

Identification and characterization of microsatellite markers in the Chagas disease vector *Triatoma infestans* (Heteroptera: Reduviidae)

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Abstract

Triatoma infestans, the main vector of Chagas disease in the southern cone countries, is the principal target of a regional elimination program. A better understanding of its dispersal, sources of reinfestation, and insecticide resistance is key to an effective control program. To address such problems, we identified and characterized 13 microsatellite loci of *T. infestans*. For each locus, primer sequences and PCR conditions are presented. Allele variability and frequency were analyzed in 59 *T. infestans* specimens from different rural communities in northwestern Argentina; nine loci were considered suitable for population genetic studies. Departure from Hardy–Weinberg equilibrium was detected in 10/13 loci with F_{IS} values ranging from 0.04 to 0.91, indicating heterozygote deficit and a possible grade of sub-structure in the sample analyzed. Presence of null alleles in some loci cannot be discarded. The present work provides a promising tool to develop a population genetic study of natural populations of *T. infestans* in tandem with field studies and analyses of bug dispersal and the reinfestation process. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Chagas disease (American Trypanosomiasis), caused by the protozoan *Trypanosoma cruzi* and transmitted by triatomine bugs, is one of the most important vector-borne diseases in Latin America, with an estimated 16–18 million people infected and 50,000 deaths annually (WHO, 2002). *Triatoma infestans* is considered the main vector of Chagas disease because of the number of *T. cruzi* infections attributed to it, its wide geographic distribution and its almost exclusive domestic or peridomestic habitat (Schofield, 1985). Consequently, since 1991, it has been the target

of a regional elimination program, the Southern Cone Initiative, which relies principally on residual spraying with pyrethroid insecticides (Schmunis et al., 1996). So far, the program has shown great success in Brazil, Uruguay and Chile, as well as in parts of Argentina, Bolivia and Paraguay (TDR, 2000).

Unfortunately, early reinfestation of human dwellings after blanket residual spraying of pyrethroids has been frequently observed regardless of the triatomine species, insecticide or setting involved (Cecere et al., 2002; Gürtler et al., 2004). In northwestern Argentina, in the absence of effective surveillance, sustained control actions and socio-economic or environmental improvements, domestic populations of *T. infestans* increased exponentially to return to baseline levels 3–5 years after spraying (Gürtler et al., 1994).

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The reestablishment of bug populations following a blanket application of insecticide may be attributed to persistence of residual insect foci or immigration of bugs. Persistence of residual populations may be explained by a failure in exposing all bugs to the insecticide, or after several sprayings, development of insecticide resistance, while immigration may be due to incidental human transport or active dispersal of the insect. Incomplete elimination of *T. infestans* following a single application of pyrethroid insecticides was considered the most likely source of reinfestation (Cecere et al., 1997; Dujardin et al., 1996; Gürtler et al., 2004). The peridomicile, where most failures in control and detection may occur, is usually the first area to be recolonized and contains the greatest abundance of *T. infestans* 1 year post-spraying (Cecere et al., 2002). Moreover, as discussed by Cecere et al. (2004), a single peridomestic structure hosting a residual focus could be the origin of reinfestation by flight-dispersing bugs to sites located within a 450 m radius. However, there is no definitive evidence to support this hypothesis.

The origin of post-spray triatomine populations and other issues, such as definition of population boundaries, geographic distribution, dispersal and gene flow, can be addressed through triatomine population studies on a site level. For these types of studies, markers with high resolution are needed. Previously available isoenzyme markers described for *T. infestans* (Dujardin and Tibayrenc, 1985) are not polymorphic enough for fine-scale population studies that require detecting differences among recently diverged populations within a species. Wing geometric morphometric studies of *T. infestans* described a spatial structuring pattern (Schachter-Broide et al., 2004), but could not provide definitive explanations for the structuring origin. Analyses of mitochondrial DNA sequences, microsatellites and RAPDs in various species of insect disease vectors and parasites have proven effective (e.g., Monteiro et al., 1999; Borges et al., 2000; Blackston et al., 2001; Lehmann et al., 2003a, 2003b).

Microsatellite loci have been previously described for triatomine *Rhodnius pallescens* (Harry et al., 1988), *Triatoma dimidiata* (Anderson et al., 2002) and very recently for *T. infestans* (García et al., 2004), but thus far, no works applying or testing those markers for population genetics studies have been published. The present work not only describes but also tests the suitability of new microsatellite loci for *T. infestans* for population genetic studies as part of a comprehensive study on the eco-epidemiology and control of Chagas disease in northern Argentina.

2. Materials and methods

2.1. Insect origins

For construction of a microsatellite library, DNA was extracted from eggs (second generation in laboratory) from a *T. infestans* colony from the Department of El Patiño,

Formosa province, Argentina, currently maintained at the Center of Vector Reference, Punilla, Argentina. For preliminary tests with microsatellite primers, DNA was extracted from fresh insect legs from a colony from Quebrada de Cautana, San Luis, Argentina. For analysis of allele variability, DNA was obtained from the legs of 59 insects captured in October 2002 in Amamá (27°12'33''S, 63°02'10''W) and 15 surrounding communities (8–20 km apart) in the Department of Moreno, Santiago del Estero province, Argentina, and preserved in 70% ethanol. Each specimen was collected from a different house.

2.2. DNA extraction

All genomic DNA extractions were performed using the Promega Wizard Genomic DNA extraction kit following the manufacturer recommendations (Promega, Madison, Wisconsin). Before extraction, legs preserved in ethanol were dried over an absorbent paper for 30 min and then in a speed-vacuum centrifuge for 15 min. DNA used in library construction was further purified with phenol/chloroform/isopropanol.

2.3. Library construction

The purified DNA was sent to the Savannah River Ecology Laboratory for microsatellite enrichment using a hybridization capture protocol based on that of Hamilton et al. (1999). In brief, the DNA was digested with *RsaI* and dephosphorylated with shrimp alkaline phosphatase, linked with the SuperSNX linker. This DNA was mixed with complementary biotinylated oligonucleotides ((TG)₁₂, (AG)₁₂, (AAG)₈, (ATC)₈, (AAC)₈, (AAT)₁₂ and (ACT)₁₂) and separated with Dynabeads. The products eluted from beads were PCR-amplified using the SuperSNX-24 primer (GTTTAAGGCCTAGCTAGCAGAATC) and TA cloned into pGEM T-easy vector (Promega). JM109 cells were transformed with the ligated products. White colonies were picked with a toothpick, touched to an agar plate and swirled in 25 µl TE. These crude DNA preparations were amplified using M13 forward and reverse primers (in a 30 µl total reaction volume of 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, pH 9, 3.1 mM MgCl₂, 200 µM each dNTP, 10 pmol/µl of each primer, 0.525 U *Taq*, and 1–2 µl of TE prep DNA) under the following PCR conditions: 5 min at 95 °C, 35 cycles of: 95 °C for 30 s, 47 °C for 30 s, 72 °C for 1 min, and extension for 10 min at 72 °C. PCR products of 150 base pair (bp) or more were sequenced on an ABI 3100 (Applied Biosystems).

2.4. Primers design

Primers were designed for sequences flanking of the microsatellites (considered five or more repeats of 2 bp) using Primer 3 software (Rozen and Skaletsky, 1998). The resulting PCR products were expected to be no longer than

Table 1

Description of loci and amplification conditions of 13 microsatellite loci in *Triatoma infestans*

Locus/GenBank accession no.	Microsatellite array	Primer sequence (5'–3')	TA (°C)	
Tinfest_ms3/AY742670	(GT) ₁₀ G (GT) ₂ GC (GT) ₂ GG (GT) ₂	F	f : GCG GAC TGA GAA AGG AAC AC	58
		R	TTC ACC GCT CGT CTA CAC AC	
Tinfest_ms5/AY742671	(CA) ₂₅	F	f : ATG CTG ATA GTC GCA ACA CG	58
		R	TCG ATC TTT TTC CCA AAT CG	
Tinfest_ms21/AY742672	(TG) ₂ TT (TG) ₁₄ GGG (GT) ₃	F	h : CGC TTT CAA CAC AAC AGG AG	59.5
		R	TTT GTT TAA TTA CAA AAT CCA TAC C	
Tinfest_ms22/AY742673	(TG) ₉	F	f : CGA GTC AAA TTT TCC ATG AGG	58
		R	CCC ATG GTG TTA CCC AAA AC	
Tinfest_ms23/AY742674	(CA) ₅ CC (CA) ₅ AA (CA) ₂	F	h : CTC TTG CTG GTT GTG CAC TG	58
		R	GTA AAC GCC ATC CTC ACA CC	
Tinfest_ms27/AY742675	(CT) ₁₃	F	h : ATG AAG CCG AAA CCA CAA AG	58
		R	GGG GAA GAG AAT GCA TTG AG	
Tinfest_ms42/AY742683	(CA) ₁₆	F	h : GAC GCT CCA GCT ATC GAT TC	58
		R	GGC CAA TTG GTT TGG TAG TG	
Tinfest_ms45/AY742676	(AC) ₁₂	F	f : ACT TTG GAG CCG TTT TTG TG	63.5
		R	CAC ATA ATG TTG CGC ACC TC	
Tinfest_ms55/AY742677	(GT) ₁₃	F	f : GAC TGA GCC ACA GCG AAG	56.5
		R	CCA CCT AGA CTC AAA AAG C	
Tinfest_ms56/AY742678	(CA) ₈ T(AC) ₃	F	f : TTC TTA TTA ACG TAA TCT C	46.3
		R	CGT GTG GTA TAT CTC TC	
Tinfest_ms64/AY742679	(CT) ₆ (GC) ₄ CT (CA) ₁₃	F	f : CTA CAG AAC AAT TCC CAC	51.8
		R	GCA GGT AAG CTG GGA G	
Tinfest_ms65/AY742680	(GA) ₁₇	F	f : GCG ATG CGA TTA CAG AGG	56.5
		R	GCT CTG TTC CTA GCT TAC C	
Tinfest_ms68/AY742681	(AC) ₅ C (CA) ₆	F	f : GAT TCA TTG TGG CAG GTC	56
		R	GCA ATG AAG GAA GAG GAG	

TA: annealing temperature in °C; F primer of each set was 5' labeled, **f**: 6-fam; **h**: hex, as indicated in bold.

400 bp to reduce the occurrence of mutations outside of the actual microsatellite. Moreover, a minimum length of 20–22 bp and a G-C base at the 3' terminal was required to assure primer stability. Flanking sequences of different clones were compared to avoid repetition of the same clones, using the Pileup program from Genetics Computer Group (Wisconsin Package Version 10.3, Accelrys Inc., Wisconsin).

2.5. Amplification

Each primer set was tested for proper amplification by PCR. Optimal annealing temperature for each set was obtained using a temperature gradient thermal cycler (BIO-RAD Laboratories, Inc.). Reaction products were visualized on a 2% agarose gel stained with ethidium bromide. The forward primer from each set that produced a unique band of the appropriate size (according to sequence data) was 5'-fluorescent labeled with HEX or 6-FAM dyes (Applied Biosystems) for detection with an automated DNA sequencer.

PCR amplification for labeled primers was performed in a 30 µl total volume reaction including 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, pH 9, 3.1 mM MgCl₂, 200 µM each dNTP, 20 ng/µl of each primer (forward was 5-labeled), 0.525 U *Taq*, and 30–50 ng template DNA. Amplifications were carried out in a thermal cycler (BIO-RAD Laboratories, Inc.) using the following reaction

conditions: Hot-start at 95 °C; 95 °C for 5 min; 28 cycles of 95 °C for 30 s; annealing temperature (see Table 1) for 30 s; 72 °C for 1 min; and a final extension step 72 °C for 18 min.

2.6. Fragment size determination

One microliter of the PCR reaction was added into a standard loading cocktail: 0.5 µl of an internal GeneScan size standard, ROX-500 (Applied Biosystems), and 10 µl of HIDI formamide (Applied Biosystems), and was then loaded on the ABI 3100 DNA sequencer (Applied Biosystems). Determination of fragment size was carried out using ABI Prism[®] GeneScan Analysis 3.7 and Genotyper 3.7 (both from Applied Biosystems). Only clear electropherograms, with one or two peaks of the expected size were considered in the analysis. All PCR of samples with electropherograms with many peaks or non-specific products were repeated until obtaining unambiguous results.

2.7. Data analysis

The calculations of allele frequencies, expected and observed heterozygosity, as well as the goodness-of-fit with Hardy-Weinberg expectations and genotypic disequilibrium tests were performed using exact tests available in Genepop Version 3.4 software (Raymond and Rousset, 1995).

Table 2

Characterization of 13 microsatellite loci for *T. infestans*; allele size and variability, sample size and compliance with Hardy–Weinberg (F_{IS})

Locus	N	Allele size range (clone size)	N_a	Most common allele (%)	H_e (H_o)	F_{IS}
Tinfest_ms3	118	160–204 (200)	8	58.5	0.60 (0.49)	0.22**
Tinfest_ms5	86	364–396 (396)	15	20.9	0.89 (0.70)	0.18**
Tinfest_ms21	74	318–320 (331)	2	87.8	0.22 (0.24)	–0.13
Tinfest_ms22	116	162–196 (177)	13	36.2	0.74 (0.71)	0.04*
Tinfest_ms23	118	148–177 (161)	5	86.4	0.25 (0.14)	0.45**
Tinfest_ms27	114	290–314 (299)	11	44.7	0.76 (0.65)	0.15*
Tinfest_ms42	110	206–246 (216)	15	20.9	0.86 (0.73)	0.16*
Tinfest_ms45	68	392–434 (412)	8	54.4	0.64 (0.06)	0.91**
Tinfest_ms55	104	217–275 (231)	12	79.8	0.36 (0.33)	0.10
Tinfest_ms56	100	156–170 (163)	7	45.0	0.72 (0.78)	–0.08
Tinfest_ms64	106	146–162 (160)	9	56.6	0.63 (0.36)	0.43**
Tinfest_ms65	98	222–266 (231)	17	26.5	0.87 (0.59)	0.32**
Tinfest_ms68	118	183–191 (188)	5	49.2	0.58 (0.29)	0.51**

N : sample size (alleles); N_a : number of alleles; H_e : expected heterozygosity; H_o : observed heterozygosity; F_{IS} : inbreeding coefficient, according to Weir and Cockerham (1984; in Genepop). * $p < 0.05$; ** $p < 0.01$ and < 0.05 after sequential Bonferroni correction.

3. Results

Of 10^4 clones of the *T. infestans* microsatellite enriched library, 96 were picked and their plasmid inserts were sequenced. Primers were designed for 33 clones and 16 were selected for labeling and subsequent screening; the others were rejected due to non-specific amplification or absent PCR products. We selected 13 microsatellite loci for further population genetic analysis (Table 1), after rejecting 3 loci due to inconsistent PCR amplification. The suitability of each locus for population genetic analysis was assessed based on the genotypes of 59 *T. infestans* specimens. To minimize the chance that the bugs represent close family relatives and bias allele variability, each specimen selected for the analysis was collected from a different house.

The mean number of alleles per locus was 8.6 (range: 2–17). The frequencies of the most common alleles ranged from 21% to 88% (Table 2, Fig. 1). These frequencies, together with the number of alleles per locus obtained, were used as indicators of the suitability of the loci for population genetics studies; those locus having at least four alleles with the frequency of the most common allele $< 75\%$ were considered polymorphic enough to be suitable for population studies in a small geographical area. Loci Tinfest_ms21, Tinfest_ms23 and Tinfest_ms55 did not meet these criteria and therefore were not considered suitable in the sample analyzed.

Expected heterozygosity ranged from 0.22 to 0.89. The probability test showed significant departures from Hardy–Weinberg equilibrium in 10 of the 13 loci with F_{IS} values ranging from 0.04 to 0.91 (Table 2). However, after applying the sequential Bonferroni correction for multiple tests, only seven showed significant departures (Table 2). All departures from H–W expectations were due to heterozygosity deficit, since all F_{IS} values were positive. The test for linkage disequilibrium (LD), which measures the departure of associations between alleles of different loci from that

expected by chance in a population with random mating, detected five significant deviations from a total of 78 tests (6.4%); however, this was not significantly higher than the fraction expected by chance (binomial test, $P > 0.2$). None of the loci pairs was significant after applying the sequential Bonferroni correction.

Our sequences were compared to previous published microsatellite loci for triatomine species, both by alignments, with Pileup program, and by searching for their primer sequences in our loci or vice versa, using Findpatterns program and allowing for 5 bp mismatches (both programs in Genetics Computer Group, Wisconsin Package Version 10.3, Accelrys Inc., Wisconsin). The sequences flanking the loci were not similar nor did the primer sequences match sufficiently to amplify. Thus, we consider our microsatellite loci different from the published sequences for *R. pallescens* (Harry et al., 1988), *T. dimidiata* (Anderson et al., 2002) and *T. infestans* (García et al., 2004).

4. Discussion

This study was designed to identify, characterize, and evaluate the suitability of microsatellite loci in *T. infestans* for population genetics studies. Thirteen loci provided clear and reproducible PCR products and 9 were moderately or highly polymorphic in a sample of 59 bugs from rural communities in NW Argentina, up to 20 km apart. The test for genotypic disequilibrium did not reveal significant departures from equilibrium. This suggests that loci are not linked and therefore each one would provide independent information on population genetics studies.

Departures from H–W equilibrium were due to heterozygote deficit, which could reflect a substructure in the sample analyzed. Our current study was designed to detect the maximum variability by pooling insects from houses of

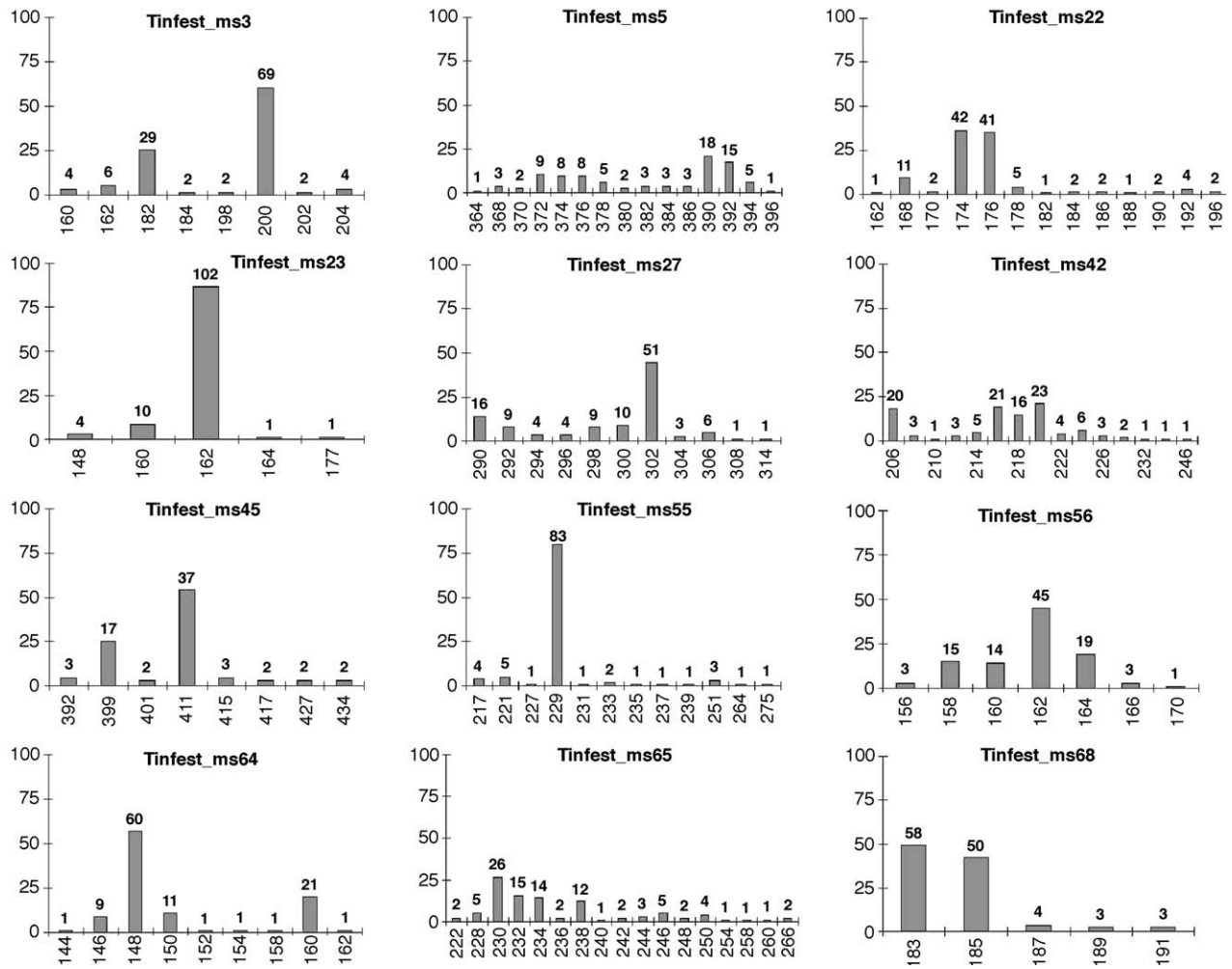


Fig. 1. Allele size and frequency of 12 new microsatellite loci described for *T. infestans*. Numbers on top of the bars are the actual number of alleles obtained in the sample analysed. Note: Graph of Tinfest_21 was not included because only two alleles were obtained and frequency of the most common is conformed in Table 2.

15 different communities, which are expected to belong to separate reproductive units. Pooling of subpopulations could result in increase homozygote frequencies as in Wahlund effect (Lehmann et al., 2003b). However, in locus Tinfest_ms45, associated with high heterozygote deficit (F_{IS} 0.91), the presence of null alleles is supported by repetitive PCR failures in specimens that amplified well with other loci, therefore, their suitability for population studies should be evaluated more carefully.

The four loci, namely Tinfest_ms21, Tinfest_ms23, Tinfest_ms45 and Tinfest_ms55, deemed unsuitable in our current study because of low polymorphism or high heterozygote deficit, may be more polymorphic and informative in other populations. Further studies on larger numbers of bugs per subpopulation, and separate sub-analyses for each population are necessary to determine whether these results reflect the sample used in this study or are consistent in these markers. Furthermore, because the loci location on the *T. infestans* genome is not known, their independence should be corroborated by determining

whether they are or not on the same chromosome, i.e. by in situ hybridization.

Having identified nine new suitable loci, we can make significant headway in the population genetic analysis of *T. infestans*. Observed polymorphisms in each locus may provide the basis for examining gene frequencies of specific alleles, establishing boundaries of local populations, and estimating the relative amount of migration and mixing that occurs between local populations. We will apply this molecular approach in tandem with geometrical morphometric analysis, to explain data of *T. infestans* populations obtained in our field studies and analyses and to test the hypotheses developed for reinfestation dynamics (Cecere et al., 2004) or flight dispersal mechanisms (Vazquez-Prokopec et al., 2004).

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