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Morphometric and molecular differentiation of a *Rhodnius robustus*-like form from *R. robustus* Larousse, 1927 and *R. prolixus* Stal, 1859 (Hemiptera, Reduviidae)

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ABSTRACT

In Triatominae, "robustus" group constitutes a cluster of species with great haplotypic divergences but high similarities at morphological and nuclear DNA levels. Given these similarities, species identification generates a frequently problematic issue. In northwestern Amazonia, *Rhodnius robustus* cohabit with an apparently new species, cryptic with *R. robustus* (Abad-Franch and Monteiro, 2005). In this region (municipality of Puerto Asís, Department of Putumayo, Colombia), we collected insects classified as *R. robustus* by traditional keys. We compared this sample with specimens of *R. robustus* from Venezuela, and of *R. prolixus* from Colombia and Venezuela. The comparisons used landmark-based geometric morphometrics, and analyses of mitochondrial cytochrome b gene and of D2 variable region of the 28S RNA. The shape of the wings from Puerto Asís specimens disclosed clear-cut divergence from the shape of the wings as found for *R. prolixus* specimens from Venezuela and Colombia, and diverged from the shape of *R. robustus* from Venezuela. Thus, morphometric analyses suggested that the Puerto Asís collection could represent a new taxon. Using *R. pallescens* as an outgroup, a tentative phylogenetic tree based on the geometry of the wing showed the *Rhodnius* from Puerto Asís more similar to the *R. prolixus* from Colombia than their congeners from Venezuela. In contrast, the molecular classification clustered Colombian *R. prolixus* and Venezuelan *R. robustus* with published GenBank sequences, but it gave the insects from Puerto Asís a basal position to the "*robustus*" group. This outcome suggests that the Puerto Asís haplotype could be the one found by Abad-Franch and Monteiro (2005). Thus, both morphometric and molecular markers used here, although differing in the phylogenetic classification of samples, could differentiate the Puerto Asís sample from the morphologically similar *R. prolixus* and *R. robustus*. This could represent a valuable help in the entomological surveillance related to the control of Chagas disease in the South of Colombia and North of Ecuador.

1. Introduction

The subfamily Triatominae (Hemiptera-Reduviidae) includes 141 species of hematophagous insects taxonomically arranged in five tribes and 15 genera (Schofield and Galvao, 2009; Jurberg et al., 2009). Most species are potential vectors of the protozoan parasite *Trypanosoma cruzi*, the causative agent of American trypanosomiasis, or Chagas disease. The '*robustus* group', a major lineage of the tribe Rhodniini (Abad-Franch and Monteiro, 2007), comprises members with principally cis-Andean (*prolixus*, *robustus*, *nasutus*, *neglectus*, *milesi*, *dalessandroi*, *domesticus*, and the *Psammolestes*) and only one trans-Andean species (*R. neivai*). *Rhodnius prolixus*, the main vector of Chagas disease in Colombia and Venezuela, is essentially a domestic species most of its range. However, this species has been found occupying the same sylvatic habitat with its closely related and morphologically similar species *R. robustus*, which has a minor epidemiological importance (Feliciangeli et al., 2007; Fitzpatrick et al., 2008). The frequently colonization of houses by sylvatic populations of *R. prolixus* and their confusion with *R. robustus,* difficult surveillance and control practices (Feliciangeli et al., 2007; Fitzpatrick et al., 2008).

R. robustus exhibits high morphological and nuclear DNA similarities with *R. prolixus* (Harry et al., 1992a,b; Harry, 1993; Barrett, 1996; Solano et al., 1996; Monteiro et al., 2000), but divergences in mitochondrial DNA and known ecological adaptations are used as an argument to considered them as different species

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Table 1
List of samples submitted to morphometric analysis. (–) indicated absent data. Environmental mean temperature: 27 °C (Puerto Asís), 27.6 °C (Coyaima).

Species	Code	Collection site		Field/ Colony	Domestic/ Sylvatic	Date collected	Females	Males
		Country	State/Municipality					
R. robustus-like	roCOL	Colombia	Putumayo/Puerto Asís	F	S	1999	7	5
	rCc	Colombia	Putumayo/Puerto Asís	C	S	1999	31	39
	roVE	Venezuela	Trujillo/Pampanito	F	S	1997	5	7
R. prolixus	prCOL	Colombia	Tolima/Coyaima	F	D	1991	5	6
			Casanare/San Luis	F	D	1995	15	14
	pCc	Colombia	Tolima/Coyaima	C	D	1991	20	20
	prVE	Venezuela	Barinas/–	F	D	–	12	18
R.pallescens	paCOL	Colombia	Diferent sites	F	S	–	25	24

(Monteiro et al., 2003). Furthermore, *R. robustus* presents five moderately divergent allopatric groups of haplotypes (I–V) with different geographical location from Amazonian region to Colombian and Venezuelan Plains (Monteiro et al., 2003; Abad-Franch et al., 2009): Haplotype I is sympatric with *R. prolixus* in the Orinoco region (Colombian and Venezuelan Plains), haplotype II is in Amazonian region from Ecuador and Brazil, haplotypes III and IV are parapatric in Northeast of Brazil (Amazon forest region) and Southern French Guyana (Pavan and Monteiro, 2007). Finally, an apparently new species, morphologically indistinguishable to *R. robustus* was found in palm trees in the Ecuadorian Amazon (Abad-Franch and Monteiro, 2005). This outcome was based on genetic distance of Kimura 2-parameters substitution model among cytochrome b (mtCyt b) gene sequences of mitochondrial DNA and the basal position of that haplotype to the lineage "*robustus*" (*R. prolixus*, *R. neglectus*, *R. robustus* and *Psammolestes* sp.).

The genetic and biogeographic heterogeneity of specimens morphologically indistinguishable from *R. robustus*, may suggest differences in ecological and evolutionary dynamics among members of the "*robustus*" group, especially in their abilities to colonize human dwellings (Abad-Franch et al., 2009). In this context, the use of inexpensive identification tools, such as the one based on Geometric Morphometrics, is of epidemiological relevance. Since the goal is to discriminate *R. prolixus* for other species morphologically similar but with less epidemiological importance, the relevant question is to discriminate rather than to classify. However, this approach requires determine whether the morphometric variation found in *Rhodnius sp.* address taxonomical differences or whether this variation correspond to plastic responses to environmental changes. Metric changes according to habitat are known to occur indeed, such as those described in the transition from sylvatic to domestic or laboratory habitat (Dujardin et al., 1997, 1998, 1999a,b,c; Jaramillo et al., 2002; Feliciangeli et al., 2007; Rodríguez et al., 2007; Caro-Riaño et al., 2009; Dujardin et al., 2009). This issue is important because morphological variation patterns of sylvatic *R. prolixus* in Colombian (Guhl, 1999, 2003; Pinto et al., 2005; Angulo-Silva, 2000) and Venezuelan Plains (Gamboa, 1963; Zeledon and Rabinovich, 1981; Feliciangeli et al., 2007; Fitzpatrick et al., 2008) may be confounded with *R. robustus,* making difficult surveillance and control practices (Feliciangeli et al., 2007; Fitzpatrick et al., 2008).

Given the importance to get a fast and low cost tool in the entomological surveillance of the vectors of Chagas disease (Dujardin et al., 2007), especially in tracking possible house re-infestation by *R. prolixus* from palm trees (Feliciangeli et al., 2007), we verified in this work the relevance of the morphometric identification approach. This was possible thanks to the concomitant application on the same specimens of molecular techniques based on nucleotide sequences comparison of a fragment of the mitochondrial cytochrome b (mtCyt b) gene and of D2 variable region of the 28S RNA (D2).

2. Materials and methods

2.1. Insects

A total of 82 Colombian specimens of *Rhodnius* from municipality of Puerto Asís, department of Putumayo, and 40 *R. prolixus* from municipalities of Coyaima and San Luis, from the departments of Tolima and Casanare, respectively, were submitted to geometric morphometric analysis and compared with other specimens previously genotyped by Monteiro et al. (2003) that included 12 *R. robustus* haplotype I from Venezuela and 18 individuals of *R. prolixus* from the same country. In addition, 24 Colombian specimens of *R. pallescens* were used as outgroup for the phylogenetic study. Comparison among taxa was performed separately for specimens obtained from the field and laboratory (descendants from those field insects). Detailed information on the samples used in this study is given in Table 1. The morphological diagnostic based on traditional keys (Lent and Wygodzinsky, 1979), assigned the Puerto Asís sample to *R. robustus* (Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos, Departamento de Protozoologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil).

One Colombian specimen of *Rhodnius* from Puerto Asís and one of *R. prolixus* were sequenced and compared with GenBank data for *R. robustus*, *R. prolixus*, *R. neglectus and Psammnolestes coreodes*. For the phylogenetic classification tree, GenBank sequences of species of "pictipes lineage" (*R. pictipes, R. brethesi, R. ecuadoriensis, R. pallescens, R. colombiensis*) were used as an outgroup (Table 2).

2.2. Geometric morphometrics

Right wings of *R. pallescens* and of all specimens reared in laboratory (*Rhodnius* from Puerto Asís and *R. prolixus* from Coyaima) were dissected and mounted by standard techniques. These wings were photographed using a Nikon 990 digital camera fitted to a Nikon SMS 800 stereomicroscope. The Colombian specimens collected in the field, are part of the biological collections of BCEI Laboratory. For this reason, their wings were only photographed leaving the specimens intact without a dissection procedure. Both *R. prolixus* and *R. robustus* from Venezuela (Pampanito), were dissected, mounted in Hoyer, and photographed ten years ago by using a digital camera that was manually adapted to one ocular of the binocular microscope. On the other hand, the taxonomical status of these two species was confirmed by genotyping of mtCyt b and D2 genes by Monteiro et al. (2003).

Nine landmarks of type I (Bookstein, 1991), were identified on each wing on digital photographs (Fig. 1). The coordinates of these landmarks were digitized by using the software COO V. 39 (Dujardin, 2010). The isometric estimator known as centroid size (CS) was used for size comparisons. CS is defined as the square root of the sum of the squared distances between the center of the con-

Table 2
List of sequences of mitochondrial (cytochrome b) and nuclear (D2 variable region of the 28S RNA) DNA used in this study.

Species	Code	Country	State/Locality	GenBank Accession Number	Gene
R.robustus-like	roCOL	Colombia	Putumayo/Puerto Asís	This study	(cytb, D2)
R.prolixus	prCOL	Colombia	Tolima/Coyaima	This study	(cytb, D2)
	prVE1	Venezuela	Trujillo/Pampanito	EF011716	(cytb)
	prVE5	Venezuela	Ortiz/Guarico	AF435862	(D2)
	prCO1	Colombia	Tolima/Coyaima	AF435860	(D2)
	prCas	Colombia	Casanare	This study	(D2)
R.robustus	roBR7	Brazil	Pará/Barcarena	AF421343	(cytb)
	roBR4	Brazil	Pará/Itupiranga	AF421342	(cytb)
				AF435857	(D2)
	roBR8	Brazil	Balbina/Amazonas	AF435859	(D2)
	roEC	Ecuador	Napo	AF421341	(cytb)
				AF435858	(D2)
	roVE1	Venezuela	Trujillo/Pampanito	AF421340	(cytb)
	roVE2	Venezuela	Trujillo/Pampanito	AF435861	(D2)
R.nasutus	naBr	Brazil	Teresina/Piaui	AF435856	(D2)
R.neglectus	neg			AF045716	(cytb)
R.pictipes	pic			AF045713	(cytb)
R.brethesi	bre			AF045714	(cytb)
R.ecuadoriensis	ecu	Ecuador		AF045715	(cytb)
R.pallescens	pal	Colombia	Caldas/Norcasia	GQ850481	(cytb)
R.colombiensis	col	Colombia	Tolima/Coyaima	FJ229360	(cytb)
Psammolestes coreodes	Pcor			AF045719	(cytb)

figuration of landmarks and each individual landmark (Bookstein, 1991). Raw coordinates were submitted to generalized Procrustes analyses (GPA) to generate "partial warp" (PW) scores (Rohlf, 1990; Rohlf and Slice, 1990) as shape variables. Both CS and shape variables were calculated using the software MOG V. 82 (Dujardin, 2010).

To examine differences in wing shape between the Puerto Asís specimens and known species, the PW were submitted to Discriminant Analysis by using the software PAD V. 98 (Dujardin, 2010). This analysis was performed by choosing those insects reared in the same laboratory given that they were photographed following the same procedure. Then, based on Mahalanobis distances of each wing to the mean shape of each group, a validated classification of the Puerto Asís specimens was performed separately for females and males. Sample sizes for field comparisons were too low to perform a reliable Discriminant Analysis. Additionally, to explore relationships among the *Rhodnius* from Puerto Asís and the previously genotyped insects of *R. prolixus* and *R. robustus* (Monteiro et al., 2003), a neighbour-joining tree (NJ; Saitou and Nei, 1987) was produced by using Procrustes distances among insects from field (PHYLIP package, neighbour module,by J. Felsenstein, http://evolution.genetics.washington.edu/phylip.html).

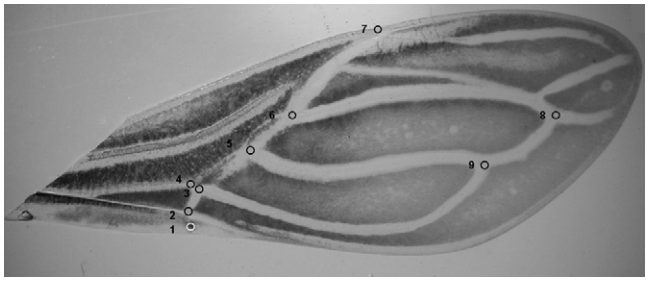

Fig. 1. Landmarks type I measured as coordinates of wings of *R. prolixus*. Numbering on the landmarks denotes the arrangement followed during digitization. Landmark 1 corresponds to the "*coaptor*" located on the "*clavum*." Landmarks 2–7 are located along the "*corium*-membrane junction" of the wing, and landmarks 8 and 9 are on the membranous part.

2.3. Genetic diversity

DNA was extracted from the legs of *R. robustus* and *R. prolixus* from Colombia following to Lyman et al. (1999). Primers based on Triatominae conserved regions of the mtCyt b gene: CYTB7432F: 5′-GGACG(AT)GG(AT)ATTTATTATGGATC-3′ and CYTB7433R: 5′-GC(AT)CCAATTCA(AG)GTTA(AG)TAA-3′ and amplification conditions previously reported by Monteiro et al. (2000, 2003) were used to amplify by PCR a 663-base pair (bp) fragment of the mtCyt b gene. Amplified PCR fragments were sent to Macrogen Inc. for sequencing. Possibility of mtDNA introgressions between the *R. prolixus* and *R. robustus* lineages were explored using sequence analysis based on the D2 variable region of the 28S rRNA (D2), which was amplified with the primers and PCR conditions described by Porter and Collins (1996).

Edition of both forward and reverse DNA strands to produce consensus sequences together multiple alignments with previous reported sequences was done using the software Bioedit V. 7.0.9.0 (Hall, 1999).

Genetic differentiation between samples was tested by cluster analysis using the NJ algorithm (Saitou and Nei, 1987) and Kimura 2-parameter distance (K2-p, Kimura, 1980). Statistical support for branches in the NJ tree was assessed by the bootstrap method (Felsenstein, 1985) with 1,000 replications. These analyses were conducted using the software MEGA V. 4 (Tamura et al., 2007).

3. Results

3.1. Geometric morphometrics

The size of both sexes of Colombian *Rhodnius* from Puerto Asís, Colombia, (average female: 9.593 mm; average male: 8.697 mm) showed a larger mean values than for *R. prolixus* (females: 8.577 mm; males: 7.730 mm), even after ten years of laboratory rearing (Fig. 2). Differences in wing shape were on the membranous part of the wing and they were more visible for males than females (Figs. 3 and 4). Colombian *Rhodnius* from Puerto Asís and *R. prolixus* differed in the location of the landmarks 8 and 9 (Fig. 3), whereas *Rhodnius* from Puerto Asís and Venezuelan *R. robustus* differed in

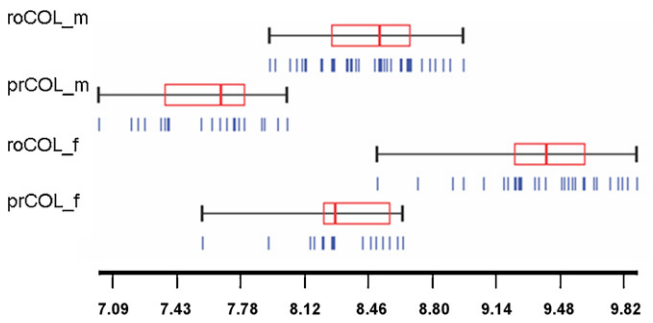

Fig. 2. Size variation (mm) in females (f) and males (m) specimens of *Rhodnius* from Puerto Asís, Colombia (roCOL) and Colombian *R. prolixus* (prCOL).

the location of the landmarks 7 and 8 (Fig. 4). These differences were found both field and laboratory insects (Fig. 4).

Wing shapes *Rhodnius* specimens from Puerto Asís were clearly different from Colombian *R. prolixus* as indicated by discriminant analysis in each sex (Fig. 5). The first discriminant function for the analysis of each sex explained 100% of the total variance and was highly significant (Wilks' lambda: $0.058_{(14,\ 34)}$ and $0.078_{(14,\ 45)}$ for females and males, respectively; $p < 0.001$). The correct attribution of taxa before and after validated classification was 100% in each sex in laboratory insects.

Additionally, field specimens of *Rhodnius* from Puerto Asís, Colombia, and field specimens of *R. robustus* from Venezuela were clustered in different groups in the NJ trees (Fig. 6). However, in this case *Rhodnius* from Puerto Asís were clustered with field specimens of Colombian *R. prolixus*.

3.2. Genetic diversity

Colombian *R. prolixus* was clustered with the conspecific specimen from Venezuela given that both exhibited a K2-p distance of 0.009 for the mtCyt b gene (Fig. 7, Table 3). The *Rhodnius* from Puerto Asís, Colombia, did not clustered with any of mitochondrial haplotype previously reported to *R. robustus* (Monteiro et al., 2003), *R. prolixus* or any species of *Rhodnius* or *Psammolestes* included in this study (Fig. 7). In fact, the *Rhodnius* from Puerto Asís, Colombia, was less divergent with *R. robustus* haplotype II from Ecuador (K2-p = 0.10); however, it was clustered in a basal position to the lineage "robustus" and it was clearly different from the lineage "pictipes."

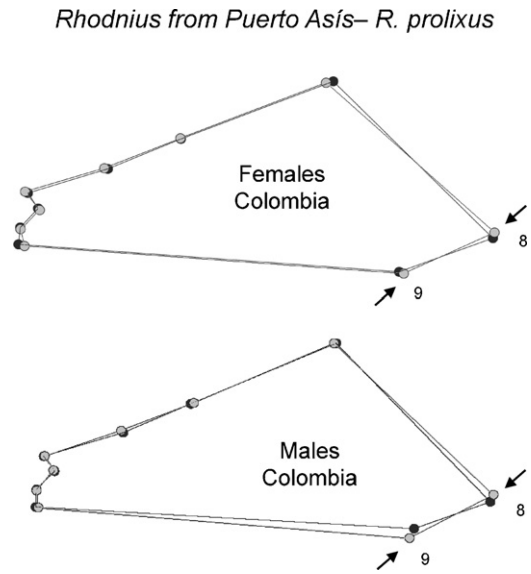

Fig. 3. Differences in wing shape between insects of *Rhodnius* from Puerto Asís (black) and *R. prolixus* (gray) from Colombia. Polygons connect residual coordinates after Procrustes superimposition to the general consensus (translation, scaling and rotating). Solid circles represent landmarks. Differences in wing shape are represented by incongruence between homologous landmarks. Numbers and arrows indicate the landmarks with apparently highest differences in wing shape.

Divergence between *Rhodnius* from Puerto Asís, and Colombian *R. prolixus* was higher (K2-p = 0.118) than between Venezuelan *R. prolixus* and *R. robustus* (K2-p = 0.034) specimens. Nuclear D2 sequence of *Rhodnius* from Puerto Asís, differed from all haplotypes previously reported for *R. robustus* (I–IV) and *R. prolixus*, in position 73 (transversion A/C). In addition, a transition G/A in position 262 was shared with haplotype IV of *R. robustus* (roBR8). Both mtCyt b as D2 nucleotide sequences indicated that *Rhodnius* from Puerto Asís did not represent a hybrid between *R. prolixus* and *R. robustus* (Tables 3 and 4).

4. Discussion

Our study demonstrated the presence in Colombia of a possible new member of the *robustus* complex; most probably

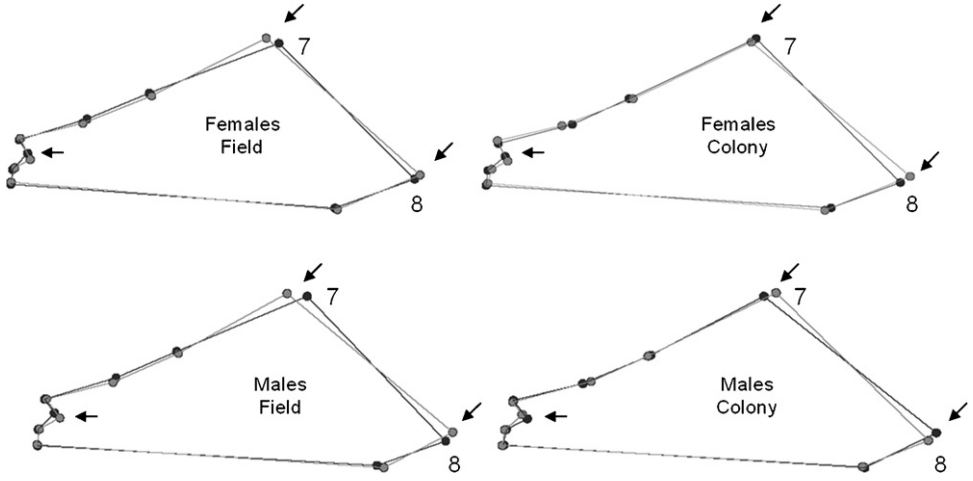

Fig. 4. Differences in wing shape between insects of *Rhodnius* from Puerto Asís, Colombia (black) and *R. robustus* fromVenezuela (gray). Polygons connect residual coordinates after Procrustes superimposition to the general consensus (translation, scaling and rotating) in specimens from field and colonies. Solid circles represent landmarks. Differences in wing shape are represented by incongruence between homologous landmarks. Numbers and arrows indicate the landmarks with apparently highest differences in wing shape.

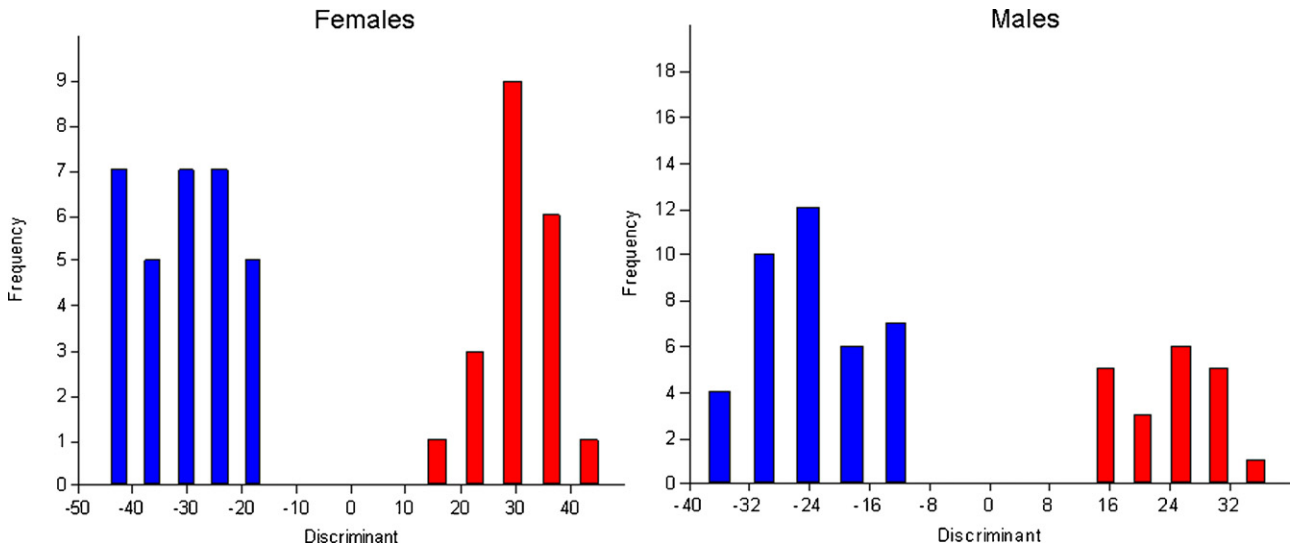

Fig. 5. Plot of the discriminant scores (one by sex) from the analysis of laboratory descendants of *Rhodnius* from Puerto Asís, Colombia, and *R. prolixus* from Colombia.

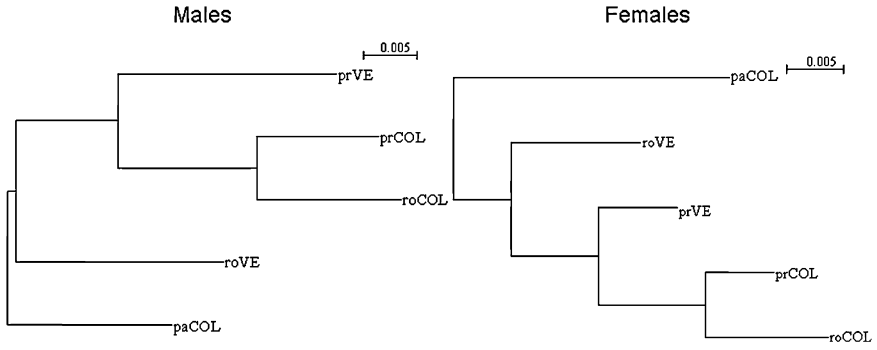

Fig. 6. Neighbour-joining trees based on Procrustes distances between the wing shape of *Rhodnius* from Puerto Asís, Colombia (roCOL), of *R. robustus* from Venezuela (roVE) and of *R. prolixus* from Colombia (prCOL) and Venezuela (prVE). *R. pallescens* from Colombia (paCOL) was used as outgroup.

the same one already described in Ecuador by Abad-Franch and Monteiro (2005). We also confirmed the ability of geometric morphometrics to discriminate this member of the *robustus* complex from the morphologically close *R. prolixus* in Colombia.

The haplotype of *Rhodnius* from Puerto Asís, Colombia was genetically more similar to the Ecuadorian haplotype of *R. robustus*. However, the genetic distance between these two haplotypes (K2-p = 0.10) and its basal position to the "*robustus*" group (82% bootstrap support), suggested that this new haplotype is genet-

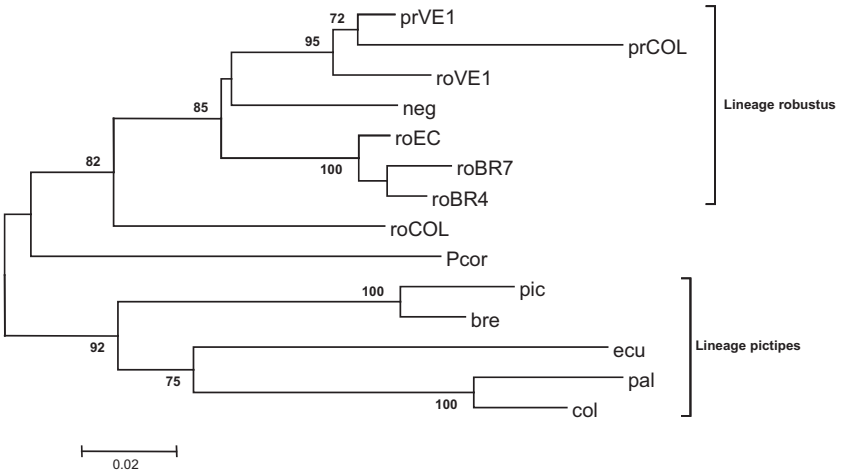

Fig. 7. Neighbour-joining tree based on Kimura 2-parameters distances between the sequences of mtCyt b of Colombian *R. prolixus* and *Rhodnius* from Puerto Asís, Colombia, and haplotypes previously published. The numbers on the branches indicate bootstrap support. BR: Brazil, EC: Ecuador, VE: Venezuela, COL: Colombia, ro: *R. robustus*, pr: *R. prolixus*, negl: *R. neglectus*, Pcor: *P. coeredes*, bre: *R. brethesi*, pic: *R. pictipes*, ec: *R. ecuadoriensis*, pal: *R. pallescens* y col: *R. colombiensis*.roCOL: *Rhodnius* from Puerto Asís, Colombia.

Table 3
Pair-wise genetic distance Kimura 2-parameters between cytochrome b haplotypes of Colombian *R. prolixus* and *Rhodnius* from Puerto Asís, Colombia, and mitochondrial haplotypes previously reported. See Table 2 for haplotype codes.

		2	3	4	5	6	7	8	9	10	11	12	13
1. roCOL													
2. roVE1	0.126												
3. roEC	0.100	0.071											
4. roBR4	0.115	0.078	0.025										
5. roBR7	0.118	0.089	0.022	0.022									
6. prVE1	0.118	0.034	0.068	0.068	0.085								
7. prCOL	0.118	0.025	0.064	0.064	0.082	0.009							
8. neg	0.138	0.082	0.082	0.082	0.100	0.064	0.061						
9. Pcor	0.167	0.159	0.163	0.179	0.192	0.155	0.159	0.155					
10. bre	0.161	0.194	0.181	0.190	0.177	0.186	0.190	0.186	0.183				
11.pic	0.177	0.199	0.198	0.216	0.194	0.203	0.207	0.203	0.175	0.038			
12. ecu	0.227	0.210	0.215	0.223	0.232	0.189	0.193	0.193	0.245	0.173	0.181		
13. pal	0.215	0.230	0.200	0.200	0.209	0.218	0.222	0.188	0.236	0.199	0.194	0.168	
14. col	0.197	0.217	0.200	0.209	0.209	0.209	0.205	0.180	0.205	0.170	0.169	0.172	0.054

ically similar to the one found by Abad-Franch and Monteiro (2005) in a specimen morphologically similar to *R. robustus* from Sucumbios, northeast Ecuadorian Amazon (Abad-Franch and Monteiro, 2005).

The geometric shape of the wing between Colombian specimens of *Rhodnius* from Puerto Asís was greatly different from *R. prolixus*, showing a total discrimination (100%, Fig. 5). Additionally, wings of *Rhodnius* from Puerto Asís were larger than those of *R. prolixus* allowing clear differentiation between the two taxa based on size only (Fig. 2). The morphometric distinction in shape between Colombian specimens of *R. prolixus* and *Rhodnius* from Puerto Asís is a relevant finding given that it may avoid inaccurate diagnostics in case of size overlapping. Given that Colombian specimens of *R. robustus* (genetically verified) were not available for this work, it remains to explore the degree of wing shape discrimination between the Puerto Asís population and the *R. robustus sensu stricto* from Colombia.

The degree of shape divergence between Puerto Asís specimens and Venezuelan *R. robustus* (Procrustes distance: 0.055 and 0.040 for males and females, respectively) was higher than those found between Puerto Asís specimens and Colombian *R. prolixus* (Procrustes distance: 0.025 and 0.016 for males and females, respectively). This result was concordant with mitochondrial DNA analysis (mtCyt b), which showed a higher genetic distance between *Rhodnius* from Puerto Asís, Colombia and *R. robustus* from Venezuela (K2-p = 0.126) than *Rhodnius* from Puerto Asís and *R. prolixus* from Colombia (K2-p = 0.118) (Table 3).

Beside this parallelism, profound differences appeared between the topologies of the morphometric and the genetic trees. The morphometric tree showed the *Rhodnius* from Puerto Asís more similar to the *R. prolixus* from Colombia than their congeners from Venezuela, while the genetic tree clustered them according to known taxonomic relationships over any geographic arrangement.

The discrepancy between geometric morphometrics and mtDNA trees could be explained in two non-exclusive ways: an artefact, or different genetic behaviors of markers. An artefact is not unlikely since different photographing and mounting techniques were used in this work for the Colombian and the Venezuelan material.

The genetic explanation to the diverging trees is not unlikely too. At the mitochondrial level there is evidence that suggest that genetic differences exists between *R. robustus* and *R. prolixus*, whereas these genetic differences are generally absent at the nuclear DNA level (Bargues et al., 2010). This discrepancy between geometric morphometrics and mtDNA classifications may suggest that shape of the wings is depending on nuclear DNA. This latter has been confirmed in some groups of insects (Roff and Mousseau, 1987; Bitner-Mathe and Klaczko, 1999; Gilchrist and Partridge, 2001; Hoffmann and Shirriffs, 2002; Ayala et al., 2011), but no such studies have been implemented for Triatominae. Such explanation would suggest that genes codifying for mtCyt b (a single gene) and wing shape (probably polygenic) may be submitted to different evolutionary forces. Given their role on metabolism, mtCyt b may be submitted to neutral changes, whereas wing shape may be submitted to selective pressure. Different evolutionary rates have been described among nuclear and mitochondrial genes (Monteiro et al., 2000; this study) and even in two mitochondrial genes in Triatominae: large subunit ribosomal RNA and mtCyt b (Lyman et al., 1999).

The hypothesis that the geometric morphometrics classification could be searched in the environment is less likely. We do not deny an environmental influence partly affecting some aspects of shape, but we argue that the same differences in wing shape found in both field and laboratory insects (Fig. 4) can not be attributed uniquely to environmental variations, furthermore if they persisted under the same controlled conditions of laboratory through 45 and 24 generations for *R. prolixus* and *Rhodnius* from Puerto Asís, respectively. If such differences were environmental induced, they should have been modified under similar conditions of rearing in the laboratory.

Table 4
Pair-wise genetic distance Kimura 2-parameters between nuclear DNA (D2 variable region of the 28S RNA) of sequences of Colombian *R. prolixus* and *Rhodnius* from Puerto Asís, Colombia, and sequences previously reported.

		2	3	4	5	6	7	8
1. prVE5								
2. roVE2	0.000							
3. prCO1	0.000	0.000						
4. roBR8	0.002	0.002	0.002					
5. roEC	0.000	0.000	0.000	0.002				
6. roBR4	0.002	0.002	0.002	0.005	0.002			
7. naBr	0.007	0.007	0.007	0.005	0.007	0.010		
8. prCas	0.002	0.002	0.002	0.005	0.002	0.002	0.010	
9. roCOL	0.005	0.005	0.005	0.002	0.005	0.007	0.007	0.007

In conclusion, our study brought convincing arguments sustaining a genetic background to the shape differences observed in *Rhodnius* sp. from Puerto Asís, Colombia. The morphometric discrimination of *Rhodnius robustus*-like from actual *R. robustus* and *R. prolixus* may constitute a useful tool in the epidemiological surveillance of Chagas disease in the south of Colombia and North of Ecuador.

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