Molecular Ecology (2007) **16**, 3361–3373 doi: 10.1111/j.1365-294X.2007.03371.x

Comparative phylogeography of *Trypanosoma rangeli* and *Rhodnius* (Hemiptera: Reduviidae) supports a long coexistence of parasite lineages and their sympatric vectors

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Abstract

To make reliable interpretations about evolutionary relationships between *Trypanosoma rangeli* lineages and their insect vectors (triatomine bugs of the genus *Rhodnius*) and, thus, about the determinant factors of lineage segregation within *T. rangeli*, we compared phylogenies of parasite isolates and vector species. Sixty-one *T. rangeli* isolates from invertebrate and vertebrate hosts were initially evaluated in terms of polymorphism of the spliced-leader gene (SL). Further analysis based on SL and SSUrRNA sequences from 33 selected isolates, representative of the overall phylogenetic diversity and geographical range of *T. rangeli*, supported four phylogenetic lineages within this species. By comparing the phylogeny of *Rhodnius* species with that inferred for *T. rangeli* isolates and through analysis of the geographical range of the isolates, we showed that there is a very significant overlap in the distribution of *Rhodnius* species and *T. rangeli* lineages. Congruence between phylogeographical analysis of both *T. rangeli* lineages and complexes of *Rhodnius* species are consistent with the hypothesis of a long coexistence of parasites and their vectors, with lineage divergence associated with sympatric species of *Rhodnius* apparently without association with particular vertebrate hosts. Separation of *T. rangeli* isolates from vectors of distinct complexes living in sympatry favours the absence of gene flow between the lineages and suggests evolution of *T. rangeli* lineages in independent transmission cycles, probably associated to specific *Rhodnius* spp. ecotopes. A polymerase chain reaction assay based on SL intergenic sequences was developed for simultaneous identification and lineage genotyping of *T. rangeli* in epidemiological surveys.

Keywords: evolution, phylogeography, *Rhodnius*, ribosomal gene, spliced-leader gene, *Trypanosoma rangeli*

Received 4 March 2007; revision received 4 March 2007; accepted 12 April 2007

Introduction

The family Trypanosomatidae (Euglenozoa: Kinetoplastida) comprises eight genera of protozoan parasites of vertebrates, invertebrates and plants. The genus *Trypanosoma* harbours parasites of all vertebrate classes and the life cycle of these parasites involves alternation between two hosts: vertebrates and diverse haematophagous invertebrates (vectors). This genus includes only three *Trypanosoma* species that infect man: *T. cruzi* and *T. rangeli* in Latin America and *T. brucei* in Africa (Stevens *et al*. 2001).

Trypanosoma rangeli infects humans, and domestic, and sylvatic mammals from Central America to southern South America sharing with *T. cruzi* the same mammalian hosts and triatomine vectors in overlapping areas. A high

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prevalence of *T. rangeli* human infections has been reported in Central America and northwest South America (D'Alessandro & Saravia 1999; Guhl & Vallejo 2003). In Brazil, only three human cases have been reported in Amazonia (Coura *et al*. 1996), whereas infection of sylvatic mammals and triatomines is very common in this region (Miles *et al*. 1983; Maia da Silva *et al*. 2004a, b) and has also been reported in southern, southeastern and central regions (Steindel *et al*. 1991; Ramirez *et al*. 2002; Gurgel-Goncalves *et al*. 2004).

In contrast to *T. cruzi* and *T. brucei*, *T. rangeli* is harmless to mammalian hosts but may cause pathogenicity to its insect vectors in which it can induce difficulties or even lethal effect in moulting and feeding. Unlike *T. cruzi* whose development in triatomines is entirely restricted to the gut, *T. rangeli* multiplies in the gut but completes its development in the insect salivary gland, where metacyclogenesis takes place as *T. brucei* in tsetse (Añez 1984; Guhl & Vallejo 2003). Genetic distances concur with life-cycle differences between *T. cruzi* and *T. rangeli* (Stevens *et al*. 1999; Maia da Silva *et al*. 2004b). Natural *T. rangeli* infection appears to be restricted to the gut in all other triatomine genera except *Rhodnius*, and its transmission through saliva inoculation during probing and feeding has been proved only for species of this genus. Isolates of *T. rangeli* of distinct geographical origin show variable behaviour in different *Rhodnius* spp., and transmission by bite is mostly restricted to local vector species, suggesting a tight evolutionary relationship between *T. rangeli* isolates and their sympatric vectors (D'Alessandro & Saravia 1999; Guhl & Vallejo 2003; Vallejo *et al*. 2003; De Stefani Marquez *et al*. 2006).

Several host–parasite associations have been investigated by examining the degree of congruence between host and parasite phylogenies. However, much less information is available regarding the co-evolutionary process in protozoan parasites than in ectoparasites and parasitic worms (Page 1991; Paterson & Banks 2001; Banks & Paterson 2005; Brooks & Ferrao 2005; Huyse *et al*. 2005). Phylogenetic analyses used to address evolutionary processes within *Trypanosoma* indicate host–parasite associations between the following trypanosomes and their insect vectors: (i) *T. brucei* and related species and the tsetse fly; (ii) *T. theileri* of artiodactyls and tabanids; (iii) *T. lewisi* and allied species of rodents and fleas; and (iv) *T. cruzi* and *T. rangeli* and triatomine bugs (Stevens *et al*. 2001; Maia da Silva *et al*. 2004b; Rodrigues *et al*. 2006).

The triatomine vectors of *T. rangeli* belong to the tribe Rhodniini (Reduvidae: Triatominae), which comprises two genera of blood-sucking bugs: *Rhodnius* and *Psammolestes*. Triatomines of the genus *Rhodnius* have a complex biogeographical history and a long history of co-evolution with palm trees in Latin America. Species of this genus are primarily associated with palm tree crowns; the distribution of the sylvatic species coincides with the distribution of palms and may be associated with particular niches in distinct palms. The genus *Psammolestes* comprises species that live in nests of birds. In contrast, triatomines of the genera *Triatoma* and *Panstrongylus* have evolved predominantly associated with terrestrial habitats such as rocks, animal burrows and hollow trees (Gaunt & Miles 2000).

The species of the genus *Rhodnius* are distributed into three complexes according to phylogenies based on zymodemes (Dujardin *et al*. 1999) and sequences of 16S mitochondrial rDNA, cytochrome *b* (cyt *b*) and 28S nuclear rRNA (Lyman *et al*. 1999; Hypsa *et al*. 2002; Monteiro *et al*. 2000, 2003; Paula *et al*. 2006). The *Rhodnius* complexes are associated with sympatric palms and have different phylogeographical structures as follows: (i) *R. pallescens* complex formed by *R. pallescens*, *R. colombiensis* and *R. ecuadoriensis*, distributed from the west of the Andes Mountains to Central America, northern and northwest South America; (ii) *R. brethesi* complex, constituted by *R. brethesi* and *R. pictipes*, *R. amazonicus* and *R. stali*, which are restricted to the Amazon region east of the Andes; and (iii) *R. prolixus* complex, formed by *R. prolixus*, *R. robustus*, *R. neglectus*, *R. nasutus*, *R. domesticus* and *R. neivai*. *R. prolixus* is the most widespread species with high prevalence in Central and northwest South America, west of the Andes. *R. pictipes* and *R. robustus* are restricted to north and northwest South America. *R. neglectus*, *R. nasutsus* and *R. domesticus* occur in the northeast and southeast of Brazil (Galvão *et al*. 2003). The genus *Rhodnius* includes three domiciled species, *R. prolixus*, *R. pallescens* and *R. ecuadoriensis*. *R. robustus* in Venezuela can enter dwellings and the peridomicile without becoming domestic, whereas in Brazil, *R. robustus*, *R. pictipes* and *R. brethesi* are considered entirely sylvatic, although there have been reports of these species inside houses and/or attacking humans (Dujardin *et al*. 1991; Coura *et al*. 1996).

A peculiar organization of the spliced-leader (SL) RNA repeats displaying the highly conserved 5S rRNA gene inserted into the SL intergenic region was observed in *T. rangeli* and few other trypanosomatids (Aksoy *et al*. 1992; Stevens *et al*. 1999; Gibson *et al*. 2000). SL sequences are valuable tools for assessing polymorphism and genetic relatedness among closely related species and are useful as taxonomic and diagnostic tools for genera, species and lineages of trypanosomatids (Souto *et al*. 1996; Teixeira *et al*. 1996; Zingales *et al*. 1998; Serrano *et al*. 1999; Ventura *et al*. 2001). Intra-specific polymorphisms of the SL gene revealed different groups of *T. rangeli* isolates (Maia da Silva *et al*. 1999; Grisard *et al*. 1999; Urrea *et al*. 2005). The existence of two groups within *T. rangeli* was also indicated by other molecular markers in addition to SL: random amplified polymorphic DNA (RAPD) patterns (Steindel *et al*. 1994), kDNA (Vallejo *et al*. 2002, 2003; Urrea *et al*. 2005) and histone H2A gene (Cuervo *et al*. 2006). In our previous studies (Maia da Silva *et al*. 2004a, b) besides confirming the existence of these two lineages, we demonstrated using RAPD

analysis and phylogeny based on small subunit (SSU) and ITSrDNA (Maia da Silva *et al*. 2004a, b) that *T. rangeli* populations are much more complex than previously supposed and are segregated into at least four phylogenetic lineages.

Analysis of a larger number of isolates from different hosts and geographical regions, utilization of additional molecular markers and association of molecular data with ecogeographical and biological traits is required to make reliable interpretations about lineage segregation within *T. rangeli* and evolutionary relationships between parasite lineages and different species of *Rhodnius*. In this study, we analysed polymorphism of the SL and ribosomal genes of *T. rangeli* by genotyping 61 isolates, and in addition, we inferred phylogenetic relationships among 26 isolates with the following aims: (i) to assess the consistency of *T. rangeli* lineages through analysis of a large collection of isolates; (ii) to confirm the concordance between lineage genotyping and phylogeny using both spliced-leader and ribosomal markers; (iii) to evaluate evolutionary aspects of *T. rangeli* by phylogeographical analysis and by comparing vector and parasite phylogenies; and (iv) to define SL sequences suitable for simultaneous diagnosis and genotyping of *T. rangeli*.

Materials and methods

Isolation of Trypanosma rangeli *from triatomines and identification and phylogeny of* Rhodnius *spp.*

Triatomines were collected from palm trees in peridomestic and sylvatic environments in Brazil, Venezuela, and Panama. Triatomines were initially identified morphologically according to Lent & Wygodzinsky (1979) (Table 1). Field-collected triatomines were dissected and their gut and salivary glands removed, gently squeezed onto glass slides containing phosphate-buffered saline, examined under a phase microscope, and samples positive for *Trypanosoma rangeli* were inoculated separately in biphasic medium for parasite isolation. To obtain pure cultures of *T. rangeli*, specimens of *Rhodnius robustus* from a laboratory colony were infected through intracoelomic inoculation of mixed cultures of *T. rangeli* and *Trypanosoma cruzi*. After ~30 days, the haemolymph and salivary glands were examined for the presence of trypanosomes, and the positive samples were inoculated in biphasic medium (Maia da Silva *et al*. 2004a).

DNA from the same insect specimens from which *T. rangeli* had been isolated was purified according to Aljanabi & Martinez (1997), and a 682-bp fragment of the mitochondrial cyt *b* gene was amplified, cloned and sequenced as before (Monteiro *et al*. 2003). Two cyt *b* sequences showing small polymorphisms compared to previously reported sequences of *Rhodnius* spp. were deposited in GenBank (Accession numbers in parentheses): *R. robustus* II, 365 from Rondonia, Brazil (EF071583); and *R. pallescens*, 366 from Panamá (EF071584). Sequences obtained from *R. prolixus* and *R. robustus* I from Venezuela were almost identical to those previously described in Monteiro *et al*. (2003). These sequences were aligned with those from reference species of *Rhodnius*: *R. brethesi* (AF045714); *R. pictipes* (AF045713); *R. pallescens* (AF045720); *R. ecuadoriensis* (AF045715); *R. robustus* I (AF421340); *R. robustus* II (AF421341); *R. robustus* III (AF421342); *R. robustus* IV (AF421343); *R. prolixus*

Table 1 *Trypanosoma rangeli* isolates, geographical and host species of origin, genotyping using SL, SL and SSUrDNA sequences employed in the phylogenetic analysis

TryCC†	Isolate	Host species		Geographical origin‡	TraSL PCR§ (bp)	GenBank Accession no.	
						SL	SSUrDNA
Lineage A							
031	S. Augustin (SA)	Human	*Homo sapiens*	Colombia	417	M62864	AJ012417
020	Macias	Human	*Homo sapiens*	Colombia	417	—	AJ012415
024	H8GS	Human	*Homo sapiens*	Honduras	417	AF083351	AY491744
591	SMH-03	Human	*Homo sapiens*	Guatemala	417	—	AY491739
594	SMH-79	Human	*Homo sapiens*	Guatemala	417	—	AY491740
529	MHOM/VE/99/CH-99	Human	*Homo sapiens*	Venezuela (Barinas)	417	—	AY491742
▲ 530	MHOM/VE/99/D-99	Human	*Homo sapiens*	Venezuela (Trujillo)	417	• EF071551	—
533	MAN/VE/00/LOBITA	Dog	*Canis familiaris*	Venezuela (Barinas)	417	• EF071552	• EF071572
220	AT-AEI	Monkey	*Saimiri sciureus*	Brazil (PA) Marajó@	417	• EF071553	AY491747
202	AT-ADS	Monkey	*Saimiri sciureus*	Brazil (PA) Marajó	417	—	AY491746
353	Maloch-05	Monkey	*Callicebus m. cupreus*	Brazil (AC) P. de Castro	417	—	AY491750
369	ROma 01	Opossum	*Didelphis marsupialis*	Brazil (RO) Monte Negro	417	• EF071554	AY491748
382	ROma 06	Opossum	*Didelphis marsupialis*	Brazil (RO) Monte Negro	417	—	AY491749
▲*	P02	Opossum	*Didelphis marsupialis*	Brazil (MG) Uberaba	417	—	—
▲*	P07	Opossum	*Didelphis marsupialis*	Brazil (MG) Uberaba	417	—	—
▲*	P18	Opossum	*Didelphis marsupialis*	Brazil (MG) Uberaba	417	—	—
▲*	P19	Opossum	*Didelphis marsupialis*	Brazil (MG) Uberaba	417	• EF071555	• EF071573

Table 1 *Continued*

TryCC†	Isolate		Host species	Geographical origin‡	TraSL PCR§ (bp)	GenBank Accession no. SL	SSUrDNA
▲*	P21	Opossum	*Didelphis marsupialis*	Brazil (MG) Uberaba	417	• EF071556	• EF071574
021	Choachi	Triatomine	*Rhodnius prolixus*	Venezuela	417	• EF071557	AJ012414
022	Palma-2	Triatomine	*Rhodnius prolixus*	Venezuela	417	• EF071558	AY491741
▲775	VE/9	Triatomine	*Rhodnius prolixus*	Venezuela (Barinas)	417	• EF071560	• EF071575
▲ 795	VE/3	Triatomine	*Rhodnius robustus* I	Venezuela (Trujillo)	417	• EF071559	• EF071576
▲ 1013	IRHO/VE/04/F45-04	Triatomine	*Rhodnius prolixus*	Venezuela (Barinas)	417	—	—
▲ 1024	IRHO/VE/05/Apure 1	Triatomine	*Rhodnius prolixus*	Venezuela (Apure)	417	—	—
▲ 1025	IRHO/VE/04/F24-04	Triatomine	*Rhodnius prolixus*	Venezuela (Barinas)	417	—	—
▲ 677	ROR-20	Triatomine	*Rhodnius robustus* II	Brazil (RO) Monte Negro	417	• EF071561	• EF071577
▲ 681	ROR-68	Triatomine	*Rhodnius robustus* II	Brazil (RO) Monte Negro	417	—	—
▲ 683	ROR-67	Triatomine	*Rhodnius robustus* II	Brazil (RO) Monte Negro	417	—	—
▲ 701	ROR-62	Triatomine	*Rhodnius robustus* II	Brazil (RO) Monte Negro	417	• EF071562	• EF071578
▲ 704	ROR-85	Triatomine	*Rhodnius robustus* II	Brazil (RO) Monte Negro	417	• EF071563	• EF071579
Lineage B							
086	AM80	Human	*Homo sapiens*	Brazil (AM) Rio Negro #	380	• EF071547	AY491766
261	AM11	Human	*Homo sapiens*	Brazil (AM) Rio Negro	380		AY491758
207	AE-AAA	Monkey	*Cebuella pygmaea*	Brazil (AC) Rio Branco	380	• EF071564	AY491752
194	AE-AAB	Monkey	*Cebuella pygmaea*	Brazil (AC) Rio Branco	380	• EF071565	AY491753
233	4–30	Monkey	*Saguinus l. labiatus*	Brazil (AC) Rio Branco	380	—	AY491756
238	5–31	Monkey	*Saguinus l. labiatus*	Brazil (AC) Rio Branco	380	—	AY491754
236	8–34	Monkey	*Saguinus f. weddelli*	Brazil (AC) Rio Branco	380	—	AY491755
205	M-12229	Monkey	*Aotus sp*	Brazil (AM) Manaus	380	—	AY491757
416	2495	Monkey	*Alouatta stramineus*	Brazil (AM) Rio Negro	380	• EF071566	AY491760
427	2570	Monkey	*Callicebus lugens*	Brazil (AM) Rio Negro	380	—	AY491751
012	Saimiri	Monkey	*Saimiri sciureus*	Brazil (AM) Manaus	380	• EF071550	AY491768
013	Preguici	Sloth	*Choloepus didactylus*	Brazil (PA) Belém	380	• EF071549	AY491767
010	Legeri	Anteater	*Tamandua tetradactyla*	Brazil (PA) Belém	380	• EF071548	AY491769
032	Legeri	Anteater	*Tamandua tetradactyla*	Brazil (PA) Belém	380	—	AY491759
▲*	4176	Triatomine	*Rhodnius brethesi*	Brazil (AM) Rio Negro	380	• EF071567	• EF071580
▲ 759	4167	Triatomine	*Rhodnius brethesi*	Brazil (AM) Rio Negro	380	—	—
▲ 760	4166	Triatomine	*Rhodnius brethesi*	Brazil (AM) Rio Negro	380	—	—
Lineage C							
014	PG	Human	*Homo sapiens*	Panama	480	• EF071568	AJ012416
328	1625	Human	*Homo sapiens*	El Salvador	480	• EF071569	AY491738
&	Bg-60	Human	*Homo sapiens*	Costa Rica	480		X62675
&	*T. leeuwenhoeki*	Sloth	*Choloepus didactylus*	Panama	480	AJ012420	AJ012412
&	RGB	Dog	*Canis familiaris*	Colombia	480	AJ01419	AJ009160
▲ 1249	Pa 482TD	Triatomine	*Rhodnius pallescens*	Panama	480	—	—
▲ 1250	Pa 476TD	Triatomine	*Rhodnius pallescens*	Panama	480	—	—
▲ 1252	Pa 480TD	Triatomine	*Rhodnius pallescens*	Panama	480	—	—
▲ 1254	Pa 487GS	Triatomine	*Rhodnius pallescens*	Panama	480	—	—
▲ 1260	Pa 479GS	Triatomine	*Rhodnius pallescens*	Panama	480	—	—
▲*	SO18	Triatomine	*Rhodnius pallescens*	Colombia (Sucre)	480	—	—
▲*	SO29	Triatomine	*Rhodnius pallescens*	Colombia (Sucre)	480	• EF071570	• EF071581
▲*	G5	Triatomine	*Rhodnius pallescens*	Colombia (Sucre)	480	• EF071571	• EF071582
Lineage D							
023	SC58	Rodent	*Echimys dasythrix*	Brazil (SC)	500	AF083350	AY491745

†TryCC, number codes of cultures cryopreserved in the Trypanosomatid Culture Collection of the Department of Parasitology, University of São Paulo; ▲ new isolates characterized in this study; *only DNA samples were available; & only sequences from GenBank were employed in this study; • sequences obtained in this study and deposited in the GenBank.

‡Geographical origin of the isolates [country (state) city]: @, Marajó Island; #, Rio Negro region. Brazilian states: MG, Minas Gerais; RO, Rondônia; AC, Acre; PA, Para; AM; Amazonas; SC, Santa Catarina.

§DNA amplified fragments (in bp) generated by TraSL-PCR assay from DNA of *T. rangeli* isolates.

(AF045718); *R. neglectus* (AF045716). Sequences of *Triatoma infestans* (AF045721), *Triatoma sordida* (AF045730) and *Triatoma dimidiata* (AF045726) were used as outgroups for Rhodniini. Alignment of cyt *b* sequences was performed using the program GENEDOC and manually refined. Maximum parsimony (MP) analysis was performed using a heuristic search strategy and the default options of PAUP* 4.0b10 (Swofford 2002). Bootstrapped MP analysis with 100 replicates was done using PAUP with parameters settings as described in Rodrigues *et al*. (2006).

Growth, identification and genotyping of T. rangeli *isolates*

Sixty-one *T. rangeli* isolates from distinct host species and geographical areas were used in this study, including 27 new isolates (Table 1). Trypanosome cultures and DNA extraction were performed as previously described (Maia da Silva *et al*. 2004a). Besides morphological identification, the new isolates were also tested using a *T. rangeli*-specific polymerase chain reaction (PCR) assay (Maia da Silva *et al*. 2004a). All new *T. rangeli* isolates were also genotyped by PCR amplification of ITS rDNA as before (Maia da Silva *et al*. 2004b).

PCR amplification of the whole SL repeat units

The location and sequences of the primers employed for PCR amplification of SL gene sequences are shown in Fig. 1. Whole SL repeats of *T. rangeli* were amplified using primers RSL1 and RSL2 (Fig. 1b) in 25-μL reaction mixtures containing 200 μM of each dNTP, 20 μM of each primer, 50 ng of DNA templates and 2.5 U of *Taq* DNA polymerase. Reactions were cycled 30 times as follows: 1 min at 94 °C, 2 min at 48 °C and 2 min at 72 °C (with an initial cycle of 3 min at 94 °C and a final cycle of 10 min at 72 °C). Amplified products were separated in a 2% agarose gel, stained with ethidium bromide, transferred to nylon membranes and hybridized with the SL201 probe complementary to *T. cruzi* SL exon sequence (Teixeira *et al*. 1996).

Standardization of TraSL-PCR for T. rangeli*-specific amplification of SL intergenic-spacer sequences*

Primers TraSL1 and TraSL2 were designed for PCR amplification (TraSL-PCR) of a *T. rangeli*-specific DNA fragment of ~380–500 bp of the intergenic spacer, which is flanked by the SL intron and 5S rRNA. These oligonucleotides are complementary to intron (TraSL1) or 5S rRNA (TraSL2) sequences, which are highly conserved within *T. rangeli* (Fig. 1c). Amplification reactions were prepared as described above for the complete repeat of the SL gene and cycled 30 times as follows: 1 min at 94 °C, 1 min at 68 °C and 1 min at 72 °C (with an initial cycle of 3 min at 94 °C and a final cycle of 10 min at 72 °C). PCR products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide.

Sequencing and data analysis of SL and SSU rDNA gene sequences

PCR-amplified whole SL repeats and SL intergenic sequences of *T. rangeli* isolates were purified from agarose gels and cloned, and at least three clones from each isolate were sequenced. Sequences corresponding to variable region of V7-V8 flanked by conserved sequences of SSUrRNA (~900 bp) from 11 new *T. rangeli* isolates were determined in this study and deposited in the GenBank (Table 1). These sequences were aligned with those from other isolates retrieved from GenBank, most previously determined by our group (Table 1) and used to infer phylogenies using the MP and maximum likelihood (ML) methods.

Alignments of SL and SSUrRNA sequences were done using GENEDOC with final adjustments by eye. MP analysis was performed using the default options of PAUP 4.0b10. ML parameters were optimized using the hierarchical likelihood test in MODELTEST 3.06 (Posada & Crandall 1998) and ML analysis was carried out using TREEPUZZLE 5.0 (Strimmer & Von Haeseler 1996). Bootstrap analyses with 100 replicates were performed for MP and ML phylogenies as before (Rodrigues *et al*. 2006). The program TREEMAP 2.02 (Page & Charleston 1998) was used to identify co-phylogenetic events between *Rhodnius* spp. and *T. rangeli* lineages. Alignments used in this study are available from the authors upon request.

Results

Identification and genotyping of new T. rangeli *isolates*

Sixty-one *Trypanosoma rangeli* isolates were examined in this study, including 32 isolates that we had studied previously using other molecular markers (Maia da Silva *et al*. 2004a, b) and 27 new isolates, with most (21) from sylvatic, peridomestic and domestic species of *Rhodnius* spp. from Venezuela, Colombia, Panama and Brazil (Table 1). Isolates investigated in this study showed morphology and behaviour in mice and triatomines compatible with *T. rangeli* (data not shown), and were selected after species confirmation using a *T. rangeli*-specific PCR assay (Maia da Silva *et al*. 2004a). All new *T. rangeli* isolates were genotyped using internal transcribed spacer (ITS) ribosomal markers and ascribed to previously defined lineages as before (Maia da Silva *et al*. 2004b): (i) isolates from the Brazilian Amazon region were genotyped as lineages A or B; (ii) Isolates from Venezuela and southeastern Brazil were ascribed to lineage A; (iii) isolates from Panama and Colombia were all genotyped as lineage C.

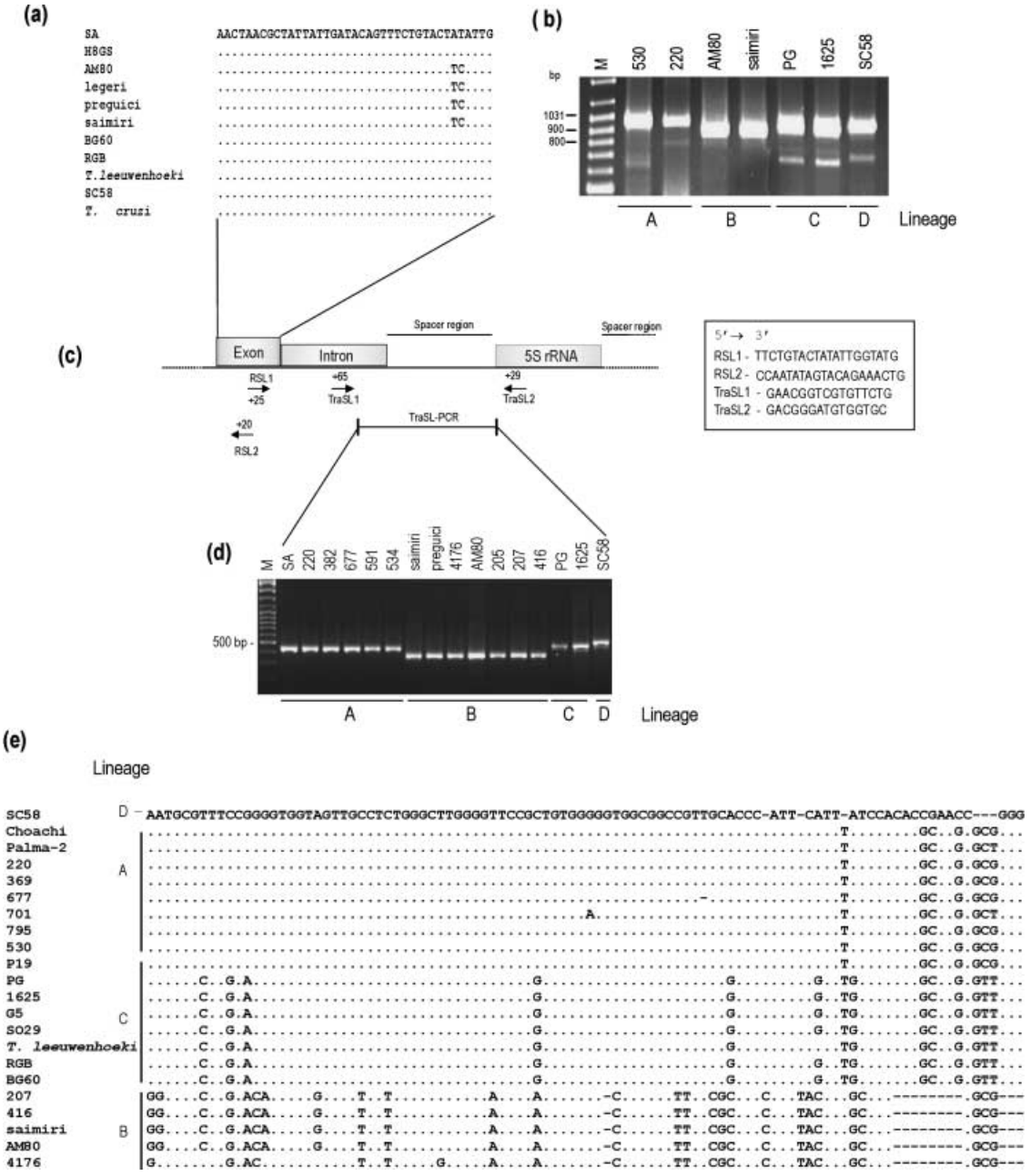

Fig. 1 (a) Alignment of the SL exon sequences from *Trypanosoma rangeli* isolates; (b) Agarose gel showing amplified fragments corresponding to whole SL repeat units of *T. rangeli* isolates stained with ethidium bromide; (c) Schematic diagram of the SL gene showing the annealing sites for oligonucleotides used as primers (in the box) used for PCR amplifications; (d) Agarose gel stained with ethidium bromide showing DNA fragments generated by TraSL-PCR using DNA from *T. rangeli* isolates of lineages A–D; (e) Alignment of SL intergenic spacer sequences of *T. rangeli* isolates from lineages A–D.

SL repeat length and sequence polymorphism among T. rangeli *isolates and allied species*

Full-length SL gene repeats of *T. rangeli* isolates amplified by PCR, determined by agarose gel electrophoresis (Fig. 1b) and confirmed by hybridization with SL exon-derived probe SL201 (data not shown), revealed variable repeat lengths compatible with those previously described for *T. rangeli* isolates. Figure 1a illustrates the size polymorphism of SL repeats within *T. rangeli*: (i) isolates from lineage A (530 and 220) had ~980-bp SL repeats, similar to previously described for *T. rangeli* SA (Murthy *et al*. 1992); (ii) isolates belonging to lineage B, represented by the isolates AM80, saimiri, preguici and legeri from wild mammals (Maia da Silva

et al. 2004a, b), showed SL repeats of ~840 bp; and (iii) members of lineage C, PG, 1625, *T. leeuwenhoeki*, present a SL repeat from ~920–955 bp, as previously reported (Stevens *et al*. 1999; Gibson *et al*. 2000). The isolate SC58 (lineage D) had a repeat length of ~915 bp (Grisard *et al*. 1999). Length variability of SL repeats in *T. rangeli* isolates is due insertion/deletion events and microsatellite repeats in the intergenic spacer (Fig. 1e).

Preliminary analysis of SL sequences from a small number of *T. rangeli* isolates suggested that this marker could also generate the same four lineages derived from RAPD and ribosomal markers (Maia da Silva *et al*. 1999). In this study, sequences of whole SL repeat units were determined for *T. rangeli* isolates AM80, saimiri, preguici and legeri (lineage B), and aligned with sequences from isolates of other lineages (GenBank): A (SA, H8GS); C (RGB, *T. leeuwenhoeki*) and D (SC58), plus the sequence of BG60 from Costa Rica (here ascribed to lineage C) (Table 1). In contrast to the high degree of conservation of the exon sequences among trypanosomatids, the aligned SL repeat sequences of *T. rangeli* isolates disclosed two different exon sequences showing minor polymorphism: one shared by lineages A, C and D, identical to that of *Trypanosoma cruzi*, and the other only present in lineage B (Fig. 1a). Alignment of full-length sequences of seven isolates from the four lineages of *T. rangeli* showed very similar intron sequences (average divergence was ~2.2%) and highly variable intergenic sequences (average divergence was ~30%). Contrasting to the exon and intron sequences, alignment of intergenic sequences was unreliable due to the very high polymorphism (Fig. 1e).

Genetic relationships between T. rangeli *isolates based on intergenic spacer sequences*

To better evaluate the intraspecific variability and genetic relationships among *T. rangeli* lineages, we selected a moderately conserved region within the SL repeat to compare several isolates (Fig. 1c). With this purpose, we sequenced amplified fragments (380–500 bp) (Fig. 1d) of intergenic sequences flanked by the SL intron and 5SrRNA sequences of 21 further isolates (Table 1) and aligned them with the sequences reported above. The SL intergenic spacer in trypanosomatids has very high interspecies variability, precluding reliable alignments of *T. rangeli* sequences even with the closely related *T. cruzi*. Based on a similarity matrix constructed using aligned intergenic sequences (Fig. 1e depicts a part of this region to illustrate lineage-specific polymorphisms), it was possible to define four groups of *T. rangeli*. According to the phylograms constructed using the MP (data not shown) or ML (Fig. 2a) methods, the same four lineages (A–D) previously determined by RAPD and ribosomal gene (Maia da Silva *et al*. 2004a, b) were also defined by SL sequences. Lineage A, constituted by isolates from Venezuela, Colombia, Guatemala and the Brazilian Amazonia, had ~98% sequence similarity; lineage B, comprising exclusively isolates from the Brazilian Amazonia, showed the most significant sequence divergence (~93–99% similarity); and lineage C, composed by isolates from Panama, Colombia, Costa Rica and El Salvador, which shared ~95% sequence similarity. Lineage B was separated for largest sequence divergences from all other lineages:

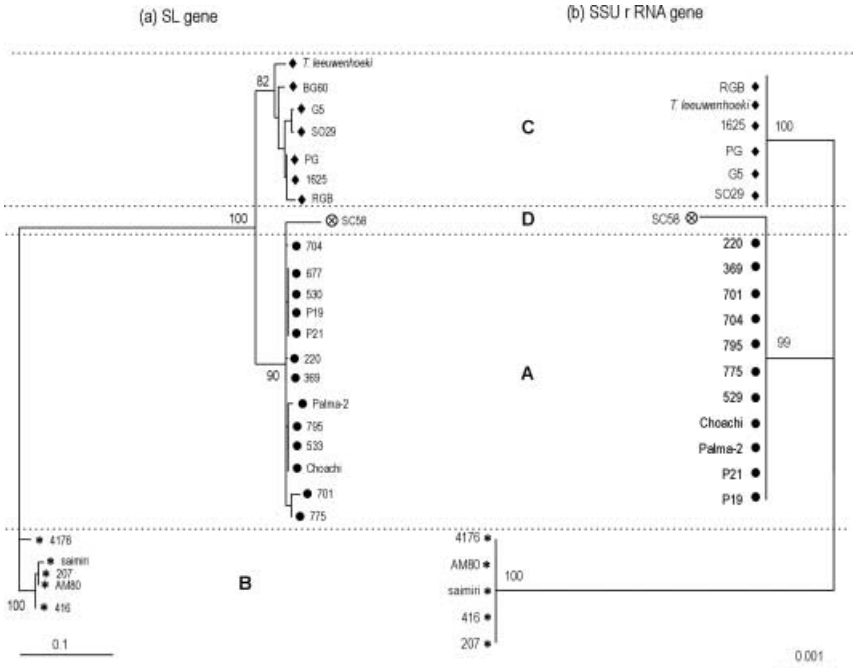

Fig. 2 Parity between phylogenetic relationships among *Trypanosoma rangeli* from lineages A (•), B (*), C (♦) and D (⊗) based on SL intergenic spacer sequences (a) and V7-V8 regions of SSU rRNA sequences (b), inferred by maximum likelihood (ML). For SL sequences alignment (–ln L = 1343.1262), the best-fit evolutionary model, as determined by MODELTEST, was K80 (Kimura 1980) with gamma distribution (α = 0.5505). For alignment of V7-V8 SSU rRNA sequences (–ln L = 1277.6781) the chosen model was K80. The numbers at nodes correspond to percentage of ML bootstrap support values derived from 100 replicates.

A (~35%), C (41.5%) and D (37%), whereas the smallest divergence separated lineage C from A (~16%) and D (~21%). Lineage D, represented by the isolate SC58 from southern Brazil, although separated by relatively small divergence from either lineages A (~16%) or C (~21%), were positioned together with lineage A (Fig. 2a).

Parity analysis between lineages of T. rangeli *defined based on spliced-leader and SSU rRNA sequences*

We previously demonstrated that SSU and ITS ribosomal sequences showed the same branching pattern of isolates within *T. rangeli*, with consistent heterogeneity segregating the isolates into four well-supported lineages (Maia da Silva *et al*. 2004b). To compare phylogenies of *T. rangeli* isolates using sequences from different genes, showing different evolutionary rates, we compare phylograms generated using SSUrRNA and intergenic SL sequences. The results showed total agreement between data from these two genes (Fig. 2).

Phylogeography and evolutionary relationships between T. rangeli *lineages and* Rhodnius *spp.*

Our data suggest an association between *T. rangeli* lineages and both geographical origin and complexes of *Rhodnius*. The geographical distribution of sylvatic species of Rhodniini varies from sylvatic species tightly associated with ecological traits of specific palms, like *R. brethesi* with the piassaba palm, to the most widespread *R. prolixus*, which besides sylvatic is domiciled in many areas (Fig. 3). The triatomines from which *T. rangeli* was isolated were classified according to morphological and molecular taxonomy. Cytochrome *b* sequences from specimens of these triatomines were compared with sequences from GenBank. Brazilian triatomines were classified as *R. robustus* II (Rondonia) or *R. brethesi* (Rio Negro, Amazonas), specimens from Venezuela were classified as *R. prolixus* (Barinas and Trujillo) or *R. robustus* I (Trujillo) and those from Panama as *R. pallescens* (Table 1). *Rhodnius* species were confirmed by a phylogenetic analysis (Fig. 3a), that included sequences described in Monteiro *et al*. (2000, 2003).

By comparing the phylogeny of *Rhodnius* species (Fig. 3a) with that inferred for *T. rangeli* isolates (Fig. 2) and by comparing the geographical range of the isolates (Fig. 3b), we showed that there is total concordance of lineages and vector species and a very significant overlap in the distribution of *Rhodnius* species and *T. rangeli* lineages. The three major lineages analysed in this study could be related to the ecogeographical structure of the Rhodniini population as follows: (i) lineage A, circulating in Venezuela, Colombia, Guatemala, Honduras and Brazil, related to both domestic and sylvatic cycles of species of the *R. prolixus* complex; (iii) lineage B, occurring in the northern Brazilian Amazon region and related to sylvatic *R. brethesi*; and (iii) lineage C, related to domestic and sylvatic cycles of the *R. pallescens* complex circulating in Panama, Costa Rica and Colombia. Accordingly, *T. rangeli* isolates from *Rhodnius* spp. from different complexes did not cluster together (Fig. 3; Table 1).

Comparison of *Rhodnius* and *T. rangeli* phylogenies revealed a total concordance of terminal taxa between *T. rangeli* lineages and complexes of their respective vector species, suggesting a long history of codivergence in these

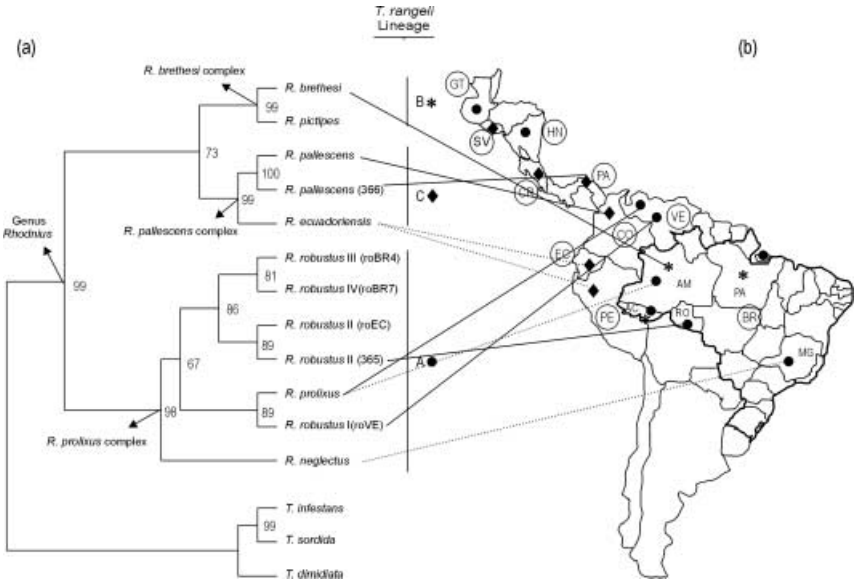

Fig. 3 Schematic representation linking the (a) phylogeography of *Rhodnius* species with (b) geographical range of *Trypanosoma rangeli* isolates from Central and South America (Table 1) ascribed to lineages A (•), B (*) or C (♦). (a) Phylogeography of *Rhodnius* was based on mitochondrial cyt *b* sequences and inferred using maximum parsimony. The continuous lines indicate the collecting location of the specimens of *Rhodnius* spp. from which we obtained the *T. rangeli* isolates. Sequences from the isolates and from their respective *Rhodnius* hosts of origin were included in the phylogenetic analysis. Dotted lines indicate data from other studies. The numbers at nodes correspond to bootstrap values derived from 100 replicates. GT, Guatemala; SV, El Salvador; HN, Honduras; CR, Costa Rica; PA, Panama; CO, Colombia; VE, Venezuela; BR, Brazil. States of Brazil: AM, Amazonas; AC, Acre; RO, Rondonia, PA, Para; MG, Minas Gerais.

host–parasite assemblages. However, comparison of *Rhodnius* and *T. rangeli* phylogenies revealed incongruent branching patterns (Fig. 3) suggesting association between *T. rangeli* lineages and *Rhodnius* spp. by evolutionary process other than co-evolution. In the *Rhodnius* phylogeny based on cyt *b*, *R. pallescens* and *R. brethesi* complexes are sister groups (Fig. 3) whereas in the phylogeny of *T. rangeli*, lineage C (*R. pallescens* complex) is sister to lineage A (*R. prolixus* complex) and not to lineage B (*R. brethesi*), which was distantly positioned (Fig. 2). Analysis done using TREEMAP2.02 program (not shown) suggested that host switching, sympatric speciation (duplication) and episodes of particular extinction (sorting) play a role in the evolutionary history of these host-parasite relationships.

Identification and genotyping of T. rangeli *isolates by TraSL-PCR based on SL intergenic sequences*

Aiming to define SL sequences that are able simultaneously to identify and genotype *T. rangeli* isolates without DNA sequencing, we developed the TraSL-PCR assay (Fig. 1d). The standardized PCR yielded DNA bands for all 58 *T. rangeli* isolates examined. Specificity of TraSL-PCR was confirmed by negative results obtained using phylogenetically related organisms and/or flagellates sharing the same vertebrate and invertebrate hosts, such as *T. cruzi* (*T. cruzi* I, *T. cruzi* II and Z3 lineages), *T. conorhini* and *Blastocrithidia* spp. (data not shown). Besides high specificity, TraSL-PCR was shown to be very sensitive, detecting down to 500 pg of DNA by ethidium bromide staining and down to 100 pg of DNA (~10–20 cells) after hybridization using the SL amplified fragment of *T. rangeli* (SA) as probe (data not shown). The TraSL-PCR amplified fragments varied in length according to the lineage of the isolate whereas bands of the same length were shared by all isolates within each lineage: A (417 bp); B (380 bp); C (480 bp) and D (500 bp) (Table 1). All 58 isolates tested could be assigned to one of the four previously defined lineages (Fig. 1; Table 1).

Discussion

Studies regarding biological and molecular features of *Trypanosoma rangeli* isolates from distinct hosts and geographical origins have shown that they form a monophyletic group more closely related to the *Schizotrypanum* species (*Trypanosoma cruzi*) than to any other trypanosome (Stevens *et al*. 1999; Maia da Silva *et al*. 2004b). Despite being very closely related, several molecular markers separated *T. rangeli* isolates into distinct lineages, apparently related to *Rhodnius* complexes (Vallejo *et al*. 2002, 2003; Maia da Silva *et al*. 2004a, b; Urrea *et al*. 2005). To better understand the phylogenetic relationships within *T. rangeli,* we analysed SL and ribosomal sequences from 36 *T. rangeli* isolates from distinct mammals plus 23 isolates from triatomine bugs.

Results from this study showed that all *T. rangeli* isolates, from Central and South America, could be genotyped using PCR assays based on either SL or ITSrDNA markers into one of the four previously reported phylogenetic lineages, supported by analyses using SSU and ITS rDNA sequences (Maia da Silva *et al*. 2004a, b). Parity between phylogenetic relationships using SSU and ITS rDNA sequences was also reported for lineages of *T. theileri* (Rodrigues *et al*. 2006) and *T. vivax* (Cortez *et al*. 2006). Congruence between SL and SSUrRNA relationships among *T. rangeli* lineages shown in this study supported four previously defined phylogenetic lineages (Maia da Silva *et al*. 2004a, b), and corroborated lineages association with complexes of *Rhodnius* (Vallejo *et al*. 2002, 2003; Maia da Silva *et al*. 2004a, b; Urrea *et al*. 2005). Parity between genotyping using rDNA and SL markers was also demonstrated for lineages of *T. cruzi* (Zingales *et al*. 1998; Brisse *et al*. 2001), *T. vivax* (Cortez *et al*. 2006) and *T. theileri* (A.C. Rodrigues, F. Maia da Silva, M. Campaner & M.M.G. Teixeira, unpublished).

Molecular phylogenies based on different sequences have indicated three major complexes of species within *Rhodnius* associated with specific ecotopes and geographical areas (Gaunt & Miles 2000; Monteiro *et al*. 2000, 2003). The *R. brethesi* complex is the most heterogeneous, has been reported in the Brazilian and Venezuelan Amazon regions, and comprises *R. brethesi* associated with piassaba palms, and *R. pictipes* with a broader distribution. The *R. pallescens* complex is the most homogeneous and has been reported in Panama and Costa Rica (Central America) and northwest region of South America. The *R. prolixus* complex comprises six species, besides genetically different populations ascribed to the same species: *R. robustus* from Venezuela (RbI) is more closely related to *R. prolixus* than to Brazilian *R. robustus*, which is distributed into three genetically and geographically separated populations: RbII (west), RbIII (north and center) and RbIV (north) (Monteiro *et al*. 2003).

According to data from this study, isolates ascribed to lineage A came from northwest South America (Venezuela and Colombia), Central America (Honduras and Guatemala) and Brazil (western and eastern Amazon region). This lineage was associated with the *R. prolixus* complex and includes isolates from *R. prolixus*, *R. robustus* I and *R. robustus* II, plus isolates from humans, dog, opossum and monkeys. The isolate SC58, from southern Brazil, was previously ascribed to lineage D based on RAPD patterns and on SSU and ITS rDNA sequences (Maia da Silva *et al*. 2004b), and took into account previous studies that separated isolates from this region from all others by isoenzymes, RAPD and SL markers (Steindel *et al*. 1991, 1994; Grisard *et al*. 1999; Maia da Silva *et al*. 1999, 2004a). *T. rangeli* in southern Brazil should be transmitted by *R. domesticus* (Steindel *et al*. 1994), which is positioned in a relatively distant branch within the *R. prolixus* complex (Monteiro *et al*. 2000; Hypsa *et al*.

2002). This putative vector agrees with the positioning of the isolate SC58 closest to lineage A in this study, contrasting to kDaNA markers that grouped this isolate with isolates of the *R. pallescens* complex (lineage C) (Vallejo *et al*. 2003; Urrea *et al*. 2005). Corroborating the hypothesis that lineage A is associated with the *R. prolixus* complex, *T. rangeli* circulating in *Didelphis marsupialis* and *R. neglectus* in southeast Brazil (Ramirez *et al*. 2002) were positioned within lineage A in this study, and also associated with *R. prolixus* using kDaNA markers (Gurgel-Goncalves *et al*. 2004).

Group B includes exclusively Brazilian isolates from humans, triatomines and wild mammals from the Brazilian Amazon region. Previous studies have shown that *R. brethesi* is the only vector for *T. rangeli* in the Rio Negro region of the northern Brazilian Amazon (Coura *et al*. 1996, 2002), and that isolates from *R. brethesi* can be identical to isolates from humans and monkeys (Maia da Silva *et al*. 2004a, b). The alleged absence of *R. brethesi* and piassava palms in areas of western (Acre) and eastern (Para) Amazon region where isolates ascribed to lineage B originate strongly suggests that *R. pictipes*, which is widespread and the closest relative species of *R. brethesi*, could, in addition to *R. brethesi*, be a vector of *T. rangeli* lineage B. We are currently trying to isolate *T. rangeli* from *R. pictipes* aiming to clarify this hypothesis.

Group C was formed by isolates from *R. pallescens* from Colombia and Panama, human isolates from Central America (Panama, Costa Rica and El Salvador), a dog isolate from Colombia and *T. leeuwenhoeki* from a sloth from Panama. Therefore, all isolates of this lineage came from within the ecogeographical range of the *R. pallescens* complex. In agreement with data using SL genes, these isolates had been previously grouped and associated with the *R. pallescens* complex using RAPD and SSU and ITSrDNA (Maia da Silva *et al*. 2004a, b). Associations between *T. rangeli* isolates from Colombia, Panama and Peru and *R. pallescens* complex were also established by kDNA and SL (mini-exon) markers (Vallejo *et al*. 2003; Urrea *et al*. 2005).

The relative patterns of *T. rangeli* grouping could be explained by the geographical structure of *Rhodnius* spp. In conformity with the distribution of *R. robustus* and *R. brethesi*, *T. rangeli* isolates from the Brazilian Amazon were segregated into lineages A and B. In general, isolates from the same geographical regions are more closely related, excepting for isolates from regions where different *Rhodnius* complexes naturally overlapped, or shared the same habitats after their human introduction. For example, isolates from the same area of Colombia were segregated into lineages A and C related to the *R. prolixus* or *R. pallescens* complexes, respectively (Vallejo *et al*. 2002, 2003; Urrea *et al*. 2005).

Complex vector–parasite interactions reflect a long evolutionary history of *T. rangeli* lineages with different *Rhodnius* complex. The congruency among ribosomal, SL, RAPD and kDaNA markers suggested that *T. rangeli* lineages evolved with a nonrandom association of independent molecular markers, pointing towards the existence of linkage disequilibrium and the absence of gene flow between recently diverged lineages. In this study, all *T. rangeli* lineages contain isolates from different host species (humans and domestic or sylvatic mammals), thus without association with particular taxon of vertebrate. The small divergence separating *T. rangeli* lineages is compatible with a recent divergence of *Rhodnius* species (Monteiro *et al*. 2003). In contrast, large genetic distances separate *T. cruzi* lineages that appear to evolve in association with their preferential mammalian hosts, which diverged a long time ago (Gaunt & Miles 2000; Yeo *et al*. 2005). Contrasting to vertebrate host-restriction reported for some trypanosomes of rabbit (Hamilton *et al*. 2005), bats (Stevens *et al*. 2001) and artiodactyls (Rodrigues *et al*. 2003, 2006), avian trypanosomes apparently lack vertebrate-host specificity (Sehgal *et al*. 2001) likewise *T. cruzi* and *T. rangeli*.

Demonstration that *T. rangeli* isolates from distinct *Rhodnius* complexes living in sympatry are separated in distinct lineages suggests evolution of *T. rangeli* lineages by a long coexistence with their sympatric vectors in independent transmission cycles. Phylogeographical analysis strongly supports an association between geographical regions and both *T. rangeli* lineages and *Rhodnius* spp. Therefore, biogeography is very important in structuring these host–parasite assemblages.

Data from this study demonstrated total concordance between different *T. rangeli* lineages and the complexes of *Rhodnius* using either SL or ribosomal markers, which suggests vector–parasite co-evolution. However, comparison of *Rhodnius* and *T. rangeli* phylogenies showed incongruent branching patterns, which indicates a lack of or little cospeciation, with host-switching indicating that the evolutionary histories of *Rhodnius* and *T. rangeli* are complex. In our analysis, *T. rangeli* lineage C (associated to *R. pallescens*) was more closely related to lineage A (*R. prolixus*) than to lineage B (*R. brethesi*), in disagreement with the close relationship of *R. pallescens* and *R. brethesi* complexes based on cyt *b* phylogeny (Monteiro *et al*. 2000, 2003). A phylogeny of the tribe Rhodniini based on zymodemes suggested a basal group comprising *R. pictipes* and *R. brethesi* originated in northern Amazonia and then dispersed northwest and south to give rise to the *R. pallescens* and *R. prolixus* complexes (Dujardin *et al*. 1999). However, *R. pictipes* was not found as the most primitive branch in other studies. The phylogeny based on the 16S mitochondrial rDNA positioned *R. pictipes*/*R. brethesi* closer to *R. prolixus* than to *R. pallescens*, which was positioned in the most basal branch of the Rhodniini (Hypsa *et al*. 2002). Therefore, depending on methods, sequences and parameters used, different topologies were obtained, placing the *R. pictipes* complex as sister group of either the *R. pallescens* or the *R. prolixus* complex (Lyman *et al*. 1999; Monteiro *et al*. 2000; Hypsa *et al*. 2002). A recent

phylogenetic interpretation of 16S mtrDNA sequence data with regard to the geographical distribution of *Rhodnius* spp. and geological events affecting the origin and diversification of Rhodniini support a complex biogeographical history of this tribe (Paula *et al*. 2006).

Apparently, a major role in the evolutionary origin of *T. rangeli* lineages is their geographical distribution and specific ecotopes and niches of *Rhodnius* species. However, the evolutionary history of Rhodniini is far from being clearly understood and the vector–parasite associations found in the present study must be interpreted with caution. A better understanding of population structure and the factors involved in lineage segregation within *T. rangeli* requires analysis of a larger number of isolates from invertebrate and vertebrate hosts from a large ecogeographical range, together with a better phylogeographical analysis of *Rhodnius*. More lineages can be described by using other molecular markers and by analysing other *Rhodnius* spp. from new geographical areas. The *T. rangeli*-specific SL-derived PCR assay that we have developed may be useful for larger surveys by allowing simultaneous identification and genotyping of *T. rangeli*.

Acknowledgements

We are grateful to Dr Erney P. Camargo and Dr Jeffrey J. Shaw for their continued encouragement, discussions and constructive criticisms in reviewing our manuscript. We thank M. C. Brigido and C. Bonvicino for blood samples and identification of monkeys, and Toby Barret and Jeffrey J. Shaw for *T. rangeli* isolates. We thank Robson C. Ferreira and Myrna G. Serrano for initial help in the SL gene sequencing. We are indebted to several collaborators and students for their inestimable help in the fieldwork and to anonymous reviewers for valuable comments and English revision that improved our manuscript. This project was supported by Brazilian grants of CNPq, FAPESP and PRONEX. Adriana C. Rodrigues and Flávia Maia da Silva are postdoctoral fellows sponsored by CNPq and CAPES, respectively.

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Flávia Maia da Silva and Angela C. V. Junqueira completed this work as part of their PhD thesis. Together with Adriana C. Rodrigues, they are currently postgraduate students working on biodiversity of trypanosomes, processes structuring biodiversity, and evolutionary history of *Trypanosoma*. Marta Campaner works as biologist at the University of São Paulo where she is responsible for the isolation and culture of trypanosomes. Gladys Crisante and Zuleima C. E. Caballero are graduate students and collaborated on fieldwork during this study. Fernando Monteiro is a research scientist interested in the systematics, phylogeography and population genetics of insect vectors of parasitic diseases. Professors Marta M. G. Teixeira, Luis E. Ramirez, José Rodrigues Coura (Brazil) and Nestor Añez (Venezuela) have a longstanding interest and experience in different aspects of trypanosome infection on humans and triatomine bugs, including transmission and disease emergence in Amazonia. Marta Teixeira is also interested in diversity, phylogeny, biogeography and phylogeography, molecular diagnosis and epidemiology of trypanosomes from all vertebrate classes and their insect vectors.