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Artículo científico

Rhodnius pallescens microsatellite markers for population genetic analysis in *Rhodnius ecuadoriensis*: preliminary assessment

Marcadores de microsatélite de *Rhodnius pallescens* para análisis genéticos poblacionales en *Rhodnius ecuadoriensis*: Evaluación preliminar

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RESUMEN.- *Rhodnius ecuadoriensis* Lent & León (Hemiptera: Reduviidae) es el prinicipal vector de la enfermedad de Chagas en Ecuador, donde la estructura genética de sus poblaciones es poco conocida. Nosotros probamos seis Repeticiones Cortas en Tamdem (RCT) de *R. pallescens* Barber en poblaciones selváticas y domésticas de R. ecuadoriensis. Dos microsatelites fueron monomórficos, dos dieron resultados ambiguos y dos fueron polimórficos (16 y 19 alelos) y fueron utilizados para análisis. Los resultados de las frecuencias alélicas, AMOVA y los pruebas Bayesianas para genética favorecen la teoría de la existencia de una sola población. Estos resultados preliminares sugieren que las poblaciones selváticas y domésticas de *R. ecuadoriensis* intercambian frecuentemente migrantes. Por consiguiente el control de la Enfermedad de Chagas requiere vigilancia entomológica continua en la costa del Ecuador.

PALABRAS CLAVES: Enfermedad de Chagas, Ecuador, microsatelites, *Rhodnius*

ABSTRACT.- *Rhodnius ecuadoriensis* Lent & León (Hemiptera: Reduviidae) is a major Chagas disease (CD) vector in Ecuador, but little is known about its population genetic structure. We tested six Short Tandem Repeats (STRs) from *R. pallescens* Barber in wild and synanthropic *R. ecuadoriensis* populations. Two STRs were monomorphic, two yielded ambiguous alleles, and two were polymorphic (16 and 9 alleles) and used for analysis. Allele frequencies, AMOVA, and a Bayesian population structure test all favored a single-population hypothesis. These preliminary results suggest that local wild andsynanthropic R. ecuadoriensis populations frequently exchange migrants; long-term CD control will therefore require continuous entomological surveillance in coastal Ecuador.

KEY WORDS: *Rhodnius*, Chagas disease, Ecuador, STRs.

INTRODUCTION

Chagas Disease (CD), caused by *Trypanosoma* cruzi and transmitted via the feces of infected triatomine bugs, is endemic in western Ecuador *Rhodnius ecuadoriensis* is the main vector in the coastal region (Grijalva et al., 2010, 2012, 2014), where it occupies both artificial and sylvatic ecotopes – mainly *Phytelephas aequatorialis* palm trees and squirrel nests (Abad-Franch et al. 2001, 2005; Grijalva et al. 2012, 2014; Suárez-Dávalos et al. 2010). Quantitative phenotypic analyses suggest that wild populations are involved in the re-infestation of insecticide-treated households (Villacís et al. 2010, Grijalva et al. 2014), which is common in central coastal Ecuador (Grijalva et al. 2011; 2014). Yet, the population dynamics of this vector species remain largely unknown; in particular, powerful molecular genetics tools have never been used for the study of inter-population relationships in *R. ecuadoriensis* as they have in other important CD vectors (e.g., Fitzpatrick et al. 2008). Here we address *R. ecuadoriensis* population connectivity in central coastal Ecuador by testing the utility of Short Tandem Repeat (STR) markers developed for the closely-related species, *R. pallescens* (Harry et al. 1998, Abad-Franch et al. 2009).

MATERIAL AND METHODS

Fieldwork was conducted in 2009-2010 in the province of Manabí, where CD is endemic and insecticide-based vector control strategies perform poorly (Grijalva et al. 2011). Triatomines were manually collected in domestic-peridomestic structures and nearby sylvatic habitats in eight localities (Fig. 1).

All collections were conducted following protocols approved by the Institutional Review Boards of Catholic University of Ecuador and Ohio University. Overall, 270 specimens were studied, 150 collected in sylvatic habitats and 120 in artificial structures. Sampling included 100 individual ecotopes (1 to 16 bugs/ecotope, median=2, interquartile range 2-3), including squirrel nests (*Sciurus stramineus*), hen nests, houses, bird nests (*Campylorrynchus fasciatus* and one *Synallaxis* sp.), mouse and rat nests, guinea pig enclosures, and timber piles. Specimens included all stages except first-instar nymphs, with adults representing about 67 % of the sample and evenly distributed in relation to sex. Hence, we are confident that our sample fairly represents local population diversity.

Figure 1. Map of Manabí province indicating the location of the sampled communities. Insert shows a map of Ecuador with the location of Manabí.

DNA was isolated and purified using DNeasy® extraction kits (Qiagen) following the manufacturer's protocol for animal tissue. Microsatellite amplification followed a protocol modified from Harry et al. (1998), using primers designed for six loci (L3, L9, L13, L25, L43, L47) of *R. pallescens*, a species phylogenetically close to *R. ecuadoriensis* (Abad-Franch et al. 2009). For PCR, we used the GoTaq® Colorless Master Mix kit (Promega), adding 6.4 µl of $MgCl_2$ 25mM, 0.4pM of each fluorochrome-labeled primer, and 10ng/µl of DNA for a final reaction volume of 25µl. PCR was carried out in a PTC-100® thermal cycler (MJ Research), with a denaturation step (95 ºC for 5 min) followed by 40 cycles of denaturation (95 ºC, 30 sec), annealing (50 ºC, 30 sec) and extension (72 ºC, 30 sec), and a final extension step (72 ºC, 5 min). For loci L13 and L43, a nested PCR was carried out using the product of the first PCR asthe template and the protocol above – except that no additional $MgCl_2$ was used.

We first screened the full microsatellite panel with 30 randomly selected bug samples to check for polymorphism over all loci. This scan revealed that two loci were monomorphic (L9 and L25) and two (L3 and L43) yielded ambiguous allele signatures that precluded confident scoring. These four loci were therefore disregarded in subsequent analyses, and only the two polymorphic markers yielding unambiguous alleles (L13 and L47) were used to analyze the full sample of triatomines. Genotyping was performed by capillary electrophoresis in an ABI 313xl with a standard G500 LIZ ladder (Applied Biosystems); .fsa files from capillary runs were then analyzed with Peak Scanner™ 1.0 for manual allele scoring. A total of 93 samples yielded no PCR product; results below are therefore based on 177 bugs, 82 from sylvatic and 95 from artificial ecotopes. Different analyses were conducted in Excel® spreadsheets, Arlequin 3.1 (Excoffier et al. 2005), and STRUCTURE 2.2 (Pritchard et al. 2000).

RESULTS

Overall, 16 alleles were identified for L13 and nine for L43; in both cases, allele frequencies were similar in wild and synanthropic populations, with only one L47 allele found at higher frequency among wild bugs (Fig. 2 and Table).

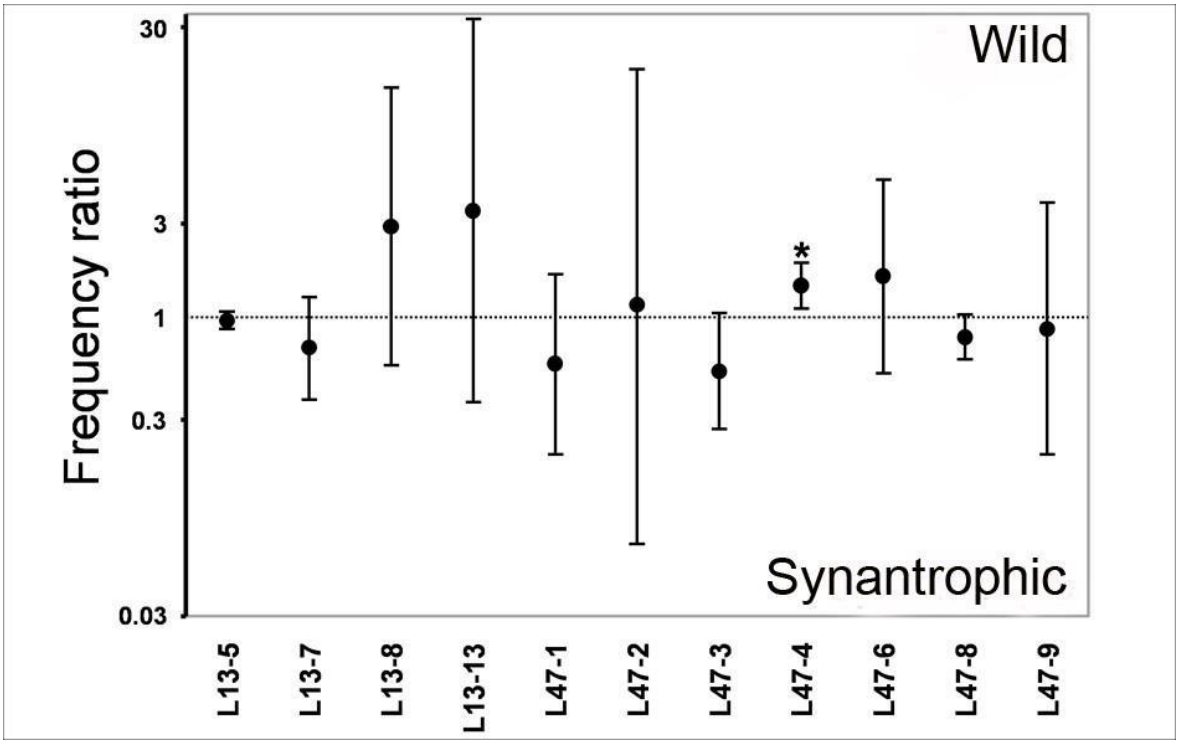

Figure 2. Allele frequency *(f)* ratios, estimated as *f*(wild)/f(synanthropic), for 11 shared alleles on two microsatellite loci (L13 and L47) in *Rhodnius ecuadoriensis* populations. Point estimates (solid circles) and 95% confidence intervals (CIs, between short horizontal lines) are shown, with allele codes given on the x-axis. The asterisk indicates the only allele (L47-4) for which we found evidence of a higher frequency in one of the populations (wild); for the rest, the fact that CIs include 1 (horizontal dotted line) indicates that allele frequencies are not significantly different at the 5% level.

Twelve rare L13 alleles, each with an overall frequency < 0.012, were only found in either wild (six alleles) or synanthropic bugs (six alleles); this was the case for just two L47 alleles, found only in wild specimens (Table). A total number of 70 synanthropic (73, 68 %) and 48 wild (58, 54 %) individuals were homozygotic for the L13 locus, with an overall observed heterozygosity HO = 33,33 %. Homozygote figures for L47 were 71, 58 % (68/95) for synanthropic and 70,73 % (58/82) for wild populations; overall observed heterozygosity was therefore HO = 28,81 %. L47 allele frequencies departed significantly from expectations under Hardy-Weinberg equilibrium among both synanthropic (expected heterozygosity HE = 72,41 %, vs. HO = 28,42 %, p < 0,0001) and wild bugs (HE = 65,89 %, vs. HO = 29,27 %, p < 0,0001), with exact p-values (Guo and Thompson 1992) estimated via a Markov chain of 100 000 steps (1 000 discarded as burn-in) implemented in Arlequin 3.1. This heterozygote deficit is likely due to allelic dropout caused by poor primer specificity, although selection acting on genes located in the same chromosome region as L47 or random drift might also contribute to this finding; our preliminary dataset does not allow for a detailed appraisal of these alternatives.

Gene diversity was slightly higher in the wild (0,799 ± 0,023) than in the synanthropic population (0,783 ± 0,021). An analysis of molecular variance (AMOVA) suggested that an overwhelming proportion of genetic variation (98,7 %) lies among individuals within populations. Both AMOVA and an exact test of population differentiation suggest that allele frequencies in both populations are

Table 1. Allele frequencies of two microsatellite loci (L13 and L47) in wild and synanthropic populations of *Rhodnius ecuadoriensis* from coastal Ecuador

Locus-allele	Wild (*N*=82)		Synanthropic (*N*=95)		Overall
	Count	Frequency	Count	Frequency	
L13-1	1	0.0061	0	0	0.0028
L13-2	2	0.0122	0	0	0.0056
L13-3	0	0	2	0.0105	0.0056
L13-4	0	0	1	0.0053	0.0028
L13-5	128	0.7805	154	0.8105	0.7966
L13-6	0	0	2	0.0105	0.0056
L13-7	15	0.0915	25	0.1316	0.1130
L13-8	5	0.0305	2	0.0105	0.0198
L13-9	0	0	1	0.0053	0.0028
L13-10	2	0.0122	0	0	0.0056
L13-11	4	0.0244	0	0	0.0113
L13-12	0	0	1	0.0053	0.0028
L13-13	3	0.0183	1	0.0053	0.0113
L13-14	1	0.0061	0	0	0.0028
L13-15	3	0.0183	0	0	0.0085
L13-16	0	0	1	0.0053	0.0028
L47-1	5	0.0305	10	0.0526	0.0424
L47-2	1	0.0061	1	0.0053	0.0056
L47-3	11	0.0671	24	0.1263	0.0989
L47-4	75	0.4573	60	0.3158	0.3814
L47-5	2	0.0122	0	0	0.0056
L47-6	7	0.0427	5	0.0263	0.0339
L47-7	1	0.0061	0	0	0.0028
L47-8	59	0.3598	86	0.4526	0.4096
L47-9	3	0.0183	4	0.0211	0.0198

comparable (F_{ST} = 0,013, exact p = 0, 09); an F_{ST}-based estimate of between-population migration suggests that about 20 reproductive individuals are exchanged per generation.

Taken together, these results all suggest that both wild and synanthropic populations belong to a single meta-population occurring across the study region. To further test this possibility, we conducted a Bayesian test of population subdivision using STRUCTURE 2.2. The analysis estimates and compares the likelihood of the data over a set of possible numbers of subdivisions (K); we run 20 iterations for each K value between 1 and 9, without a *priori* assignment of individuals to putative clusters. ΔK was then calculated following Evanno et al. (2005): the most likely number of subdivisions corresponds to the K value that maximizes ΔK. In our case, as shown in Fig. 3, there was strong support for the existence of a single genetic cluster (K=1).

DISCUSSION

We have presented the first population-level analysis of microsatellite loci in *R. ecuadoriensis*, a locally important CD vector that frequently re-invades and re-colonizes insecticide-treated households. The present study is a first step towards more detailed appraisals, which should include further polymorphic loci and the design of species-specific primer pairs (e.g., Fitzpatrick et al. 2008). The development of specific markers for R. ecuadoriensis is needed to improve our understanding of the vector's population dynamics, especially as wild populations seem to play a key role in CD transmission (Grijalva et al. 2014).

Even if still preliminary, the results of our analyses are suggestive of a single meta-population scenario in which *R. ecuadoriensis* migrants frequently move between sylvatic and artificial ecotopes – at a frequency high enough to homogenize both gene

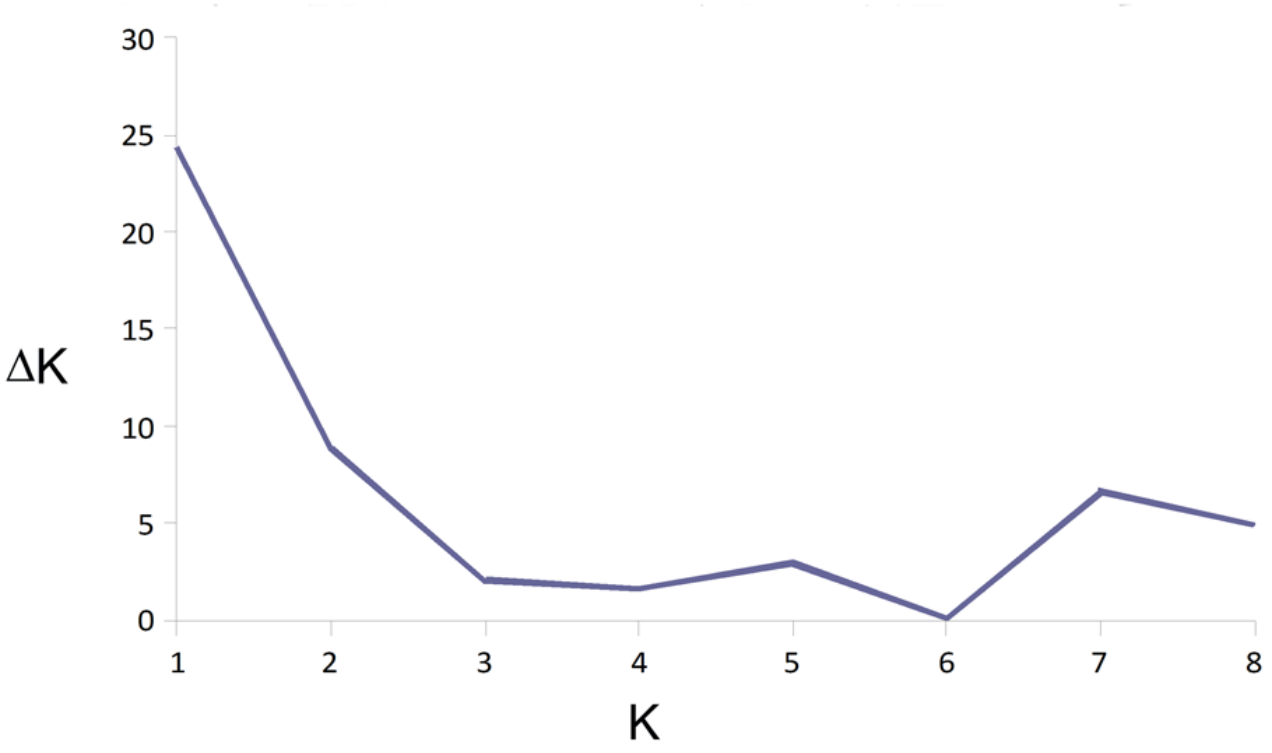

Figure 3. Estimation of the number of genetic subdivisions in *Rhodnius ecuadoriensis* populations from central western Ecuador. *K*, putative number of subdivisions; Δ*K*, Bayesian statistic derived from the rate of change in the log-probability of the data, given each *K* value. The peak value of Δ*K* at *K*=1 suggests that all specimens probably belong to a single meta-population.

pools. This agrees with ecological and quantitative phenotypic assessments showing both frequent re-infestation of treated households (Grijalva et al., 2011, 2014) and a lack of morphological or morphometric differentiation of wild and synanthropic specimens (Villacís et al. 2010).

In line with previous findings (Grijalva et al. 2011), our data thus suggest that longitudinal surveillance will be a key requirement of long-term CD control in central coastal Ecuador. Entomological surveillance usually performs better when the community takes on an active role in reporting infestation (Abad-Franch et al. 2011); however, a timely, professional response of vector control services is also needed, and this should include not only insecticide application but also environmental management of peridomestic ecotopes prone to harbor R. ecuadoriensis breeding colonies – particularlypalm trees and chicken coops in our study setting (Abad-Franch et al. 2005, Grijalva et al. 2011).

CONCLUSIONS

- Only two of six microsatellites developed for *R. pallescens* showed polymorphic loci in *R. ecuadoriensis* populations.

- Results of our analyses are suggesting only one genetic cluster is present in populations of *R. ecuadoriensis* in the province of Manabi, where migrants appear to move between sylvatic and artificial ecotopes.

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