Phylogeny of *Trypanosoma* (*Megatrypanum*) *theileri* and related trypanosomes reveals lineages of isolates associated with artiodactyl hosts diverging on SSU and ITS ribosomal sequences

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SUMMARY

SSU ribosomal sequences of trypanosomes from Brazilian cattle and water buffalo were used to infer phylogenetic relationships between non-pathogenic *T. theileri* and allied species parasitic in artiodactyls. *T. theileri* trypanosomes from distinct geographical regions in Brazil and from other countries were tightly clustered into the 'clade *T. theileri*' distant from the '*T. brucei* clade' of pathogenic parasites of artiodactyls, and also distinct from trypanosomes of other mammals. The existence of this monophyletic assemblage (*T. theileri* clade) composed only by isolates from artiodactyl species justifies the continued recognition of the subgenus *T. (Megatrypanum)* with *T. theileri* as its type species. Phylogenies based on SSU and ITS1 ribosomal sequences produced the same branching pattern with isolates from different mammalian hosts clustered in 5 lineages: A, related to water buffalo; B, C and D, to cattle; E, to fallow deer. The pattern of host specificity allied to some congruence between host and parasite phylogenies suggested association of these trypanosomes with their respective hosts. Segregation of cattle isolates into three lineages revealed an overall geographical structure. Moreover, positioning of trypanosomes infecting tabanids in the *T. theileri* clade is consistent with the role of these flies as important vectors of these trypanosomes.

Key words: *Trypanosoma theileria*, *Megatrypanum*, Artiodactyla, cattle, buffalo, phylogeny, evolution, ribosomal gene, tabanid flies.

INTRODUCTION

Trypanosoma (*Megatrypanum*) *theileri* parasitic in cattle is the type species of the subgenus *T.* (*Megatrypanum*) (Hoare, 1972), a heterogeneous group of large trypanosomes infecting almost all mammalian orders (Hoare, 1972; Wells, 1976). Molecular phylogenies have indicated that the subgenus *T.* (*Megatrypanum*) is polyphyletic (Stevens *et al.* 1999, 2001). Hoare (1972) suggested that morphologically similar trypanosomes infecting domestic and wild ruminants should all be classified as *T. theileri*, and proposed the abolition of several synonyms of *T. theileri* created for trypanosomes infecting different Bovidae species and cattle breeds. Isolates from Cervidae species have either been classified as *T. theileri* or as separate species based on morphology (Kingston and Morton, 1975; Dirie *et al.* 1990*a*, *b*; Bose *et al.* 1993).

T. theileri trypanosomes share mammalian host restriction, worldwide distribution, contaminative transmission by tabanids and lack of pathogenicity. All attempts to infect mammals other than cattle with *T. theileri* have failed (Hoare, 1972; Wells, 1976). A *T. theileri*-like parasite of cervids was not infective to cattle (Kingston and Morton, 1975) and bovid *T. theileri* were not infective to deer (Bose *et al.* 1987). We showed that *T. theileri* trypanosomes from cattle and water buffalo living together clustered according to host species by RAPD analysis, suggesting some degree of host specificity of these trypanosomes (Rodrigues *et al.* 2003).

T. theileri is distributed worldwide from the tropics to near the Arctic Circle, with higher prevalence in tropical and neotropical areas. Trypanosomes morphologically indistinguishable from one another and from *T. theileri* have been described infecting Bovidae, Antilocapridae, Tragulidae and Cervidae with cattle accounting for the largest number of reports (Hoare, 1972; Wells, 1976; Schlafer, 1979; Rodrigues *et al.* 2003).

T. theileri is considered non-pathogenic, causing a chronic and cryptic infection usually only

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detectable in blood cultures. Many cases indicate its potential pathogenicity in concurrent infections with *Babesia*, *Theileria*, *Anaplasma* or piroplasms, or associated with stress, poor nutrition and gestation (Wells, 1976; Hussain *et al.* 1985; Braun *et al.* 2002). All trypanosomes that are pathogenic to artiodactyls are of African origin and positioned in the '*T. brucei* clade', cyclically transmitted by tsetse flies and mechanically by other biting flies. *T. theileri* and '*T. brucei* clade' trypanosomes share artiodactyl host species, have overlapping distributions and bovids carrying mixed trypanosome infection are commonly found in the field. All the '*T. brucei* clade' trypanosomes show broad host-specificity, infecting a much wider range of vertebrates than their 'natural' host range, i.e. the hosts they evolved in, which were probably wild artiodactyls, e.g. suids and antelopes (Logan-Henfrey *et al.* 1992; Gibson *et al.* 2001; Stevens *et al.* 2001). In Latin America, cattle, buffalo and cervids have been reported infected by *T. theileri* and related species (Hoare, 1972; Wells, 1976; Rodrigues *et al.* 2003). '*T. brucei* group' parasites have been imported into Latin America where they are always mechanically transmitted; *T. vivax* is widespread in cattle (Ventura *et al.* 2001); *T. evansi* infects horses, dogs and wild mammals (Ventura *et al.* 2002) and cattle (Herrera *et al.* 2004).

Tabanidae (Diptera) flies, which are thought to be the most important vectors of *T. theileri* occur worldwide and are also mechanical vectors for salivarian trypanosomes (Hoare, 1972; Krinsky, 1976; Foil, 1989). Development of *T. theileri* in tabanids is typical of stercorarian trypanosomes; i.e., restricted to the gut with transmission by penetration of metacyclic trypomastigotes in the faeces through the bite wound of the vector, mucosa, or by ingestion of faeces or of the vector itself by the host (Wells, 1976; Bose *et al.* 1987; Bose and Heister, 1993). The presence of metacyclic trypomastigotes in the salivary glands suggests that ticks can transmit *T. theileri* and *T. cervi* by bite, a route peculiar to salivarian trypanosomes (Krinsky and Burgdorfer, 1976; Morzaria *et al.* 1986).

Phylogenetic relationships among *T. theileri* trypanosomes have never been addressed in the previous studies (Stevens *et al.* 1999, 2001; Hamilton *et al.* 2005) by comparing several isolates from different host species and geographical origin. In this work, we analysed SSU and ITS ribosomal sequences of these trypanosomes aiming (a) to infer phylogenetic relationships among isolates from water buffalo, never included before in phylogenetic studies, with isolates from cattle and fallow deer, (b) to assess the genetic polymorphism among Brazilian isolates from cattle and water buffalo from distant geographical regions and (c) to analyse relationships of these non-pathogenic with pathogenic trypanosomes parasitic in artiodactyls.

MATERIALS AND METHODS

Trypanosoma *spp. used in this study, isolation and growth of* T. theileri *isolates*

In this study we compared 34 *T. theileri* isolates, 18 from cattle (*Bos taurus*) and 12 from water buffaloes (*Bubalus bubalis*), most isolates (30) came from Brazilian regions: (a) North, State of Rondônia (RO), Amazonia; (b) Central, Mato Grosso do Sul State (MS), Pantanal; (c) Southeast, São Paulo State (SP); (d) Southern, Paraná (PR) and (e) Rio Grande do Sul (RS) States (Table 1; Fig. 2). The largest geographical distance ($\sim 3\cdot600$ km) is between RO and RS (Fig. 2B). Trypanosomes from water buffaloes were from Southeast (SP) and Central (Pantanal, MS) regions. Most cattle isolates were recovered from zebu-cattle (Nelore breed, Indian origin), except some isolates from SP and PR States, which are from taurine-cattle (Holstein breed, European origin), and the isolates from RO State, which are all from cross-breed (zebu and taurine) cattle. Trypanosomes were isolated and cultured as before (Rodrigues *et al.* 2003). *Trypanosoma* sp D30 was isolated from the fallow deer *Dama dama* in Germany (Bose *et al.* 1993).

PCR amplification, sequencing of SSU and ITS ribosomal genes, and restriction analysis

DNA of cultured trypanosomes was obtained by classical phenol-chloroform extraction. A ~ 900 bp DNA fragment containing V7-V8 regions of SSU rRNA gene was PCR amplified and products from 3 independent reactions were pooled and automatically sequenced as before (Maia da Silva *et al.* 2004). DNA fragments corresponding to whole ITS rDNA (ITS1, 5·8S and ITS2 sequences) or to only ITS1 rDNA sequences were obtained by PCR, cloned and 2–3 clones from each isolate were sequenced as before (Maia da Silva *et al.* 2004). For restriction analysis, whole ITS amplified DNA were digested with *Bsh* I enzyme and analysed on 2·0% agarose gel stained with ethidium bromide. DNA of *T. vivax*, *T. b. brucei* and *T. evansi* were obtained as before (Ventura *et al.* 2001, 2002). Nucleotide sequence data reported in this paper are available in the GenBank database under the Accession numbers listed in the Table 1.

DNA preparation and PCR amplification of DNA from T. theileri *trypanosomes infecting tabanids*

Crude preparations of DNA templates were obtained from individual digestive tract of dried preserved specimens of *Tabanus* sp using the method described by Aljanabi and Martinez (1997). Tabanids feeding on cattle and horses were caught using small nets in 2 regions from where *T. theileri* isolates were also obtained: MS (24 specimens) and SP (12) States.

Table 1. *Trypanosomes parasitic in artiodactyls and tabanids ascribed to the clade* Trypanosoma theileri *in this study and phylogenetic lineages defined based on polymorphic SSU and ITS ribosomal sequences*

Trypanosome	Host origin	Geographical origin		Lineages defined based on ribosomal gene			GenBank Accession number	
		Country	Region	SSU/ITS1[a] sequences	ITS1[b] (lengh in bp)	Whole ITS[c] PCR-RFLP	SSU	ITS1
Tthb2	Buffalo[1]	Brazil	Southeast	A	A ($\sim$330)	A	—	—
Tthb3	Buffalo	Brazil	Southeast	A	A ($\sim$330)	A	AY773674	—
Tthb4	Buffalo	Brazil	Southeast	A	A (289)[d]	A	AY773675	AY773701
Tthb5	Buffalo	Brazil	Southeast	A	A ($\sim$330)	A	—	—
Tthb6	Buffalo	Brazil	Southeast	A	A (289)[d]	A	AY773676	AY773699
Tthb7	Buffalo	Brazil	Southeast	A	A ($\sim$330)	A	—	—
Tthb8	Buffalo	Brazil	Southeast	A	A ($\sim$330)	A	—	—
Tthb9	Buffalo	Brazil	Southeast	A	A ($\sim$330)	A	—	—
Tthb10	Buffalo	Brazil	Southeast	A	A ($\sim$330)	A	—	—
Tthb11	Buffalo	Brazil	Southeast	A	A ($\sim$330)	A	—	—
Tthb12	Buffalo	Brazil	Center	A	A (289)[d]	A	AY773677	AY773702
Tthb13	Buffalo	Brazil	Center	A	A (289)[d]	A	AY773678	AY773703
Tthc2	Cattle[2]	Brazil	Southeast	B	B (292)[d]	B	AY773679	AY773707
Tthc3	Cattle	Brazil	Southeast	B	B (292)[d]	B	AY773681	AY773698
Tthc11	Cattle	Brazil	Southeast	B	B ($\sim$335)	B	AY773685	—
Tthc22	Cattle	Brazil	Southeast	B	B ($\sim$335)	B	AY773688	—
T. theileri ATCC	Cattle	USA		B	B (292)[d]	B	AY773680	AY773700
T. theileri KM	Cattle	Japan		B	B (289)[d]	B	AB007814[e]	AB007814[e]
Tthc15	Cattle	Brazil	Center	B	B ($\sim$335)	B	AY773686	—
Tthc16	Cattle	Brazil	Center	B	B ($\sim$335)	B	AY773687	AY773708
Tthc8	Cattle	Brazil	Southern	B1	B (296)[d]	B	AY773682	AY773704
Tthc9	Cattle	Brazil	Southern	B1	B (296)[d]	B	AY773683	AY773705
Tthc10	Cattle	Brazil	Southern	B1	B (296)[d]	B	AY773684	AY773706
Tthc5	Cattle	Brazil	Center	C	C (299)[d]	C	AY773689	AY773711
Tthc12	Cattle	Brazil	Center	C	C (299)[d]	C	AY773690	AY773712
Tthc13	Cattle	Brazil	Center	C	C ($\sim$340)	C	AY773691	—
Tthc14	Cattle	Brazil	Center	C	C (295)[d]	C	AY773692	AY773709
Tthc17	Cattle	Brazil	Center	C	C ($\sim$340)	C	AY773693	—
Tthc18	Cattle	Brazil	Northern	C	C (299)[d]	C	AY773694	AY773713
Tthc19	Cattle	Brazil	Northern	C	C (295)[d]	C	AY773695	AY773710
Tthc20	Cattle	Brazil	Northern	C	C ($\sim$340)	C	AY773696	—
Tthc21	Cattle	Brazil	Northern	C	C ($\sim$340)	C	AY773697	—
T. theileri K127	Cattle	Germany		D	ND ND	ND	AJ009164[e]	—
T. theileri TREU124	Cattle	Scotland		D	ND ND	ND	AJ009163[e]	—
T. sp D30	Deer[3]	Germany		E	E (274)[d]	ND	AJ009165[e]	AY773714
Tab1.1cl2	tabanid	Brazil	Center	ND	ND ND	ND	—	—
Tab1.2cl2	tabanid	Brazil	Center	ND	ND ND	ND	AY971802[f]	—
Tab12.1cl2	tabanid	Brazil	Center	ND	ND ND	ND	AY971803[f]	—
Tab12.2cl1	tabanid	Brazil	Center	ND	ND ND	ND	—	—

Trypanosomes are cryopreserved in the Trypanosomatid Culture Collection of the Department of Parasitology, University of São Paulo, Brazil. Tthc, isolates from cattle; Tthb, isolates from water buffalo.
1, water buffalo, *Bubalus bubalis*; 2, cattle, *Bos taurus* – taurine and zebu breeds; 3, fallow deer, *Dama dama*.
[a] Lineages derived from phylogenetic trees based on V7-V8 SSU and ITS1 ribosomal sequences.
[b] Lineages defined based on length of PCR amplified ITS1 sequences determined on agarose gel.
[c] Lineages defined by RFLP analysis of whole amplified ITS digested with *Bsh* I enzyme.
[d] Length of ITS1 determined by sequencing of PCR amplified DNA.
[e] SSU rRNA sequences recovered from Genbank.
[f] Sequences corresponding only to V7 region of SSU rRNA.
ND, Not Determined.

All DNA preparations were submitted to *T. theileri* specific PCR-Tth625 assay (Rodrigues *et al.* 2003) and samples positive in this assay were used as template for amplification of the V7 region of the SSU rRNA ($\sim$500 bp) using the primers 609F and 1156R (Maia da Silva *et al.* 2004), with annealing temperature of 56 °C and adding 5 μg of BSA to reaction mixtures. Probably due to poor quality of DNA templates, only small DNA fragments (V7SSU rRNA) could be amplified. PCR products were
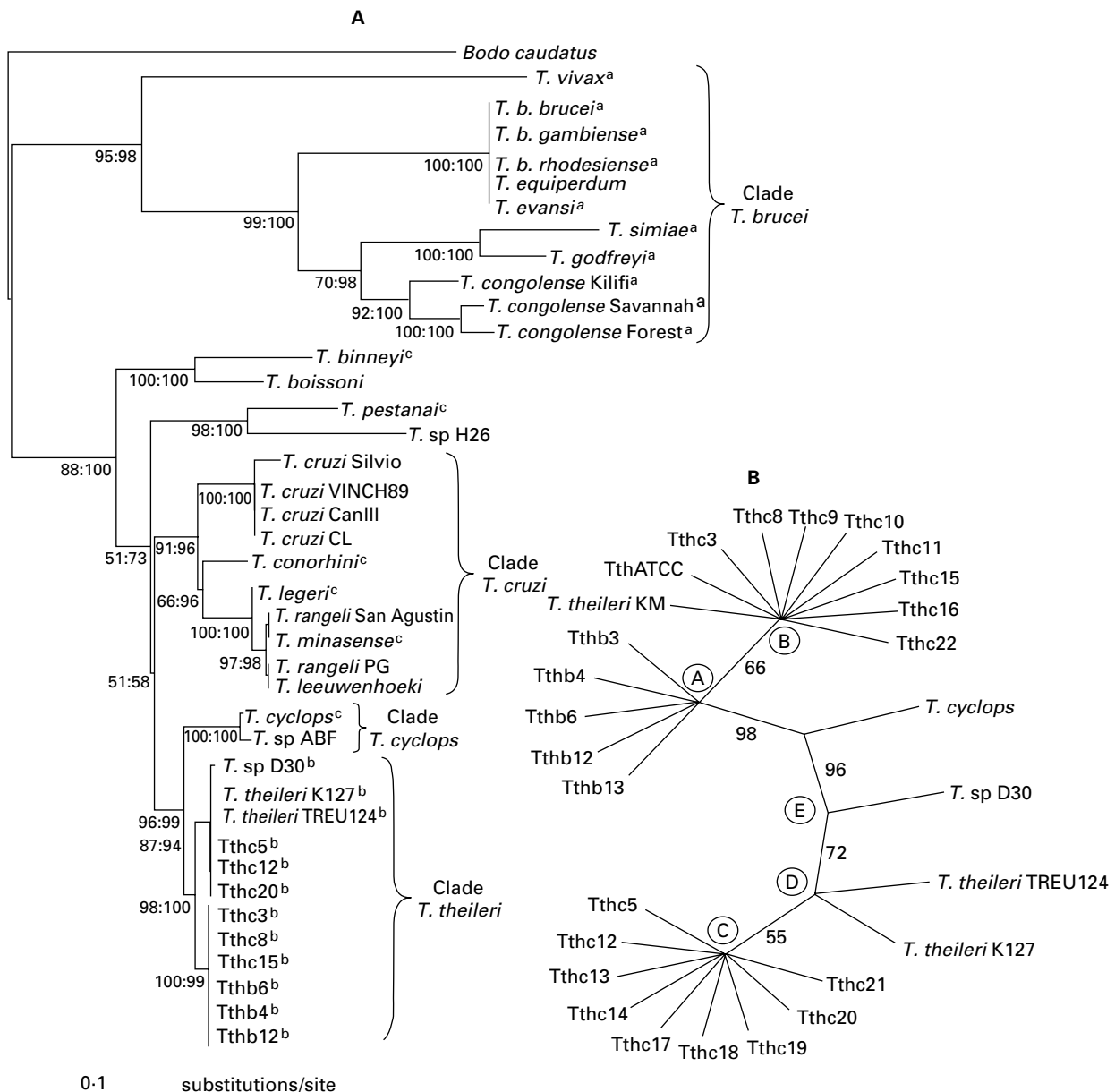
A

B

Fig. 1 (A) Phylogenetic tree of 39 trypanosome species from artiodactyls and several other mammalian orders based on Maximum Likelihood analysis of SSU rRNA (V7-V8) sequences: a, pathogenic species parasites of artiodactyls of clade *Trypanosoma brucei*; b, non-pathogenic species parasites of artiodactyls of clade *T. theileri*; c, *T. (Megatrypanum)* spp. from non-artiodactyl hosts (−lnL 5642.1548). The numbers at nodes correspond to percentage of bootstrap values (Maximum Likelihood: Maximum Parsimony) derived from 100 replicates. (B) Maximum Likelihood analysis of SSU rRNA (V7-V8) sequences from 27 isolates of the clade *T. theileri*. *T. cyclops* was used as outgroup for clade *T. theileri* (−lnL 1385.6146). The numbers correspond to percentage bootstrap support values from 100 replicates. A-E corresponds to the 5 lineages of isolates disclosed by analysis of clade *T. theileri*.

cloned and 3–5 clones from each sample were sequenced as before (Maia da Silva *et al*. 2004).

Alignment of SSU and ITS1 ribosomal sequences and phylogenetic inferences

Partial SSU rRNA sequences (730 bp of V7-V8 regions) from *T. theileri* trypanosomes determined in this study were aligned with sequences of cattle isolates from Japan and Europe, and with the sequence from the cervid trypanosome (D30) from GenBank

(Table 1). Sequences from other trypanosomes from artiodactyls were also retrieved from GenBank (Accession number): *T. b. brucei* (AL359782); *T. b. gambiense* (AJ009141); *T. b. rhodesiense* (AJ009142); *T. vivax* (U22316); *T. congolense* Kilifi (AJ009144); *T. congolense* Savannah (AJ009146); *T. congolense* Forest (AJ009145); *T. simiae* (AJ009162); *T. godfreyi* (AJ009155); *T. equiperdum* (AJ009153); *T. evansi* (D89527). We also included in the alignment sequences from trypanosomes of several other mammalian orders (GenBank): *T. pestanai*
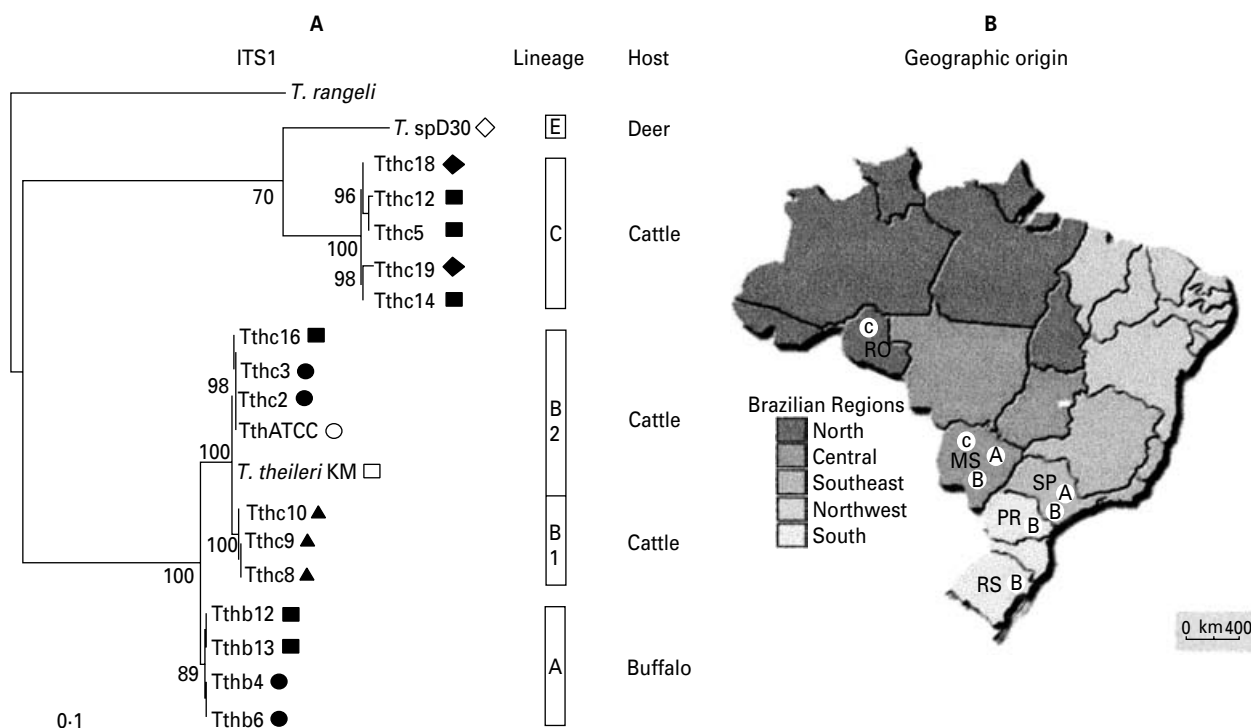
Fig. 2. (A) Phylogeny based on Maximum Likelihood analysis using ITS1 rDNA sequences from isolates representative of all lineages (excepting D) disclosed using SSU rRNA sequences) (Fig. 1) of *Trypanosoma theileri* from cattle (B and C), and *T. theileri*-related trypanosomes from water buffalo (A) and fallow deer (E) previously defined using SSU rRNA sequences (Fig. 1). *T. rangeli* was used as outgroup. Symbols on each isolate correspond to geographical origin of *T. theileri*. (B) Geographical origin of Brazilian isolates, Southeast (●), South (▲), Central (■) and North (◆) regions. North American (○), European (◇) and Japanese (□) isolates. The numbers correspond to percentage of ML bootstrap support values derived from 100 replicates.

(AJ009159); *T.* sp H26 (AJ009169); *T. binneyi* (AJ132351); *T. cruzi* VINCH89 (AJ009149); *T. cruzi* Sylvio X10 (AJ009147); *T. cruzi* CLBR (AF245383); *T. cruzi* CanIII (AJ009148); *T. rangeli* PG (AJ012416); *T. rangeli* San Augustin (AJ01 2417); *T. minasense* (AJ012413); *T. leeuwenhoeki* (AJ012412); *T. legeri* (AY491769); *T. conorhini* (AJ012411); *T. cyclops* (AJ250743); *T.* sp ABF (AJ620564). The sequence of *T. boissoni* (U39580) from fish was also included in the alignment and the sequence of *Bodo caudatus* was used as outgroup for *Trypanosoma*.

Alignments were made using as guide the general alignment of the rRNA database (http://rrna.uia. ac.be/) with subsequent manual adjustment. Maximum parsimony (MP) and maximum-likelihood (ML) analysis were based on 1162 well-aligned characters and carried out using PAUP 4.0b10. The ML model and parameters were estimated using the hierarchical likelihood test implemented in the program Modeltest, version 3.06 (Posada and Crandall, 1998). The best-fit evolutionary model for the likelihood analysis determined by Modeltest was Tamura and Nei with gamma distribution shape. MP and ML analysis and bootstrapping with 100 replicates for both analyses were done as described (Hamilton *et al.* 2004, 2005). Previous analysis demonstrated that phylogenetic trees inferred using

V7-V8 sequences of SSU rRNA disclosed the same branching pattern and all major clades of trypanosomes (Maia da Silva *et al.* 2004) compared to trees generated using larger SSU rRNA sequences (Hamilton *et al.* 2004). Alignments used in this study are available from the authors upon request.

A second alignment was done to analyse the clade *T. theileri* using 17 V7-V8 sequences of SSU rRNA from Brazilian isolates of *T. theileri* from cattle (17 isolates) and water buffaloes (5 isolates), plus 5 isolates from cattle from USA, Japan, Germany and Scotland (Table 1). Sequences corresponding to V7-SSU rRNA (minimum 500 bp) obtained from crude preparations of trypanosomes infecting tabanids, all from MS State, were aligned with sequences from trypanosomes of clades *T. theileri* and *T. cyclops*, the last including 2 trypanosomes from wallaby, *T.* sp ABF (GenBank Accession number AJ620564) and *T.* sp 10 (AJ620571), and 1 isolate from leech, TL.AV.43cl1 (AJ620571). Restricting the alignment to closely related trypanosomes allowed characters in more variable regions to be aligned with higher confidence and included in the ML analysis (K80 evolutionary model) of these very closely related taxa.

Alignment of ITS sequences, similarity matrices and phylogeny (ML) were done as before (Maia da Silva *et al.* 2004). The evolutionary model

determined by Modeltest was F81 with gamma distribution shape. Alignment did not reveal significant polymorphism among ITS1 sequences of clones from the same isolate.

RESULTS

Phylogeny of T. theileri *and related trypanosomes based on SSU rRNA sequences*

Phylogenetic relationships among trypanosomes parasitic in Artiodactyla and species infecting different mammalian orders were inferred by comparison of SSU rRNA sequences (V7-V8 regions). We analysed 27 *T. theileri* isolates and related trypanosomes and 11 isolates of 7 species of salivarian trypanosomes parasites of artiodactyls: *T. vivax*; *T. congolense*; *T. b. brucei*; *T. evansi*; *T. equiperdum*; *T. simiae*; *T. godfreyi*. The large genetic distances among the several trypanosome species used to infer the phylogeny of *Trypanosoma*, contrasted with the homogeneity of *T. theileri* trypanosomes prevented visualization of the branching pattern within the *T. theileri* clade. For this reason, after analysis using all isolates (data not shown) we selected 12 representatives of all major groups to represent the *T. theileri* clade in the phylogeny of *Trypanosoma* (Fig. 1A). Species from all major clades of mammalian trypanosomes, parasites of almost all other mammalian orders, were included in this analysis. Members of the *T. theileri* clade were tightly clustered into a strongly supported (100% bootstrap) monophyletic assemblage. Similar strongly supported topologies were obtained for phylogenetic trees inferred using both ML (Fig. 1A) and MP (data not shown) methods and the bootstrap values varied only slightly (Fig. 1A). The clade *T. theileri* was separated from all other trypanosomes of the subgenus *T. (Megatrypanum)* infecting other mammalian orders (*T. conorhini, T. legeri, T. minasense, T. pestanai,* and *T. binneyi*). The *T. theileri* clade was closest to *T. cyclops* (∼89% similarity) and *T. cruzi* (∼87% similarity). The '*T. brucei* clade', comprising all salivarian trypanosomes, was very distinct from the '*T. theileri* clade'.

Grouping of T. theileri *trypanosomes according to polymorphism on the SSU ribosomal sequences*

To clarify the branching pattern within the *T. theileri* clade and the phylogenetic relationships among the closely related trypanosomes of this clade (Fig. 1A), 17 sequences from Brazilian isolates of *T. theileri* from cattle and 5 from buffaloes were compared with sequences from cattle isolates from USA, Japan, Germany and Scotland (Table 1). *T. cyclops* was used as outgroup. Despite the high similarity (99·9–97·8%), the SSU rRNA (V7-V8 regions) segregated isolates of *T. theileri* clade into 2 major subclades (Fig. 1B). One subclade was formed by 2 clusters

comprising isolates from water buffaloes (lineage A) and from cattle (lineage B). The second subclade was subdivided in 3 branches (lineages C, D and E). Lineages C and D contain only cattle isolates and lineage E 1 isolate from deer (Fig. 1B). Thus, the 5 isolates from water buffaloes (lineage A) were tightly clustered together (100% similarity) whereas Brazilian isolates from cattle were divided in 2 clusters (lineages B and C) separated from European cattle isolates (D). The sequence divergence among the 3 groups of cattle isolates ranged from 0·5 to 1·2%, with higher heterogeneity within lineage C. The trypanosome from deer (D30), the only sample available from Cervidae, was on a separate branch (lineage E) closest to isolates from European cattle (Fig. 1).

Congruence of lineages defined by analysis of polymorphism of ITS and SSU ribosomal sequences

Length and sequence polymorphisms in the ITS rDNA sequences were analysed to evaluate the consistency of grouping within the clade *T. theileri*. The aligned whole ITS of the Brazilian cattle (Tthc3) and buffalo (Tthb6) isolates with sequences from cattle isolates from USA and Japan showed significant sequence divergence on ITS1 (6·1%) and ITS2 (2·6%), whereas sequences of 5·8S were identical.

To compare phylogenies of *T. theileri* and related species using sequences with different evolutionary rates, highly conserved SSU were compared with more variable ITS1 sequences from isolates representative of clusters previously defined by SSUrRNA analysis (Fig. 1B). Isolates from lineage D (Europe) were not available to be included in this analysis. The topology of the ITS1 dendrogram (Fig. 2A) was the same as that generated by SSU analyses (Fig. 1B), with total congruency of topologies using both MP (data not shown) and ML methods, showing the same two major monophyletic assemblages of isolates, one formed for A and B, and the other for C, D and E branches.

In agreement with the SSU rRNA data, lineages A and B showed the smallest distance based on ITS1 sequences whereas the largest distances separated lineages A and C (Fig. 2A). In contrast to the homogeneity (100% similarity) among buffalo isolates (lineage A), a significant polymorphism was showed by cattle isolates within both lineages B and C. Lineage B showed 2·7% of divergence intralineage and was divided into 2 sublineages: B1, consisted of isolates from the Southern region sharing 100% of similarity, and B2, comprising isolates from the Southeast region also sharing 100% similarity, plus the isolates from USA and Japan, the most divergent within this lineage. Lineage C was the most heterogeneous, with intra-lineage divergence ranging from 1·2% to 5·5% (average of 3·3%).

Considering the average sequence divergence among isolates of the same host species, cattle isolates were separated from water buffalo and cervid isolates by 26% and 35%, respectively, whereas isolates from buffalo were separated from the cervid isolate by 38% of sequence divergence. Besides significant similarity between isolates from cattle and buffalo (lineages A and B) living together, the largest genetic distances separated Brazilian cattle isolates from North (C) and South (B1) Brazil (Fig. 1B) suggested higher divergence between more geographically distant populations or independent introductions. The existence in Pantanal (Central Brazil) of the lineages detected in Southeast (SP) and Northern (RO) regions (Table 1, Figs 1 and 2A) is consistent with relative geographical distances (Fig. 2B) and the known regular transport of cattle among these regions.

Detection and sequence analysis of trypanosomes of T. theileri *clade infecting tabanids*

Tabanid specimens positive for *T. theileri* and related trypanosomes were screened using *T. theileri*-specific PCR-Tth625 that generated a 500 bp DNA band specific for these trypanosomes (Rodrigues *et al.* 2003). Of 36 tabanid specimens, 22 were positive for *T. theileri*. DNA fragments corresponding to the V7 region of SSU rRNA from 6 specimens positive for *T. theileri* trypanosomes selected by PCR-Tth625 were cloned and at least 3 clones were sequenced for each sample. Alignment of 13 sequences of very good quality, obtained from 6 tabanid specimens from MS State, and sharing greatest (minimum 98%) similarity to *T. theileri* according to BLAST searches, disclosed small polymorphism with the 4 different sequences obtained represented by the following clones: Tab1.1cl2, Tab1.2cl2, Tab12.1cl2, Tab12.2cl1. All selected DNA sequences from tabanid specimens were tightly clustered in the clade *T. theileri* (100% of bootstrap) (Fig. 3).

Length and restriction fragment length polymorphism of ITS rDNA as a tool for diagnosis and genotyping of trypanosomes of clade T. theileri

To standardize a method for easy identification and lineage typing of *T. theileri* trypanosomes, without DNA sequencing and only requiring a small amount of DNA, we evaluated (a) PCR amplification of whole ITS or ITS1 sequences and (b) PCR-RFLP of ITS DNA. The length of PCR-amplified whole ITS or ITS1 of trypanosomes from cattle, buffalo and fallow deer was similar in 2% agarose gels, and is consequently suitable for differentiation of members of the *T. theileri* clade from other trypanosomes parasitic in ruminants (Fig. 4). The difference in size of PCR product was insufficient to reliably identify *T. theileri* groups by size alone; however, it was
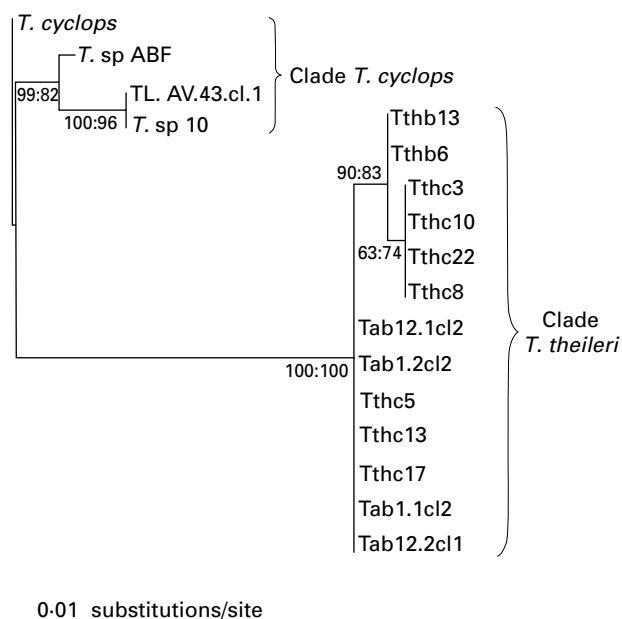

Fig. 3. Relationships among *Trypanosoma theileri* trypanosomes from vertebrate host, cattle (Tthc) and water buffalo (Tthb), and invertebrate (*Tabanus* spp) (Tab) hosts based on alignment of V7SSU rRNA sequences using ML analysis. Bootstrap values (Maximum Likelihood: Maximum Parsimony) were derived from 100 replicates.

possible to discriminate *T. theileri* from the other species tested by size (Fig. 4; Table 1). Three banding patterns were identified in the PCR-RFLP gels, each one corresponding to one lineage previously defined for *T. theileri* of Brazilian cattle (B and C) and buffalo (A) isolates (Fig. 4). The restriction pattern of *Trypanosoma* sp (D30) from deer differed from those of cattle and buffalo isolates (data not shown).

DISCUSSION

Phylogenetic relationships among trypanosomes parasitic in artiodactyls were inferred for non-pathogenic *T. theileri* from cattle, related trypanosomes from water buffalo and fallow deer, and pathogenic trypanosomes parasitic in artiodactyls (African trypanosomes). *T. theileri* and allied species were tightly clustered into a well-supported *T. theileri* clade by analysis of SSU and ITS DNA sequences, and were distinct from all *T. (Megatrypanum)* of other mammalian orders, confirming the previously reported polyphyly of this subgenus (Stevens *et al.* 1999, 2001). Since *T. theileri* is the type species of *T. (Megatrypanum)* (Hoare, 1972) the name *T. (Megatrypanum)* should only apply to members of the *T. theileri* clade and not to other species that have previously been included in this subgenus.

Divergence on SSU and ITS rDNA sequences separated trypanosomes of artiodactyls of clade
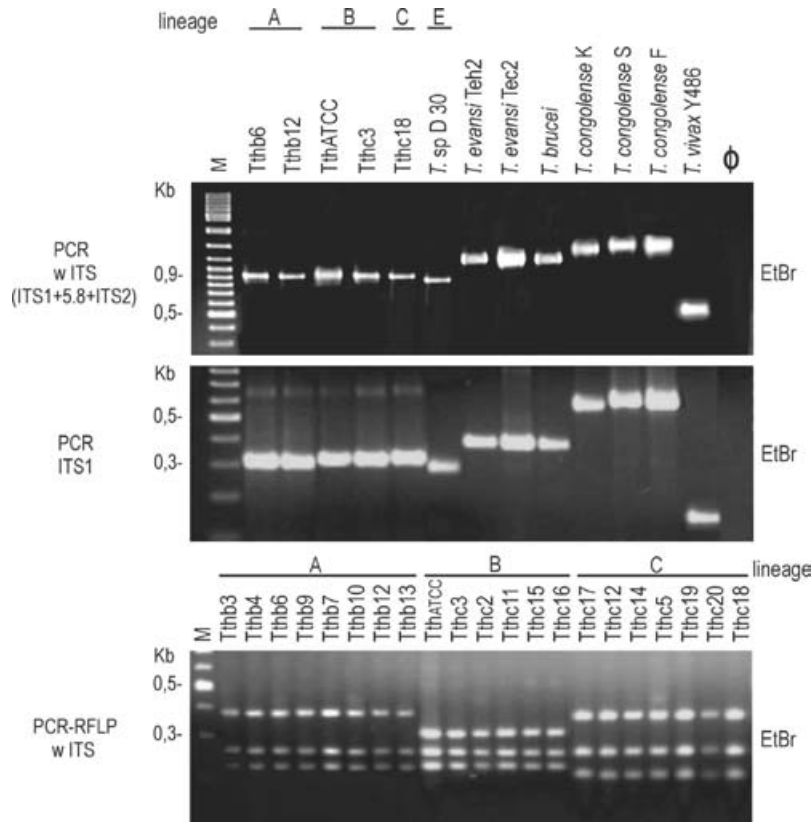
Fig. 4. Agarose gels (2%) stained with ethidium bromide (Etbr) of amplified ITS rDNA sequences from isolates of *Trypanosoma theileri* and allied species and salivarian species of trypanosome parasitic in ruminants. PCR-wITS, length polymorphism of PCR amplified whole ITS DNA sequence (ITS1 + 5·8S + ITS2) and PCR-ITS1, exclusive amplification of ITS1. Negative controls were done using a mixture of DNA from cattle and water buffalo. PCR-RFLP, analysis of PCR amplified whole ITS DNA from trypanosomes of clade *T. theileri*, selected to illustrate distinct lineages within clade *T. theileri*, digested with *Bsh* I enzyme used for lineage genotyping.

T. theileri into 5 lineages. Isolates from the three host species examined in this study never clustered together, even when they were collected from the same locations, suggesting a high degree of host specificity. Lineage A was related to water buffalo, lineages B, C and D to cattle and lineage E to fallow deer. However, the significance of host-parasite associations must be interpreted with caution, particularly when the number of isolates examined from different host species is small and when experimental tests of host-specificity have not been carried out. Although cattle and water buffalo generally live in dry and swampy areas, respectively, segregation of trypanosomes from these hosts living on the same farms and sharing the same pastures indicated lack of host switching and is consistent with host restriction of these trypanosomes. We have never found animals with mixed lineage infections (Rodrigues *et al*. 2003). Although only one isolate from deer was available for this study, it was recognized as a separated lineage (E) based on data from this and from previous studies consistent with host specificity (Kingston and Morton, 1975; Krinsky, 1976; Dirie *et al*. 1990 *a*, *b*; Bose *et al*. 1993). The molecular data showing distinct groups of parasites in different species is

consistent with the lack of cross-infectivity in experimental studies in field conditions, all biochemical studies and different trypanosomes isolated from distinct artiodactyl species, while those from the same hosts were indistinguishable (Dirie *et al*. 1990 *a*, *b*; Bose *et al*. 1993; Rodrigues *et al*. 2003).

Only trypanosomes of Bovidae and Cervidae, artiodactyls of the Pecora suborder (former Ruminantia), were positioned in the clade *T. theileri*. There are some old reports of these trypanosomes in chevrotain (ruminant of the suborder Tragulina) and camels (Tylopoda, primitive ruminants) (cited in Wells, 1976). However recent surveys using morphological and molecular approaches detected salivarian trypanosomes in camels and suids (Gibson *et al*. 2001), but never *T. theileri*. Broader analysis of isolates from other artiodactyls is required to determine the range of vertebrate host species of the *T. theileri* clade.

Small sequence divergence indicated that host-associated lineages are recently diverged and could be correlated to host phylogeny (Hassanin and Douzery, 1999). Isolates from cattle and buffaloes (Bovidae) are more related to each other than to Cervidae isolate. Lineage B of cattle trypanosomes

and lineage A of buffalo isolates share the same geographical regions. Lineage B showed to be more related to lineage A than to other lineages of cattle parasites from more distant regions. Zebu cattle and water buffaloes are both from India and recently introduced in Brazil. Only analysis of trypanosomes from Indian water buffaloes could clarify if differences arose before these animals arrived in Brazil and the relationships between isolates from these hosts. Asian (*Bubâlus bubalis*) and African (*Syncerus caffer*) buffaloes are infected by *T. theileri*-like, and although these trypanosomes from buffaloes were never compared, both differed from cattle isolates (Dirie *et al*. 1990*a*; Rodrigues *et al*. 2003). The large genetic distance separating artiodactyl trypanosomes suggests an ancient divergence of *T. theileri* and the *T. brucei* clade. Artiodactyls originated in the northern hemisphere in the early Eocene (54,8–33,7 mybp) and by the end of the Oligocene had radiated into a number of families (Hassanin and Douzery, 1999). All data suggested that *T. theileri* trypanosomes are recently diverged, compatible with a recent divergence of their host species. *T. theileri* probably evolved with ruminant artiodactyls and dispersed with the artiodactyl radiation in the Miocene and, more recently, by humans transporting livestock around the world.

Our results using a *T. theileri*-specific PCR assay (Rodrigues *et al*. 2003) indicated a high prevalence of *T. theileri* trypanosomes infecting tabanids, thus confirming for the first time using a molecular method the role of these flies as important invertebrate hosts and, probably, as the most important vectors of these trypanosomes. Sequences of V7SSU rRNA of trypanosomes infecting tabanids were tightly clustered together within the clade *T. theileri*. Moreover, these sequences clustered together with those of cattle isolates from the same geographical region (MS State). Tabanids may have been in existence since the Eocene/Oligocene (Martins-Neto, 2003). The developmental cycle of *T. theileri* in tabanids and transmission ability of these flies had been well demonstrated through experimental infections (Wells, 1976; Bose *et al*. 1987; Bose and Heister, 1993). The data strongly suggest that *T. theileri* of bovids are transmitted by tabanid species, which are catholic feeders, whereas host-specific hippoboscid flies are important vectors for cervid trypanosomes (Wells, 1976; Foil, 1989; Bose *et al*, 1993). We are currently analysing a larger number of tabanids from distinct regions in order to understand vector-parasite relationships within the *T. theileri* clade.

The *T. theileri* clade is clearly a complex taxon comprising lineages associated with host species and geographical origin, and is constituted by different populations that appear to have evolved with non-random association of independent molecular markers (clonal structure). However, more biological and molecular data from isolates from different artiodactyls, from widespread geographical origins, will be required for a fuller investigation of host-parasite relationships and to determine segregation factors of *T. theileri* lineages.

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