# Two main clusters within *Trypanosoma cruzi* zymodeme 3 are defined by distinct regions of the ribosomal RNA cistron

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SUMMARY

*Trypanosoma cruzi* is currently classified into 2 major phylogenetic lineages, *T. cruzi* I and II, that correlate with the formerly described zymodeme 1 and 2, respectively. Another isoenzymic group (zymodeme 3–Z3) was also described. In this study, we analysed the genetic diversity among Z3 isolates of the Brazilian Amazon by restriction fragment length polymorphism of the intergenic transcribed spacers (ITSs) of the ribosomal RNA cistron and the size of the divergent domain D7 of the 24Sα rRNA gene. DNAs from 12 *T. cruzi* Z3 isolates obtained from humans (2), *Panstrongylus geniculatus* (1), and *Rhodnius brethesi* (9) were submitted to PCR amplification of the ITSs plus the 5·8S rDNA. The PCR products were digested with 4 distinct endonucleases and the profiles analysed by a numerical methodology. The phenetic dendrogram revealed a clear dichotomy in the Z3 group, defining 2 groups that were named Z3-A and Z3-B. Dimorphism was also found in the band sizes of the amplified D7 divergent domain of the 24Sα rDNA, which showed a perfect correlation with the ITSs clustering. The organization of the ribosomal cistron was investigated by Southern blotting and shown to be conserved in the genome of the 2 Z3 groups. This study shows that the rDNA cistron allows the definition of 2 distinct subclusters in Z3 isolates.

Key words: *Trypanosoma cruzi*, zymodeme 3, Amazon region, Internal Transcribed Spacers, mini-exon gene, ribosomal RNA cistron.

## INTRODUCTION

*Trypanosoma cruzi*, the causative agent of Chagas disease, infects several million people in Central and South America. In the chronic phase, patients may be asymptomatic or present cardiac and/or digestive alterations. The distinct clinical features of Chagas disease have been attributed to the variability of the host immune response and to the genetic heterogeneity of the parasite (Macedo & Pena, 1998).

Although described, hitherto, as a single taxon, *T. cruzi* shows remarkable differences in biological and genetic parameters (Tibayrenc & Ayala, 1988). The *T. cruzi* strains that circulate in the domestic and sylvatic transmission cycles were originally phenotyped by enzyme electrophoresis profiles into 2 major clusters: zymodeme 1 (Z1) circulating mostly in the sylvan environment; and zymodeme 2 (Z2) present in the domestic cycle of the protozoan and encompassing the vast majority of the infected human hosts in Southern Cone countries (Miles *et al*. 1977). A third isoenzyme group of *T. cruzi* was further characterized as zymodeme 3 (Z3) (Miles *et al*. 1978). This enzymatic variant, originally described in the Amazon region, was also associated with the *T. cruzi* sylvatic transmission cycle, infecting armadillos, the terrestrial marsupial *Monodelphis brevicaudata*, the triatomine *Panstrongylus geniculatus* and was rarely isolated from humans (Barrett *et al*. 1980; Miles *et al*. 1981*a*; Povoa *et al*. 1984).

Recently, a plethora of genetic loci, such as mini-exon, 24Sα ribosomal RNA gene (rDNA), random polymorphic amplified DNAs (RAPD), and chromosomal markers have revealed 2 different lineages in *T. cruzi* that correlate with the aforementioned zymodemes: *T. cruzi* I (Z1) and II (Z2) (Souto *et al*. 1996; Fernandes *et al*. 1998*a*; Tibayrenc, 1995; Satellite Meeting, 1999).

Focusing on the study of Chagas disease in the Amazon region, several *T. cruzi* isolates have been recently characterized as Z3, based on isoenzyme

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patterns and on an insertion in the non-transcribed spacer of the mini-exon gene (Fernandes *et al*. 1998*b*, 2001). Although this genetic marker distinguishes the Z3 group from Z1 and Z2, it does not allow characterization of intra-group polymorphisms since the mini-exon gene has proved to be a slowly evolving gene. On the other hand, rDNA and ribosomal spacers are suitable markers for group discrimination.

In trypanosome species, the ribosomal cistron exhibits an unusual organization (Arruda *et al*. 1990). An internal transcribed spacer (ITS1) separates the coding region of the 18S subunit (SSU) and the 5·8S rDNA. Another ITS (ITS2) separates the 5·8S rDNA from the 24S$\alpha$ rDNA. The analysis of the restriction fragment length polymorphism (RFLP) of the ITS-rDNA (ITS1 + 5·8S rDNA + ITS2) has been used to investigate relationships between *Leishmania* species and genetic evolutionary links between *Leishmania* and *Endotrypanum* (Cupolillo *et al*. 1995, 1998, 2000). The same approach was applied to establish the genetic relationship between *T. cruzi* isolates. The phenetic dendrogram resultant from RFLP profiles clustered several isolates into the 2 *T. cruzi* major phylogenetic lineages (Fernandes *et al*. 1999*a*). In the present study, the same genetic tool was employed to analyse genetic similarity among *T. cruzi* Z3 stocks derived from the Amazon region revealing a subdivision into 2 groups. This subdivision was confirmed by a dimorphic pattern of a specific domain of the 24S$\alpha$ rDNA and the genomic organization of 3 independent genetic markers.

## MATERIALS AND METHODS

### *Parasites and Z3 typing*

Twelve *T. cruzi* Z3 stocks, previously typed by a differential amplification of part of the non-transcribed spacer of the mini-exon gene were used in this study (Fernandes *et al*. 2001). Two stocks were isolated from humans, 1 from *Panstrongylus geniculatus* and 9 from *Rhodnius brethesi*. All the isolates were from the Amazon region: 10 isolates from the district of Barcelos, located on the left margin of the Negro River and 2 isolates from other localities in the Amazonas State (Table 1). Four reference clones were used as controls, Silvio X10 cl1 and Dm 28c (*T. cruzi* I), CL Brener and Esmeraldo cl3 (*T. cruzi* II). All the isolates were maintained in LIT medium at 28 °C. Epimastigote cultures at the end of the log phase were centrifuged at 4000 ***g***. The cells were washed with saline solution (0·9 %), resuspended in 1 ml of TE (10 mM Tris–HCl, pH 8·0, 10 mM EDTA, pH 8·0) and incubated at 56 °C for 2 h with 100 $\mu$g/ml of proteinase K and 0·5 % SDS (sodium dodecyl sulfate). The DNA of the lysed cells was extracted with phenol : chloroform (1 : 1) and precipitated after the addition of sodium acetate and ethanol (Sambrook, Fritsch & Maniatis, 1989).

### *PCR amplification of the ITS-rDNA*

The ITS1 + 5·8S rDNA + ITS2 region was amplified using the oligonucleotides and conditions described elsewhere (Cupolillo *et al*. 1995). The thermal profile used was: 1 cycle of 94 °C/4 min; 35 cycles of 94 °C/1 min; 55 °C/1 min; 72 °C/2 min and 1 last cycle of 72 °C/10 min. The PCR products were analysed by 2 % agarose gel electrophoresis and further independently digested with 4 restriction enzymes (*Bst*UI, *Sau*3AI, *Rsa*I and *Hae*III). The restriction fragments were electrophoresed in 12·5 % polyacrylamide gels in a GenePhor® apparatus (Amersham Pharmacia Biotech).

### *PCR amplification of the D7 domain of the 24S$\alpha$ rDNA*

The divergent domain D7 of the 24S$\alpha$ rDNA gene was PCR amplified with the primers D71 (5′-AAGGTGCGTCGACAGTGTGG-3′) and D72 (5′-TTTTCAGAATGGCCGAACAGT-3′) as previously described (Souto *et al*. 1996). The reaction products were run in 7·5 % polyacrylamide gels and stained with ethidium bromide.

### *Southern blot*

Genomic DNA (2·5 $\mu$g) from the isolates was subjected to *Pst*I digestion, electrophoresed in 0·8 % agarose gels and transferred to nylon membranes (Hybond-N; Amersham Parmacia Biotech) according to standard procedures (Sambrook *et al*. 1989). The Southern blot was hybridized with different probes: 24S$\alpha$ rRNA gene from *T. cruzi* (G101p10 probe, Arruda *et al*. 1990) and 2 expression sequence tags (EST) from CL Brener: TENF 462 (GenBank Accession no. AA532107) and TENF 552 (GenBank Accession no. AA676033). The ESTs were obtained from a normalized cDNA library of epimastigote forms of CL Brener, the reference organism of the *T. cruzi* Genome Project (Zingales *et al*. 1997) and were cloned in the plasmid pT7T318D (Amersham Pharmacia Biotech) flanked by T3 and T7 promoters (Ürmenyi *et al*. 1999). The ESTs sequences were amplified by PCR employing T3 and T7 primers (Amersham Pharmacia Biotech) with 30 cycles of 3 temperatures (94 °C/1 min; 55 °C/1 min; 72 °C/1 min). The amplification product was ethanol precipitated and resuspended in TE. The probes were labelled with [$\alpha$-$^{32}$P]dATP by the random primed synthesis method (GIBCO-BRL). The hybridization was carried out for 16 h at 67 °C in 3 × SSC/0·1 % SDS/0·1 % Ficoll/0·1 % polyvinylpyrrol-

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Table 1. Information on isolates used in this study

| Isolates | Host origin | Mini-exon typing | Clinical presentation | Geographical origin* |
|---|---|---|---|---|
| Field isolates | | | | |
| 3663 | *Panstrongylus geniculatus* | Z3 | – | Manaus |
| 3869 | Human | Z3 | Acute phase | Caraurari |
| 4166 | *Rhodnius brethesi* | Z3 | – | Barcelos |
| 4167 | *Rhodnius brethesi* | Z3 | – | Barcelos |
| 4176 | *Rhodnius brethesi* | Z3 | – | Barcelos |
| 4181 | *Rhodnius brethesi* | Z3 | – | Barcelos |
| 4182 | *Rhodnius brethesi* | Z3 | – | Barcelos |
| 4183 | *Rhodnius brethesi* | Z3 | – | Barcelos |
| Rb3 | *Rhodnius brethesi* | Z3 | – | Barcelos |
| Rb8 | *Rhodnius brethesi* | Z3 | – | Barcelos |
| Rb10 | *Rhodnius brethesi* | Z3 | – | Barcelos |
| JJ | Human | Z3 | Indeterminate form | Barcelos |
| Reference clones | | | | |
| Dm28c | *Didelphis marsupialis* | *T. cruzi* I | – | Venezuela |
| Silvio X10 cl1 | Human | *T. cruzi* I | Acute phase | Pará, Brazil |
| Esmeraldo cl3 | Human | *T. cruzi* II | Acute phase | Bahia, Brazil |
| CL Bremer | *Triatoma infestans* | *T. cruzi* II | – | Rio Grande do Sul, Brazil |

* All field stocks were isolated in the Brazilian Amazon region. The district is indicated.

idone/100 $\mu$g/ml salmon sperm DNA. The final wash of the blots was carried out in 0·1 % × SSC/ 0·1 % SDS at 67 °C for 30 min. The membranes were exposed to X-ray film. Before rehybridizing blots, probes were removed by boiling in a solution of 0·5 % SDS.

### *Phenetic analysis*

The RFLP profiles of the ITS-rDNA were analysed by a numerical methodology. A similarity matrix was constructed using the simple matching coefficient and the phenogram generated by the UPGMA algorithm using the NTSYS program (Exeter Software, Setauket, NY).

## RESULTS

*Trypanosoma cruzi* Z3 isolates present an insertion in the non-transcribed spacer of the mini-exon gene (Fernandes *et al*. 1998*b*). Using a multiplex PCR that is able to type *T. cruzi* strains into *T. cruzi* I, II and Z3, it was possible to characterize all the 12 isolates described in Table 1 (Fernandes *et al*. 2001). Indeed, all of them yield a 150 bp product that is peculiar to Z3 stocks (Fig. 1). Furthermore, 3 stocks (3663, 3869 and 4181) were previously submitted to isoenzyme characterization and proved to be Z3 (Fernandes *et al*. 2001).

In order to characterize the genetic relationships among the Z3 parasites, RFLP analysis of the ITS1 + 5·8S + ITS2 of the rDNA locus was performed (Fig. 2A). Computer-assisted analysis of the RFLP profiles generated a phenetic dendrogram that shows a clear dichotomy in the Z3 group (Fig. 2B). One cluster (Z3-A) is represented by 3663, 3869, 4176, and 4182 stocks and the other (Z3-B) by 4166, 4167, 4181, 4183, Rb3, Rb8, Rb10, and JJ stocks. In the Z3-B subgroup, all the stocks were isolated in the same district of Barcelos. The genetic distance between the 2 subgroups shows a low level of genetic heterogeneity (mean 0·72 ± 0·13).

A previous report indicated that PCR amplification of the divergent domain D7 of the 24S$\alpha$ rDNA of a wide sample of *T. cruzi* isolates produced DNA products of different molecular sizes: 110 bp product (*T. cruzi* I), 125 bp product (*T. cruzi* II) and the presence of both products (group 1/2) (Souto *et al*. 1996; Zingales *et al*. 1998). Searching for an eventual correlation of the Z3 dichotomy found on the basis of RFPL of the ITS – rDNA, the amplification pattern of the D7 domain was also investigated. For this purpose, DNA of the 12 Z3 stocks was submitted to PCR amplification employing D71 and D72 primers. As controls, DNA of Dm28c (*T. cruzi* I) and CL Brener (*T. cruzi* II) was also amplified. Data shown in Fig. 3 indicate that a 110 bp product is obtained for the 4 Z3-A isolates and products smaller than 125 bp are generated for the 8 Z3-B stocks (Table 2). DNA sequencing estimated the molecular size of these products as 117 bp (Kawashita *et al*. 2001). Accordingly, the isolates presenting this specific amplification product will be denominated as group 3-B.

The genomic organization of 24S$\alpha$ rRNA genes was analysed in all the isolates. Nuclear DNA was digested with *Pst*I, since this enzyme cleaves the 24S$\alpha$ subunit of the rDNA cistron of *T. cruzi* (Arruda *et al*. 1990), and the Southern blot was hybridized with a probe containing the 24S$\alpha$ rDNA gene of the Y strain (Arruda *et al*. 1990). A previous report showed that this probe hybridizes with a

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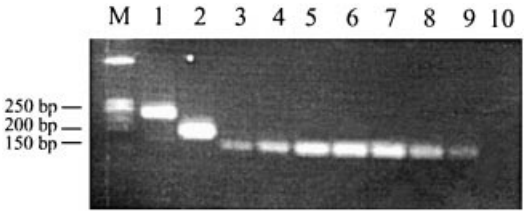


Fig. 1. Agarose gel electrophoresis of PCR products corresponding to a hypervariable region of the mini-exon gene non-transcribed spacer that are able to characterize *Trypanosoma cruzi* I (200 bp); II (250 bp) and Z3 (150 bp). M. Molecular weight markers correspond to $\phi$X DNA digested with *Hae*III. Lanes: CL Brener (1); Dm 28c (2); 4167 (3); JJ (4); 3663 (5); 4182 (6); 3869 (7); 4166 (8); Rb 3 (9); Negative control where no DNA was added to the reaction (10).

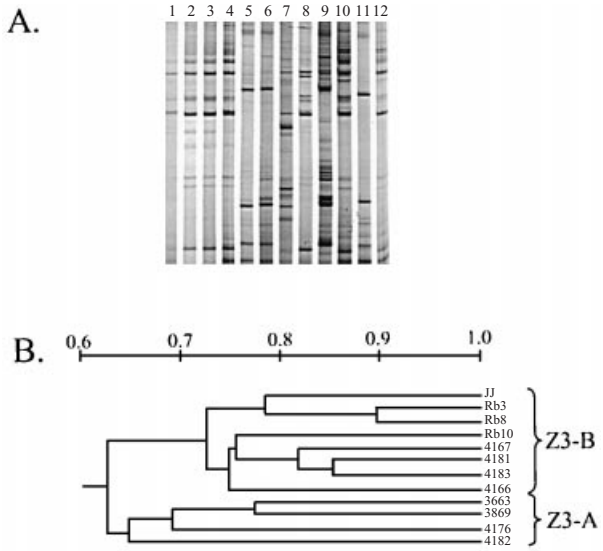


Fig. 2. RFLP analysis of the ITS-rDNA of *Trypanosoma cruzi* Z3 isolates. (A) PCR products digested with *Bst*UI were submitted to polyacrylamide gel electrophoresis and silver stained. Lanes: JJ (1); Rb3 (2); Rb8 (3); Rb10 (4); 3663 (5); 3869 (6); 4166 (7); 4167 (8); 4176 (9); 4181 (10); 4182 (11); 4183 (12). (B) Phenetic dendrogram generated after RFLP analysis (4 distinct enzymes) of the ITS-rDNA by a numerical methodology. Similarity matrix constructed using the simple matching coefficient and phenogram generated by the UPGMA algorithm (NTSYS program – Exeter Software, Setauket, NY). The strains are described on the right.

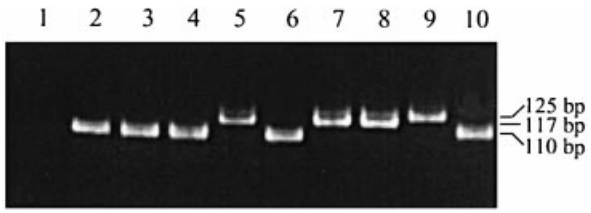


Fig. 3. PCR amplification of the D7 domain of the 24S$\alpha$ rDNA with primers D71 and D72. Lanes: negative control where no DNA was added to the reaction (1); *Trypanosoma cruzi* isolates: 4182 (2); 3663 (3); 3869 (4); 4181 (5); 4176 (6); Rb 8 (7); JJ (8); CL Brener (9); Dm28c (10). Molecular sizes (in bp) are indicated.

single band of 3·8 kb in *T. cruzi* II strains, with a band of 3·6 kb in *T. cruzi* I isolates, and with both bands in isolates of group 1/2 (Souto *et al*. 1996). Data in Fig. 4A indicate that the 24S$\alpha$ rDNA probe hybridizes with a 3·6 kb band in 11-field isolates from Amazonas. The lack of hybridization of the probe with DNA of JJ stock (Fig. 4A, lane 3) was attributed to an experimental failure of DNA digestion with the restriction enzyme as visualized in the ethidium bromide-stained gel (data not shown). For samples corresponding to 4167 and 4183 (Fig. 4A, lanes 7 and 9) an additional band of approximately 3·7 and 3·65 kb, respectively, shows a faint hybridization with the 24S$\alpha$ rDNA probe. This result could be attributed to minor sequence variations among the repetitive units of the ribosomal cistrons.

The hybridization pattern of a probe corresponding to EST TENF 462 in the Southern blot of *Pst*I-digested genomic DNA of the isolates is shown in Fig. 4B. This probe hybridizes with 2 fragments of 3·18 kb and 1·93 kb in Esmeraldo cl3 (Fig. 4B, lane 1) and CL Brener (not shown), with 2 fragments of 2·44 kb and 1·93 kb in Silvio X10 cl1 (Fig. 4B, lane 2) and Dm28c (not shown). In 7 of the Z3 isolates, this probe hybridizes with 2 bands of 3·18 kb and 1·93 kb, whereas in 5 isolates the sizes of these bands are 2·44 kb and 2·0 kb. Data are summarized in Table 2.

After removal of the 462 probe, the membrane was hybridized with EST TENF 552 probe. Fig. 4C shows that the probe hybridizes with a 3·5 kb band in Esmeraldo cl3 and with a 4·9 kb band in Silvio X10 cl1. In CL Brener, 3·5 kb and 6·4 kb fragments were detected (not shown). In 8 Z3 isolates, hybridization with a 6·4 kb band was observed, whereas in 4 isolates the hybridization was obtained with a 4·9 kb fragment. In the Rb8 isolate, the 552 probe hybridized with strong intensity with a 4·9 kb fragment and weakly with a 6·4 kb band. Data are summarized in Table 2.

## DISCUSSION

In this study, the phylogenetic relationships among 12 Z3 isolates from the Amazonas State (North of Brazil) were investigated based on the intergenic transcribed spacers of the ribosomal RNA cistron and the divergent D7 domain of 24S$\alpha$ rRNA gene. RFLP analysis of the ITS1 + 5·8S + ITS2 region showed that Z3 isolates can be divided into 2 discrete groups, named Z3-A and Z3-B. The same dichotomy obtained by the ITS-rDNA approach was also evident when the size of the D7 domain of the 24S$\alpha$ rDNA was investigated. In fact, PCR amplification of this region produced DNA products of 110 bp and 117 bp, respectively, in Z3-A and Z3-B isolates. The sequences of these amplification products have been determined by our group and aligned with previously published D7-24S$\alpha$ rDNA sequences of other *T.*

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Table 2. Amplification products of the D7 domain of 24Sα rDNA and genomic organisation of three DNA sequences

| Isolates* | PCR product of D7 (bp) | Hybridization bands (kb) | | |
|---|---|---|---|---|
| | | 24SαrDNA | EST TENF 462 | EST TENF 552 |
| Field isolates Z3-A | | | | |
| 3663 | 110 | 3·6 | 2·44; 2·0 | 4·9 |
| 3869 | 110 | 3·6(faint) | 2·44; 2·0 | 4·9 |
| 4176 | 110 | 3·6 | 2·44; 2·0 | 4·9 |
| 4182 | 110 | 3·6 | 2·44; 2·0 | 6·4 |
| Field isolates Z3-B | | | | |
| JJ | 117 | N.D. | 3·18; 1·93 | 6·4 |
| Rb3 | 117 | 3·6 | 3·18; 1·93 | 6·4 |
| Rb8 | 117 | 3·6 | 2·44; 2·0 | 4·9; 6·4(faint) |
| Rb10 | 117 | 3·6 | 3·18; 1·93 | 6·4 |
| 4167 | 117 | 3·6; 3·7(faint) | 3·18; 1·93 | 6·4 |
| 4181 | 117 | 3·6 | 3·18; 1·93 | 6·4 |
| 4183 | 117 | 3·6; 3·65(faint) | 3·18; 1·93 | 6·4 |
| 4166 | 117 | 3·6 | 3·18; 1·93 | 6·4 |
| Reference clones | | | | |
| Dm28c | 110 | 3·6 | 2·44; 1·93 | 4·9 |
| Sylvio X10 cl1 | 110 | 3·6 | 2·44; 1·93 | 4·9 |
| Esmeraldo cl3 | 125 | 3·8 | 3·18; 1·93 | 3·5 |
| CL Brener | 125 | 3·8 | 3·18; 1·93 | 3·5; 6·4 |

* Field isolates have been divided into Z3-A and Z3-B subgroups according to Fig. 2B.

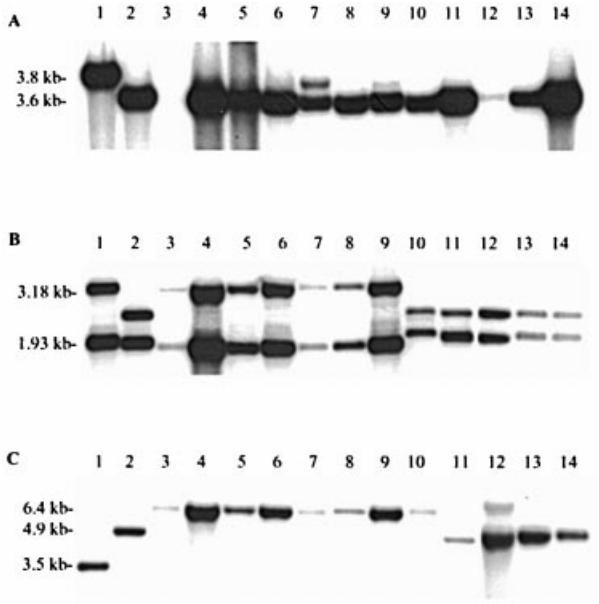


Fig. 4. Southern blot of *Pst*I-digested DNA from *Trypanosoma cruzi* isolates. Hybridization with (A) 24Sα rDNA gene; (B) EST TENF 462; (C) EST TENF 552. (A) Lanes: CL Brener (1); Dm28c (2); JJ (3); Rb 3 (4); Rb 8 (5); Rb 10 (6); 4167 (7); 4181 (8); 4183 (9); 4166 (10); 3663 (11); 3869 (12); 4176 (13); 4182 (14). (B and C) Lanes: Esmeraldo cl3 (1); Silvio X10 cl1 (2); JJ (3); Rb 3 (4); Rb 10 (5); 4166 (6); 4167 (7); 4181 (8); 4183 (9); 4182 (10); 4176 (11); Rb 8 (12); 3663 (13); 3869 (14). Molecular size markers in kb are indicated.

*cruzi* stocks (Kawashita *et al*. 2001). Phylogenies inferred from these datasets confirmed the presence of 2 phylogenetic clusters in Z3.

It has been shown that *T. cruzi* I and *T. cruzi* II present differences in the overall structure of the rDNA cistron (Souto *et al*. 1996). The organization of the ribosomal cistron of 11 Z3 stocks was investigated by Southern blot analysis of *Pst* I-digested genomic DNA and hybridization with the 24Sα rDNA probe. The data indicate a conserved pattern in all the stocks with a single hybridization band of 3·6 kb. This pattern was shown to be characteristic of *T. cruzi* I (Souto *et al*. 1996). The genomic organization of other transcribed genes corresponding to 2 ESTs of CL Brener was also investigated. In contrast to the conserved organization of 24Sα rRNA gene, each EST gave dimorphic hybridization patterns, which show a good correlation with the Z3-A and Z3-B division. Few exceptions were verified, such as the pattern obtained for Rb8 isolate. This result could be tentatively explained considering that a mixed population of strains is represented in this stock. In addition, in the Z3-A subgroup the hybridization pattern of TENF 552 is conserved in 3 isolates (3663, 3869 and 4176) whereas 4182 stock gives the pattern characteristic of the Z3-B subgroup.

Zymodeme 3 presents a wide distribution in the Amazon region. It has also been reported in Bahia State (Brazil) (Barrett *et al*. 1980), in endemic Chagas disease areas of Venezuela (Miles *et al*. 1981*b*) and Colombia (Saravia *et al*. 1987), and also in the USA (Brisse, Barnabe & Tibayrenc, 2000). Z3 is predominantly sylvatic, with current evidence suggesting a primitive association with armadillos, and other nest-building mammals, especially opossums (Miles

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& Cibulskis 1986). So far, Z3 has been isolated from few triatomine species: *P. geniculatus*; *Rhodnius robustus* and *R. brethesi*. The description of human Z3 isolates is sporadic and stocks were obtained from patients in the acute phase of Chagas disease or in the chronic indeterminate form (Miles & Cibulskis, 1986). Extensive clinical surveys in Central Brazil never managed to find Z3 isolates (Luquetti *et al.* 1986). In addition, we could not detect Z3 in sylvatic mammals in Southeast Brazil (Fernandes *et al.* 1999*b*). These data suggest that Z3 is predominant in the Amazon Basin and North up to the USA.

In our study, 2/12 Z3 stocks from Amazonas State were obtained by xenodiagnosis from Chagas disease seropositive individuals. Interestingly, these stocks do not belong to the same Z3 subgroup. Stock 3869 (Z3-A) was isolated from a patient with typical symptoms of acute Chagas disease and the other stock (**JJ**, Z3-B) from an asymptomatic individual, with no electrocardiogram alteration. These patients were from distinct districts. The remaining 10 Z3 stocks were isolated from triatomines. Stock 3663 (group Z3-A) was isolated from *P. geniculatus* and 9 stocks distributed into the 2 Z3 subgroups were obtained from *R. brethesi*, the main vector of the parasite in the Negro River region of Amazonas (Coura, Barrett & Naranjo, 1994).

The characterization of *T. cruzi* isolates into Z3 has traditionally been performed phenotypically by multilocus enzyme electrophoresis (Miles *et al.* 1978; Barrett *et al.* 1980; Miles *et al.* 1981*a*). Initial evidence of the existence of 2 groups within Z3 was obtained when *T. cruzi* stocks from Pará State (Brazil) were analysed (Miles *et al.* 1981*a*). Stocks with typical Z3 isoenzyme patterns were observed along with stocks presenting Z3 profile and Z1 aspartate aminotransferase (ASAT) character. The subdivision of Z3 into 2 phylogenetic clusters was confirmed on the basis of MLEE and RAPD data (Brisse *et al.* 2000). This analysis also supported the existence of the 2 major groups of *T. cruzi*. The branch harbouring Z2 isolates was partitioned into 5 delineated phylogenetic clusters, 2 of them representing Z3 stocks (2a and 2c) (Brisse *et al.* 2000). Subcluster 2a included Can III (Z3 reference stock, Miles *et al.* 1978), whereas subcluster 2c included the Z3/Z1ASAT stocks (Miles *et al.* 1981*a*). The possibility of correspondence of the 2 Z3 groups described herein with the subgroups previously reported must be considered (Miles *et al.* 1981; Brisse *et al.* 2000). Unfortunately, we do not have in our laboratory Z3 stocks representative of the subclusters 2a or 2c mentioned above, to check this hypothesis. In addition, in our experimental conditions the resolution of ASAT activity by electrophoresis in agarose gels does not produce reliable results.

The phylogenetic position of Z3 has been a matter of debate. Numerical taxonomy based on 24 isoenzyme profiles suggested that Z3 is more related to Z1 than Z2 (Ready & Miles, 1980). On the other hand, the analysis of riboprinting patterns, although disclosing a tendency towards a bi-polar grouping of Z1 and Z2, could not clearly determine the position of Z3. In fact, 1 Z3 stock was included into the Z2-group, whereas 3 Z3 isolates remained intermediate between Z1 and Z2 (Stothard *et al.* 1998).

Phylogenetic analyses based on band patterns, such as those obtained by MLEE, RAPD and RFLP may not identify particular groups of strains or give confounding results. This may happen because these markers evolve too fast to resolve such deep divergences. To address this issue our group has analysed the phylogenetic relationships of *T. cruzi* strains using slowly evolving markers, such as rDNA sequences. Phylogenies inferred from complete sequences of the 18S rDNA and of the D7 region of the 24S$\alpha$ rDNA of strains representative of *T. cruzi* I and II and isolates of Z3-A (3663 and 3869) and Z3-B (4166 and 4167) confirmed the *T. cruzi* major clades (Briones *et al.* 1999; Kawashita *et al.* 2001). The cluster containing *T. cruzi* I stocks also includes 2 other subgroups that correspond to Z3-A and Z3-B. This analysis indicates that Z3 is closer to Zl (*T. cruzi* I) as previously described (Ready & Miles, 1980, Fernandes *et al.* 2000) and disagrees with the proposal of Brisse *et al.* (2000) that allocates Z3 closer to Z2 (*T. cruzi* II). Furthermore, it shows that Z3-A and Z3-B have distinct phylogenetic origins (Kawashita *et al.* 2001).

Typing of *T. cruzi* isolates has as a major objective the identification of groups of strains that can be associated with different epidemiological characteristics and/or clinical manifestations of Chagas disease. No specific association could be established between the Z3 subgroups and specific epidemiological features, probably due to the low number of studied isolates.

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