Trypanosoma cruzi: ancestral genomes and population structure

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Although the genome of Trypanosoma cruzi *has been completely sequenced, little is known about its population structure and evolution. Since 1999, two major evolutionary lineages presenting distinct epidemiological characteristics have been recognised:* T. cruzi *I and* T. cruzi *II. We describe new and important aspects of the population structure of the parasite, and unequivocally characterise a third ancestral lineage that we propose to name* T. cruzi *III. Through a careful analysis of haplotypes (blocks of genes that are stably transmitted from generation to generation of the parasite), we inferred at least two hybridisation events between the parental lineages* T. cruzi *II and* T. cruzi *III. The strain CL Brener, whose genome was sequenced, is one such hybrid. Based on these results, we propose a simple evolutionary model based on three ancestral genomes,* T. cruzi *I,* T. cruzi *II and* T. cruzi *III. At least two hybridisation events produced evolutionarily viable progeny, and* T. cruzi *III was the cytoplasmic donor for the resulting offspring (as identified by the mitochondrial clade of the hybrid strains) in both events. This model should be useful to inform evolutionary and pathogenetic hypotheses regarding* T. cruzi.

Key words: *Trypanosoma cruzi* - Chagas disease - genome - sex - evolution - pathogenesis

Two major anniversaries will dominate the biomedical sciences in Brazil in 2009: the bicentennial of the birth of Charles Darwin (and 150 year anniversary of the publication of "On the Origin of Species") and the 100th anniversary of Carlos Chagas' first report of the disease that carries his name. There is one nexus between these two great scientists; Adler (1959) and others after him proposed that Charles Darwin suffered from Chagas disease, which he might have contracted in Mendoza, Argentina in 1853. Independent of the truth or fiction of this hypothesis (which probably will never be settled), Darwin and Chagas will be linked together in the present article, where *Trypanosoma cruzi* and Chagas disease are analysed from an evolutionary point of view.

The ecobiology of *T. cruzi*

T. cruzi may be transmitted through wild hemiptera in a cycle that generally involves wild mammals (the "sylvatic" cycle) and may also be transmitted by home-dwelling hemiptera in a cycle 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, the regions that originated North and South America, respectively (Briones et al. 1999). Its first contact with humans occurred much more recently, in the late Pleistocene (20,000-15,000 years ago), when humans first peopled the Americas. Thus, *Homo sapiens* is a very recent new host for *T. cruzi*. Convincing molecular evidence indicates the presence of *T. cruzi* DNA in mummies exhumed in Northern Chile and Southern Peru dating as far back as 9,000 years BP (Aufderheide et al. 2004).

The conventional mode of transmission of *T. cruzi* to humans is through the faeces of infected hematophagous triatomine bugs. Alternative modes of infection include blood transfusion, congenital transmission from infected mothers and the ingestion of contaminated foods. Thanks to intensive programmes of triatomine control, vectorial infection has been virtually abolished in Brazil and Argentina (Dias et al. 2002). Improved screening of blood-donors and early detection and treatment of congenital cases have also contributed to a decrease in the number of new cases. However, it would be a mistake to think that Chagas disease has been controlled. High levels of vector-borne transmission are still apparent in many areas and several countries where *T. cruzi* is endemic have yet to develop serious large-scale surveillance and intervention programmes (Dias et al. 2002). The migration of infected individuals presents the risk of new transmission in previously non-endemic regions, such as the United States (US) (Kirchhoff 1993). Furthermore, the ancient and wide-ranging sylvatic cycle maintains an enormous reservoir of the parasite that represents a threat to humans.

Recent studies have shown that in a non-endemic area of the Brazilian Atlantic coastal rainforest, 50% of the triatomine vectors and marsupials *Didelphis marsupialis* and *Philander opossum* (Jansen et al. 1999) as well as 52% of the golden lion tamarins and several other species of New World primates (Lisboa et al. 2004) were naturally infected with *T. cruzi*. Moreover, in the US *T. cruzi* has been found in 11.4% of opossums and 22% of

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raccoons, together with infected triatomine bugs in the state of Georgia (Pung et al. 1995). In certain areas of that state, up to 43% of the raccoons were infected (Pietrzak & Pung 1998). Closer to the human domestic environment, Bradley et al. (2000) have shown that 3.6% of 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 (Maguire et al. 1986) or by the ingestion of foods contaminated by hemipteran faeces or crushed insects (Shikanai-Yasuda et al. 1991, da Silva Valente et al. 1999). Thus, a complete understanding of the population structure of *T. cruzi*, especially the sylvatic cycle, will be indispensable for controlling the disease.

Genetics and genomics of *T. cruzi*

T. cruzi is diploid, with differentially-sized homologous chromosome pairs (Pedroso et al. 2003). Its genome has been recently sequenced (El-Sayed et al. 2005) and is estimated to be between 106.4-110.7 Mb in size (diploid). At least 50% of the *T. cruzi* genome is repetitive sequence, consisting mostly of large gene families of surface proteins, retrotransposons and subtelomeric repeats.

T. cruzi exhibits extensive and well-characterised intraspecific genetic diversity (Macedo & Pena 1998, Tibayrenc 2003). Two major evolutionary lineages of the parasite, named *T. cruzi* I and *T. cruzi* II, have been identified (Satellite Meeting 1999). These lineages are very divergent, as revealed by several biological and molecular markers including isozymes (Miles 1978) and polymorphism in 24Sα rDNA (GenBank accession nº L19411) and mini-exon gene (GenBank accession nº X62674) sequences (Fernandes et al. 1999). *T. cruzi* I and *T. cruzi* II strains belong predominantly to distinct ecological environments: the sylvatic and domestic transmission cycles of Chagas disease, respectively (Briones et al. 1999, Zingales et al. 1999). *T. cruzi* I strains are characterised as containing zymodeme Z1 (a zymodeme is a group of strains that have the same isozyme profile), 24Sα rDNA and mini-exon group 2, and induce low parasitism in human Chagas patients. In contrast, *T. cruzi* II strains are characterised as containing zymodeme Z2, 24Sα rDNA and mini-exon group 1, and cause human infections with high parasitemia in classic endemic areas (Zingales et al. 1999). In Brazil and Argentina, at least, *T. cruzi* II strains appear to be primarily responsible for the tissue lesions seen in Chagas disease (Di Noia et al. 2002, Freitas et al. 2005).

However, there are some parasite strains that cannot be properly grouped into any one of these two major lineages. Among these unclassified strains are those belonging to zymodeme Z3 (23) and hybrid strains characterised as rDNA group 1/2 (Souto et al. 1996, Stolf et al. 2003). Using isozymes and random amplification of polymorphic DNA (RAPD) typing, Brisse et al. (2000, 2001) proposed an alternative subdivision of *T. cruzi* strains into two major groups or DTUs (Discrete Typing Units) I and II. DTUI is a homogenous group corresponding to *T. cruzi* I. DTU II, however, is further subdivided into five sub-lineages (DTU IIa-e), each comprising one of the following reference strains: CanIII cl1 (IIa), Esmeraldo cl3 (IIb), M5631 cl5 (IIc), MN cl2 (IId) and CL Brener (IIe). DTU IIb corresponds to the major *T. cruzi* II lineage (Brisse et al. 2001). The extensive genetic diversity within each of these clades or sub-lineages can be unraveled by analyses of microsatellites and several other genomic markers (Macedo et al. 2004).

Reproduction of *T. cruzi*

Although capable of recombination in vitro (Gaunt et al. 2003), *T. cruzi* reproduces predominantly by binary fission. Consequently, its diploid nuclear genotype is transmitted *en bloc* to its progeny. Thus, *T. cruzi* presents extreme degrees of linkage disequilibrium, as shown through isozymes (Tibayrenc et al. 1986) and microsatellites (Oliveira et al. 1998), and exhibits a predominantly clonal population structure. Indeed, *T. cruzi* has been considered a prototypical clonal eukaryotic pathogenic microorganism (Tibayrenc & Ayala 2002).

The occurrence of hybridisation in natural populations of *T. cruzi* was suggested by isozyme analyses (Bogliolo et al. 1996, Carrasco et al. 1996), restriction fragment length polymorphism of housekeeping genes (Higo et al. 2000), RAPD (Brisse et al. 2003) and genotype variations observed at chromosomal level (Brisse et al. 2003, Pedroso et al. 2003, Sturm et al. 2003) and has been confirmed by nucleotide sequencing (Machado and Ayala 2001, Augusto-Pinto et al. 2003). This discovery proved that sexual events definitely have occurred and have shaped the genetic 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 (Brisse et al. 2003). Genotyping of nuclear markers in *T. cruzi* has thus far been limited to the characterisation of multi-locus genotypes. To understand the evolutionary history of the species, it is desirable to dissect multi-locus genotypes into their constituent haploid genome blocks (haplotypes).

Haplotype studies in *T. cruzi*

For our haplotype studies, we initially typed a large number of monoclonal strains of *T. cruzi* with five nuclear CA-repeat microsatellites (Freitas et al. 2006). For our analysis we used as a metric the minimum number of mutational steps necessary to transform the microsatellite profile of one strain into that of another, assuming a stepwise mutation model for the evolution of the microsatellites. From the pairwise distance between the strains, we built a genetic distance matrix, which is pictorially depicted by the multidimensional scaling (MDS) plot shown in Fig. 1. 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. (2000): MDS-cluster A corresponds to DTU I or *T. cruzi* I, MDS-cluster C to DTU IIb or *T. cruzi* II, MDS-cluster B to DTU IIc and MDS-cluster BH to the DTU IId and IIe. Only three strains fell outside the four clusters: CanIII (DTU IIa), Dog Theis and 402.

It should be noted once more that *T. cruzi* is essentially a diploid organism. The multidimensional plot shown in Fig. 1 is based on the joint microsatellite typing of all the

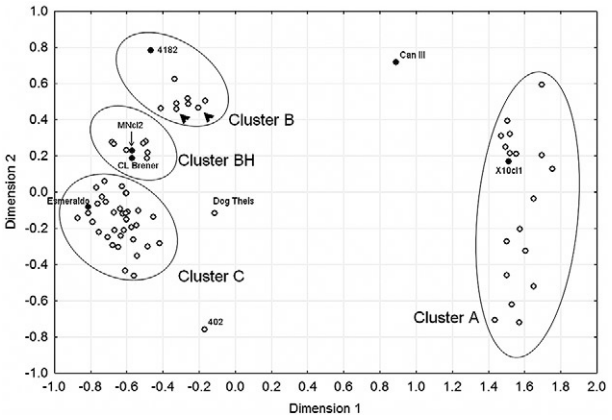

Fig. 1: multidimensional scaling plot of 75 *Trypanosoma cruzi* strains genotyped for five microsatellites. Only outliers and the prototypical strains of Brisse et al. (2000) 11 are named in the plot. Arrowheads indicate strains 222 and 115. The strains in the regions delimited by ellipsoids are: multidimensional scaling (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 B: 115, 222, 226, 231, 3663, 3869, 4182, M5631cl5; MDS-cluster BH: M6241cl6, 167, 1022, cl82, CL Brener, MNcl2, NR, SC43 cl1, SO3, Tulacl2; 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. Modified from Freitas et al. (2006).

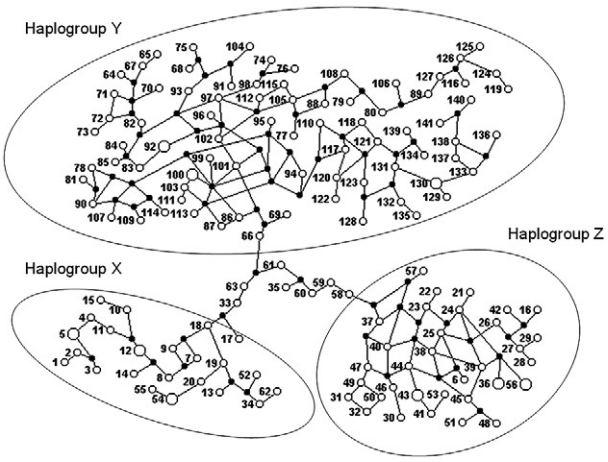

Fig. 2: neighbor-joining trees obtained from the sequences of three mitochondrial genes of *Trypanosoma cruzi*: cytochrome oxidase subunit II (COII) (Freitas et al. 2006), cytochrome b (CYb) (Brisse et al. 2003) and nicotinamida adenina dinucleotídeo dehydrogenase subunit 1 (ND1) (Machado & Ayala 2002). The numbers in the three main branches indicate the percentage bootstrap values. For COII, the strains displayed are the following: clade A: 1004, 1006, 1502, Col18/05, Cuica, Cutia, RBI, RbII, SilvioX10; clade B: sublineage IIc - 222, 231, 3869, M6241, Mn, sub-lineages 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, Mas Cl1, MPD, TU18, Y. For CYb, the strains are as follows: clade A: Cuica, SC13, Tehuentepec, X10; clade B: sublineage IIc - M5631, M6241, X109/2, sub-lineages 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, sub-lineages 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. Modified from Freitas et al. (2006).

homologous chromosomes; in other words, this map is based on multi-local diploid genotypes of microsatellites. However, much more information could be acquired by separating the microsatellite alleles that are in each of the two homolog chromosomes, i.e., the two haplotypes.

If we were analysing a sexually reproducing organism, we would obtain two haplotypes for each chromosome pair. However, because of the predominant binary fission asexual reproduction of *T. cruzi*, extreme levels of linkage disequilibrium are present. Thus, the obtained haplotypes in fact represent whole haploid genomes, like the haploid gametes that unite during fertilisation to form a diploid organism.

To obtain the haplotypes, we ran the combined results of the microsatellite genotypes and 24Sα rRNA gene polymorphisms through PHASE software (Stephens et al. 2001). This powerful programme uses a Bayesian strategy to estimate haplotypes from population genotype data. The programme identified no fewer than 141 different haplotypes in the 75 strains tested, corresponding to a haplotypic diversity of 0.993 (Freitas et al. 2006). The identified haplotypes were then subjected to a median joining analysis using NETWORK 3.1 software (Bandelt et al. 1999). The resulting multitude of plausible trees is best expressed by a network that displays alternative potential evolutionary paths (Fig. 2). Although the data are complex, three haplotypic clusters are clearly identifiable. We refer to these clusters as haplogroups X, Y and Z. The three haplogroups are connected by long and unique paths, emphasising 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. We assigned these haplotypes to a fourth haplogroup that we called "I" for indeterminate.

We then assigned two haplogroups to each of the 75 strains to constitute a haplogroup "genotype" (Table). The results were very simple. All strains belonging to the DTU I lineage (MDS-cluster A) (Fig. 1) proved to be Z/Z, i.e., had two haplotypes belonging to haplogroup Z. Likewise, all the strains of DTU IIb (MDS-cluster C) (Fig. 1) had Y/Y genotypes and those of DTU IIc (MDS-cluster B) (Fig. 1) had X/X genotypes (Freitas et al. 2006). Thus we were able to identify three large groups of ancestral *T. cruzi* strains. The first had haplogroup "genotype" Z/Z, corresponding to *T. cruzi* I. The second had haplogroup "genotype" Y/Y and clearly corresponded to *T. cruzi* II. The most significant finding of our study was the identification of the X/X cluster, which corresponds exactly to the *T. cruzi* strains that belong to zymodeme Z3 found by Miles et al. (1978) and which do not fit into the dichotomous *T. cruzi* I/*T. cruzi* II model proposed in

TABLE

Nuclear and mitochondrial markers of *Trypanosoma cruzi*

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/X
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: restriction fragment length polymorphism typing of the *T. cruzi* cytochrome oxidase subunit II (COII) gene; *b*: clusters of strains generated by multidimensional scaling analysis; *c*: haplotypes inferred by PHASE; *d*: clusters of haplotypes in the Network. Modified from Freitas et al. (2006).

1999. We suggest that this is a third phylogenetic lineage of *T. cruzi* and should be designated *T. cruzi* III.

Interestingly, the strains in cluster BH all had X/Y genotypes, confirming their hybrid nature and indicating that they were derived from recombination events between ancestral types Y/Y and X/X, i.e., *T. cruzi* II and *T. cruzi* III. Due to the way that PHASE identifies haplotypes, proximity of haplotype numbers is highly correlated with genetic proximity. Hybrid strains 167, 1022, 182, CL Brener and Tulacl2 have genotypes 4/99, 2/102, 5/108, 5/100 and 3/103, respectively, and form one group, while strains MNcl2, NR, SC43cl1, and SO3 have genotypes 52/133, 55/130, 54/129 and 54/130 and form another group. Notably, these groups are equivalent to sub-lineages IIe and IId of Brisse et al. (2000). This suggests that at least two independent hybridisations occurred, presumably followed by clonal microdifferentiation.

The classification of *T. cruzi* into three major phylogenetic trunks does not exhaust all ancestral possibilities. The strains Can III [genotype I/I, cytochrome oxidase subunit II (COII) B], Dog Theis (genotype I/I, COII C), 402 and Mas1cl1 (both genotype I/Y, COII C) share haplotypes with haplogroup I. Three of these four strains are located outside MDS clusters in Fig. 1A, which may suggest the existence of yet other phylogenetic lineages in *T. cruzi*.

Mitochondrial DNA studies

The extreme levels of linkage disequilibrium in the genome of *T. cruzi* not only cause the association of all nuclear markers into haploid genotypes, but also create strong associations between nuclear markers and mitochondrial genotypes. The latter are known to be

uniparental (by convention, the maternal gamete is the mitochondrial donor) and functionally haploid. Indeed, Gaunt et al. (2003) have shown that the hybridisation of *T. cruzi* strains involves only nuclear genomes and that mitochondrial fusion does not occur.

We studied the sequences of large portions of the maxicircle-encoded COII gene (Freitas et al. 2006) as well as cytochrome b (Brisse et al. 2003) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (Machado & Ayala 2001). The neighbour-joining trees generated for each of these regions are shown in Fig. 3. All trees was had very similar topologies, consisting of three tightly clustered sets of strains, separated by very large genetic distances, permitting the straightforward allocation of *T. cruzi* strains into three mitochondrial clades. For simplicity, we called these clades clusters A, B, and C.

It was most rewarding to observe that our MDS clusters (Fig. 1) corresponded perfectly to their homonymous mitochondrial clades, i.e., MDS cluster A was composed of the same strains that belonged to haplogroup Z/Z and to mitochondrial clade A, MDS cluster C was composed of the same strains that belonged to haplogroup Y/Y and to mitochondrial clade C and MDS cluster B was composed of the same strains that belonged to haplogroup X/X and to mitochondrial clade B. Thus, the results of the mitochondrial sequencing strongly confirm the existence of the *T. cruzi* III phylogenetic group.

The MDS cluster BH contained the strains belonging to the hybrid sub-lineages IId and IIe (Brisse et al. 2000), all of which fall within mitochondrial clade B. Thus, the strains of sub-lineages IId and IIe are not only produced by hybridisations between *T. cruzi* II and *T. cruzi* III, but both have the same mitochondrial donor, *T. cruzi* III.

An evolutionary model

Based on our results, we propose that three ancestral genomes, *T. cruzi* I, *T. cruzi* II and *T. cruzi* III (Fig. 4), existed in the distant past. It is interesting to note that this proposal matches the initial suggestion made by Miles et al. (1978) almost 30 years ago on the basis of isozyme studies. It is likely that *T. cruzi* II and *T. cruzi* III had overlapping ecological niches and thus the conditions necessary for hybridisation were in place. At least two hybridisation 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.

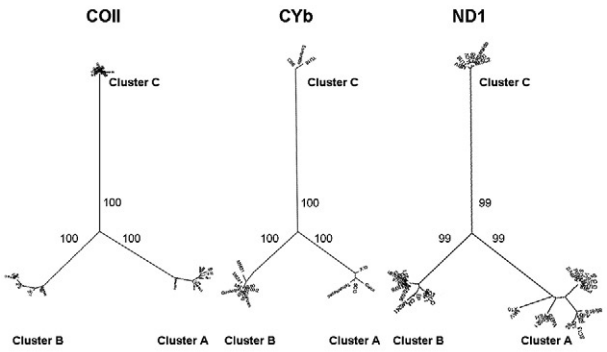

Fig. 3: median joining network of the haplotypes identified by the PHASE software.

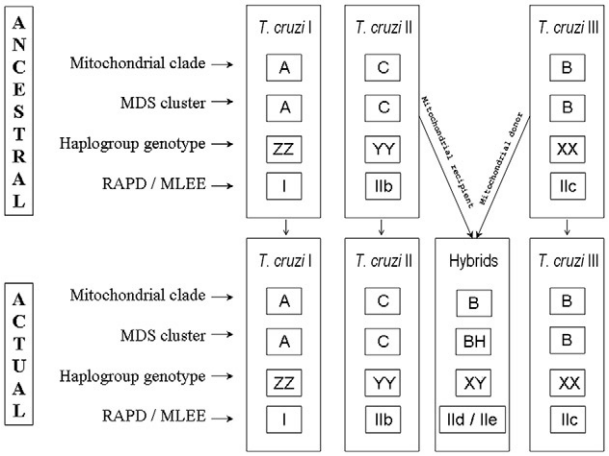

Fig. 4: diagram depicting the proposed model for the evolution of *Trypanosoma cruzi* strains. Modified from Freitas et al. (2006).

The existence of strains that cannot be accommodated into this scenario, i.e., CanIII [sub-lineage IIa of Brisse et al. (2000)] and Dog Theis, suggests additional complexity in the evolutionary history of *T. cruzi*. Other alternative models have been proposed for its population structure (Westenberger et al. 2005), but we will not discuss them fully here as they are not based on the novel high resolution haplotypic analysis presented in our work (Freitas et al. 2006).

The fact that the same population structure of *T. cruzi* is predicted with different molecular markers, such as isozymes (Miles et al. 1978), RAPD (Brisse et al. 2000, 2003), microsatellites (Oliveira et al. 1998) and several other nuclear sequences (Fernandes et al. 1999, Zingales et al. 1999, Machado & Ayala 2001, August-Pinto et al. 2003), and mitochondrial markers (Machado & Ayala 2001, Brisse et al. 2003, Freitas et al. 2006), bears witness to its extreme stability. Although it has been shown conclusively in our study and also by others (Machado & Ayala 2001, Brisse et al. 2003) that hybridisation events have occurred in the evolutionary history of *T. cruzi*, they seem to have been subsequently stabilised by strong clonal propagation (Macedo & Pena 1998, Tibayrenc 2003).

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