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Authors

Telleria, J
Barnabé, C
Ayala, FJ
et al.

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Phylogenetic character mapping of RADES Probing, a new marker for exploring the clonal evolution of expressed coding sequences in *Trypanosoma cruzi*, the agent of Chagas disease

J. Telleria [a], C. Barnabé [a], F.J. Ayala [b], M. Tibayrenc [a,*]

[a] Maladies Infectieuses et Vecteurs Ecologie, Génétique, Evolution et Contrôle (MIVEGEC; Institut de Recherche pour le Développement 224 – Centre National de la Recherche Scientifique 5290 – Universités de Montpellier 1 et 2), IRD Center, BP 64501, 34394 Montpellier Cedex 5, France
[b] Department of Ecology and Evolutionary Biology, University of California at Irvine, Irvine, CA 92697, USA

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ABSTRACT

We have tested a new genetic marker, RADES Probing (RADES-P), on a standard sample of 19 laboratory-cloned stocks of *Trypanosoma cruzi*, the agent of Chagas disease. This set of stocks, fully characterized using multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD), is representative of this parasite's main genetic subdivisions. RADES-P consists in hybridizing RAPD profiles with probes composed of the products of random amplified differentially expressed sequences (RADES). The profiles thus obtained uncover only expressed coding sequences that are as well present on RAPD gels. Direct visual examination and the banding record show that these RADES-P profiles are different of, and not redundant with, both RAPD and RADES patterns obtained on the same set of stocks with the same primers. Phylogenetic character mapping (PCM) of the RADES-P polymorphism fairly confirms the known population structure and phylogenetic diversity of *T. cruzi*. This suggests that the impact of clonal evolution on *T. cruzi* has been predominant enough over the long term to carve the polymorphism of all types of DNA sequences, including polymorphisms of expressed coding sequences, although these sequences are subject to natural selection.

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1. Introduction

Chagas disease is a parasitic infection caused by *Trypanosoma cruzi*. Although increasingly better controlled than in the past, *T. cruzi* infections remain an important public health problem in most Latin American countries; it is the source of blood transfusion accidents in the United States; and it is now spreading in Europe, mainly in Spain. The available treatment is highly toxic and it is poorly efficient in the chronic phase of the disease. No vaccine is available. (Moncayo and Silveira, 2010). Basic research, including evolutionary studies, is therefore sorely needed so that effective control measures (efficient drugs, vaccination) can be designed.

Members of our research group and others have conducted long-term investigations of the molecular evolution of *T. cruzi* (reviewed in Tibayrenc et al., 2010). Moreover, the parasite's genome has been fully sequenced (El-Sayed et al., 2005), which makes *T. cruzi* one of the pathogenic agents whose genetic polymorphism and population structure are best known (Tibayrenc et al., 2010). Although the higher branch order of this parasite's genetic subdivisions remains in question, there is a consensus that *T. cruzi* is subdivided into six discrete genetic lineages or discrete typing units (DTUs, Tibayrenc, 1998), numbered Tc I-VI (Zingales et al., 2012).

T. cruzi exhibits a complex pattern of reticulate evolution, combining predominant clonal evolution and severely restrained recombination with occasional bouts of hybridization (Tibayrenc et al., 1986; Tibayrenc and Ayala, 2012). However, the actual impact of natural selection on this parasite's genetic polymorphism has been insufficiently explored. Moreover, some aspects of *T. cruzi*'s molecular evolution remain to be investigated.

A powerful approach for elucidating an organism's patterns of molecular evolution is phylogenetic character mapping (PCM; Avise, 2004), which explores the relationships between a newly explored character and a known phylogeny. We have previously used this strategy by comparing the phylogenies obtained from multilocus enzyme electrophoresis (MLEE) with those obtained with random primed amplified polymorphic DNA (RAPD) (Tibayrenc et al., 1993). In a previous paper (Telleria et al., 2004), we explored the relationships between the diversity patterns uncovered by MLEE and RAPD, on the one hand, with those obtained with random amplified differentially expressed sequences (RADES; Murphy and Pellé, 1994), on the other hand. Moreover, we have recently used a PCM approach to explore protein expression in *T. cruzi* (Telleria et al., 2010).

* Corresponding author. Present address: Department of Ecology and Evolutionary Biology, University of California at Irvine, Irvine, CA 92697, USA.
E-mail address: Michel.Tibayrenc@ird.fr (M. Tibayrenc).

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In the present work, we use PCM in order to analyze the relationships between this parasite's known phylogenetic diversity and the polymorphism of expressed coding sequences revealed by a new genetic marker, RADES probing (RADES-P), based on the hybridization of RAPD profiles with probes obtained from RADES amplification products.

2. Material and methods

2.1. T. cruzi stocks

We investigated a standardized set of 19 stocks. These stocks were laboratory-cloned with verification of the actual cloning under the microscope. They have been fully characterized by multilocus enzyme electrophoresis (MLEE) at 22 genetic loci (Barnabé et al., 2000) and by random amplified polymorphic DNA (RAPD) with 20 different decameric primers (Brisse et al., 2000). The genetic identity of these stocks is verified several times a year to detect accidental mixtures. For the present study, the stocks were again fully characterized by MLEE/RAPD typing (Telleria et al., 2004). These stocks are representative of the six discrete typing units (DTUs), or "near-clades" (Tibayrenc and Ayala, 2012) that subdivide *T. cruzi* and have been sampled in diversified ecosystems and hosts (Table 1). Their biological features have been explored in different studies in our Montpellier laboratory (see Revollo et al., 1998; Pinto et al., 2000; Toledo et al., 2004, for example). The stocks were cultivated in liver infusion tryptone (LIT) medium supplemented with 10% fetal calf serum at 28 °C.

2.2. Random amplification polymorphic DNA (RAPD) profiles

We used four kits of decameric primers whose sequence was randomly selected, namely E3, B1, B18 and R8, from Operon Technologies® (Alameda, CA), in order to amplify the 19 stocks included in the present study. These 4 primers were selected because they are enough to permit a clear distinction between *T. cruzi* near-clades (Brisse et al., 2000). 20 ng of DNA isolated from each stock were amplified in a solution comprising Tris-HCl 10 mM, $MgCl^2$ 1.5 mM, KCl 50 mM, pH 8.3, dNTPs 0.1 mM, each primer 0.2 μM and 0.9 Taq DNA polymerase units (Boeringer Mannheim), for a total volume of 60 μl. The amplification was performed using a PCR thermocycler PTC 100 (MJ Research Inc.®, USA) with the following parameters: initial DNA denaturation step (94°C for 5 min), 45 cycles at 94°C for 1 min, 36°C for 1 min, 72°C for 2 min and final extension at 72°C for 7 min. RAPD amplification products were separated using a 1.6% agar gel for 15 min in standard TE buffer and stained with 200 μg/l ethidium bromide.

2.3. Random amplified differentially expressed sequence (RADES) profiles and probes

We isolated messenger RNA of the 19 stocks using a technique derived from the RADES (random amplified differentially expressed sequences) technique. Magnetic beads (Dynabeads®), tailed by poly (dt25nt) that captures the tail poly (A), were used. We performed reverse transcription by AMV reverse transcriptase (15 U/μg of mRNA, Promega®) using oligo (dT) primer (dT18nt) in the presence of dNTP and with ribonuclease inhibitor (RNAsin). The mixture was incubated at 42°C for 60 min. Subsequently, the cDNA products were purified by the Qiagen® PCR purification kit. To obtain a sufficient quantity, it was necessary to amplify the cDNA by PCR using two primers, one 23-nt primer based on the conserved mini-exon sequence (5′-GATACAGTTTCTGTACTATATTG-3′) and one 18-nt oligo dT primer (dT 18) complementary to the 3′ end. The cDNAs were processed by RAPD amplification, using the same four decameric primers (E3, B1, B18 and R8) from Operon Technology® as for the RAPD amplification used for total DNA, under the same technical conditions.

In order to obtain hybridization probes, 1 μg of RADES amplification product was labeled with Digoxigenein (DIG)-11-dUTP, (Dig-Nick translation Mix®, Roche) according to the manufacturer's recommendations. Probes were not generated from all stocks. Four different probes were used against the RAPD products of four different primers. We used the same 4 primers (E3, B1, B18 y R8), for the RADES amplification, the RAPD amplification, and the hybridization. The Rades products were used as probes to hybridize the RAPD products amplified with the same 4 primers

2.4. Hybridization and detection conditions

The RAPD amplification profiles were transferred after alkali denaturation (0.5N NaOH, 1.5M NaCl for 5 min) onto Hybond membrane® (Amersham, USA) in a vacuum device. DNA fixation was performed using a thermoblock at 90 °C for 10 min. Hybridization of the RAPD profiles with the RADES probes (RADES Probing) was performed using the DIG system® (Boeringer, USA) at 65 °C overnight in a rotating oven. The membranes were washed three times in a buffer, stringency of SSC 2X, 0.1 SDS, SSC 1X, 0.1 SDS,

Table 1
Geographical origin, host distribution and genetic identification (DTU) of the *Trypanosoma cruzi* stocks.

N° stock	Code	DTU	Host	Country and locality
T. cruzi (1)	Cutia Cl1	I	*Dasyprocta aguti*	Brazil (Espiritu Santo, Colatina)
T. cruzi (2)	SP104 cl1	I	*Triatoma spinolai*	Chile (Region IV, Combarbala)
T. cruzi (3)	P209 cl93	I	Human	Bolivia (Sucre)
T. cruzi (4)	92122102R	IV	*Procyon lotor*	USA (Georgia, Statesboro, Bulloch)
T. cruzi (5)	CanIIIcl1 z3	IV	Human	Brazil (Belém)
T. cruzi (6)	Tu18 cl93	II	*Triatoma infestans*	Bolivia (Tupiza)
T. cruzi (7)	CBB cl 3	II	Human	Chile (Region IV, Tulahuen)
T. cruzi (8)	Mas cl1	II	Human	Brazil (Brasilia)
T. cruzi (9)	IVV cl4	II	Human	Chile (Region IV, Cuncumen)
T. cruzi (10)	CM17	III	*Dasypus sp.*	Colombia (Meta, Carimaga)
T. cruzi (11)	M5631 cl5	III	*Didelphis novemcinctus*	Brazil (Selva Terra)
T. cruzi (12)	M6241 cl6	III	Human	Brazil (Belém)
T. cruzi (13)	MN cl 2	V	Human	Chile (Region IV, Illapel)
T. cruzi (14)	Bug2148 cl1	V	*Triatoma infestans*	Brazil (Rio Grande do Sul)
T. cruzi (15)	SO3 cl5	V	*Triatoma infestans*	Bolivia (Potosi)
T. cruzi (16)	SC43 cl1	V	*Triatoma infestans*	Bolivia (Santa Cruz)
T. cruzi (17)	Cl Brener	VI	*Triatoma infestans*	Brazil (Rio Grande do Sul)
T. cruzi (18)	P63 cl1	VI	*Triatoma infestans*	Paraguay (Makthlawaiya)
T. cruzi (19)	Tula cl2	VI	Human	Chile (Region IV, Tulahuen)

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SSC 0.5X, 0.1 SDS, each time at 65°C for 15 min. Detection was performed with the antibody antidigoxigenin-AP (150 mU) and colored with NBT and BCIP for 1 h.

2.5. Data analysis

The RADES Probing (RADES-P) profiles were processed following the same procedures used for MLEE, RAPD and RADES (Telleria et al., 2004). Genetic variability was evaluated by computing Jaccard genetic distances (Jaccard, 1908) from a matrix based on the presence or absence of bands. Phylogenetic relationships among stocks were depicted by neighbor joining (NJ) and the unweighted pair group method of arithmetic average (UPGMA) distance trees, (Sneath and Sokal, 1973). Bootstrap analysis and construction of majority rule consensus trees were carried out using the Seqbot Mix programme and Consensus of the PHYLIP package (Felsenstein, 2004). The consensus tree was drawn with TreeView (Page, 1996). The correlation among the genetic distances obtained with all the markers used in the present study (RADES Probing, RADES, RAPD, MLEE) was estimated using the nonparametric Mantel test (Mantel, 1967).

3. Results

Fig. 1 shows the profiles of the 19 stocks obtained with the RAPD amplification (A), the RADES amplification (B) and the hybridization profile (C), all obtained with the B1 primer. The RADES products were used as probes to hybridize the RAPD products amplified with the same B1 primer. We have previously shown (Telleria et al., 2004) that RAPD and RADES profiles are not redundant to each other. As shown on Fig. 1 C, RADES probing (RADES-P) profiles proved to be different from, and not redundant with, both RAPD and RADES profiles (Fig. 1 A and B), which had been previously described (Telleria et al., 2004). It can be seen in the profiles that many bands present in the RAPD and/or RADES profiles are absent in the RADES-P profiles; and the reciprocal: bands present in the RADES-P profiles are not found in RAPD and/or RADES profiles. Moreover, the profiles generated by different probes obtained from different stocks using the same primer are also different from each other (data not shown).

RADES-P diversity therefore appears not to be redundant with either RAPD or RADES variability. Nevertheless, the tree generated from RADES-P profiles (Fig. 2) shows clustering patterns that are similar to those derived from MLEE, RAPD and RADES diversity. This result is confirmed by the Mantel correlation tests (Mantel, 1967), which show highly significant correlations between genetic distances obtained from MLEE, RAPD, RADES and RADES-P (Table 2).

4. Discussion

The strong correlation between genetic distances obtained with independent sets of genetic markers, which have distinct DNA sequence targets, is significant evidence of linkage disequilibrium or nonrandom association between different sets of genetic mark-

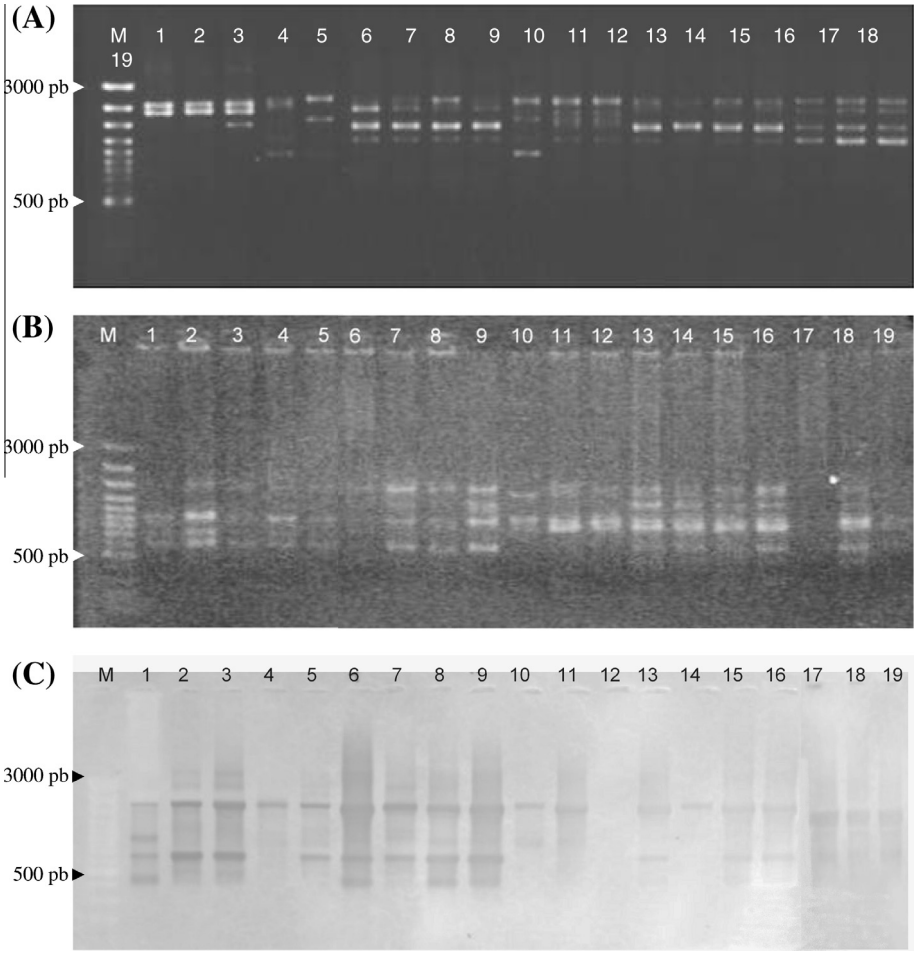

Fig. 1. Profiles for 19 *Trypanosoma cruzi* stocks for (A) Random Amplified Polymorphic DNA (RAPD) obtained with the primer B1; (B) mRNA RADES obtained with the primer B1 and (C) hybridization between RADES and RAPD amplification products, both obtained with the primer B1. On top are the stock numbers as in Table 1.

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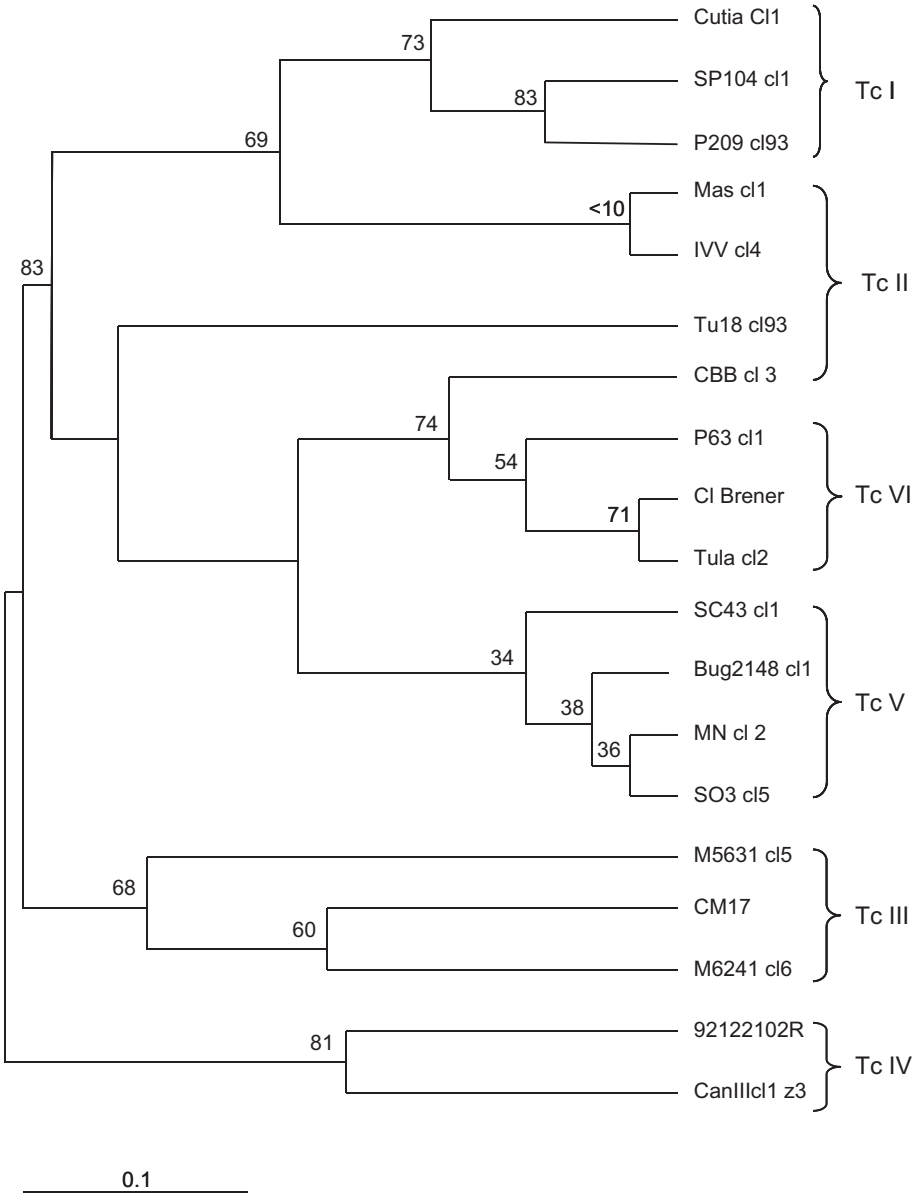

Fig. 2. UPGMA dendrogram, based on the RADES probing analysis, showing the genetic relationships of 19 stocks of *Trypanosoma cruzi* (Table 1). Six DTUs are distinguished, confirming the well known genetic structure of *T. cruzi*. The bootstrap values are indicated at the nodes of the tree.

Table 2
Mantel Test of correlation between genetic distances generated from MLEE, RAPD, RADES and RADES-P.

	RAPD	RADES	RADES-P	MLEE
RAPD	0	–	–	–
RADES	$R = 0.55$ $p < 10^{-4}$	0	–	–
RADES-P	$R = 0.69$ $p < 10^{-4}$	$R = 0.51$ $p < 10^{-4}$	0	–
MLEE	$R = 0.60$ $p < 10^{-4}$	$R = 0.49$ $p < 10^{-4}$	$R = 0.66$ $p < 10^{-4}$	0

ers (test g; Tibayrenc et al., 1990). A strong linkage disequilibrium means that there are severe restrictions to genetic recombination, which is therefore taken as circumstantial evidence for predominant clonal evolution (Tibayrenc et al., 1990; Tibayrenc and Ayala, 2012). When the correlation is extreme, phylogenetic trees with similar or identical clustering patterns are generated, as has been evidenced between MLEE and RAPD trees in the case of *T. cruzi* (Tibayrenc et al., 1993; Brisse et al., 2000).

RADES profiles (Murphy and Pellé, 1994) show the amplification products by randomly selected decameric primers of the cDNAs of mRNAs, corresponding to expressed coding sequences. The target sequences are therefore in principle highly different from the RAPD sequences, which concern all possible sequences, including noncoding genes, as well as genomic rearrangements. However, once the cDNA is obtained, the technique for RADES and RAPD is the same. When using the same primers, it was impossible to rule out *a priori* that RADES and RAPD profiles would overlap to a certain extent. It so happens that we obtained a totally different result (Telleria et al., 2004). RADES bandings were quite different from RAPD bandings for the same primers and the same stocks (see Fig. 1). However, although their banding patterns were by no means redundant, RAPD and RADES diversities were closely related, with a highly significant correlation between their genetic distances and identical clustering patterns in phylogenetic trees. We have interpreted this to be confirmation of the predominance of clonal evolution in *T. cruzi* (Telleria et al., 2004).

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In the present study, we aimed at exploring in greater depth the relationships between the diversity stemming from expressed coding sequences (RADES) on one hand, and all types of sequences, including nonexpressed coding and noncoding sequences (RAPD) on the other hand. Instead of using overall amplification profiles, we probed RAPD banding patterns with RADES products corresponding to expressed coding sequences. One could have expected the banding profiles obtained to be partly or totally redundant with RADES banding patterns. However, the results are different. These RADES-P banding patterns seem to be different from, and not redundant with, both RAPD and RADES banding patterns. However, as is the case between (i) MLEE and RAPD (Tibayrenc et al., 1993; Barnabé et al., 2000) and (ii) RAPD and RADES (Telleria et al., 2004), we have now observed that genetic distances between either RAPD or RADES and RADES-P are highly correlated, as shown by correlation tests (Table 2) as well as by clustering pattern similarities in the phylogenetic tree (Fig. 2).

Phylogenetic character mapping (PCM, Avise, 2004) consists in exploring the relationships between a newly investigated character and a known phylogeny. PCM of RADES-P on the known consensus phylogeny previously inferred from MLEE, RAPD and RADES shows a close match between RADES-P phylogeny and the previously known phylogeny. This result is an additional manifestation of the strong linkage disequilibrium that links together most parts of *T. cruzi*'s genome, including expressed coding sequences, nonexpressed coding sequences, noncoding sequences, and genomic rearrangements. This does not mean that sequences uncovered by RAPDs RADES and RADES-P do not overlap at all. But it does mean that the main molecular targets of these three markers have radically dissimilar evolutionary patterns. Moreover, it is worth emphasizing that the linkage evidenced here concerns the whole species *T. cruzi* (the six DTUs) and does not deal with possible within-DTU linkages. This extreme linkage is visible in protein expression, which proves to be correlated with the parasite's phylogenetic diversity (Telleria et al., 2010). So, although this parasite is capable of occasional bouts of genetic hybridization that probably have a notable impact on an evolutionary scale (Gaunt et al., 2003), our results are another confirmation of the profound impact of clonal evolution on its genomic diversity. This result should be emphasized all the more, since RADES-P reveals the polymorphism of expressed coding sequences, which are subject to natural selection. One could have expected, therefore, that natural selection might interfere with the phylogenetic profile of these stocks, which is not the case. This, again, illustrates predominant clonal evolution in *T. cruzi*'s evolutionary history.

Isoenzyme profiles are revealed by probing overall biological extracts with specific biochemical reactions that visualize the activity of given enzymes. Therefore, on overall electrophoreses of crude biological extracts, the isoenzyme profiles reveal the migration of any given enzyme. Similarly, RADES-P reveals where expressed coding sequences have migrated on overall electrophoreses of randomly amplified DNAs, which include all types of sequences. We, therefore, consider RADES-P as a new marker to be explored in depth for evolutionary patterns.

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