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Multilocus phylogeographical analysis of *Trypanosoma* (*Megatrypanum*) genotypes from sympatric cattle and water buffalo populations supports evolutionary host constraint and close phylogenetic relationships with genotypes found in other ruminants ☆

Herakles A. Garcia [a,b], Adriana C. Rodrigues [a], Franjo Martinkovic [c], Antonio H.H. Minervino [d], Marta Campaner [a], Vânia L.B. Nunes [e], Fernando Paiva [f], Patrick B. Hamilton [g], Marta M.G. Teixeira [a,*]

[a] *Departamento de Parasitologia, Universidade de São Paulo, Instituto de Ciências Biomédicas II, Av. Prof. Lineu Prestes 1374, 05508-900 São Paulo, SP, Brazil*
[b] *Departamento de Patología Veterinaria, Faculdad de Ciencias Veterinarias, Universidad Central de Venezuela, Maracay, Venezuela*
[c] *Department for Parasitology and Parasitic Diseases, Veterinary Faculty, University of Zagreb, Zagreb, Croatia*
[d] *Instituto de Biodiversidade e Floresta, Universidade Federal do Oeste do Pará, PA, Brazil*
[e] *Centro de Ciências Biológicas, Agrárias e da Saúde, Universidade para o Desenvolvimento do Estado e da Região do Pantanal, Campo Grande, MS, Brazil*
[f] *Departamento de Parasitologia Veterinária, Universidade Federal do Mato Grosso do Sul, MS, Brazil*
[g] *Biosciences, College of Life and Environmental Sciences, University of Exeter, United Kingdom*

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ABSTRACT

Species of the subgenus *Trypanosoma* (*Megatrypanum*) have been reported in cattle and other domestic and wild ruminants worldwide. A previous study in Brazil found at least four genotypes infecting cattle (*Bos taurus*), but only one in water buffalo (*Bubalus bubalis*). However, the small number of isolates examined from buffalo, all inhabiting nearby areas, has precluded evaluation of their diversity, host associations and geographical structure. To address these questions, we evaluated the genetic diversity and phylogeographical patterns of 25 isolates from water buffalo and 28 from cattle from four separate locations in Brazil and Venezuela. Multigene phylogenetic analyses of ssrRNA, internal transcribed spacer of rDNA (ITSrDNA), 5SrRNA, glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH), mitochondrial cytochrome *b* (Cyt *b*), spliced leader (SL) and cathepsin L-like (CATL) sequences positioned all isolates from sympatric and allopatric buffalo populations into the highly homogeneous genotype TthIA, while the cattle isolates were assigned to three different genotypes, all distinct from TthIA. Polymorphisms in all of these sequences separated the trypanosomes infecting water buffalo, cattle, sheep, antelope and deer, and suggested that they correspond to separate species. Congruent phylogenies inferred with all genes indicated a predominant clonal structure of the genotypes. The multilocus analysis revealed one monophyletic assemblage formed exclusively by trypanosomes of ruminants, which corresponds to the subgenus *T.* (*Megatrypanum*). The high degree of host specificity, evidenced by genotypes exclusive to each ruminant species and lack of genotype shared by different host species, suggested that the evolutionary history of trypanosomes of this subgenus was strongly constrained by their ruminant hosts. However, incongruence between ruminant and trypanosome phylogenies did not support host–parasite co-evolution, indicating that host switches have occurred across ruminants followed by divergences, giving rise to new trypanosome genotypes adapted exclusively to one host species.

1. Introduction

Trypanosoma (*Megatrypanum*) is a complex of phylogenetically related species and genotypes nested within a monophyletic assemblage formed by trypanosomes that apparently only infect species of Ruminantia (former Artiodactyla). *Trypanosoma* (*Megatrypanum*) *theileri* is a prevalent and cosmopolitan parasite of cattle from the tropics to near the Arctic Circle (Hoare, 1972; Wells, 1976). Species of the subgenus *T.* (*Megatrypanum*) are also highly prevalent in other livestock species such as water buffalo, sheep and goats, but not pigs or horses, and there are numerous reports in wild bovids, such as African buffalo and antelopes (e.g., duikers, sitatunga, nyala, kudu) and European and North American bovids (e.g., elk, bison, moose, caribou) and cervids (mules and fallow, red, rein and roe deer), South American brocket deer and sika deer

☆ *Note:* The nucleotide sequences reported in this paper are available in GenBank under Accession Numbers listed in Table 1.

* Corresponding author. Tel.: +55 11 3091 7268; fax: +55 11 3091 7417.
E-mail address: mmgteix@icb.usp.br (M.M.G. Teixeira).

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in Japan (D'Alessandro and Wells, 1971; Hoare, 1972; Matthews et al., 1977; Hoffmann et al., 1984; Dirie et al., 1990a; Böse et al., 1993; Lefebvre et al., 1997; Rodrigues et al., 2003, 2006; Hatama et al., 2007; Hamilton et al., 2009; Johnson et al., 2010; Gibson et al., 2010; Neumüller et al., in press). Nothing is known about these trypanosomes in terms of wildlife conservation and reservoirs with regard to livestock infections.

Trypanosoma theileri infections of cattle are generally chronic and asymptomatic and remain so for years, with the parasites rarely being detected in peripheral blood and evading immune responses through mechanisms that are completely unknown (Hoare, 1972; Wells, 1976). *Trypanosoma theileri* is assumed to be non-pathogenic, although infection with this species has been linked to pathogenicity in cattle concomitantly infected with other haemoparasites or subjected to severe stress. Apparently, parasitaemia can increase in immunocompromised animals, triggering the pathogenicity of *T. theileri*, and these infections can result in anaemia and abortion and the death of both the newborn calf and the cow, with parasites dispersing throughout several organs and even the CNS (Ward et al., 1984; Seifi, 1995; Braun et al., 2002; Villa et al., 2008).

According to traditional taxonomy, *T. theileri* of cattle represents the type-species of the subgenus *T.* (*Megatrypanum*) (Hoare, 1972), which was phylogenetically validated as a clade exclusively harbouring trypanosomes of ruminants tightly clustered with *T. theileri* (Rodrigues et al., 2006). Except for *Trypanosoma melophagium* and *Trypanosoma theodori*, which are associated with sheep and goats, respectively, trypanosomes from bovids other than cattle have been classified as *T. theileri*-like and, recently, as new lineages/genotypes of *T. theileri* (Wells, 1976; Rodrigues et al., 2006, 2010a; Hamilton et al., 2009; Gibson et al., 2010; Neumüller et al., in press). Trypanosomes of the subgenus *T.* (*Megatrypanum*) found in bovids and cervids are transmitted by tabanid flies, whereas those infecting sheep and goats are transmitted by host-specific hippoboscid flies (Hoare, 1972; Böse and Heister, 1993). Ticks have been also implicated in the transmission of *T. theileri* (Shastri and Deshpande, 1981; Latif et al., 2004).

Experimental infections and field observations have suggested that *T.* (*Megatrypanum*) spp. are not infective for non-ruminants; *T. theileri* of cattle are unable to cross-infect sheep and goats and vice versa. Taking into account host and vector restrictions, trypanosomes from sheep, goats and deer were classified as *T. melophagium*, *T. theodori* and *Trypanosoma cervi*, respectively (Hoare, 1972; Kingston and Morton, 1975; Wells, 1976). Recently, a trypanosome from a sheep ked was molecularly characterised and classified as *T. melophagium* (Gibson et al., 2010). Although natural and laboratory infections have indicated restricted host ranges, classification of either all trypanosomes from ruminants as *T. theileri* or validation of additional species is questionable. Because water buffalo trypanosomes have not been evaluated regarding their ability to infect other bovid species and due to limited field epidemiological data being available, they have been classified as *T. theileri*-like, or simply as genotype TthIA of *T. theileri* (Rodrigues et al., 2003, 2006, 2010a,b).

The level of host specificity is a key determinant of parasite evolution and has profound implications for their ability to infect new vertebrate hosts and be transmitted by different vectors and, hence, for their population dynamics (Dick and Patterson, 2007; Poulin, 2010). Phylogenetic assemblages of trypanosomes may generally be associated with closely related host species, of one order or genus, or even a single species in natural infections (Rodrigues et al., 2006; Hamilton et al., 2007; Maia da Silva et al., 2007, 2010; Viola et al., 2009; Cavazzana et al., 2010).

According to recent phylogenies, *T.* (*Megatrypanum*) from cattle, water buffalo, antelopes (duiker and sitatunga) and deer constitute a monophyletic assemblage of trypanosomes exclusive to ruminant hosts from a broad geographical range in Europe, South America, Asia and Africa. However, limited numbers of samples and the fact that most of the available data are restricted to ssrRNA sequences, which have proven to be too conserved to discriminate relationships between closely related species of *T.* (*Megatrypanum*), have hindered the evaluation of their real genetic diversity and host-associated genotypes (Rodrigues et al., 2006, 2010a; Hatama et al., 2007; Hamilton et al., 2009).

We previously described internal transcribed spacer (ITS) rDNA, spliced leader (SL) and cathepsin L-like (CATL) polymorphisms that are able to discriminate *T. theileri* from cattle and water buffalo, suggesting that each host species is likely to be infected by different cryptic species/genotypes circulating in the same areas. However, the majority of isolates described in previous studies were from cattle and included only a few buffalo isolates from a limited geographic range (Rodrigues et al., 2003, 2006, 2010a). Phylogeographical patterns provide a valuable framework for investigating evolutionary and ecological factors shaping genetic diversity. However, the process of parasite diversification and host association is often confounded by underlying geographical patterns of host distribution. A clear picture of the evolutionary histories of parasites can only emerge from understanding the spatial structure of hosts and parasites and detecting cryptic genetic diversity (Brooks and McLennan, 2002; Dick and Patterson, 2007; Nieberding et al., 2008; Bordes et al., 2009).

The goals of this study were (i) to understand associations between trypanosomes of the subgenus *T.* (*Megatrypanum*) and their hosts through phylogeographical analysis of isolates from sympatric and allopatric water buffalo and cattle populations across distant regions and (ii) to build a more robust phylogeny through multilocus phylogenetic analysis to resolve relationships and determine genotype/species boundaries among closely related trypanosomes of *T.* (*Megatrypanum*) from cattle, water buffalo, antelopes, deer and sheep.

2. Materials and methods

2.1. Survey of T. (Megatrypanum) spp. in buffalo and cattle from Brazil and Venezuela

A survey of *T.* (*Megatrypanum*) in blood samples from cattle (137 samples from taurine and zebuine breeds of *Bos taurus* and crosses of these breeds) and water buffalo (226 samples from the Murrah breed of *Bubalus bubalis*) was performed by haemocultures. Samples were collected from 1999 to 2009 in three locations in Brazil: Vale do Ribeira (Southeast region, São Paulo State – SP) ($n = 96$ for buffalo and $n = 54$ for cattle), Amazonia (North region, Pará State, PA) ($n = 64$ and 20 for buffalo and cattle, respectively), and Pantanal (Central region, Mato Grosso do Sul, MS) ($n = 16$ and 23). In Venezuela, samples were obtained from buffalo and cattle of the States of Yaracuy ($n = 15$ and 20) and Cojedes ($n = 35$ and 20).

The haemocultures were evaluated weekly by microscopic examination from the 10 to 30 days p.i., and positive cultures were transferred to monolayers of insect cells (Rodrigues et al., 2003, 2006). After successive passages, the established cultures were cryopreserved in the Trypanosomatid Culture Collection (TCC), Department of Parasitology of the University of São Paulo, Brazil (Table 1).

2.2. Trypanosomes of the subgenus T. (Megatrypanum) characterised in this study

In this study, we characterised Brazilian and Venezuelan trypanosomes from water buffalo and cattle grazing together from four separate locations over a large geographic range (over

Table 1
Trypanosomes nested in the subgenus *Trypanosoma* (*Megatrypanum*) used in this study and phylogenetic lineages defined based on analysis of V7–V8 ssrRNA, internal transcribed spacer 1 (ITS1), spliced leader (SL), cathepsin L-like (CATL), glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) and cytochrome *b* (Cyt *b*) sequences.

Trypanosoma theileri isolates[a]	TCC	Host origin	Geographic Origin	Lineages based on DNA sequences[b]				GenBank Acession numbers[d]					
				RFLP[c] ITS1/SL	Cyt *b*	gGAPDH	ITS/ SL	V7-V8 ssrRNA	gGAPDH	Cyt *b*	CATL	ITS1	SL
Water buffalo													
TthbV9	–	Buffalo	VE Northwest (Y, San Felipe)	IA	IA	–	IA	HQ664902	–	HQ664756	HQ664736	HQ664814	HQ664860
TthbV13	–	Buffalo	VE Center (C, El Baul)	IA	IA	–	IA	HQ664903	–	HQ664766	HQ664737	HQ664815	HQ664861
TthbV15	–	Buffalo	VE Center (C, El Baul)	IA	IA	IA	IA	HQ664904	HQ664791	HQ664767	HQ664738	HQ664816	HQ664862
Tthb1	063	Buffalo	BR Southeast (SP, Vale do Ribeira)	IA	–	–	IA	–	–	–	–	–	–
Tthb2	–	Buffalo	BR Southeast (SP, Pariquera–açu)	IA	IA	–	IA	–	–	HQ664757	GU299375	–	–
Tthb3	163	Buffalo	BR Southeast (SP, Pariquera-açu)	IA	IA	–	IA	AY773674	–	HQ664758	GU299388	–	–
Tthb4	162	Buffalo	BR Southeast (SP, Jacupiranga)	IA	IA	–	IA	AY773675	–	HQ664752	GU299378–GU299380	AY773701	–
Tthb5	164	Buffalo	BR Southeast (SP, Registro)	IA	–	–	IA	–	–	–	–	–	–
Tthb6	165	Buffalo	BR Southeast (SP, Jacupiranga)	IA	IA	–	IA	AY773676	–	HQ664753	–	AY773699	GQ162118
Tthb7	169	Buffalo	BR Southeast (SP, Eldorado)	IA	–	–	IA	–	–	–	–	–	–
Tthb8	167	Buffalo	BR Southeast (SP, Eldorado)	IA	–	–	IA	–	–	–	–	–	–
Tthb9	168	Buffalo	BR Southeast (SP, Jacupiranga)	IA	IA	–	IA	–	–	HQ664759	GU299383	–	–
Tthb10	166	Buffalo	BR Southeast (SP, Eldorado)	IA	IA	IA	IA	HQ664895	HQ664784	HQ664760	GU299381	HQ664808	HQ664857
Tthb11	170	Buffalo	BR Southeast (SP, Eldorado)	IA	IA	–	IA	–	–	HQ664761	–	–	–
Tthb12	–	Buffalo	BR Center (MS, Dourados)	IA	–	–	IA	AY773677	–	–	HQ664732	AY773702	–
Tthb13	–	Buffalo	BR Center (MS, Dourados)	IA	IA	–	IA	AY773678	–	HQ664762	HQ664733	AY773703	GQ162121
Tthb14	1858	Buffalo	BR North (PA, Santarém)	IA	IA	IA	IA	HQ664896	HQ664785	HQ664763	–	HQ664809	HQ664859
Tthb15	–	Buffalo	BR North (PA, Santarém)	IA	–	–	IA	–	–	–	–	–	–
Tthb16	–	Buffalo	BR North (PA, Santarém)	IA	–	IA	IA	HQ664897	HQ664786	–	–	–	–
Tthb17	1856	Buffalo	BR North (PA, Santarém)	IA	IA	IA	IA	HQ664898	HQ664787	HQ664754	HQ664734	HQ664810	–
Tthb18	–	Buffalo	BR North (PA, Santarém)	IA	–	–	IA	–	–	–	–	–	–
Tthb19	1855	Buffalo	BR North (PA, Santarém)	IA	IA	IA	IA	HQ664899	HQ664788	HQ664755	HQ664735	HQ664811	–
Tthb20	1857	Buffalo	BR North (PA, Santarém)	IA	IA	IA	IA	HQ664900	HQ664789	HQ664764	–	HQ664812	–
Tthb21	–	Buffalo	BR North (PA, Santarém)	IA	–	–	IA	–	–	–	–	–	–
Tthb22	1859	Buffalo	BR North (PA, Santarém)	IA	IA	IA	IA	HQ664901	HQ664790	HQ664765	–	HQ664813	–
Cattle													
Tthc1	–	Cattle	BR Southeast (SP, Jacupiranga)	IB	IB	IB	IB	HQ664905	HQ664792	HQ664768	GU299400	HQ664817	HQ664863
Tthc2	171	Cattle	BR Southeast (SP, Jacupiranga)	IB	IB	–	IB	AY773679	–	HQ664769	GU299397; GU299398	AY773707	HQ664864
Tthc3	161	Cattle	BR Southeast (SP, Eldorado)	IB	IB	IB	IB	AY773681	HQ664793	HQ664770	HQ664739	AY773698	GQ162125
Tthc8	–	Cattle	BR Southeast (RS, Porto Alegre)	IC	–	–	IC	AY773682	–	–	–	–	–
Tthc9	–	Cattle	BR Southeast (PR,	IC	–	–	IC	AY773683	–	–	–	–	–

(*continued on next page*)

Table 1 (*continued*)

Trypanosoma theileri isolates[a]	TCC	Host origin	Geographic Origin	Lineages based on DNA sequences[b]				GenBank Acession numbers[d]					
				RFLP[c] ITS1/SL	Cyt *b*	gGAPDH	ITS/ SL	V7-V8 ssrRNA	gGAPDH	Cyt *b*	CATL	ITS1	SL
			Londrina)										
Tthc10	–	Cattle	BR Southeast (PR, Londrina)	IC	–	–	IC	AY773684	–	–	–	–	–
Tthc11	–	Cattle	BR Southeast (SP, Eldorado)	IB	–	–	IB	AY773685	–	–	–	–	–
Tthc22	–	Cattle	BR Southeast (SP, Araçatuba)	IB	–	–	IB	AY773688	–	–	–	–	–
Tthc5	–	Cattle	BR Center (MS, Dourados)	IIB	–	–	IIB	AY773689	–	–	GU299371	AY773711	–
Tthc12	–	Cattle	BR Center (MS, Miranda)	IIB	–	–	IIB	AY773690	–	–	–	–	–
Tthc13	–	Cattle	BR Center (MS, Miranda,)	IIB	IIB	–	IIB	AY773691	–	HQ664771	–	–	–
Tthc14	–	Cattle	BR Center (MS, Miranda)	IIB	–	–	IIB	AY773692	–	–	GU299368	–	–
Tthc15	–	Cattle	BR Center (MS, Miranda)	IB	–	–	IB	AY773686	–	–	–	–	–
Tthc16	–	Cattle	BR Center (MS, Miranda)	IB	–	–	IB	AY773687	–	–	GU299395	–	–
Tthc17	360	Cattle	BR Center (MS, Miranda)	IIB	IIB	–	IIB	AY773693	–	HQ664772	–	–	–
Tthc28	1458	Cattle	BR Center (MS, Miranda)	IIB	IIB	–	IIB	GQ176155	–	HQ664774	GU299359	–	–
Tthc29	1459	Cattle	BR Northeast (RN, Mossoró)	IIB	IIB	IIB	IIB	GQ176156	HQ664799	HQ664773	–	HQ664818	–
Tthc30	1460	Cattle	BR Northeast (RN, Mossoró)	IIA	–	IIA	IIA	GQ176157	HQ664801	–	GU299354–GU299355	–	GQ162134
Tthc32	1462	Cattle	BR Northeast (RN, Mossoró)	IIA	–	IIA	IIA	GQ176158	HQ664802	–	GU299351–GU299352	–	HQ664882; GQ162136
Tthc37	1787	Cattle	BR North (PA, Catanhal)	IIA	–	IIA	IIA	GQ176159	HQ664803	–	GU299347–GU299348	–	HQ664883
Tthc38	1788	Cattle	BR North (PA, Catanhal)	IIB	IIB	–	IIB	GQ176160	–	HQ664775	–	–	HQ664866; HQ664867
Tthc39	–	Cattle	BR North (PA, Santarem)	IIB	IIB	IIB	IIB	HQ664906	HQ664794	HQ664776	HQ664740	HQ664823	HQ664868; HQ664869, HQ162160
Tthc40	–	Cattle	BR North (PA, Santarem)	IIB	IIB	IIB	IIB	HQ664907	HQ664795	HQ664777	HQ664741	HQ664825	HQ664870–HQ664872; HQ162164
Tthc41	–	Cattle	BR North (PA, Santarem)	IIB	IIB	IIB	IIB	HQ664908	HQ664796	HQ664778	HQ664742	HQ664827	HQ664873–HQ664875
TthcV2	–	Cattle	VE Center (C, El Baul)	IIB	IIB	–	IIB	HQ664909	–	HQ664779	HQ664743	HQ664829	HQ664878; HQ664879
TthcV4	–	Cattle	VE Northwest (Y, San Felipe)	IIB	IIB	IIB	IIB	HQ664910	HQ664797	HQ664780	HQ664744	HQ664830	HQ664880; HQ664881
TthcV5	–	Cattle	VE Center (A, San Fernando)	IIB	–	IIB	IIB	HQ664911	HQ664798	–	–	–	–
TthcV6	–	Cattle	VE West (M, Mérida)	IIB	–	–	IIB	–	–	–	–	HQ664831	–
K127	–	Cattle	Germany	–	–	–	–	AJ009164	AJ620282	–	–	–	–
TREU124	–	Cattle	Scotland	–	–	IIB	IIB	AJ009163	HQ664800	–	–	–	HQ664876; HQ664877
Tth KM	–	Cattle	Japan	–	–	–	–	AB007814	–	–	–	–	–
TthATCC	–	Cattle	EUA	IB	–	–	IB	–	–	–	GU299391	–	–
Other T. theileri and T. theileri-like sequences used in this study													
CepCamp4	–	Duiker	Cameroon	–	–	–	–	FM202491	HQ664804	HQ664782	HQ664748	HQ664832–HQ664836	HQ664889
CepCamp5	–	Duiker	Cameroon	–	–	–	–	FM202490	HQ664805	HQ664783	HQ664749–HQ664751	HQ664837–HQ664841	HQ664888
SitaBip1	–	Sitatunga	Cameroon	–	–	–	–	FM202489	FM164792	HQ664781	HQ664745–HQ664747	HQ664842–HQ664844	HQ664886; HQ664887
TspD30	–	Deer	Germany	–	–	–	IIC	AJ009165	HQ664806	–	GU299415–GU299417	AY773714	HQ664884; HQ664885

Trypanosoma melophagium	–	Sheep ked	Scotland	–	–	–	–	FN666409	–	–	–	–	FN666410	
T. melophagium	1993	Sheep ked	Croatia	–	–	–	–	HQ664912	–	–	–	–		HQ664890–HQ664892

Underlined accessions numbers are sequences determined in the present study.
TCC, code number at Trypanosomatid Culture Collection from the Department of Parasitology, University of São Paulo, ICBII, Brazil.
Nd, sequences not determined, isolates were genotyped using one or more sequences from the different molecular markers used in this study: SL/ITS1/V7-V8ssrRNA/CATL and Cyt *b* genes.
[a] Tthc, isolates from Brazilian cattle; TthcV, isolates from Venezuelan cattle; Tthb, isolates from Brazilian water buffalo; TthbV, isolates from Venezuelan water buffalo.
[b] Lineages derived from phylogenetic trees based on SL/ITS1/V7V8SSU/CatL-like/CytB DNA sequences.
[c] Lineages defined by RFLP analysis of whole amplified ITS and SL digested with *Bsh*1236 restriction endonuclease.
[d] SL, Ribosomal, CatL-like, CytB and gGAPDH sequences accession number in GenBank.

5,000 km). Buffalo isolates ($n = 13$) previously obtained from Vale do Ribeira and Pantanal (Rodrigues et al., 2006) were compared with 12 new isolates, three of which were from Venezuela and nine were from Amazonia (PA) (Table 1). *Trypanosoma theileri* from cattle from the USA, Germany, Scotland and Japan were used for comparison, as well as trypanosomes from other ruminants: *T. melophagium* (from sheep keds) from Scotland (Gibson et al., 2010) and Croatia (Martinkovic et al., in press); two isolates from blue duikers (*Cephalophus monticola*) and one from sitatunga (*Tragelaphus spekei*) antelopes from Cameroon, Africa (Hamilton et al., 2009), and one isolate from German fallow deer (*Cervus dama*) (Böse et al., 1993) (Table 1).

2.3. Diagnosis and genotyping of T. (Megatrypanum) isolates

All new isolates included in this study were confirmed as *T.* (*Megatrypanum*) based on the morphology of epimastigote cultured forms and by a specific PCR assay targeting Cathepsin L (CATL) sequences using DNA prepared from primary and/or established cultures. Diagnosis of bovids infected with *T.* (*Megatrypanum*) was also performed using the same PCR assay and DNA templates from blood samples collected from the field (Rodrigues et al., 2010a). All new isolates were genotyped through PCR–RFLP of ITSrDNA and SL sequences as before (Rodrigues et al., 2006, 2010a).

2.4. PCR amplification and sequencing of multiple genes

DNA of cultured trypanosomes was extracted using the phenol–chloroform method. DNA from blood and primary cultures (~10 days) was purified using the Wizard DNA Clean-Up System (Promega). Distinct sequences were obtained: V7–V8 region of ssrRNA (~840 bp); ITS1rDNA (~300 bp) (Rodrigues et al., 2006); glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) (~847 bp); (Hamilton et al., 2007); ~400 bp corresponding to transcribed exon and intron plus ~270 bp of the intergenic region of SL gene repeats; the amplified SL repeat sequence contains the 5S rRNA gene (Rodrigues et al., 2010a); cytochrome *b* gene (Cyt *b*) (~449 bp) (Cavazzana et al., 2010) and CATL gene (~477 bp) (Rodrigues et al., 2010b). PCR products were purified using a Spin-X® kit (Costar®) and cloned with a TA-Cloning® kit (Invitrogen). Sequences of three to five clones of each gene from each isolate were determined, and those representing polymorphism among and within isolates were used for phylogenetic inferences and deposited in GenBank (Table 1).

2.5. Phylogenetic inferences

The sequences of seven genes from *T.* (*Megatrypanum*) spp. employed in this study were determined herein or in our previous studies (Rodrigues et al., 2006, 2010a,b), and those from the other trypanosomes included in the phylogenetic analyses were obtained from GenBank (Table 1). To make phylogenetic inferences, sequences were aligned using the ClustalX program (Thompson et al., 1994) and refined manually. We created 10 alignments: (A) V7-V8 ssrRNA sequences from isolates of cattle ($n = 25$ isolates), buffalo ($n = 27$), antelopes ($n = 3$), sheep keds ($n = 2$) and fallow deer ($n = 1$); (B) gGAPDH sequences of isolates from buffalo ($n = 8$), cattle ($n = 13$), fallow deer ($n = 1$), antelopes ($n = 3$) and 28 sequences from other trypanosomes and trypanosomatids of other genera; (C) ITS1rDNA sequences of isolates from buffalo ($n = 13$), cattle ($n = 14$), antelopes ($n = 3$) and fallow deer ($n = 1$); (D) Cyt *b* sequences of buffalo ($n = 16$), cattle ($n = 10$) and antelope ($n = 3$) isolates; (E) SL gene sequences from buffalo ($n = 7$), cattle ($n = 8$), antelope ($n = 3$), fallow deer ($n = 1$) and sheep ked ($n = 1$) isolates; (F) 5S rRNA sequences of isolates from buffalo

($n = 7$) and cattle ($n = 8$); (G) CATL sequences of isolates from buffalo ($n = 12$), cattle ($n = 17$), antelopes ($n = 3$) and fallow deer ($n = 1$); (H) combined V7-V8 ssrRNA and gGAPDH sequences; (I) combined ITS1rDNA and SL sequences; and (J) concatenated sequences of all six genes. All sequences determined herein were deposited in GenBank (Table 1). All alignments are available from the authors by request.

Phylogenetic inferences were assessed using maximum likelihood (ML), maximum parsimony (MP), neighbour-joining (NJ) and/or Bayesian methods, as described previously (Rodrigues et al., 2006; Viola et al., 2009; Cavazzana et al., 2010). MP and bootstrap analyses were performed using PAUP* 4.0b10 with 100 replicates of a random additional sequence, followed by branch swapping (RAS-TBR). ML analyses were performed using RAxML v.7.0.4 (Stamatakis, 2006), and tree searches were conducted using GTRGAMMA with 500 MP starting trees. Model parameters were estimated in RAxML over the duration of the tree search. Nodal support was estimated using 500 bootstrap replicates in RAxML with GTR+GAMMA and MP starting trees. Bayesian analysis was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). For tree searches, we employed GTR+GAMMA and a proportion of invariable sites. The first 25.0% of the trees from 100,000,000 generations were discarded as burn-in. Phylogenetic networks were constructed using the Neighbour-Net method with Kimura 2 parameters implemented in Splits Tree4 V4.10 (Huson and Bryant, 2006). Internode support was estimated by performing 100 bootstrap replicates using the same parameters optimised for network inferences.

3. Results

3.1. Survey of T. (Megatrypanum) isolates in water buffalo and cattle

The haemocultures of blood samples from buffalo and cattle showed trypanosome prevalences varying with host species and geographical origin. The highest prevalences were 40.6% for buffalo and 31.5% for cattle sharing pastures in Vale do Ribeira where animals have no access to large water bodies. In Amazonian lowland regions, where animals share extensive grasslands on floodplains and have access to large rivers (at flood times, animals are transferred to upland pastures), the prevalence rates were 14.1% for buffalo and 25% for cattle. In the Pantanal, a region where animals live in large swamp areas, the prevalences were 12.5% for buffalo and 30.4% for cattle. In Venezuela, the prevalences were 6.0% for buffalo and 5.0% for cattle. The buffalo and cattle examined in all regions share pastures and management conditions. Tabanids are abundant throughout the year in all cases. However, differences in animal health conditions, the size of herds and grazing areas, access to large water bodies where buffaloes are protected against haematophagous insects, are all factors that may account for variations in the prevalence of trypanosomes. These factors were not evaluated in this study.

The number of isolates established in the cultures was limited to 30% of positive primary haemocultures (Table 1). All positive haemocultures only exhibited *T.* (*Megatrypanum*), as identified by morphological and molecular diagnosis (Rodrigues et al., 2003, 2010b).

3.2. Barcoding and phylogenetic positioning of trypanosomes from ruminants

The identity and genetic diversity of the *T.* (*Megatrypanum*) isolates from buffalo and cattle were initially assessed through barcoded V7–V8 ssrRNA sequences of new isolates from Venezuela ($n = 7$) and Brazil ($n = 45$), including isolates from southeastern ($n = 19$), central ($n = 10$) and northern ($n = 17$) regions. These sequences were compared with those from other isolates characterised previously in a study that demonstrated that these small sequences is a valuable targets for DNA barcoding aiming at the species/genotypes identification of trypanosomes of the subgenus *T.* (*Megatrypanum*) (Rodrigues et al., 2006), and for other trypanosomes including *Trypanosoma rangeli* and species of the subgenera *Trypanosoma* (*Schizotrypanum*) and *Trypanosoma* (*Herpetosoma*) (Maia da Silva et al., 2007, 2010; Cavazzana et al., 2010). All buffalo isolates shared identical V7–V8 ssrRNA sequences corresponding to the TthIA genotype, which is highly related to TthIB of cattle (0.14% divergence). The other cattle isolates were assigned to the TthIIA and TthIIB genotypes, and the genotypes from other ruminants varied according to the host species. A dendrogram of the barcoded sequences showed two major clusters corresponding to the lineages TthI (cattle and buffalo) and TthII (cattle, antelopes, deer and sheep keds), but the relationships among the genotypes within these lineages could not be resolved due to the high conservation of ssrRNA genes (Fig. 1A).

We determined the entire gGAPDH sequences for eight buffalo and 11 cattle isolates from Brazil and Venezuela that were selected to represent the diversity shown in the barcoding analysis, in addition to isolates from blue duikers, fallow deer and cattle (Treu124) from Scotland. These sequences were aligned with sequences of trypanosomes from German cattle and African sitatunga (Table 1). Regardless of the phylogenetic methods used, all *T. theileri* isolates clustered tightly together into a clade corresponding to the subgenus *T.* (*Megatrypanum*) formed by two strongly supported major phylogenetic lineages. All buffalo isolates shared identical sequences, while the cattle isolates were assigned to genotypes TthIB and TthIIA/B. The divergence between the gGAPDH sequences from the TthI and TthII isolates was 7.5%, indicating that TthI trypanosomes belong to at least one new species separated from the trypanosomes nested within the TthII lineage. Cattle genotype TthIB was separated from the closest genotype, TthIA, of buffalo by 1.1% divergence. A divergence of 1.5% separated the TthIIA and TthIIB cattle genotypes. The reference isolate of *T. theileri* (Treu124) was assigned to TthIIB and largely diverged from trypanosomes from other hosts: by 7.8% for buffalo (genotype IA), 4.7% for fallow deer (genotype IIC), 3.0% for sitatunga and 5.0% for duiker (two isolates of blue duiker diverging by 3.3%). Analysis of gGAPDH sequences clearly separated all of the isolates within the subgenus *T.* (*Megatrypanum*) into host-specific clades, with at least one genotype associated with each host species and no genotype shared by different host species.

Phylogenies inferred using combined ssrRNA and gGAPDH gene sequences resulted in topologies very similar to those derived from independent datasets (data not shown) but showed higher support values for the clades corresponding to the subgenus *T.* (*Megatrypanum*) and the lineages TthI and TthII (Fig. 1B). Nevertheless, low intra-lineage bootstrap values indicated that this analysis still did not clearly resolve the relationships among closely related trypanosomes of the *T.* (*Megatrypanum*) subgenus from distinct hosts.

3.3. Phylogeography of trypanosomes from water buffalo and cattle living in sympatry and allopatry

The phylogeographical patterns of water buffalo and cattle isolates under conditions of host sympatry and allopatry were assessed by comparing isolates from four collection sites: Amazonia, Vale do Ribeira and the Pantanal, in Brazil, and two regions in Venezuela (Fig. 2). A total of 25 isolates from buffalo and 28 from cattle were compared using polymorphic sequences of ITS-rDNA, which we previously demonstrated are useful to resolve genotypes within *T.* (*Megatrypanum*) (Rodrigues et al., 2006). All buffalo isolates shared identical genotyping patterns based on

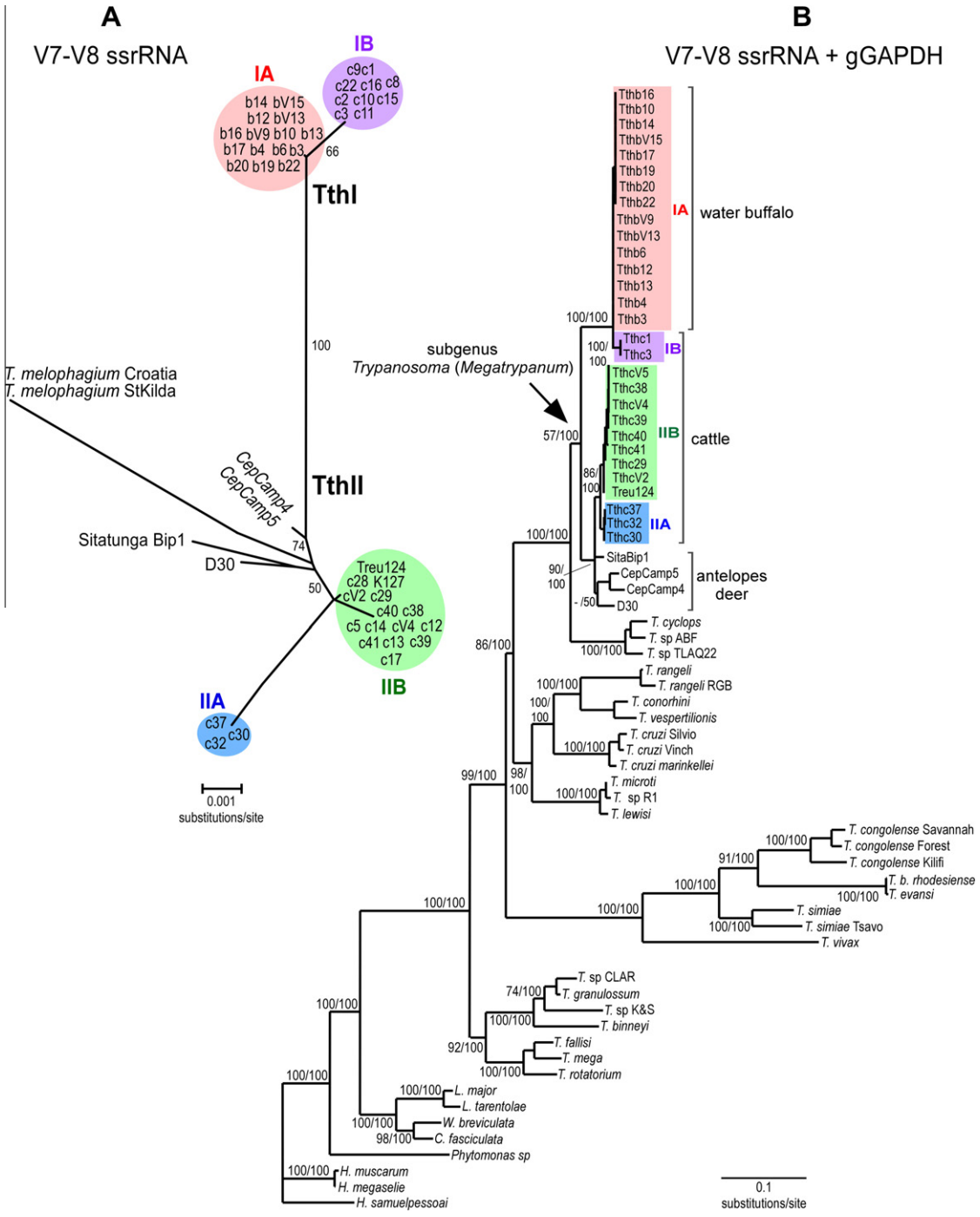

Fig. 1. Barcoding and phylogenetic positioning of trypanosomes from ruminants. (A) Dendrogram of barcoded V7-V8 ssrRNA sequences from trypanosomes of the subgenus *Trypanosoma* (*Megatrypanum*) from water buffalo (15 isolates), cattle (25), deer (01), duiker (02) and sitatunga (01) antelopes and *Trypanosoma melophagium* from sheep keds (02). Neighbour-joining analysis using the Kimura 2 parameter algorithm and nodal support estimated with 500 bootstrap replicates. (B) Phylogenetic tree based on concatenated V7–V8 ssrRNA and glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) sequences from 33 isolates representing all genotypes nested within *T.* (*Megatrypanum*) and 28 species representative of all other major clades of the genus *Trypanosoma*. Trypanosomatids of other genera were used as outgroups. Alignment of 1,960 characters was employed for maximum likelihood (ML) and Bayesian (B) inferences, and the numbers at nodes refer to ML/B support values derived from 500 replicates.

PCR–RFLP analysis of whole ITSrDNA (Table 1) and ITS1rDNA sequences. Cattle and buffalo isolates were always separated, with the smallest divergences observed between isolates of sympatric animals from the Vale do Ribeira (~11%, TthIB), whereas the largest divergences separated isolates from buffalo and cattle of TthIIA/B (~46%) from the Pantanal and Amazonia (Fig. 2A). Phylogeographical analysis using Cyt *b* sequences also supported the highly homogeneous genotype TthIA as being exclusive to water buffalo (Fig. 2B) and diverging ~6.0% from TthIB and ~15% from TthIIB.

Nuclear and mitochondrial genes have different inheritance modes and evolution rates, and comparison of these genes can reveal different evolutionary histories. However, comparison of phylogenetic analyses using nuclear and Cyt *b* sequences, which are the first from the mitochondrial genome to be determined for try-

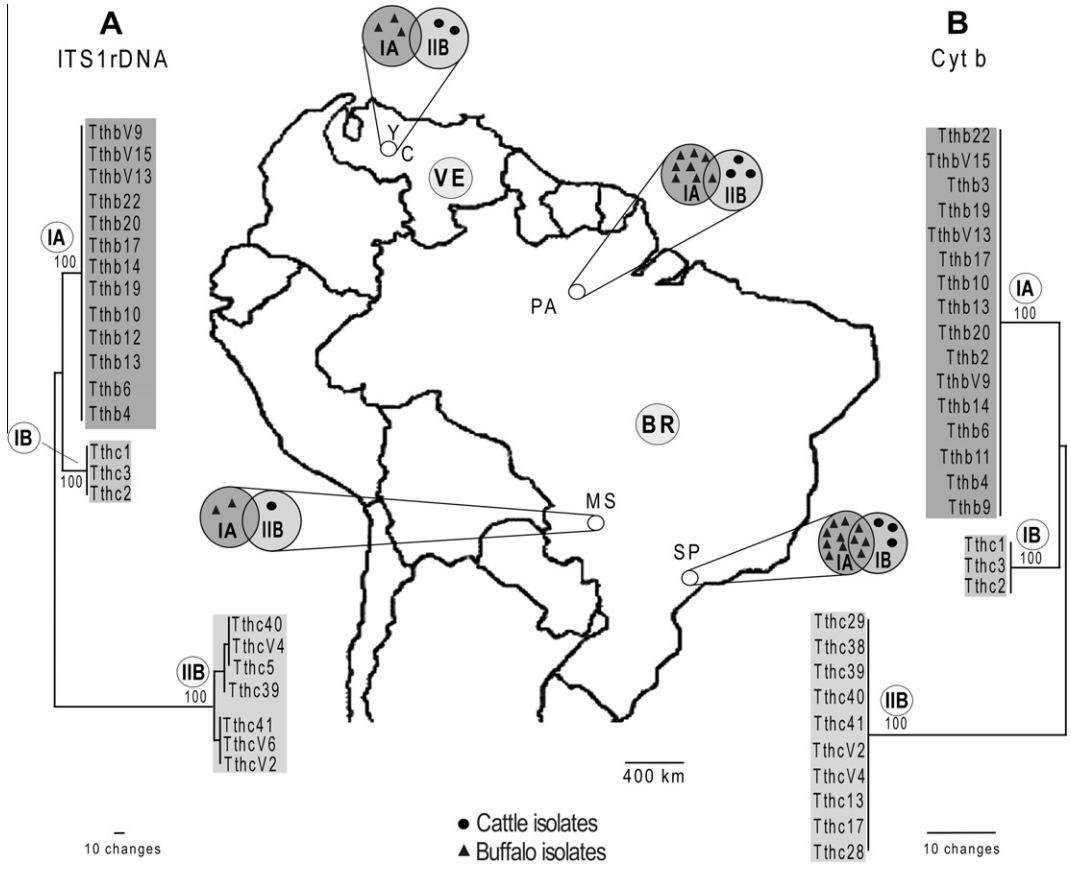

Fig. 2. Comparative phylogeographical analyses based on internal transcribed spacer 1 (ITS1) rDNA (A) and cytochrome *b* (Cyt *b*) (B) sequences of *Trypanosoma* (*Megatrypanum*) genotypes from cattle and water buffalo. Collection sites are shown on the map: BR, Brazil (states of SP, São Paulo; MS, Mato Grosso do Sul; PA, Pará) and VE, Venezuela (Y, Yaracuy; C, Cojedes). Symbols correspond to the genotypes and number of isolates from buffalo (TthIA genotype) and cattle (TthIB/IIB genotypes) included in the analysis. Maximum parsimony analyses of (A) ITS1rDNA sequences (356 characters, 179 parsimony informative) of 22 isolates and (B) Cyt *b* sequences (449 characters, 79 parsimony informative) of 29 isolates. Numbers at nodes indicate bootstrap values derived from 500 replicates.

panosomes of the *T.* (*Megatrypanum*) subgenus, showed congruent branching patterns. All buffalo isolates clustered tightly together, independent of their geographic origin and sympatric or allopatric conditions, whereas the cattle genotypes appeared to be geographically structured (Fig. 2).

3.4. Genotyping and phylogenetic relationships among isolates of *T.* (*Megatrypanum*)

Genotyping of buffalo and cattle isolates was also conducted using PCR–RFLP analysis of SL gene repeats. All of the isolates from buffalo examined in this and in previous studies exhibited the same restriction patterns, which were clearly distinguishable from the profiles generated for the isolates from cattle and deer (Rodrigues et al., 2006, 2010a) (Table 1).

To improve our understanding of the relationships and population structure within *T.* (*Megatrypanum*) by comparing isolates from all available host species, we analysed polymorphisms in the SL sequences of isolates from buffalo, cattle, antelopes, deer and sheep keds (Table 1). None of the SL sequences of isolates from distinct species shared significant similarities. The two isolates of *T. melophagium* from Scotland and Croatia were identical in their SL transcript sequences (Fig. 3A). Small divergences were found in the SL sequences among Venezuelan (~0.34%) and Brazilian (~0.76%) buffalo isolates. SL was the most polymorphic marker, exhibiting the largest divergences separating the host-associated genotypes and even isolates of the same genotypes. However, in agreement with all other molecular markers compared here, SL sequences were more homogeneous for buffalo (~1.4% divergence) than for cattle (~12%) isolates.

We previously showed that *T.* (*Megatrypanum*) from cattle and buffalo present a copy of the 5S rRNA gene inserted into the intergenic region of the SL repeats (Rodrigues et al., 2010a). Here, we demonstrated that all *T.* (*Megatrypanum*) spp. investigated share this peculiarity. The 5S rRNA that are highly conserved among trypanosomes in general displayed unexpected divergence, even between the closely related ThIA and ThIB genotypes (~1.5% divergence), with a high divergence (~14%) separating the TthI and TthII lineages.

The hypothesis of host-associated genotypes was also supported by analysis of genes encoding CATL enzymes from buffalo and cattle isolates. The results corroborated the high homogeneity (~0.5%) observed within the buffalo TthIA genotype, which was separated by ~1.8% and ~8.6% from the ThIB and TthIIB genotypes found in sympatric cattle, respectively. Distinct genotypes were confirmed for the isolates from deer, duikers and sitatunga (Fig. 3B). Sequences of CATL encoding genes, which have been demonstrated to be valuable for diagnosis and genotyping of *T.* (*Megatrypanum*), were determined from trypanosomes of primary haemocultures to assess whether they contained more than one genotype, as we previously demonstrated in blood samples and primary cultures from Brazilian and Thai cattle (Rodrigues et al., 2010a,b; Garcia et al., 2011). Results showed a single genotype infecting buffalo in Brazil and Venezuela.

Genealogies of the SL and CATL sequences inferred using one to three cloned sequences of each isolate showed polymorphisms

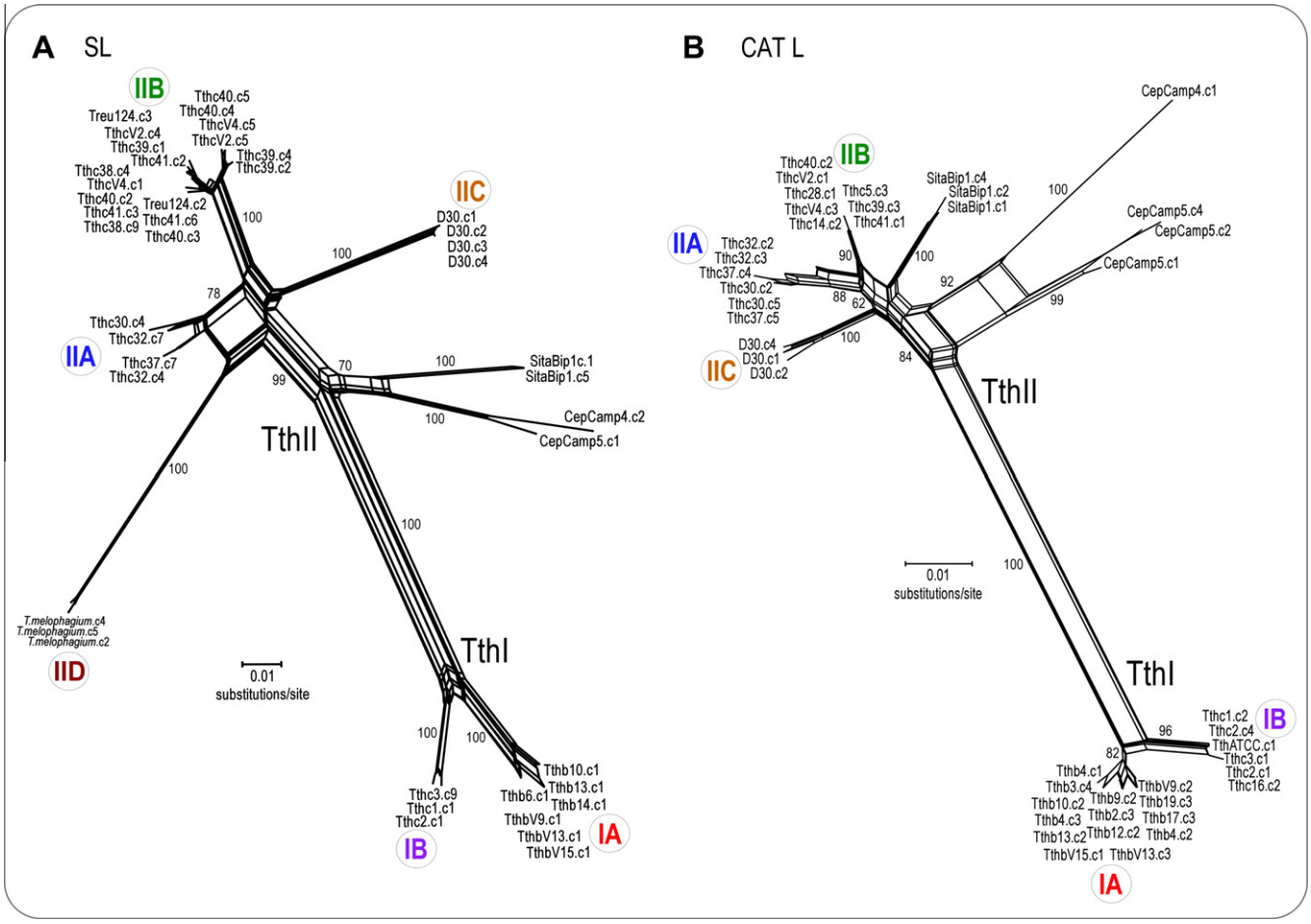

Fig. 3. Network genealogies of spliced leader (SL) and cathepsin L (CATL) gene sequences of *Trypanosoma* (*Megatrypanum*) genotypes found in cattle, water buffalo, deer, sitatunga and duiker antelopes and sheep inferred using the Neighbour-Net method with the K2P parameter. (A) Genealogy of 41 cloned SL sequences (intron and partial intergenic region – 555 characters) from 22 isolates. (B) Genealogy of 44 CATL cloned sequences (477 characters) from 32 isolates. The codes used for the sequences indicate the codes for the isolates, followed by the numbers of cloned sequences.

within isolates of the same genotypes and even among distinct copies of both SL and CATL gene repeats in the same isolates that were not revealed by analyses of other genes. Small polymorphisms were even detected for the highly homogeneous TthIA genotype. Reticulated network genealogies of SL and CATL sequences suggested that some recombination process had occurred, especially among TthII genotypes, which was also recently suggested for TthI isolates based on CATL analysis of field blood samples from Thai cattle (Garcia et al., 2011). Nevertheless, the divergences were smaller among sequences from one genotype than from distinct genotypes, and the networks confirmed the genotype arrangement demonstrated by other genes (Figs. 1–3).

3.5. Predominant clonal structure of genotypes and host-species associations of trypanosomes of the subgenus T. (Megatrypanum) supported by multilocus and concatenated gene analyses

Multilocus phylogenetic analyses presented highest bootstrap values supporting independent genotypes of *T.* (*Megatrypanum*). The parity of the genotypes defined by all seven loci investigated suggested a predominant clonal structure of the populations within this subgenus. High divergences indicated that these trypanosomes varied according to their host species, supporting the existence of other species besides *T. theileri* of cattle, represented here by cattle TthIIB isolates from Scotland, which diverged greatly from the trypanosomes of other ruminants and other cattle genotypes. Our findings supported an independent status for the trypanosome from water buffalo, genotype TthIA, which was separated from *T. theileri* by large divergences: 7.5% for gGAPDH, 15.3% for Cyt *b*, ~35% for SL, and ~39% for ITSrDNA. The cattle genotype TthIB was separated from its closest genotype, TthIA, by divergences of 1.1% for gGAPDH, 6.0% for Cyt *b*, ~9.2% for SL, and ~11% for ITS1rDNA.

In addition to analyses using independent sequences, different combinations of sequences were used with the aim of obtaining robustness regarding the relationships within *T.* (*Megatrypanum*). Combined analyses using ssrRNA and gGAPDH sequences resulted in phylogenetic trees showing branching patterns with low support values that were unable to clearly resolve these relationships (Fig. 1B). In contrast, analyses restricted to polymorphic ITS1rDNA and SL sequences (Fig. 4A) or including seven DNA sequences of 17 isolates from distinct host species (Fig. 4B) showed identical clustering patterns with strong support values supporting the host species-associated genotypes: water buffalo (genotype IA), cattle (IB, IIA, IIB), deer (IIC), sheep (IID), sitatunga (IIE) and blue duikers (IIF, IIG). More than one genotype was found in cattle and blue duikers corroborating high genotype repertoire in ruminants, even from the same species and geographic origin.

4. Discussion

This study was conducted to assess the genetic diversity, host association and population structure of *T. theileri* and related trypanosomes comprising the subgenus *T.* (*Megatrypanum*). All

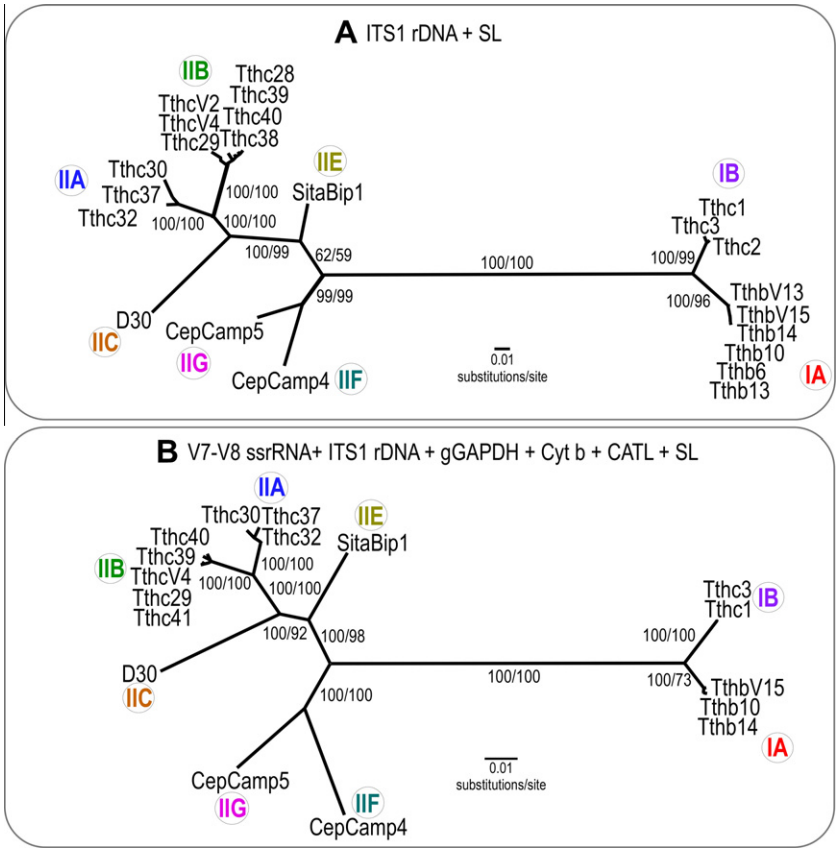

Fig. 4. Phylogenetic analyses of the subgenus *Trypanosoma* (*Megatrypanum*) using concatenated DNA sequences. (A) Internal transcribe spacer 1 (ITS1) rDNA and spliced leader (SL) sequences (872 characters) of 24 trypanosomes. (B) V7-V8 ssrRNA, ITS1rDNA, glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH), cytochrome *b* (Cyt *b*), cathepsin L (CATL) and spliced leader (SL) sequences (3,358 characters) from 17 trypanosomes representative of two lineages and all major genotypes (IA-B, IIA-C, IIE-G) found in domestic and wild ruminants. Both alignments were employed for maximum likelihood (ML) and Bayesian (B) inferences, and the numbers at nodes refer to ML/B support values derived from 500 replicates.

trypanosomes from ruminants that have thus far been included in phylogenetic trees have clustered with *T. theileri*, except the pathogenic species of African origin (Rodrigues et al., 2006). To our knowledge no systematic analysis had previously been performed to investigate the genetic diversity or quantify the degree of phylogenetic association among *T.* (*Megatrypanum*) spp. from distinct host species and geographic origins. Relationships among closely related trypanosomes in this subgenus based on high polymorphic sequences (ITSrDNA and SL) revealed four genotypes in cattle and only one in water buffalo (Rodrigues et al., 2006, 2010a,b). Here, we conducted a comprehensive sampling of isolates from buffalo and cattle living either in sympatry or allopatry and compared them with other trypanosomes from cattle, deer, antelopes and sheep ked, using a multilocus analysis based on independent and concatenated data, including genes with different evolutionary rates and, hence, reflecting variable timescales in the evolution of the trypanosomes.

Data from this study supported the existence of at least 10 phylogenetically defined genotypes within the subgenus *T.* (*Megatrypanum*): one genotype was associated with water buffalo, four with cattle, two with blue duikers, one with sitatunga, one with fallow-deer and one with sheep keds, whereas previous studies revealed six genotypes in this subgenus (Hamilton et al., 2009; Rodrigues et al., 2006, 2010a,b; Gibson et al., 2010; Garcia et al., 2011). All buffalo isolates were assigned to genotype TthIA, which differed from all genotypes found in sympatric and allopatric cattle and in other ruminant species. *Trypanosoma melophagium* isolates from sheep keds from Scotland and Croatia belong to the same genotype that differs from both genotypes that infect cattle in these two countries (Gibson et al., 2010; Martinkovic et al., in press). Trypanosomes from German deer and cattle were found to be different, while isolates from deer in Croatia and Germany share high similarity. Our findings agreed with previous studies of *T.* (*Megatrypanum*) isolates. Based on the comparison of zymodemes of bovid and cervid trypanosomes, a new species, *T. cervi*, was proposed for a North American deer trypanosome (Matthews et al., 1977; Böse et al., 1993). Zymodeme analyses have also distinguished trypanosomes of African buffalo (*Sincerus caffer*), bison, moose and deer, while moose isolates from Europe and North America share similar patterns (Dirie et al., 1990b; Böse et al., 1993).

All of the previous and new data indicate the existence of different *T.* (*Megatrypanum*) spp. and provide evidence of host–parasite associations. All of the genotypes observed within this subgenus appear to be host-specific. Although it is likely that some genotypes would be originated through host switching events, they are unlikely to be able to infect all ruminants to which they are exposed because even animals living in sympatry do not share the same parasite genotype. These results suggest the existence of high host specificity and that the evolution of these ruminant parasites appears to be constrained by their hosts. However, the host range of any parasite might not solely be a consequence of vertebrate host associations but can also be determined, at least in part, by vector-host interactions (Brooks and McLennan, 2002; Hellgren et al., 2008). *Trypanosoma theileri* is spread among bovids by tabanids, which are flies that, despite exhibiting preferences, feed on blood from a range of species, facilitating host switching of parasites across host species. We previously demonstrated, by PCR,

the presence of trypanosome genotypes associated with buffalo and cattle in the same tabanid species, suggesting that they can be transmitted by the same vectors (Rodrigues et al., 2010b). However, several species of tabanids can be found in all farms investigated, and the detection of mixed infections does not warrant that different genotypes infect and are cyclically transmitted by the same tabanid species. The parasite population transmitted by vectors is expected to reflect pressures from both host and vector defenses. The understanding of the relationships between tabanid species and *T.* (*Megatrypanum*) spp. and ruminant species require investigation.

Similarity among parasites generally decreases with increasing geographical distances between host populations (Poulin, 2010). However, in the present study, we showed that host species, but not geographical distance, appears to be a determinant of most *T. theileri* genotypes. All data associated the TthIA genotype with water buffalo, independent of the presence on the same farm of cattle infected with other genotypes sharing the same pastures and regardless of geographical distances. All of our findings support TthIA as a highly homogeneous genotype that is presently restricted to water buffalo and is widely dispersed in Brazil and Venezuela. The genotype TthIA is closest to cattle TthIB genotype, which have been reported from Brazil, Venezuela, the USA, Japan, Korea, Thailand and Croatia, while TthII isolates have been found in Brazil, Venezuela, Germany and Scotland (Rodrigues et al., 2006, 2010a,b; Garcia et al., 2011).

Domestic bovids are transported across large distances, favouring dispersion and homogenisation of their associated parasites. The history of the Brazilian cattle began with the Portuguese colonisation in Brazil. The first cattle herds were introduced from Portugal to the Southeastern region in 1534, followed by introduction in the Northeast of animals from Portugal and North Africa. Animals from these two regions developed as native breeds until the importation of zebu cattle from India in 1868. The domestic Asian water buffalo (*B. bubalis*) is widespread throughout Asia, South America, southern Europe, North Africa and Australia. This species was imported from India to Brazilian Amazonia in 1895 and today large herds, most of Murrah breed, live in the lower floodplains of the Amazonian region (Mariante and Cavalcante, 2000; Sheikh et al., 2006).

In general, sympatric and phylogenetically related hosts sharing ecological niches exhibit similar parasite communities determined by similar resources, host physiology and phylogeny. However, host switches may weaken these evolutionary links, and some species can infect host species across small or large phylogenetic distances, especially when vectors are involved in parasite transmission (Brooks and McLennan, 2002; Dick and Patterson, 2007; Bordes et al., 2009). This appears to be the case for *T.* (*Megatrypanum*) spp., which are apparently only infective for ruminants. However, although the lack of cross-infectivity of these trypanosomes among sympatrically related hosts provides strong evidence of host restriction, incongruence between the phylogenies of ruminants and trypanosomes of *T.* (*Megatrypanum*) suggest host switches and do not support host–parasite co-evolution. Host fitting, a process by which parasites should be able to colonise and adapt to new host species as a result of traits they already possess, may have played an important role in the evolution of *T.* (*Megatrypanum*) spp., as has also been indicated for several parasites and other trypanosomes (Brooks and McLennan, 2002; Hamilton et al., 2007).

We found no direct associations between the phylogenetic distances among parasites and their hosts; some isolates from cattle (TthI) are closest to isolates from buffalo, while other cattle isolates (TthII) are closest to isolates from sheep and deer. The close relationships between cattle and buffalo trypanosomes within the TthI lineage suggested that a recent host switch had occurred (available data do not allow us to hypothesise whether TthIB diverged from ThIA or vice versa), followed by divergence giving rise to a new genotype adapted exclusively to one host species. A major driver for parasite selection, and hence an important determinant of host restriction, is the specificity of immune response, which is dependent on the host MHC phenotype. Compatibility of the parasites and immune-signalling molecules of their hosts, avoiding an immunological response, plays an important role in the parasite selection and evolution (Dick and Patterson, 2007). Our findings highlight the need for studies of the interactions between these trypanosomes and the innate immune defences of ruminant hosts.

The results from the multilocus and phylogeographical analyses performed in this study support the hypothesis of a separate genotype of *T.* (*Megatrypanum*) for each ruminant species. Additional isolates from different host species need to be characterised to improve our knowledge of the genotype repertoire infecting ruminants globally. At present, the number of isolates from hosts other than cattle and water buffalo is too small to warrant reliable host associations. Confirmation of restriction of the TthIA genotype to water buffalo and its existence outside of South America also require further study, especially of trypanosomes infecting water buffalo in Asia, from where they were introduced to Brazil (Sheikh et al., 2006). Overall, the results from this study support the conclusion that the evolution of trypanosomes of the *T.* (*Megatrypanum*) subgenus may be strongly constrained by their hosts.

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References

Bordes, F., Morand, S., Kelt, D.A., Van Vuren, D.H., 2009. Home range and parasite diversity in mammals. Am. Nat. 173, 467–474.

Böse, R., Heister, N.C., 1993. Development of *Trypanosoma* (*Megatrypanum*) *theileri* in tabanids. J. Eukaryot. Microbiol. 40, 788–792.

Böse, R., Petersen, K., Pospichal, H., Buchanan, N., Tait, A., 1993. Characterization of *Megatrypanum* trypanosomes from European Cervidae. Parasitology 107, 55–61.

Braun, U., Rogg, E., Walser, M., Nehrbass, D., Guscetti, F., Mathis, A., Deplazes, P., 2002. *Trypanosoma theileri* in the cerebrospinal fluid and brain of heifer with suppurative meningoencephalitis. Vet. Rec. 150, 18–19.

Brooks, D.R., McLennan, D.A., 2002. The Nature of Diversity: An Evolutionary Voyage of Discovery. University of Chicago Press, Chicago, Illinois.

Cavazzana Jr., M., Marcili, A., Lima, L., Maia da Silva, F., Junqueira, A.C.V., Veludo, H.H., Viola, L.B., Campaner, M., Nunes, V.L., Paiva, F., Coura, J.R., Camargo, E.P., Teixeira, M.M.G., 2010. Phylogeographical, ecological and biological patterns shown by nuclear (ssrRNA and gGAPDH) and mitochondrial (Cyt *b*) genes of trypanosomes of the subgenus *Schizotrypanum* parasitic in Brazilian bats. Int. J. Parasitol. 40, 345–355.

D'Alessandro, A., Wells, E.A., 1971. Trypanosome infections in the family Cervidae. Trans. R. Soc. Trop. Med. Hyg. 65, 845–846.

Dick, C.W., Patterson, B.D., 2007. Against all odds: explaining high host specificity in dispersal-prone parasites. Int. J. Parasitol. 37, 871–876.

Dirie, M.F., Bornstein, S., Wallbanks, K.R., Molyneux, D.H., Steen, M., 1990a. Comparative studies on *Megatrypanum* trypanosomes from cervids. Trop. Med. Parasitol. 41, 198–202.

Dirie, M.F., Bornstein, S., Wallbanks, K.R., Stiles, J.K., Molyneux, D.H., 1990b. Zymogram and life-history studies on trypanosomes of the subgenus *Megatrypanum*. Parasitol. Res. 76, 669–674.

Garcia, H.A., Kamyingkird, K., Rodrigues, A.C., Jittapalapong, S., Teixeira, M.M.G., Desquesnes, M., 2011. High genetic diversity in field isolates of *Trypanosoma theileri* assessed by analysis of cathepsin L-like sequences disclosed multiple and new genotypes infecting cattle in Thailand. Vet. Parasitol. 180, 363–367.

Gibson, W.C., Pilkington, J.G., Pemberton, J.M., 2010. *Trypanosoma melophagium* from the sheep ked *Melophagus ovinus* on the island of St. Kilda. Parasitology 137, 1799–1804.

Hamilton, P.B., Gibson, W.C., Stevens, J.R., 2007. Patterns of co-evolution between trypanosomes and their hosts deduced from ribosomal RNA and protein-coding gene phylogenies. Mol. Phylogenet. Evol. 44, 15–25.

Hamilton, P.B., Adams, E.R., Njiokou, F., Gibson, W.C., Cuny, G., Herder, S., 2009. Phylogenetic analysis reveals the presence of *Trypanosoma cruzi* clade in African terrestrial mammals. Infect. Genet. Evol. 9, 81–86.

Hatama, S., Shibahara, T., Suzuki, M., Kadota, K., Uchida, I., Kanno, T., 2007. Isolation of a *Megatrypanum* trypanosome from sika deer (*Cervus nippon yesoensis*) in Japan. Vet. Parasitol. 149, 56–64.

Hellgren, O., Bensch, S., Malmqvist, B., 2008. Bird hosts, blood parasites and their vectors-associations uncovered by molecular analyses of blackfly blood meals. Mol. Ecol. 17, 1605–1613.

Hoare, C.A., 1972. The Trypanosomes of Mammals: A Zoological Monograph. Blackwell Scientific Publications, Oxford, pp. 123–141.

Hoffmann, M., Büscher, G., Friedhoff, K.T., 1984. Stercorarian trypanosomes from deer (Cervidae) in Germany. J. Protozool. 31, 581–584.

Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23, 254–267.

Johnson, D., Harms, N.J., Larter, N.C., Elkin, B.T., Tabel, H., Wei, G., 2010. Serum biochemistry, serology, and parasitology of boreal caribou (*Rangifer tarandus caribou*) in the Northwest Territories, Canada. J. Wildl. Dis. 46, 1096–1107.

Kingston, N., Morton, J.K., 1975. *Trypanosoma cervi* sp. n. from elk (*Cervus canadensis*) in Wyoming. J. Parasitol. 61, 17–23.

Latif, A.A., Bakheit, M.A., Mohamed, A.E., Zweygarth, E., 2004. High infection rates of the tick *Hyalomma anatolicum anatolicum* with *Trypanosoma theileri*. Onderstepoort J. Vet. Res. 71, 251–256.

Lefebvre, M.F., Semalulu, S.S., Oatway, A.E., Nolan, J.W., 1997. Trypanosomiasis in woodland caribou of northern Alberta. J. Wildl. Dis. 33, 271–277.

Maia da Silva, F., Junqueira, A.C., Campaner, M., Rodrigues, A.C., Crisante, G., Ramirez, L.E., Caballero, Z.C., Monteiro, F.A., Coura, J.R., Añez, N., Teixeira, M.M.G., 2007. Comparative phylogeography of *Trypanosoma rangeli* and *Rhodnius* (Hemiptera: Reduviidae) supports a long coexistence of parasite lineages and their sympatric vectors. Mol. Ecol. 16, 3361–3373.

Maia da Silva, F., Marcili, A., Ortiz, P.A., Epiphanio, S., Campaner, M., Catão-Dias, J.L., Shaw, J.J., Camargo, E.P., Teixeira, M.M.G., 2010. Phylogenetic, morphological and behavioural analyses support host switching of *Trypanosoma* (*Herpetosoma*) *lewisi* from domestic rats to primates. Infect. Genet. Evol. 10, 522–529.

Mariante, A.S., Cavalcante, N., 2000. Animais do descobrimento, raças domésticas da história do Brasil. Embrapa-Cenargen, Brasília, pp. 14–90.

Martinkovic, F., Matanović, K., Rodrigues, A.C., Garcia, H.A., Teixeira, M.M.G, in press. *Trypanosoma* (*Megatrypanum*) *melophagium* in the sheep ked *Melophagus ovinus* from organic farms in Croatia: phylogenetic inferences support restriction to sheep and sheep keds and close relationship with trypanosomes from other ruminant species. J. Eukaryot. Microbiol.

Matthews, M.J., Kingston, N., Morton, J.K., 1977. *Trypanosoma cervi* Kingston and Morton, 1975 from mule deer, *Odocoileus hemionus*, in Wyoming. J. Wildl. Dis. 13, 33–39.

Neumüller, M., Nilsson, K., Påhlson, C., in press. *Trypanosoma* spp. in Swedish game animals. Parasitol. Res. doi:10.1007/s00436-011-2462-9.

Nieberding, C.M., Durette-Desset, M.C., Vanderpoorten, A., Casanova, J.C., Ribas, A., Deffontaine, V., Feliu, C., Morand, S., Libois, R., Michaux, J.R., 2008. Geography and host biogeography matter for understanding the phylogeography of a parasite. Mol. Phylogenet. Evol. 47, 538–554.

Poulin, R., 2010. Decay of similarity with host phylogenetic distance in parasite faunas. Parasitology 137, 733–741.

Rodrigues, A.C., Campaner, M., Takata, C.S., Dell' Porto, A., Milder, R.V., Takeda, G.F., Teixeira, M.M.G., 2003. Brazilian isolates of *Trypanosoma* (*Megatrypanum*) *theileri*: diagnosis and differentiation of isolates from cattle and water buffalo based on biological characteristics and randomly amplified DNA sequences. Vet. Parasitol. 116, 185–207.

Rodrigues, A.C., Garcia, H.A., Batista, J.S., Minervino, A.H., Góes-Cavalcante, G., Maia da Silva, F., Ferreira, R.C., Campaner, M., Paiva, F., Teixeira, M.M.G., 2010a. Characterization of spliced leader genes of *Trypanosoma* (*Megatrypanum*) *theileri*: phylogeographical analysis of Brazilian isolates from cattle supports spatial clustering of genotypes and parity with ribosomal markers. Parasitology 137, 111–122.

Rodrigues, A.C., Garcia, H.A., Ortiz, P.A., Cortez, A.P., Martinkovic, F., Paiva, F., Batista, J.S., Minervino, A.H., Campaner, M., Pral, E.M., Alfieri, S.C., Teixeira, M.M.G., 2010b. Cysteine proteases of *Trypanosoma* (*Megatrypanum*) *theileri*: cathepsin L-like gene sequences as targets for phylogenetic analysis, genotyping diagnosis. Parasitol. Int. 59, 318–325.

Rodrigues, A.C., Paiva, F., Campaner, M., Stevens, J.R., Noyes, H.A., Teixeira, M.M.G., 2006. Phylogeny of *Trypanosoma* (*Megatrypanum*) *theileri* and related trypanosomes reveals lineages of isolates associated with artiodactyl hosts diverging on SSU and ITS ribosomal sequences. Parasitology 132, 215–224.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Seifi, H.A., 1995. Clinical trypanosomosis due to *Trypanosoma theileri* in a cow in Iran. Trop. Anim. Health Prod. 27, 93–94.

Shastri, U.V., Deshpande, P.D., 1981. Hyalomma anatolicum anatolicum (Koch, 1844) as a possible vector for transmission of *Trypanosoma theileri*, Laveran, 1902 in cattle. Vet. Parasitol. 9, 151–155.

Sheikh, P.A., Merry, F.D., McGrath, D.G., 2006. Water buffalo and cattle ranching in the Lower Amazon Basin: comparisons and conflicts. Agric. Syst. 87, 313–330.

Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 11, 4673–4680.

Villa, A., Gutierrez, C., Garcia, E., Moreno, B., Chacón, G., Sanz, P.V., Büscher, P., Touratier, L., 2008. Presence of *Trypanosoma theileri* in Spanish cattle. Ann. NY Acad. Sci. 1149, 352–354.

Viola, L.B., Almeida, R.S., Ferreira, R.C., Campaner, M., Takata, C.S., Rodrigues, A.C., Paiva, F., Camargo, E.P., Teixeira, M.M.G., 2009. Evolutionary history of trypanosomes from South American caiman (*Caiman yacare*) and African crocodiles inferred by phylogenetic analyses using SSU rDNA and gGAPDH genes. Parasitology 136, 55–65.

Ward, W.H., Hill, M.W., Mazlin, I.D., Foster, C.K., 1984. Anaemia associated with a high parasitaemia of *Trypanosoma theileri* in a dairy cow. Vet. J. 61, 324.

Wells, E.A., 1976. Subgenus *Megatrypanum*. In: Lumsden, W.H.R., Evans, D.A. (Eds.), Biology of the Kinetoplastida. Academic Press, London, pp. 257–275.