**Sex, Subdivision, and Domestic Dispersal of Trypanosoma cruzi Lineage I in Southern Ecuador**

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**Abstract**

**Background:** Molecular epidemiology at the community level has an important guiding role in zoonotic disease control programmes where genetic markers are suitably variable to unravel the dynamics of local transmission. We evaluated the molecular diversity of Trypanosoma cruzi, the etiological agent of Chagas disease, in southern Ecuador (Loja Province). This kinetoplastid parasite has traditionally been a paradigm for clonal population structure in pathogenic organisms. However, the presence of naturally occurring hybrids, mitochondrial introgression, and evidence of genetic exchange in the laboratory question this dogma.

**Methodology/Principal Findings:** Eighty-one parasite isolates from domiciliary, peridomiciliary, and sylvatic triatomines and mammals were genotyped across 10 variable microsatellite loci. Two discrete parasite populations were defined: one predominantly composed of isolates from domestic and peridomestic foci, and another predominantly composed of isolates from sylvatic foci. Spatial genetic variation was absent from the former, suggesting rapid parasite dispersal across our study area. Furthermore, linkage equilibrium between loci, Hardy-Weinberg allele frequencies at individual loci, and a lack of repeated genotypes are indicative of frequent genetic exchange among individuals in the domestic/peridomestic population.

**Conclusions/Significance:** These data represent novel population-level evidence of an extant capacity for sex among natural cycles of *T. cruzi* transmission. As such they have dramatic implications for our understanding of the fundamental genetics of this parasite. Our data also elucidate local disease transmission, whereby passive anthropogenic domestic mammal and triatomine dispersal across our study area is likely to account for the rapid domestic/peridomestic spread of the parasite. Finally we discuss how this, and the observed subdivision between sympatric sylvatic and domestic/peridomestic foci, can inform efforts at Chagas disease control in Ecuador.

**Introduction**

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is the most important parasitic infection in Latin America [1]. An estimated 10 million people carry the infection, while another 90 million live at risk [2]. This vector-borne zoonosis causes severely debilitating and potentially deadly disease in more than a third of infected people [3]. Mucosal or abrasion contact with the infected faeces of hematophagous triatomine bugs constitutes the major mode of transmission [2].

Chagas disease is endemic to several regions in Ecuador, including the warm inter-Andean valleys of the southern province of Loja, where the main vectors are *Rhodius ecuadoriensis*, *Triatoma carrioni*, *Panstrongylus chinai*, and *Panstrongylus rufotuberculatus* [4,5]. Loja Province is currently targeted by the Ecuadorian Chagas Disease Control Program. Complementing disease prevention efforts, recent progress has been made in understanding local vector dynamics [5–7]. However, parasite molecular epidemiology could also play a role in guiding effective intervention measures.

Molecular diversity was first recognised in *T. cruzi* in the early 1970s [8]. Six major genetic subdivisions, known as discrete typing units (DTUs), are currently recognised (TcI–TcVI [9]), with distributions loosely defined by geography, transmission cycle, and ecology [1]. TcI predominates in northern South America, causes significant human disease [10,11] and occurs in both domestic and sylvatic cycles of parasite transmission. Of major interest to those planning sustainable control strategies in this region is the extent to which these cycles are connected [12–14]. The provision of such data relies on the evaluation of molecular diversity ‘hidden’ at the sub-DTU level [15–17].
Author Summary

Trypanosoma cruzi is transmitted by blood sucking insects known as triatomines. This protozoan parasite commonly infects wild and domestic mammals in South and Central America. However, triatomines also transmit the parasite to people, and human infection with T. cruzi is known as Chagas disease, a major public health concern in Latin America. Understanding the complex dynamics of parasite spread between wild and domestic environments is essential to design effective control measures to prevent the spread of Chagas disease. Here we describe T. cruzi genetic diversity and population dynamics in southern Ecuador. Our findings indicate that the parasite circulates in two largely independent cycles: one corresponding to the sylvatic environment and one related to the domestic/peridomestic environment. Furthermore, our data indicate that human activity might promote parasite dispersal among communities. This information is key for the design of control programmes in Southern Ecuador. Finally, we have encountered evidence of a sexual reproductive mode in the domestic T. cruzi population, which constitutes a new and intriguing finding with regards to the biology of this parasite.

Hypervariable molecular markers, like microsatellites, have given new and unprecedented insight into the population genetics of other important parasitic zoonoses [18–22]. For the first time, specific hypotheses regarding parasite dispersal and reproduction can be addressed. However, the validity of molecular epidemiological data depends heavily on study design. Numerous confounders, including biased sampling (e.g., sampling only one host in a heteroxenous transmission system [23]), population subdivision in both space and time (leading to Wahlund effects [24]), and low sample size all influence the estimation of key population genetic parameters. Historically, such biases have acted as an impediment to obtaining useful epidemiological information from parasite molecular data, and, particularly in T. cruzi, to resolving the frequency of sex in natural populations.

Here we present microsatellite data for 10 variable loci amplified from a large number of Tc1 isolates collected from domestic, peridomestic, and sylvatic hosts and vectors in and around several adjacent communities in Loja Province, Ecuador. We evaluate evidence for genetic subdivision between transmission cycles, anthropogenic dispersal of parasites between communities, and panmixia among a subset of strains.

Methods

Study area and sampling

Sixteen communities in Loja Province, southern Ecuador, were sampled (Figure 1). These communities were located at altitudes less than 2,200 m and were representative of the ecological diversity of the province. Trypanosomases were isolated from triatomines and small mammals (rodents and opossums) captured at domestic (within dwellings), peridomestic (near dwellings and/or associated with human activities, e.g., crop stores, chicken roosts, wood and rock piles), and sylvatic (more than 20 meters from dwellings) foci (Table S1). Written informed consents from the head of the houses for domiciliary bug searches and capture of mammals near houses were obtained. These documents have been approved by the institutional review board from National Institute of Health (NIH), Ohio University (OU) and Pontifical Catholic University of Ecuador (PUCE). Vertebrates were euthanized to obtain samples; all procedures were carried out in strict accordance with the protocol approved by the Ohio University Institutional Animal Care and Use Committee (IACUC). The Ohio University IACUC adheres to the guidelines in the United States Government Code of Federal Regulations (CFR), Title 9, Chapter 1, Subchapter A- Animal Welfare Parts 1–3 and the United States Health Research Extension Act of 1985, Public Law 99–150 “Animals in Research”.

Molecular identification of T. cruzi and lineage genotyping

Trypanosome species was determined by PCR amplification of the kinetoplast minicircle region as in Vallejo et al. [25]. Discrete Typing Units (DTU) genotyping was achieved by assaying a combination of three nuclear loci as described by Lewis et al. [26].

Microsatellite analysis

Ten previously identified polymorphic microsatellite loci were studied (Table S2) [16]. These loci are distributed across seven T. cruzi chromosomes and include two groups of physically linked markers [27]. Allelic products were amplified using previously described reaction conditions [16]. Allele sizes were determined using an automated capillary sequencer (AB3730, Applied Biosystems, UK) in conjunction with a fluorescently tagged size standard and were manually checked for errors. All isolates were typed “blind” to control for user bias. By reference to a representative panel of strains, no cross reactivity was identified between T. rangeli and the microsatellite primers used in this study. Population-level genetic diversity was assessed using sample size corrected allelic richness (\(\text{AR}\)) in FSTAT v2.9.3.2 [28] and number of private (population specific) alleles per locus (\(\text{PA}\)). \(\text{F}_S\), a measure of the distribution of heterozygosity within and between individuals, was estimated per locus per population in FSTAT v2.9.3.2. \(\text{F}_S\) can vary between \(-1\) (all loci homozygous for the same alleles) and \(+1\) (all loci homozygous for different alleles). \(\text{F}_S=0\) indicates Hardy-Weinberg allele proportions. The extent of population subdivision between isolates from different transmission cycles was estimated using \(\text{F}_{ST}\) in ARLEQUIN v3.1 and statistical significance assessed via 10,000 random permutations of alleles between populations [29]. Similarly, within-population subdivision was examined in ARLEQUIN v3.1, in this case using a hierarchial Analysis of Molecular Variance (AMOVA). Population-level heterozygosity indices were also calculated in ARLEQUIN v3.1 and associated significance levels for \(p\) values derived after sequential Bonferroni correction to minimise the likelihood of Type I errors [30]. Individual-level pair-wise distances were estimated using \(\text{D}_{AS}\) (1-proportion of shared alleles at all loci vs. \(n\) [31] under an IAM and \(\delta\mu^2\) [32] under an SMM in MICROSAAT [33]. \(\text{D}_{AS}\) values form the basis of the dendrogram in Figure 2. To accommodate multi-allelic loci, a script was written in Microsoft Visual Basic to make multiple random diploid re-samplings of each multilocus profile (software available on request). Individual-level genetic distances were calculated as the mean across multiple re-sampled datasets. A Mantel’s test for the effect of isolation by distance within populations (pair-wise genetic vs. geographic distance) was implemented in Genalex 6 using 10,000 random permutations [34]. Linkage disequilibrium indices, pair-wise \(R_{\text{GDD}}\) and multilocus \(I_{\text{AX}}\), were calculated in LINKDOS [35] and MULTILOCUS1.3b [36], respectively. Multiple diploid re-samplings were also made to evaluate the influence of multi-allelic loci on \(I_{\text{AX}}\), the results of which are shown in Table 1. Assignment of individuals to populations was made by reference to the topology of the \(\text{D}_{AS}\) derived tree. Secondarily, this model-free population assignment was corrob-
rated using STRUCTURE (Figure S1) [37]. Sample affiliations are listed in Table S1.

**Results**

Eighty-one isolates of *T. cruzi* were obtained from triatomines and mammals. All were genotyped as TcI. Kinetoplast analysis detected the presence of mixed infection with *T. rangeli* in nine isolates from the sylvatic environment (Table S1). In total, a dataset of 1,637 alleles was derived across all loci, excluding missing data (Table S3). Multiple (≥3) alleles were observed at 3.08% of loci.

**Isolate clustering and population subdivision by transmission cycle**

We evaluated patterns of clustering and subdivision among parasite strains in the Loja samples based upon their microsatellite profiles. To identify genetically distinct groups we relied on three
lines of evidence: neighbor-joining analysis based on pair-wise genetic distance; model-based population assignment (STRUCTURE); and the statistical significance of the fixation index $F_{ST}$. The deepest and most robust (56.5%) internal branching within the neighbor-joining tree constructed from pair-wise genetic distance values ($D_{AS}$) supported the delineation of two populations (Figure 2 and Table 1). No pattern or diversification by host or vector was observed within these populations. The observed bipartite subdivision was unaffected by the presence of multi-allelic loci (100% congruence, Figure 2) and was used as a means to define the populations examined in later analyses (See Table 1). Sample allocation between these two populations was...
Table 1. Population genetic parameters for domestic/peridomestic and sylvatic populations of *T. cruzi* in Loja Province, Ecuador.

| Population       | N  | PL | PA  | MA/S | A_s±SE | H_o±p  | H_e±p  | % PL H_o H_e | % PL H_o H_e | F_95±SE | I_A±p  | Median I_A | % PL PLD±p |
|------------------|----|----|-----|------|--------|--------|--------|-------------|-------------|---------|--------|------------|------------|
| Domestic/Peridomestic | 18 | 9  | 0.8 | 0.67 | 3.851±0.527 | 0.478 | 0.478 | 0           | 0           | 0.003±0.084 | 0.170 | 0.13 | 3.5       | 5.5        |
| Sylvatic         | 55 | 63 | 2.0 | 0.24 | 4.583±0.504 | 0.367 | 0.457 | 11.1        | 44.4        | 0.184±0.098 | 0.562 | <0.001 | 38.5      |            |

N = Number of isolates in population.
G = Number of multilocus genotypes per population.
PL = Number of polymorphic loci.
PA = Mean number of private alleles per locus.
MA/S = Mean number of multiple (3+) alleles per sample.
H_o±p = Mean observed heterozygosity ± standard error, calculated in FSTAT (28).
H_e±p = Mean expected heterozygosity ± standard error, calculated in FSTAT (28).
% PL H_o H_e = Percentage of loci demonstrating significant heterozygosity deficit or excess.
F_95±SE = Fst over loci ± standard error, calculated in FSTAT (28).
I_A±p = Mean FIS over loci ± standard error, calculated in FSTAT (28).
Median I_A = Median FIS over loci, calculated in Linkage (35).
% PL PLD = Median FIS over loci ± standard error, calculated in FSTAT (28).

Genetic diversity

Following the identification of two genetically distinct groups of parasite strains circulating in this endemic area, the genetic diversity of each was evaluated and compared. Estimates of allelic richness (A_s) did not demonstrate dramatic difference between LOJ_A_Dom/Peri and LOJ_A_Sylv (Table 1); both populations showed considerable genetic diversity. More private alleles per locus (PA) were found in the larger and marginally more diverse sylvatic population (PA = 2.0; Table 1). In conjunction with its apparent genetic distance from other South American TcI populations (Figure 2), the lack of private alleles within LOJ_A_Dom/Peri (PA = 0.8) suggests diversification of this population from a local source.

Geographic dispersal within populations

In light of the role played by transmission cycles in structuring the local parasite population, we compared the rate of parasite dispersal within LOJ_A_Dom/Peri with that within LOJ_A_Sylv. This rate is inversely proportional to the amount of spatial structure (or isolation by distance (IBD)) in the population. Interestingly, tests for IBD among individuals from LOJ_A_Dom/Peri and LOJ_A_Sylv showed statistically significant and epidemiologically important differences between these two populations. Infinite allele models (IAMs) of microsatellite mutation intrinsically overestimate genetic distances between closely related isolates as compared to stepwise mutational models (SMMs). To circumvent possible bias we chose to test for IBD under both. Strong evidence for spatial structure in LOJ_A_Sylv existed regardless (D_xy = 0.265, P < 0.0001; δ2 = R_xy = 0.177, p = 0.001). Among isolates from LOJ_A_Dom/Peri no spatial structure was evident from either measure (D_xy = 0.100, p = 0.164; δ2 = R_xy = 0.05, p = 0.384). Results are summarised in Figure 3 and strongly suggest more rapid parasite dispersal among domestic and peridomestic foci than that occurring between sylvatic locales at the same spatial scale.

Compelling evidence for sex in LOJ_A_Dom/Peri

Several approaches were employed to estimate the rate of genetic recombination within the parasite populations identified in Loja. Multiple indicators suggested frequent sex among trypano-
somes of \textit{LOJA}_{Dom/Peri}. Pair-wise inter-locus linkage ($R_{GGD}$) was infrequent (5.3\% Table 1) even among physically linked loci (3/4 physically linked locus pairs, those on the same chromosome, were not statistically linked) and despite abundant allelic diversity available within this population for inter-correlation (the statistical power of $R_{GGD}$ drops dramatically with decreasing population-level genetic diversity). Infrequent pair-wise linkage is consistent with the lack of significance attributable to the index of association ($I_A$) (median $p=0.13$, $P=0.05$ in 89\% of 1000 resampled diploid datasets; Table 1), and with the null hypothesis of random mating that must be assumed. Additionally, tests for deficit or excess of heterozygosity in this population showed no significant deviation from Hardy-Weinberg expectations, reflected by mean values for the inbreeding coefficient ($F_{IS}$) across loci that approximate zero (Table 1). Finally, repeated multilocus genotypes, indicative of clonal reproduction, were absent from this population while present in \textit{LOJA}_{Sub}. Other aspects of \textit{LOJA}_{Sub} diversity pointed to predominant clonality, especially strong pair-wise (38.5\% of locus pairs) and multilocus linkage ($I_A$, $P<0.001$) in all diploid resampled datasets (Table 1), but also strong deviation from Hardy-Weinberg levels of heterozygosity under all three measures employed (Table 1). Consistent with spatial structure identified in this population, however, an AMOVA conducted across isolates from San Jacinto and Bramaderos, which make up the majority of \textit{LOJA}_{Sub} strains (Figure 1 and Table S1), did demonstrate significant but weak $F_{ST}$ ($F_{ST}=0.173$, $P<0.0001$, 16,000 permutations), evidence that a Wahlund effect could be depressing heterozygosity. Correspondingly, estimates of linkage disequilibrium might also be somewhat inflated by subdivision in this population [38], and it is difficult to reject the possibility that recombination may occur in the sylvatic populations at a micro-geographic scale.

Discussion

This study constitutes a first attempt to understand the population dynamics of \textit{T. cruzi} at a local scale using high-resolution molecular markers. The sample includes isolates from different transmission cycles, vectors, hosts, and adjacent communities. This arrangement aims to minimise sample bias and maximise the resulting molecular epidemiological inference. However, all field studies are affected by the natural abundance of hosts and vectors in different transmission cycles, and we cannot claim a perfect dataset. Nonetheless, we can report strong evidence for parasite diversification by transmission cycle, human involvement in parasite dispersal, and the possibility of sex in one parasite population.

The presence of the \textit{T. cruzi} lineage I in southern Ecuador is consistent with reports of this DTU throughout northern South America [10,39,40]. In our study, as in other studies, sub-DTU level diversity of the parasite occurred independently of vector and host [16,17,41]. Instead, we found evidence that transmission cycle (domestic, peri-domestic, or sylvatic) is likely to be the major driver behind parasite differentiation, apparently a phenomenon common to \textit{T. cruzi} populations across much of northern South America [15,16] but never before studied on a local scale. On the basis of our data, we suggest that widespread, internationally distributed TcI subgroups associated with specific transmission cycles may not exist. A lack of connectivity between \textit{LOJA}_{Dom/Peri} and domestic TcI from Venezuela, \textit{VEN}_{Dom} (Figure 2) exemplifies this. Furthermore, clear cross-propagation of parasites between transmission cycles (Figure 2) and few private alleles in \textit{LOJA}_{Dom/Peri} (Table 1) suggests that these domestic groups are likely to emerge and diversify from local sylvatic sources.

\textit{T. cruzi} is the only stercorarian trypanosome of medical importance [42]. Natural transmission efficiency by this route (contamination with vector feces) is very low. The rate of transmission from infected \textit{Triatoma infestans} to humans in Argentina, for example, is estimated at approximately one in 650 bites [43]. As with \textit{R. prolixus} in Venezuela [14], \textit{R. ecuadoriensis}, a major disease vector in Loja, occurs at high frequency in both domestic and sylvatic locales [7]. Our data suggest that even if vector invasion from sylvatic foci is common, as in Venezuela [14], associated transmission of parasites to domestic foci is too infrequent to break up population subdivision. Where cross-propagation does occur, circumstantial evidence incriminates synanthropic mammals as the link between transmission cycles. \textit{Dipetalogaster maruapiensis} infected with parasites from the \textit{LOJA}_{Dom/Peri} group were found at both peridomestic (Isolate Numbers IN 9 and 13, Figure 2) and sylvatic locales (IN 6 and 17, Figure 2). Furthermore, a \textit{R. rattus} individual captured at a peridomestic site was found infected with a \textit{LOJA}_{Sub}, strain (IN 31, Figure 2). Finally \textit{P. chinai} and \textit{T. carinii} adults and nymphs, so far thought to be exclusively domestic and peridomestic triatomin species in Loja (IN 27,28,58,68 and 81, Figure 2) [5], were found infected with a \textit{LOJA}_{Sub}, strain, likely as a result of contact with invasive sylvatic mammals. This blurring of the lines between transmission cycles is likely to mirror local environmental change, where human activity is driving land-use transformation.

Parasite sampling in Loja was undertaken across an area only 50 km in radius (Figure 1). However, this area encompassed several ecological zones punctuated by high mountains (>2,500 m in elevation) and deep interconnecting valleys. Spatial genetic diversification among sylvatic isolates is an expected outcome given such barriers to host and vector migration (Figure 1 and 3). Conversely, parasites belonging to the \textit{LOJA}_{Dom/Peri} group lack this signature, a finding possibly linked to rapid anthropogenic dispersal in the form of infected individuals, livestock, or passively transported vectors and/or small peridomestic mammals. \textit{T. cruzi} has, until recently, been a paradigm for clonal population structure in pathogenic organisms [44,45]. However, the presence of naturally occurring hybrids [46], mitochondrial introgression [46], and a capacity for genetic exchange in the laboratory [47] has challenged this dogma. The frequent observation of linkage disequilibrium in \textit{T. cruzi} may partially stem from cryptic population subdivision (temporal, spatial, and/or genetic) to which linkage statistics are intrinsically sensitive [38]. Frustratingly, if assignment software with intrinsic Hardy-Weinberg assumptions (e.g., STRUCTURE [48] or BAPS [49]) is used to account for subdivision prior to linkage analysis, the resulting populations will be sorted to maximise adherence to Hardy-Weinberg allelic frequencies, with artifactual sexuality a possible result [21,50]. Fortunately, in our study, the status of \textit{LOJA}_{Dom/Peri} as a stable deme is corroborated by distance-based, model-free assignment, as well as STRUCTURE. In conjunction with Hardy-Weinberg allele frequencies at individual loci, we consider, therefore, that linkage equilibrium among isolates from \textit{LOJA}_{Dom/Peri} Represents strong evidence for frequent genetic exchange among field isolates of \textit{T. cruzi}. We believe that the relatively small sample size of \textit{LOJA}_{Dom/Peri} (n = 18) does not affect this conclusion, partly due to the ample genetic diversity present in this population, but also because the lack of spatial subdivision in this group suggests frequent contact and opportunity for mixis. Thus it constitutes exactly the group of strains between which genetic exchange might be expected to occur. We cannot rule out the possibility that genetic exchange may also occur in the sylvatic cycle, if the role that substructure found in \textit{LOJA}_{Sub} played in inflating linkage statistics $I_A$ and $R_{GGD}$ could be taken into
account. However, more focused high-density sample collection from multiple individual localities would be required to address such a hypothesis. Furthermore, we cannot infer the cellular mechanism of genetic recombination events on the basis of these data. Hardy-Weinberg allelic frequencies are consistent with classical meiosis. However, the lack of haploid life stages so far observed in T. cruzi are not consistent with classical meiosis, nor are the genetic exchange events so far observed in *vitro* [47].

Molecular epidemiology at this scale has an important guiding role to play in Chagas disease control programmes. Future efforts in Loja province must account for inter-domiciliary and inter-community parasite dispersal. This includes sustained surveillance and coordinated region-wide spraying campaigns to eliminate local vector re-invasion sources and community education to target passive triatomine dispersal routes. It is also clear that the role of synanthropic mammals cannot be overlooked as these represent an important potential link between sylvatic and domestic foci.

We have shown that microsatellite markers, adequate sample sizes, and associated population statistics give fundamental insight into the genetic exchange in *T. cruzi*. Our results, skewed toward samples from the vector, intuitively imply that the vector may be a site of genetic exchange, as is the case for *T. brucei* [51] and *Leishmania magra* [52]. The data also indicate, not surprisingly, that the majority of events probably occur within a *T. cruzi* lineage between epidemiologically linked strains, and these events have therefore historically been difficult to detect. The intriguing mechanisms of genetic exchange in *T. cruzi* warrant further investigation of their functional, adaptive, and epidemiological significance.

**Supporting Information**

**Figure S1** Clustering of populations based on STRUCTURE analysis. Results from structure analysis corresponded with the structure defined by $D_{AS}$ values. Both analyses identified two distinct populations among Ecuadorian samples. Ten replicates per value $k$ were made assuming a no-admixture model, and with a burn in of 100,000 followed by 1,000,000 interactions of the algorithm. Delta $k$ calculated according to Evanno et al. [37].

| Table S1 | Location, habitat, and lineage of *Trypanosoma* isolated in Loja Province, Ecuador. |
|----------|-----------------------------------------------------------------------------------|
| Found at | do:10.1371/journal.pntd.0000915.s002 (0.15 MB DOC) |

| Table S2 | *Trypanosoma cruzi* primers employed in this study. |
|----------|-----------------------------------------------------|
| Found at | do:10.1371/journal.pntd.0000915.s003 (0.04 MB DOC) |

| Table S3 | Allele sizes (in base pairs) from 10 loci analyzed (fluorescent dye) from 81 *T. cruzi* isolates from Loja Province, Ecuador. |
|----------|-------------------------------------------------------------------------------------------------------------------|
| Found at | do:10.1371/journal.pntd.0000915.s004 (0.30 MB DOC) |

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**Author Contributions**

Conceived and designed the experiments: SOM MJG. Performed the experiments: SOM MSL MJG. Analyzed the data: SOM MSL JAC MG. Wrote the paper: SOM MSL JAC MAM MJG.

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