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*

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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
prevalence of T. rangeli human infections has been reported in Central America and northwest South America (D’Alessandro & Saravia 1999; Guhl & Vallemão 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 mating 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 (Anfinsen 1984; Guhl & Vallemão 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 & Vallemão 2003; Vallemão 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 phylogeneies. 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 2001; Paterson & Banks 2001; Banks & Paterson 2005; Teixeira et al. 1996). 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 (Reduviidae: 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 zymo-demes (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. nasutus 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 (Vallemão 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 ITS rDNA (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 Trypanosoma 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.

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 | Mi62864 | 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 | • EFO71551 | — |
| 533           | MAN/VE/00/LOBITA | Dog | Canis familiaris | Venezuela (Barinas) | 417 | • EFO71552 | • EFO71572 |
| 220           | AT-AEI        | Monkey | Saimiri sciureus | Brazil (PA) Marajo® | 417 | • EFO71553 | AY491747 |
| 202           | AT-ADS        | Monkey | Saimiri sciureus | Brazil (PA) Marajo | 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 | • EFO71554 | 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 | • EFO71555 | • EFO71573 |

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| 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 prolitis    | Venezuela         | 417                  | • EF071557 • AJ012414 |
| 022    | Palma-2 | Triatomine    | Rhodnius prolitis    | Venezuela         | 417                  | • EF071558 • AY491741 |
| ▲775   | VE/9    | Triatomine    | Rhodnius prolitis    | 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 prolitis    | Venezuela (Barinas) | 417                      |                  |
| ▲1024  | IRHO/VE/05/Apure 1 | Triatomine | Rhodnius prolitis    | Venezuela (Apure) | 417                      |                  |
| ▲1025  | IRHO/VE/04/F24-04 | Triatomine | Rhodnius prolitis    | 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                     |                  |
| ▲704   | ROR-85  | Triatomine    | Rhodnius robustus II | Brazil (RO) Monte Negro | 417                     | • EF071562 • EF071578 |
|        |         |              |                      |                  |                      |
| Lineage B |        |              |                      |                  |                      |
| 086    | AM80    | Human         | Homo sapiens         | Brazil (AM) Rio Negro # | 380            | • EF071547 • AY491766 |
| 261    | AM11    | Human         | Homo sapiens         | Brazil (AM) Rio Negro | 380            | • EF071554 • AY491758 |
| 207    | AE-AAB  | Monkey        | Cebuella pygmea      | Brazil (AC) Rio Branco | 380            | • EF071564 • AY491752 |
| 194    | AE-AAB  | Monkey        | Cebuella pygmea      | Brazil (AC) Rio Branco | 380            | • EF071565 • AY491753 |
| 233    | 4–30    | Monkey        | Saginus i. labiatus  | Brazil (AC) Rio Branco | 380            | —                  |
| 238    | 5–31    | Monkey        | Saginus i. labiatus  | Brazil (AC) Rio Branco | 380            | —                  |
| 236    | 8–34    | Monkey        | Saginus f. weddelli  | Brazil (AC) Rio Branco | 380            | —                  |
| 205    | M-12229 | Monkey        | Aotus sp             | Brazil (AM) Mauana | 380            | —                  |
| 416    | 2495    | Monkey        | Alouatta stramineus  | Brazil (AM) Rio Negro | 380            | • EF071566 • AY491760 |
| 427    | 2570    | Monkey        | Callicebus lugens    | Brazil (AM) Rio Negro | 380            | —                  |
| 012    | Saimiri | Monkey        | Saimiri sciureus     | Brazil (AM) Mauana | 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 |
| ▲*     | 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       | 380            | • EF071569 • AY491738 |
| &      |         |              |                      |                  |                      | X62675 |
| &      | T. leeuwenhoeki | Sloth       | Choloepus didactylus | Costa Rica        | 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 dasylirix    | Brazil (SC)       | 500            | AR883350 • 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.

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Reactions were cycled 30 times as follows: 1 min at 94 °C, 1 min at 72 °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.

Sequential 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 TreePrE 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, b) and were selected after species confirmation using a T. rangeli-specific PCR assay (Maia da Silva et al. 2004a, b). 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. 2004a, b): (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.
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, sainiri, preguiç and legeri from wild mammals (Maia da Silva...
et al. 2004a, b), showed SL repeats of ~840 bp; and (iii) members of lineage C, FG, 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, pregui 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 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. 1b) 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.

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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. 2).

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 domiciliated 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
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 TREEMAPR version 2.02 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 ITS rDNA 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. 2004a, 2004b) 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 ectoparas 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.
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 \textit{R. pallescens} complex (lineage C) (Vallejo et al. 2003; Urrea et al. 2005). Corroborating the hypothesis that lineage A is associated with the \textit{R. prolixus} complex, \textit{T. rangeli} circulating in Didelphis marsupialis and \textit{R. neglectus} in southeast Brazil (Ramirez et al. 2002) were positioned within lineage A in this study, and also associated with \textit{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 \textit{R. brethesi} is the only vector for \textit{T. rangeli} in the Rio Negro region of the northern Brazilian Amazon (Coura et al. 1996, 2002), and that isolates from \textit{R. brethesi} can be identical to isolates from humans and monkeys (Maia da Silva et al. 2004a, b). The alleged absence of \textit{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 \textit{R. pictipes}, which is widespread and the closest relative species of \textit{R. brethesi}, could, in addition to \textit{R. brethesi}, be a vector of \textit{T. rangeli} lineage B. We are currently trying to isolate \textit{T. rangeli} from \textit{R. pictipes} aiming to clarify this hypothesis.

Group C was formed by isolates from \textit{R. pallescens} from Colombia and Panama, human isolates from Central America (Panama, Costa Rica and El Salvador), a dog isolate from Colombia and \textit{T. leeuwenhoeki} from a sloth from Panama. Therefore, all isolates of this lineage came from within the ecogeographical range of the \textit{R. pallescens} complex. In agreement with data using SL genes, these isolates had been previously grouped and associated with the \textit{R. pallescens} complex using RAPD and SSU and ITSrDNA (Maia da Silva et al. 2004a, b). Associations between \textit{T. rangeli} isolates from Colombia, Panama and Peru and \textit{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 \textit{T. rangeli} grouping could be explained by the geographical structure of \textit{Rhodnius} spp. In conformity with the distribution of \textit{R. robustus} and \textit{R. brethesi}, \textit{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 \textit{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 \textit{R. prolixus} or \textit{R. pallescens} complexes, respectively (Vallejo et al. 2002, 2003; Urrea et al. 2005).

Complex vector–parasite interactions reflect a long evolutionary history of \textit{T. rangeli} lineages with different \textit{Rhodnius} complex. The congruency among ribosomal, SL, RAPD and kDaNA markers suggested that \textit{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 \textit{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 \textit{T. rangeli} lineages is compatible with a recent divergence of \textit{Rhodnius} species (Monteiro et al. 2003). In contrast, large genetic distances separate \textit{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 \textit{T. cruzi} and \textit{T. rangeli}.

Demonstration that \textit{T. rangeli} isolates from distinct \textit{Rhodnius} complexes living in sympatry are separated in distinct lineages suggests evolution of \textit{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 \textit{T. rangeli} lineages and \textit{Rhodnius} spp. Therefore, biogeography is very important in structuring these host–parasite assemblages.

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

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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.