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Ancestral Genomes, Sex, and the Population Structure of *Trypanosoma cruzi*

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Acquisition of detailed knowledge of the structure and evolution of *Trypanosoma cruzi* populations is essential for control of Chagas disease. We profiled 75 strains of the parasite with five nuclear microsatellite loci, 24Sα RNA genes, and sequence polymorphisms in the mitochondrial cytochrome oxidase subunit II gene. We also used sequences available in GenBank for the mitochondrial genes cytochrome B and NADH dehydrogenase subunit 1. A multidimensional scaling plot (MDS) based in microsatellite data divided the parasites into four clusters corresponding to *T. cruzi* I (MDS-cluster A), *T. cruzi* II (MDS-cluster C), a third group of *T. cruzi* strains (MDS-cluster B), and hybrid strains (MDS-cluster BH). The first two clusters matched respectively mitochondrial clades A and C, while the other two belonged to mitochondrial clade B. The 24Sα rDNA and microsatellite profiling data were combined into multilocus genotypes that were analyzed by the haplotype reconstruction program PHASE. We identified 141 haplotypes that were clearly distributed into three haplogroups (X, Y, and Z). All strains belonging to *T. cruzi* I (MDS-cluster A) were Z/Z, the *T. cruzi* II strains (MDS-cluster C) were Y/Y, and those belonging to MDS-cluster B (unclassified *T. cruzi*) had X/X haplogroup genotypes. The strains grouped in the MDS-cluster BH were X/Y, confirming their hybrid character. Based on these results we propose the following minimal scenario for *T. cruzi* evolution. In a distant past there were at a minimum three ancestral lineages that we may call, respectively, *T. cruzi* I, *T. cruzi* II, and *T. cruzi* III. At least two hybridization events involving *T. cruzi* II and *T. cruzi* III produced evolutionarily viable progeny. In both events, the mitochondrial recipient (as identified by the mitochondrial clade of the hybrid strains) was *T. cruzi* II and the mitochondrial donor was *T. cruzi* III.

Citation: Freitas JM, Augusto-Pinto L, Pimenta JR, Bastos-Rodrigues L, Gonçalves VF, et al. (2006) Ancestral genomes, sex, and the population structure of *Trypanosoma cruzi*. PLoS Pathog 2(3): e24.

Introduction

The parasite protozoan *Trypanosoma cruzi* causes Chagas disease, a malady that afflicts almost 20 million people in South America and Central America, with more than 20,000 deaths reported each year [1,2]. Two different ecosystems exist for *T. cruzi:* one related to wild hemiptera and generally involving wild mammals (the "sylvatic" cycle), and another dependent on home-dwelling hemiptera and primarily involving humans and household animals (the so-called "domestic" cycle). The connection between the two ecosystems is made by infected rats, mice, bats, marsupials, and other feral mammals. It is estimated that the parasite emerged as a species well over 150 million years ago, originally infecting primitive mammals dispersed throughout Laurasia and Gondwanaland, regions that originated North and South America, respectively [3]. The first contact with humans occurred much more recently, in the late Pleistocene, 15,000–20,000 years ago, when humans first peopled the Americas—thus, *Homo sapiens* is a very recent new host for *T. cruzi*. There is convincing molecular evidence for the presence of *T. cruzi* DNA in mummies exhumed in Northern Chile and Southern Peru and dating as far back as 9,000 years before the present day. [4].

The conventional mode of transmission of *T. cruzi* to humans is by the feces of infected hematophagous triatomine bugs. Alternative modes of infection include blood transfusion, congenital transmission from infected mothers, and ingestion of contaminated foods. Thanks to intensive programs of triatomine control, vectorial infection has been virtually abolished in Brazil, Chile, Uruguay, and Argentina [5]. Moreover, improved screening of blood donors to reduce the likelihood of transfusional transmission and early detection and treatment of congenital cases have added to this success. It would be, however, a mistake to think that Chagas disease has been controlled. High levels of vector-borne transmission are still apparent in many areas, and several of the endemic countries have yet to develop serious large-scale surveillance and intervention programs [5]. Also, migrations of infected individuals offer a risk of new

Editor: John Boothroyd, Stanford University, United States of America

Received October 13, 2005; **Accepted** February 21, 2006; **Published** March 31, 2006

DOI: 10.1371/journal.ppat.0020024

Abbreviations: *COII*, cytochrome oxidase subunit II; *CYb*, cytochrome b; MDS, multidimensional scaling plot; *ND1*, NADH dehydrogenase subunit 1; NJ, neighbor-joining; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment-length polymorphism

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Synopsis

The parasite protozoan *Trypanosoma cruzi* causes Chagas disease, a malady that afflicts almost 20 million people in South America and Central America. Although the genome sequencing of *T. cruzi* has been recently completed, little is known about its population structure and evolution. Since 1999, two major evolutionary lineages presenting distinct epidemiological characteristics have been recognized in the parasite: *T. cruzi* I and *T. cruzi* II, the latter being much more associated with severe chronic cases of the disease. We describe new and important aspects of the population structure of the parasite, especially the characterization of a third ancestral lineage that we propose to call *T. cruzi* III. Through careful dissection of the genetic constitution of blocks of genes that are stably transmitted from generation to generation of the parasite we deduced at least two occurrences of the formation of hybrid strains from the parental lineages *T. cruzi* II and *T. cruzi* III, including the strain CLBrener, whose genome was sequenced. We did not find any hybrids originating from *T. cruzi* I. A fascinating finding was that both hybrids studied had the same mitochondrial DNA type as the *T. cruzi* III ancestral lineage, which was quite different from *T.cruzi* II.

transmission in previously nonendemic regions, such as the United States [6]. Furthermore, the ancient and wide-ranging sylvatic cycle constitutes an enormous reservoir of parasites that represents a threat for humans.

Recent studies have shown that in a nonendemic area of the Brazilian Atlantic coastal rainforest 50% of the triatomine vectors and of the marsupials *Didelphis marsupialis* and *Philander opossum* [7] as well as 52% of the golden lion tamarins and several other species of New World primates [8] were naturally infected with *T. cruzi*. Moreover, in the United States *T. cruzi* has been found in 11.4% of opossums and 22% of the raccoons, together with infected triatomine bugs in the state of Georgia [9]. In certain areas of that state 43% of the raccoons were infected [10]. Closer to the human domestic environment, Bradley et al. [11] have shown that 3.6% of the rural hunting dogs in Oklahoma were seropositive for *T. cruzi*. Human infection from the sylvatic environment can occur either from sudden migration of hemiptera to the human environment, forced by the destruction of forests [12] or by the ingestion of foods contaminated by the feces of hemipterae or by crushed insects [13,14]. Thus, a complete understanding of the population structure of *T. cruzi*, especially the sylvatic cycle, will be indispensable for controlling the disease.

T. cruzi is diploid, with different-sized homologous chromosome pairs [15]. Its genome has been recently sequenced [16], and its size (diploid) has been estimated between 106.4 and 110.7 Mb. At least 50% of the *T. cruzi* genome is made up of repetitive sequences, consisting of large gene families of surface proteins, retrotransposons, and subtelomeric repeats.

There is extensive and well-characterized intraspecific genetic diversity in *T. cruzi* (reviewed in [17,18]). Two major evolutionary lineages of the parasite, named *T. cruzi* I and *T. cruzi* II, have been identified [19]. These lineages are very divergent as revealed by several biological and molecular markers, including isozymes, 24Sα rDNA, and mini-exon gene polymorphisms [20]. *T. cruzi* I and *T. cruzi* II strains belong predominantly to distinct ecological environments: respectively, the sylvatic and domestic transmission cycles of Chagas disease [3,21]. *T. cruzi* I strains are characterized by zymodeme Z1 (a zymodeme is a group of strains that have the same isozyme profile), 24Sα rDNA group 2, and mini-exon group 2, and induce low parasitism in human chagasic patients. In contrast, *T. cruzi* II strains are characterized by zymodeme Z2, 24Sα rDNA and mini-exon group 1, and cause human infections with high parasitemia in classic endemic areas [21]. At least in Brazil, *T. cruzi* II strains appear to be exclusively responsible for tissue lesions in Chagas disease [22]. Additionally, there are some parasite strains that cannot be properly grouped into any one of these two major lineages. Among these unclassified strains are those identified as belonging to zymodeme Z3 [23] and other hybrid strains characterized as rDNA group 1/2 [24,25]. Using isozymes and random amplified polymorphic DNA (RAPD) typing, Brisse et al. [26] proposed that *T. cruzi* II strains could be partitioned into five phylogenetic sublineages (IIa-e), each comprising one of the following reference strains: CanIII cl1 (IIa), Esmeraldo cl3 (IIb), M5631 cl5 (IIc), MN cl2 (IId), and CLBrener (IIe). In contrast, *T. cruzi* I strains could not be further subdivided. Within each of these clades or sublineages, there is extensive genetic diversity that can be unraveled by analyses with microsatellites and several other genomic markers (reviewed in [27]).

Although capable of recombination in vitro [28], *T. cruzi* reproduces predominantly by binary fission and consequently its diploid nuclear genotype is transmitted *en bloc* to the progeny. Thus, the parasite presents extreme degrees of linkage disequilibrium, as shown through isozymes [29] and microsatellites [30], and exhibits a predominantly clonal population structure. Indeed, *T. cruzi* still has been considered the paradigm for clonal eukaryotic pathogenic microorganisms [31]. The occurrence of hybrid strains in natural populations of *T. cruzi* was suggested by isozyme analyses [32,33], restriction fragment-length polymorphism (RFLP) of housekeeping genes [34], RAPD [35], and genotype variations observed at chromosomal level [15,35,36], and has been confirmed using nucleotide sequences [37,38]. Their discovery proved that sexual events definitely have taken place in the past and have shaped the genetical structure of current *T. cruzi* populations. However, such genetic exchange events seem to have been rare enough to allow the propagation of clonal genotypes over long periods of time and wide geographical regions [35]. Because of the linkage disequilibrium, genotyping of nuclear markers in *T. cruzi* has thus far been limited to characterization of multilocus genotypes. Therefore, to understand the evolutionary history of the species it would be desirable to dissect the multilocus genotypes into their constituent haploid genome blocks. We wish to report that we have achieved this, revealing the existence of ancestral haplogroups and repeated hybridization events in *T. cruzi*.

Results

We have typed 75 strains of *T. cruzi* (Table 1) with five nuclear CA-repeat microsatellites (Table S1). We assumed a stepwise mutation model for the evolution of microsatellites and used the minimum number of mutational steps necessary to transform one strain microsatellite profile into another to build a genetic distance matrix. The multidimensional scaling (MDS) plot shown in Figure 1 provided, with excellent fit

Table 1. Characteristics of *T. cruzi* Strains Analyzed

Strains	Host/Vector	Origin	Cycle	Mini-Exon Type[a]	Zymodeme[b]	Isozyme Type[c]	RAPD/MLEE Lineage[c]	Major Lineage[d]
A83	D.m	F.G.	Sylvatic	2	ND	ND	ND	TcI
A87	D.m	F.G.	Sylvatic	2	ND	ND	ND	TcI
Be62	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
CanIII cl1	Human	PA/Brazil	Domestic	3[e]	Z3	27	IIa	Hybrid[f]
CLBrener	T.i	RS/Brazil	Domestic	1	Z2	43	IIe	Hybrid[f,g]
Colombiana	Human	Colombia	Domestic	2	ND	ND	ND	TcI
Col18/05	Human	Colombia	Domestic	2	ND	ND	ND	TcI
CPI11/94	Human	Colombia	Domestic	1	ND	ND	ND	TcII
CPI95/94	Human	PI/Brazil	Domestic	1	ND	ND	ND	TcII
Cutia	D.a	ES/Brazil	Sylvatic	2	Z1	19	I	TcI
Cuíca	P.o.	SP/Brazil	Sylvatic	2	Z1	19	I	TcI
Dog Theis	ND	ND	ND	1	ND	ND	ND	TcII
D7	D.m	RJ/Brazil	Sylvatic	2	ND	ND	ND	TcI
Esmeraldo	Human	MG/Brazil	Domestic	1	Z2	30	IIb	TcII
Gamba cl1	D.g	SP/Brazil	Sylvatic	2	Z1	19	I	TcI
Gil	Human	MG/Brazil	Domestic	ND	ND	ND	ND	TcII[g]
GLT564	L.r	RJ/Brazil	Sylvatic	1	ND	ND	ND	TcII
GLT593	L.r	RJ/Brazil	Sylvatic	1	ND	ND	ND	TcII
GMS	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
GOCH	Human	GO/Brazil	Domestic	1	ND	ND	ND	TcII
Ig539	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
JAF	Human	MG/Brazil	Domestic	ND	ND	ND	ND	TcII[g]
JG	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
JHF	Human	MG/Brazil	Domestic	ND	ND	ND	ND	TcII[g]
JSM	Human	MG/Brazil	Domestic	ND	ND	ND	ND	TcII[g]
Mas1 cl1	Human	MG/Brazil	Domestic	1	Z2	32	IIb	TcII
Mn cl2	Human	Chile	Domestic	1	Z2	39	IId	Hybrid[f,g]
MPD	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
M5631 cl5	D.n	PA/Brazil	Domestic	3[e]	Z3	36	IIc	TcIII
M6241 cl6	Human	PA/Brazil	Domestic	3[e]	Z3	35	IIc	TcIII
NR cl3	Human	Chile	Domestic	1	Z2	39	IId	Hybrid[f,g]
OPS27/94	Human	PI/Brazil	Domestic	1	ND	ND	ND	TcII
Rb1	R.b	AM/Brazil	Sylvatic	2	ND	ND	ND	TcI
Rb2	R.b	AM/Brazil	Sylvatic	2	ND	ND	ND	TcI
Rb6	R.b	AM/Brazil	Sylvatic	2	ND	ND	ND	TcI
SC43 cl1	T.i	MG/Brazil	Domestic	1	Z2	39	IId	Hybrid[g]
SE	Human	AM/Brazil	Sylvatic	2	ND	ND	ND	TcI
SilvioX10 cl1	Human	PA/Brazil	Domestic	2	Z1	17	I	TcI
SO3	T.i	Bolivia	Domestic	1	Z2	39	IId	Hybrid[f,g]
Tu18 cl2	T.i	MG/Brazil	Domestic	1	Z2	32	IIb	TcII
Tula cl2	Human	Chile	Domestic	2	Z2	43	IIe	Hybrid[f,g]
Y	Human	SP/Brazil	Domestic	1	ND	ND	ND	TcII
84Ti	Human	MG/Brazil	Domestic	ND	ND	ND	ND	TcII[g]
84	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
115	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcIII
167	Human	MG/Brazil	Domestic	ND	ND	ND	ND	Hybrid[g]
169/1	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
182	Human	MG/Brazil	Domestic	1	ND	ND	ND	Hybrid[g]
200pm	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
207	Human	MG/Brazil	Domestic	ND	ND	ND	ND	TcII[g]
209	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
222	Human	MG/Brazil	Domestic	ND	ND	ND	ND	TcIII
226	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcIII
231	Human	MG/Brazil	Domestic	ND	ND	ND	ND	TcIII
239	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
402	ND	ND	ND	ND	ND	ND	ND	ND
577	Human	GO/Brazil	Domestic	1	ND	ND	ND	TcII
578	Human	GO/Brazil	Domestic	1	ND	ND	ND	TcII
580	Human	GO/Brazil	Domestic	1	ND	ND	ND	TcII
581	Human	GO/Brazil	Domestic	ND	ND	ND	ND	TcII[g]
803	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
1001	D.m	MG/Brazil	Sylvatic	2	ND	ND	ND	TcI
1004	T.i	MG/Brazil	Domestic	2	ND	ND	ND	TcI
1005	T.i	MG/Brazil	Domestic	1	ND	ND	ND	TcII
1006	T.i	MG/Brazil	Domestic	2	ND	ND	ND	TcI
1014	P.m	MG/Brazil	Domestic	1	ND	ND	ND	TcII
1022	P.m	MG/Brazil	Domestic	1	ND	ND	ND	Hybrid[g]
1043	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
1502	D.m	AM/Brazil	Sylvatic	2	ND	ND	ND	TcI

Table 1. Continued.

Strains	Host/Vector	Origin	Cycle	Mini-Exon Type[a]	Zymodeme[b]	Isozyme Type[c]	RAPD/MLEE Lineage[c]	Major Lineage[d]
1523	D.m	AM/Brazil	Sylvatic	2	ND	ND	ND	TcI
1931	Human	MG/Brazil	Domestic	1	ND	ND	ND	TcII
3663	R.b	AM/Brazil	Sylvatic	3[e]	Z3	ND	ND	TcIII
3869	Human	AM/Brazil	Sylvatic	3[e]	Z3	ND	ND	TcIII
4182	R.b	AM/Brazil	Sylvatic	3[e]	Z3	ND	ND	TcIII
183744	Human	GO/Brazil	Domestic	1	ND	ND	ND	TcII

[a]Revised by Zingales et al. [21].
[b]Described by Miles et al. [23].
[c]Described by Tibayrenc et al. [43] and Brise et al. [26].
[d]TcI, TcII, and TcIII are abbreviations for the major lineages of *T. cruzi.* TcII was characterized in this work.
[e]Mini-exon type 3 associated with Z3 strains.
[f]The hybrid characteristics of these strains were based on the data described by Brisse et al. [35] and Machado and Ayala [37].
[g]The hybrid characteristic of these strains were based on data described in this work.
D.a, *Dasyprocta aguti;* D.g, *Didelphis azarae;* D.m, *Didelphis marsupialis;* D.n, *Didelphis novemcinctus;* F.G., French Guyana; L.r, *Leonthopitecus rosalia;* MLEE, multilocus enzyme electrophoresis; P.o., *Philander opossum;* P.m, *Panstrongylus megistus;* R.b, *Rhodnius brethesi;* T.i, *Triatoma infestans;* and ND, not determined.
DOI: 10.1371/journal.ppat.0020024.t001

(stress = 0.08), a visual representation of the distance matrix. Four clusters are clearly visible and identified by ellipsoids in the MDS plot. The identity of the clusters is revealed by the presence of the prototypical strains of Brisse et al. [26]: MDS-cluster A corresponds to *T. cruzi* I, MDS-cluster C to *T. cruzi* IIb, MDS-cluster B to *T. cruzi* IIc, and MDS-cluster BH to the IId and IIe sublineages. Only three strains fell outside the four clusters: CanIII (sub-lineage IIa), Dog Theis, and 402.

In 41 of the 75 strains we sequenced a 290–base pair region of the maxicircle-encoded cytochrome oxidase subunit II *(COII)* gene encompassing 44 variable positions (Figure 2). The sequenced data were used to generate a neighbor-joining (NJ) tree that is shown in Figure 3. It is clear that there are three tightly clustered sets of strains, separated by very large genetic distances, permitting straightforward allocation of *T. cruzi* strains into three mitochondrial clades that can also be simply identified by variation in just two AluI RFLP sites (Figure 2), which were then scored for all 75 strains (Table 2). Our MDS clusters corresponded perfectly to these mitochondrial clades, with the exceptions of MDS-clusters B (sublineage IIc) and BH (thus called because it contains the hybrid sublineages IId and IIe), both of which fall within mitochondrial clade B. To confirm our finding, we also built NJ trees for sequences obtained from GenBank of two other mitochondrial genes, cytochrome b *(CYb)* [35] and NADH dehydrogenase subunit 1 *(ND1)* [37]. The *CYb* and *ND1* trees

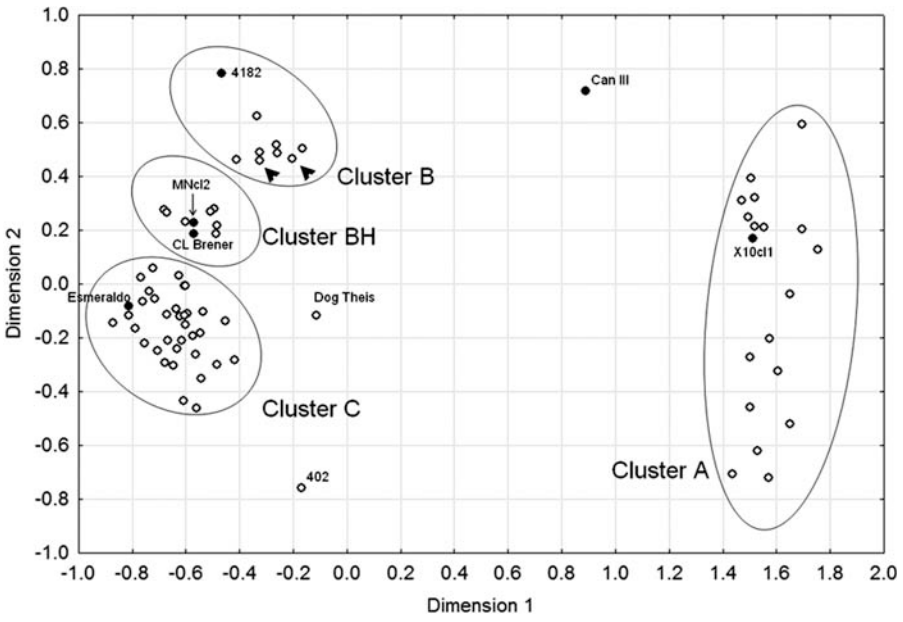

Figure 1. Multidimensional Scaling Plot of 75 *T. cruzi* Strains Genotyped for Five Microsatellites

Only outliers and the prototypical strains of Brisse et al. [26] are named in the plot. Arrowheads indicate strains 222 and 115. The strains in the areas delimited by ellipsoids are the following: MDS-cluster A: 1001, 1004, 1006, 1502, 1523, A83, A87, Col18/05, Colombiana, Cuíca, Cutia, D7, Gambacl1, Rb1, Rb2, Rb6, SE, X10cl1, 402, Mas1cl1, 84, 207, 209, 239, 577, 578, 580, 581, 803, 1005, 1014, 1043, 1931, 183744, 169/1; MDS-cluster C: 200pm, 84Ti, Be62, CPI11/94, CPI95/94, Esmeraldo, Gil, GLT564, GLT593, GMS, GOCH, Ig539, JAF, JG, JHF, JSM, MPD, OPS27/94, Tu18 cl11, Y; MDS-cluster B: 115, 222, 226, 231, 3663, 3869, 4182, M5631cl5; MDS-cluster BH: M6241cl6, 167, 1022, c182, CLBrener, MNcl2, NR, SC43 cl1, SO3, Tulacl2.
DOI: 10.1371/journal.ppat.0020024.g001

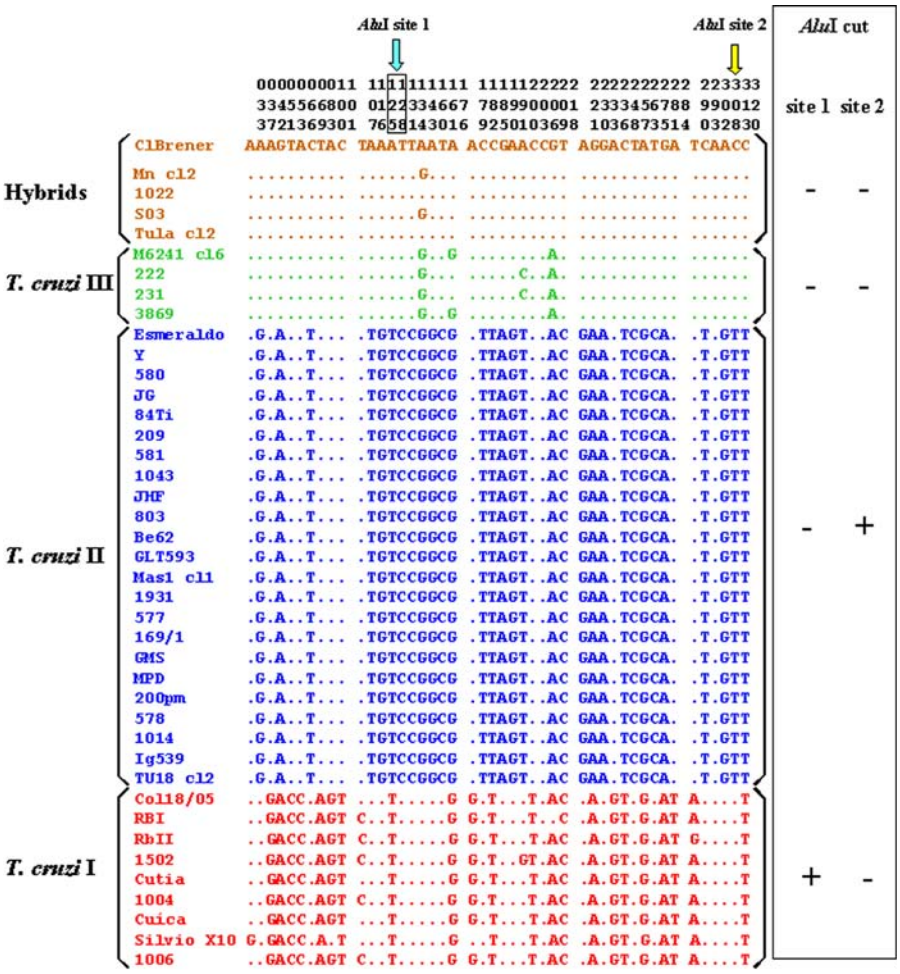

Figure 2. Sequence of a 290–Base Pair Fragment of the Mitochondrial *COII* Gene of 41 Strains of *T. cruzi*

Only the variable nucleotides are shown. *T. cruzi* III refers to sublineage IIc, and "Hybrids" indicate the strains belonging to sublineages IId and IIe of Brisse et al [26]. Two AluI restriction sites are indicated. RFLP analysis of these two sites allows unambiguous classification of *T. cruzi* strains to the three mitochondrial clades as shown on the right hand side.

DOI: 10.1371/journal.ppat.0020024.g002

had very similar topology to that of the *COII* tree (all with extremely high bootstrap values for the three main branches), confirming that sublineages IIc, IId, and IIe indeed belong to the same mitochondrial clade (Figure 3). We tested this notion further using analysis of molecular variance [39]. By partitioning the variability within and between mitochondrial clades we found that for *COII*, *CYb*, and *ND1*, respectively, 97%, 91%, and 68% of the genetic variability was found among clades.

We also typed all strains for the polymorphism of the D7 divergent domain of the 24Sα rRNA gene (Table S1) and combined the results with the microsatellites into multilocus genotypes that were analyzed with the PHASE software [40]. We identified 141 different haplotypes corresponding to a haplotypic diversity of 0.993. The identified haplotypes were then subjected to a median joining analysis using the NETWORK 3.1 software [41]. The resulting multitude of plausible trees is best expressed by a network that displays alternative potential evolutionary paths (Figure 4). Three haplotypic clusters are clearly identifiable: we called them haplogroups X, Y, and Z. Within these haplotypic clusters there is extensive reticulation because of the stepwise recurrent nature of microsatellite mutations [42]. However, the three haplogroups are connected by long and unique paths, emphasizing the great genetic distance between them. Seven haplotypes (numbers 33, 35, 58, 59, 60, 61, and 63) belong to these "bridges" and hence could not be assigned to any of the haplogroups—they were lumped into a haplogroup "I" (for indeterminate). We could then assign to each of the 75 strains a haplogroup genotype (Table 2). All strains belonging to the *T. cruzi* I lineage (MDS-cluster A in Figure 1) proved to be Z/Z (i.e., had two haplotypes belonging to haplogroup Z). Likewise, all the strains in MDS-cluster C (Figure 1) had Y/Y genotypes and those in MDS-cluster B had X/X genotypes. The strains in cluster BH all had X/Y genotypes confirming their hybrid nature. Strains Can III (genotype I/I, *COII* B), Dog Theis (genotype I/I, *COII* C), 402, and Mas1cl1 (both genotype I/Y, *COII* C), and M6241cl16 (genotype I/X, *COII* B) presented haplotypes of haplogroup I. It is noteworthy that three of these five strains are the ones outside MDS clusters in Figure 1A.

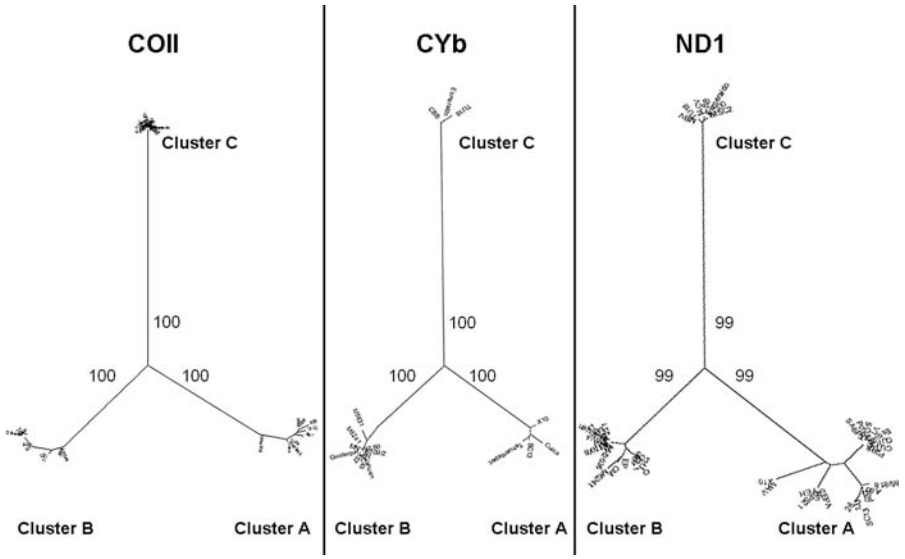

Figure 3. NJ Trees Obtained from the Sequences of Three Mitochondrial Genes of *T. cruzi: COII, CYb,* and *ND1*

The numbers in the three main branches indicate the percentage bootstrap values. For *COII* the strains the following clades are displayed: clade A: 1004, 1006, 1502, Col18/05, Cuica, Cutia, RBI, RbII, SilvioX10; clade B: sublineage IIc—222, 231, 3869, M6241, Mn, sublineages IId and IIe—CLBrener, 1022, SO3, Tula; clade C: 1014, 1043, 169/1, 1931, 200pm, 209, 577, 578, 580, 581, 803, 84Ti, Be62, Esmeraldo, GLT593, GMS, Ig539, JG, JHF, Mas1, MPD, TU18, Y. For *CYb* the strains are as follows: clade A: Cuica, SC13, Tehuentepec, X10; clade B: sublineage IIc—M5631, M6241, X109/2, sublineages IId and IIe—92.80, CL, Guateque, MN, SC43, Tulahuen, X57; clade C: CBB, Esmeraldo, TU18. For *ND1* the strains are as follows: clade A: 133, 26, 85/818, A80, A92, CEPA, CUICA, CUTIA, Esquilo, MAV, OPS21, P0AC, P209, SABP3, SC13, SO34, TEH, V121, Vin, X10; clade B: sublineage IIc—CM, M6241, X109/2, X110/8, X9/3, sublineages IId and IIe—CL, 86/2036, 86–1, EPP, P251, P63, PSC-O, SO3, Tulahuen, VMV4; clade C: CBB, Esmeraldo, MBV, MCV, MSC2, TU18, X-300.
DOI: 10.1371/journal.ppat.0020024.g003

Discussion

The population structure of *T. cruzi* is far from being completely understood. Although the existence of two major lineages in this species is well accepted, uncertainties about the existence or not of a third major ancestral group have been raised [15,35,36]. For instance, strains belonging to zymodeme Z3 or to rDNA group 1/2 could not be classified into either *T. cruzi* I or *T. cruzi* II [19]. Likewise, other strains (such as SC43) that present incongruities between the rDNA (group 2) and mini-exon (group 1) typing cannot be allocated into any of the two major lineages [24]. One of the major goals of this work was to investigate the genetic relationships among these "unclassifiable" strains.

Our first strategy was to perform the phylogenetic analysis of *T. cruzi* populations by using microsatellite data. Albeit extremely variable, these DNA markers allowed us to reliably identify four significant major clusters of strains (MDS clusters A, B, C, and BH in Figure 1). MDS-cluster A corresponds to *T cruzi* I and MDS-cluster C to classical *T. cruzi* II or *T. cruzi* IIb as named by Brisse et al. [26]. MDS-cluster B contains strains classified as Z3 and assigned to the IIc sublineage [26]. Finally, the strains within MDS-cluster BH were known to belong to the putative hybrid isozyme clonets 39 or 43 as proposed by Tibayrenc [43] and later classified as IId and IIe sublineages by Brisse et al. [26] (see Table 1).

Nucleotide sequencing and AluI RFLP analysis of a 290-bp stretch of the mitochondrial *COII* gene demonstrated that all strains enclosed in our microsatellite clusters B and BH (Z3 and hybrid strains) belonged to the same mitochondrial clade B. Sequences of two other mitochondrial genes, *CYb* [35] and *ND1* [37], obtained from GenBank, amply confirmed this observation by showing that indeed hybrid strains (sublineages IId and IIe) and Z3 strains (sublineage IIc) were grouped together into the same mitochondrial clade B. This same conclusion had been reached earlier [35,37].

Gaunt et al. [28] have shown that the hybridization of *T. cruzi* strains involves only nuclear genomes, without mitochondrial fusion. Here, we clearly demonstrated that the mitochondrial clade B is a third major phylogenetic division of *T. cruzi*, distinct from *T. cruzi* I (mitochondrial clade A) and *T. cruzi* II (mitochondrial clade C) major lineages. We have also shown that the strains with hybrid molecular markers in their nuclear genomes have a distinct mitochondrial genome (genotype B).

The analyses with all studied nuclear markers identified 141 different haplotypes that could be clustered into three haplogroups. All strains belonging to the *T. cruzi* I major lineage (MDS-cluster A in Figure 1) proved to be Z/Z (i.e., had two haplotypes belonging to haplogroup Z). Likewise, all the strains in MDS-cluster C (Figure 1) had Y/Y genotypes and those in MDS-cluster B had X/X genotypes. Thus, our data do not corroborate the suggestion made by Sturm et al. [36] that sublineage IIc (MDS-cluster B) is a hybrid. In contrast, the strains in MDS-cluster BH all had X/Y genotypes, confirming their hybrid character. Because of the way that PHASE identifies haplotypes, proximity of haplotype numbers is highly correlated with genetic proximity. Hybrid strains 167, 1022, 182, CLBrener, and Tulacl2 have, respectively, genotypes 4/99, 2/102, 5/108, 5/100, and 3/103, forming one group, while strains MNcl2, NR, SC43cl1, and SO3 have genotypes 52/133, 55/130, 54/129, and 54/130, and form another (notice equivalence with sublineages IIe and IId of Brisse et al. [26]). This indicates that at least two independent hybridizations occurred, presumably followed by clonal microdifferentiation.

Based on these results we propose the following minimal scenario for the evolution of *T. cruzi* populations (Figure 5). In the distant past there were at least three ancestral clades (MDS clusters A, C, and B in Figure 1) that we may call,

Table 2. Nuclear and Mitochondrial Markers of *T. cruzi* Inferred in This Work

Strains	*COII*[a]	Clusters[b]	Haplotypes[c]	Haplogroups[d]
A83	A	A	25/26	Z/Z
A87	A	A	27/28	Z/Z
Col18/05	A	A	21/56	Z/Z
Colombiana	A	A	24/56	Z/Z
Cuíca	A	A	31/49	Z/Z
Cutia	A	A	44/47	Z/Z
D7	A	A	6/29	Z/Z
Gamba cl1	A	A	30/46	Z/Z
Rb1	A	A	36/57	Z/Z
Rb2	A	A	16/36	Z/Z
Rb6	A	A	38/41	Z/Z
SE	A	A	48/53	Z/Z
SilvioX10cl1	A	A	32/50	Z/Z
1001	A	A	43/43	Z/Z
1004	A	A	23/37	Z/Z
1006	A	A	22/40	Z/Z
1502	A	A	39/45	Z/Z
1523	A	A	42/51	Z/Z
115	B	B	7/19	X/X
222	B	B	1/20	X/X
226	B	B	9/18	X/X
231	B	B	11/17	X/X
3663	B	B	8/10	X/X
3869	B	B	13/14	X/X
4182	B	B	15/62	X/I
M5631cl5	B	B	12/12	X/X
M6241cl6	B	B	33/34	X/X
167	B	BH	4/99	X/Y
182	B	BH	5/108	X/Y
1022	B	BH	2/102	X/Y
CLBrener	B	BH	5/100	X/Y
Mncl2	B	BH	52/133	X/Y
NR	B	BH	55/130	X/Y
SC43 cl1	B	BH	54/129	X/Y
SO3	B	BH	54/130	X/Y
Tula cl2	B	BH	3/103	X/Y
CanIII cl1	B	Outlier	58/59	I/I
Be62	C	C	88/105	Y/Y
CPI95/94	C	C	110/114	Y/Y
Esmeraldo	C	C	109/135	Y/Y
Gil	C	C	77/79	Y/Y
GLT564	C	C	97/98	Y/Y
GLT593	C	C	74/131	Y/Y
GMS	C	C	90/131	Y/Y
GOCH	C	C	81/126	Y/Y
Ig539	C	C	123/125	Y/Y
JAF	C	C	67/92	Y/Y
JG	C	C	82/92	Y/Y
JHF	C	C	83/84	Y/Y
JSM	C	C	65/95	Y/Y
Mas1 cl1	C	C	63/136	Y/I
MPD	C	C	64/76	Y/Y
OPS27/94	C	C	134/141	Y/Y
Tu18 cl11	C	C	137/138	Y/Y
Y	C	C	86/87	Y/Y
84	C	C	66/78	Y/Y
84Ti	C	C	71/73	Y/Y
169/1	C	C	85/94	Y/Y
200pm	C	C	112/115	Y/Y
207	C	C	75/139	Y/Y
209	C	C	69/101	Y/Y
239	C	C	80/96	Y/Y
577	C	C	89/124	Y/Y
578	C	C	122/127	Y/Y
580	C	C	117/118	Y/Y
581	C	C	113/116	Y/Y
803	C	C	72/119	Y/Y
1005	C	C	100/106	Y/Y
1014	C	C	68/93	Y/Y
1043	C	C	107/128	Y/Y
1931	C	C	91/104	Y/Y
183744	C	C	120/121	Y/Y
CPI11/94	C	C	111/140	Y/Y
Dog Theis	C	Outlier	60/61	I/I
402	C	Outlier	35/70	Y/I

[a]RFLP typing of the *T. cruzi COII* gene.
[b]Clusters of strains generated by MDS analysis.
[c]Haplotypes inferred by PHASE.
[d]Clusters of haplotypes in the network.
DOI: 10.1371/journal.ppat.0020024.t002

respectively, *T. cruzi* I, *T. cruzi* II, and *T. cruzi* III. It is interesting to note that this proposal matches the initial suggestion made by Miles et al. [23] almost 30 years ago on the basis of isozyme studies. Most likely, *T. cruzi* II and *T. cruzi* III had overlapping ecological niches, and thus the conditions necessary for hybridization were in place. At least two hybridization events produced evolutionarily viable progeny. In both events, the cytoplasmic donor for the resulting offspring (as identified by the mitochondrial clade of the hybrid strains) was *T. cruzi* III. From the haplotype reconstitutions we can estimate the parentage of a hybrid strain. For instance, CLBrener, the reference strain for the recently completed *T. cruzi* genome sequencing [16], has genotype 5/100. Its most likely mitochondrial recipient was a strain proximate to 1005 (genotype 100/106), while the most likely mitochondrial donor was a close relative of strains 222 and 115, which are very near each other in Figure 1 (arrowheads). The existence of strains that cannot be accommodated into this scenario (i.e., CanIII [sublineage IIa of Brisse et al. [26]] and Dog Theis) indicates that the evolutionary history had additional complexities. However, our simple model (depicted in Figure 5) should be useful for proposing and testing evolutionary and pathogenetic hypotheses.

The fact that the same population structure of *T. cruzi* can be envisaged with different molecular markers, such as isozymes [23], RAPD [26,35], microsatellites [30], and several sequence-based nuclear [20,21,37,38] and mitochondrial ([35,37], this study) markers, bears witness to its extreme stability. Although, as shown conclusively in our study and also by others [35,37], hybridization events clearly did occur in the evolutionary history of *T. cruzi,* they seem to have been only occasional and to have been subsequently stabilized by strong clonal propagation (reviewed in [17,18]).

Materials and Methods

***T. cruzi* isolates.** *T. cruzi* stocks (75) isolated from both domestic and sylvatic transmission cycles were analyzed (Table 1). DNA from the parasites were kindly provided by Dr. Égler Chiari from the Departamento of Parasitologia, Universidade Federal de Minas Gerais (Belo Horizonte, Brazil); Dr. José Rodrigues Coura and Dr. Ana Maria Jansen-Franken, from the Departamento de Medicina Tropical and the Departamento de Protozoologia, Fundação Oswaldo Cruz (Rio de Janeiro, Brazil), respectively; and Dr. M. Tibayrenc from the Centre d'Études sur le Polymorphisme des Microorganismes (Montpellier, France).

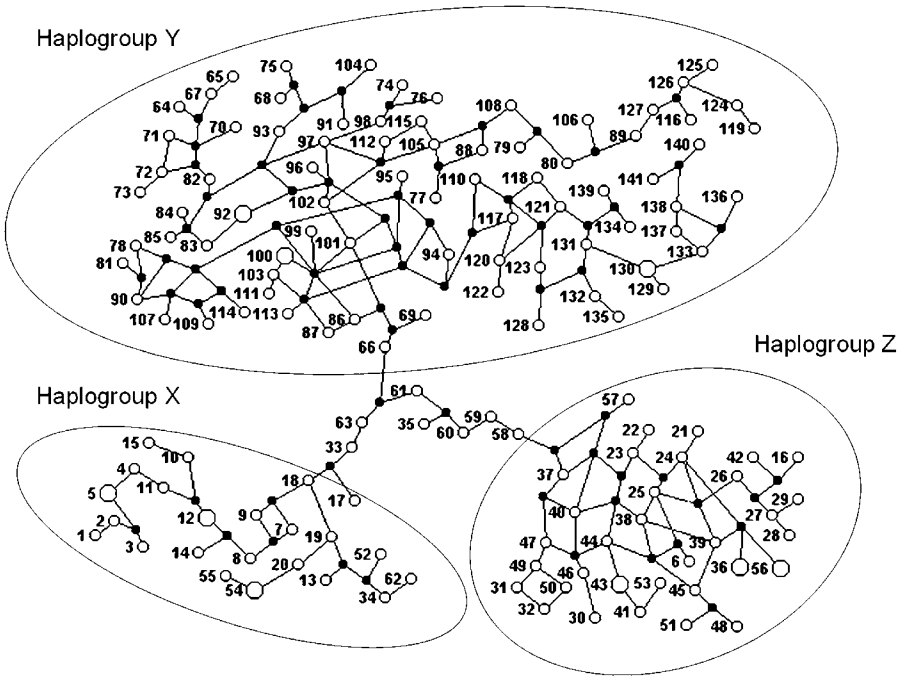

Figure 4. Median Joining Network of the Haplotypes Identified by the PHASE Software
DOI: 10.1371/journal.ppat.0020024.g004

Nuclear genetic typing. Amplification of five previously described microsatellite loci, denominated SCLE10, SCLE11, MCLE01, MCLF10, and MCLG10, was performed as previously described [30]. After the PCR, the amplified microsatellites were loaded on a 6% denaturing polyacrylamide gel and analyzed on an ALF sequencer (GE Healthcare, Milwaukee, Wisconsin, United States) using the Allelinks software (GE Healthcare). To determine the allele size the samples were directly compared with the band sizes from an allelic ladder prepared by amplification of an artificial mixture of DNA from 60 *T. cruzi* strains.

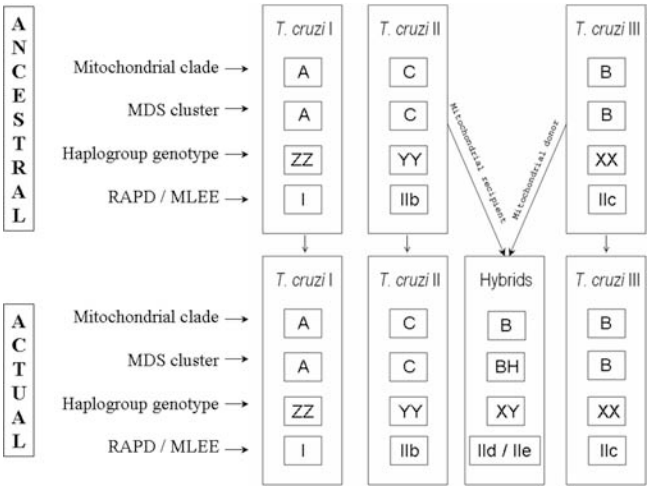

Figure 5. Diagram Depicting the Proposed Model for the Evolution of *T. cruzi* Strains

The mitochondrial clade was typed by RFLP of the *COII* maxicircle gene, the MDS clusters were established by multidimensional scaling of microsatellite data, the haplogroups were established by haplotype estimation from multilocal genotypes followed by median joining network analysis, and the RAPD/multilocus enzyme electrophoresis typing was obtained from Brisse et al. [26].
DOI: 10.1371/journal.ppat.0020024.g005

Amplification of the D7 divergent domain of the 24Sα rRNA gene was achieved by PCR with D71 fluorescent (5′-AAGGTGCGTCGA-CAGTGTGG-3′) and D72 (5′-TTTTCAGAATGGCCGAACAGT-3′) primers following protocols described previously [24]. The amplification products were also analyzed in ALF sequencer and allele sizes determined by the Allelinks software.

Mitochondrial genetic typing. Amplification of the mitochondrial *COII* gene [37] was performed using the primers TcMit31 (5′-TAAATAATATATATTGTACATGAG-3′) and TcMit40 (5′-CTRCATTGYCCATATATTGT-3′). Total DNA (1–10 ng) were used in each PCR reaction in the following condition: 30 s denaturation at 94 °, primer annealing for 2 min at 48 °, and primer extension for 2 min at 72 °, in a total of 30 cycles. The amplified products were purified and sequenced using primer TcMit31 and the cycle sequencing with Thermo-Sequenase (ETKit; GE Healthcare) using the thermal cycling program recommended in the kit. The sequencing products were purified and run on a MegaBACE capillary sequencer (GE Healthcare). After Phred, Phrap, and Consed analyses, the sequences were trimmed to have equal length (290 base pairs). All bases sequenced had Phred values above 30 [44].

Based on the restriction map of *COII* sequences, the AluI restriction endonuclease was chosen to perform RFLP analyses in the mitochondrial *COII* gene. After PCR amplification, the amplicons were submitted to enzyme digestion for 16 hours according to instructions provided by the manufacturer (Promega, Madison, Wisconsin, United States). Digested products were analyzed on polyacrylamide gel electrophoresis and silver stained.

Sequences for the mitochondrial *CYb* gene [35] and the *ND1* (37) were obtained from GenBank.

Construction of distance matrices, multidimensional scaling, and NJ trees. Based on the microsatellite results, a distance matrix between the strains was constructed as described previously [30]. In order to provide a visual representation of the distance matrix we used the multidimensional scaling plot using the software Statistica Version 6.0 [45]. Analyses of molecular variance for the mitochondrial sequences were performed using the Arlequin v.2.0 software using 1,000 permutations [46].

NJ trees were obtained separately for the *COII, CYb,* and *ND1* sequences with the MEGA v. 3.1 software [47] using the Kimura 2 parameter and 500 replications for the bootstrap statistics.

Haplotype inference and network construction. Haplotypes were reconstructed from the 75 *T. cruzi* populations by using a Bayesian coalescent theory-based method contained in PHASE software (Version 2.0.2 for Linux) [40]. The type of polymorphism (SNP or

multiallelic with stepwise mutation mechanism for rDNA and microsatellite data, respectively) is taken into account in PHASE. For the analyses the default parameters of the program were used, with additional runs up to 10,000 permutations. These were the best-tested conditions, giving highly reproducible results. The resultant haplotypes were then arranged in a network by using the Median Joining analysis [41], available in NETWORK 3.1 software provided by Fluxus Technology (http://www.fluxus-engineering.com).

Supporting Information

Table S1. Typing of rDNA Group and Allele Sizes (in bp) of Five Microsatellite Loci

Found at DOI: 10.1371/journal.ppat.0020024.st001 (105 KB DOC).

Accession numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for the genes and gene products discussed in this paper are 24S rDNA (L19411), mini-exon gene (X62674), *COII* (AF359041 and DQ343715–DQ343753), *CYb* (AJ130921, AJ130931–AJ130938, AJ439719–AJ439727), and *ND1* (AF359009, AF359011–AF359029, AF359031–AF359053).

Acknowledgments

We are grateful to Dr. José Rodrigues Coura and Dr. Ana Maria Jansen-Franken, from the Departamento de Medicina Tropical and the Departamento de Protozoologia, FIOCRUZ (Rio de Janeiro, Brazil), respectively, and Dr. M. Tibayrenc from the Centre d'Études sur le Polymorphisme des Microorganismes (Montpellier, France) for kindly providing DNA from *T. cruzi* strains. We thank Neuza A. Rodrigues and Katia B. Gonçalves for expert technical assistance.

Author contributions. SMRT, AMM, CRM, and SDJP conceived and designed the experiments. JMdF, LAP, JRP, LBR, and VFG performed the experiments. JMdF, LAP, JRP, LBR, VFG, and SDJP analyzed the data. EC, ACVJ, and OF contributed reagents/materials/analysis tools. AMM, CRM, and SDJP wrote the paper.

Funding. Supported by CNPq-Brazil, PRONEX, and World Health Organization. The research of SMRT and CRM was supported, in part, by an International Research Scholars Grant from the Howard Hughes Medical Institute.

Competing interests. The authors have declared that no competing interests exist. ■

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