

Short communication

Microsatellite markers from the Chagas disease vector, *Rhodnius prolixus* (Hemiptera, Reduviidae), and their applicability to *Rhodnius* species

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Received 2 November 2007; received in revised form 12 January 2008; accepted 15 January 2008

Available online 19 January 2008

Abstract

Ten microsatellites were isolated and characterized from a partial genomic library of *Rhodnius prolixus*, the principal Chagas disease vector in Venezuela, Colombia and Central America. These polymorphic molecular markers could be particularly useful in Chagas disease control initiatives. A wider applicability of the primer-pairs isolated was shown, from 6 to 10 loci being amplifiable in five out of the ten *Rhodnius* species tested, namely *R. domesticus*, *R. nasutus*, *R. neglectus*, *R. neivai* and *R. robustus*. Interestingly, all the loci were amplified in the latter. These markers may be of interest to trace the colonization of human dwellings from triatomine sylvatic populations in order to better define epidemiological risk patterns.

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Keywords: *Rhodnius prolixus*; *Rhodnius robustus*; Microsatellites; Cross-species amplification; Chagas disease

1. Introduction

The *Rhodnius* genus (Hemiptera, Reduviidae, Triatominae), distributed from Central America to Argentina, comprise 16 species (Galvao et al., 2003), all vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease. It is the most important parasitic disease in Latin America in terms of its impact on national economies and public health systems. Serological data indicate that well over 12 million people are infected with 90 million people living at risk and 45,000 deaths per year (Miles et al., 2003). Due to the lack of vaccines, chemoprophylaxis or

therapy suitable for large-scale use, the interruption of the epidemiological cycle relies upon vector control. Among *Rhodnius* species, *R. prolixus* is the primary target of vector control initiatives in Venezuela and Colombia where it is the principal vector of *T. cruzi*. In Central America, following the interventions of the Central American Initiative against Chagas disease, *R. prolixus* rather is reduced to isolated populations and is slated for eradication (Ponce, 2007).

Effective control requires a good understanding of the epidemiological cycles with a global ecological approach, including a reliable analysis of the genetic structure of vector populations and estimation of their dispersal abilities using appropriate polymorphic markers such as microsatellites (Harry et al., 2000). In *Rhodnius* species, such markers are available for *R. pallescens* (Harry et al., 1998), but were still lacking for *R. prolixus*. Recently, microsatellite loci have been described for two species of *Triatoma* genus, namely *T.*

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dimidiata (Anderson et al., 2002) and *T. infestans* (Garcia et al., 2004; Marcet et al., 2006), vectors of Chagas disease in southern countries of Latin America.

2. Materials and methods

For construction of the microsatellite library, DNA was extracted from *R. prolixus* from the University of Antioquia strain, Colombia. The taxonomic status of the specimens was confirmed by performing Blast procedure using *cytb* sequences. *cytb* sequences of our specimens (EU363822) matched unequivocally with *R. prolixus cytb* sequences previously deposited in GenBank (e.g. highest match (98%) with EF011726.1, *E*-value = 0.00, 403 nucleotides). In order to avoid misidentification of specimens, the samples used for cross-amplification studies were obtained from the reference strains of the Laboratory of Entomology of the Oswaldo Cruz Institution (FIOCRUZ, Brasil) except *R. pallescens* which came from the University of Antioquia strains, Colombia (Table 1). Moreover, both *R. robustus* and *R. pallescens* samples were completed by wild specimens collected in French Guyana and in a natural palm tree forest in San Onofre, Colombia (Romaña et al., 1999), respectively. The taxonomic status of *R. robustus* (EU363824) and *R. pallescens* (EU363823) wild specimens were also confirmed by performing Blast procedures using *cytb* sequences (e.g. highest match (98%) with AF42134.1, *E*-value = 0.00, 417 nucleotides for *R. robustus* and highest match (98%) with EF071584.1, *E*-value = 0.00, 435 nucleotides, for *R. pallescens*).

Protocols for DNA extraction and genomic library construction followed Estoup et al. (1993). After DNA extraction from *R. prolixus* (University of Antioquia strain, Colombia) and digestion with *Sau3A* (Eurogentec, Liège, Belgium), restriction fragments were cloned in pUC18 vector (Pharmacia). The screening procedure was performed with a mix of six DIG labelled oligonucleotide probes (Boehringer): (TG)₁₀, (TC)₁₀, (CAC)₅CA, CT(CCT)₅, CT(ATCT)₆ and (TGTA)₆TG. Isolated clones were sequenced using dye

terminator chemistry (DIG Nucleic Acid Detection Kit, Boehringer).

PCR were performed using a Biometra thermal cycler, with 30 cycles (denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 45 s). Annealing temperatures and Mg²⁺ concentration are given in Table 2 according to the studied loci. The reaction solution (10 µL) contained 1 µL PCR buffer (50 mM KCl, 0.1% Triton X-100, 10 mM Tris–HCl, pH 9.0), 0.44 µL of GTP, CTP, TTP (1.66 mM), 1.2 µL of ATP (50 mM), 1 µL of alpha-33P-ATP (1 mCi/100 µl), 0.2 µL (10 mg/mL) BSA, 0.4 µL of forward and reverse primers (10 µM), 0.4 units of *Taq* polymerase (Boehringer), and about 10 ng of template DNA.

3. Results

The screening procedure was performed on about 6000 clones. Over the 56 positive clones isolated, 40 were sequenced and 10 pairs of primers were designated. All the loci were polymorphic in *R. prolixus*, the number of alleles per locus ranging from 2 to 5.

With this microsatellite set, we performed a cross-priming study in 10 *Rhodnius* species. Two sets of species could be distinguished (Table 2): (1) *R. nasutus*, *R. neglectus*, and *R. robustus*, that are species of the *R. prolixus* complex plus *R. neivai* and *R. domesticus* in which 6–10 loci were amplifiable and (2) *R. pallescens*, *R. ecuadoriensis*, *R. pictipes*, and *R. stali*, with 1–2 amplifiable loci. In *R. brethesi* none of the loci gave reliable PCR products.

4. Discussion

The *R. prolixus* genome showed a low density of microsatellites and, consequently, an extensive work of screening was performed to finally obtain less than 1% of positive clones (0.93%). Comparatively, using the same method, microsatellites density reached 2.3% of positive clones in *R. pallescens*, a Chagas disease vector (Harry et al., 1998) as well as 3.5% and only 0.83% for honey bee and bumble-bee, respectively in Hymenoptera (Estoup et al., 1993).

At the time of this study, only laboratory strains of *R. prolixus* provided by the Unidad de Ecoepidemiología y control biológico (Universidad de Antioquia, Colombia) were available, a constraint for the evaluation of the level of polymorphism that could be revealed using these microsatellite markers. Actually, the genetic variability is generally lower for strains than for natural populations. This might be due to diverse causes, such as genetic drift resulting from a drastic decrease of the effective size (i.e. a bottleneck) or inbreeding resulting from artificial selection (Hartl and Clark, 1988). However, all loci were polymorphic. Thus, even tested on laboratory strains, isolated microsatellites seem good candidates for fine-scale population genetic studies on natural populations in which a higher polymorphism is expected. Indeed, these polymorphic molecular markers which are neutral, codominant and Mendelian, could be extremely useful in Chagas disease control initiatives, allowing to identify and

Table 1
Origin of the samples used in the study

Species	Origin	<i>N</i>
<i>R. prolixus</i> complex		
<i>R. nasutus</i>	Piauí, Brazil (Fiocruz)	4
<i>R. neglectus</i>	Sylvatic, Município Formoso, Goiás, Brazil (Fiocruz)	3
<i>R. prolixus</i>	Venezuela (University of Antioquia, Colombia)	5
<i>R. robustus</i>	Para, Brazil (Fiocruz)	3
	French Guyana (Field collected)	2
Other species		
<i>R. brethesi</i>	Amazona, Brazil (Fiocruz)	2
<i>R. domesticus</i>	UFSC Santa Catalina, Brazil (Fiocruz)	3
<i>R. ecuadoriensis</i>	Lourdes, El Oro, Ecuador (Fiocruz)	4
<i>R. neivai</i>	Maracay, Venezuela (Fiocruz)	2
<i>R. pallescens</i>	Vegachi, Antioquia, Colombia (Fiocruz)	4
	San Onofre Sucre, Colombia (Field collected)	3
<i>R. pictipes</i>	Para, Brazil (Fiocruz)	3
<i>R. stali</i>	Alto Beni, Bolivia (Fiocruz)	4

Table 2

Primers and characteristics of the microsatellites isolated from *Rhodnius prolixus* and results of cross-species amplification performed in 10 *Rhodnius* species

Locus GenBank Accession	Primers (5'–3') <i>R. prolixus</i>	Cloned allele size (bp) (size range)	Repeat array	T_m (°C)	PCR [MgCl ₂]	<i>R. pro</i> <i>N</i> = 5	<i>R. nas</i> <i>N</i> = 4	<i>R. neg</i> <i>N</i> = 3	<i>R. rob</i> <i>N</i> = 5	<i>R. bre</i> <i>N</i> = 2	<i>R. dom</i> <i>N</i> = 3	<i>R. ecua</i> <i>N</i> = 4	<i>R. nei</i> <i>N</i> = 2	<i>R. pal</i> <i>N</i> = 4	<i>R. pic</i> <i>N</i> = 3	<i>R. stal</i> <i>N</i> = 4
R4 DQ364967	F: AAGTGGTAAAAATGAAAATATTCC R: CCGGTAAGACGCAGAGTAC	258 (252–258)	(GT) ₂ T ₂ (GT) ₁₇	50	1.5	+	+	+	+	–	+	–	+	–	–	–
R8 DQ364968	F: ATGGCAACTTTAATTTCAAGTATTC R: TCTGACGAAACGCCACTG	203 (203–205)	(GT) ₈	50	1.5	+	+	+	+	–	+	+	+	+	–	–
R11 DQ364969	F: CTCCAAGCATCCAGCTTCTC R: CAATGACCACCTGGTCACG	228 (222–230)	(GA) ₁₅	48	1.5	+	+	+	+	–	–	–	+	–	–	–
R13 DQ364970	F: TACATCTTCAATAATCATCACACAC R: AAGTAAATGAATGAATGCCC	192 (190–192)	(AC) ₅ GC(AC) ₁	48	1.5	+	+	–	+	–	+	–	+	–	–	–
R17 DQ364971	F: TCCAATGTGTTAAAAATGGCAGTTCTG R: ACGCGCTGGTTGCTCGG	232 (230–234)	(CT) ₃ (CA) ₁₀	48	1.5	+	–	–	+	–	+	+	+	+	–	–
R26 DQ364972	F: AGAAGGAATCTATCCACTTTTCGC R: CCTCGCTATCAGCTGCTACG	120 (112–120)	(CA) ₃ CT(CA) ₂	54	1.2	+	–	+	+	–	+	–	+	+	+	+
R29 DQ364973	F: ATCAAAGCCAAACGCTGTTG R: GCAAATACCTGCGCTATTTTCTC	187 (183–199)	(GT) ₂ GC(GT) ₈	54	1.5	+	+	–	+	–	–	–	–	–	–	–
R30 DQ364974	F: GATCCAGGCAGTTTTCTTAAGTG R: CAATGGAACAAGAATTTAGTGAGG	220 (218–220)	(GT) ₈	54	1.2	+	+	+	+	–	–	–	+	–	–	–
R31 DQ364975	F: TGTGGTAAGTCTGTGTAGAAGG R: TCTGTTGGTCCAGACACGG	131 (129–133)	(GT) ₁₁	52	1.5	+	+	+	+	–	+	–	–	–	+	–
R32 DQ364976	F: TGAGCTTATCCACTGGCTTCAG R: CAGGAGTGGGCCATTTC	226 (224–226)	(GT) ₁₀	52	1.5	+	–	–	+	–	+	–	+	–	–	–
<i>N</i> loci						10	7	6	10	0	7	2	8	3	2	1

R. rob: *R. robustus*, *R. nas*: *R. nasutus*, *R. neg*: *R. neglectus*, *R. bre*: *R. brethesi*, *R. dom*: *R. domesticus*, *R. ecua*: *R. ecuadoriensis*, *R. nei*: *R. neivai*, *R. pal*: *R. pallescens*, *R. pic*: *R. pictipes*, *R. sta*: *R. stali* with the loci isolated from *R. prolixus* (*R. pro*). *N* is the sample size (see Table 1 for insect origin). The clone allele size and size range of amplified microsatellites is given for *R. prolixus*. *N* loci is the total of loci that amplified for all the individuals tested.

characterize *R. prolixus* populations that persistently re-infest insecticide-treated households.

In Venezuela, two vectors could compromise Chagas' disease eradication; first, *R. prolixus* by the reinvasion of the houses from sylvatic populations (Feliciangeli et al., 2007) and second, *R. robustus* attracted by artificial light that could have a putative role as extradomestic vector (Feliciangeli et al., 2002). Effectively, most of the *Rhodnius* species have sylvatic habits, often living in palm tree crowns and bird nests and have a large geographic distribution (e.g. *R. prolixus*, *R. pictipes*, *R. robustus*, *R. stali*). However, the precise geographic distribution of some *Rhodnius* species remains unclear due to difficulties in their identification because of their close morphological similarity. There is evidence of such confusion for the four species *R. prolixus*, *R. robustus*, *R. neglectus* and *R. nasutus*, which were grouped into the *prolixus* complex and more especially for the two species *R. prolixus* and *R. robustus*. Isoenzymes data (Harry et al., 1993; Solano et al., 1996; Monteiro et al., 2002) confirmed the lack of discrimination between the *R. prolixus*–*R. robustus* species pair but recently Pavan and Monteiro (2007) have separated *R. prolixus* from *R. robustus* using a multiplex PCR assay. However, studies based on mitochondrial *cytb* gene and haplotype comparisons suggest that *R. robustus* represents a species complex (Monteiro et al., 2003). The identification of these two vectors is of importance since *R. robustus* could be responsible for Chagas disease transmission in western Venezuela (Feliciangeli et al., 2002). It is noteworthy that for *R. robustus*, all the microsatellites isolated from *R. prolixus* were here successfully amplified.

Moreover, tests for cross-species amplification showed a wider applicability of the primer-pairs isolated from *R. prolixus*, reliable population genetics could be performed in four additional *Rhodnius* species (i.e. *R. nasutus*, *R. neglectus*, *R. neivai*, *R. domesticus*) for which a minimum of five loci were amplifiable. Furthermore, investigating the conservation of primer sequence, cross-species-amplification tests could be informative about the species taxonomical proximity. However, relatedness study should be achieved with caution when amplifying microsatellites in species other than ones from which they were isolated because of the possible occurrence of null alleles. Nevertheless, the results of cross-species amplification support the taxonomical proximity of some species especially between the *prolixus* complex species, as previously deduced, for example, from quantitative morphometry (Harry, 1993a, 1994) antennal sensilla patterns (Catala and Schofield, 1994), isoenzymes (Harry, 1993b; Chavez et al., 1999) and recently from mitochondrial and nuclear sequences (Monteiro et al., 2000, 2003; Lyman et al., 1999).

Appropriate genetic markers are now available to assess how gene flows occur between bug populations and especially to trace the colonization of human dwellings from triatomine sylvatic populations in order to better define epidemiological risk patterns. But, only the understanding of the correlation between ecological genetics and habitats characteristics of triatomines will enable to predict the migration pathways of the vectors (Harry et al., 2000). The isolation of microsatellite markers for the main vector of Chagas disease in north Latin

America, and applicable in six other related *Rhodnius* species constitute a major step in the global strategy of vector control.

Acknowledgements

The authors thank J. Calle (Universidad de Antioquia, Colombia) for *Rhodnius prolixus* samples and J.-M. Beranger for Guyanese *R. robustus* samples. We thank L. Dupont for the reading of the manuscript.

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