiata* ITS-2 groups.

1.2. Materials and Methods

1.2.1. Insects Collection

A total of 15 experimental crosses were performed. The 30 bugs used were field collected during 2008 and the geographical origin of each one is shown in the Table. Some bugs were donated by community members who brought them to our laboratory.

1.2.2. Crossbreeding experiments

Experimental crosses were planned on the basis of the geographical origin of the insects (Figure). The selected areas represent different epidemiological scenarios for *T. dimidiata* in Guatemala. Jutiapa is an area with high house infestation, and where the infested houses are usually made of adobe or mud and stick construction (Bustamante et al., 2009). Alta Verapaz and Izabal are areas with low infestation (Nakawaga et al., 2005) where the bugs live in the sylvatic areas (palm trees, caves) and can visit the houses, which are usually made of wooden boards. The Yaxha Archeological site in Peten is an entirely sylvatic area where the bugs live in the subtropical forest (Monroy et al., 2003). We also used two bugs from Amatitlan, an area located only 30 km south of Guatemala City; here we have observed that bugs visit well-constructed houses close to surrounding coffee plantations. Previous studies had shown that ITS-2 group 1A could be found in Jutiapa, ITS-2 groups 2 and 3 could be found in Peten (Bargues et al., 2008; Dorn et al., 2009); and ITS-2 group 1A could be found in Alta Verapaz and Izabal (Dorn et al., 2009). The sylvatic bugs from Peten are the only ones belonging to ITS-2 group 3 in Guatemala. Originally we planned to make reciprocal crosses between insects from Peten with insects from the other locations. However, only a few nymphs were collected in Peten and when these hatched to adults, only females were obtained. Sylvatic bugs from this area are difficult to obtain given that special permission from the Guatemalan Institute of Anthropology and History is needed, and extensive field work is required to recover very few bugs.

All bugs used for the crosses were field collected as nymphs. These insects were kept separately in the insectary of the Laboratory of “Entomología Aplicada y Parasitología” at San Carlos University (Guatemala), in an incubator (at 24–27°C, relative humidity 65 ± 5%) and mice were offered weekly as a blood meal source. Within two days after the bugs reached the adult stage, a male and a female were placed together in a plastic vial with folded filter paper. Each vial was checked daily for eggs. Eggs were transferred to new vials and hatching was observed daily for 122 days. For each cross we determined the number of eggs laid and the eggs hatched (Table). All bugs that died either in the nymph or adult stages were preserved in 5% glycerol/95% ethanol. After the final parental cross, the offspring were followed until adult stage and some F1 individuals were crossed to evaluate their fertility.

1.2.3. Genetic analyses

Following the crosses, some of the preserved bugs (22/30) were sent to Loyola University (New Orleans, USA) for ITS–2 sequencing. DNA was isolated from three to five legs of each bug using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. ITS–2 amplification and sequencing were performed as in Dorn et al. (2009). The bugs were placed in ITS–2 groups using the criterion of a 99% match with the corresponding haplotype in GenBank (Benson et al., 2013). The bugs that were not sequenced (8/30) were assigned to a group based on the observed patterns from the same locality.

1.3. Results and Discussion

The crosses between group 1A females and group 2 males (four crosses: C1–C4, Table) produced the highest number of eggs laid (average 144 ± 48.08 eggs) and a high percentage of these eggs hatched ($79.40 \pm 12.85\%$). The reciprocal cross, i.e., group 2 females with group 1A males (four crosses: C5–C8, Table) were also quite successful, producing an average of 120.50 ± 44.08 eggs with ~50% of hatching success. These results suggest that there are no reproductive barriers between these populations identified genetically as groups 1A and 2 by the ITS–2 marker. The single cross between group 2 individuals (C9, Table) originating from different habitats (sylvan and domestic) produced 110 eggs with 31.82% hatching success, suggesting no reproductive isolation among bugs including in the same genetic group from different habitats. Some of the offspring of these crosses reached the adult stage and a few insects were used to make F1 crosses; these crosses did produce eggs (C1, C5–C7, Table).

The crosses among group 3 females with males in either group 1A or 2 (six crosses: C10–C15, Table) produced an average of 85.67 ± 30.26 eggs, but failed to produce viable eggs. These results support reproductive isolation between ITS–2 group 3 individuals and individuals belonging to the other two groups (1A and 2) in Guatemala. The lack of viable eggs suggests the existence of a pre–zygotic isolation in which either no sperm transfer occurs or there is gametic mortality or incompatibility following sperm transfer (Templeton, 1989). However, it is still necessary to conduct the reciprocal crosses (group 3 males with females from both groups 1A and 2) to confirm the existence of a complete pre–zygotic isolation among these groups.

This type of pre–zygotic isolation could indicate an important genetic divergence of group 3 with groups 1A and 2 and it is in agreement with the chromosomal and mitochondrial genetic differences detected between them (Panzeria et al., 2006; Bargues et al., 2008; Dorn et al., 2009; Monteiro et al., 2013). These differences led to the suggestion that group 3 is a new species named *T. sp. aff. dimidiata* (Bargues, et al., 2008). However, our results are in apparent conflict with the existence of natural adult hybrids between group 2 and 3 reported in the Yucatan Peninsula (Herrera–Aguilar et al., 2009; Nouvellet et al., 2011). This discrepancy could be explained by genetic differences between groups 3 individuals from Yaxha (Guatemala) and Yucatan (Mexico), which are not detectable by ITS–2 sequences. To date, only chromosomal differences were reported between these populations. Bugs from Yucatan (Mexico) and Yaxha (Guatemala) present different chromosomal forms, named cytotypes 2 and 3 respectively, with marked differences in the C–heterochromatin contents of the X₁ chromosome (Panzeria et al., 2006). Perhaps this cytogenetic variation represents a mechanism of genetic isolation which would explain both the reported natural hybrids between Yucatan and group 2 individuals (both of them lacking heterochromatin on the X₁ chromosome) as well as the non–viability of eggs produced from matings between individuals from Yaxha (with heterochromatin in the X₁) and individuals from groups 1A and 2 (without heterochromatin in the X₁ chromosome). In order to test the hypothesis of

reproductive barrier based on chromosomal differences, it will be important to conduct crossbreeding experiments between individuals from Yucatan and Yaxha as well as among Yucatan and groups 1A and 2 individuals.

Although we were unable to perform the reciprocal crosses, we believe that the preliminary information generated is important to shed light on the issue of *T. dimidiata* as a species complex or a polytypic species. We hope our results will spark interest in further experiments to better clarify the taxonomic status of *T. dimidiata* populations.

In summary, the lack of reproductive isolation between groups 1A and 2 supports that they belong to the same biological species. These two groups are sympatric and given that they are the most epidemiologically important (they seasonally visit or colonize human dwellings), it is significant that they can potentially interbreed. This preliminary study also suggests that the *T. dimidiata* sylvan population found in Yaxha, Guatemala is likely a separate biological species based on its reproductive isolation. It is already considered divergent at the species level based on a nuclear marker (ITS-2 group 3) and an mtDNA marker (cyt b), and shows distinct chromosomal characteristics (cytotype 3). The sum total of the evidence therefore suggests it is a sibling species within the *T. dimidiata* species complex.

Acknowledgments

We appreciate the technical assistance of Johana Maradiaga. We also want to thank Daniel Aquino and the Instituto de Antropología e Historia de Guatemala for authorizing the collection of bugs at Sitio Arqueológico Yaxha–Nakum–Naranjo, Flores, Peten, Guatemala. This research has been supported in part by: SENACYT/CONCYT/ FODECYT Grant 044–2007 (San Carlos University), RELCON/CHEMO/Barcelona Grant (San Carlos University), National Institutes of Health (USA) Grant 1R15 A1079672–01A1, a Richard Frank Grant (Loyola University New Orleans, USA) and “Programa de Intercambio Científico” between Universidad de San Carlos (Guatemala) and Udelar (Uruguay).

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Results of cross breeding experiments using *Triatoma dimidiata* field collected in Guatemala, and classified into three groups based on ITS-2 sequences.

Table

Cross	Female Department, Municipality, Habitat	Female ITS-2 Group / Haplotype	Male Department, Municipality, Habitat	Male ITS-2 Group / Haplotype	Number of Eggs Laid	Number of Eggs Hatched	Success (percentage of hatched eggs)	Offspring reached adult stage	F1 crosses produced eggs?
C1	Guatemala, Amatitlan, S. ^a	1A / H2	Izabal, Los Amates, S.	2 / H18	77	58	75.32	Yes	Yes
C2	Jutiapa, Comapa, D. ^b	1A / H1	Izabal, Los Amates, S.	2 / H18	142	128	90.14	Yes	Not tested
C3	Jutiapa, Comapa, D.	1A ^c	Izabal, Los Amates, S.	2 / H18	173	109	63.01	Yes	Not tested
C4	Jutiapa, Comapa, D.	1A ^c	Izabal, Los Amates, S.	2 ^c	184	164	89.13	Yes	Not tested
C5	Izabal, Los Amates, S.	2 / H18	Jutiapa, Comapa, D.	1A ^c	141	57	40.43	Yes	Yes
C6	Izabal, Los Amates, S.	2 / H18	Jutiapa, Comapa, D.	1A ^c	72	51	70.83	Yes	Yes
C7	Izabal, Los Amates, S.	2 / H18	Guatemala, Amatitlan, S.	1A ^c	171	91	53.22	Yes	Yes
C8	Izabal, Los Amates, S.	2 / H18	Alta Verapaz, San Cristobal, D.	1A / H1	98	31	31.63	Yes	Not tested
C9	Izabal, Los Amates, S.	2 / H18	Alta Verapaz, San Cristobal, D.	2 / H18	110	35	31.82	Yes	Not tested
C10	Peten, Flores, Yaxha, S.	3 / H33	Jutiapa, Comapa, D.	1A / H1	79	0	0.00	–	–
C11	Peten, Flores, Yaxha, S.	3 / H33	Jutiapa, Comapa, D.	1A / H1	70	0	0.00	–	–
C12	Peten, Flores, Yaxha, S.	3 ^c	Alta Verapaz, San Cristobal, D.	1A / H1	113	0	0.00	–	–
C13	Peten, Flores, Yaxha, S.	3 / H33	Izabal, Los Amates, S.	2 / H18	118	0	0.00	–	–
C14	Peten, Flores, Yaxha, S.	3 / H33	Izabal, Los Amates, S.	2 ^c	37	0	0.00	–	–
C15	Peten, Flores, Yaxha, S.	3 / H33	Izabal, Los Amates, S.	2 / H18	97	0	0.00	–	–

^aD= domestic;

^bS= sylvan;

^cITS-2 group inferred.