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Genotyping of *Trypanosoma cruzi*: Systematic Selection of Assays Allowing Rapid and Accurate Discrimination of All Known Lineages

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Abstract

Trypanosoma cruzi, the agent of Chagas disease, can be subdivided into six discrete typing units (DTUs), TcI, TcIIa, TcIIb, TcIIc, TcIIId or TcIIe, each having distinct epidemiologically important features. Dozens of genetic markers are available to determine the DTU to which a *T. cruzi* isolate belongs, but there is no consensus on which should be used. We selected five assays: three polymerase chain reaction (PCR)-restriction fragment length polymorphisms based on single nucleotide polymorphisms (SNPs) in the *HSP60*, Histone *H1*, and *GPI* loci, and PCR product size polymorphism of the LSU rDNA and mini-exon loci. Each assay was tested for its capacity to differentiate between DTUs using a panel of 48 genetically diverse *T. cruzi* clones. Some markers allowed unequivocal identification of individual DTUs, however, only by using a combination of multiple markers could all six DTUs be resolved. Based upon the results we recommend a triple-assay comprising the LSU rDNA, *HSP60* and *GPI* markers for reliable, rapid, low-cost DTU assignment.

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi*, causative agent of Chagas disease, is harbored by at least 10 million people in Latin America and is estimated to cause ~13,000 deaths per year.¹ *T. cruzi* is endemic across the vast majority of Latin America and into the southern states of the United States, but Chagas disease occurs primarily in areas where human populations come into contact with domiciliated triatomine vector species. Furthermore, blood transfusion and congenital transmission can lead to cases of Chagas disease including cases outside Latin America. Control campaigns have resulted in reduced levels of *T. cruzi* transmission across much of the endemic area, yet significant challenges remain.^{2,3} These include re-infestation of houses by vector species^{4,5} and out-breaks associated with oral transmission caused by triatomine contamination of foods and drinks.⁶⁻⁸

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Trypanosoma cruzi shows extremely high levels of genetic diversity, and a plethora of genetic markers can be used to stratify the species into various subdivisions, with greater or lesser levels of resolution depending on the markers used. Typing of genetic polymorphisms at relatively conserved loci can define major genetic subdivisions,^{9,10} whereas analysis of highly variable loci such as microsatellites¹¹⁻¹³ or kDNA minicircle sequences^{14,15} allows higher level resolution, potentially even to the level of profiles that are specific to individual strains.

An understanding of the genetic diversity of any microbial pathogen is crucial, especially for epidemiologic research and for diagnostic, evolutionary, and basic biological studies. Historically, study of *T. cruzi* genetic diversity has been hampered by a lack of standardized typing methods and the use of various alternative nomenclatures^{16,17} (Table 1). In some cases, this has led to confusion in the literature and made comparison between different studies problematic. For the purposes of molecular epidemiology, a useful conceptual development has been that of the discrete typing unit (DTU), which groups strains on the basis of shared characteristics of multilocus genotypes but without making explicit assumptions about their evolutionary relatedness.¹⁸ For *T. cruzi*, multilocus genotyping has consistently shown six distinct DTUs, TcI, TcIIa, TcIIb, TcIIc, TcIIId, and TcIIe,^{19,20} each having distinct epidemiologic and evolutionary aspects. Although many typing systems are in use for *T. cruzi*, there is a lack of data regarding comparison of different methods, particularly with respect to the relatively undersampled DTUs TcIIa and TcIIc, which are only occasionally present in domestic settings.

Two of the most commonly used *T. cruzi* genotyping assays exploit sequence variability in the D7 divergent domain of the 24Sα rRNA locus (LSU rDNA) and in the non-transcribed intergenic region of the *SL*-RNA (mini-exon) array. This permits discrimination of some of the different lineages by simple visualization of differences in polymerase chain reaction (PCR) product size^{9,10,20-22} (Table 2). Using the rDNA target, some lineages are easily genotyped because they give single band profiles, for example, 110 bp for TcI/IIc or 125 bp for TcIIb/IIe. TcIIId strains are typically characterized by the presence of both of these bands, although the larger band can be weak or absent entirely.^{10,20} Furthermore, TcIIa does not have a single characteristic band size; some isolates, including the reference strain CanIII c11, give a band smaller than 125 bp, estimated to be either 120 or 117 bp,²¹ whereas TcIIa isolates from North America [TcIIa(NA)] seem to be characterized by a 130 bp band.²⁰ Regarding the mini-exon, early studies showed a multiplex PCR assay easily differentiated TcI (350 bp) from TcIIb/IIId/IIe (300 bp).^{9,10} Using this assay to characterize ZIII isolates (TcIIa and TcIIc) has proven to be less straightforward; some authors report a lack of amplification,²⁰ whereas others have successfully amplified products of 400 bp for TcIIa and 250 bp for TcIIc.^{23,24} Others recommend the use of modified protocols using lineage-specific primers to allow discrimination of ZIII isolates.^{14,25}

A number of PCR-restriction fragment length polymorphism (RFLP) protocols have been described,^{26,27} but to date, they have only been tested on a limited number of isolates, and it is not clear which are most suitable for standardized, widespread application. We selected three of these assays that, when used in combination, showed the potential to identify TcI, TcIIa, TcIIb, TcIIc, and a joint TcIIId/TcIIe group. We set out to compare the performance of these three PCR-RFLP assays with the LSU rDNA and mini-exon genotyping assays using a large cohort of *T. cruzi* clones representing all six DTUs. We show that combining the LSU rDNA assay with two of the PCR-RFLP assays allows the simple, rapid, and low-cost resolution of all known *T. cruzi* DTUs.

MATERIALS AND METHODS

Parasite stocks and extraction of genomic DNA

A panel of 48 *T. cruzi* biological clones representing all six DTUs was assembled (Table 3). They originate from diverse localities in endemic areas and consist of isolates from sylvatic and domestic transmission cycles; their sources include triatomine vectors, mammal hosts, and infected humans; full details of their origins are given elsewhere.²⁸ Parasites were cultivated in supplemented RPMI-1640 liquid medium (Sigma, Gillingham, UK) at 28°C as described previously.²⁹ Total genomic DNA was prepared from logarithmic phase cultures using standard phenol:chloroform protocols or alternatively using the Genra Puregene Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's protocols.

PCR product size polymorphism assays

All strains were characterized by PCR amplification of the D7 divergent domain of the 24Sα rRNA gene (LSU rDNA) and the non-transcribed spacer of the mini-exon gene using standard protocols.^{10,20} Amplification reactions contained 0.2 mmol/L of each dNTP, 1.5 mmol/L MgCl₂, 1 pmol/μL of each primer, 1 Unit of *Taq* DNA polymerase (Bioline, London, UK) and 10–100 ng gDNA. For the LSU rDNA, PCR primers D71 and D72 were used, and amplifications were performed using an initial denaturation step of 94°C for 3 minutes and then 27 amplification cycles (94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute), followed by a final elongation step at 72°C for 5 minutes.¹⁰ For the mini-exon PCRs, a pool of three primers, TC, TC1, and TC2, was used, and amplifications were performed using an initial denaturation step of 94°C for 3 minutes and then 27 amplification cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds), followed by a final elongation step at 72°C for 5 minutes.¹⁰ LSU rDNA PCR products were separated by gel electrophoresis in 3% agarose gels and mini-exon products in 1.5% gels. For expected product sizes, see Table 2.

PCR-RFLP assays

All strains were characterized by restriction enzyme digestion of PCR products from the amplification of three target loci, as first described by Westenberger and others,²⁷ with minor modifications to the protocol. The following target/restriction enzyme combinations were used: heat shock protein 60 (*HSP60*)/*EcoRV*, histone *H1*/*Aa*I, and glucose-6-phosphate isomerase (*GPI*)/*Hha*I. Amplification reactions were as above but contained 2 mmol/L MgCl₂, and the primer pairs used were as follows: HSP60_for and HSP60_rev (for *HSP60*),³⁰ H1_for and H1_rev (for histone *H1*),³⁰ and GPI_for and GPI_rev (for *GPI*).³¹ Amplifications were performed using a touchdown PCR strategy comprising an initial denaturation step of 3 minutes at 94°C, followed by four cycles (94°C for 30 seconds, 64°C for 30 seconds, 72°C for 1 minute), followed by 28 cycles (94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute), and then a final elongation step at 72°C for 10 minutes. PCR products were checked on 1.5% agarose gels and if necessary purified using the Qiaquick gel extraction kit (Qiagen) to remove non-specific products. For restriction enzyme digestion, 10 μL of PCR product (typically ~1 μg) was digested in a reaction containing 0.25 U/μL of the appropriate restriction enzyme, i.e., *EcoRV* (Promega, Southampton, UK), *Hha*I (NEB, Hitchin, UK), or *Aa*I (NEB, Hitchin, UK), 100 ng/μL BSA, and 1× quantity of the manufacturer's recommended reaction buffer in a total volume of 20 μL. The digestion reactions were incubated for 4 hours at 37°C, after which 5 μL of the reaction was used for restriction fragment size analysis using either 1.5% agarose gels (*GPI/Hha*I) or 3% gels (*HSP60/EcoRV* and histone *H1/Aa*I). For expected product sizes, see Table 2.

RESULTS

LSU rDNA PCR product size polymorphism

All samples were readily genotyped at this locus (Figure 1; Table 3). As expected, all TcI and TcIIc strains gave 110-bp bands and all TcIIb and TcIIe strains gave 125-bp bands. TcIIId strains always had the 110-bp band; usually, the 125-bp band was also present, producing the characteristic double band profile. However, the intensity of the 125-bp band was variable across independent replicates and was sometimes not visible (Figure 1A). CanIII c11 and three other TcIIa strains gave the expected intermediate-sized product (Figure 1B; Table 3), considered in this study to be 117 bp in accordance with Kawashita and others, 21 rather than 120 bp.²⁰ One South American TcIIa strain, Saimiri3 c11, generated the 125-bp normally characteristic for TcIIb/IIe as shown previously.²⁰ The TcIIa(NA) strains gave 130-bp bands, as found previously for other strains of the same origin.²⁰ In summary, genotyping of this locus confirmed its general utility as a discriminatory marker for *T. cruzi* lineages; however, it does require very small differences in band size (5 bp) to be resolved, which can be technically challenging.

Mini-exon PCR product size polymorphism

All samples were genotyped using PCR amplification of the mini-exon gene (Figure 1C; Table 3). In total, eight isolates were typed as TcIIc (250-bp products), 24 as TcIIb/d/e (300 bp), eleven as TcI (350 bp), and three as TcIIa (400 bp). Compared with the original multiple locus enzyme electrophoresis (MLEE) and/or random amplified polymorphic DNA (RAPD) genotyping, most strains gave product sizes as expected. One strain, Saimiri3 c11, gave a product estimated to be 380 bp (Figure 1C); a previous study had recorded no amplification for this strain.²⁰ Another, 10R26, gave no amplification despite repeated attempts, although this strain has previously been reported to contain a TcI-like sequence.³⁰

It should be noted that for a number of the strains previously typed as TcIIc (Table 3), the mini-exon exhibited a lack of reproducibility across repeated experiments: the diagnostic 250-bp band was often faint, and multiple non-specific bands were observed more frequently compared with other strains. As an example, profiles showing bands at both 250 and 300 bp were observed for ARMA13 c11 and SABP19 c11 (Figure 1C; Table 3). Difficulty in genotyping TcIIc strains by mini-exon PCR is in keeping with previous findings.²⁰ Nevertheless, in this study, the diagnostic 250-bp band was not observed for any other strains and thus was considered to be indicative of a TcIIc genotype.

The TcIIa strains showed heterogeneity in the size of the mini-exon amplification product and also frequently presented non-specific bands. Besides the two strains already mentioned (10R26 and Saimiri3 c11), CanIII c11, 92122102R, and StC10R c11 did give products of 400 bp, but X10610 c15 and ERA c12 produced bands of 350 bp, normally characteristic of TcI.

PCR-RFLP assays

A recent study detailed the development of six PCR-RFLP assays for genotyping *T. cruzi* lineages.²⁷ According to the data presented for a panel of 26 *T. cruzi* isolates (ten of which are also in our panel), there was no single assay that could split the strains into more than three groups of genotypes. However, by combining the data from three of these assays (*HSP60* digested with *EcoRV*, histone *H1* digested with *AatII*, and *GPI* digested with *HhaI*), all DTUs except TcIIId/IIe would be predicted to have unique multiple assay profiles. To validate the potential of this typing scheme, the three PCR-RFLP assays were applied to all samples.

HSP60—Minimal non-specific PCR products for *HSP60* were observed, and this meant that the digestion reaction could be performed directly on PCR products without the need for purification, thus reducing the time needed for this assay. An example of RFLP profiles generated by this assay is shown in Figure 2. The genotype group designations (TcI/IIa/IIb, TcIIc, or TcIIId/IIe)²⁷ were applied to the entire cohort of samples. Across the whole panel of *T. cruzi*, the genotype assignments showed an exact correlation with those predicted by other genotyping methods (Table 3). Notably, this assay proved to be a reliable method to discriminate TcIIc strains from all other lineages.

Histone H1—The PCR-RFLP of histone *H1* was performed for all samples and produced the same three profiles (TcI/IIc, TcIIb, or TcIIa/IIId/IIe) identified by Westenberger and others²⁷ (Figure 2; Table 2). Digestion of unpurified PCR products, in some cases, generated some non-specific bands, predominantly one ~320 bp in size. Such bands, however, did not hamper detection of the diagnostic bands. Comparison of the genotype assignments with expectations showed that 45 of 48 samples in this study gave RFLP profiles consistent with these expectations. Exceptions were Saimiri3 c11, StC10R c11, and 92122102R, all of which gave TcI/IIc profiles rather than the expected TcIIa/IIId/IIe profile. This single assay reliably resolved DTU TcIIb from all others.

GPI—As for *HSP60*, the digestion reaction for *GPI* amplification products could be performed directly without the need for purification. All the *T. cruzi* isolates in this study gave RFLP profiles (Figure 2) that could easily be assigned to the possible genotype groups (TcI/IIc, TcIIa/IIb, or TcIIId/IIe) identified by Westenberger and others.²⁷ Genotypes were consistent with those expected for each DTU based on other markers for 46 of 48 samples (Table 3). Exceptions were StC10R c11 and 92122102R, both of which gave TcI/IIc profiles rather than the expected TcIIa/IIb profile.

DISCUSSION

For many years, MLEE was the method of choice for resolving *T. cruzi* subgroups. With the advent of direct genetic typing, a range of PCR-based assays capable of delineating *T. cruzi* subdivisions to varying extents were developed and readily applied. However, there has been a tendency for the number of loci used for typing to be reduced to only two that are widely used (LSU rDNA and mini-exon) or various additional loci that are only used by a small number of laboratories. This creates the problem of reduced discriminatory power and/or difficulty in comparing work in different laboratories using different typing systems. Although high-resolution genetic typing can now be achieved using multilocus sequence typing (MLST)^{27,32,33} or multilocus microsatellite typing (MLMT),^{11,13,34} these methods are impractical for simple DTU assignment. For that objective, PCR-RFLP assays hold much promise given the low resource requirement. We selected three PCR-RFLP assays based on data from an analysis of 26 strains showing that they had good potential for differentiating between *T. cruzi* DTUs.²⁷ We set out to test the performance of these assays for typing a large cohort of cloned *T. cruzi* isolates and compared them to the commonly used mini-exon and LSU rDNA typing assays.

Typing of the LSU rDNA allowed discrimination of the following groups: TcI/TcIIc, TcIIb/TcIIe, and most TcIIId samples. Brisse and others²⁰ showed that this marker also allowed resolution of two groups within TcIIa corresponding to strains from North America and South America because of unique profiles for each of these groups. This result was also observed here for four additional samples, strengthening the likelihood that there are conserved differences at this locus between North and South American TcIIa strains, and in keeping with some molecular data indicating a significant divergence.^{32,35,36} However, caution is needed when drawing such conclusions, as exemplified by the finding that another

TcIIa strain, Saimiri3 c11, has a TcIIb/TcIIe profile.²⁰ There are additional concerns for the resolution of TcIIc because the double band profile that specifies TcIIc was not always observed. A single band at this locus is a feature that has also been noted for a small number of other TcIIc strains including Sc43 c11,^{10,20} for which the double band profile was detected in this study. This could be a result of differences in experimental conditions or genuine genetic differences between the stocks of the same name in the different laboratories. A drawback of this genotyping method is the requirement to distinguish between bands that are only 5 bp different in size, which can be technically challenging unless appropriate reference strains are used as standards in each analysis.

The mini-exon marker reliably discriminated DTU TcI and a combined group of TcIIb, TcIIc, and TcIIe. Typing of DTUs TcIIc and TcIIa was less reliable, in keeping with previous reports.²⁰ Repeated assays were often needed to confirm TcIIc profiles, and in some cases TcI-like bands were observed, raising the possibility of misclassifications. TcIIa strains gave a number of different profiles, which in some cases would lead to incorrect DTU assignment if this marker was used alone. One reason for the variable TcIIc and TcIIa profiles is the presence of insertions and/or deletions within the target locus, influencing the efficiency of primer binding.²⁰ Such indels have been characterized in two TcIIc isolates and a TcIIa isolate,²⁵ but the data presented here suggest that these may not be conserved features within and/or between these sublineages. Furthermore the mini-exon locus exhibits significant secondary structure,³⁷ which could also adversely affect amplification in some cases. Although the mini-exon assay is clearly inferior to the others tested here in terms of its reliability, direct sequencing of the *SL-rRNA* locus does have potential for the characterization of intra-DTU diversity, as, for example, shown by a recent study of TcI strains in Colombia.³⁸

The three PCR-RFLP assays are clearly useful additions to the repertoire of available *T. cruzi* genotyping protocols. The data presented here are mostly consistent with expectations based on the original study.²⁷ If each assay is considered separately, there are two cases where unique profiles that are specific to a single DTU were observed. First, the *HSP60/EcoRV* assay reliably discriminated TcIIc strains, and second, the histone *H1/AaII* assay generated TcIIb-specific profiles. The application in combination of the three RFLP markers assessed in this study proved to reliably discriminate all strains into the four non-hybrid DTUs and a fifth combined TcIIc/IIe hybrid group, agreeing with the results from the analysis of Westenberger and others²⁷ of 26 strains. However, exceptions to this general rule occurred in the case of the histone *H1/AaII* assay for TcIIa(NA) strains and Saimiri3 c11 and also with the *GPI/HhaI* assay, again with TcIIa(NA) samples. These discrepancies, caused by point mutations in a relevant restriction site, may or may not reflect a more substantial overall divergence between such North American strains and other members of TcIIa; full sequencing of the target loci will be needed to resolve this question. Overall, these assays were simple to perform and, although they require an extra experimental step and additional reagents compared with the mini-exon or LSU rDNA assays, they seem to be less subject to equivocal results. On the other hand, only LSU rDNA is capable of separating TcIIc and TcIIe samples.

None of the individual markers tested here allowed complete DTU resolution, and in any case, reliance on a single marker would be inadvisable because of the consequent loss of resolution and the potential influence of genetic exchange on some lineages. Using a combination of multiple assays, therefore, permits more reliable DTU assignment. Brisse and others²⁰ proposed a multiple assay system based on a combination of mini-exon, LSU rDNA and 18S rRNA (SSU rDNA) PCR product size polymorphism assays. Although this strategy does permit assignment into each of the six DTUs, the mini-exon assay seems to

lack reproducibility in some cases, and several of the assignments depend on the absence, rather than the presence, of bands, which is inadvisable.

The data presented here show that the application of another combination of markers, including PCR-RFLPs, can achieve the same level of resolution with all assignments depending on the presence of specific band sizes. Only the LSU rDNA marker and the mini-exon are able to distinguish TcIIa(NA) from TcI, so one of these assays should be included. The results of this study lead to a strong preference for LSU rDNA because it allows separation of TcIIId from TcIIe and is much more reliable for typing of TcIIa and TcIIc. Follow-up with RFLP of *HSP60* could resolve all six DTUs. However, each of the RFLPs relies on the presence or absence of either one or two SNPs, which may be affected by mutations in as yet untested strains. It would therefore seem sensible to include a second PCR-RFLP assay in addition to *HSP60/EcoRV*. The *GPI/HhaI* assay has two advantages over histone *H1/AaII*: first, the absence of non-specific bands, and second, a larger, and therefore more easily visible, smallest digestion product.

In this study, the combination of LSU rDNA PCR with *HSP60/EcoRV* and *GPI/HhaI* PCR-RFLPs reliably determined the “correct” DTU for 45 of the 48 cloned isolates. The first exception was that the two TcIIa strains from North America gave TcI/Ic-type RFLP profiles, although they could still be identified by the characteristic 130bp LSU rDNA PCR product. Second, strain Saimiri3 c11, which has been typed as TcIIa by MLEE and RAPD,¹⁹ had atypical profiles for several of the single-locus genotyping assays used here. The existence of a minority of strains that do not fit comfortably into the DTU concept, should, however, be viewed as an interesting feature of the species rather than an inadequacy of a genotyping system that works most of the time. Complications caused by the existence of such rare isolates or others from as yet unsampled populations are likely to be unavoidable without the application of more markers. Observation of unexpected multilocus genotypes could indicate as yet undiscovered lineages or recombinant strains that warrant further study, for instance by MLST. MLST has not only allowed the identification of recombination in *T. cruzi*^{32,33} but also in other eukaryotic pathogens, including *Leishmania* spp.,^{39,40} *Giardia lamblia*,⁴¹ *Toxoplasma gondii*,⁴² and *Candida* spp.^{43,44} Nevertheless, as a tool for simple DTU assignment, our data show that a triple-assay comprising LSU rDNA, *HSP60/EcoRV*, and *GPI/HhaI* (Figure 3) represents a good compromise of type-ability of most strains, adequate discriminatory power, reproducibility, and cost, as well as minimal sample material and time requirements.

Further development of multiple locus PCR-RFLP systems will require testing of many more isolates to prove the reliability of each target/enzyme combination. Testing of additional targets is clearly warranted, particularly of ones capable of discriminating between TcIIId and TcIIe and any that unequivocally identify TcIIa. Details of other RFLP markers capable of similar levels of resolution were published during the course of this study, including an assay targeted to the *GP72* gene using the restriction enzyme *TaqI*, which does seem to provide discrimination between TcIIId and TcIIe.²⁶ These authors also proved the potential of using PCR-RFLPs to detect diversity of *T. cruzi* in both clinical and field samples.

In this study aimed at validating genotyping assays, we used a panel of cloned, laboratory cultivated strains. The utility of these assays in practice, however, is subject to the complication of mixed infections, which are well documented in both vectors and mammal hosts, including humans.⁴⁵⁻⁴⁷ Depending on the strains present in such cases, mixed genotype profiles could be observed. For example, mixtures of TcIIb and TcIIc would generate TcIIId/IIe profiles for the RFLP assays tested here. It may also be necessary to distinguish between *T. cruzi* and *Trypanosoma rangeli*, and this can be done either

morphologically, or genetically using an assay that exploits PCR product size differences in the large subunit rRNA gene (LSU rDNA).⁴⁸ Furthermore, if there is reason to suspect an isolate belongs to the closely related, bat host-restricted subspecies *Trypanosoma cruzi marinkellei*, it can be identified by its unique 135-bp band for the LSU rDNA PCR assay.²⁰

The strains that are used for testing of typing systems need to be carefully considered. Although there are abundant isolates described from domestic transmission cycles, the diversity of *T. cruzi* in sylvatic settings is less well understood. Indeed, the predominantly sylvatic DTUs TcIIa and TcIIc are often poorly represented in many types of study, including those aimed at characterizing genetic markers. Reducing this sample bias is also particularly important because the success of control strategies targeted at domestic transmission means that the epidemiology of Chagas disease is changing, and adventitious transmission of *T. cruzi* from sylvatic sources (i.e., TcI, TcIIa, and TcIIc) is seen as increasingly important.² This is exemplified by cases of acute Chagas disease caused by enzootic transmission in the Brazilian Amazon, which may become increasingly frequent as migration more often brings humans into contact with sylvatic sources of *T. cruzi*.⁶ Reliable and reproducible genotyping protocols will aid characterization of new isolates and should contribute to a coordinated research effort across multiple disciplines.

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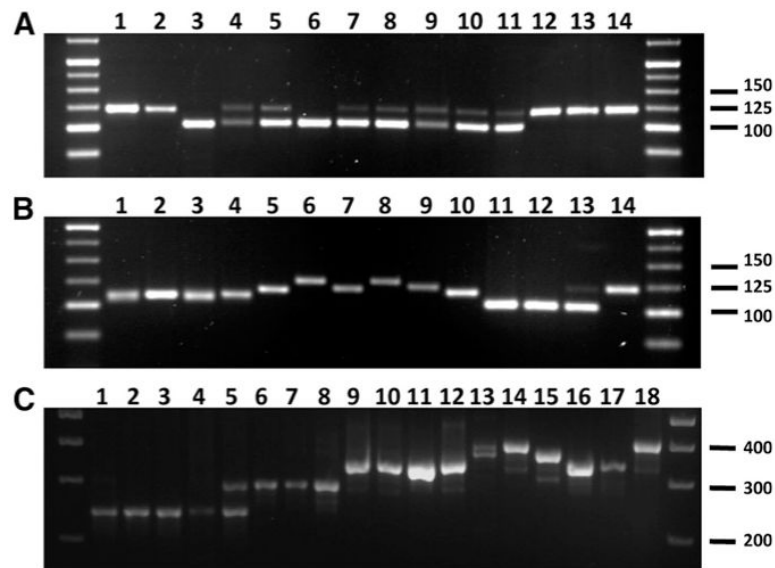


Figure 1.

Example of LSU rDNA and mini-exon PCR product size polymorphism genotyping assay profiles. **A**, LSU rDNA—Lanes: 1, Rita cl5 (TcIIb); 2, Chaco23 col4 (TcIIb); 3, ARMA18 cl3 (TcIIc); 4, Sc43 cl1 (TcIIId); 5, 92-80 cl2 (TcIIId); 6, Bug2148 cl1 (TcIIId), note absence of 125-bp band; 7, Chaco2 cl3 (TcIIId); 8, PAH179 cl5 (TcIIId); 9, Para6 cl4 (TcIIId); 10, Para4 cl3 (TcIIId); 11, Vinch101 cl1 (TcIIId); 12, EPV20-1 cl1 (TcIIe); 13, P251 cl7 (TcIIe); 14, VFRA1 cl1 (TcIIe). **B**, LSU rDNA—Lanes: 1, C8 cl1 (TcI); 2, SAXP18 cl1 (TcI); 3, JR cl4 (TcI); 4, B187 cl10 (TcI); 5, 10R26 (TcIIa); 6, 92122102R [TcIIa(NA)]; 7, CanIII cl1 (TcIIa); 8, StC10R cl1 [TcIIa(NA)]; 9, Saimiri3 cl1 (TcIIa); 10, ERA cl2 (TcIIa); 11, JA2 cl2 (TcIIc); 12, SABP19 cl1 (TcIIc); 13, Vinch101 cl1 (TcIIId); 14, LHVA cl4 (TcIIe). Note that comparison of Lanes 8–11 shows the four distinct product sizes: 130, 125, 117, and 110 bp. **C**, Mini-exon—Lanes: 1, M5631 cl5 (TcIIc); 2, JA2 cl2 (TcIIc); 3, ARMA18 cl3 (TcIIc); 4, 85/847 cl2 (TcIIc); 5, SABP cl1 (TcIIc); 6, VFRA1 cl1 (TcIIe); 7, Chaco2 cl3 (TcIIId); 8, Esm cl3 (TcIIb); 9, X10/1 (TcI); 10, B187 cl10 (TcI); 11, JR cl4 (TcI); 12, 92101601P cl1 (TcI); 13, CanIII cl1 (TcIIa); 14, 92122102R [TcIIa(NA)]; 15, Saimiri3 cl1 (TcIIa); 16, X10610 cl5 (TcIIa); 17, ERA cl2 (TcIIa); 18, StC10R cl1 [TcIIa(NA)].

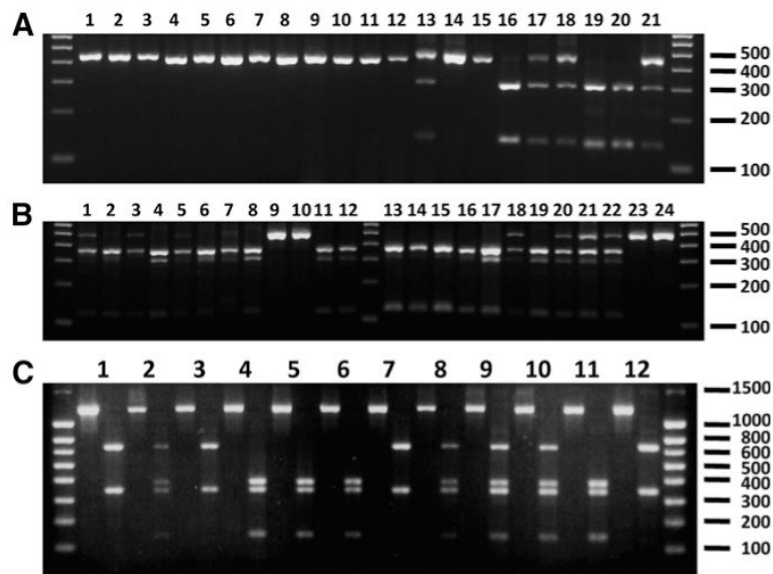


Figure 2.

Examples of PCR-RFLP genotyping profiles. **A**, *HSP60*: digestion products only are shown. Lanes: 1, X10610 cl5 (TcIIa); 2, Saimiri3 cl1 (TcIIa); 3, ERA cl2 (TcIIa); 4, JR cl4 (TcI); 5, 10R26 (TcIIa); 6, StC10R cl1 [TcIIa(NA)]; 7, CanIII cl1 (TcIIa); 8, X10/1 (TcI); 9, 92122102R [TcIIa(NA)]; 10, CJ005/PII (TcI); 11, B187 cl10 (TcI); 12, Chile C22 cl1 (TcI); 13, 92-80 cl2 (TcIIId); 14, Rita cl5 (TcIIb); 15, Pot7a cl1 (TcIIb); 16, ARMA18 cl3 (TcIIc); 17, PAH179 cl5 (TcIIId); 18, VFRA1 cl1 (TcIIe); 19, SABP19 cl1 (TcIIc); 20, M6241 cl6 (TcIIc); 21, Vinch101 cl1 (TcIIId). **B**, *Histone H1*: digestion products from unpurified PCR products are shown. Lanes: 1, X10610 cl5 (TcIIa); 2, Saimiri3 cl1 (TcIIa); 3, ERA cl2 (TcIIa); 4, JR cl4 (TcIIc); 5, 10R26 (TcIIa); 6, StC10R cl1 [TcIIa(NA)]; 7, CanIII cl1 (TcIIa); 8, X10/1 (TcI); 9, Pot7b cl5 (TcIIb); 10, Rita cl5 (TcIIb); 11, JA2 cl2 (TcIIc); 12, ARMA13 cl1 (TcIIc); 13, CJ007/PI (TcI); 14, B187 cl10 (TcI); 15, SAXP18 cl1 (TcI); 16, 92122102R [TcIIa(NA)]; 17, SABP19 cl1 (TcIIc); 18, Para4 cl3 (TcIIId); 19, CM25 cl2 (TcIIc); 20, PAH179 cl5 (TcIIId); 21, Sc43 cl1 (TcIIId); 22, Chaco17 col1 (TcIIe); 23, Tu18 cl2 (TcIIb); 24, Chaco23 col4 (TcIIb). **C**, *GPI*: each pair of lanes shows undigested PCR product followed by restriction digest products. Lanes: 1, SAXP18 cl1 (TcI); 2, VFRA1 cl1 (TcIIe); 3, StC10R cl1 [TcIIa(NA)]; 4, 10R26 (TcIIa); 5, Pot7a cl1 (TcIIb); 6, Rita cl5 (TcIIb); 7, JA2 cl2 (TcIIc); 8, Para4 cl3 (TcIIId); 9, Vinch101 cl1 (TcIIId); 10, Chaco9 col15 (TcIIe); 11, CanIII cl1 (TcIIa); 12, ARMA18 cl3 (TcIIc).

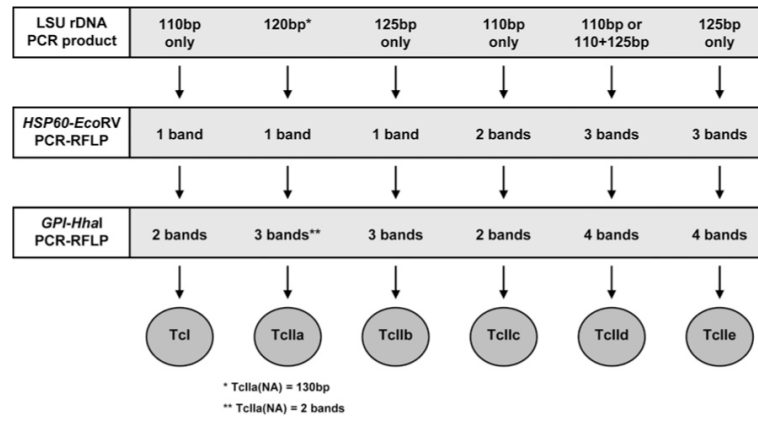


Figure3.
Recommended triple-assay for discriminating *T. cruzi* DTUs.

Table 1

Comparison of selected *T. cruzi* subdivision nomenclature schemes

Term	Criterion	Subdivision nomenclature										Reference	
		TcI	TcIIa	TcIIb	TcIIc	TcId	TcIe	TcIf	TcIg	TcIh	TcIi		
DTU	RAPD and MLEE	ZI	ZIII	ZII	ZIII-ASAT I	Heterozygous ZII	Heterozygous ZII						19
Zymodeme	MLEE	1-25	26-29	30-34	35-37	38,39	40-43						49
Clonet	MLEE	2	2'	1	2'	1/2	1	1	1	1	1	1	50
Lineage	rDNA size polymorphism	2	2'	1	2'	1	1	1	1	1	1	1	10
Lineage	<i>SL</i> -RNA gene sequences	2	2'	1	2'	1	1	1	1	1	1	1	9,25
Group	Consensus of experts	<i>T. cruzi</i> I	<i>T. cruzi</i> *	<i>T. cruzi</i> II	<i>T. cruzi</i> *	<i>T. cruzi</i> *	<i>T. cruzi</i> *	<i>T. cruzi</i> *	<i>T. cruzi</i> *	<i>T. cruzi</i> *	<i>T. cruzi</i> *	<i>T. cruzi</i> *	51
Zymodeme	PCR-RFLP of rDNA loci		ZIII-B		ZIII-A								22
Major lineage	Multilocus microsatellites, rDNA size polymorphism, mtDNA sequences	TcI		TcII	TcIII	Tc Hybrid	Tc Hybrid	Tc Hybrid	Tc Hybrid	Tc Hybrid	Tc Hybrid	Tc Hybrid	34

* Group left undefined.

Table 2

Genotype assignment of PCR amplification product sizes (bp) 9,10,20,21,24

Target (enzyme)	Expected PCR product (digestion product) band sizes (bp)						
	TcI	TcIIa	TcIIb	TcIIc	TcIIId	TcIIe	
Mini-exon	350	400,* or none [†]	300	250,* or none [†]	300	300	
LSU rDNA	110	117 [‡] or 120 [‡] or 125 [§] or 130 [¶]	125	110	110 or 110 + 125 ^{**}	125	
<i>HSP60/EcoRV</i>	432-462 (432-462)	432-462 (432-462)	432-462 (432-462)	432-462 (314 + 148-118)	432-462 (432-462 + 314 + 148-118)	432-462 (432-462 + 314 + 148-118)	
Histone <i>HII/AatII</i>	486 (364 + 122)	486 (486 + 364 + 122)	486 (486)	486 (364 + 122)	486 (486 + 364 + 122)	486 (486 + 364 + 122)	
<i>GPI/HhaI</i>	1,264 (817 + 447)	1,264 (490 + 447 + 253)	1,264 (490 + 447 + 253)	1,264 (817 + 447)	1,264 (817 + 490 + 447 + 253)	1,264 (817 + 490 + 447 + 253)	

* According to Yeo and others.²⁴[†] According to Brisse and others.²⁰[‡] According to Kawashita and others.²¹[§] For one strain, Saimiri3, according to Brisse and others. 20[¶] For three strains of North American origin, according to Brisse and others. 20^{**} Double band pattern observed for most isolates; 125-bp band exhibits variable intensity (see text).

Table 3

Summary of genotypes

Strain	Genotype (method)*	PCR product size polymorphism				PCR-RFLP	
		Mini-exon	LSU rDNA	HSP60	HI	HI	GPI
X10/1	Tel (MLEE)	Tel ^{††}	Tel/Hc/Ild ^{††}	Tel/Ha/IHb [§]	Tel/Hc [§]	Tel/Hc ^{§§}	Tel/Hc ^{§§}
C8 cl1	Tel (MLEE)	Tel	Tel/Hc/Ild	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
P1 (CJ007)	Tel (MLEE, RAPD)	Tel	Tel/Hc/Ild	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
PH (CJ005)	Tel (MLEE, RAPD)	Tel	Tel/Hc/Ild	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
B187 cl10	Tel (rDNA, mini-exon)	Tel	Tel/Hc/Ild	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
Chile C22 cl1	Tel (MLEE)	Tel	Tel/Hc/Ild	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
SAXP18 cl1	Tel (MLEE)	Tel	Tel/Hc/Ild	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
92101601P cl1	Tel (MLEE, RAPD)	Tel [†]	Tel/Hc/Ild ^{††}	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
JR cl4	Tel (RAPD)	Tel	Tel/Hc/Ild	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
X10610 cl5	Tella (RAPD)	Tel	Tella	Tel/Ha/IHb	Tella/Ild/Ile	Tella/Ild/Ile	Tella/IHb
92122102R	Tella (rDNA)	Tella ^{††}	Tella(NA) ^{††}	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
StC10R cl1	Tella (MLEE, RAPD)	Tella	Tella(NA)	Tel/Ha/IHb	Tel/Hc	Tel/Hc	Tel/Hc
Can III cl1	Tella (MLEE)	Tella ^{††††}	Tella ^{††}	Tel/Ha/IHb [§]	Tella/Ild/Ile [§]	Tella/Ild/Ile [§]	Tella/IHb [§]
10 R26	Tella (MLEE)	Tel^{†††}	Tella [†]	Tel/Ha/IHb	Tella/Ild/Ile	Tella/Ild/Ile	Tella/IHb
Saimiri 3 cl1	Tella (MLEE, RAPD)	380^{†††}	TcHb/Hc[†]	Tel/Ha/IHb	Tel/Hc	Tella/Ild/Ile	Tella/IHb
ERA cl2	Tella (RAPD)	Tel	Tella	Tel/Ha/IHb	Tella/Ild/Ile	Tella/Ild/Ile	Tella/IHb
Esan cl3	TelHb (MLEE)	TelHb/Ild/Ile ^{††}	TcHb/Hc/Ile ^{††}	Tel/Ha/IHb [§]	TelHb [§]	TelHb [§]	Tella/IHb [§]
Por7a cl1	TelHb (MLEE)	TelHb/Ild/Ile	TcHb/Hc/Ile	Tel/Ha/IHb	TelHb	TelHb	Tella/IHb
Por7b cl2	TelHb (MLEE)	TelHb/Ild/Ile	TcHb/Hc/Ile	Tel/Ha/IHb	TelHb	TelHb	Tella/IHb
Rita cl5	TelHb (MLEE)	TelHb/Ild/Ile	TcHb/Hc/Ile	Tel/Ha/IHb	TelHb	TelHb	Tella/IHb
Tu18 cl2	TelHb (MLEE, RAPD)	TelHb/Ild/Ile ^{††}	TcHb/Hc/Ile ^{††}	Tel/Ha/IHb [§]	TelHb [§]	TelHb [§]	Tella/IHb [§]
CBB cl2	TelHb (MLEE)	TelHb/Ild/Ile ^{††}	TcHb/Hc/Ile	Tel/Ha/IHb [§]	TelHb [§]	TelHb [§]	Tella/IHb [§]
IVV cl4	TelHb (MLEE, RAPD)	TelHb/Ild/Ile	TcHb/Hc/Ile	Tel/Ha/IHb	TelHb	TelHb	Tella/IHb
Chaco23 col4	TelHb (MLEE)	TelHb/Ild/Ile	TcHb/Hc/Ile	Tel/Ha/IHb	TelHb	TelHb	Tella/IHb
M5631 cl5	TelHc (MLEE)	TelHc ^{††}	Tel/Hc/Ild/Ile ^{††}	TelHc [§]	TelHc [§]	Tel/Hc [§]	Tel/Hc [§]

Strain	Genotype (method)*	PCR product size polymorphism				PCR-RFLP		
		Mini-exon	LSU rDNA	HSP60	HI	GPI		
M6421 cl6	TcIc (MLEE)	TcIc [‡]	TcI/Ic/Ild [‡]	TcIc [§]	TcI/Ic [§]	TcI/Ic [§]	TcI/Ic [§]	
JA2 cl2	TcIc (MLEE)	TcIc	TcI/Ic/Ild	TcIc	TcI/Ic	TcI/Ic	TcI/Ic	
ARMA13 cl1	TcIc (MLEE)	TcIc	TcI/Ic/Ild	TcIc	TcI/Ic	TcI/Ic	TcI/Ic	
ARMA18 cl3	TcIc (MLEE)	TcIc	TcI/Ic/Ild	TcIc	TcI/Ic	TcI/Ic	TcI/Ic	
CM25 cl2	TcIc (MLEE, RAPD)	TcIc [‡]	TcI/Ic/Ild [‡]	TcIc	TcI/Ic	TcI/Ic	TcI/Ic	
85/847 cl2	TcIc (rDNA)	TcIc [‡]	TcI/Ic/Ild [‡]	TcIc	TcI/Ic	TcI/Ic	TcI/Ic	
SABP19 cl5	TcIc (MLEE)	TcIc	TcI/Ic/Ild	TcIc	TcI/Ic	TcI/Ic	TcI/Ic	
Se43cl1	TcIld (MLEE)	TcIIb/Ild/Ile [‡]	TcI/Ic/Ild [‡]	TcIld/Ile [§]	TcIIa/Ild/Ile [§]	TcIld/Ile [§]	TcIld/Ile [§]	
92-80 cl2	TcIld (MLEE)	TcIIb/Ild/Ile [‡]	TcI/Ic/Ild [‡]	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
Para4 cl3	TcIld (MLEE)	TcIIb/Ild/Ile	TcI/Ic/Ild [‡]	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
Para6 cl4	TcIld (MLEE)	TcIIb/Ild/Ile	TcI/Ic/Ild [‡]	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
Chaco2 cl3	TcIld (MLEE)	TcIIb/Ild/Ile	TcI/Ic/Ild [‡]	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
Vinch101 cl1	TcIld (MLEE)	TcIIb/Ild/Ile	TcI/Ic/Ild [‡]	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
Bug2148 cl1	TcIld (rDNA, mini-exon)	TcIIb/Ild/Ile [‡]	TcI/Ic/Ild [‡]	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
PAH179 cl5	TcIld (MLEE)	TcIIb/Ild/Ile	TcI/Ic/Ild	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
CL Brener	TcIc (MLEE, RAPD)	TcIIb/Ild/Ile [‡]	TcIIb/Ile [‡]	TcIld/Ile [§]	TcIIa/Ild/Ile [§]	TcIld/Ile [§]	TcIld/Ile [§]	
Chaco17 col1	TcIc (MLEE)	TcIIb/Ild/Ile	TcIIb/Ile	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
Chaco9 col15	TcIc (MLEE)	TcIIb/Ild/Ile	TcIIb/Ile	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
Tula cl2	TcIc (MLEE)	TcIIb/Ild/Ile [‡]	TcIIb/Ile [‡]	TcIld/Ile [§]	TcIIa/Ild/Ile [§]	TcIld/Ile [§]	TcIld/Ile [§]	
P251 cl7	TcIc (MLEE)	TcIIb/Ild/Ile	TcIIb/Ile	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
EPV20-1 cl1	TcIc (MLEE)	TcIIb/Ild/Ile	TcIIb/Ile	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
LHYA cl4	TcIc (MLEE)	TcIIb/Ild/Ile	TcIIb/Ile	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	
VFR-A1 cl1	TcIc (MLEE)	TcIIb/Ild/Ile	TcIIb/Ile	TcIld/Ile	TcIIa/Ild/Ile	TcIld/Ile	TcIld/Ile	

Bold text indicates unexpected or incongruent genotypes. The indicated studies did not report the same genotypes in all cases (see text).

* For strain origins and references, see Lewis and others (2009). 28

[‡] Previously typed by Souto and others (1996). 10

[‡] Previously typed by Brisse and others (2001). 20

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§ Previously typed by Westenberger and others (2005), 27

¶ Previously typed by Roellig and others (2008), 52

** Secondary band ~390 bp observed.

†† No amplification; TcI group previously reported.³⁰

‡‡ 380 bp, Saimiri3 cII unique mini-exon product size.