

***Trypanosoma cruzi*: Genetic Structure of Populations and Relevance of Genetic Variability to the Pathogenesis of Chagas Disease**

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Chagas disease, caused by the protozoan Trypanosoma cruzi, has a variable clinical course, ranging from symptomless infection to severe chronic disease with cardiovascular or gastrointestinal involvement or, occasionally, overwhelming acute episodes. The factors influencing this clinical variability have not been elucidated, but it is likely that the genetic variability of both the host and the parasite are of importance. In this work we review the genetic structure of T. cruzi populations and analyze the importance of genetic variation of the parasite in the pathogenesis of the disease under the light of the histotropic-clonal model.

Key words: *Trypanosoma cruzi* - genetic variability - mismatch repair - population structure - pathogenesis - Chagas disease

Chagas disease is a chronic illness caused by the protozoan *Trypanosoma cruzi* afflicting over 18 million people in the three Americas, from Southern Argentina to the Southern United States. *T. cruzi* is transmitted to humans by the feces of infected hematophagous triatomine bugs, blood transfusion, congenitally from infected mothers, or more infrequently by organ transplantation, contaminated food or laboratory accidents (Lambrecht 1965, Prata, 2001).

Under natural conditions the life cycle of *T. cruzi* alternates between an invertebrate host, one of more than 130 hemiptera from the family Reduviidae, and a vertebrate host that can be any one of more than 100 mammalian species from different orders (Lambrecht 1965, Zeledon & Rabinovich 1981). The geographical distribution of the triatomines and the vertebrate hosts associated with the insects' preferences for specific blood sources define two transmission cycles of *T. cruzi*: a sylvatic cycle involving different species of triatomid bugs and wild animals and a domestic/peridomestic cycle in which household animals and human beings act as reservoirs.

The human disease has a very variable clinical presentation. Following the parasite infection, there is a short acute phase characterized by an abundant parasitemia, but frequently very mild and unspecific symptoms that difficult the recognition of the contagion. In less than 5% of the cases, fatal neurological and/or cardiac complications of the acute phase can be observed. The infection then proceeds to a chronic phase, with scarce parasitemia and an unpredictable clinical course that ranges from an absence of symptoms to severe disease with cardiovascular and/or gastrointestinal involvement (Prata 2001). The

heart pathology is characterized by chronic myocarditis that frequently leads to cardiomegaly, congestive heart failure and arrhythmias (Tanowitz 1992). In the digestive forms of the disease, dilatation of the esophagus and/or colon (megaesophagus and megacolon, respectively) may be observed in advanced stages (Lopes et al. 1989, Adad et al. 1991). Pathologically, both the heart and digestive tract exhibit focal lymphocytic infiltrates (Brenner 1992), with scarring and loss of myocardial cells in the cardiopathy (Palacios-Pru et al. 1989) and neuronal degeneration with denervation in megaesophagus and megacolon (Köeberle, 1968, Adad et al. 1991).

Geographical variations in the prevalence of clinical forms and morbidity of Chagas disease have been reported. In Brazil, the asymptomatic or indeterminate form is the most common (60-70%), followed by the cardiac and digestive forms (20-30% and 8-10%, respectively); the cardio-digestive form is less frequent. However in the Central Brazil and in Chile, the digestive form of Chagas disease predominates, while it is practically non-existent in Venezuela and Central America (Luquetti et al. 1986, Dias 1992). The reason for this geographical heterogeneity and why different patients develop different clinical forms remain unexplained. It certainly cannot be explained by major genetic differences between the human populations that inhabit these areas. Thus, it is generally believed that they are primarily determined by genetic variation of *T. cruzi*. However, a possible role for environmental, nutritional, and immunological aspects of the host cannot be discarded.

It is interesting to observe that the relative importance attributed to parasite-related versus human-related variable has changed in the evolution of pathogenetic explanations for Chagas disease. Indeed, historically, we can discern three main phases. The first one, which we might call "parasite phase", corresponds to the period immediately following discovery of the disease, when there was no questions as to the primary pathogenetic role of *T. cruzi*. In this period there was an intense, albeit fruitless, search for biological and biochemical methods and mark-

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ers capable of establishing a link between the variability of the parasite and the clinical form of the disease. The failure of these endeavors, associated with the scarcity of parasites in the blood and tissues of chronic symptomatic patients led to the “autoimmune phase”. In this second period the pathogenetic importance of the parasite was relegated to a second plane, except as providing cross-reacting epitopes capable of unleashing autoimmune reactions (Schmunis 1987, Brener 1987, Kalil & Cunha-Neto 1996). The discovery of more sensible techniques for *T. cruzi* detection rescued the primary role of the parasite in the pathogenesis of Chagas disease. We are now experiencing the third wave, the “genomic phase”, in which the illness is seen as the product of the interaction of two highly variable and coevolving genomes: the parasite genome and the human genome.

THE GENOMIC PHASE

The genomic phase of the pathogenesis of Chagas disease started in the early 90's when the presence of *T. cruzi* in chronic infected tissues was revealed by application of powerful immunohistochemical techniques developed in the past two decades (Higuchi et al. 1993). However, it was the application of the sensitive polymerase chain reaction (PCR) that provided definitive molecular evidence that clearly tied tissue lesions to the presence of the parasite (Jones et al. 1993, Brandariz et al. 1995, Vago et al. 1996a). These new studies highlighted the primary role of *T. cruzi* in the pathogenesis of Chagas disease and set the stage for establishing the notion that genomic variation of *T. cruzi* might influence the course of the disease.

THE GENETIC VARIABILITY OF *T. CRUZI*

Different studies including biological, biochemical and molecular ones have demonstrated that *T. cruzi* is a very heterogeneous taxon (reviewed by Macedo & Pena 1998, Macedo et al. 2001, Devera et al. 2003). In the present review we will recapitulate some of these studies, with emphasis in those that have contributed to a better understanding of the *T. cruzi* population structure and its role for the pathogenesis of Chagas disease.

ZYMODEMES

The first experimental method that demonstrated the extent of genetic diversity of *T. cruzi* was the analysis of electrophoretic variants of cellular enzymes (isoenzymes). Based on the isozymic variability of six loci Miles et al. (1977, 1978, 1980) proposed the existence of three isoenzymatic clusters (zymodemes Z1, Z2, and Z3) of *T. cruzi*. Epidemiological studies demonstrated that Z1 and Z3 were associated with the sylvatic cycle and Z2 with the domestic transmission cycle (Miles et al. 1978, 1980, Barret et al. 1980). Carneiro et al. (1990) identified four isoenzymatic groups (ZA, ZB, ZC, and ZD) when analyzing eight isoenzyme loci. Subsequent studies using 15 isoenzyme loci predictably evidenced much higher genetic diversity, and 43 genotypes were proposed (Tibayrenc et al. 1986, Tibayrenc & Ayala 1988). Numerical taxonomic analysis of these data later suggested the existence of two major highly heterogeneous phylogenetic lineages of *T. cruzi* that differed in several biological properties (Tibayrenc

1995). More recently, Brisse et al. (2000) identified five lower phylogenetic subdivisions within one of the lineages and proposed the existence of six principal discrete *T. cruzi* groups (so-called lineages I, IIa, IIb, IIc, IId, IIe).

SCHIZODEMES

The first studies of DNA polymorphism in *T. cruzi* were published in 1980 by Morel et al. who reported on restriction fragment length polymorphism (RFLP) typing of kDNA minicircles. Parasite populations displaying identical or very similar kDNA minicircle restriction patterns were called schizodemes. kDNA RFLP analyses unraveled an unexpected amount of genetic diversity in *T. cruzi*, although some correlation was seen with the isozyme classification, demonstrating that linkage disequilibrium extended even between different (nuclear and mitochondrial) genome compartments (Tibayrenc & Ayala 1987). Together with cloning experiments, kDNA restriction analysis demonstrated for the first time that single strains of *T. cruzi* could contain two or more distinct clones (Morel et al. 1980). The existence of these multiclonal *T. cruzi* strains was confirmed later by several groups using different techniques (Deane et al. 1984, Marques-Araújo & Chiari, 1988, Carneiro et al. 1990, Macedo et al. 1992, Oliveira et al. 1998, 1999).

LOW STRINGENCY SINGLE SPECIFIC PRIMER (LSSP)-PCR

An alternative and more sensitive way to study the polymorphism of the kDNA is the LSSP-PCR method (Vago et al. 1996a). In this technique a previously purified DNA amplicon is subjected to PCR using a single specific primer under very low stringency conditions. The primer hybridizes specifically to its complementary region and also nonspecifically to multiple sites within the fragment, in a sequence-dependent manner, thus producing a highly complex set of reaction products that can be resolved by electrophoresis to generate ‘gene signatures’ (Pena et al. 1994, Pena & Simpson, 1996). LSSP-PCR has been successfully applied to the ~ 330 bp variable DNA fragment of *T. cruzi* kDNA minicircles to produce the ‘kDNA signatures’, allowing for the first time to profile parasites present in the tissues of chronically infected patients (Vago et al. 1996b). The initial idea of the differential tissue tropism playing fundamental importance in determining the pathogenesis of Chagas disease, proposed as early as 1911 (Vianna 1911) was revitalized when it was shown that parasites presenting different genetic profiles could be found in distinct tissues (esophagus and heart) of the same patient (Vago et al. 2000). Moreover, in a study of doubly infected mice, we found a strong correlation between the strain profile (JG or Coll.7G2) and the type of lesion in the heart and rectal tissues (Andrade et al. 1999).

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

The PCR-based RAPD technique and the less used but closely related simple sequence repeat anchored primer PCR (SSR-PCR) have provided a sensitive approach for *T. cruzi* profiling and constitute useful tools for establishing genetic relationship among isolates (Dias Neto et al. 1993, Steindel et al. 1993, Tibayrenc et al. 1993, Oliveira et al. 1997, Brisse et al. 2000). Complex and strain-specific

banding patterns were evidenced for different *T. cruzi* populations, although no correlation with clinical aspects has been found (Oliveira 1997). However, when we compare the RAPD profiles of strains isolated from patients in the chronic phase of the disease, with isolates from patients in the acute phase, and with *T. cruzi* populations obtained from sylvatic vectors and mammals, a clear increasing gradient in variability is observed (Table I). These results are compatible with the idea that not all *T. cruzi* strains are capable of establishing effective infections in *Homo sapiens* and that humans thus function as a filter in selecting more adapted sub-populations (Macedo & Pena 1998). Even in the domestic environment, the probability of acquiring Chagas disease from one bite of an infected bug has been estimated to be only about one in a thousand (Prata 2001).

RAPD patterns have been used in numerical taxonomy studies. A strong correlation has been observed between RAPD profiles and zymodemes (Steindel et al. 1993, Tibayrenc et al. 1993). Moreover, RAPD profiling was also utilized for corroborating the *T. cruzi* taxon division in two major lineages (Tibayrenc 1995, Souto et al. 1996).

MINI EXON POLYMORPHISM

The mRNA of trypanosomes is unique in that most, if not all, mature species bear an identical 35-bp splicer leader (SL) at their 5'-termini (Borst & Greaves 1986). This SL is encoded in blocks of tandemly repeated units, the mini-exon genes (ME). Comparative alignment of ME from different *T. cruzi* strains showed the presence of highly conserved sequences regions of 39 bp (the exons), similar regions of 73 bp with more than 98% of identity (corresponding to the introns) and divergent intergenic regions (less than 59% of similarity) (Murthy et al. 1992, Souto et al. 1996) (Fig. 1). By using a multilocal PCR strategy it was possible to cluster *T. cruzi* strains into two groups according to the amplification products. Strains that yielded 300 bp products were designated as belonging to group 1 and those that yielded 350 bp products were designated as belonging to group 2 (Souto et al. 1996). Subsequent analysis of mini-exon gene sequences of Z3 strains recognized a sub-clade inside of group 2 characterized by a ~ 50 bp insertion/deletion in the non-transcribed spacer region of the mini-exon (Fernandes et al. 1998). New sets of primers were designed to amplify the Z3 strains (Fernandes et al. 2001).

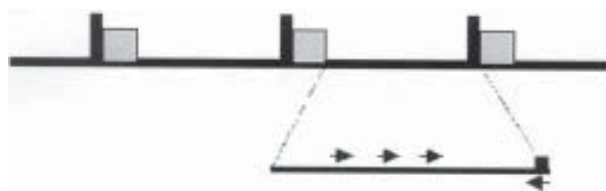


Fig. 1: schematic representation of the *Trypanosoma cruzi* mini-exon (ME) locus. The ME locus is organized as tandem repeated units in which the exon region are conserved throughout the kinetoplastida (black boxes). Intron regions are indicated by the grey boxes and the non-transcribed spacer is represented by the black connecting line. ME typing is based on a multiplex polymerase chain reaction using an exon-specific primer (right arrow) and lineage-specific primers located in the spacer region.

rDNA POLYMORPHISMS

In trypanosomes, the ribosomal cistron exhibits a singular organization. Both the small subunit (SSU) and the large subunit (LSU) are considerably larger than the typical eukaryotic rRNA species. Particularly interesting is the LSU that contains two-high-molecular weight RNAs (24S α /24S β) and six low-molecular-weight RNA (S₁-S₆) components (Leon et al. 1978, Hernandez et al. 1990, Arruda et al. 1990). The rDNA cistron is constituted of repeated sequences in which the coding regions of the SSU and the LSU are separated by two internal transcribed spacers. The ITS 1 separates the coding region of the 18S subunit and the 5.8 S rDNA, and the ITS2 separates 5.8 S rDNA sequence from the 24S rDNA (Fig. 2). Comparison between the 24S α rRNA gene of *T. cruzi* and other trypanosomatids showed high homology except for discrete regions. The most divergent domain (D7) is a segment of about 100 bp located at the 3' end of *T. cruzi* gene (Arruda et al. 1990, Souto & Zingales 1993). Initially, PCR amplification of a sequence from the D7 domain revealed a dimorphism when DNA from different *T. cruzi* strains was used (Souto & Zingales 1993). Three different groups or lineages of *T. cruzi* strains could be detected. Strains from lineage 1 gave a 125 bp product and another group of strains (lineage 2) gave a 110 bp product. A third group presenting both PCR products and denoted group 1/2 was later detected (Souto et al. 1996).

TABLE I

Analysis of genetic variability of 58 *Trypanosoma cruzi* strains by random amplified polymorphic DNA according to their origin

Sample	Number of strains	Average number of bands per track	Average number of shared bands in different strains (\pm s.d.)	Average proportion of shared bands
Strains isolated from non-human sources	11	45.7 \pm 7.8	10.2 \pm 8.9	22%
Strains isolated from patients with acute Chagas disease	11	55.0 \pm 10.3	21.5 \pm 13.9	39%
Strains isolated from patients with asymptomatic chronic Chagas disease	14	55.4 \pm 6.7	28.1 \pm 16.8	51%
Strains isolated from patients with symptomatic chronic Chagas disease	22	56.9 \pm 4.4	30.1 \pm 11.8	53%

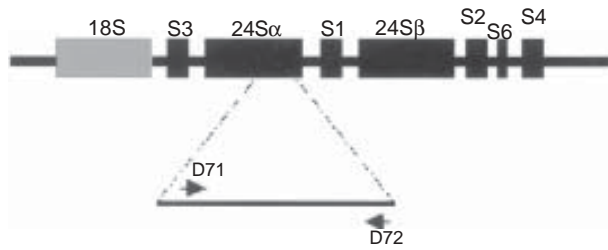


Fig. 2: schematic organization of *Trypanosoma cruzi* rDNA cistron. The small subunit (SSU) is indicated by a grey box and the large subunit (LSU) by black boxes. The rDNA cistron is constituted of repeated sequences in which the coding regions of the SSU and the LSU are separated by two internal transcribed spacers (ITS). The ITS 1 separates the coding region of the 18S subunit and the 5.8 S rDNA, and the ITS2 separates 5.8 S rDNA sequences from the 24S rDNA. rDNA typing is performed using primers D71 and D72 that amplifies a dimorphic region at the 3' end of the 24S α rRNA gene.

The phylogenetic relationship of strains from the rDNA group 1, 2, and 1/2 is still under intense investigation. Studies on RAPD and mini-exon profiling had shown that the strains of the D7 rDNA group 1/2 were more genetically related to the rDNA lineage 1 than rDNA lineage 2 (Souto et al. 1996). However Zingales et al. (1999) sequenced both alleles of the D7 rDNA from a strain of group 1/2 and while the alignment of the sequence type 1 placed this strain among lineage 1 strains, the sequence type 2 did not allow the clear position of the strain in the tree. In fact, depending on the algorithm used group 1/2 in some tree seemed to be more related to lineage 1 and in the other trees to lineage 2.

Additionally, when comparing 18S and D7-24S α rDNA sequences, Kawashita et al. (2001) found that *T. cruzi* could be organized into four riboclades. The riboclade 1 (rDNA group 1) could be separated from the others, but the riboclade 2 (containing the strains rDNA group 2) proved to be a sister group of the two additional riboclades 3 and 4, which grouped representative strains from zymodeme Z3 and rDNA group 1/2.

More recently, extra D7 PCR products of 117/119 bp (Pimenta 2002) or 120/130 pb (Brisse et al. 2001) were found. Brisse et al. (2001) combine data from 18S, D7 region of 24S α rDNA and mini-exon data to reinforce their hypothesis of a subdivision of the *T. cruzi* into six lineages (I, IIa, IIb, IIc, IID, IIE).

KARYOTYPING

The chromatin of *T. cruzi* is poorly condensed during mitosis and distinct chromosomes cannot be visualized by microscopic methods. The development of the pulsed-field gel electrophoresis (PFGE) technique made possible the identification of 20 chromosomal bands ranging from 450 Kb to 3500 Kb for CL Brener, the model clone of the *T. cruzi* genome initiative (Santos et al. 1997, Porcile et al. 2003). However, the intensities of different chromosomal bands vary in ethidium bromide stained gels, indicating that the chromosome number may be possibly much higher than 20 (Santos et al. 1997).

Annealing studies with several DNA probes show

hybridization signals in pairs, presumably in homologous chromosomes, which in many cases differ in size. In fact extensive chromosomal size polymorphism has been revealed among different strains and even between clones of the same strain of *T. cruzi* (Henriksson et al. 1995, Alves et al. 1996). This polymorphism can be seen in most trypanosomatids and has been attributed to expansion/contraction of tandem repeats (Dujardin et al. 1995). Moreover, differences in ploidy for one or more chromosomes (aneuploidy) have been also observed (reviewed by Henriksson et al. 1996).

Attempts have been made to reconstruct the evolutionary history of *T. cruzi* by using the absolute chromosomal size difference index (aCSDI) as a genetic distance among the isolates. This method assumes that the genomic distance between two organisms is the sum of the size differences between their homologous. The phenetic trees obtained had defined initially two (Henriksson et al. 2002) and later three (Pedroso et al. 2003) major clusters in *T. cruzi* encompassing respectively the strains from D7 rDNA group 1, 2 and 1/2.

MICROSATELLITE ANALYSES

The disclosure of the *T. cruzi* microsatellites (polymorphic tandem repeats of 2-6 bases long simple motifs) has provided a new tool for the analysis of the population structure of the parasite (Oliveira et al. 1998). These DNA markers are extremely polymorphic and are dispersed throughout the parasite's nuclear genome. The microsatellite analysis of different *T. cruzi* strains and clones constituted a simple screening test to estimate if a *T. cruzi* strain is a monoclonal or a multiclonal population (Oliveira et al. 1998, 1999, Macedo et al. 2001). By using microsatellite analyses it was demonstrated that the percentage of multiclonal (or aneuploidy) populations decrease progressively as we compare strains isolated from the sylvatic cycle with those isolated from man, confirming the previous idea of a human filter selecting more adapted strains (Table II- Oliveira et al. 1998, 1999, Macedo et al. 2001).

The *T. cruzi* microsatellite profile also has been used for phylogenetic investigations. In initial studies involving 53 *T. cruzi* populations isolated from different geographical areas, the tree topology indicated clustering of the strains in three major branches. The first two of these branches contained respectively the strains belonging to D7 rDNA group 1/mini-exon group 1 and those of D7 rDNA group 2/mini-exon group 2. The third branch was less homogeneous and enclosed strains from the D7 rDNA

TABLE II

Proportion of *Trypanosoma cruzi* strains with more than two microsatellite peaks according to their origin

Sample	Total number	Strains with more than two peaks
Strains isolated from non-human sources	40	23 (58%)
Strains isolated from patients with chronic Chagas disease	79	8 (10%)
Total	119	21 (18%)

group 1/2 together with some strains presenting D7 rDNA type 2, but mini-exon type 1 (Oliveira et al. 1999, Macedo et al. 2001). It was proposed that strains presenting D7 rDNA type 2 could be split into two distant subgroups with different origins, and that one of these subgroups could be more related to the strains from group 1/2 (Souto et al. 1996, Oliveira et al. 1998, Macedo et al. 2001). Further work using microsatellite analysis demonstrated that the third cluster may correspond to hybrid *T. cruzi* strains (Augusto-Pinto et al. manuscript in preparation).

OTHER GENE POLYMORPHISMS

Using two different nuclear genes (trypanothione reductase - TR and dihydrofolate reductase-thymidylate synthase - DHFR-TS) and a region of the mitochondrial DNA partially encompassing the maxicircle-encoded genes (cytochrome oxidase subunit II - COII and NADH dehydrogenase subunit 1 - ND1) Machado and Ayala (2001) proposed the division of *T. cruzi* strains into four different clades, named A, B, C, and D. Clade A included all DNA sequences from strains belonging zymodeme 1 (Z1). However, the DNA sequences obtained from strains of Z2 and Z3 were not monophyletic and were clustered into two (mitochondrial data) or three (nuclear data) distinct clades (B, C, and D).

Augusto-Pinto et al. (2003) cloned and sequenced the mismatch repair mutS homologue 2 (MSH2) of *T. cruzi*, which proved to be polymorphic. Examination of 13 different strains identified three clusters of sequences that could be used for dividing the taxon into three distinct clades (A, B, and C). It was shown that strains belonging to clade A correspond to those presenting D7 rDNA 2, mini-exon 2, and zymodeme Z1 (that were initially named as group 2 by Souto et al. 1996, but latter renamed *T. cruzi* I, see about). Similarly strains belonging to clade C correspond to those strains sharing D7 rDNA 1, mini-exon 1, zymodeme Z2 and initially refereed as group 1 by Souto et al. (1996) and latter *T. cruzi* II. Into the third clade B were grouped the hybrid strains such as those presenting D7 rDNA 1/2 or zymodeme Z3.

THE MAJOR LINEAGES IN *T. CRUZI*

As pointed out previously, *T. cruzi* is a very polymorphic species and the overall population structure is far from being completely understood. Until the end of 80's, the prevalent idea was that *T. cruzi* strains could not be grouped into few discrete groups that might represent natural taxa (Morel et al. 1980, Tibayrenc et al. 1986). Instead, a multiclonal population structure was proposed, with the different clones evolving from a very ancient ancestral, basically by clonal reproduction (Tibayrenc et al. 1986, Zhang et al. 1988). However the identification of a strong correlation between different DNA markers such as mini-exon, D7-24S rDNA, RAPD and microsatellites generated a consensus about the existence of two major phylogenetic lineages within the taxon (Tibayrenc 1995, Souto et al. 1996, Nunes et al. 1997, Zingales et al. 1998). The basic dichotomy of the species was further correlated with a plethora of epidemiological, biochemical, biological and molecular markers (Fernandes et al. 1998, Oliveira et al. 1998, Macedo et al. 2001, Henriksson et al.

2002, reviewed by Momen 1999, Zingales et al. 1999, and Buscaglia & Di Noia 2003), but the nomenclature of the major groups became very confusing. In 1999, in an effort to homogenize the nomenclature, the two major lineages were renamed *T. cruzi* I and *T. cruzi* II (Satellite Meeting 1999, Table III). At the time, putative hybrid strains such as those belonging to D7- rDNA group 1/2 (Souto et al. 1996), clonet 39 (Tibayrenc 1995) and Z3 could not be unequivocal classified as *T. cruzi* I or *T. cruzi* II. Thus it was established that these strains should remain identified as *T. cruzi* without any other specification, until new insights could clarify the phylogenetic relationship of the group (Satellite Meeting 1999).

IS *T. CRUZI* A SINGLE SPECIES?

One important question that arises is the significance of the division of *T. cruzi* into discrete major lines. Should be *T. cruzi* I and *T. cruzi* II considerate as separated sub-species or even different species?

The modern concept of a species is that of a population that is simultaneously a reproductive community, an ecological unit and a genetic unit. Obviously, basically clonal asexual species such as *T. cruzi* cannot be defined in reproductive terms and thus the concept of genetic unity, in the sense of a shared genome, becomes critical. The question here is how much intraspecific genomic variability can be allowed before we start splitting the species into different ones. In attending this question Briones et al. (1999) analyzed the rDNA cistron and found that *T. cruzi* I and II constitute monophyletic groups with a genetic distance compatible with a coalescence time of 88 million years before present. This genetic distance is comparable to that separating different *Leishmania* species. They proposed also that *T. cruzi* emerged as a species over 150 million years ago and that the major groups remained separated from each other during the insulation of South America (from 100 to 38 MYR ago), thus favoring their cryptic speciation. According to this view, *T. cruzi* I was autochthonous to South America and co-evolved with marsupials and edentates, whereas *T. cruzi* II co-evolved in North American placental mammals (Briones et al. 1999).

One might alternatively try to define a single species based on the fact that *T. cruzi* clones have similar life cycles and cause Chagas disease. However even this latter criterion falls apart when we consider the clinical pleiotropism of the disease. The evolutionary diversification of *T. cruzi* pre-dated the appearance of *H. sapiens* by several tens of millions of years. Indeed, the first contact of *T. cruzi* with humans seems to have occurred when immigrants from Central Siberia peopled the Americas, less than 15,000 years ago, in the late Pleistocene (Santos et al. 1999). Thus, specific adaptation to human hosts, to whatever extent it exists, must have evolved independently in separate lineages, and may not have evolved at all in some *T. cruzi* (Macedo & Pena 1998). Several findings strengthen this point of view. *T. cruzi* I strains are preferentially encountered in the sylvatic cycle of transmission of Chagas disease while *T. cruzi* II strains are strongly associated with the domestic cycle, at least in Brazil (Fernandes et al. 1998, 1999, Zingales et al. 1998). *T. cruzi*

II is apparently more associated with primates and particularly with human infections while *T. cruzi* I, which seems to be more adapted to marsupials, is only occasionally isolated from humans and even then in regions where *T. cruzi* is enzootic. Until now all parasites isolated from soropositive individuals from endemic regions in Brazil belong to *T. cruzi* II major lineage. (Fernandes et al. 1998, 1999, Zingales et al. 1998, reviewed by Zingales et al. 1999). Recent unpublished results obtained in our laboratory have shown that all parasites typed directly from affected heart and/or esophagus tissues of Brazilian patients with clinical forms of the disease are from the *T. cruzi* II lineage (J Freitas, AM Macedo, manuscript in preparation).

THE ORIGIN OF THE NATURAL HYBRID STRAINS

The occurrence of genetic exchange with homologous recombination has just recently been demonstrated for *T. cruzi* (Stothard et al. 1999, Gaunt et al. 2003). However, in the last two decades evidence for some kind of genetic exchange in *T. cruzi* was brought to light (reviewed by Tait 1980, Gibson & Stevens 1999). Most of the evidence was based on the identification of putative natural hybrid strains by isoenzyme analyses (Bogliolo et al. 1996, Higo et al. 2000), RAPD (Carrasco et al. 1996), karyotyping and/or gene sequencing (Sturn et al. 2003).

What is the origin of the natural hybrid populations detected in *T. cruzi*? An interesting observation is that most of them, if not all, (see below) correspond to those strains that could not be appropriately classified into *T. cruzi* I or *T. cruzi* II major lines (satellite Meeting 1999, Momen 1999). The list of such unclassifiable *T. cruzi* populations includes strains presenting both alleles for the locus D7 24S α rDNA (group 1/2 - Souto et al. 1996), strains from clonot 39 (Tibayrenc 1995, Tibayrenc & Ayala 1998), strains belonging to zymodeme Z3 (Fernandes et al. 1998, 1999) and strains showing discrepant alleles for different loci, for example, D7 type 2 minixon type 1 (Souto et al. 1996). There were no putative hybrids among the strains clearly classified as *T. cruzi* I or *T. cruzi* II, except for CL Brener. Another possibly hybrid is the Tulauhén strain. Here, the complication is that there is more than one strain circulating among different laboratories with the same name; some of them are genuinely *T. cruzi* I, but others are *T. cruzi* II or hybrids (Souto et al. 1996, Brisse et al. 2001, Augusto-Pinto et al. 2003). CL Brener, the clone chosen for the *T. cruzi* genome project, was initially classified as *T. cruzi* II, on the bases of D7 and mini-exon gene typing (Souto et al. 1996). However, several lines of evidence have demonstrated that CL Brener is in fact a hybrid strain presenting characteristics from both major lines and being genetically much more closer to those strains belonging to D7 rDNA group 1/2 than to *T. cruzi* II (Brisse et al. 2001, Pedroso et al. 2003, Augusto et al. 2003).

More recently there was an effort to classify the hybrid strains into the *T. cruzi* I or *T. cruzi* II major lineages. However this turned out to be a more difficult task than initially expected, since depending on the polymorphic marker used, the hybrid strains seemed to be closer related to one or other major lineage. As an example we can cite the strains belonging to the D7 group 1/2 (Souto et al.

1996). As mentioned before, studies on RAPD and mini-exon profiling showed that these strains seemed to be genetically closer to *T. cruzi* II than *T. cruzi* I. However, sequence analyses of the alleles of D7 or 18S rDNAs put the 1/2 strains together with *T. cruzi* I or *T. cruzi* II depending on the algorithm used (Zingales et al. 1999, Kawashita et al. 2001). In order to better accommodate the hybrid strains within *T. cruzi* II, Brisse et al. (2000, 2001) proposed a subdivision of this major lineage into five lower subgroups (named IIa, IIb, IIc, IId, and IIe). However this strategy did not take into account the fact that depending on the characteristic analyzed the hybrids seem to be more related to the *T. cruzi* I or even very different from both major lines (Souto et al. 1996, Zingales et al. 1999, Machado & Ayala 2001, 2002, Augusto-Pinto et al. 2003). An alternative viewpoint now emerging is that the hybrid strains could constitute a third major lineage on *T. cruzi* taxon, maybe the *T. cruzi* III lineage (Machado & Ayala 2001, 2002, Augusto-Pinto et al. manuscript in preparation).

Gaunt et al. (2003) have shown evidence that the hybridization of *T. cruzi* strains occurs only with nuclear genome without mitochondrial fusion, so if the natural hybrid strains have the same evolutionary lineage the hybrids will also have the same type of mitochondrial DNA. Machado and Ayala (2001, 2002) demonstrated that the mitochondrial clade B is constituted by at least two Z3 strains and other hybrid strains from isoenzyme type 39 and 43. Recently, Augusto-Pinto et al. (manuscript in preparation) showed that all strains presenting hybrid molecular markers in their nuclear genomes have a specific mitochondrial genome distinct from both the *T. cruzi* I and *T. cruzi* II major lineages. These results suggest that the hybrid *T. cruzi* strains might represent a truly diverging phylogenetic lineage of *T. cruzi*, conceivably originating from an ancient recombination event between *T. cruzi* I and *T. cruzi* II strains, rather than from multiple recent hybridization episodes.

CLONAL POPULATION STRUCTURE VERSUS SEXUAL REPRODUCTION IN *T. CRUZI*

Analyses of several genetic loci among different parasite populations have demonstrated that *T. cruzi* populations present drastic departures from Hardy-Weinberg expectations and strong linkage disequilibrium (reviewed in Ayala 1993). These findings have been interpreted as suggestive that *T. cruzi* has a clonal population structure in which sexual reproduction is rare or absent. In this perspective each clone represents an independent lineage that divides by binary division and evolves by mutation only from an ancient ancestral (Tibayrenc et al. 1986, Zhang et al. 1988).

One expectation of this would be that *T. cruzi*, as an asexual organism, should present the "Meselson effect", i.e., in the absence of sexual recombination, the two alleles at any given locus evolve independently from the moment of setting up the asexual lineage and nothing prevents them from diverging. Indeed, a great allele divergence has been found in the asexual Bdelloid rotifers (Welch and Meselson 2000). Analyzing the DNA sequence of four different genes from Bdelloid, Welch & Meselson (2000) showed that the genes presented allele divergence

as great as 54% in degenerate sites (third positions in codons, where any of the four bases can be found in the DNA without changing the amino acid in the correspondent protein). In *T. cruzi* however, microsatellite data show an excess of homozygosity (Oliveira et al. 1998). Indeed, a recent study on gene sequencing did not detect any significant divergence of the two alleles in strains belonging to *T. cruzi* I or *T. cruzi* II lineages (Machado & Ayala 2001). Contrasting with the asexual rotifers, allele divergence in *T. cruzi* was only observed in nuclear genes of some strains presenting hybrid isoenzyme specimens and was better attributed to hybridization events rather than a signal of ancient asexuality. Therefore it has been proposed that one or more of four different possible mechanisms, automixing, sexuality (gene exchange), gene conversion and mitotic recombination, could be involved in decreasing the *T. cruzi* allele divergence (Machado & Ayala 2001).

Until now there is no evidence for automixing in *T. cruzi*. Conversely, Gaunt et al. (2003), have demonstrated that genetic exchange can occur in the parasite. Working with two strains of *T. cruzi* I carrying different drug-resistance markers, they were able to recover six double-drug-resistant progeny clones from the mammalian stage of the life cycle. The hybrids showed a fusion of parental genotypes with loss of alleles and homologous recombination. Isoenzyme and karyotype analyses have confirmed that the putative hybrid clones really shared characters from both parental populations.

As point out above, there are many evidences for natural occurrence of hybrid strains in *T. cruzi*, but in relation to the “Meselson effect”, the hybrid strains do not explain the high homozygosity observed in *T. cruzi*. Instead

initially the presence of hybrids would be expected to increase the divergence between the alleles in the same locus. Of course, a meaningful question is which is the frequency of the “recombination events” between strains from the *T. cruzi* I and *T. cruzi* II lineages, since excess homozygosity could result from either clonality or from aneuploidy with subsequent allele loss (i.e. from hemizygosity, which is experimentally indistinguishable from homozygosity). We cannot rule out the possibility that cell fusion followed by allele loss may be a common feature in these strains. In this case *T. cruzi* would not have a typical asexual reproduction and the absence of the “Meselson effect” could be explained. However, this possibility seems *a priori* unlikely, and other mechanisms should be entertained.

Homogenizing mechanisms like mitotic recombination or genetic conversion could limit the “Meselson effect”, decreasing the genetic diversity within and between individuals. Schon and Martens (2003) showed that the ancient asexual ostracod species *Darwinula stevensoni* shows very low nucleotide divergence in three nuclear regions. Likelihood permutation tests confirmed the presence of gene conversion in the multi-copy internal transcribed spacer sequence, but rejected rare or cryptic forms of sex as a general explanation for the low genetic diversity in the organism (Schon & Martens 2003).

The human Y chromosome is another example of genetic conversion as homogenizing mechanism (Skaletsky et al. 2003). It presents a male-specific region (MSY), which is a mosaic of heterochromatic sequences and three classes of euchromatic sequences: X-transposed, X-degenerate and ampliconic. There are evidences that the X-degenerate and ampliconic sequences were transmitted exclusively

TABLE III
Biological and molecular markers used for characterization of *Trypanosoma cruzi* sub-division ^a

<i>T. cruzi</i> I	<i>T. cruzi</i> II	Hybrid	Reference
Type III	Type II	Type I	Andrade (1974)
Strain-group 1	Strains-group 2		Miles et al. (1977)
Type 1	Type 2		Miles et al. (1978)
Zymodeme 1	Zymodeme A	Zymodeme B	Romanha et al.(1979)
Group 1	Zymodeme 2	Zymodeme 3	Barret et al. (1980)
Type 2 (PNA)	Group 2		Ebert (1982)
Braz ZI	Type 1 (WGA)		Schottelius (1982)
Group A	Braz Z2	Bol Z2	Tibayrenc & Miles (1983)
Chilean Z1	GroupB		Zillman & Ebert (1983)
Isoenzyme strain (IS) 1	Chilean Z2a	Chilean Z2b	Miles et al. (1984)
Zymodeme 17	IS 2e	IS 2	Tibayrenc et al. (1984)
DNA group 1	Zymodeme 30	Zymodeme 39	Tibayrenc & Ayala (1988)
Ribodeme II	DNA group 2		Muhlplfordt & Berger (1990)
Group I	Ribodeme I		Clark & Pung (1994)
Lineage II	Group II	Genotype 39	Tibayrenc (1995)
Biodeme III	Lineage I	Group 1/2	Souto et al. (1996)
Group II	Biodeme II	Biodeme I	Andrade & Magalhães (1997)
1st Major Clade	Group I		Nunes et al. (1997)
Clade A	2nd Major Clade	Lower Clade	Tibayrenc (1998)
Clade A	Clade C	Clade B	Machado & Ayala (2001)
	Clade C	Clade B	Augusto-Pinto et al. (2003)

^a: modified from Momen (1999)

through the male germline, and neither participated in meiotic crossing over with a homologous counterpart. In the Y chromosome the shutting down of X-Y crossing over during evolution triggered a monotonic decline in gene function. This model is corroborated by the presence, in the MSY's X-degenerate sequences, of decayed, intron-bearing pseudogenes. However, in the ampliconic regions, which present functional genes, there is an abundant Y-Y gene conversion as a homogenizing mechanism that permitted this region to overcome the problem of sexual absence. This process might provide a mechanism for conserving gene functions across evolutionary time in the absence of crossing over (Skaletsky et al. 2003).

It remains to be established if genetic conversion and/or mitotic recombination are important phenomena for homogenization of gene sequences in *T. cruzi*. In this aspect, it was shown that *T. cruzi* cells present atypical high resistance to ionizing radiation (IR), which could be associated with recombination-based DNA repair (Takeda et al. 1986, Amato-Neto et al. 1996).

MECHANISM OF *T. CRUZI* GENETIC VARIATION?

Another intriguing characteristic of *T. cruzi* is how it achieves remarkable heterogeneity among the strains and even clones, paradoxically maintaining at same time high levels of individual homozygosity. One possibility that is unlikely, but cannot be discarded, is that the homozygosity is artefactual and in reality represents hemizygosity occurring in aneuploid clones. Ignoring this possibility for the moment, and working within the paradigm of a clonal structure for the *T. cruzi* populations, one could then expect that mutation and DNA repair would play a role.

The function of mismatch repair (MMR) in decreasing the mutation rate is well understood (reviewed by Hsieh 2001). It is clear that the loss of this mechanism plays a determinant role in some types of human cancers (Peltomaki 2003). However, pathogenic microorganisms presenting defective MMR have been isolated in a proportion much higher than initially expected and it has been argued that an increased mutation rate could permit greater host adaptability for these organisms (LeClerc et al. 1996).

As already discussed above, the sequence of the *T. cruzi* MSH2 homologue gene identified tree haplogroups: A (*T. cruzi* I), B (hybrid strains) and C (*T. cruzi* II) (Augusto-Pinto et al. 2003). The MMR efficiency was further investigated among strains belonging to each haplogroup by analyzing two phenotypes: microsatellite instability (in the presence of hydrogen peroxide) and cisplatin resistance. Microsatellite instability was observed only for strains belonging to haplogroups B and C, but not in haplogroup A. Furthermore cells from haplogroups B and C were considerably more resistant to cisplatin treatment, a characteristic known to be conferred by deficiency of mismatch repair in eukaryotic cells (Fujieda et al. 1998). Altogether, these data suggest that strains belonging to haplogroups B and C may have decreased mismatch repair ability at stress conditions and consequently higher mutations rates when compared with those belonging to haplogroup A (Augusto-Pinto et al. 2003). Up to now the mutation rates for each of the *T. cruzi* major lineages are unknown, but it is noteworthy that

haplogroups B and C, which apparently have decreased mismatch repair efficiency, are involved in most cases of human infection (Buscaglia & Di Noia 2003).

CLINICAL IMPLICATIONS OF *T. CRUZI* GENETIC VARIABILITY

Humans could be considered a recent accident in the evolutionary history of *T. cruzi*. It is estimated that *T. cruzi* emerged as a species over 150 million years ago and the first contact with man is supposed to be much more recent, in the late Pleistocene, approximately 15,000 years ago, when man first peopled the Americas (Briones et al. 1999, Macedo et al. 2001). Thus, it is natural suppose that not all *T. cruzi* strains can infect humans and cause Chagas disease. As pointed out above, it is increasingly evident that in Brazil the human disease is much more associated with *T. cruzi* II strains, although some *T. cruzi* I and *T. cruzi* III could be eventually involved. However, it is not clear yet what factors determine the different clinical forms of the disease. Although we cannot discard the influence of patient-associated factors, an important role of the parasite genetics is nowadays recognized (Vago et al. 1996). On the other hand, many studies have been unsuccessful in correlating specific elements of the variability of the parasites with the clinical characteristics of the disease (Morel et al. 1980, Macedo et al. 1992). One possible explanation for this is that strains of *T. cruzi* represent swarms of clones that may present symbiotic relationships, but also compete fiercely for available resources. In addition, because of biological polymorphism, different clones from a strain may present tropism for different tissues (cardiac muscle, myoenteric plexuses in the esophagus and rectum, etc.), and thus an important factor determining the clinical course of disease might be the specific 'constellation' of infecting clones in the swarm and their specific tropisms. Since most of genetic profiling techniques used for the genetic characterization of *T. cruzi* require parasite isolation from patient blood and growth in laboratory animals or cultures, there is ample opportunity for clonal selection and consequently the trypanosome populations available for analysis can differ from those actually causing the tissue lesion. This scenario constitutes the core of what we have called the 'clonal-histotropic model' of Chagas disease (Macedo & Pena 1998, Macedo et al. 2001). Our recent demonstration that it is possible to achieve genetic typing of *T. cruzi* directly from clinical tissues by LSSP-PCR (Vago et al. 1996a, b, 2000), rDNA (J Freitas et al. manuscript in preparation) or microsatellite analysis (J Pimenta, AM Macedo, manuscript in preparation) provides invaluable tools to study the molecular epidemiology of Chagas disease. When applied to experimentally double infected mice with JG and Col1.7G2 populations, these techniques revealed a perfect concordance between the *T. cruzi* DNA profile obtained from the hearts with the JG strain and from the recta and the clone Col 1.7G2 of the same animals (Andrade et al. 1999). Similarly Vago et al. (2000) clearly demonstrated that different *T. cruzi* population could be associated to the heart or esophagus of single patients. Taking together these results open new vistas for a better understanding of the pathogenesis of the Chagas disease.

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