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Pioneer study of population genetics of *Rhodnius ecuadoriensis* (Hemiptera: Reduviidae) from the central coastand southern Andean regions of Ecuador

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Abstract

Effective control of Chagas disease vector populations requires a good understanding of the epidemiological components, including a reliable analysis of the genetic structure of vector populations. *Rhodnius ecuadoriensis* is the most widespread vector of Chagas disease in Ecuador, occupying domestic, peridomestic and sylvatic habitats. It is widely distributed in the central coast and southern highlands regions of Ecuador, two very different regions in terms of bio-geographical characteristics. To evaluate the genetic relationship among *R. ecuadoriensis* populations in these two regions, we analyzed genetic variability at two microsatellite loci for 326 specimens (n = 122 in Manabí and n = 204 in Loja) and the mitochondrial cytochrome *b* gene (*Cyt b*) sequences for 174 individuals collected in the two provinces (n = 73 and = 101 in Manabí and Loja respectively). The individual samples were grouped in populations according to their community of origin. A few populations presented positive F_{IS}, possible due to Wahlund effect. Significant pairwise differentiation was detected between populations within each province for both genetic markers, and the isolation by distance model was significant for these populations. Microsatellite markers showed significant genetic differentiation between the populations of the two provinces. The partial sequences of the *Cyt b* gene (578 bp) identified a total of 34 haplotypes among 174 specimens sequenced, which translated into high haplotype diversity (Hd = 0.929). The haplotype

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distribution differed among provinces (significant Fisher's exact test). Overall, the genetic differentiation of *R. ecuadoriensis* between provinces detected in this study is consistent with the biological and phenotypic differences previously observed between Manabí and Loja populations. The current phylogenetic analysis evidenced the monophyly of the populations of *R. ecuadoriensis* within the *R. pallescens* species complex; *R. pallescens* and *R. colombiensis* were more closely related than they were to *R. ecuadoriensis*.

Keywords

Chagas disease; *Rhodnius pallescens* species complex; *Rhodnius ecuadoriensis*; Ecuador; Population genetics; Microsatellite markers; Cytochrome *b*; Panmictic unit

1. Introduction

The hemipteran species of the subfamily Triatominae are vectors of the protozoan parasite *Trypanosoma cruzi*, the etiological agent of Chagas disease. According to the latest WHO data updated on March 2017, this disease currently affects about 6–7 million people, mostly on the American continent (WHO, 2017). At least 151 Triatominae species have been reported worldwide (Schofield and Galvão, 2009; Justi and Galvão, 2017) from which 16 were described in Ecuador, and at least seven are likely vectors of Chagas disease to humans (Abad-Franch et al., 2001; Villacís et al., 2010) among which the most important Chagas disease vectors are *Triatoma dimidiata* and *Rhodnius ecuadoriensis*. Populations of *R. ecuadoriensis* are widely distributed in the central coast and southern Andean regions from Ecuador (Villacís et al., 2010; Grijalva et al., 2015), and northern Peru (Cuba-Cuba et al., 2002). In Ecuador these occupy domestic, peridomestic as well as sylvatic habitats (Suarez-Davalos et al., 2010; Villacís et al., 2010), and abundant sylvatic populations can be found in nests of the Guayaquil squirrel (*Sciurus stramineus*) and the fasciated wren bird (*Campylorhynchus fasciatus*) in both the coastal and southern Andean regions (Grijalva and Villacís, 2009; Suarez-Davalos et al., 2010; Grijalva et al., 2010, 2012).

Among the Triatominae, populations of the same species frequently present distinctive phenotypic expression across their geographical distribution (Catalá et al., 2005; Abrahan et al., 2008; Hernandez et al., 2008; Dujardin et al., 2009; Schofield and Galvão, 2009; Villacís et al., 2010). Morphometric studies based on antennal phenotype and wing geometry of *R. ecuadoriensis* demonstrated strong phenotypic differences between Manabí (central coast) and Loja (southern Andean) provinces (Villacís et al., 2010), which correlated with differences in size and behavior also observed between individuals from the two provinces (Villacís et al., 2008, 2010). The results of the morphometric analysis pointed to an incipient speciation process between the populations of these provinces and the hypothesis of disruptive selection acting upon *R. ecuadoriensis* was proposed (Villacís et al., 2010).

Molecular tools, such as mitochondrial cytochrome *b* gene (*Cyt b*) sequences and microsatellite markers have the advantage of addressing species phylogeny and population genetics (Mas-Coma and Bargues, 2009). Previous studies with the *Cyt b* of several species of the Rhodniini tribe revealed two significant clusters, one including the species belonging to the *Rhodnius. prolixus* complex (*R. prolixus*, *R. robustus*, *R. neglectus* and *R. nasutus*),

another one clustering the species of the *R. pallescens* complex (*R. colombiensis*, *R. ecuadoriensis* and *R. pallescens*). However other species were not clustered in any significant group, and the complete hierarchy of species in the genus *Rhodnius* remains to be elucidated (Monteiro et al., 2000). Among several studies on species of the genus *Triatoma* using mitochondrial sequences, genetic structure of *T. infestans* populations collected before and after spraying was elucidated with *Cyt b* sequences (Quisberth et al., 2011). Moreover, with a different mitochondrial gene (*COI*), studies detected the genetic differentiation of three Colombian *Triatoma dimidiata* populations from different ecogeographical regions (Gómez-Palacio et al., 2015). For *R. ecuadoriensis*, a first study based on *Cyt b* sequences of some Ecuadorian and Peruvian populations (four from Ecuador and one from Peru) found that Peruvian bugs present a markedly divergent haplotype, and suggest an incipient process of speciation within *R. ecuadoriensis* (Abad-Franch et al., 2003, 2004; Abad-Franch and Monteiro, 2005). Moreover, cytogenetic analysis of the chromosomal location of ribosomal genes in *R. ecuadoriensis* populations also suggests differentiation between Peruvian and Ecuadorian specimens (Pita et al., 2013). Nevertheless, in both studies the exact origin of the populations of Ecuador remains elusive, so it is not known whether the Peruvian population is distinguished from those of central coast, or southern Andean of Ecuador.

Microsatellites are short DNA sequences that are repeated in tandem at one or more locations in the genome (Hartl, 2000). Microsatellite loci have been described for several triatomine species: *T. dimidiata* (Anderson et al., 2002), *T. infestans* (García et al., 2004; Marcet et al., 2006), *T. brasiliensis* (Harry et al., 2009), *T. pseudomaculata* (Harry et al., 2008a), *R. prolixus* (Harry et al., 2008b) as well as for *R. pallescens* (Harry et al., 1998; Díaz et al., 2014). The latter were successfully assayed in individuals of other species within the *R. pallescens* complex. A study of *R. nasutus* using microsatellites demonstrated high genetic differentiation between two groups of specimens collected in different ecotopes (different palm tree species in two sites with distinct abiotic features) (Dias et al., 2011). A similar study of *R. pallescens* evaluated allelic polymorphism in field and laboratory individuals (Gómez-Sucerquia et al., 2009), suggesting genetic differences between them were due to a strong founder effect occurring in laboratory colonies. Moreover, microsatellite markers applied to Venezuelan *R. prolixus* populations to understand the house infestation process revealed a lack of genetic structure between sylvatic and domestic ecotopes, indicative of unrestricted gene flow between the bugs in both ecotopes (Fitzpatrick et al., 2008). Similarly, high levels of gene flow were demonstrated between wild and intra-peridomestic populations of *T. infestans* in the Bolivian Andes (Brenière et al., 2013).

In this context, the present study aimed to perform preliminary exploration of the genetic diversity and structure of *R. ecuadoriensis* populations at the regional level, comparing several communities in two provinces (Loja and Manabí) from the central coast and southern Andean regions of Ecuador, using both microsatellites and *Cyt b* gene sequences as genetic markers.

2. Materials and methods

2.1. Geographic origin and collection of R. ecuadoriensis

The two areas studied are located in the central coast (Manabí province) and southern Andean (Loja province) regions in Ecuador (Fig. 1). The central coast region has both subtropical dry and tropical humid climates, a flat landscape with some low hills, and mostly agglomerate habitat. Agriculture is the main economic activity with a predominance of sugar cane, oranges, bananas, yucca, corn and rice. In addition, some palms such as cade or tagua (*Phytelephas aequatorialis*) and coconuts are cultivated. The walls and floor of the houses in this region are for the most part constructed with bamboo cane "caña guadúa" (*Guadua angustifolia*) or wood, the roof is commonly made of cade palm fronds, with a few made of zinc (Black et al., 2007). The samples studied were collected in six communities, ranging from 65 to 400 m in elevation, located in a single county (Fig. 1). In contrast, the southern Andean region has a dry temperate climate, with high mountains and scattered habitat. The vegetation is dominated by bushes and prickly and herbaceous plants (Sierra, 1999). The main agricultural crops are corn, kidney beans, yucca, papaya, peanuts, bananas and coffee. Houses are typically made of adobe walls, with a dirt floor, and the roof is made of ceramic tile (Black et al., 2007; Grijalva et al., 2015; Nieto-Sanchez et al., 2015). The *R. ecuadoriensis* specimens studied from Loja province were collected in 11 communities in five counties (Fig. 1); these communities ranged from 710 to 1601 m in elevation (Grijalva et al., 2015).

Triatomines were collected in domiciles (D), peridomiciles (P) and sylvatic areas (S) in both provinces, during summer times between 2005 and 2008. In domiciles and peridomiciles, the bugs were manually collected by two-person teams of trained field workers from the National Chagas Disease Control Program, using the one-man-hour method as previously described (Grijalva et al., 2005, 2011, 2015). In sylvatic areas, manual searches were performed around the communities of Bejuco and Maconta Abajo in Manabí (Suarez-Davalos et al., 2010) and La Ciénega, Naranjo Dulce, Santa Rosa and Galápagos in Loja (Grijalva and Villacís, 2009; Grijalva et al., 2012, 2015). Triatomines were collected under the collection permit numbers: 002–17IC-FAU-DNBAPVS/MA and 010-IC-FAU-DNBAPVS/MA.

2.2. DNA extraction and PCR amplification

DNA was extracted from one to three legs of *R. ecuadoriensis* individuals, using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer's recommendations for DNA isolation from animal tissue. The DNA concentration was determined using a NanoDrop 1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Wilmington, DE, USA) and stored at −20 °C.

Six previously described microsatellite markers (L3, L9, L13, L25, L43, L47), specific to *R. pallescens* and recommended to *R. ecuadoriensis* (Harry et al., 1998) were evaluated with 70 current *R. ecuadoriensis* individuals and amplification conditions were modified when necessary for optimization. These markers were amplified in a 15-μl reaction volume containing 1.2 μl dNTPs (2.5 mM), 1.5 μl GoTaq® 5× Buffer, 0.5 μl $MgCl_2$ (25 mM), 0.105

µl GoTaq® DNA polymerase ([5 u/µl] Promega®), 3.75 µl each primer (20 ng/µl reverse; 5 µM fluorescently labeled forward) and 10 ng of DNA template. Amplifications were performed in a thermocycler (Bio-Rad, Hercules, CA, USA) with a first step at 94 °C for 5 min; followed by 30 cycles (94 °C for 30 s, the annealing temperature according to each primer set for 30 s, 72 °C for 1 min) and a final elongation step at 72 °C for 10 min. One microliter of PCR product mixed with 10 µl of HiDi Formamide and 0.5 µl of standard weight marker 500 ROX™ (Applied Biosystems, Van Allen Way, Carlsbad, CA, USA) was analyzed on an automated DNA sequencer (Applied Biosystems® 3130xL Genetic Analyzer, Foster, CA, USA). Allele fragment lengths for each sample were determined using GeneMapper 4.0 (Applied Biosystems®).

The *Cyt b* gene fragment was amplified with the primers CYTB7432F (5′-GGAC-G(AT)GG(AT)ATTTATTATGGATC) and CYTB7433R (5′ GC(AT)CCAATTCA(AG) GTTA(AG)TAA) (Monteiro et al., 2003), using a 10-ng template of the DNA isolated from each insect in a 25-µl reaction volume: 2 µl dNTPs (2.5 mM each), 5.0 µl 5× buffer GoTaq, Promega® 1 µl of $MgCl_2$ (25 mM) Promega®, 0.125 µl Go Taq DNA polymerase (5 u/µl, Promega®) and 1 µl of each primer (10 pmol/µl).

Amplifications were carried out in a thermocycler (Bio-Rad, Hercules, CA, USA) under the following conditions: one step at 94 °C for 5 min; followed by 35 cycles: 94 °C for 30 s, 47 °C for 30 s, 72 °C for 1 min and a final elongation step at 72 °C for 10 min. PCR products were purified with MultiScreen PCR purification plates (Merck Millipore, Billerica, MA, USA) following the manufacturer's recommendations. Both fragment strands were sequenced on an automated DNA sequencer (Applied Biosystems® 3500xL Genetic Analyzer).

2.3. Data analysis

Individual microsatellite multilocus genotypes were obtained for 326 *R. ecuadoriensis* specimens. These were grouped into 17 populations according to their community of origin (Table 1). However, only 12 collection sites had sufficient samples (at least six bugs collected) to be considered for subpopulation analyses (Table 2). The significance of the microsatellite genotype association between pairs of loci in the overall sample (linkage disequilibrium) was tested using the Fstat 2.9.3.2 software (Goudet, 2001). Analysis of genetic variability per locus, per population and per subpopulation and overall loci consisted of following descriptive indexes estimated using Fstat: the number of alleles (Nall), the allelic richness (aRich), which is a measurement of the number of alleles independent of sample size, the unbiased expected heterozygosity (He) and the observed heterozygosity (Ho). To explore deviations from Hardy Weinberg (HW) expectations within populations, subpopulations and within each locus, the F_{IS} index, which measures the heterozygote deficit or excess, was tested after 2400 random allele permutations among individuals within the samples using Fstat. The isolation by distance model between populations was tested by the Mantel test using the Cavalli-Sforza and Edwards (1967) genetic distance between the populations estimated with the R software (R Core Team, 2016). The level of geographical genetic structuring among populations was estimated through hierarchical analyses of molecular variance (AMOVA), with the Arlequin software v. 3.5.1.2 (Excoffier et al., 2005).

The pairwise F_{ST} were computed with Arlequin and differentiation between populations and subpopulations was tested by permutation procedures of multilocus genotypes between populations or between subpopulations (1000 permutations).

A Bayesian approach was implemented to determine the number of genetic clusters that best fit the data set using STRUCTURE v. 2.2 (Pritchard et al., 2000; Falush et al., 2007). Several runs with a burn-in period of 50,000 iterations, 10,000 iterations in length, with variable genetic clusters (k = 2–17) were carried out to identify the best assignment; this procedure places individuals in k clusters chosen in advance and membership coefficients are calculated for each individual in inferred clusters. The “admixture” (individuals may have ancestry from various populations) and “no admixture” (each individual comes from one of the k populations) models for the ancestry of individuals were applied to the set of data with the “Allele Frequencies Correlated” option (meaning that allele frequencies in the different populations are most probably similar) while the other parameters were left at their default values. These models were also tested with the LOCPRIOR option, which allows clustering by using sampling locations of individuals as prior information. This option is more appropriate for data sets with few markers and small sample sizes. Fifteen independent runs for each k were produced to assess the consistence of the results across runs, which is one of the criteria to estimate the best k (Pritchard et al., 2000). The k fitting best with the current set of data was selected using the Structure Harvester web v06.94 (Earl and VonHoldt, 2012).

The *Cyt b* gene fragment was obtained for a total of 174 specimens captured in 19 different communities in Manabí and Loja. SeqMan Software (DNAstar Lasergene 8, v8.1.2) (Kleywegt, 2005) was used to assemble and align forward (F) and reverse (R) chromatograms, and to correct them to obtain the consensus sequence for each individual. MEGA 5 software (Tamura et al., 2011) was used to align multiple sequences and to study the phylogenetic relationships among sequences. Standard genetic variability (haplotype diversity, Hd and nucleotide diversity, *Pi*) and differentiation among sequences were evaluated using DnaSP version 5.1 software (Librado and Rozas, 2009). The Fisher’s exact test for testing the null hypothesis of independence of the haplotype distribution between the two provinces or between habitats was performed with R software. The least complex phylogenetic trees (maximum parsimony) was constructed using the program NETWORK v.4.6.1.2 (available at http://www.fluxus-engineering.com/sharenet.htm) with the following options: median-joining algorithm that allows nucleotide multistate characters, same weight for each site (set to 10), epsilon factor set to the same value as the highest site weight (10) as recommended by the program’s authors and the others option set at the default. Post processing option (maximum parsimony calculation) was used to draw the networks containing all the shortest trees. External rooting of the network was applied using *R. pallenscens* sequence as an outgroup (accession number, KC543512).

For the construction of a phylogenetic tree, all available GenBank sequences of the *Rhodnius* genus were downloaded. Those that aligned with over at least 500 bp of our sequences were retained, limiting the number of sequences per species to a maximum of five different haplotypes. A total of 26 GenBank haplotypes of the *Rhodnius* genus were included in the study: *R. ecuadoriensis* (accession numbers KC543507-510), *R. pallescens*

(KC543512, FJ229359, JQ686687, JQ686684, GQ850481) *R. colombiensis* (FJ229360, KC543506), *R. pictipes* (JX273157, FJ887792), *R. stali* (FJ887790-91, KT805172), *R. prolixus* (KP126733, KC543514, EF011726, EF011723), *R. neglectus* (JX273156), *R. nasutus* (JX273155), *R. robustus* (JX273158, KC543515, EF071583, EF011722). Also, two haplotypes of *T. infestans* (HQ848648, JN006796) were added as an outgroup. The best-fitting substitution model for the data set was determined using JMODELTEST 0.1 (Posada, 2008). To build the tree, the maximum composite likelihood method was used. The reliability of the inferred tree was tested by the bootstrap resampling technique with 100 replicates.

3. Results

3.1. Microsatellite analysis and population structure

3.1.1. Variability and Hardy Weinberg equilibrium in R. ecuadoriensis populations—Of the six published sets of microsatellite primers recommend for *R. ecuadoriensis* (Harry et al., 1998), two were discarded because of inconsistent amplification (L3 and L43), two others gave monomorphic results for a subsample of 70 individuals (L9 and L25) and were not included in the analysis, and two were polymorphic (L13 and L47). Data from these last two microsatellite loci were analyzed for a total of 326 *R. ecuadoriensis* specimens.

The overall loci amplification success was >95%, with 96.3% for L13 and 96.9% for L47. No significant association between the two loci genotypes among the overall data and within each population was found, a result supporting the statistical independence of the two loci. A summary of the genetic variability per locus and population is presented in Table 1. No private allele was registered throughout the populations for the either loci. The allelic richness was similar for the two loci, varied from 1.6 to 4.3 for L13 (3.0 ± 0.8) and from 1 to 4.6 (2.5 ± 1.2) for L47 locus. Significant deviations from HW expectations, overall loci, due to a heterozygote deficit (positive F_{IS}) were observed in each province (all Manabí, all Loja) and overall specimens (all Loja + Manabí) (Table 1). When each population was analyzed separately, three of the 17 (MBJ, LCG, and LSS) presented a significant deviation from HW expectations due to a heterozygote deficit (Table 1). To determine if deficits in heterozygote could be due to Wahlund effect, F_{IS}-values were calculated per subpopulations corresponding to collection site (a single GPS point), which presumes the smallest panmictic unit. Only 12 subpopulations of different habitats including at least six specimens were available from the total current sample, and none of them were in HW disequilibrium (Table 2).

3.1.2. Genetic differentiation between populations and subpopulations—For the following analysis only 12 of the 17 community populations with ten and more specimens were included (eight populations from Loja and four from Manabí, see Table 1). A significant F_{ST} value of 0.17 ($p < 10^{-4}$) was observed among the populations (AMOVA). Moreover, of a total of 67 pairwise F_{ST} population comparisons, 73.1% were significant. In the provinces, the F_{ST} values between pairwise populations were also significant for 66.6% in Manabí and 76.2% in Loja. The correlation between the geographic and genetic distance

matrices calculated between the 12 populations, testing the isolation by distance model, was significant (r = 0.83, $p < 10^{-3}$). F_{ST} values were also calculated between the 12 subpopulations (habitats) analyzed in Table 2, and most of them were significant (73.1%).

3.1.3. Geographical and ecological genetic differentiation—A hierarchical AMOVA procedure using the two loci and the 12 populations that included at least 10 individuals was applied, using the following hierarchies: between provinces, populations within a province, individuals within populations and among individuals throughout the sample (Table 3). Most of the genetic variation was assigned to differences among individuals within the total sample (59.2% of the total variation). The variation attributed to differences between the two geographic groups (18.3%) was significant, reflecting the genetic differentiation among the populations of the two provinces. Moreover, although lower, the variation attributed to differences between the populations within the groups (4.4%) was significant (Table 3), reflecting diversity within the provinces. Considering the 12 subpopulations two types of hierarchies were evaluated grouping them according to (i) their province of origin, and (ii) their habitat (peridomicile versus sylvatic area) (Table 4). Significant genetic differentiation between provinces was obtained, but no between habitats.

The best model detected by STRUCTURE, with all models evaluated, to explain the genetic structure of the total sample (including all specimens) was for a k value of 2 (Fig. 2), indicating that all individuals could be grouped into two significantly distinct genetic clusters. Indeed, the first cluster included most of the individuals from Loja province and the second, most of those from Manabí; few individuals (~8%) had admixture genotypes.

3.2. Mitochondrial Cyt b gene variability in R. ecuadoriensis

3.2.1. Indices of genetic diversity in communities and provinces—Partial sequences (578 bp) of the *Cyt b* gene were obtained for a total of 174 specimens of *R. ecuadoriensis* from the provinces of Manabí (73) and Loja (101). As expected for intraspecific mitochondrial sequences, no insertion or deletions, “indels” were observed.

Table 5 summarizes the genetic diversity obtained among all the sequences, within each community and within each province. The high haplotype diversity observed within the entire set of sequences (174 specimens) (Hd = 0.93 for 34 haplotypes identified) was quite similar within the set of sequences from each province. The overall nucleotide diversity expressed by the *Pi* value was 0.017 ranging from 0.0003 to 0.02 among populations. The large majority of the nucleotide substitutions were parsimoniously informative (46 of 49 variable sites). Of the 49 nucleotide substitutions, 17 (34.7%) were shared between the two provinces. The genetic diversity was further evaluated by community for seven and four populations from Loja and Manabí provinces, respectively, considering at least five specimens per community (Table 5). Although more than one haplotype was observed in all populations, haplotype diversity was highly variable among them, ranging from 0.182 in the LSS to 0.917 in the MBJ population.

3.2.2. Geographical Cyt b haplotype distribution in Manabí and Loja provinces—The geographic distribution of 34 haplotypes among the different communities is presented in Table 5. Of them, seven (H01, H04, H06–H08, H13, H16)

comprised 70% of the 174 sequences. Most of the other haplotypes (20 haplotypes) were detected in only one or two specimens. There were 18 *Cyt b* haplotypes identified in Loja and 19 in Manabí, and only three were found in both provinces (H04, H08 and H26). The Fisher's exact test revealed a significantly different distribution of the haplotypes between the two provinces ($p < 0.01$).

A median joining network was constructed with all the *R. ecuadoriensis* sequences under study with the option of external rooting with one sequence of *R. pallescens* as the outgroup (Fig. 3). The network was composed of only two shortest trees and the topology of the two trees did not differ. In network representations, the nodes corresponded to haplotypes or to hypothetical sequences (median vector, mv) linking the current haplotypes; internal nodes are assumed to be ancestral while terminal nodes are assumed to be recent (Posada and Crandall, 2001). The position in relationship with the root in the network suggests that the most ancestral *R. ecuadoriensis* sequences would be the haplotypes H19, H16 found in Manabí. Moreover, most of the haplotypes only found in Loja have a derivated node position (peripheral) from the haplotypes H26 and H08, which were found in both provinces.

The sampling was selected primarily to assess the genetic diversity of *R. ecuadoriensis*, and therefore was not well suited to analyze the data according to habitat. However, considering the three communities with sufficient number of specimens collected in a single collection site (Table 6), it was found that the haplotype distribution was significantly different between domicile and sylvatic area in one community of Loja ($p < 0.05$) and not significantly different between peridomicile and domiciles together versus sylvatic area in two communities of Manabí (Fisher's exact test).

3.3. Phylogenetic analysis of R. ecuadoriensis based on Cyt b sequences of the Rhodnius genus

The sequences of the 34 haplotypes identified in this work were aligned with 26 GenBank sequences of nine *Rhodnius* species including the four sequences of *R. ecuadoriensis* available in GenBank and two sequences of *T. infestans* selected as the outgroup. The best model of evolution for this data set was Hasegawa-Kishino-Yano with discrete gamma distribution and the I option (HKI + G + I). All 34 haplotypes obtained here clustered with the four *R. ecuadoriensis* sequences previously deposited in GenBank with a bootstrap of 100% (Fig. 4). This cluster included the haplotypes H07 and H22 that differ clearly from the others. At the upper level of the tree, two significant clusters were observed, the first included *R. ecuadoriensis*, *R. pallescens* and *R. colombiensis*, corresponding to the *R. pallescens* complex, the second included the species of the *R. prolixus* complex: *R. robustus*, *R. neglectus*, *R. nasutus* and *R. prolixus*. The two other species that were included in the analysis, *R. pictipes* and *R. stali* that belong to the *R. pictipes* complex, clustered together with a lower bootstrap value of 81%. Within the *R. pallescens* complex, *R. pallescens* and *R. colombiensis* were more closely related than they were to *R. ecuadoriensis*.

4. Discussion

Transmission of *Trypanosoma cruzi* to humans is a complex phenomenon that involves interplay between the parasites, vectors, reservoirs and humans. Environmental factors, type

of dwelling and other anthropogenic factors play important roles in the creation of optimal conditions for disease transmission (WHO, 2002; Black et al., 2007; Grijalva et al., 2010; Ocaña-Mayorga et al., 2010; Nieto-Sanchez et al., 2015).

The rural areas in the southern Andean region of Ecuador are among the poorest in the country; most people live in substandard housing, under conditions that influence the presence of vectors indoors in Chagas endemic areas. This context combined with reports of widespread infestation in sylvatic areas (Grijalva et al., 2012) and frequent reports of re-infestation after control intervention (Grijalva et al., 2015) indicate a need for monitoring effectiveness of vector control in this province. A similar situation probably occurs in northern Peru where *R. ecuadoriensis* has been reported, in an environment and human habitat conditions that are very similar to Loja (Cuba-Cuba et al., 2002).

In Manabí the situation is quite different. The presence of *R. ecuadoriensis* is mostly reported in the sylvatic and peridomestic habitats and not indoors (Suarez-Davalos et al., 2010; Grijalva et al., 2011). The socioeconomic level is higher than in Loja, dwellings are modest, with guadúa cane walls, giving little opportunity for insects to hide and the common palm roofs are increasingly replaced with zinc. Nevertheless, the peridomestic areas offer abundant microhabitats for triatomines. The molecular evidence presented here, although preliminary, indicate that gene flow is likely occurring between domestic/peridomestic and sylvatic habitats within Manabí (see below). In this context, vector transmission is more likely caused by occasional incursions of triatomines in the domestic habitat rather than vector–human contact with established colonies inside the house.

4.1. Genetic variability, panmictic unit and spatial structuring of R. ecuadoriensis populations

The analysis herein performed is based on two microsatellite loci and sequences of a single gene; therefore the results must be interpreted cautiously.

The first step of the analysis was to determine the panmictic unit within *R. ecuadoriensis* through analysis of microsatellite data. When the individuals were grouped in populations according to their community of origin, pooling bugs captured in different habitats (domestic, peridomestic or sylvatic), most of the populations were in HW equilibrium, but the absence of rejection of the null hypothesis may be due to the low number of loci applied. Nonetheless, with only these two loci, HW disequilibrium were detected in the overall sample and in a few communities of both provinces, all due to heterozygote deficit, and probably caused by Wahlund effect. Interestingly, the analysis of subpopulations composed of individuals captured at a single collection site did not reveal any HW disequilibrium. This result shows that the panmitic unit may be at the individual structure/house level rather than at the community, as previously observed for other species of triatomines (Marcet et al., 2006; Brenière et al., 2012).

The second step was to explore the genetic differentiation between the two provinces, which was clearly supported by the results of both microsatellite and *Cyt b* sequence analyses. The hypothesis of regional genetic differentiation of *R. ecuadoriensis* is supported by previous observations of several phenotypic differences between the populations of Loja and Manabí

(Villacís et al., 2010) which includes the high differentiation of wing shape, considered a characteristic determined by genetic factors (Dujardin et al., 2009). In addition, the behavior traits of these regional populations are very different; in Manabí *R. ecuadoriensis* is more associated with sylvatic habitat, while in Loja this species tends toward domiciliation (Grijalva et al., 2005, 2015). A speciation process between the two geographical populations may be proposed to the extent that it is supported by a genetic differentiation between these populations in addition to several contrasting morphological and biological properties.

4.2. Origin of R. ecuadoriensis and dispersion

According to the nucleotide diversity measurement for *Cyt b*, gene fragments showed that *R. ecuadoriensis* exhibits a similar genetic variability (*Pi* = 0.017) than wild *T. infestans* (*Pi* = 0.014), although the sample of the latter species was from a larger geographic area (Waleckx et al., 2011). However, if including samples considering the complete geographical distribution of *R. ecuadoriensis* (i.e. from Esmeraldas (northern Ecuador) to northern Peru (Cuba-Cuba et al., 2002)), the genetic diversity of the species could be significantly increased. The phylogenetic tree including the available sequences species of *Rhodnius*, shows a clear clustering of *R. ecuadoriensis* and distinction from the rest of the species. Consistent with previous reports, *R. ecuadoriensis* shares common ancestry with *R. pallescens* and *R. colombiensis* (Díaz et al., 2014), although *R. ecuadoriensis* reflects higher divergence from the other two species. The geographic distribution of *R. ecuadoriensis* is limited to the Pacific region, separated from *R. pallescens* and *R. colombiensis* by the Andes in Colombia and Panama. The divergence time of *R. ecuadoriensis* from the two other species was estimated to have occurred in the late Miocene period (~11 to 7 Mya) (Díaz et al., 2014).

The genetic diversity within a species is greater in ancestral populations than in more recent ones, and measuring the genetic diversity across the species distribution can help determine its origin (Avise et al., 1987; Emerson et al., 2001). In this sense, the current data shows a similar genetic diversity among Manabí and Loja populations, but the Manabí sample was spatially limited to only one county (Portoviejo) and the Loja sample was more geographically extended, including all the counties of this province. Moreover, the relationship between the haplotypes was explored through the construction of a network using *R. pallescens* as outgroup. Indeed, its topology favored the hypothesis that the Manabí populations were more ancestral than those from Loja. Indeed, the majority of Loja haplotypes are derived by a few mutations from 2 haplotypes (H26, H08) found in both Manabí and Loja. The divergent haplotype (H07) found only in Loja, differed substantially from the other haplotypes. Interestingly, this divergent haplotype was only found in two communities (LHY and LEX), located close to each other (7 km) in the same valley. One hypothesis to explain this divergent haplotype is a possible earlier wave of dispersion from Manabí to Loja (by birds or mammals) followed more recently by other waves (by human and others). Expanded sampling could help define whether there are intermediate haplotypes between H07 and the others observed and also determine the actual distribution of this haplotype, or if it is limited to this valley.

4.3. Population genetics and ecology of R. ecuadoriensis populations

Genetic markers as well as phenotypic markers have been proposed to analyze movements of triatomines between habitats. In the present study, although the topic was explored, the available samples only allowed us to partially address this question. The populations collected in domiciles and sylvatic areas in Loja were genetically differentiated; this is in agreement with a situation of ancient colonization, and posterior restriction of gene flow between domestic and sylvatic habitats, from which genetic drift has been the main process for differentiation. In Manabí, the populations of these two habitats were not genetically differentiated, which would suggest that active gene flow occurs among them, probably through sporadic incursion of sylvatic populations to peridomiciles and domiciles. However the limited number of samples could be affecting these results, which should be reevaluated with a larger sample set including more sites of each habitat type with enough individuals collected per site.

Finally, we recommend additional collections of *R. ecuadoriensis* over its entire distribution area, including samples from Peru, to conduct population genetic studies at a major geographic scale. Also in the future, additional markers must be introduced: new microsatellites are needed for *R. ecuadoriensis*, and genes previously used such as the mitochondrial ones *COI* and the large subunit ribosomal-16S RNA gene (LSUrRNA) or nuclear ones such as D2 region of the 28S nuclear RNA can be further considered (Monteiro et al., 2003; Calleros et al., 2010; Brenière et al., 2017). Therefore, additional studies should be conducted in Peru to better understand the distribution and ecology of endemic triatomine species and to further implement an integral and coordinated vector control program considering specific heterogeneity.

4.4. Conclusion

The current results showed that *R. ecuadoriensis* populations are highly structured in space (significant genetic differentiation between communities), in both provinces evaluated. This means that, like other triatomine species, the panmictic unit could be limited to triatomine colonies. The results support the hypothesis of genetic differentiation between the Manabí and Loja population previously proposed after the observations of biological and phenotypical differences. The differentiation between the provinces could be explained by geographic distance. The idea of an incipient speciation process between populations of these two provinces remains open. Finally, to improve the knowledge of the *R. ecuadoriensis* genetic structure, ad-hoc designed sampling is required to guide fieldwork.

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References

Abad-Franch F, Monteiro FA, 2005. Molecular research and the control of Chagas disease vectors. An. Acad. Bras. Cienc 77, 437–454. [PubMed: 16127551]

Abad-Franch F, Paucar A, Carpio C, Cuba CA, Aguilar HM, Miles MA, 2001. Biogeography of Triatominae (Hemiptera: Reduviidae) in Ecuador: implications for the design of control strategies. Mem. Inst. Oswaldo Cruz 96, 611–620. [PubMed: 11500757]

Abad-Franch F, Monteiro FA, Patterson JS, Miles MA, 2003. Phylogenetic relationships among members of the Pacific *Rhodnius* lineage (Hemiptera: Reduviidae: Triatominae). Infect. Genet. Evol 2, 244–245.

Abad-Franch F, Monteiro FA, Aguilar VHM, Miles MA, 2004. Population-level mitochondrial DNA sequence diversity in *Rhodnius ecuadoriensis*. Programme and Abstracts. IX European Multicolloquium of Parasitology, Valencia, Spain, p. 201.

Abrahan L, Hernandez L, Gorla D, Catala S, 2008. Phenotypic diversity of *Triatoma infestans* at the microgeographic level in the Gran Chaco of Argentina and the Andean valleys of Bolivia. J. Med. Entomol 45, 660–666. [PubMed: 18714865]

Anderson JM, Lai JE, Dotson EM, Cordon-Rosales C, Ponce C, Norris DE, Beard CB, 2002. Identification and characterization of microsatellite markers in the Chagas disease vector *Triatoma dimidiata*. Infect. Genet. Evol 1, 243–248. [PubMed: 12798021]

Avise JC, Arnold J, Ball RM, Beringham E, Lamb T, Neigel JE, Reeb CA, Saunders NC, 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu. Rev. Ecol. Syst 18, 489–522.

Black CL, Ocana S, Riner D, Costales JA, Lascano MS, Dávila S, Arcos-Terán L, Seed JR, Grijalva MJ, 2007. Household risk factors for *Trypanosoma cruzi* seropositivity in two geographic regions of Ecuador. J. Parasitol 93, 12–16. [PubMed: 17436937]

Brenière SF, Waleckx E, Magallón-Gastélum E, Bosseno MF, Hardy X, Ndo C, Lozano-Kasten F, Barnabé C, Kengne P, 2012. Population genetic structure of *Meccus longipennis* (Hemiptera, Reduviidae, Triatominae), vector of Chagas disease in West Mexico. Infect. Genet. Evol 12, 254–262. [PubMed: 22142488]

Brenière SF, Salas R, Buitrago R, Brémond P, Sosa V, Bosseno MF, Waleckx E, Depickère S, Barnabé C, 2013. Wild populations of *Triatoma infestans* are highly connected to intra-peridomestic conspecific populations in the Bolivian Andes. PLoS One 8 e80786. [PubMed: 24278320]

Brenière SF, Condori EW, Buitrago R, Sosa LF, Macedo CL, Barnabé C, 2017. Molecular identification of wild triatomines of the genus *Rhodnius* in the Bolivian Amazon: strategy and current difficulties. Infect. Genet. Evol 51, 5–9.

Calleros L, Panzera F, Bargues MD, Monteiro FA, Klisiowicz DR, Zuriaga MA, Mas-Coma S, Pérez R, 2010. Systematics of *Mepraia* (Hemiptera-Reduviidae): cytogenetic and molecular variation. Infect. Genet. Evol 10, 221–228. [PubMed: 20018255]

Catalá S, Sachetto C, Moreno M, Rosales R, Salazar-Schetrino PM, Gorla D, 2005. Antennal phenotype of *Triatoma dimidiata* populations and its relationship with species of phyllosoma and protracta complexes. J. Med. Entomol 42, 719–725. [PubMed: 16363154]

Cavalli-Sforza LL, Edwards AWF, 1967. Phylogenetic analysis: models and estimation procedures. Am. J. Hum. Genet 19, 233–257. [PubMed: 6026583]

Cuba-Cuba CA, Abad-Franch F, Roldán RJ, Vargas VF, Pollack VL, Miles MA, 2002. The triatomines of northern Peru, with emphasis on the ecology and infection by trypanosomes of *Rhodnius ecuadoriensis* (Triatominae). Mem. Inst. Oswaldo Cruz 97, 175–183. [PubMed: 12016438]

Dias FB, Paula AS, Belisário CJ, Lorenzo MG, Bezerra CM, Harry M, Diotaiuti L, 2011. Influence of the palm tree species on the variability of *Rhodnius nasutus* Stål, 1859 (Hemiptera, Reduviidae, Triatominae). Infect. Genet. Evol 11, 869–877. [PubMed: 21335104]

Díaz S, Panzera F, Jaramillo-O N, Pérez R, Fernández R, Vallejo G, Saldaña A, Calzada JE, Triana O, Gómez-Palacio A, 2014. Genetic, cytogenetic and morphological trends in the evolution of the *Rhodnius* (Triatominae: Rhodniini) trans-Andean group. PLoS One 9, 1–12.

Dujardin JP, Costa J, Bustamante D, Jaramillo N, Catalá S, 2009. Deciphering morphology in Triatominae: the evolutionary signals. Acta Trop. 110, 101–111. [PubMed: 19026978]

Earl DA, vonHoldt BM, 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour 4, 359–361.

Emerson BC, Paradis E, Thébaud C, 2001. Revealing the demographic histories of species using DNA sequences. Trends Ecol. Evol 16, 707–716.

Excoffier LG, Laval G, Schneider S, 2005. ARLEQUIN (version 3.0): an integrated software package for population genetics data analysis. Evol. Bioinformatics Online 1, 47–50.

Falush D, Stephens M, Pritchard JK, 2007. Inference of population structure using multilocus genotyupe data: dominant markers and null alleles. Mol. Ecol. Notes 7, 574–578. [PubMed: 18784791]

Fitzpatrick S, Feliciangeli MD, Sanchez-Martin MJ, Monteiro FA, Miles MA, 2008. Molecular genetics reveal that Silvatic *Rhodnius prolixus* do colonise rural houses. PLoS Negl. Trop. Dis 2, e210. [PubMed: 18382605]

García BA, Zheng LO, Pérez de Rosas AR, Segura EL, 2004. Primer note. Isolation and characterization of polymorphic microsatellite loci in the Chagas' disease vector *Triatoma infestans* (Hemiptera: Reduviidae). Mol. Ecol. Notes 4, 568–571.

Gómez-Palacio A, Arboleda S, Dumonteil E, Townsend Peterson A, 2015. Ecological niche and geographic distribution of the Chagas disease vector, *Triatoma dimidiata* (Reduviidae: Triatominae): evidence for niche differentiation among cryptic species. Infect. Genet. Evol 36, 15–22. [PubMed: 26321302]

Gómez-Sucerquia LJ, Triana-Chávez O, Jaramillo-Ocampo N, 2009. Quantification of the genetic change in the transition of *Rhodnius pallescens* Barber, 1932 (Hemiptera: Reduviidae) from field to laboratory. Mem. Inst. Oswaldo Cruz 104, 871–877. [PubMed: 19876559]

Goudet J, 2001. Fstat (Version 2.9.3.2): a Program to Estimate and Test Gene Diversities and Fixation Indices. Lausanne University, Lausanne, Switzerland.

Grijalva MJ, Villacís AG, 2009. Presence of Rhodnius ecuadoriensis in sylvatic habitats in the southern highlands (Loja Province) of Ecuador. J. Med. Entomol 46, 708–711. [PubMed: 19496445]

Grijalva MJ, Palomeque-Rodriguez FS, Costales JA, Davila S, Arcos-Terán L, 2005. High household infestation rates by synanthropic vectors of Chagas disease in southern Ecuador. J. Med. Entomol 42, 68–74. [PubMed: 15691011]

Grijalva MJ, Palomeque FS, Villacís AG, Black CL, Arcos-Terán L, 2010. Absence of domestic triatomine colonies in an area where Chagas disease is considered endemic in the coastal region of Ecuador. Mem. Inst. Oswaldo Cruz 105, 677–681. [PubMed: 20835616]

Grijalva MJ, Villacís AG, Ocana-Mayorga S, Yumiseva CA, Baus EG, 2011. Limitations of selective deltamethrin application for triatomine control in central coastal Ecuador. Parasit. Vectors 4, 20. [PubMed: 21332985]

Grijalva MJ, Suarez-Davalos V, Villacís AG, Ocaña-Mayorga S, Dangles O, 2012. Ecological factors related to the widespread distribution of Sylvatic *Rhodnius ecuadoriensis* population in southern Ecuador. Parasit. Vectors 5, 17. [PubMed: 22243930]

Grijalva MJ, Villacís AG, Ocaña-Mayorga S, Yumiseva CA, Moncayo AL, Baus EG, 2015. Comprehensive survey of domiciliary triatomine species capable of transmitting Chagas disease in southern Ecuador. PLoS Negl. Trop. Dis 9, e0004142. [PubMed: 26441260]

Harry M, Poyet G, Romaña CA, Solignac M, 1998. Isolation and characterization of microsatellite markers in the bloodsucking bug *Rhodnius pallescens* (Heteroptera, Reduviidae). Mol. Ecol 7, 1784–1786. [PubMed: 9859210]

Harry M, Dupont L, Romaña C, Demanche C, Mercier A, Livet A, Diotaiuti L, Noireau F, Emperaire L, 2008a. Microsatellite markers in *Triatoma pseudomaculata* (Hemiptera, Reduviidae, Triatominae), Chagas' disease vector in Brazil. Infect. Genet. Evol 8, 672–675. [PubMed: 18571993]

Harry M, Roose CL, Vautrin D, Noireau F, Romaña CA, Solignac M, 2008b. Microsatellite markers from the Chagas disease vector, *Rhodnius prolixus* (Hemiptera, Reduviidae), and their applicability to Rhodnius species. Infect. Genet. Evol 8, 381–385. [PubMed: 18304894]

Harry M, Dupont L, Quartier M, Diotaiuti L, Walter A, Romana C, 2009. New perspectives for population genetics of Chagas' disease vectors in the Northeastern Brazil: isolation of polymorphic microsatellite markers in *Triatoma brasiliensis*. Infect. Genet. Evol 9, 633–637. [PubMed: 19460330]

Hartl DL, 2000. A Primer of Populations Genetics. Sinauer Associates Inc., Sunderland, Massachutsetts, USA, p. 2.

Hernandez L, Abrahan L, Moreno M, Gorla D, Catala S, 2008. Phenotypic variability associated to genomic changes in the main vector of Chagas disease in the southern cone of South America. Acta Trop. 106, 60–67. [PubMed: 18328454]

Justi SA, Galvão C, 2017. The evolutionary origin of diversity in Chagas disease vectors. Trends Parasitol. 33, 42–52. [PubMed: 27986547]

Kleywegt GJ, 2005. SeqMan (Version 6): an Assembling and Editing Sequencing Projects Using Capillary Sequence Data from the ABI 3130 and 3730 Sequencing Platform. Uppsala University, Uppsala, Sweden (Unpublished program).

Librado P, Rozas J, 2009. DnaSP (version 5.10): a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452. [PubMed: 19346325]

Marcet PL, Lehmann T, Groner G, Gurtles RE, Kitron U, Dotson EM, 2006. Identification and characterization of microsatellite markers in the Chagas disease vector *Triatoma infestans* (Heteroptera: Reduviidae). Infect. Genet. Evol 6, 32–37. [PubMed: 16376838]

Mas-Coma S, Bargues MD, 2009. Populations, hybrids and the systematic concepts of species and subspecies in Chagas disease triatomine vectors inferred from nuclear ribosomal and mitochondrial DNA. Acta Trop. 110, 112–136. [PubMed: 19073132]

Monteiro FA, Wesson DM, Dotson EM, Schofield CJ, Beard CB, 2000. Phylogeny and molecular taxonomy of the Rhodniini derived from mitochondrial and nuclear DNA sequences. Am.J.Trop. Med. Hyg 62, 460–465. [PubMed: 11220761]

Monteiro FA, Barrett TV, Fitzpatrick S, Cordon-Rosales C, Feliciangeli D, Beard CB, 2003. Molecular phylogeography of the Amazonian Chagas disease vectors *Rhodnius prolixus* and *R. robustus*. Mol. Ecol 12, 997–1006. [PubMed: 12753218]

Nieto-Sanchez C, Baus EG, Guerrero D, Grijalva MJ, 2015. Positive deviance study to inform a Chagas disease control program in southern Ecuador. Mem. Inst. Oswaldo Cruz 110, 299–309. [PubMed: 25807468]

Ocaña-Mayorga S, Llewellyn MS, Costales JA, Miles MA, 2010. Sex, subdivision, and domestic dispersal of *Trypanosoma cruzi* lineage I in Southern Ecuador. PLoS Negl. Trop. Dis 4, e915. [PubMed: 21179502]

Pita S, Panzera F, Ferrandis I, Galvão C, Gómez-Palacio A, Panzera Y, 2013. Chromosomal divergence and evolutionary inferences in Rhodniini based on the chromosomal location of ribosomal genes. Mem. Inst. Oswaldo Cruz 108, 376–382.

Posada D, 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol 25, 1253–1256. [PubMed: 18397919]

Posada D, Crandall KA, 2001. Intraespecific gene genealogies: trees grafting into networks. Trends Ecol. Evol 16, 37–45. [PubMed: 11146143]

Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multiloccus genotype data. Genetics 155, 945–959. [PubMed: 10835412]

Quisberth S, Waleckx E, Monje M, Chang B, Noireau F, Brenière SF, 2011. "Andean" and "nonAndean" ITS-2 and mtCytB haplotypes of Triatoma infestans are observed in the Gran Chaco (Bolivia): population genetics and the origin of reinfestation. Infect. Genet. Evol 11, 1006–1014. [PubMed: 21457795]

R Core Team @manual, 2016. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, URL (http://www.R-project.org).

Schofield CJ, Galvão C, 2009. Classification, evolution, and species groups within the Triatominae. Acta Trop. 110, 88–100. [PubMed: 19385053]

Sierra R, 1999. Propuesta preliminar de un sistema de clasificación de vegetación para el Ecuador Continental. Proyecto INEFAN/BIRF y Ecociencia, Quito.

Suarez-Davalos V, Dangles O, Villacís AG, Grijalva MJ, 2010. Microdistribution of sylvatic triatomine populations in central-coastal Ecuador. J. Med. Entomol 47, 80–88. [PubMed: 20180312]

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol 28, 2731–2739. [PubMed: 21546353]

Villacís AG, Arcos-Teran L, Grijalva MJ, 2008. Life cycle, feeding and defecation patterns of *Rhodnius ecuadoriensis* (Lent & Leon 1958) (Hemiptera: Reduviidae: Triatominae) under laboratory conditions. Mem. Inst. Oswaldo Cruz 103, 690–695. [PubMed: 19057820]

Villacís AG, Grijalva MJ, Catala SS, 2010. Phenotypic variability of *Rhodnius ecuadoriensis* populations at the Ecuadorian central and southern Andean region. J. Med. Entomol 47, 1034–1043. [PubMed: 21175051]

Waleckx E, Salas R, Huamán N, Buitrago R, Bosseno MF, Aliaga C, Barnabé C, Rodriguez R, Zoveda F, Monje M, Baune M, Quisberth S, Villena E, Kengne P, Noireau F, Brenière SF, 2011. New insights on the Chagas disease main vector *Triatoma infestans* (Reduviidae, Triatominae) brought by the genetic analysis of Bolivian sylvatic populations. Infect. Genet. Evol 11, 1045–1057. [PubMed: 21463708]

WHO, 2002. Control of Chagas Disease. Second Report of the WHO Expert Committee. WHO Technical Report Series. WHO, Ginebra, Suiza.

WHO, 2017. Chagas Disease (American trypanosomiasis) fact sheet. http://www.who.int/mediacentre/factsheets/fs340/en/.

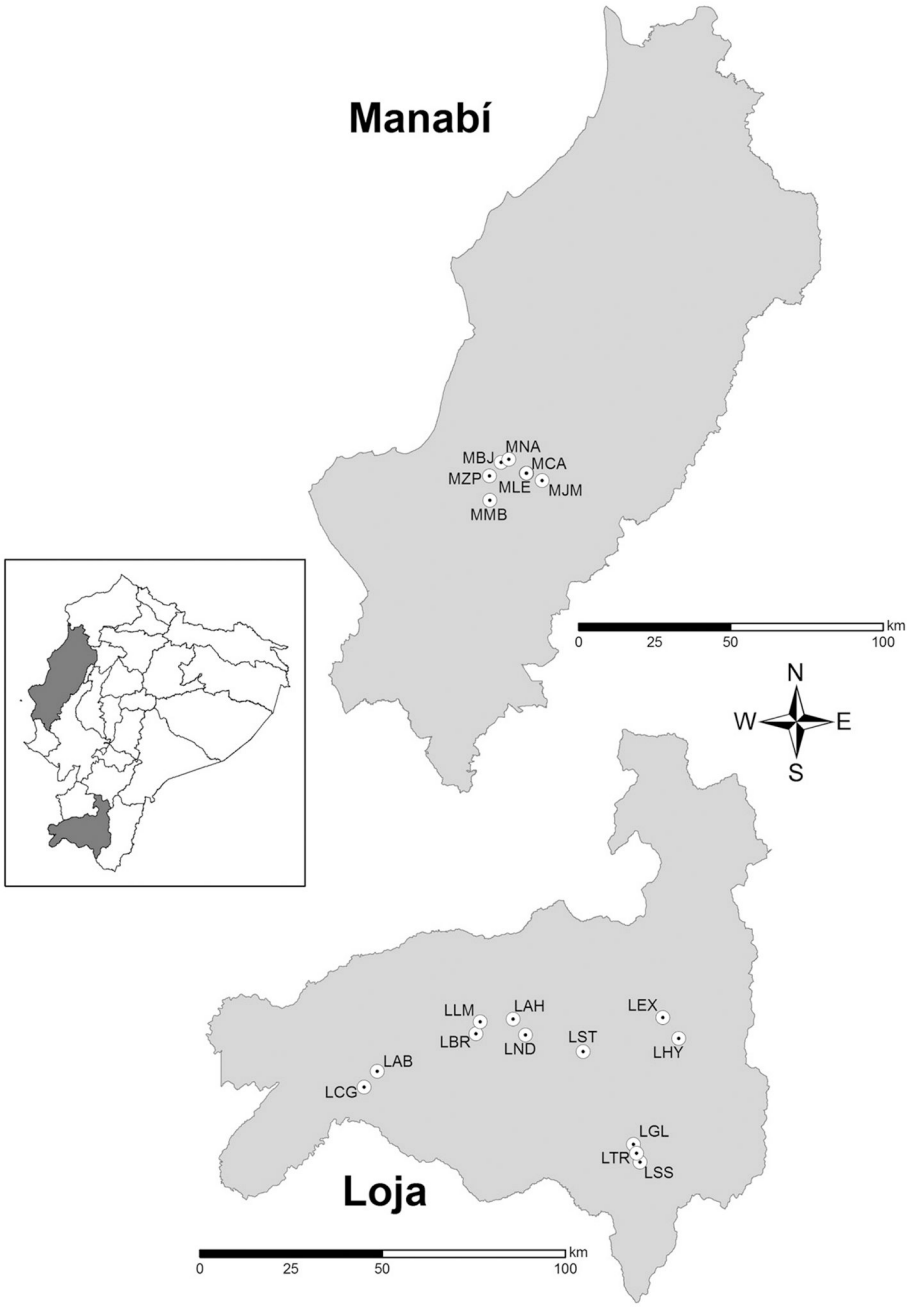

Fig. 1.

Location of samples. Map of Ecuador (in box) and location of the communities where the *R. ecuadoriensis* were collected in the central coast (Manabí province) and southern Andean (Loja province) regions. The codes in the maps indicate the communities. Manabí province: MBJ, Bejuco; MCA, Cruz Alta; MJM, Jesús María; MMB, Maconta Abajo; MNA, Naranjo Adentro; MZP, Zapallo, MLE, La Encantada. Loja province: LAB, Algarobillo; LAH, Ashimingo; LBR, Bramaderos; LCG, La Ciénega; LEX, La Extensa; LGL, Galápagos; LHY, El Huayco; LND, Naranjo Dulce; LSS, Santa Rosa; LST, Santa Ester; LTR, Tuburo, LLM, El Limón.

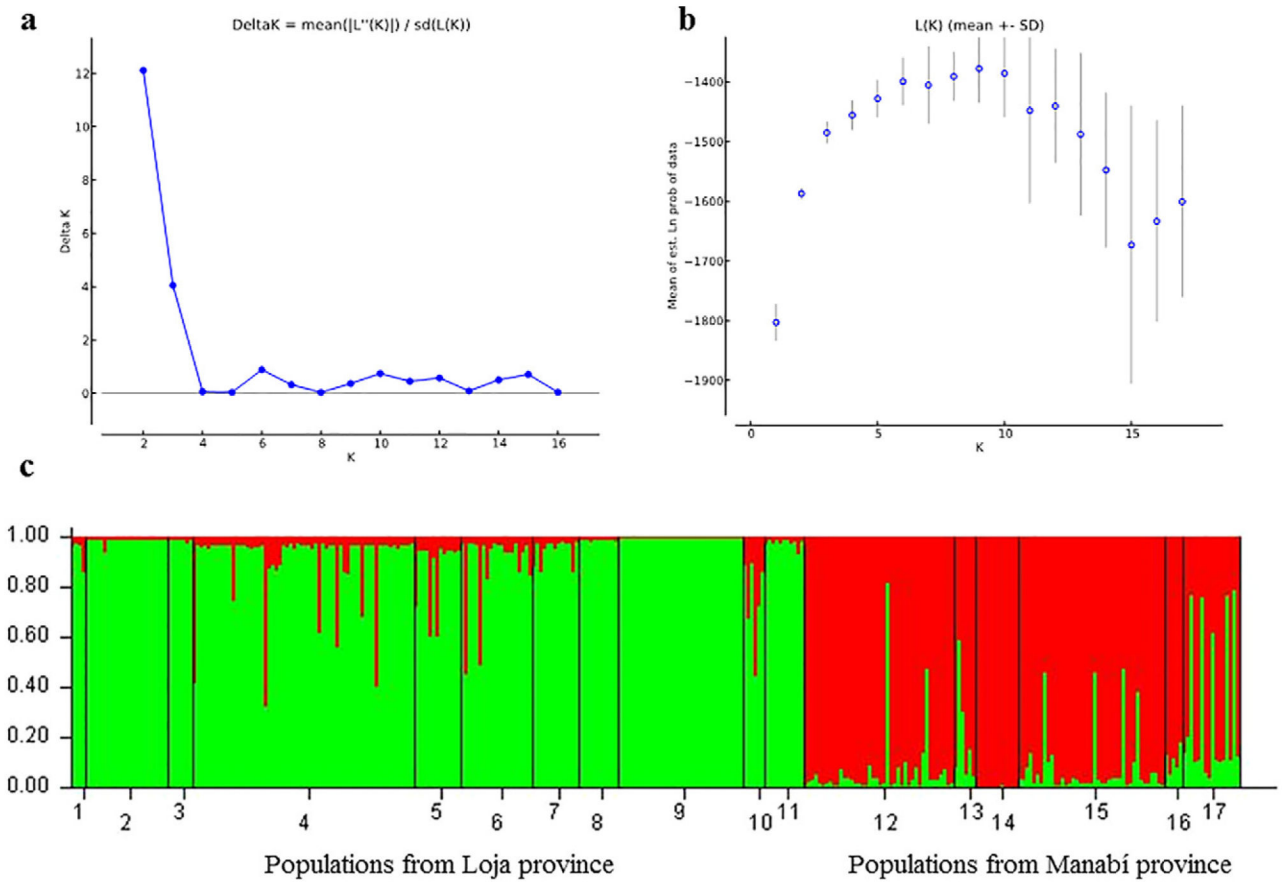

Fig. 2.

Result of STRUCTURE analyses. a) Delta K value (Delta K = mean ((|L″(K)|)/SD(L(K))) plotted for K values from 1 to 17 in Structure Harvester software for *R. ecuadoriensis*. b) Mean of estimated Ln probability of data, plotted for K values from 1 to 17 in Structure Harvester software for *R. ecuadoriensis*. c) Bar plot estimates of membership coefficients for each *R. ecuadoriensis* individual, in each of two inferred clusters (k = 2; best fitting with the current set of data). Each individual in the data set is represented by a single vertical line, which is partitioned into different colored segments representing the individual membership estimate in each of the inferred clusters. The current bar plot is one of those obtained with the admixture model and the prior location option, the other options being left as default. Each number in the bar plot correspond to one population: 1, LAB; 2, LAH; 3, LBR; 4, LCG; 5, LEX; 6, LGL; 7, LHY; 8, LND; 9, LSS; 10, LST; 11, LTR; 12 MBJ; 13, MCA; 14, MJM; 15, MMB; 16 MNA; 17, MZP.

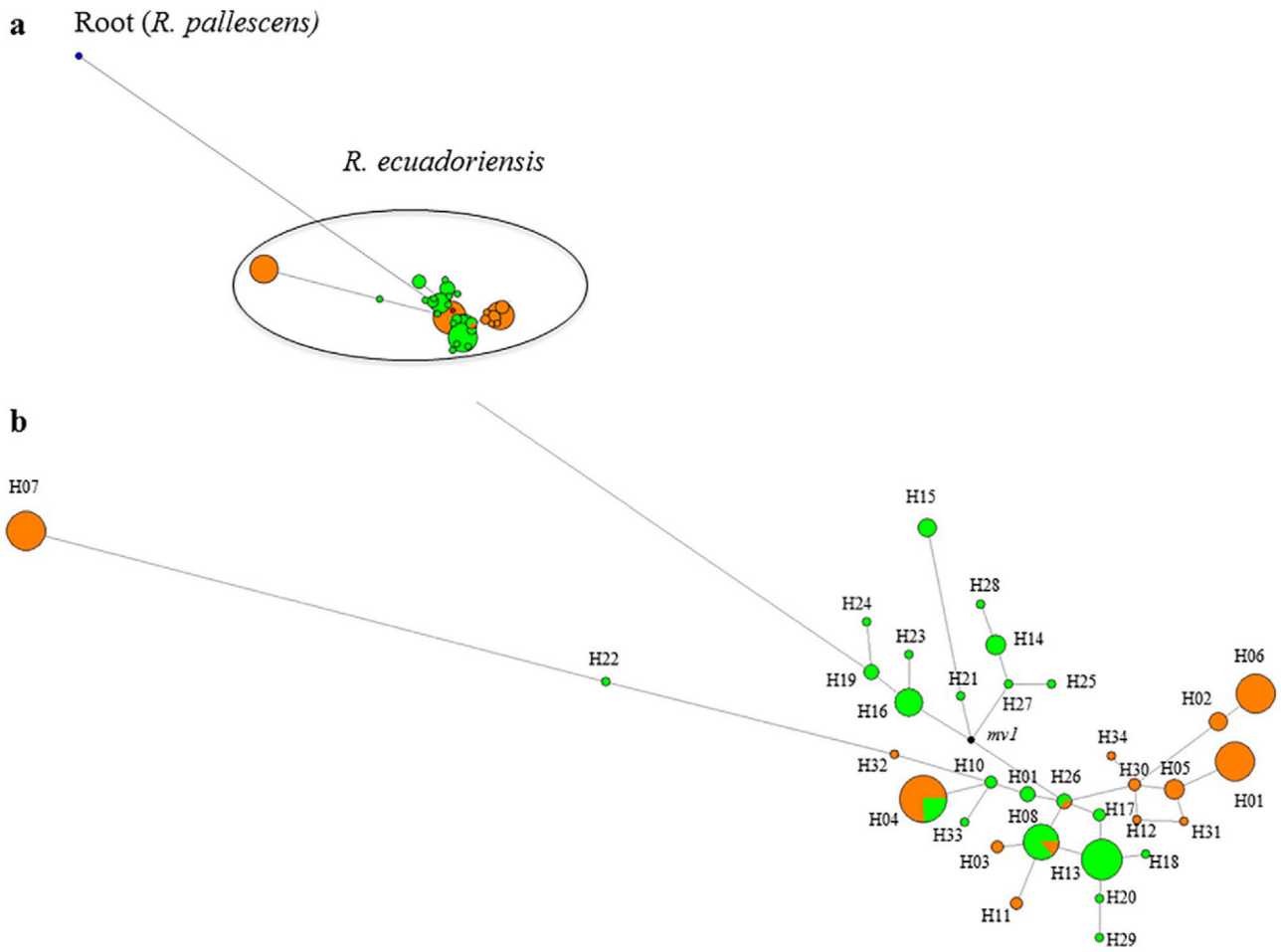

Fig. 3.

Cyt b gene median joining network. The network was resolved from 34 haplotypes found in *R. ecuadoriensis* collected in two provinces (Manabí and Loja) and two haplotypes of *R. pallescens* used as the outgroup. Black circles are median vectors (mv) that are hypothesized sequences not found in the sample and required to connect the existing haplotypes. The other cycles are nodes corresponding to one haplotype found in the current data set; their size is proportional to the number of haplotypes that form the node. Blue nodes are haplotypes of *R. pallescens*, green and orange nodes are *R. ecuadoriensis* haplotypes from Manabí and Loja, respectively. a) General figure of the network; b) enlarged figure that shows only the *R. ecuadoriensis* haplotypes.

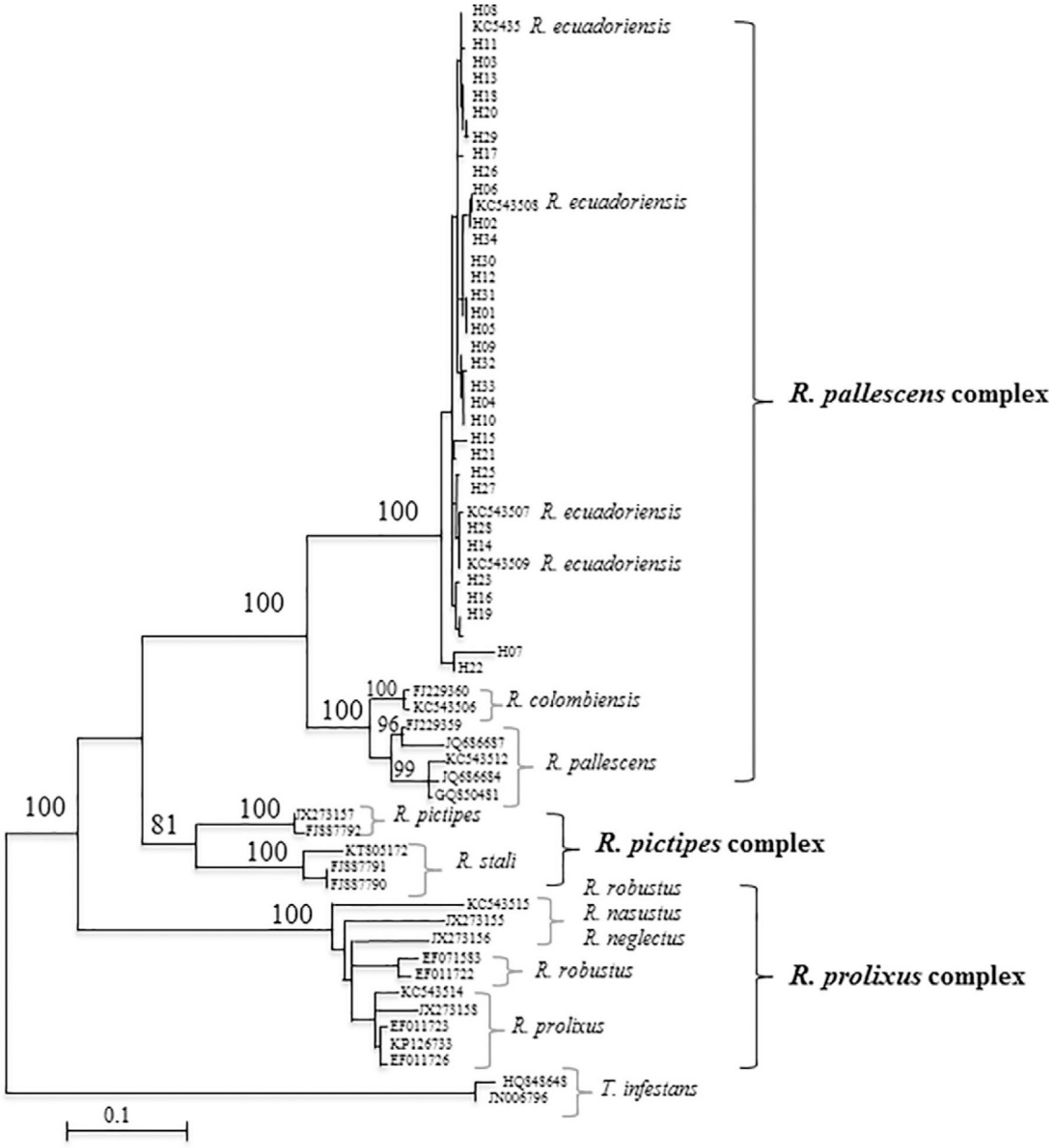

Fig. 4.

Cytochrome b gene phylogenetic analysis using maximum likelihood method. The analysis includes the current *R. ecuadoriensis* haplotypes (from Loja and Manabí provinces) and GenBank sequences of *Rhodnius* genus with their accession number. The alignment was 578 bp long and the analysis included 62 nucleotide sequences. The best model of evolution for this data set of sequences was Hasegawa-kishino-Yano with a discrete gamma distribution and the I option (HKI + G + I). The values next to the branches represent the percentage of trees in which the associated sequences clustered together (only values N 75% are shown).

Table 1

Summary of the genetic variability for two microsatellite loci in 17 populations of *R. ecuadoriensis* with different community origins in Manabí and Loja provinces.

	Manabí populations per community (code number)							**Loja populations per community (code number)**												
	MBJ	**MCA**	**MJM**	**MMB**	**MNA**	**MZP**	**All Manabí**	**LAB**	**LAH**	**LBR**	**LCG**	**LEX**	**LGL**	**LHY**	**LND**	**LSS**	**LST**	**LTR**	**All Loja**	**All Loja + Manabí**
Capture sites																				
Domicile	7	0	0	4	0	0	11	0	8	2	1	10	6	0	2	5	2	4	40	51
Peridomicile	13	6	12	12	5	16	64	4	15	5	25	4	12	13	4	22	4	5	113	177
Sylvatic	22	0	0	25	0	0	47	0	0	0	35	0	2	0	5	7	0	2	51	98
Total	42	6	12	41	5	16	122	4	23	7	61	14	20	13	11	34	6	11	204	326
Locus L13																				
N	41	6	12	41	5	16	121	4	22	7	57	13	18	13	10	34	4	11	193	314
Nall	8	4	6	7	3	3	8	2	5	2	5	5	5	3	3	4	4	5	7	8
aRich	4.44	3.57	4.29	3.70	3	2.56	8	2	3.07	1.57	2.54	3.21	3.13	2.21	1.8	2.29	4.0	3.32	6.24	7.9
F_{IS}	**0.52**[a]	0.35	0.38	0.26	0.75	0.50	**0.44**[a]	1	−0.09	0.07	**0.74**[a]	0.60	0.25	−0.13	−0.03	**0.84**[a]	0.00	0.48	**0.46**[a]	**0.48**[a]
He	0.81	0.77	0.81	0.73	0.8	0.50	0.76	0.5	0.58	0.14	0.41	0.57	0.59	0.34	0.19	0.38	0.75	0.69	0.46	0.62
Ho	0.39	0.5	0.5	0.54	0.2	0.25	0.43	0	0.64	0.14	0.14	0.23	0.44	0.38	0.2	0.06	0.75	0.36	0.25	0.32
Locus L47																				
N	41	6	12	40	5	16	120	4	23	7	56	13	20	13	10	33	6	11	196	316
Nall	6	4	6	6	5	4	6	2	2	1	4	2	3	2	1	2	3	2	4	6
aRich	3.76	3.33	4.58	3.83	4.4	3.64	6.00	2.00	1.32	1.00	2.27	1.99	1.70	2.00	1.00	1.71	2.58	1.92	3.61	6.00
F_{IS}	−0.07	0.29	0.00	0.12	−0.29	0.18	0.06	0.00	−0.02	NA	0.14	−0.71	−0.06	−0.85	NA	**0.87**[a]	−0.15	−0.25	0.06	0.18
He	0.73	0.7	0.00	0.74	0.77	0.76	0.76	0.25	0.08	0.00	0.39	0.49	0.19	0.5	0.00	0.24	0.43	0.36	0.31	0.55
Ho	0.78	0.5	0.83	0.65	1.00	0.63	0.72	0.25	0.09	0.00	0.34	0.85	0.20	0.92	0.00	0.03	0.50	0.45	0.29	0.46
Overall loci																				
F_{IS}	**0.24**[a]	0.32	0.19	0.19	0.24	0.31	**0.25**[a]	0.67	−0.09	0.00	**0.47**[a]	−0.01	0.17	−0.56	−0.03	**0.86**[a]	−0.06	0.23	**0.30**[a]	**0.34**[a]
He	0.77	0.71	0.81	0.73	0.77	0.63	0.76	0.34	0.33	0.07	0.4	0.53	0.39	0.43	0.097	0.31	0.59	0.52	0.39	0.58
Ho	0.58	0.5	0.67	0.60	0.60	0.44	0.57	0.12	0.36	0.07	0.22	0.43	0.32	0.65	0.1	0.04	0.62	0.41	0.27	0.42

N = number of individuals; Nall = number of alleles; aRich = allelic richness; He and Ho = expected (unbiaised index) and observed heterozygosity.

[a] In bold significant deviation from Hardy Weinberg expectations ($p \leq$ to adjusted nominal level); See Fig. 1 for the full name of each community.

Table 2

F_{IS}-values for two microsatellite loci in 12 subpopulations of *R. ecuadoriensis* of different habitats.

Code of subpopulation	Province origin	Community origin[a]	Habitat	Collection site	N	F_{IS}-value[b]
BJN41	Manabí	MBJ10	Sylvatic	Squirrel nest	6	0.27
JM301	Manabí	MJM	Peridomicile	Chicken nest	7	0.30
MBN6	Manabí	MMB	Sylvatic	Rat nest	6	−0.16
ZP01	Manabí	MZP	Peridomicile	Rat nest	7	0.12
ZP02	Manabí	MZP	Peridomicile	Opossum nest	7	0.51
AH202	Loja	LAH	Peridomicile	Opossum nest	12	−0.29
CG406	Loja	LCG	Peridomicile	Pigeon nest	22	0.39
CG001	Loja	LCG	Sylvatic	Squirrel nest	16	0.25
EX401	Loja	LEX	Domestic	Guinea pig livestock	6	0.14
GL108	Loja	LGL	Peridomicile	Chicken nest	10	0.12
HY306	Loja	LHY	Peridomicile	Chicken nest	6	−0.61
SS602	Loja	LSS	Peridomicile	Chicken nest	11	0.88

N = number of individuals.

[a] See Fig. 1 for the full names and localization of the communities.

[b] No value was significant.

Table 3

Hierarchical Molecular Variance Analysis (AMOVA) of microsatellite data at two loci from 12 *R. ecuadoriensis* populations of different communities collected in Loja and Manabí provinces.

Source of variation	Sum of squares	Degree of freedom	Variance components	% of variation	Fixation indices	*p*-Value
Between provinces[a]	34.70	1	0.12 Va	18.3	*FCT* = 0.18	<10–4
Among populations within provinces	16.47	10	0.02 Vb	4.4	*FSC* = 0.04	<10–3
Among individuals within populations	176.82	274	0.12 Vc	19.0	F_{IS} = 0.24	<10–4
Individuals within total population	112.00	286	0.39 Vd	59.2	FIT = 0.41	<10–4

[a]Includes eight populations from Loja (LAH,LCG,LEX,LGL,LHY,LND, LSS, and LTR) and four from Manabí (MBJ, MJM, MMB, and MPZ), see Fig. 1 for the full names of the communities.

Table 4

Hierarchical Molecular Variance Analysis (AMOVA) of microsatellite data at two loci from 12 *R. ecuadoriensis* subpopulations of different habitats collected in Manabí and Loja provinces.

Source of variation	Sum of squares	Degree of freedom	Variance components	% of variation	Fixation indices	*p*-Value
Geographical level						
Between provinces[a]	10.28	1	0.09 Va	16.10	*FCT* = 0.16	<10–4
Among subpopulations within provinces	14.21	10	0.05 Vb	7.75	*FSC* = 0.09	<10–4
Among individuals within subpopulations	55.87	102	0.09 Vc	14.79	F_{IS} = 0.19	<10–4
Individuals within total population	42.00	112	0.37 Vd	61.37	FIT = 0.39	<10–4
Ecological level (peridomicile vs sylvatic)						
Between habitats[b]	0.82	1	−0.03 Va	−5.1	*FCT* = −0.05	>0.05
Among subpopulations within habitats	22.75	9	0.10 Vb	20.08	*FSC* = 0.19	<10–4
Among individuals within subpopulations	52.5	97	0.09 Vc	16.90	F_{IS} = 0.20	<10–4
Individuals within total population	39.00	108	0.36 Vd	68.17	*FIT* = 0.32	<10–4

[a]Includes five subpopulations from Manabí and seven from Loja.

[b]Includes eight subpopulations from peridomiciles and three from sylvatic areas (the subpopulation from domestic habitat was not included); see also Table 2 for the information of the subpopulations.

Table 5

ty indices of Cyt b 578 bp gene fragment in the Rhodnius ecuadoriensis from different communities in Manabí and Loja provinces.

rs	Communities in Manabí province[a]								Communities in Loja province[a]												Overall samples
	MBJ	MCA	MJM	MMB	MNA	MZP	MLE	Overall Manabí	LAB	LAH	LBR	LCG	LEX	LGL	LHY	LND	LSS	LST	LTR	LLM	Overall Loja	
	24	2	6	30	3	7	1	73	1	16	3	28	11	8	12	2	11	3	7	1	101	174
e	578	578	578	578	578	578	578	578	578	578	578	578	578	578	578	578	578	578	578	578	578	578
	19	(−)	6	15	(−)	14	(−)	29	(−)	10	(−)	13	28	7	32	(−)	1	(−)	11	(−)	37	49
te	3	(−)	5	5	(−)	9	(−)	11	(−)	4	(−)	1	4	3	3	(−)	1	(−)	2	(−)	0	0
ve	16	(−)	1	10	(−)	5	(−)	18	(−)	6	(−)	12	24	4	29	(−)	0	(−)	9	(−)	37	46
ic	0.01016	(−)	0.00381	0.00586	(−)	0.00906	(−)	0.00821	(−)	0.00386	(−)	0.00640	0.01515	0.00426	0.02498	(−)	0.00031	(−)	0.00906	(−)	0.02001	0.01656
c	0.917	(−)	0.800	0.809	(−)	0.667	(−)	0.878	(−)	0.450	(−)	0.775	0.345	0.464	0.439	(−)	0.182	(−)	0.905	(−)	0.870	0.926
s	11	2	4	10	2	3	1	19	1	5	2	10	3	3	3	1	2	2	5	1	18	34
e n	H01							0		12		1			2	2			1	1	19	19
	H02							0		1		2					1				4	4
	H03							0				2									2	2
	H04	2		1	1	2		6	1	1	1	13	1						2		19	25
	H05							0				3						2			5	5
	H06							0						6			10		2		18	18
	H07							0					9		9						18	18
	H08	3		3	5	1		1	13					1				1			2	15
	H09							0		1		1							1		3	3
	H010							0				1									1	1

rs	Communities in Manabí province[a]								Communities in Loja province[a]													Overall samples
	MBJ	MCA	MJM	MMB	MNA	MZP	MLE	Overall Manabí	LAB	LAH	LBR	LCG	LEX	LGL	LHY	LND	LSS	LST	LTR	LLM	Overall Loja	
H011								0				2									2	2
H012								0			1										1	1
H013	4		1	12		2		19													0	19
H014	4			1				5													0	5
H015	4							4													0	4
H016	1	1		3		4		9													0	9
H017				2				2													0	2
H018				1				1													0	1
H019				3				3													0	3
H020			1					1													0	1
H021	1							1													0	1
H022						1		1													0	1
H023								1													0	1
H024				1				1													0	1
H025		1						1													0	1
H026	2							2				1									1	3
H027	1							1													0	1
H028				1				1													0	1
H029	1							1													0	1
H030								0				1		1							2	2
H031								0				1									1	1
H032								0					1								1	1
H033								0											1		1	1
H034								0		1											1	1
Total	24	2	6	30	3	7	1	73	1	16	2	28	11	8	11	2	11	3	7	1	101	174

licable.

for the full names and localization of the communities.

Table 6

Cyt b haplotype distribution of Rhodnius ecuadoriensis collected in different habitats in two communities in Manabí and one in Loja provinces.

Haplotype	Loja		Manabí					
	LCG		MBJ			MMB		
	D	S	P	D	S	P	D	S
H01	0	1						
H02	0	2						
H03	0	2						
H04	8	5		1	1			1
H05	3	0						
H08				1	2	2	1	2
H09	0	1						
H10	0	1						
H11	0	2						
H13			1		3	2	1	9
H14			2		2			1
H15			4					
H16				1	3			
H17						1		
H18								1
H19					2			1
H21			1					
H23					1			
H24								1
H26	0	1			2			
H27					1			
H28						1		
H29				1				
H30	1	0						
H31	0	1						
Total	12	16	8	4	17	6	2	16
p-Value for Fisher Exact test (D/S)	0.035							
p-Value for Fisher Exact test (P + D/S)			0.83			0.43		

Habitats from where the specimens were collected, peridomicile (P), domicile (D) and sylvatic area (S); see Fig. 1 for the localization of the communities.