Phylogenetic diversity of bat trypanosomes of subgenus Schizotrypanum based on multilocus enzyme electrophoresis, random amplified polymorphic DNA, and cytochrome b nucleotide sequence analyses

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Received 13 August 2002; received in revised form 3 September 2002; accepted 7 September 2002

Abstract

Trypanosome stocks isolated from bats (Chiroptera) and belonging to the subgenus Schizotrypanum were analyzed by multilocus enzyme electrophoresis (MLEE) at 22 loci, random amplified polymorphic DNA (RAPD) with 14 primers and/or cytochrome b nucleotide sequence. Bat trypanosomes belonged to the species Trypanosoma cruzi marinkellei (10 stocks), Trypanosoma domicili (four stocks) and Trypanosoma vespertilionis (three stocks). One T. rangeli stock and seven stocks of T. cruzi sensu stricto, the agent of Chagas disease, were included for comparison. The homology of several RAPD fragments shared by distinct species was verified by hybridization. The sequence of a 516-nucleotide portion of the maxicircle-encoded cytochrome b (CYb) coding region was determined in representative stocks of the species under study. Phylogenetic analysis of the data confirmed the previous taxonomic attribution of these bat trypanosomes based on biological, epidemiological and ecological features. However, a new finding was that within T. cruzi marinkellei two major subdivisions could be distinguished, T.c.m. I, found in the spear-nose bats Phyllostomus discolor and Phyllostomus hastatus, and T.c.m. II, from P. discolor. In addition, the T. c. marinkellei K stock from a short-tailed bat (Carollia perspicillata) was distantly related to these two subdivisions, and the monophyly of T. c. marinkellei is unclear based on the present data. Based on the present sample, the European species T. dionisii and T. vespertilionis appeared to be more homogeneous. RAPD and CYb data both suggested the monophyly of a group composed of T. cruzi and the two major subdivisions of T. cruzi marinkellei. This study shows that MLEE, RAPD and CYb can be used for taxonomic assignment and provide valuable phylogenetic information for strains and taxa within the subgenus Schizotrypanum. An evolutionary scenario in which the broad host-range parasite T. cruzi would be derived from a bat-restricted trypanosome ancestor is discussed.

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Keywords: Bat trypanosome; Schizotrypanum; Trypanosoma cruzi marinkellei; Trypanosoma domicili; Trypanosoma vespertilionis; Multilocus enzyme electrophoresis; Random amplified polymorphic DNA; Phylogeny; Taxonomy; Evolution

1. Introduction

The subgenus Schizotrypanum includes several trypanosome species that are difficult to discriminate by morphological examination (Hoare, 1972). The type species of the subgenus is Trypanosoma cruzi sensu stricto, the agent of Chagas disease. T. cruzi infects man and a wide variety of mammalian hosts. In contrast, all other species traditionally classified as Schizotrypanum are restricted to bats (order Chiroptera). Strains of T. (Schizotrypanum) vespertilionis and T. (Schizotrypanum) domicili from European bats have been distinguished from other Schizotrypanum species by MLEE, buoyant density and polypeptide profiles (Baker et al., 1978; Taylor et al., 1982). Other species have been distinguished mainly by epidemiological and biological characteristics: T. (Schizotrypanum) hedrcki and T. (Schizotrypanum) myoti in North America (Bower and Woo, 1981), and T. pteropi and T. hippocideri from Australian bats. The subspecies T. cruzi marinkellei was described for South and Central American bat-restricted trypanosomes, mainly after isoenzyme and DNA buoyant density criteria (Baker et al., 1978). T. cruzi s.s., the agent of Chagas disease, should accordingly become the nominate subspecies T. cruzi cruzi, but usage has retained the binominal denomination T. cruzi and we will follow it herein. Recent molecular phylogenetic data based on...
the SSU rRNA indicated that the broad host-range trypanosome T. rangeli and the rat trypanosome T. conorhini should be reclassified in the subgenus Schizotrypanum, and that T. brevovenenhoedi and possibly also T. minasense are host-range variants of T. rangeli (Stevens et al., 1999b).

Multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD) have been previously used to characterize T. cruzi isolates (Miles et al., 1977; Tibayrenc et al., 1993; Brisse et al., 1998a,b; Barnabé et al., 2000). Unlike T. cruzi sensu stricto, no RAPD data was reported from other Schizotrypanum species and only a few isoenzyme data were reported by some authors (Baker et al., 1978; Ebert, 1983; Tibayrenc and Le Ray, 1984; Teixeira et al., 1993; Steindel et al., 1998). Only two of them used genetic distances (Tibayrenc and Le Ray, 1984; Steindel et al., 1998) to differentiate species. In the present work, we attempted a phylogenetic analysis based on MLEE and RAPD data for a more complete set of bat trypanosome stocks and with a broader set of markers than previously used. In addition, with the goal to further establish the phylogenetic relationships among Schizotrypanum taxa, we determined the nucleotide sequence of a portion of the cytochrome b (CTb) gene in representative stocks.

2. Materials and methods

2.1. Origin of the stocks

Origins of the stocks and the markers used for each of them are given in Table 1. The study sample included 10 stocks previously attributed to T. cruzi marinkellei, four stocks attributed to T. donovani, three stocks attributed to T. vespertilionis, 10 stocks representative of the whole phylogenetic diversity of T. cruzi, and one T. rangeli stock.

2.2. MLEE protocol

The stocks were grown at 28 °C either in liver infusion tryptose medium (LIT) supplemented with 10% fetal calf serum or in diphasic blood-agar medium. They were harvested and twice washed in PBS pH 7.2 by centrifugation at 2800 × g and 4 °C for 20 min and the parasite pellets were stored at −70 °C until use. MLEE protocols fully described by Ben Abderrazak et al. (1993) were used with slight modifications, on cellulose acetate plates (Helena Laboratories, Beaumont, Texas). Twenty enzyme systems were analyzed: aconitate hydratase (E.C.4.2.1.3, ACON), alanine aminotransferase (E.C.2.6.1.2, ALAT), NAD (P) H dehydrogenase (quinone) (E.C.1.6.99.2, DIA), glyceraldehyde 3-phosphate dehydrogenase (phosphorylating).

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Table 1
Origins of the 28 Trypanosomatidae stocks surveyed in the present work

<table>
<thead>
<tr>
<th>Species and subdivision</th>
<th>Code</th>
<th>Country</th>
<th>Locality</th>
<th>Host</th>
<th>Date</th>
<th>Marker</th>
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<tr>
<td>T. cruzi I</td>
<td>Tri12</td>
<td>Mexico</td>
<td>nd</td>
<td>Triatoma spp.</td>
<td>1938</td>
<td>M</td>
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<td>T. cruzi I</td>
<td>SC13</td>
<td>Colombia</td>
<td>San Carlos</td>
<td>Rhodius pallescens</td>
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<td>R</td>
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<td>X101</td>
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<td>Belem</td>
<td>Human</td>
<td>1988</td>
<td>R + M</td>
</tr>
<tr>
<td>T. cruzi Ia</td>
<td>CAN E11</td>
<td>Brazil</td>
<td>Belem</td>
<td>Human</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>T. cruzi Ib</td>
<td>Esm13</td>
<td>Brazil</td>
<td>Bahia</td>
<td>Human</td>
<td>1971</td>
<td>R</td>
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<td>T. cruzi Ic</td>
<td>M6241</td>
<td>Brazil</td>
<td>Belem</td>
<td>Human</td>
<td>nd</td>
<td>R</td>
</tr>
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<td>T. cruzi IId</td>
<td>SC412</td>
<td>Brazil</td>
<td>Santa Cruz</td>
<td>Triatoma infestans</td>
<td>1981</td>
<td>M</td>
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<tr>
<td>T. cruzi IId</td>
<td>Mex12</td>
<td>Chile</td>
<td>Region IV</td>
<td>Human</td>
<td>nd</td>
<td>R</td>
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<tr>
<td>T. cruzi IId</td>
<td>Tuc12</td>
<td>Chile</td>
<td>Tolubina, Región IV</td>
<td>Human</td>
<td>nd</td>
<td>M</td>
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<td>CBReiter</td>
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<td>Rio Grande do Sul</td>
<td>Triatoma infestans</td>
<td>1980</td>
<td>R + M</td>
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<td>B3</td>
<td>Brazil</td>
<td>Sao Felipe, Bahia</td>
<td>Phyllostomus discolor</td>
<td>1974</td>
<td>R + M</td>
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<tr>
<td>T. cruzi IId</td>
<td>B7</td>
<td>Brazil</td>
<td>Sao Felipe, Bahia</td>
<td>Phyllostomus discolor</td>
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</tr>
<tr>
<td>T. cruzi IId</td>
<td>M116</td>
<td>Brazil</td>
<td>Abatetuba, Para</td>
<td>Phyllostomus hastatus</td>
<td>1969</td>
<td>R + M</td>
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<td>Brazil</td>
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<td>M29-03</td>
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<td>B34</td>
<td>Brazil</td>
<td>Sao Felipe, Bahia</td>
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<td>M1999</td>
<td>Venezuela</td>
<td>Caracas</td>
<td>Phyllostomus discolor</td>
<td>1985</td>
<td>R + M</td>
</tr>
<tr>
<td>T. cruzi IId III ?</td>
<td>Z</td>
<td>Costa Rica</td>
<td>Rio Virilla, San José</td>
<td>Carollia perspicillata</td>
<td>1968</td>
<td>R + M</td>
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<tr>
<td>T. donovani</td>
<td>P2</td>
<td>UK</td>
<td>East Anglia</td>
<td>Pipedilina pipedilina</td>
<td>1971</td>
<td>R</td>
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<tr>
<td>T. donovani</td>
<td>P3</td>
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<td>East Anglia</td>
<td>Pipedilina pipedilina</td>
<td>1971</td>
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<td>East Anglia</td>
<td>Pipedilina pipedilina</td>
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<td>R + M</td>
</tr>
<tr>
<td>T. donovani</td>
<td>P1 (1MI)</td>
<td>Belgium</td>
<td>Antwerpen</td>
<td>Pipedilina pipedilina</td>
<td>1958</td>
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<tr>
<td>T. vespertilionis</td>
<td>N6</td>
<td>UK</td>
<td>East Anglia</td>
<td>Nystalus norvegicus</td>
<td>1971</td>
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<tr>
<td>T. vespertilionis</td>
<td>P9</td>
<td>UK</td>
<td>Rollisby, Norfolk</td>
<td>Pipedilina pipedilina</td>
<td>1972</td>
<td>R + M</td>
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<tr>
<td>T. vespertilionis</td>
<td>P14</td>
<td>UK</td>
<td>Rollisby, Norfolk</td>
<td>Pipedilina pipedilina</td>
<td>1972</td>
<td>R + M</td>
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<tr>
<td>T. rangeli</td>
<td>Xeno-X11</td>
<td>Colombia</td>
<td>Ubaque, Cundinamarca</td>
<td>Human</td>
<td>1982</td>
<td>R</td>
</tr>
</tbody>
</table>

* R: RAPD and M: MLEE.
(E.C.1.2.1.12, GAPDH), glutamate dehydrogenase (NADP+) (E.C.1.4.1.4, GDH-NADP), aspartate aminotransferase (E.C.2.6.1.1, GOT), glucose-6-phosphate 1-dehydrogenase (E.C.1.1.1.49, 6GDH), glucose-6-phosphate isomerase (E.C.5.3.1.9, GPI), isocitrate dehydrogenase (NADP+) (E.C.1.1.1.42, IDH), leucyl aminopeptidase (E.C.3.4.11.1, LAP), malate dehydrogenase (E.C.1.1.1.37, MDH), malate dehydrogenase (oxaloacetate decarboxylating, NADP+) (E.C.1.1.1.40, ME), mannose-6-phosphate isomerase (E.C.5.3.1.8, MPI), purine-nucleoside phosphorylase (E.C.2.4.2.1, NH), tripeptidyl aminopeptidase (or peptidase B) (E.C.3.4.11.4, PEP-1, substrate: leucyl-leucyl-leucyl-leucine), cytosol non-specific dipeptidase (or peptidase A) (E.C.3.4.13.18, PEP-2, substrate: leucyl- alanine), phosphoglucose dehydrogenase (decarboxylating) (E.C.1.1.1.44, 6PGD), phosphoglucomutase (E.C.5.2.2.1, PGM), superoxide dismutase (E.C.1.15.1.1, SOD).

This gives a total of 22 genetic loci, since both the DIA and ME systems each exhibit the activity of two distinct loci.

2.3. RAPD protocol and hybridization

Fourteen decameric primers previously selected for giving reproducible and readable results (Brisse et al., 2000) were used: A10: TTGATCGCAT; A15: TTGCGAACCC; F4: GTGTGATCAG; N9: TGCCGGCTTG; N13: AGCGTGAG; H9262: TGGGTCCCTC; and U16: CTGCGCTGGA. The amplification reactions were performed as previously described (Brisse et al., 1991). The RAPD fragments were used as probes were eluted from the gel and labelled with [-32P]-dCTP using a Random Priming kit (Boehringer) according to the supplier’s recommendations. Membranes were washed at intermediate stringency (0.1% SSC, 0.1% SDS at RT) before autoradiography.

2.4. PCR and sequence determination

Sequence of the 5736-nt fragment TRCKPMAX (GenBank U43567) from the T. cruzi stock Tulahuen (Ochs et al., 1996) was used to design primers for amplification of the 5’ half of CYb: p18 (5’-GACAGGATTTGAGAGGAGAGAGTG-3’) and p20 (5’-CAACCTATCTCACAATGGATCG-3’). The first nucleotide of primers p18 and p20 corresponded to positions 949 and 1670 of TRCKPMAX, respectively. Reaction conditions were the same as in the RAPD protocol, with 200nM of both p18 and p20 primers. Thirty-five cycles (94°C, 1 min; 50°C, 30 s; 72°C, 90 s) followed by a final elongation step (5 min, 72°C) were performed. Sequence determination of PCR products was carried out with the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) on an ABI-373 Automated DNA Sequencer.

2.5. MLEE and RAPD data analyses

For each enzyme system, a number starting with 1 for the fastest anodic band coded all reproducible bands. All bands were taken into account, except for the central band of three-banded patterns interpreted as heterozygous, which does not correspond to a distinct allele. For RAPD profiles, the numbering of DNA bands started with 1 for the heaviest cathodic band. Several indices of genetic variability were estimated. (i) The mean genetic diversity (H, Nei, 1973), calculated by \( H = \frac{\sum n_i}{n(n-1)} \), where \( n \) is the number of loci and \( h_i = 1 - \frac{\Sigma x_i^2}{n} \), where \( x_i \) is the relative frequency of the \( i \)th electromorph at the \( j \)th locus. (ii) The polymorphism rate, i.e. the proportion of polymorphic loci as a percentage of the total number of loci; a locus was considered polymorphic when the frequency of the more frequent genotype did not exceed 95%; otherwise, the locus was considered monomorphic. (iii) Jaccard's distance (D; Jaccard, 1908) was used to estimate the genetic divergence among stocks, based on the following formula: \( D = 1 - (a/(a + b + c)) \), where \( a \) is the number of bands that are common to the two compared genotypes, \( b \) the number of bands present in the first genotype and absent from the second and \( c \) the number of bands absent from the first genotype and present in the second. The unweighted pair group method with arithmetic averages (UPGMA, Sneath and Sokal, 1973) and neighbor joining method (NJ tree, Saitou and Nei, 1987) were used to cluster the stocks from the Jaccard’s distance matrix. A cladistic analysis (Wagner’s maximum parsimony method) was undertaken by using the presence/absence of each band as the elementary character. Robustness of the nodes on the phylogenetic trees obtained was estimated by bootstrap analysis (Felsenstein, 1985) with 100 replicates. The package PHYLIP (Felsenstein, 1993) was used for cladistic analysis and to design UPGMA or NJ trees.

2.6. CYb sequence analysis

The 516-nt sequence of a non-edited portion of the coding region the CYb gene from 16 trypanosomes was aligned without gaps. Nucleotide composition, nucleotide substitution and phylogenetic analyses were performed using the
program DAMBE version 4.0.75 (Xia, 2000). The neighbor joining method was used based on the Tamura and Nei (1993) model of evolution with random input order and 1000 bootstrap replicates, using T. dionisi P7 as an outgroup. The unrooted tree was drawn using TreeView version 1.6.6 (Page, 1996). Similar topologies and bootstrap values were obtained using parsimony analysis (DNAMP function in DAMBE). Substitution saturation analysis was performed using DAMBE version 4.0.75, by plotting the amount of transitions and transversions against the estimated TN93 genetic distance.

3. Results

3.1. Genetic heterogeneity based on MLEE and RAPD data

Eighteen stocks were analyzed by MLEE, 24 by RAPD and 14 stocks by both markers (Table 1). With MLEE, 17 different multilocus genotypes were observed among the 18 stocks since only 2 stocks of T. cruzi marinkellei (B3 and B7) presented the same genotype. MLEE genetic diversity was very high, ranging from 0.55 (locus Nh) to 0.87 (locus Pgm), mean genetic diversity, $H = 0.73$. All loci were polymorphic ($P = 1.00$), the number of different isoenzyme patterns observed at each locus ranged from 3 (Me2) to 14 (Pgm), with a mean of 6.13. Jaccard’s genetic distances ranged from 0.04 to 0.99 (mean genetic distance, MD = 0.75 ± 0.21). With RAPD, each of the 24 analyzed stocks presented a distinct genotype (see Fig. 1). RAPD genetic diversity was higher than MLEE diversity, ranging from 0.81 (primer A15) to 0.93 (primer U11), mean genetic diversity, $H = 0.89$. All primers were polymorphic ($P = 1.00$), the number of different multibanded patterns ranged from 10 (primer U3) to 18 (primer U7), with a mean of 13.5. Jaccard’s genetic distances ranged from 0.03 to the maximum possible 1.0 (mean genetic distance, MD = 0.82 ± 0.22). Several RAPD fragments were apparently shared by T. cruzi and other species,
Fig. 2. Random amplified polymorphic DNA (RAPD) analysis with primer U14. (A) Profiles obtained for 28 stocks: 4 T. rangeli (lanes 2–5), 5 T. dionisii (lanes 6–10), 3 T. marinkellei II (lanes 11–15), stock Z (lane 16), 6 T. marinkellei I (lanes 17–22), and T. cruzi reference stocks (lanes 23–29). Fragment U14 is apparently shared by T. cruzi and T. marinkellei. (B) Autoradiogram after transfer onto a nylon membrane and hybridization with fragment U14 from the T. cruzi stock SC13 (lane 23) used as probe, that shows the homology across stocks of T. cruzi and stocks of T. marinkellei II.

as illustrated on Fig. 2A. However, such fragments are suitable for deducing phylogenetic relationships only if they really correspond to homologous genetic characters. In order to investigate this question, hybridization experiments were performed. Fig. 2B shows the results obtained using as a probe fragment U14-a from stock T. cruzi SC13. The results demonstrated that fragments U14-a are homologous in T. cruzi and T. cruzi marinkellei II. Homology of a RAPD fragment obtained with primer N19 and shared between T. cruzi, T. cruzi marinkellei I, stock Z, and T. dionisii was also confirmed (data not shown).

3.2. Cluster analysis of MLEE and RAPD data

The same clusters were identified by MLEE and RAPD markers, by using both UPGMA and NJ trees. Three clusters
corresponded respectively to T. cruzi sensu stricto, T. dionisii and T. vespertilionis, while T. cruzi marinkellei was clearly subdivided into two subclusters which we have called T. c. marinkellei I and T. c. marinkellei II (see Fig. 1). T. dionisii and T. vespertilionis were placed in external positions in all trees, and T. cruzi sensu stricto stocks were related to the T. c. marinkellei subclusters, but with a different branching according to the marker used, MLEE or RAPD (Fig. 1). Only two stocks, previously attributed to T. cruzi marinkellei (stock ‘Z’) and T. vespertilionis (stock N6) did not cluster with their respective species: stock Z fell in an external position while stock N6 always clustered with T. cruzi marinkellei II. The consensus trees built from RAPD or MLEE data by using the Wagner’s parsimony method were similar to NJ or UPGMA trees, with a slightly modified branching (data not shown). With RAPD, the five clusters were supported by high bootstrap values respectively 100, 100, 98, 100 and 92 for T. vespertilionis, T. dionisii, T. c. marinkellei I, T. c. marinkellei II and T. cruzi sensu stricto. With MLEE, only three clusters were well supported by bootstrap values, T. dionisii, T. c. marinkellei I, and T. c. marinkellei II with 100, 99 and 98, respectively. The node that clustered together T. cruzi I and T. marinkellei II and I was differently supported by bootstrap values according to the marker, 35 for MLEE and 80 for RAPD (Fig. 1).

3.3. MLEE and RAPD pattern analysis

One or two alleles could be identified for 21 out of 22 loci (suggesting homozygous or heterozygous genotypes, respectively). Differently, the SOD enzymatic system showed many multibanded patterns for all stocks with no possibility of allelic interpretation. The percentages of multibanded patterns were highly conserved across all taxa. A total of 177 sites were variable, 109 of them being phylogenetically informative. An excess of A/G transitions (1738) was observed compared to C/T transitions (1564), although the A/G content was only 40%. Plotting of the numbers of transitions and transversions against the estimated Tamura and Nei, 1993 (TN93) genetic distance showed a limited amount of transitions’ substitution saturation. Within T. cruzi, CYb uncorrected percent divergence was as high as 11%, observed between stock IlB-Emesv and other stocks. For comparison, this value is identical to the variation of the same sequence between Leishmania tarentolae and L. mexicana. Percent divergence was slightly higher between T. cruzi and T. c. marinkellei (for example, ranging from 11 to 13% between M1117 and T. cruzi stocks), and the divergence from T. cruzi I of T. dionisii, T. vespertilionis and T. rangeli was still higher (15–17%). The unrooted tree deduced from CYb sequences is shown on Fig. 3. Very similar branching and bootstrap values were obtained using maximum parsimony analysis (not shown). The results show that stock groupings within T. cruzi and T. cruzi marinkellei subgroups is in agreement with MLEE and RAPD data. The only exception

3.4. Cytochrome b analysis

The sequence of a 516 nt portion of the S′ portion of the non-edited coding region of the cytochrome b gene was established in 14 strains representative of the major branches of the MLEE and RAPD phylogenetic trees. The sequence of the Z stock could not be established, since the amplification yielded a low intensity product, perhaps due to some variability in the primer regions. Sequences of the T. cruzi stocks IlB-Emesv and IlD-SC43 were identical and only one was kept for further analysis. The 516 nt were aligned without insertion/deletions. An excess of base T was observed as the proportion of this base ranged from 0.45 to 0.51, whereas base C was underrepresented, with its proportion ranging from 0.06 to 0.09. The base proportions were highly conserved across all taxa. A total of 177 sites were variable, 109 of them being phylogenetically informative. An excess of A/G transitions (1738) was observed compared to C/T transitions (1564), although the A/G content was only 40%. Plotting of the numbers of transitions and transversions against the estimated Tamura and Nei, 1993 (TN93) genetic distance showed a limited amount of transitions’ substitution saturation. Within T. cruzi, CYb uncorrected percent divergence was as high as 11%, observed between stock IlB-Emesv and other stocks. For comparison, this value is identical to the variation of the same sequence between Leishmania tarentolae and L. mexicana. Percent divergence was slightly higher between T. cruzi and T. c. marinkellei (for example, ranging from 11 to 13% between M1117 and T. cruzi stocks), and the divergence from T. cruzi I of T. dionisii, T. vespertilionis and T. rangeli was still higher (15–17%). The unrooted tree deduced from CYb sequences is shown on Fig. 3. Very similar branching and bootstrap values were obtained using maximum parsimony analysis (not shown). The results show that stock groupings within T. cruzi and T. cruzi marinkellei subgroups is in agreement with MLEE and RAPD data. The only exception

Fig. 3. Unrooted neighbor joining tree deduced from CYb sequences based on the Tamura and Nei (1993) model of evolution. The numbers at the forks indicate the bootstrap values obtained after 1000 replicates with random input order of the stocks.
are stocks Ille-CL Brener and Ild-SC43 c1 which cluster together with M6241 c6, a fact that has been shown to be due to the hybrid origin of lineages Ild and Ile (Machado and Ayala, 2001; Brisse et al., 2002). Interestingly, the association of T. cruzi and T. cruzi marinkellei I and II is strongly supported (bootstrap value: 94%). The branching order of these three taxa was not resolved, however, and neither was that between T. dionisii, T. vespertilionis and T. rangeli.

4. Discussion

Bat trypanosomes of the subgenus Schizotrypanum are very difficult to distinguish morphologically (Hoare, 1972), although T. dionisii may be recognizable by the presence of long thin trypomastigotes in culture (Baker et al., 1978). Our results show that using genetic markers such as isoenzymes or RAPD, the distinction of bat Schizotrypanum species is clearly achieved, and the present phylogenetic analysis agrees with the species attribution previously proposed by Baker et al. (1978). The only exception was the case of stock N6, previously attributed to T. vespertilionis, which appeared closely related to T. c. marinkellei. This can most probably be attributed to a mislabeling of this stock or to a culture contamination by a T. c. marinkellei stock. T. dionisii, T. vespertilionis and the different subdivisions of T. c. marinkellei could each be identified by numerous specific MLEE and RAPD characters that will be useful for taxonomic attribution of unknown stocks. The European trypanosome species T. dionisii and T. vespertilionis correspond to distantly-related genetic clusters identified by numerous specific MLEE and RAPD characters (17 and 8, respectively). Their low level of polymorphism found in the present study must be taken with caution, since the stocks were isolated from limited geographic areas (Belgium and UK), and more isolates should be analyzed to evaluate the actual levels of genetic diversity within these species. Contrasting with the case of European species, the American bat trypanosome T. cruzi marinkellei was found to be highly heterogeneous. It appears to be constituted by two main groups that we named T. c. marinkellei I and II, and perhaps a third group represented so far only by one stock (stock Z). The peculiarity of the Z stock was already noticed by Baker et al. (1978), who observed little resemblance between its enzyme patterns to those of any other T. cruzi marinkellei stocks. Taylor et al. (1982) also reported that the Z stock showed certain specific features not present in other stocks. Apart from the specific case of this Z stock, the two major T. c. marinkellei groups observed by us were each supported by high bootstrap values with RAPD and MLEE markers (Fig. 1). Each of the two major groups represents a genetically heterogeneous population and was geographically widespread (Table 1). T. c. marinkellei was found in two distinct species of bats. Thus, the clustering of T. c. marinkellei into two major groups does not appear to be explained by host or geographic factors, but seems to represent a true genetic structure, a situation that is comparable to the case of T. cruzi (Tibayrenc, 1995; Brisse et al., 2000; Barnabé et al., 2000).

At the high levels of phylogenetic divergence, the resolving power of MLEE and RAPD decreases dramatically, due to too fast a molecular clock which increases the risk of homoplasy. Consequently, the higher branching levels of the cluster hierarchy on Fig. 1 must be considered cautiously and are probably not reliable, especially in the MLEE tree. The fact that the two RAPD fragments tested proved to be homologous shows that at least some phylogenetic signal is retained in the comparison of RAPD data across distinct taxa.

Given these limitations, and the inverse problem of too limited polymorphism found among 18S rRNA sequences from bat trypanosomes (Stevens et al., 1999a), we have used the faster-evolving gene cytochrome b to further investigate the genetic distinctness and phylogenetic relationships among Schizotrypanum taxa. Phylogenetic relationships among cytochrome b sequences have recently been shown to be congruent with rRNA promoter sequence data, with the unique exception of hybrid genotypes within T. cruzi (Brisse et al., 2002). Cytochrome b phylogenetic analysis fully supported the high distinctness among T. cruzi, T. rangeli, bat trypanosome species, and the two major groups of T. cruzi marinkellei. All taxa appeared to be roughly equidistant, with the exception of T. cruzi and the two major groups of T. cruzi marinkellei, which appeared to be more closely related and formed a branch that had a high bootstrap support (96%). A common ancestry of T. cruzi and T. cruzi marinkellei was also suggested by 18S rRNA data (Stevens et al., 1999a). Although not as robustly supported, RAPD data also suggested that T. cruzi and T. c. marinkellei (Fig. 1) pertain to the same phylogenetic subdivision. These results may suggest a new evolutionary scenario for the broad host-range species T. cruzi. Indeed, the fact that T. cruzi is phylogenetically linked to a bat-restricted trypanosome clade suggests that the adaptation of T. cruzi to a wide variety of hosts is a derived character that was acquired from a bat-restricted ancestor. Comparative studies of T. c. marinkellei, other bat schizotrypanosomes and T. cruzi could shed light on the molecular mechanisms of this adaptation. However, this scenario depends on the hypothesis that the putative common ancestor of T. cruzi and T. c. marinkellei was restricted to bats, which remains to be confirmed, in particular by establishing firmly the hierarchical relationships of the other members of subgenus Schizotrypanum, including non-bat-restricted species such as T. rangeli and T. conorhini (Stevens et al., 1999b). In the present study, these hierarchical relationships could not be established. Whether this is due to a particular pattern of variation of the gene CYb, or to a rapid evolutionary radiation of the different members of subgenus Schizotrypanum, remains to be determined.

The present phylogenetic analyses do not contradict, but also do not support, the proposal of a subspecies status (Baker et al., 1978) for American bat-restricted trypanosomes belonging to T. cruzi marinkellei I and II, as it is not established whether these two groups form a
monophyletic unit or not. The external position of the Z stock poses the problem of its phylogenetic and taxonomic relationships with other Trypanosoma stocks. Only a survey of a broader range of stocks will make it possible to settle the issue.

References


