Infection, Genetics and Evolution 51 (2017) 1–9

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Molecular identification of wild triatomines of the genus *Rhodnius* in the Bolivian Amazon: Strategy and current difficulties

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ARTICLE INFO

Article history:
Received 16 November 2016
Received in revised form 2 March 2017
Accepted 3 March 2017
Available online 06 March 2017

Keywords:
Triatominae
Chagas
Molecular genetics
Gene
Sequencing

ABSTRACT

The Amazon region has recently been considered as endemic in Latin America. In Bolivia, the vast Amazon region is undergoing considerable human migrations and substantial anthropization of the environment, potentially renewing the danger of establishing the transmission of Chagas disease. The cases of human oral contamination occurring in 2010 in the town of Guayaramerín provided reasons to intensify research. As a result, the goal of this study was to characterize the species of sylvatic triatomines circulating in the surroundings of Yucumo (Beni, Bolivia), a small Amazonian city at the foot of the Andes between the capital (La Paz) and Trinidad the largest city of Beni. The triatomine captures were performed with mice-baited adhesive traps mostly settled in palm trees in forest fragments and pastures. Species were identified by morphological observation, dissection of genitalia, and sequencing of three mitochondrial gene fragments and one nuclear fragment. Molecular analysis was based on (i) the identity score of the haplotypes with GenBank sequences through the BLAST algorithm and (ii) construction of phylogenetic trees. Thirty-four triatomines, all belonging to the *Rhodnius* genus, of which two were adult males, were captured in palm trees in forest fragments and pastures (overall infestation rate, 12.3%). The morphology of the phallic structures in the two males confirmed the *R. stali* species. For the other specimens, after molecular sequencing, only one specimen was identified with confidence as belonging to *Rhodnius robustus*, the others belonged to one of the species of the *Rhodnius pictipes* complex, probably *Rhodnius stali*. The two species, *R. robustus* and *R. stali*, had previously been reported in the Alto Beni region (edge of the Amazon region), but not yet in the Beni department situated in the Amazon region. Furthermore, the difficulties of molecular characterization of closely related species within the three complexes of the genus *Rhodnius* are highlighted and discussed.

1. Introduction

Chagas disease, caused by *Trypanosoma cruzi* and mostly transmitted by blood-sucking bugs of the Triatominae subfamily, is a parasitic disease that may be fatal. According to World Health Organization (WHO) estimates, six to seven million people worldwide are infected by the parasite (WHO, 2016). In the Amazon region, Chagas disease can be considered as mostly enzootic because the parasite circulates among a wide number of wild mammal species, and foci of pre-adaptation or adaptation of triatomine vectors to the domicile or peridomicile are scarce, such as *Triatoma maculata* and *Panstrongylus geniculatus* in the Brazilian Amazon (Fe et al., 2009; Luitgards-Moura et al., 2005), *Rhodnius stali* in Alto Beni in Bolivia (on the edge of the Amazon Basin) (Justi et al., 2010) and *Rhodnius prolixus* in the Orinoco River of Venezuela (Aguilar et al., 2007). Some species of the genus *Rhodnius*, mainly found in forest habitat, have been implicated in *T. cruzi* transmission to humans, either attacking humans to take a blood meal, such as *Rhodnius brethesi* in the Brazilian state Amazonas (Coura et al., 1994) or flying to peridomestic and domestic areas where they can form small colonies, such as *Rhodnius neglectus* in Brazil (Garcia-Zapata et al., 1985). The latter process may be the first step in adapting to domestic habitat and could be the strategy used to increase their epidemiological role (Dujardin et al., 2000). *R. stali* is probably responsible for transmission of Chagas disease in indigenous communities in the Alto Beni region (Justi et al., 2010; Matias et al., 2003). Recently, at the foot of the Andes, on the edge of the Amazon Basin, *P. geniculatus* and *Rhodnius robustus* adults naturally infected with *T. cruzi* and collected

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http://dx.doi.org/10.1016/j.meegid.2017.03.002
1567-1348/

from houses were described (Rojas-Cortez et al., 2016). In 2010, an outbreak by oral contamination was reported for the first time in Bolivia, (Guayaramerín, Beni department) (Santalla Vargas et al., 2011), showing that the Amazon has to be evaluated for the risk of Chagas disease transmission. In this context, a first exploration for the presence of triatomines was conducted in the surroundings of the small city of Yucumo, the largest Amazonian city on the road from La Paz (Andean capital) to Trinidad (centre of the Bolivian Amazon). The captures were done in forest fragments and pastures by positioning traps mostly in palm trees, and the species of the nymphs and adults were identified through morphology and genetic markers.

2. Material and methods

2.1. Biological material

The study was conducted near the small city of Yucumo in the province of General Jose Ballivian, Department of Beni (15° 08′43.3″S, 67° 02′ 03.01″W) in October 2012, by a group of researchers from the IRD, the FIOCRUZ and the local staff of the School of Pharmaceutical Sciences and Biochemistry (UMSA). The presence of triatomines was examined in six areas, two situated in forest fragments, two in pastures and two in peridomicile areas. Access to these forest and pasture lands was requested from their owners, who accompanied us in the captures. At the request of two owners, additional traps were positioned in palm trees in their peridomicile. In both cases the palm trees were very close to the house (<50 m). Triatomines were captured using 345 mice-baited adhesive traps (Noireau et al., 1999) positioned in 83 different sites. More precisely, 66 sites were palm trees, the large majority of traps being positioned at the crown ($n = 293$), some in an epiphyte attached to the trunk ($n = 11$), and one between roots. Eight other sites were leafy trees, and the traps were positioned in a hole ($n = 17$) or between branches ($n = 6$). Also, nine sites were dead trees where 17 traps were positioned in holes. Most of the traps were positioned in the crown of palm trees (293/345, 84.9%). In both forest fragments, the sites were selected at four distant locations 50–100 m along a track, and at each location the sites were 5–20 m apart. In pastures, traps were also mostly positioned in palm trees, virtually the only structure offering habitat to triatomines. These palm trees were dispersed, and two pasture locations were sampled. The choice of palm trees was not restricted to a species or to the size of palm trees, but the palm trees with a crown > 10 m high were not sampled because it was impossible to reach the crown with a ladder. The traps were positioned in the afternoon and inspected the next morning. Table 1 summarizes the locations and types of landscape where the explorations were carried out and the number of traps used. The geolocation of the sites is contained in the KMZ file 1 and can be viewed on GoogleMaps. In this file, yellow tags are palm tree sites, green tags are other sites and red tags are positive sites (all palm trees). The white tag indicates the location of Yucumo. Additionally, a positive palm tree was felled and dissected with the help of the owner.

2.2. Morphology of phallic structures

The structures of the male genitalia of the two captured male specimens were compared with a specimen of *R. pictipes* from Oriximiná, Pará, Brazil, and the morphological characterization was based on previous descriptions (Lent et al., 1993; Meneguetti et al., 2016). The genital capsule was subjected to a softening process with 10% KOH at room temperature for 1 h. The phallic structures were removed using tweezers and observed in a stereomicroscope.

2.3. DNA extraction and PCR

The triatomines collected (adults and nymphs) were transported alive to the laboratory, photographed and ranked according nymph stage and putative species by morphological analysis. The DNA of each bug was extracted from three or four legs, except for the first to third larval stages for which DNA was obtained from whole insects. After grinding the samples, 200 µl of 2% CTAB reagent and 20 µl of proteinase K at 20 mg/ml were added and the mixtures were incubated overnight at 37 °C. Then DNA extraction was performed with chloroform, followed by isopropanol DNA precipitation and wash in 70% ethanol. Dried DNA samples were diluted in distilled water, and the concentration was determined by measuring the optical density at 260 nm.

Four gene fragments were amplified. Three were mitochondrial, *cytochrome B* (*Cytb*), the *large subunit ribosomal-16S RNA* gene (*LSU-rRNA*), *NADH dehydrogenase subunit I* (*Nd1*), and one nuclear gene, the *D2 variable region of the 28S RNA* gene (*D2*). Previously defined protocols were used as such or slightly modified (Lyman et al., 1999; Mas-Coma and Bargues, 2009; Monteiro et al., 2003; Monteiro et al., 2000). The primers used were: (i) for *Cytb*, Cytb7432F (GGA CGW GGW ATT TAT TAT GGA TC) and Cytb7433R (GCW CCA ATT CAR GTT ART AA) (Monteiro et al., 2003); (ii) for *LSU-rRNA* LRN 13393 (CAT CTG TTT AWC AAA RAC AT) and LRJ 12966 (AAA AAA ATT ACG CTG TTA TCC CTA AAG TAA) (Monteiro et al., 2000); (iii) for *Nd1* that we designed RhodND1F (GAR CGS AGG GTY TTG GGM TA) and RhodND1R (CAA RGT CCC CCG AAC YCA AA); and (iv) for *D2*, D2F (GCG AGT CGT GTT GCT TGA TAG TGC AG) and D2R (TTG GTC CGT GTT TCA AGA CGG G) (Fitzpatrick et al., 2008). The PCR products were sent to the sequencing

Table 1
Summary of the triatomine collection using mice-baited adhesive traps in the surroundings of Yucumo city, Beni, Bolivia.

Capture place	Landscape	Explored site	Code site[a]	Palm tree				Leafy tree[b]		Dead tree[b]		Collected triatomines	Stage[c]
				Number	Traps	Positive site	Positive traps	Number	Traps	Number	Traps		
Yucu 01	Forest fragment	24	S01-S024	18	70	3	4	2	3	4	7	4	4 N5
Yucu 02	Pasture field	16	S025-S40	15	75	1	1	1	5	0	0	1[d]	1 N3
Yucu 03-1	Forest fragment	23	S41-S63	13	57	3	5	5	15	5	10	19	2 N1, 8 N2, 6 N3, 3 N4
Yucu 03-2	Peridomicile	4	S64-S67	4	20	0	0	0	0	0	0	0	–
Yucu 04-1	Pasture field	13	S68-S80	13	75	1	2	0	0	0	0	2	1 N3, 1 N5
Yucu 04-2	Peridomicile	3	S81-S83	3	8	0	0	0	0	0	0	0	–
	Total	83	S01-S83	66	305	8	12	8	23	9	17	26	2 N1, 8 N2, 8 N3, 3 N4, 5 N5

[a] Correspond to the KLM file that can be open with GoogleMaps.
[b] No triatomine was captured.
[c] N1-N5: larval stages 1 to 5.
[d] This is the only specimen molecularly identified as *R. robustus*.