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Infestation of peridomestic *Attalea phalerata* palms by *Rhodnius stali*, a vector of *Trypanosoma cruzi* in the Alto Beni, Bolivia

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Summary

OBJECTIVES To determine (i) whether peridomestic *Attalea phalerata* palms in fragmented human-occupied areas of the Alto Beni, Bolivia, are infested by triatomines; (ii) the specific status of triatomines captured in the area; and (iii) the rate of natural *Trypanosoma cruzi* infection among those triatomines.
METHODS One hundred and twenty-five live-bait traps were used to sample 47 *A. phalerata* palms in three Alto Beni localities. Active search for vectors was also performed in 10 chicken coops and three rice storage units. Only *Rhodnius* specimens were found. As nymphs of closely related *Rhodnius* species are morphologically undistinguishable, and because of controversy in the literature regarding which *Rhodnius* species occur in Bolivia, collected insects were identified through molecular taxonomy. Phylogenetic analyses of DNA sequences obtained for a fragment of the mitochondrial cytochrome *b* gene and for the nuclear ITS-2 ribosomal region were used as molecular markers. Natural infection rates were determined using a pair of primers that PCR-amplify a 330-bp fragment of the parasite's kDNA.
RESULTS Twelve nymphs were captured in five *A. phalerata* palms (from two of the three localities studied), and an adult was collected from a chicken coop in Iniqua (and morphologically identified as *Rhodnius stali*). All nymphs (as well as the adult) were molecularly identified as *R. stali* based on the two molecular markers used. A single nymph was found to be infected with *T. cruzi*.
CONCLUSIONS *Attalea phalerata* palms represent an important sylvatic ecotope occupied by *R. stali* in the Alto Beni region of Bolivia, where there are signs of *T. cruzi* transmission to humans, despite the preliminary indication of low level of natural infection of the vectors.

keywords *Rhodnius stali*, Chagas disease, Bolivia, cytochrome *b*, ITS-2, molecular taxonomy

Introduction

Chagas disease still represents a serious public health problem in Bolivia, where 28% of the population is believed to be infected (Moncayo 2003). In 1991, the Southern Cone Initiative was founded by the governments of Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay to interrupt transmission by the main domestic vector species in the region, *Triatoma infestans*. The Initiative led Bolivia in 1996 to launch its own national control programme (Dias 2007). Although control activities in Bolivia have made substantial progress (PNCCH 2007), recent reports describing the existence of abundant sylvatic populations of this vector distributed throughout the country might put control achievements at risk (Noireau *et al.* 2005). Sylvatic populations of native vector species are a major challenge for insecticide control activities, as they might act as sources of migrants that will re-colonise previously treated human dwellings (Schofield *et al.* 2006).

Rhodnius stali is another Chagas disease vector with a large distribution in Bolivia (Cortez 2007). The species was described based on museum specimens labelled as *Rhodnius pictipes* (Lent *et al.* 1993), a closely related and morphologically similar taxon also thought to occur in Bolivia (Abad-Franch & Monteiro 2007).

A recent serological survey of human populations of the Alto Beni region (a forest region located in the Andean foothills at the north of the department of La Paz) revealed that 60 of 2002 individual samples (2.9%) were reactive against anti-*Trypanosoma cruzi* antibodies (Depickère, personal communication). This observation initially came as a surprise, as this region is not included in the known geographical distribution of *T. infestans*. However, those seroprevalence rates seem to be the rule in similar ecological settings, as suggested by Aguilar *et al.* (2007),

in a recent reappraisal of Chagas disease epidemiology in the Amazon region.

Earlier field expeditions to the area led to the collection of *Rhodnius* specimens from both domestic and peridomestic environments. All samples were identified as *R. stali* after morphometric comparisons with reference *R. stali* specimens from the Chapare, Bolivia and *R. pictipes* from Pará, Brazil (Matias *et al.* 2003). The finding of 21 *R. stali* eggs adhered to a single branch (all branches were examined) of a *motacu* palm tree (*Attalea phalerata*) located near an infested house (no information is given on the number of trees inspected) led to the suggestion that this palm tree could be the natural ecotope for *R. stali* (Matias *et al.* 2003).

The purpose of this work was to investigate whether *A. phalerata* palms indeed represent a sylvatic ecotope occupied by *R. stali* populations in the Alto Beni region, and to estimate the potential epidemiological risk this species brings to local human populations through the determination of its natural infection rate by *T. cruzi*.

Materials and methods

Collection sites

The Alto Beni is a region of tropical rain forest located on the transition zone between humid lowlands and the drier highlands (Abad-Franch & Monteiro 2007). The wet season begins in October and lasts until May, when there is a transition to the dry season. Annual precipitation ranges from 1300 to 1600 mm, and the mean annual temperature is 25.5 °C.

The localities of Caranavi, Entre Rios and Iniqua (~370 m a.s.l.) were elected for vector sampling based on the existence of previous reports on the presence of domestic *Rhodnius* populations, provided by the Bolivian National Chagas Control Program. Forty-seven *A. phalerata*, the predominant palm species present in inhabited areas, were sampled with 125 live-bait traps (Abad-Franch *et al.* 2000; Noireau *et al.* 2002) to detect *Rhodnius* infestation. Palms located 5–50 m from the nearest house were selected as they are the most likely sources of the domestic *Rhodnius* populations. Preference was given to those with more abundant organic matter and epiphytic plants on their crowns, as they are likely to host larger *Rhodnius* populations (Abad-Franch *et al.* 2005). Also 10 chicken coops and three rice storage units were searched.

Molecular identification of vectors

As *Rhodnius* nymphs are unsuitable for accurate taxonomic identification based on morphology alone, they were identified through molecular taxonomy based on two markers of proven efficacy in discriminating *Rhodnius* species: a fragment of the mitochondrial cytochrome *b* gene (cyt *b*), and the nuclear ribosomal second internal transcribed spacer (ITS-2). Species identity was determined based on phylogenetic comparisons with reference samples of the species (and populations) used by Matias *et al.* (2003): one *R. stali* from the Chapare, Cochabamba Department, Bolivia (16.404.96 S; 65.375.23 W), and one *R. pictipes* from Abaetetuba, Pará state, Brazil (14.346.22 S; 48.522.73 W).

DNA was extracted from either nymphs (whole insect) or adults (head and thorax only) as described in Aljanabi and Martinez (1997). Sequencing was carried out using the primers described by Monteiro *et al.* (2003), for the cyt *b* fragment, and Marcilla *et al.* (2001), for the ITS-2 region (cloned with the Promega pGEM® T Easy Vector System kit).

Neighbour-joining phylogenetic reconstructions for both markers were performed based on Kimura 2-parameter (K2-p) distance matrices, with 1000 bootstrap replications, using the MEGA 4.0 software (Tamura *et al.* 2007). A *Rhodnius robustus* specimen from El Torno, Santa Cruz Department, Bolivia (18.064.14 S; 63.293.91 W), was used as outgroup.

Trypanosoma cruzi infection

To further evaluate the epidemiological role that these insects might play in the Alto Beni region, *T. cruzi* infection rates were determined based on the amplification of a 330-bp fragment of the parasite's kDNA, as described by Wincker *et al.* (1994).

Results

The sampling of 47 *A. phalerata* palm trees led to the capture of 12 *Rhodnius* nymphs in five palms (10.64%), from two of the three studied sites (Table 1). A single adult was obtained from a chicken coop in Iniqua and morphologically identified as *R. stali* according to Lent *et al.* (1993). No triatomines were found in any of the three rice storage units searched.

Two cyt *b* haplotypes were found among the 13 collected samples. Both the Chapare reference specimen and the Iniqua adult presented the same haplotype (*GenBank* accession number FJ887790), which was also detected in 75% of the collected nymphs; the remaining nymphs presented a second haplotype that differed from the former by a single transitional substitution (*GenBank* accession number FJ887791). Two collected *Rhodnius* nymphs, the *R. pictipes* reference specimen, and the

Table 1 Information on the samples used in this study

Species	Collection sites	Geographic coordinates	Number of palms		Number of specimens collected	Number of specimens sequenced		Haplotype	
			Investigated	Infested		cyt *b*	ITS-2	cyt *b*	ITS-2
Rhodnius stali	Caranavi*	15.842.02*S*; 67.568.19*W*	29	4	10 (8N1, 1N4, 1N5)	10	2	St1, St2	A, B
	Entre Rios*	15.830.22*S*; 67.580.19*W*	7	1	2 (N1)	2	–	St2	–
	Iniqua*	15.819.16*S*; 67.595.67*W*	11	–	1 (Ad)†	1	–	St2	–
	Chapare‡	16.404.96*S*; 65.375.23*W*	–	–	1	1	–	St2§	–
Rhodnius robustus	El Torno¶	18.064.14*S*; 63.293.91*W*	–	–	1	1	1	Rob**	Rob**
Rhodnius pictipes	Abaetetuba††	14.346.22*S*; 48.522.73*W*	–	–	1	1	1	Pic§	Pic§

N1, first stage nymph; N4, fourth stage nymph; N5, fifth stage nymph; Ad, adult specimen.
*Alto Beni Province, Department of La Paz, Bolivia.
†Specimen found inside a chicken coop.
‡Department of Cochabamba, Bolivia.
§Reference specimens.
¶Department of Santa Cruz, Bolivia.
**Outgroup.
††Pará state, Brazil.

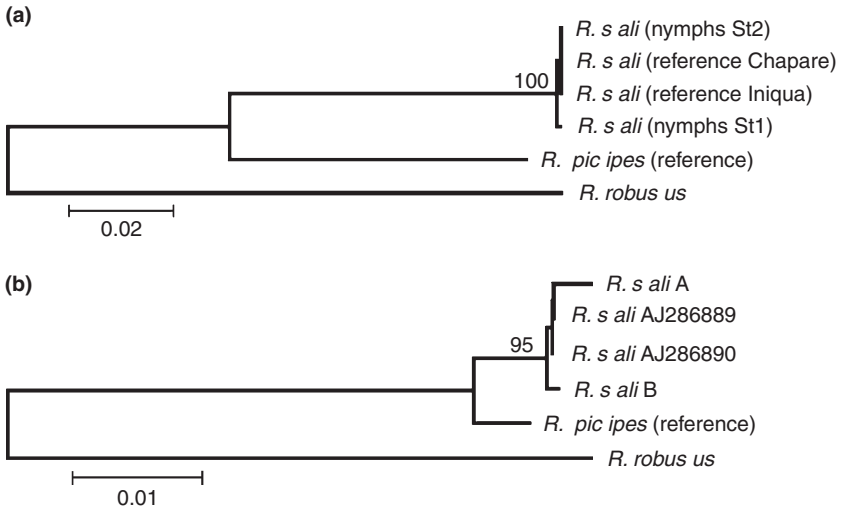

Figure 1 (a) Cyt *b* Neighbour-joining phylogenetic tree, based on Kimura 2-parameter (K2-p) distances, with 1000 bootstrap replications. Note that collected nymphs present haplotypes either very similar (St1) or identical (St2) to those of the reference *Rhodnius stali* specimens, while clearly diverge (K2P ~ 0.12) from the reference *Rhodnius pictipes*. (b) ITS-2 Neighbour-joining phylogenetic tree, based on K2-p distances, with 1000 bootstrap replications. As it was not possible to sequence the reference *R. stali* for this marker, we used *GenBank* sequences AJ286889 and AJ286890 as references for comparison. Letters 'A' and 'B' correspond to different haplotypes obtained for the two *R. stali* specimens sequenced.

R. robustus outgroup, were successfully cloned and sequenced for the ITS-2 region. We were unable to generate good quality sequences for the reference *R. stali* from the Chapare, and thus alternatively used two *R. stali* ITS-2 sequences available in *GenBank* (AJ286889 and AJ286890), determined for insects from the same region (Alto Beni).

The phylogenetic reconstructions obtained for the two molecular markers revealed the formation of a monophyletic clade comprised of the sequences obtained from the

Figure 2 *Rhodnius stali*-infested *Attalea phalerata* palm adjacent to a rural house. This scenario poses constant risk for local human populations in the Alto Beni region of Bolivia, as infected insects fly from the palms into the houses where they establish domestic colonies to, at night, blood-feed on the sleeping occupants.

nymphs and the *R. stali* reference sequences (Figure 1). Moreover, the genetic distances observed between them were consistent with an intraspecific relationship (K2-$p_{cyt\ b}$ = 0.002 and K2-$p_{ITS\text{-}2}$ = 0.002). Evidently, distance values obtained when nymph sequences are compared with *R. pictipes* reference sequences are much higher, consistent with their interspecific relationship (K2-$p_{cyt\ b}$ = 0.12 and K2-$p_{ITS\text{-}2}$ = 0.011). Therefore, the results obtained for the two molecular markers used clearly demonstrate that collected nymphs belong to the species *R. stali*.

Of the 13 analysed samples, a single first stage nymph from Entre Rios resulted positive for *T. cruzi* infection (7.7%).

Discussion

Since the 1980s *R. stali* (then regarded as a phenotypic variant of *R. pictipes*) is known to be undergoing a domiciliation process in the Alto Beni region of Bolivia. Its natural ecotope, however, was never confidently determined (Tibayrenc & Le Pont 1984; Matias *et al.* 2003). We have identified the peridomestic *motacu* palm, *A. phalerata*, as an important natural ecotope for this species in the region (Figure 2). Such palms often occur near houses where their leaves are used as rooftops, and their fruits used to feed livestock (mainly pigs). However, these findings should not be regarded as definitive for this species throughout its entire distribution, as we searched exclusively *motacu* palms, the main palm species in the studied areas. Although a close ecological association between *R. stali* and *A. phalerata* has been suggested by Abad-Franch & Monteiro (2007), *R. stali* have also been collected on *Astrocaryum murumuru* and *Oenocarpus bataua* palms in the more humid Chapare region (Noireau, personal observation).

Twelve *Rhodnius* nymphs were collected from five *motacu* palms in Caranavi and Entre Rios, and genetically compared with reference samples. To generate trustworthy results and overcome the limitations inherent to single-locus mtDNA *barcoding* methods, we used both mitochondrial and nuclear markers.

The ascertainment of a given specimen to a biological species *via* molecular taxonomy depends on the answers to two basic questions: (i) which reference taxon does the query sample form a monophyletic group with? and (ii) what is the magnitude of the genetic distances observed between that sample and the other sequences in the group?

The genetic distance values obtained indicate whether the relationship represents intraspecific variation (and thus indicates conspecificity) or interspecific variation (which would be a sibling species relationship). Although *Rhodnius* cyt *b* population level divergences do not exceed 1.5%, and interspecific values are usually above 2.3% (Monteiro *et al.* 2003), there are no clearly established cut-off values for the ITS-2 marker for this genus (Mas-Coma & Bargues, 2009).

The phylogenetic reconstructions obtained for the two molecular markers used revealed the formation of a monophyletic clade comprised of the sequences obtained from the nymphs and the *R. stali* reference sequences. Moreover, the genetic distances between them were consistent with an intraspecific relationship (K2-$p_{cyt\ b}$ = 0.002 and K2-$p_{ITS\text{-}2}$ = 0.002). Evidently, distance values obtained when nymph sequences are compared with *R. pictipes* reference sequences are much higher, consistent with their interspecific relationship (K2-$p_{cyt\ b}$ = 0.12, and K2-$p_{ITS\text{-}2}$ = 0.011). Therefore, the results obtained for the two molecular markers used clearly demonstrate that collected nymphs belong to the species *R. stali*. This method could also play an important role in the identification of triatomine eggs, which are a major indicator of ecotope (household, palm) colonisation.

Approximately 60–80% of the Bolivian territory is considered to be endemic for Chagas disease (Incosur 2001, Moncayo 2003). There is an important overlap

between this endemic area and the estimated geographical distribution of the main vector, *T. infestans*. However, the existence of endemic areas (such as the Alto Beni) that are *T. infestans* free, calls attention to the epidemiological relevance that other potential vector species, such as *R. stali*, might have in maintaining regional antropozoonotic disease transmission cycles. The single *R. stali* adult obtained from a chicken coop in Iniqua illustrates the point: it most likely represents a migrant (coloniser) that flew in from a palm of the adjacent sylvatic ecotope, as there was no evidence of the existence of *Rhodnius* colonies inside the coop.

A possible explanation for the low number of collected specimens is technical failure. As the collection took place during the dry season, the dirt roads were covered with a fine powder, which would become airborne with the regular flow of traffic; this dust would adhere to the sticky tapes used on the traps, possibly compromising the captures (in fact, we did occasionally observe first stage nymphs walking with no difficulty over the tapes).

The demonstration that *A. phalerata* palms are abundant in human-occupied areas, together with the detection of a *T. cruzi* infected nymph in the immediate vicinity of a rural house (with local human *T. cruzi* seroprevalence around 2.9%), comprises an epidemiological scenario that deserves close examination by Bolivian public health authorities. The data here presented indicates that increased surveillance is required in certain Chagas disease endemic areas regardless of the absence of *T. infestans*.

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Chagas disease control efforts?