Multilocus Sequence Typing (MLST) for Lineage Assignment and High Resolution Diversity Studies in Trypanosoma cruzi

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Abstract

Background: Multilocus sequence typing (MLST) is a powerful and highly discriminatory method for analysing pathogen population structure and epidemiology. Trypanosoma cruzi, the protozoan agent of American trypanosomiasis (Chagas disease), has remarkable genetic and ecological diversity. A standardised MLST protocol that is suitable for assignment of T. cruzi isolates to genetic lineage and for higher resolution diversity studies has not been developed.

Methodology/Principal Findings: We have sequenced and diplotyped nine single copy housekeeping genes and assessed their value as part of a systematic MLST scheme for T. cruzi. A minimum panel of four MLST targets (Met-III, RB19, TcGPXII, and DHFR-TS) was shown to provide unambiguous assignment of isolates to the six known T. cruzi lineages (Discrete Typing Units, DTUs TcI-TcVI). In addition, we recommend six MLST targets (Met-II, Met-III, RB19, TcMPX, DHFR-TS, and TR) for more in depth diversity studies on the basis that diploid sequence typing (DST) with this expanded panel distinguished 38 out of 39 reference isolates. Phylogenetic analysis implies a subdivision between North and South American TcIV isolates. Single Nucleotide Polymorphism (SNP) data revealed high levels of heterozygosity among DTUs TcI, TcII, TcIIId and, for three targets, putative corresponding homozygous and heterozygous loci within DTUs TcI and TcIII. Furthermore, individual gene trees gave incongruent topologies at inter- and intra-DTU levels, inconsistent with a model of strict clonality.

Conclusions/Significance: We demonstrate the value of systematic MLST diplotyping for describing inter-DTU relationships and for higher resolution diversity studies of T. cruzi, including presence of recombination events. The high levels of heterozygosity will facilitate future population genetics analysis based on MLST haplotypes.

Introduction

Trypanosoma cruzi is the causative agent of Chagas disease and the most important parasitic infection in Latin America. Approximately 8 million people are thought to be infected [1]. Chagas disease is a zoonosis: T. cruzi infects many mammal species [2] and is transmitted to humans primarily by the infected faeces of haematophagous triatomine bugs coming into contact with mucosal membranes or broken skin. Transmission may also be by blood transfusion, congenitally or, rarely, by ingestion of food contaminated by infected triatomine faeces [3].

T. cruzi is monophyletic but genetically heterogeneous with at least six phylogenetic lineages (discrete typing units, DTUs) previously designated TcI, and TcIIa-e. A recent review meeting on T. cruzi intraspecific nomenclature reached an international consensus that these six DTUs should be renamed (former nomenclature in parenthesis): TcI (TcI), TcII (TcIIb), TcIII (TcIII), TcIV (TcIIId), TcV (TcIIa), TcVI (TcIIc), to remove the presumptive sublineage designations within TcII [4]. Here we will adopt this new consensus nomenclature.

TcI and TcII are the most genetically distant groups. The evolutionary ancestry of TcIII and TcIV is presently a debated issue. Based on sequencing of individual nuclear genes Westeringber et al. [5] suggested an ancient hybridisation event occurred between TcI and TcII followed by a long period of clonal propagation leading to the extant DTUs TcIII and TcIV. Alternatively, de Freitas et al. [6] suggested that TcIII and TcIV have a separate evolutionary ancestry with mitochondrial sequences that are similar to each other but distinct from both TcI and TcII. Less controversially it is clear, using an array of molecular markers [6,7,8] that TcV and TcVI are hybrid lineages sharing haplotypes from both TcII and TcIII, with both DTUs retaining...
Author Summary

The single-celled parasite Trypanosoma cruzi occurs in animals and insect vectors in the Americas. When transmitted to humans it causes a major public health problem, Chagas disease (American trypanosomiasis). T. cruzi is genetically diverse and currently split into six groups, known as Tcl to TcVI. Multilocus sequence typing (MLST) is a method used for studying the population structure and diversity of pathogens. MLST involves sequencing the DNA of several different genes and comparing the sequences between isolates. MLST has not yet been developed and systematically applied to T. cruzi. Here, we sequence nine T. cruzi genes, selecting a panel of four for lineage assignment and six for higher resolution studies of genetic diversity. Our results showed that one of the T. cruzi genetic groups is further subdivided into North and South American subpopulations. Furthermore, comparative analyses of the gene sequences gave new evidence of genetic exchange in T. cruzi. Application of MLST for assigning field isolates of T. cruzi to genetic groups and for detailed investigation of diversity provides a valuable approach to understanding the taxonomy, population structure, genetics, ecology and epidemiology of this important human pathogen.

the mitochondrial genome of TcIII. Production of experimental hybrids [9] proved that T. cruzi has an extant capacity for genetic exchange (at least within Tcl) but the mechanism of recombination is not fully understood, appearing to involve nuclear fusion followed by genome erosion leading to relatively stable aneuploid hybrids, a process distinct from classical meiosis [10].

There is currently no consensus discriminatory typing method applicable to T. cruzi. Previous methods for characterisation have included multilocus enzyme electrophoresis [11,12,13,14], PCR amplification of single gene loci, [15] and various PCR based assays [16,17]. Lewis et al. [18] recommended the use of a triple-assy comprising SSU rDNA, HSP60 and GPI markers, allowing reliable and low cost typing to DTU level. Microsatellite typing (MLMT) provides a high resolution method for fine scale population genetics analysis [19]. What is still lacking is an unequivocally reproducible and standardised method that can simultaneously distinguish the known genetic lineages, describe inter-DTU relationships, and define high resolution intra-DTU diversity for population genetics studies.

Multilocus sequence typing (MLST) has been applied to a variety of bacterial [20,21] and yeast species [22,23] and typically involves sequencing internal fragments of six single copy housekeeping genes per strain [24]. The method is potentially highly discriminatory, when sequence polymorphisms within each housekeeping locus are categorised as distinct alleles. Sequence data for all loci are usually concatenated to produce a unique allelic profile (sequence type, ST). A major advantage of MLST analysis is that sound sequence data are unambiguous and suitable for population structure and epidemiological studies. Results are particularly relevant if easily accessible international databases are constructed such as MLST.net [25] which contains MLST typing schemes and data for a growing number of pathogens.

MLST was originally designed for haploid species, T. cruzi is diploid organism and as such heterozygosity renders MLST analysis more complicated. Heterozygosity from electropherograms can be inferred by a double peak (with two bases) at the same variable bi-allelic site [23]. One consequence of multiple bi-allelic sites is that of ambiguous allelic phase within loci and also ambiguous combinations of alleles across separate loci. However, it is possible for diploid sequence data, (without phase resolution) to be modified, concatenated across multiple loci [26] and applied in distance based phylogenetic methods for lineage assignment (see Materials and Methods). From these data one can also infer evolutionary relatedness, and detect gene mosaics, within or between homozygous gene loci [27]. A MLST approach specific to T. cruzi was first utilised by Machado and Ayala using two nuclear loci [8] in their study of the genetic recombination in natural T. cruzi populations and this is now being expanded by others to additional targets [28]. However the use of multiplex target genes is not recommended due to the possibility of non-identical paralogous copies confusing phylogenetic signals. The repetitive nature of the T. cruzi genome has previously hampered the search for suitable single copy targets; at least 50% of the T. cruzi genome consists of tandemly repeated genes [29]. However, the recent publication of a draft chromosomal level assembly by Weatherly et al. [30] has enabled both the determination of copy number and chromosomal distribution of markers to be established with reasonable confidence. A standardised panel of suitable MLST gene loci has yet to be developed for T. cruzi. Here we address this omission by sequencing and assessing 9 nuclear targets and evaluating them with a cohort of reference strains representing the known lineages. We demonstrate the potential of this formalised MLST for describing lineage assignment, describing inter-DTU relationships and for high resolution population genetic analysis of T. cruzi.

Materials and Methods

Isolates

A panel of 39 individual isolates (Table 1) was assembled, consisting of cloned reference strains encompassing all of the known DTUs (Table 1) spanning wide geographical and varied ecological origins. Isolates were previously characterised to DTU level by amplified fragment length polymorphisms (RFLP) in the D7 divergent domain of the 24Sx rRNA, and restriction fragment length polymorphism (RFLP) in the heat shock protein 60 (HSP60) and GPI genes [18]. Parasites were cultivated in supplemented RPMI liquid medium at 28°C, as described previously [31]. Genomic DNA was prepared from logarithmic phase cultures using DNeasy kits (Qiagen, UK).

Choice of loci

Initially, 11 genes were investigated. This number was subsequently reduced to 9 single copy targets after screening for reliability of PCR amplification. The excluded genes were trypanothione-dependent glutathione I (gene ID, Tc00.104705351 0659.240) and cyclophilin (gene ID, Tc00.1047053510947.50). Targets were verified by PCR amplification and sequences submitted for BLAST (blastsn) analyses hosted at NCBI. Copy number of targets was verified by submission of gene IDs to TriTrypDB 2.2 (http://TriTrypDB.org). The 9 single copy gene fragments amplified for MLST analysis were ascorbate-dependent haemoperoxidase (TaAPX), dihydrofolate reductase-thymidylat synthase (DHFR-TS), glutathione-dependent peroxidase II (TcGPXII), mitochondrial peroxidase (TcMPX), trypanothione reductase (TR), RNA-binding protein-19 (RB19), metacyclin-III (Met-III), metacyclin-III (Met-III) and LYT1. The ratio of non-synonymous to synonymous amino acid changes (dN/dS) was calculated according to the Nei-Gojobori method [32] using SNAP software available at http://www.hiv.lanl.gov, [33] to infer relative selection pressures. Genes possessing a dN/dS ratio <1 meet the criteria for stabilising selection for the conservation of
metabolic function. Taylor & Fisher [34] recommended the incorporation of some loci with a dN/dS ratio of >1 in order to obtain sufficient sequence diversity.

**Table 1.** Cohort of reference clonal isolates representing the six known *T. cruzi* lineages (DTUs).

| Strain     | DTU | Origin                  | Host          |
|------------|-----|-------------------------|---------------|
| C8 cl1     | TcI | La Paz, Bolivia         | *Triatoma infestans* |
| X101       | TcI | Belém, Brazil           | *Homo sapiens* |
| JR cl4     | TcI | Anzoategui, Venezuela   | *Homo sapiens* |
| PI (C007)  | TcI | Carajas, Brazil         | *Didelphis marsupialis* |
| PII (C005) | TcI | Carajas, Brazil         | Unidentified triatomine |
| B187 cl10  | TcI | Pará State, Brazil      | *Didelphis marsupialis* |
| SAXP18 cl1 | TcI | Majes, Peru             | *Didelphis marsupialis* |
| 92101601P cl1 | TcI | Georgia, U.S.A.        | *Didelphis marsupialis* |
| Esn cl3    | TcII| São Felipe, Brazil      | *Homo sapiens* |
| Pot7a cl1  | TcII| San Martin, Paraguay    | *Triatoma infestans* |
| Pot7b cl2  | TcII| San Martin, Paraguay    | *Triatoma infestans* |
| Tu18 cl2   | TcII| Tupiza, Bolivia         | *Triatoma infestans* |
| Chaco23 col4 | TcII | Chaco, Paraguay         | *Triatoma infestans* |
| M5631 cl5  | TcII| Marajo, Brazil          | *Dasyus novemcinctus* |
| M6421 cl6  | TcII| Belém, Brazil           | *Dasyus novemcinctus* |
| ARMA 18 cl3 | TcII | Campi Lorro, Paraguay  | *Dasyus novemcinctus* |
| ARMA 13 cl1 | TcII | Campi Lorro, Paraguay  | *Dasyus novemcinctus* |
| JA2 cl2    | TcII| Amazonas, Brazil        | Unknown       |
| CM25 cl2   | TcII| Carimagua, Colombia     | *Dasyusprocta fuliginosa* |
| 85/847 cl2 | TcII| Alto Beni, Bolivia      | *Dasyus novemcinctus* |
| SABP19 cl5 | TcII| Peru                    | *Triatoma infestans* |
| SC10R cl1  | TcV | Georgia, U.S.A.         | *Procyon lotor* |
| 92122102R  | TcV | Georgia, U.S.A.         | *Procyon lotor* |
| 10826      | TcV | Santa Cruz, Bolivia     | *Aotus Sp.*    |
| Canil cl1  | TcV | Belém, Brazil           | *Homo sapiens* |
| Saimiri cl1 | TcV | Venezuela               | *Saimiri sciureus* |
| PARA4 cl3  | TcV | Paraguarí, Paraguay     | *Triatoma infestans* |
| PARA6 cl4  | TcV | Paraguarí, Paraguay     | *Triatoma infestans* |
| Sc43 cl1   | TcV | Santa Cruz, Bolivia     | *Triatoma infestans* |
| 92–80 cl2  | TcV | Santa Cruz, Bolivia     | *Homo sapiens* |
| Chaco2 cl3 | TcV | Chaco, Paraguay         | *Triatoma infestans* |
| Vinch101 cl1 | TcV | Limari, Chile          | *Triatoma infestans* |
| PAH179 cl5 | TcV | Chaco, Argentina        | *Homo sapiens* |
| CL Brener  | TcVI| Rio Grande do Sul, Brazil| *Triatoma infestans* |
| Tula cl2   | TcVI| Tuleuhuen, Chile        | *Homo sapiens* |
| P251 cl7   | TcVI| Cochabamba, Bolivia     | *Homo sapiens* |
| EPV20-1 cl1 | TcVI | Chaco, Argentina        | *Triatoma infestans* |
| LHVA cl4   | TcVI| Chaco, Argentina        | *Triatoma infestans* |
| VFRA1 cl1  | TcVI| Francia, Chile          | *Triatoma infestans* |

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**PCR amplification**

Primers and annealing temperatures for PCR amplification are given in Table 2. For *DHFR-T3* and *TR*, cyclic amplifications were performed with an initial denaturation step for three minutes at 94°C, followed by 30 amplification cycles (94°C for 30 seconds, 58°C for 1 minute, 72°C for 2 minutes). Annealing temperatures were 55°C for *TaAPX*, 50°C for *TaAPX* and 62°C for *TaGPXII*. Reaction conditions for *Met-II*, and *RB19* were as follows: 3 minutes at 94°C, followed by 30 amplification cycles (94°C for 30 seconds, 53°C for 30 seconds, 72°C for 45 seconds; annealing temperatures were 51°C for *Met-III* and 56°C for *LIT1*). All reactions had a final ten minute elongation step at 72°C. Each 20 μl total reaction volume contained: 125 ng genomic DNA, 1 μl of each primer (20 pmol/μl), 2 μl dNTPs (2 mM), 0.8 μl (50 mM) MgCl₂, and 5U *Taq* (BIO-21086, Bioline, UK).

PCR products were purified with QIAQuick PCR purification kits (Qiagen) or SureClean (Bioline, UK). Bi-directional sequencing was performed with Big Dye Terminator Cycle Sequencing V3.1 (Applied Biosystems) in ABI PRISM 377 DNA Sequencers (Applied Biosystems) according to the manufacturer’s protocol. Gene fragments were sequenced in both directions (5’ and 3’) with the PCR primers described in Table 2. Additionally, in the case of *LIT1*, *Met-II*, *Met-III*, *DHFR-T3* and *TR* internal primers were used to obtain full sequence coverage. Sequence data were assembled manually in BioEdit v7.0.9.0 sequence alignment editor software (Ibis Biosciences, USA) and ambiguous peripheral regions of aligned sequences discarded to produce unambiguous partial gene sequences for each isolate. Chromatograms were examined visually in both directions and in most instances the results easily interpreted as heterozygous when two peaks in a chromatogram overlap. Re-sequencing was undertaken if results were ambiguous.

**Strain differentiation by diploid sequence typing**

The number of sequence types (STs) for each gene fragment was identified from SNP data across the panel of isolates and the discriminatory power (DP, genotypes recovered per unique isolate tested) for each gene determined. STs were conjoined across gene fragments in order to identify a diploid sequence type (DST) and to assess overall discriminatory power (DP). DSTs were applied to eBURST software to infer evolutionary relationships and founders. The eBURST algorithm [http://eburst.mlst.net] identifies related sequences and predicts a founding genotype (based on the most overrepresented genotype) with variants identified depending on the number of different loci.

Phylogenetic analysis was performed on modified sequences using the methodology described by Tavanti et al. [26] which renders diploptic data suitable for use in MEGA analysis [35] to produce distance based dendrograms. Briefly, variable loci between isolates can be considered either homozygous or heterozygous, assuming that *T. cruzi* between isolates can be considered either homozygous or heterozygous, assuming that homozygous variable locus scored as A (adenine) was heterozygous, assuming that...
### Table 2. Details of gene targets.

| Gene   | Gene ID     | Chromosome Number | Primer Sequence (5'→3') | Annealing Temp. (C) | Amplicon Size (bp) | Sequence Start 5' | Sequence Start 3' | Fragment Length (bp) |
|--------|-------------|-------------------|--------------------------|---------------------|--------------------|-------------------|-------------------|----------------------|
| LYT1   | Tc00.1047053508045.40 | 22 | CAACCTGCCTTCCTTGCCTCC (20) | 56 | 804 | TTGTGAG | GGTGGA | 691 |
| Met-II | Tc00.1047053510889.28 | 6 | TGTGTGACCTTGCCTCTTC (20) | 53 | 702 | CATTTTC | TTTGCA | 390 |
| Met-III| Tc00.1047053510943.44 | 36 | GTGGAACGCTTTGCTTCC (20) | 51 | 824 | TTACCTCG | TTTCCTTG | 619 |
| RB19   | Tc00.1047053507515.60 | 29 | GCCCTCACCCTTCGCTTCC (20) | 53 | 408 | GTGTGCG | CCCAGCT | 350 |
| TcAPX  | Tc00.1047053506193.60 | 36 | GGGAAGCTTTCACTTGCTTTC (30) | 55 | 1500 | GCGAGTC | GGCGCCG | 799 |
| TcMPX  | Tc00.1047053509153.90 | 27 | CGCTGTTTAAGATCCGTGCC (22) | 58 | 1473 | GGGGGA | GACGCTTC | 437 |
| DhFR-TS| Tc00.1047053511019.99 | 35 | GGCAAGCGCTTTGCTTTC (20) | 62 | 487 | TGGCGCC | ATTCGGC | 487 |
| TcGPXII| Tc00.104705351019.99 | 37 | ACTTGAGCCAGGTGTGAGT (21) | 58 | 1290 | TGCTATG | TACGAAGG | 602 |

**Note:**
- Primer sequences are provided for each gene target.
- The table lists the annealing temperature, amplicon size, and sequence details for each target.
- Fragment lengths are calculated based on the primer sequences provided.
Analysis of recombination at the level of individual genes was applied to isolates with unambiguous phase applied through the software package RDP3 [36] incorporating the following methods: RDP [37], Bootscanning [38], GENECONV [39], Maximum Chi Square method [40,41], the Chimaera method [40], the Sister Scanning Method [42], the 3SEQ method [43], the Reticulate compatibility matrix method [44] and the TOPAL DSS method [45,46].

**Results**

**Chromosomal localisation of markers**

Gene IDs were confirmed by BLAST searches and submitted to the TriTrypDB 1.3 (http://TriTrypDB.org) to identify chromosomal location. Locations for each of the the genes under study are shown in Table 2. Two pairs of genes were physically linked: genes LYT1 (367579–369237) and TcMPX (P:120685–121365) on chromosome 22, and MET-III (P:945156–945731) and TcAPX (P:1142201–1143187) on chromosome 36. The remaining genes were on independent chromosomes. The ratios of nonsynonymous to synonymous amino acid substitutions, resulting from sequence polymorphisms, were 1.0 or less for 7 genes, two genes possessed dN/dS ratios of above 1 (Table 3).

**Nucleotide polymorphisms and amino acid changes**

The 304 nucleotide polymorphisms among the nine sequenced fragments resulted in 121 nonsynonymous changes in amino acids encoded by sequence-variable triplets. Up to three different amino acids were present in different isolates at the same locus across the panel. A deletion of 3 nucleotides (AAA = Lysine at position 179–181) was present in C8, X10/1 and SAXP18 (TcI) within LYT1. Within TcMPX, a single non-synonymous nucleotide polymorphism resulted in a conservative amino acid change between an aspartic acid residue and a glutamic acid residue was evident (position 21). All TcV representatives possessed glutamic acid but all other panel isolates (including TcVI) possessed aspartic acid residues at the corresponding position.

**Discriminatory power of MLST targets by diploid sequence typing**

Diploid sequence typing using 9 genes was able to discriminate 38 of 39 isolates (DP 0.97, Table 4). Importantly, using only 6 genes (Met-II, Met-III, RB19, TcMPX, DHFR-TS and TR) the same number of DSTs could be identified. Only two isolates, Sc43 and Vinch101, shared a DST (29). Both are positioned within the DTU TcV which is known to possess a particularly homogenous population structure by microsatellite analysis [10]. All other isolates, notably even those those within the relatively homogenous clade TcVI, produced a unique isolate specific DST. Discriminatory power was determined for each of the 9 gene fragments (Table 4), in decreasing power, as follows: Met-II, Met-III and RB19 (0.59), LYT1 (0.56), TR (0.54), DHFR-TS and TcGPXII (0.49), TcMPX (0.41), and TcAPX (0.30). No single gene was able to distinguish all 39 reference strains. Met-II, Met-III and RB19, taken individually, were the most discriminatory genes, all three identifying 24 separate genotypes from the panel of 39. TcAPX was the least resolutive marker distinguishing 15 of 39 isolates (DP 0.38). Table 4 shows in detail the number of STs (sequence types) that each individual gene fragment resolved, and also the derived DSTs obtained from the concatenation of all 9 STs for each isolate. Table 4 also indicates the reduced panel of 6 genes required to obtain the same 38 DSTs. eBURST analysis of the genotypes and DSTs for 39 T. cruzi isolates from the panel revealed one cluster of 7 DSTs within TcV as the only related set to emerge from this analysis. Vinch101 (Limari, Chile) and Sc43 (Santa Cruz, Bolivia), both DST 29 (Table 4) are the predicted genetic founders of isolates of all other Tc-V isolates. The remaining isolates across other DTUs appeared as unrelated singletons (isolates that do not belong to any cluster). Overall the results reveal that diploid sequence typing using just 6 genes is highly discriminatory.

**Intra DTU diversity**

Table 5 describes the levels of diversity seen in each gene fragment represented as the number of variable sites per DTU (VS), the number of genotypes differentiated per DTU (GT) and the discriminatory power for each gene fragment for each DTU (DP). The number of genotypes identified at the intra DTU level, varied widely and is correlated to the SNP diversity within each gene fragment. The most and least discriminatory genes for each of the six DTUs are also shown in detail in Table 5. LYT1 and RB19 genes revealed the most diversity for TcI discriminating 7 of 9 isolates (DP 0.88). Two genes DHFR-TS and Met-III gave the highest resolution for TcII (DP 0.80), generally considered to be a relatively homogenous clade, and revealed surprising discriminatory ability, distinguishing 4 of 5 isolates. A single gene (RB19) was able to distinguish all 8 reference isolates within TcIII (DP 1.0). Six of 9 genetic loci were able to discriminate individually the full panel of 5 TcIV reference strains (DP 1.0). DTUs Tc-V and Tc-VI have previously been shown to have homogenous population

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**Table 3. Properties of nine T. cruzi MLST targets.**

| Gene Fragment | No. Of Polymorphic Sites | No. Of Genotypes | No. Of Genotypes/Polymorphism (Typing Efficiency) | Ratio Of Nonsynonymous To synonymous Changes |
|---------------|--------------------------|------------------|-----------------------------------------------|------------------------------------------|
| LYT1          | 47                       | 22               | 0.47                                          | 0.833                                    |
| Met-II        | 51                       | 24               | 0.47                                          | 0.880                                    |
| Met-III       | 50                       | 24               | 0.48                                          | 0.440                                    |
| RB19          | 27                       | 24               | 0.89                                          | 0.129                                    |
| TcAPX         | 27                       | 14               | 0.62                                          | 2.04                                     |
| TcMPX         | 15                       | 16               | 1.06                                          | 0.061                                    |
| DHFR-TS       | 32                       | 19               | 0.59                                          | 0.088                                    |
| TcGPXII       | 27                       | 18               | 0.66                                          | 0.502                                    |
| TR            | 28                       | 21               | 0.75                                          | 1.964                                    |

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structures with low intralineage diversity. Nevertheless, TcAPX resolved three of 5 isolates within TcV (DP 0.43) and TcMPX 5 of 6 (DP 0.83) isolates within DTU VI.

Phylogenies and incongruence
In addition to the derivation of DSTs, neighbor-joining trees for individual and concatenated gene fragments were constructed, representatives of which are shown in Figures 1 and 2. Concatenation using all of the original 9 gene fragments generated phylogenies with the expected DTU assignments for all 39 isolates with much higher bootstrap values than for individual genes (supporting information, Figure S1); there were no unexpected outliers. However, bootstrap support distinguishing TcV and VI was low (21%), but to distinguish between these DTUs concatenation of just two genes (DHFR-TS and LYT1 sequences) generated robust lineage assignment, and also two separate clusters within

| Table 4. Sequence types (STs) and diploid sequence types (DSTs) for nine gene fragments. |
|---------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Strain                          | DTU | DST | LYT1 (ST) | Met-II* | Met-III* | RB19* | TcAPX | TcMPX* | DHFR-TS | TcGPXII | TR* |
| C8 cl1                          | TcI | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   |
| X10/1                           | TcI | 2   | 2   | 2   | 1   | 2   | 2   | 1   | 1   | 2   | 1   |
| JR cl4                          | TcI | 3   | 3   | 3   | 2   | 3   | 3   | 1   | 1   | 3   | 1   |
| PII (C007)                      | TcI | 4   | 4   | 1   | 1   | 4   | 2   | 1   | 1   | 1   | 2   |
| PII (C005)                      | TcI | 5   | 5   | 4   | 3   | 1   | 2   | 1   | 1   | 1   | 3   |
| B187 cl10                       | TcI | 6   | 6   | 4   | 1   | 5   | 2   | 1   | 1   | 4   | 4   |
| SXP18 cl1                       | TcI | 7   | 1   | 1   | 1   | 6   | 2   | 1   | 1   | 1   | 5   |
| 92101601P cl1                   | TcI | 8   | 7   | 1   | 4   | 7   | 2   | 1   | 2   | 3   | 6   |
| Esm cl3                         | TcII| 9   | 8   | 5   | 5   | 8   | 4   | 2   | 3   | 5   | 7   |
| Po7a cl1                        | TcII| 10  | 8   | 5   | 6   | 8   | 4   | 3   | 4   | 5   | 7   |
| Po7b cl2                        | TcII| 11  | 8   | 5   | 8   | 4   | 3   | 4   | 5   | 8   |
| Tu18 cl2                        | TcII| 12  | 9   | 6   | 7   | 9   | 5   | 2   | 5   | 5   | 8   |
| Chaco23 cl4                     | TcII| 13  | 10  | 7   | 8   | 9   | 4   | 2   | 6   | 6   | 8   |
| M6361 cl5                       | TcIII| 14 | 11  | 8   | 9   | 10  | 6   | 4   | 7   | 7   | 9   |
| M6421 cl6                       | TcIII| 15 | 11  | 9   | 10  | 11  | 7   | 4   | 7   | 8   | 10  |
| ARMA18 cl3                      | TcIII| 16 | 12  | 10  | 10  | 12  | 6   | 5   | 7   | 8   | 11  |
| ARMA13 cl1                      | TcIII| 17 | 12  | 9   | 11  | 13  | 6   | 5   | 7   | 9   | 11  |
| JA2 cl2                         | TcIII| 18 | 13  | 11  | 12  | 14  | 6   | 4   | 8   | 10  | 10  |
| CM25 cl2                        | TcIII| 19 | 14  | 12  | 13  | 15  | 7   | 4   | 7   | 11  | 12  |
| 85/847 cl2                      | TcIII| 20 | 15  | 11  | 14  | 16  | 6   | 4   | 9   | 12  | 13  |
| SABP19 cl5                      | TcIII| 21 | 16  | 13  | 15  | 17  | 6   | 6   | 7   | 7   | 11  |
| SC10R cl1                       | TcIV| 22  | 17  | 14  | 16  | 18  | 8   | 7   | 10  | 13  | 14  |
| 92122102R                       | TcIV| 23  | 17  | 15  | 17  | 18  | 8   | 8   | 11  | 14  | 15  |
| 8R26                            | TcIV| 24  | 18  | 16  | 18  | 19  | 9   | 9   | 12  | 15  | 16  |
| Can III cl1                     | TcIV| 25  | 19  | 17  | 19  | 20  | 10  | 10  | 13  | 16  | 17  |
| Sainiri3 cl1                    | TcIV| 26  | 20  | 18  | 20  | 20  | 11  | 11  | 14  | 17  | 18  |
| PARA4 cl3                       | TcIV| 27  | 21  | 19  | 21  | 21  | 12  | 11  | 15  | 18  | 19  |
| PARA6 cl4                       | TcIV| 28  | 21  | 20  | 22  | 21  | 12  | 11  | 15  | 18  | 19  |
| SC43 cl1                        | TcIV| 29  | 21  | 19  | 22  | 21  | 13  | 11  | 16  | 18  | 19  |
| 92-80 cl2                       | TcIV| 30  | 21  | 19  | 22  | 21  | 13  | 11  | 15  | 18  | 19  |
| Chaco2 cl3                      | TcIV| 31  | 21  | 19  | 22  | 21  | 13  | 11  | 16  | 18  | 19  |
| Vinch101 cl1                    | TcIV| 32  | 21  | 19  | 22  | 21  | 13  | 11  | 16  | 18  | 19  |
| PAH179 cl5                      | TcIV| 33  | 21  | 19  | 22  | 21  | 14  | 12  | 16  | 18  | 19  |
| CL Briner                       | TcIV| 34  | 21  | 21  | 23  | 22  | 13  | 13  | 17  | 18  | 19  |
| Tula cl2                        | TcVI| 35  | 22  | 21  | 24  | 23  | 13  | 14  | 18  | 18  | 19  |
| P251 cl7                        | TcVI| 36  | 22  | 22  | 23  | 22  | 13  | 15  | 18  | 18  | 19  |
| EPV20-1 cl1                     | TcVI| 37  | 22  | 23  | 23  | 22  | 14  | 8   | 18  | 18  | 19  |
| LHVA cl4                        | TcVI| 38  | 22  | 24  | 23  | 24  | 15  | 16  | 19  | 18  | 21  |
| Discriminatory Power            | Discriminatory Power | 0.97 | 0.56 | 0.62 | 0.62 | 0.62 | 0.38 | 0.41 | 0.49 | 0.49 | 0.54 |

*Indicate six genes required to identify the same number of DSTs as the full panel of 9 genes.

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Table 5. Intra-lineage diversity.

| SNP per DTU | Isolates | VS | GT | DP |
|-------------|----------|----|----|----|
| TcI         | 8        | 8  | 7  | 0.88|
|             | 7        | 7  | 0.88|
|             | 6        | 6  | 0.88|
| TcII        | 5        | 4  | 0.60|
|             | 3        | 3  | 0.60|
|             | 2        | 2  | 0.60|
| TcIII       | 8        | 4  | 0.50|
|             | 3        | 3  | 0.50|
|             | 2        | 2  | 0.50|
| TcIV        | 5        | 2  | 0.40|
|             | 1        | 1  | 0.40|
|             | 0        | 0  | 0.40|
| TcV         | 7        | 0  | 0.00|
|             | 1        | 1  | 0.00|
|             | 0        | 0  | 0.00|

Abbreviations in each of the gene fragment columns are as follows: VS = No variable sites per DTU, GT = Number of genotypes per DTU, DP = Discriminatory power. Numbers in bold indicate highest DP values per DTU. *All SNP variation observed within the DTU originated from a single isolate.

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Specifically, concatenated sequences of intralineage diversity. Other genes (for example TcAPX, TcMPX, Met-II, and TcIII) generated trees with high bootstrap values for both lineage assignment and comparatively high bootstraps for intralineage diversity. Conversely, TcMPX produced the least phylogenetically informative dendrograms with low associated bootstrap values.

Choice of loci for lineage assignment

The minimum number of genes required for phylogenetic assignment to DTU level was investigated according to the following criteria. Firstly, the minimum number of genes required to produce phylogenetically robust bootstrap support. Secondly, to choose those genes that produced the most consistent unambiguous sequences. Thirdly, to choose genes requiring the minimum number of internal primers. Using these criteria a minimum combination of 4 genes (Met-III, RB19, TcGPXII and DHFR-TS) enabled all reference isolates to be assigned to DTU level. Specifically, concatenated sequences of Met-III, RB19 and TcGPXII assigned isolates to DTUs TcI-TcIV (Figure 2A). Separately, DHFR-TS (the only gene requiring internal primers) distinguished the DTUs TcV and TcVI (Figure 2B, insert). Internal primers for Met-III, RB19 and TcGPXII were not required after initial optimisation (Table 2).

Intralineage phylogenies

Generally, bootstrap support within DTUs using concatenated sequences was lower than between DTUs (Figure 1) which is expected in data originating from gene fragments with non-contiguous topologies at the intralineage level. In most instances intralineage topology of individual gene dendrograms was generally low and/or not contiguous across loci (Figure 1). However, certain observations deserve closer scrutiny. Importantly, two distinct clusters within TcIV separated isolates from North America and South America. This genetic partitioning was detected in 8 out of 9 gene individual targets in the present study, indicating a robust cladistic subdivision within TcIV. Interestingly, two TcI isolates SAXP18 (Peru, Didelphis marsupialis) and C3 (Bolivia, Triatoma infestans) were isolated from silvatic and domestic sources respectively, and are genetically similar suggesting present or past overlapping transmission cycles. Relatively high bootstrap support for isolates of the TcII clade did not reveal evidence of geographical clustering and genetically similar isolates originated from a single isolate.
from distant localities. Within TcIII at least two clusters were seen (in concatenated data); silvatic isolates (from Dasypus novemcinctus) in the Paraguayan Chaco clustered with a domestic strain (SABP19) isolated from a domestic T. infestans in Peru. The 8 TcIII isolates included in our panel showed considerable diversity. However, numbers are insufficient to examine population substructuring.

Intralineage recombination

Analysis for the presence of mosaic alleles, at the level of individual genetic loci was performed using RDP [37], applied to a total of 240 representative sequences of known allelic phase (single SNP heterozygous and homozygous sequence profiles) encompassing DTUs TcI, TcII, TcIII and TcIV across the 9 genetic loci. We found no evidence of allelic mosaics within individual genes. However, diploptic SNP data revealed for three genes (LYT1, TcGPXII and TcMPX) that putative donor homozygous SNP profiles and the corresponding heterozygous profiles were present as shown in Figure 3. Specifically, within LYT1 the heterozygous isolate (PII) and putative donors (B187 and PI) were identified within TcI. For TcGPXII, 2 heterozygous isolates (ARMA18 and M6421) possess SNP profiles of the putative donor isolates CM25 and 85/847 (TcIII). Lastly, for TcMPX, SABP19 was heterozygous at a single locus with potential donors in the same DTU (TcIII). In each instance individual isolates containing heterozygous and donor SNPs were present only in a single gene and were not contiguous across loci.

SNP data and loss of heterozygosity

Our panel of isolates included 13 representatives of DTUs V and VI, which are known to be genetic hybrids of TcII and TcIII [8].

Figure 1. Phylogenetic DTU incongruence between two individual gene trees (TcI, TcIII and TcIV). *loss of heterozygosity in isolates at the Met-II locus (EPV20-1, TcVI: Para 6, TcV). doi:10.1371/journal.pntd.0001049.g001
Across all 9 loci the expected allelic heterozygous profile was observed in most individual isolates. However, at four gene loci complete LOH was detected for individual isolates within the hybrid lineages. The observations are as follows: within Met-III, two TcVI isolates (Tula cl2 and P251 cl7) possessed a TcII-like allele but not a TcIII-like allele as did an individual TcV isolate for Met-II (PARA6 cl4). In contrast a single TcVI isolate at the locus RB19 (VFRA cl1) possessed only the TcIII-like allele. At the TcMPX locus, all TcV isolates (with the exception of PAH179) appeared to have lost the TcIII-like allele and 2 representatives of TcVI (P251 and LHVA) lost the TcII-like allele; the remaining 4 TcVI isolates retained heterozygosity. LOH among individual isolates in genetic loci could be clearly visualised when dendrograms were constructed, for example, hybrid isolates PARA6 (TcV) and EPV20-1 (TcVI) for Met-II clustered within TcII (Figure 1). There was no continuity of allelic loss across different genes for individual isolates. For example, the two aforementioned isolates exhibiting homozygosity in Met-III were heterozygous in TcAPX, which are linked on chromosome 36. It is therefore evident that homozygosity has been maintained elsewhere on the same chromosome. Taken together the results indicate a return to homozygosity in 6.24% of TcV and TcIV (combined) isolates when considering the four affected gene fragments.

Discussion

Discriminatory power and diploid sequence typing

Diploid sequence typing of 39 reference isolates indicates that together the nine genes under study were highly discriminatory, 38 of 39 reference isolates generated a unique DST. It was apparent that the number of STs identified for each gene fragment varied considerably (Table 5) despite the fact that all but two of the genes (TcAPX and TR) were under stabilising selection. However, by using just 6 of the original panel of nine genes the same 38 DSTs could be identified. The reduced panel consisted of those genes with the highest overall DPs (Met-II, Met-III and RB19) with additional genes (TcMPX, DHFR-TS and TR) resolving further STs for the genetically homogenous DTUs TcV and TcVI. The reduction in the number of genes would represent a considerable saving in sequencing effort if applied to new samples. Furthermore, the reduced panel consists primarily of genes that are easily amplified and sequenced. The derived STs and DSTs applied to eBURST software, designed specifically for MLST data to infer evolutionary relationships and genetic founders, identified one cluster encompassing all of the TcV reference isolates. The inferred founder of this group (DST29) was found in 2 isolates (Vinch101 and Sc43). Both of these isolates were originally isolated.
from domestic *T. infestans* from Chile and Bolivia respectively. The ability to differentiate isolates within *TcV* is an important development, as this DTU is found throughout the Southern Cone countries in domestic cycles. Currently other methods to demonstrate diversity within *TcV* have been lacking. The high number of singletons observed in remaining DTUs was surprising, although this is probably a consequence of reference isolates spanning diverse spatial and ecological origins and diversity found within our panel. It is likely that high intensity localised sampling would reveal more easily genetic relationships between isolates. High numbers of singletons are often observed in other MLST typing schemes (for example *Candida* species) when applied to small datasets. Furthermore distance based dendrograms have been shown to correlate well with clonal clusters generated by eBURST when applied to large numbers [27]. It is also worth noting that high rates of singletons are typical of populations with a high rate of recombination relative to mutation [27]. Although eBURST is an established software for diplotyping and haplotyping pathogens [47,48] until analysis has been applied to a larger number of sympatric *T. cruzi* isolates it is difficult to evaluate the usefulness of the analysis as a tool for predicting founders and clusters in DTUs other than *TcV*, and other approaches should be considered in parallel.

Phylogenies and incongruence

A separate approach for the analysis of diplotypic MLST data is to generate phylogenetic trees utilising distance based methods and modified sequences, adapted for diplotyping. At the level of individual gene fragments, isolates were generally assigned to the predicted clades, although bootstrap support varied widely and no single gene was able to assign all isolates robustly. A noticeable observation was that of incongruent topologies in individual gene trees and in particular between DTUs *TcI*, *TcIII*, and *TcIV*. At the intra DTU level there was incongruence between dendrograms (Figure 1). The bootstraps supporting incongruence varied widely between genes and DTUs and similar patterns of incongruence have been previously observed in nuclear genes [5,49]. Specifically, our data revealed that DTUs *TcIII* and *TcIV* possessed split affinities to DTUs *TcI* and *TcII*. For 4 genes, DTUs *TcIII* and *TcIV* showed greater affinity to *TcI*. These split affinities of *TcIII* and *TcIV* to *TcI* and *TcII* are in broad agreement with the spectrum of polymorphism observed by Westenberger et al. [5]. Such incongruence is not immediately compatible with a model of strict clonality; genealogical relationships where sequences vary in topological position is a classical marker in populations that have undergone recombination.

Choice of loci for lineage assignment

Sequence data concatenated across all 9 genes produced robust phylogenetic assignment for all DTUs except for *TcV* and *TcVI*, which could be resolved using two concatenated genes (*DHFR-TS* and *LYT1* sequences). Importantly, this number could subsequently be reduced to a combination of just 4 genes (*Met-III*, *RB19*, *TcGPXII* and *DHFR-TS*) while maintaining high bootstrap support. The first three aforementioned gene sequences were concatenated to differentiate DTUs *TcI*-*TcIV*, with *DHFR-TS* dendrograms separately able to distinguish DTUs *TcV* and *TcVI*. Notably concatenated data implies a cladistic subdivision within *TcIV*, separating North American from South American isolates. Bootstrap support is a requirement for any MLST scheme, but of equal importance are reliable PCR amplifications which generate unambiguous sequences in both forward and reverse directions. All four targets meet this criterion. *Met-III*, *RB19* and *TcGPXII* do not require internal primers, although some were used initially in
the optimisation process, and generate small amplicons (824 bp, 408 bp and 487 bp respectively). DHFR-TS alone requires the use of internal sequencing primers. LYT1 was considered to be an alternative candidate, also able to distinguish TcV and TcVI, but it was technically more difficult to obtain sequences in the forward direction despite multiple attempts at optimisation.

**Intralineage recombination**

SNP data revealed that for three genes (LYT1, TeGPXII and TcMPX) putative donor homozygous SNP profiles and the corresponding heterozygous profiles, were present within TcI (a single heterozygous isolate) and TcIII (1 heterozygous isolate) one tentative explanation of the presence of heterozygous SNPs is that of a recombination signature within these DTUs. In the present paper examination 240 sequences (of known allelic phase) using an array of recombination detection algorithms did not detect evidence of allic mosaic. This result is not unexpected as diplotyping is not as sensitive as haplotyping for detecting mosaic’s or investigating the sexual reproduction in evolutionary history [50]. Future recombination analysis will include more refined phase resolution of sequences heterozygous at two or more loci, typically not required for diplotypic MLST typing schemes. However, haplotypes can be derived from current isolates reconstructed by the program PHASE [51], or more recently fastPHASE [http://deptps.washington.edu/ucw-ke expres license/ assets/fastphase/], by cloning, or by allelic specific PCR [52]. In a sexually reproducing population the frequencies the genotypes (defined by haplotypes) should be in Hardy-Weinberg equilibrium. New software to analyse haplotype data include those incorporating Bayesian methodologies; Structure [53] and Beast [54] reconstruct phylogenies with epidemiological and evolutionary informative results.

**Loss of heterozygosity**

LOH observed in 4 gene fragments affecting the hybrid lineages TcV and TcIV has potential significant consequences for MLST and lineage assignment. For example, TcMPX hybrid isolates (TcV) would be incorrectly assigned if judgment was based on a single locus due to LOH (Figure 1). There was no continuity of allelic loss across different genes for individual isolates, linked genes (chromosome 36) were homozygous in TcI and TcIII for single isolates. Incongruent topologies and the corresponding heterozygous profiles, were present within DTUs TcI and TcIII for single isolates. Incongruent topologies and the intra and inter DTU level is not consistent with a model of strict clonality. The high levels of heterozygosity detected in the gene targets offers the potential for a future deeper level of analysis of population genetics based on haplotypes.

**Supporting Information**

**Figure S1** Concatenation and lineage assignment. Unrooted neighbor-joining diplotypic tree showing p-distance for 9 concatenated gene fragments (A). Concatenation of LYT1 and DHFR-TS discriminate between DTUs V and VI (inset). Concatenated diplotypic tree using a reduced panel of 4 gene fragments (B). Concatenation of Met-III, RB19, TeGPXII and DHFR-TS assign isolates to DTUs TcI-TcIV. DHFR-TS differentiates DTUs V and VI (inset). Found at: doi:10.1371/journal.pntd.0001049.s001 (0.41 MB DOC)

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**Author Contributions**

Conceived and designed the experiments: MY MAM. Performed the experiments: MY LAM. Analyzed the data: MY LAM. Contributed reagents/materials/analysis tools: MY LAM MSL NTA TB PD HJC. Wrote the paper: MY MAM. Input into design of experiments and technical advice: ILM.

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