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Evidence of substantial recombination among *Trypanosoma cruzi* II strains from Minas Gerais

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ABSTRACT

Due to the scarcity of evidence of sexuality in *Trypanosoma cruzi*, the causative agent of Chagas disease, it has been general accepted that the parasite reproduction is essentially clonal with infrequent genetic recombination. This assumption is mainly supported by indirect evidence, such as Hardy–Weinberg imbalances, linkage disequilibrium and a strong correlation between independent sets of genetic markers of *T. cruzi* populations. However, because the analyzed populations are usually isolated from different geographic regions, the possibility of population substructuring as generating these genetic marker imbalances cannot be eliminated. To investigate this possibility, we firstly compared the allele frequencies and haplotype networks using seven different polymorphic loci (two from mitochondrial and five from different nuclear chromosomes) in two groups of TcII strains: one including isolates obtained from different regions in Latin America and the other including isolates obtained only from patients of the Minas Gerais State in Brazil. Our hypothesis was that if the population structure is essentially clonal, Hardy–Weinberg disequilibrium and a sharp association between the clusters generated by analyzing independent markers should be observed in both strain groups, independent of the geographic origin of the samples. The results demonstrated that the number of microsatellite loci in linkage disequilibrium decreased from 4 to 1 when only strains from Minas Gerais were analyzed. Moreover, we did not observed any correlation between the clusters when analyzing the nuclear and mitochondrial loci, suggesting independent inheritance of these markers among the Minas Gerais strains. Besides, using a second subset of five physically linked microsatellite loci and the Minas Gerais strains, we could also demonstrate evidence of homologous recombination roughly proportional to the relative distance among them. Taken together, our results do not support a clonal population structure for *T. cruzi*, particularly in TcII, which coexists in the same geographical area, suggesting that genetic exchanges among these strains may occur more frequently than initially expected.

1. Introduction

Trypanosoma cruzi, the casual agent of Chagas disease, is a heterogeneous species as extensively demonstrated in several biological, biochemical, and molecular studies (Devera et al., 2003; Macedo and Pena, 1998; Macedo et al., 2001). Since 2009, to standardize the taxon nomenclature, the *T. cruzi* strains have been divided into six discrete taxonomic units (DTUs), TcI–TcVI (Zingales et al., 2009). The epidemiological relevance of these DTUs or even the subdivision of some of them or inclusion of a new one (Tcbat) is still under debate (Zingales et al., 2012; Pinto et al., 2012). However, TcII and its direct derived hybrids, TcV and TcVI, seem to be associated with more severe cases of Chagas disease in Southern Cone countries.

Despite considerable progress in cellular and molecular biology and in evolutionary genetics within recent decades, the debate

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regarding the population structure and reproductive mode of *T. cruzi* is far from being settled. The current theory, which is known as "the clonal theory of parasitic protozoa", was proposed by Tibayrenc and Ayala in 1990, which stated that *T. cruzi* and other protozoans undergo predominant clonal propagation with very little, if any, sexuality (Tibayrenc et al., 1990). For many years, the primary basis of this theory has been the observation of large deviations from Hardy–Weinberg (H–W) expectation and the apparent linkage disequilibrium (LD) between genotypes at different loci, some of which involved genomic separate compartments, such as the nucleus and mitochondria (Tibayrenc et al., 1986, 1991; Tibayrenc and Ayala, 2002).

Although prevalent in the literature, the clonal hypothesis has been challenged by many authors (Bastien et al., 1992; Bogliolo et al., 1996; Gaunt et al., 2003; Freitas et al., 2006). For instance, conflicting findings, obtained by our and other groups questioned the extent of this theory. Using microsatellite markers to study *T. cruzi* populations, we observed systematic deviations between the genotypic and allelic frequency expected by Hardy–Weinberg equilibrium. However, unlike an excess of heterozygosity naturally expected in populations in which recombination events are rare, an excess of homozygosity was observed (Oliveira et al., 1998; Balloux et al., 2003; Bengtsson, 2003). These findings were inconsistent with a population of parasites operating under an essentially clonal reproduction because asexual organisms exhibit the Meselson Effect (Mark Welch and Meselson, 2000).

Moreover, the occurrence of *T. cruzi* hybrid strains presenting evidence of sorting of homologous chromosomes and homologous recombination has been also identified (Gaunt et al., 2003; Freitas et al., 2006; Tomazi et al., 2009; Venegas et al., 2009; Westenberger et al., 2005; Carranza et al., 2009; Brisse et al., 2003; Carrasco et al., 1996; Sturm et al., 2003). And, at least for in vitro experiments, the recombination events in these parasites involve fusion of nuclear genotypes, homologous recombination, allelic loss, and uniparental inheritance of mitochondrial genotypes (Gaunt et al., 2003).

Despite these observations, many questions remain regarding the occurrence and frequency of recombination between current assumed asexual parasites such as *T. cruzi*. Indeed, most studies on population structures of *T. cruzi* have compared parasite populations isolated from distant geographical locations or at different times of isolation, which may have failed in detecting genetic recombination events among the strains due to bias of the sample selections. It is well known that if two or more subpopulations have different allele frequencies, the heterozygosity of the overall population is reduced, despite the subpopulations being in Hardy–Weinberg equilibrium. This is easily achieved, for example, by the existence of geographical barriers that prevent meetings and thus, the flow of genes among subpopulations, leading to a phenomenon known as the Wahlund effect (Poulin and Morand, 1999).

To verify this hypothesis, herein we compared the molecular data obtained from TcII strains isolated from both distant geographic areas throughout Latin America and only Minas Gerais, a Brazilian Southern state. In this study, our aim was to investigate whether geographical proximity would increase the probability of detecting evidence of sexual recombination among the strains. Our results demonstrated that when only TcII isolates from Minas Gerais were analyzed, four of the five microsatellite loci entered into H–W equilibrium, suggesting that the apparent deviation observed in previous studies could be due to geographical isolation of the analyzed strains.

2. Methods

2.1. T. cruzi populations analyzed

A total of 88 *T. cruzi* isolates were analyzed in this study (Table 1). Sixty of them were recently isolated from chronic patients, 58 from natives or residents in Minas Gerais (Southeast Brazil) and two from Bahia (Northeast Brazil). In addition, genotyping data from 28 other *T. cruzi* isolated in different times and different places in Latin America (Freitas et al., 2006), were also used for the calculation of HW and LD imbalances.

Table 1
T. cruzi II[a] strains and clones analyzed in this study.

Strain/clone[a]	Origin	Strain/clone[a]	Origin
002 B	MG/Brazil	154 a	MG/Brazil
003 a	MG/Brazil	162 a	MG/Brazil
005 B	MG/Brazil	128 a	BA/Brazil
007 B	MG/Brazil	38	MG/Brazil
009 B	MG/Brazil	013 a	MG/Brazil
010 B	MG/Brazil	016 B	MG/Brazil
011 B	MG/Brazil	192 a	MG/Brazil
012 a	MG/Brazil	JG	MG/Brazil
012 B	MG/Brazil	022 b	BA/Brazil
013 B	MG/Brazil	003 B	MG/Brazil
019 a	MG/Brazil	002 a	MG/Brazil
020 B	MG/Brazil	005 a	MG/Brazil
023 B	MG/Brazil	006 a	MG/Brazil
024 B	MG/Brazil	021 B	MG/Brazil
025 B	MG/Brazil	097 a	MG/Brazil
026 B	MG/Brazil	188 a	MG/Brazil
029 a	MG/Brazil	**Be62**[b]	MG/Brazil
031 a	MG/Brazil	Esmeraldo[b]	MG/Brazil
037 a	MG/Brazil	**GMS**[b]	MG/Brazil
044 a	MG/Brazil	**Ig539**[b]	MG/Brazil
045 a	MG/Brazil	Mas1 cl1[2]	MG/Brazil
050 b	MG/Brazil	**MPD**[2]	MG/Brazil
053 a	MG/Brazil	Tula cl2[b]	MG/Brazil
055 a	MG/Brazil	**84**[b]	MG/Brazil
058 a	MG/Brazil	**169/1**[b]	MG/Brazil
065 b	MG/Brazil	**200pm**[b]	MG/Brazil
067 a	MG/Brazil	**209**[b]	MG/Brazil
079 a	MG/Brazil	**239**[2]	MG/Brazil
083 a	MG/Brazil	**803**[b]	MG/Brazil
085 a	MG/Brazil	**1005**[b]	MG/Brazil
090 a	MG/Brazil	**1014**[b]	MG/Brazil
092 a	MG/Brazil	**1043**[b]	MG/Brazil
094 a	MG/Brazil	**1931**[b]	MG/Brazil
103 a	MG/Brazil	**GOCH**[2]	GO/Brazil
105 a	MG/Brazil	**577**[2]	GO/Brazil
109 a	MG/Brazil	**578**[2]	GO/Brazil
110 a	MG/Brazil	**580**[2]	GO/Brazil
115 b	MG/Brazil	183744[b]	GO/Brazil
116 a	MG/Brazil	CPI95/94[b]	PI/Brazil
120 a	MG/Brazil	OPS27/94[b]	PI/Brazil
129 a	MG/Brazil	GLT564[b]	RJ/Brazil
132 a	MG/Brazil	GLT593[b]	RJ/Brazil
138 a	MG/Brazil	**Y**[b]	SP/Brazil
146 a	MG/Brazil	CPI11/94[b]	Colombia

[a] *T. cruzi* strains were identified as *T. cruzi* II as described (D'Avila et al., 2009).
[b] The data of these strains were obtained from Freitas et al. (2006).

T. cruzi isolation from patients and all of the procedures were performed with the informed consent of the participants and approved by the Ethics Committee 087/99 of UFMG, Belo Horizonte, MG, Brazil.

The parasites DNA was extracted using the phenol:chloroform protocol (Macedo et al., 1992) and used as a template for the PCR assays.

2.2. T. cruzi DTU genotyping

All of the 88 isolates of *T. cruzi* were genotyped as TcII, using the triple assay as recommended by Macedo and colleagues (D'Avila et al., 2009) to discriminate the six *T. cruzi* DTUs. Initially, the strains were analyzed using PCR-RFLP of the COII gene followed by digestion with *Alu* I (de Freitas et al., 2006). The second step consists of a PCR of internally transcribed spacer leader (ITS) gene