Randomly amplified polymorphic DNA analysis of *Trypanosoma rangeli* and allied species from human, monkeys and other sylvatic mammals of the Brazilian Amazon disclosed a new group and a species-specific marker

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SUMMARY

We characterized 14 trypanosome isolates from sylvatic mammals (9 from primates, 1 from sloth, 2 from anteaters and 2 from opossum) plus 2 human isolates of Brazilian Amazon. These isolates were proven to be *Trypanosoma rangeli* by detection of metacyclic trypomastigotes in the salivary glands of triatomines and by a specific PCR assay. Polymorphism determined by randomly amplified polymorphic DNA (RAPD) revealed that most (12) of the Brazilian *T. rangeli* isolates from the Amazon differed from those of other geographical regions, thus constituting a new group of *T. rangeli*. Four Brazilian isolates clustered together with a previously described group (A) that was described as being composed of isolates from Colombia and Venezuela. Isolates from Panama and El Salvador form another group. The isolate from Southern Brazil did not cluster to any of the above-mentioned groups. This is the first study that assesses the genetic relationship of a large number of isolates from wild mammals, especially from non-human primates. A randomly-amplified DNA fragment (Tra625) exclusive to *T. rangeli* was used to develop a PCR assay able to detect all *T. rangeli* groups.

Key words: *Trypanosoma rangeli*, monkey, South American trypanosomes, Brazilian Amazon, *Herpetosoma*, RAPD, genetic relatedness, wild mammals, PCR, diagnosis.

INTRODUCTION

Trypanosoma rangeli lacks mammalian host specificity being found in humans, domestic and wild mammals. It is widely distributed in Central, Northern and Western South America, where it is sympatric with *Trypanosoma cruzi*, sharing vertebrate and invertebrate (triatomine bugs) hosts. This species has several unique characteristics regarding development on both vertebrate and invertebrate hosts, being considered non-pathogenic for mammalian hosts whereas damaging to insect vectors. Different from *T. cruzi*, intracellular stages (amastigote) were not detected in vertebrate hosts of *T. rangeli* (D'Alessandro, 1976; D'Alessandro & Saravia, 1992, 1999; Guhl & Vallejo, 2003).

Although transmission of *T. rangeli* is mainly by the inoculation of triatomine saliva containing metacyclic trypomastigotes during bug feeding, which is typical of salivarian trypanosomes, it can also be transmitted via vector faeces, a feature of stercorarian trypanosomes (D'Alessandro & Saraiva, 1999). This species was classified within the subgenus *Herpetosoma* of the Stercoraria Section (Hoare, 1972). However, due to this unusual transmission, its relationship with *T. brucei*, a typical salivarian species, has been questioned (Ãnez, 1982). Phylogenetic studies based on small subunit of ribosomal RNA gene (SSU rRNA) sequences tightly positioned *T. rangeli* within Stercoraria, close to *T. cruzi* and distant from all the salivarian species (Stevens *et al.* 1999, 2001). Populations of *T. rangeli* exhibited a significant degree of genetic polymorphism that permitted their partition in two main groups, apparently related to geographical origin, based on kDNA (Vallejo *et al.* 2002); isoenzymes and RAPD patterns (Steindel *et al.* 1994) and mini-exon gene sequences (Grisard, Campbell & Romanha, 1999).

T. rangeli has been sporadically reported in Brazil, especially in wild mammals and triatomines of the Amazon region (Deane *et al.* 1972; Shaw, 1985; Miles *et al.* 1983; Ziccardi & Oliveira, 1998; Ziccardi *et al.* 2000). Few confirmed human *T. rangeli* cases have also been reported in this region (Coura *et al.* 1996). In Brazil, outside Amazon, confirmed *T. rangeli* was related in triatomines, wild rodents and opossums in Southern and Southeast Regions (Steindel *et al.* 1991; Ramirez *et al.* 1998, 2002).

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While the taxonomic position of isolates from human and triatomines is well defined, the status of *T. rangeli*-like trypanosomes from wild mammals has not been investigated. *T. rangeli* has been reported in non-human primates, edentates, marsupials, carnivores and rodents (Hoare, 1972; D'Alessandro, 1976; Miles *et al*. 1983; D'Alessandro & Saravia, 1992, 1999; Marinkelle, 1976; Dereure *et al*. 2001). There is a high prevalence of trypanosomes in neotropical monkeys (Marinkelle, 1976), with several reports from Brazilian Amazon (Deane *et al*. 1972; Ziccardi & Oliveira, 1998; Ziccardi *et al*. 2000), Colombia (Marinkelle, 1976; D'Alessandro & Saravia, 1992); Panama (Sousa, Rossan & Baerg, 1974; Sousa & Dawson, 1976) and French Guyana (de Thoisy *et al*. 2001).

Sylvatic mammals described as natural hosts of *T. rangeli* can be infected by other *Herpetosoma* spp., by *T. cruzi*, and by species of subgenera *Megatrypanum*. On the basis of their resemblance to *T. rangeli* in blood and/or in culture, mammalian host restriction and different behaviour in distinct triatomine species, some trypanosomes from non-human primates, sloths, anteaters and opossums have been identified as *T. rangeli*-like or allied species: *T. cebus*; *T. diasi*; *T. saimiri*; *T. myrmecophage*; *T. preguici*; *T. mesnilbrimonti*; *T. mycetae*; *T. leewennhoeki*, etc. (Hoare, 1972; D'Alessandro, 1976; Marinkelle, 1976; Shaw, 1985; D'Alessandro & Saravia, 1992). However, there are controversies about those species and their taxonomic positions remain to be revised. We do not known if there are trypanosomes genetically closer to *T. rangeli* which deserve specific status within the subgenus *Herpetosoma* or if the so-called *T. rangeli*-like trypanosomes are indeed different organisms worthy of specific names.

In this study, we isolated *T. rangeli* from sylvatic mammals of Brazilian Amazon, especially from non-human primates, and characterized them by comparing morphology and behaviour in triatomine bugs and mice. These isolates were also analysed by the RAPD method with the following purposes: (i) to evaluate the polymorphism among isolates from wild mammals and from man of Brazilian Amazon and their relatedness with those from other geographical origins; (ii) to investigate if the populations from sylvatic animals differed according to their host-species and/or geographical origin; (iii) to define the relationships of *T. rangeli* with *T. rangeli*-like and allied trypanosomes; (iv) to identify taxonomic markers for *T. rangeli*.

MATERIALS AND METHODS

Isolation and growth of trypanosomes

Trypanosomes were obtained by culture of the peripheral blood of wild mammals (Table 1) from Pará, Amazonas, Rondonia and Acre States of Brazilian Amazon (Fig. 1). Sylvatic animals were manipulated with authorization and according to the Brazilian Institute of Environment (IBAMA – Instituto Brasileiro de Meio Ambiente) recommendations. Isolates from anteaters and sloth had been previously obtained (Shaw, 1985), while those from monkeys and opossums were recently isolated. Isolation was done using BAB-LIT medium, consisting of Blood Agar Base (BAB-DIFCO) containing 15% sheep blood as a solid phase with an overlay of LIT medium supplemented with 15% foetal bovine serum (FBS), with incubation at 28 °C. Selected isolates were also co-cultivated with monolayers of $LLCMK_2$ and Hela cells in DMEM medium (GIBCO) containing 10% FSB. All *T. rangeli* isolates and related species were grown in BAB-LIT, by incubation at 28 °C. The other trypanosome species and *Blastocrithidia culicis* were grown in LIT medium with 10% FBS, at 28 °C. All trypanosomatids are cryopreserved in the Trypanosomatid Culture Collection (TCC) of the Department of Parasitology, University of São Paulo, Brazil.

Infection of triatomine bugs and mice

Fifth instar nymphs of triatomines (*Rhodnius neglectus*, *R. prolixus* and *Triatoma infestans*) were inoculated intracoelomically with stationary-phase cultures of *T. rangeli* (Hecker, Schwarzenbach & Rudin, 1990). The infected triatomines (~30 for each culture) were fed on normal mice every 15 days, and 20, 30, and 60 days p.i., haemolymph (H), digestive tube (DT) and salivary glands (SG) were smeared on glass sides, fixed with methanol and Giemsa stained. Balb/c mice were infected by inoculation (i.p.) of stationary-phase cultures containing metacyclic forms or metacyclics from SGs of triatomines ($\sim 10^6$ cells/mouse) and also by the bite of infected bugs. Mice blood samples were examined weekly from 5 to 90 days p.i. by the microhaematocrit method. *R. neglectus* was used for the xenodiagnosis of experimentally infected mice.

Light microscopy of trypanosomes from culture, triatomines and mice

Thin blood smears made from infected mice, naturally infected opossums, H, DT and SG contents of triatomines, and smears of logarithmic and stationary-phase cultures were fixed in methanol and Giemsa-stained for light microscopy.

DNA preparation and RAPD fingerprinting

DNA was extracted from cultured trypanosomes using the phenol/chloroform method. Crude preparations of DNA templates from smears of

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Table 1. *Trypanosoma rangeli* isolates and allied trypanosomes used in this study, geographical and host species of origin, and distribution in groups based on the RAPD-derived patterns and dendrogram (Fig. 3)

TryCC*	Organism†	Host origin		Geographical origin country (state) city§	Group RAPD
031	San Augustin (SA)	Man	*Homo sapiens*	Colombia	A
020	Macias	Man	*Homo sapiens*	Venezuela	A
022	Palma-2	Triatomine	*Rhodnius prolixus*	Venezuela	A
024	H8GS	Man	*Homo sapiens*	Honduras	A
220	AT-AEI	Monkey	*Saimiri sciureus*	Brazil (PA) Marajó Island	A
202	AT-ADS	Monkey	*Saimiri sciureus*	Brazil (PA) Marajó Island	A
369	ROma01	Opossum	*Didelphis marsupialis*	Brazil (RO) Monte Negro	A
382	ROma06	Opossum	*Didelphis marsupialis*	Brazil (RO) Monte Negro	A
086	AM80	Man	*Homo sapiens*	Brazil (AM) Rio Negro	B
261	AM11	Man	*Homo sapiens*	Brazil (AM) Rio Negro	B
205	M12229	Monkey	*Aotus* sp	Brazil (AM) Manaus	B
207	AE-AAA	Monkey	*Cebuella pygmaea*	Brazil (AC) Rio Branco	B
194	AE-AAB	Monkey	*Cebuella pygmaea*	Brazil (AC) Rio Branco	B
233	4-30	Monkey	*Saguinus labiatus labiatus*	Brazil (AC) Rio Branco	B
238	5-31	Monkey	*Saguinus labiatus labiatus*	Brazil (AC) Rio Branco	B
236	8-34	Monkey	*Saguinus fuscicollis weddelli*	Brazil (AC) Rio Branco	B
013	*T. preguici*	Sloth	*Choloepus didactylus*	Brazil (PA) Belém	B
010	*T. legeri*	Anteater	*Tamandua tetradactyla*	Brazil (PA) Belém	B
032	*T. legeri*	Anteater	*Tamandua tetradactyla*	Brazil (PA) Belém	B
014	PG	Man	*Homo sapiens*	Panamá	C
328	1625	Man	*Homo sapiens*	El Salvador	C
023	SC58	Rodent	*Echimys dasythrix*	Brazil (SC)	D
012	*T. saimiri*	Monkey	*Saimiri sciureus*	Brazil (AM) Manaus	NG

* TryCC, Numbers of the trypanosome cultures in the Trypanosomatid Culture Collection, Department of Parasitology, ICB, USP, São Paulo, Brazil.
† Original denomination of the isolates.
§ Geographical origin of the trypanosomes: Brazilian States: (AM), Amazonas; (PA), Pará; (RO), Rondonia; (AC), Acre; SC, (Santa Catarina).
NG, Organism not grouped in any, of the above groups by RAPD analysis.

triatomines (SG, DT and H) on glass-slides were obtained as described (Serrano *et al*. 1999), except for the use of 0·5% Tween 20 instead of 0·02% SDS. We initially tested 15 primers to amplify DNA from 4 *T. rangeli* isolates and then we selected the 625 (CCGCTGGAGC), 601 (CCGCCCACTG), 606 (CGGTCGGCCA) and 639 (ATCGAGCACC), that yielded the most discriminating RAPD patterns to analyse all isolates. Amplifications were performed in 50 μl reaction volumes using 50 ng of DNA, 2.5 U of *Taq* DNA polymerase, 0·2 mM each dNTP and 200 pM of each primer, for 34 cycles as follows: 1 min at 95 °C, 2 min at 37 °C and 2 min at 72 °C. The amplified products were separated on 2·0% agarose gels and stained with ethidium bromide. The molecular sizes of DNA fragments were determined using GeneRuler DNA Ladder Mix (MBI Fermentas).

RAPD data analysis

Digitized gel images were analysed using the RFLPscan Plus software (version 3.0, Scanalytics CSP Inc., Billerica, MA, USA). The discrete character matrix was analysed by RAPDistance software (version 1.03) for the calculation of genetic distances, using the Jaccard similarity coefficients, to produce a pairwise matrix, which was used to construct dendrograms based on the UPGMA and Neighbour-joining (NJ) methods as before (Serrano, Camargo & Teixeira, 1999). Bootstrap values (100 replicates) were calculated with the PHYLIP package SEQBOOT program.

Southern and slot blot hybridization analysis

Amplified fragments generated by primer 625 were separated in 2% agarose gels and transferred to Nylon membrane (Hybond-N, Amersham Pharmacia). Slot blots of genomic DNA (2·0 μg) were prepared as described (Ventura *et al*. 2000). A RAPD-derived DNA fragment generated by primer 625 (Tra625) from *T. rangeli* (Choachi) DNA was purified (Spin-X, Costar), labelled by random primed synthesis with [α-^{32}P] dCTP (Ready to Go kit, Amersham Pharmacia) and used as probe. Slot blots were pre-hybridized for 1 h and hybridized with Tra625 probe, at 37 °C, for 16–18 h in 2 × SSC, 2% SDS, 4·0 mM sodium pyrophosphate, and 40 mg/ml of salmon sperm DNA. Membranes were washed 3 times for 15 min each in 1 × SSC, 2% SDS and 4 mM sodium pyrophosphate at 55 °C.

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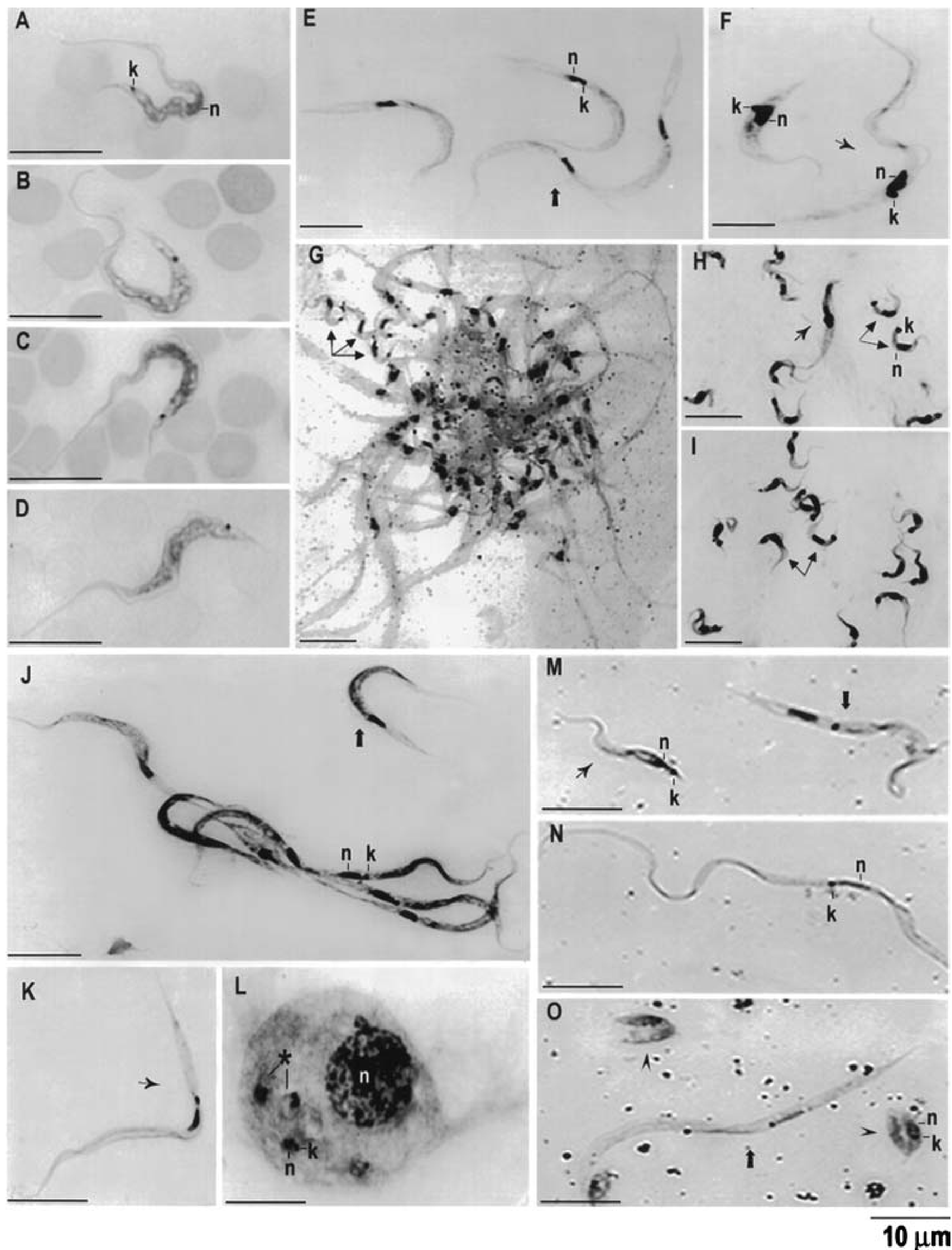

Fig. 1. Light microscopy of Giemsa-stained blood and culture smears of *Trypanosoma rangeli* and related species. Bloodstream trypomastigotes of (A) naturally infected opossum and (B) mouse experimentally infected with culture forms of isolate 369 obtained from the same opossum. Bloodstream trypomastigotes on blood smears of mouse experimentally infected with *T. preguici* (C) and *T. legeri* (D). Culture smears showing epimastigotes (E) and trypomastigotes (F) of isolate 194 (from monkey). Mass of epimastigotes and metacyclic trypomastigotes in the salivary glands of *R. prolixus* infected with *T. legeri* (G). Metacyclic trypomastigotes in the salivary glands of *R. neglectus* infected with monkey isolate 205 (H) or with human isolate AM80 (I). Haemolymph of *R. neglectus* infected with isolate 205 showing (J) epimastigotes, (K) trypomastigotes and (L) 'amastigote' and spheromastigote inside haemocytes. Digestive tube smears of *Rhodnius prolixus* infected with isolate 194 showing trypo- and epimastigote (M,N), spheromastigotes and epimastigotes (O). Spheromastigote (➢); metacyclic trypomastigote (⟶); 'amastigote' (✱); trypomastigote (➔); epimastigote (➡).

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Development of the specific T. rangeli *PCR assay (PCR-Tra625)*

Tra625 DNA fragments from selected trypanosomes were excised and purified from agarose gels, cloned (pGEM kit, Promega) and the nucleotide sequences of 2 clones from each isolate were determined by automated sequencing. The primers Tra625a and Tra625b (Fig. 5A) were designed based on the sequence of the Tra625 fragment for PCR amplification of a 300 bp DNA fragment (PCR-Tra625. Amplifications were done in 25 μl reaction volumes using 50 ng of DNA, 2.5 U of *Taq* DNA polymerase, 0·2 mM each dNTP and 8 μM of each primer through 30 cycles as follows: 1 min at 95 °C, 1 min at 66 °C and 1 min at 72 °C, with an initial cycle of 3 min at 95 °C, and a final extension cycle of 10 min at 72 °C.

RESULTS

Isolation of trypanosomes from sylvatic mammals and identification of T. rangeli *isolates*

In the present study trypanosomes were isolated by haemoculture of several sylvatic mammals from different regions of the Brazilian Amazon (Table 1; Fig. 1). No culture forms were obtained that were compatible with members of the subgenus *Megatrypanum* (Hoare, 1972). Haemocultures showing mixed infections of trypanosomes resembling *T. rangeli* and *T. cruzi* were inoculated in triatomines to recover *T. rangeli* from SG, thus separating them from *T. cruzi*. All new cultures were analysed regarding their development in triatomine and mouse to distinguish isolates of *T. rangeli* or related species. The PCR described by Vargas *et al*. (2000) were used for molecular diagnosis of *T. rangeli* (data not shown).

Growth in culture and light microscopy of trypanosomes

Blood forms were rarely observed in the natural hosts of the isolates. In anteaters, large blood forms compatible with *Megatrypanum* species were seen (Shaw, 1985), whereas blood forms typical of *Herpetosoma* were seen in opossums (Fig. 1A). Culture forms could be detected after 5–20 days of haemoculture, with growth and morphological features of isolates being essentially identical. The incapacity of the isolates to multiply within mammalian cells was demonstrated by co-cultivation with monolayers of $LLCMK_2$ and Hela cells at 37 °C, using *T. cruzi* (G) as a control of cell infection.

To illustrate the morphological features of isolates from wild mammals we selected isolates from different host species representative of distinct genetic groups: *T. rangeli* from monkeys (194, 205 and 220) and from opossum (369), *T. legeri*, *T. saimiri* and *T. preguici* (Table 1; Fig. 1). For comparative purposes, the human *T. rangeli* isolate (AM80) (Coura *et al*. 1996) was included (Fig. 1). Light microscopy of Giemsa-stained smears from recently obtained cultures revealed highly pleomorphic epi- (Fig. 1E) and trypomastigotes (Fig. 1F). After 20–30 days, logarithmic-phase cultures showed mostly long and slender epimastigotes (Fig. 1E) whereas small metacyclics typical of *T. rangeli* were observed in stationary-phase cultures (data not shown). Length and shape of body, size and position of kinetoplast, and undulant membrane of all isolates are compatible with those described for *T. rangeli* (Hoare, 1972; D'Alessandro & Saraiva, 1992).

Behaviour and morphology of T. rangeli *isolates in triatomine bugs and in Balb/c mice*

All new *T. rangeli* isolates developed in DT, H and SG of experimentally infected *R. neglectus*, especially when parasites were inoculated into the hemocoel (~90% of SG infection) rather than feeding on infected mice (xenodiagnosis). After about 30 days p.i., many insects died, confirming the pathogenicity of *T. rangeli* for its vector. DT of the triatomines showed large epi- and trypomastigotes, spheromastigotes and few short trypomastigotes, quite different from those of the SG (Fig. 1M–O). After the 10th day, long and slender epimastigotes, sometimes in the form of huge masses, short epimastigotes and trypomastigotes were seen free in the H (Fig. 1J, K) whereas 'amastigotes' or spheromastigotes were found inside haemocytes (Fig. 1L). After the 20th day p.i., few long epi- and trypomastigotes were present in the SG, in addition to a great number of metacyclic trypomastigotes with a large subterminal kinetoplast (Fig. 1G–I). Thus, triatomine infections developed as typically described for *T. rangeli* (Hecker, Schwarzenbach & Rudin, 1990).

Although the infection rate and the ability to produce metacyclic trypomastigotes in the SG was higher in *R. neglectus*, our new isolates of groups A and B also reach the SG's of *R. prolixus* and *T. infestans*. The behaviour of *T. saimiri* in triatomine bugs was identical to *T. rangeli*. Exceptions were the old cultures of *T. legeri* and *T. preguici* that, despite flagellates, could be frequently observed in the H, showed few metacyclics in the SG of only ~5% of the infected bugs, and exclusively in *R. neglectus*.

Metacyclics from both stationary cultures and from SG of triatomines infected with all new isolates infected Balb/c mice. The levels of parasitaemia were always very low, detectable by microhaematocrit from 3 to 15 days p.i. and lasting in general less than 1 month. The morphology of trypomastigotes seen in mouse blood (Fig. 1B, C, D) was typical of *T. rangeli*, and identical to forms present in the blood of naturally infected animals (Fig. 1A). Infection in mice was assessed by haemocultures (BAB/LIT) and by xenodiagnosis (*R. neglectus*).

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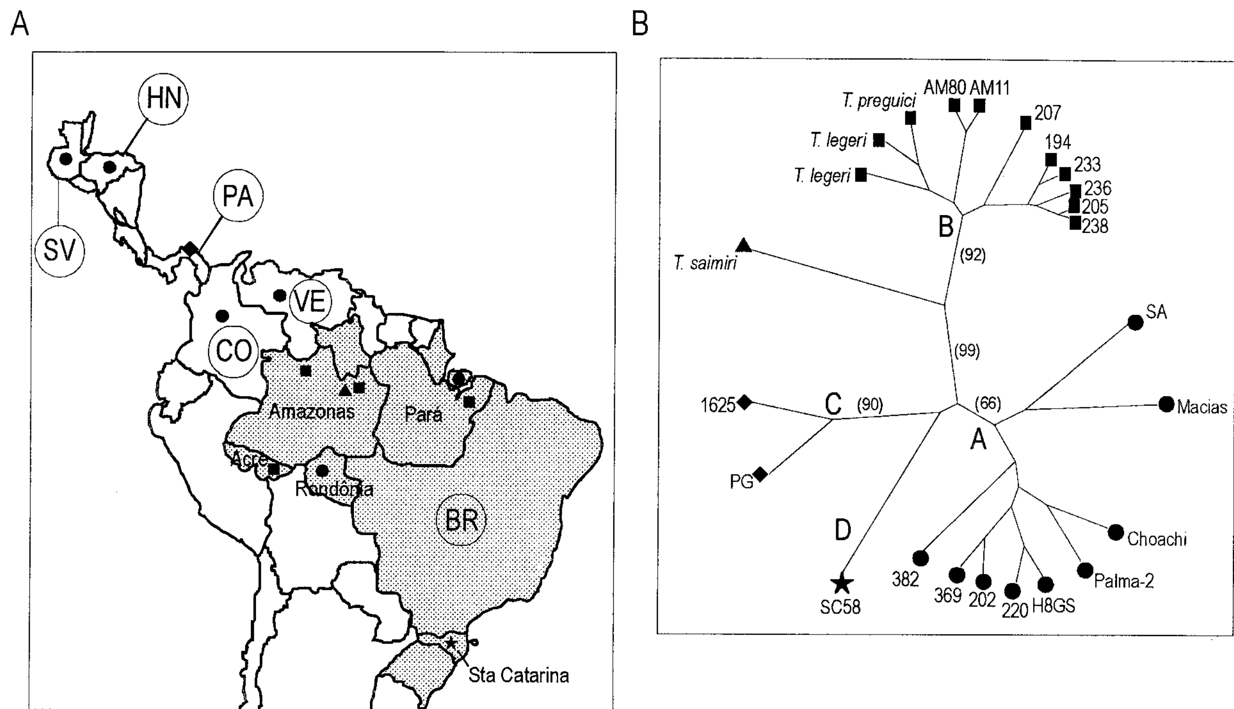

Fig. 2. (A) Geographical origin of *Trypanosoma rangeli* isolates and allied species employed in this study. Countries are indicated within circles: SV, El Salvador; HN, Honduras; PA, Panama; VE, Venezuela; CO, Colombia; BR, Brazil. Amazonas; Pará; Rondônia and Acre are states from Brazilian Amazon. (B) Genetic distance dendrogram based on RAPD patterns constructed using the UPGMA method. The numbers in parentheses refer to the bootstrap values of the clusters in 100 replicates. The organisms were segregated into groups A (●), B (■), C (◆), and D (★). *T. saimiri* (▲) was not grouped.

Clustering of T. rangeli *isolates based on analysis of RAPD patterns*

The polymorphism among *T. rangeli* populations from sylvatic mammals compared with isolates from humans and triatomines of different geographical origin was assessed by RAPD patterns. The branching patterns of UPGMA (Fig. 2B) and Neighbour-joining (data not shown) dendrograms were identical and supported by significant bootstrap values. *T. rangeli* isolates and related species were segregated into 2 major groups (A and B), besides 2 minor groups (C and D). Group A, which was composed of Northwest South American (Colombia and Venezuela) and Central American (Honduras) isolates, and from Brazilian isolates from Marajó Island, Pará (202 and 220), and Rondônia (369 and 382). Group B was composed exclusively of isolates from Acre, Amazônia and Pará States of the Brazilian Amazon. *T. saimiri*, although closer to group B was not included in this group due to the small percentage of RAPD bands shared with members of this group (Table 2). Isolates of group C (from Panama and El Salvador) and the isolate SC58 (South Brazil) constantly showed different RAPD patterns with all primers investigated (Fig. 3).

Topology of dendrogram (Fig. 2B) and similarity indexes determined by number of shared RAPD fragments indicated a close genetic relationship among populations within the groups contrasting with the low similarity between groups. The average similarity index of ∼58% and ∼64% were detected within groups A and B, respectively, whereas the similarity between these groups was only ∼13% (Table 2). The dendrogram revealed significant variability within groups A and B, especially in the last group which clearly demonstrated subclusters of organisms that could also be grouped by 639-RAPD patterns (Fig. 3).

Table 2. Percentage similarity of Randomly Amplified Polymorphic DNA (RAPD) bands shared within and among groups of *Trypanosoma rangeli* isolates and allied species

(A–D correspond to branches observed in the dendrogram of the Fig. 3.)

Group	A	B	C	D
A	58·72			
B	12·72	70·17		
C	31·29	20·81	71·88	
D	24·60	15·17	35·66	N.D.
T. saimiri	9·80	32·06	11·01	17·65

N.D., Similarity index of the group D was not determined because in this study it was used only the isolate (SC58) representing this group.

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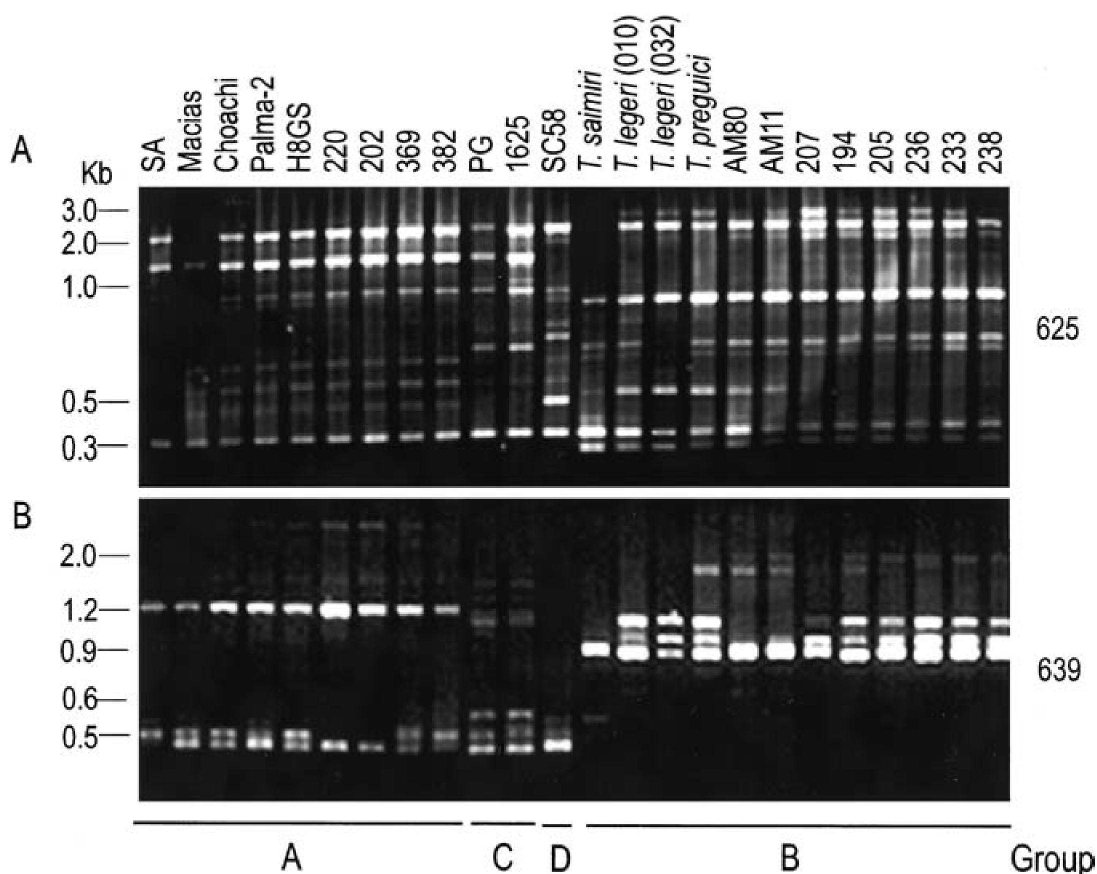

Fig. 3. Agarose gels (2%) stained with ethidium bromide showing RAPD patterns generated from DNA of *Trypanosoma rangeli* isolates and allied species using primers 625 and 639, selected to illustrate the genetic polymorphism (A) and grouping pattern (B) among the organisms.

Isolates from Panamá and El Salvador had a high similarity (72%) and formed the group C that was always separated from the others by significant divergences (21–36%). Similarly, isolate SC58, from a rodent of Southern Brazil was always distinct from the others and ascribed into group D (Fig. 2B, Table 2).

Characterization and evaluation of the species specificity of Tra625 sequence

The patterns generated by primer 625 revealed a DNA fragment (Tra625 of ~0·3 kb) shared by all *T. rangeli* isolates (Fig. 3A) but not by 7 other trypanosome species (Fig. 4A). To investigate whether the same-sized Tra625 fragment generated from DNA of all *T. rangeli* isolates and allied species consists of a conserved species-specific DNA sequence, the fragment taken from the isolate SA was used as probe. There was no cross-hybridization with any species except *T. rangeli* and allied species using both Southern Blot of amplified fragments generated by primer 625 (Fig. 4B) and slot blots of genomic DNA (Fig. 4C). Sequences of Tra625 fragments from 5 isolates representing the distinct groups (Choachi, SC58, *T. saimiri*, 220 and PG) were identical (Fig. 5A) and when submitted to Blast search of NCBI showed no significant similarity with any known sequences. The sequence data from Tra625 fragments have been submitted to GenBank and assigned to the following Accession numbers: Choachi (AY362188); SC58 (AY362190); *T. saimiri* (AY362192), 220 (AY362191) and PG (AY362189).

Evaluation of RAPD patterns as a taxonomic tool for identification of T. rangeli *groups*

Of the patterns generated by all primers, those obtained using primer 639 showed the clearest division of the isolates into the 4 groups (Fig. 3B), in a total concordance with the dendrogram (Fig. 2B), enabling *T. rangeli* isolates to be ascribed into their respective groups, without using other markers. Moreover, the small heterogeneity of 639-RAPD patterns within group B could be correlated with subclusters within this group also revealed by dendrogram branching pattern (Fig. 2B).

Standardization, specificity and sensitivity of the PCR-Tra625

To avoid the RAPD shortcomings with respect to reproducibility, sensitivity and requirement for purified and well-preserved DNA templates, we

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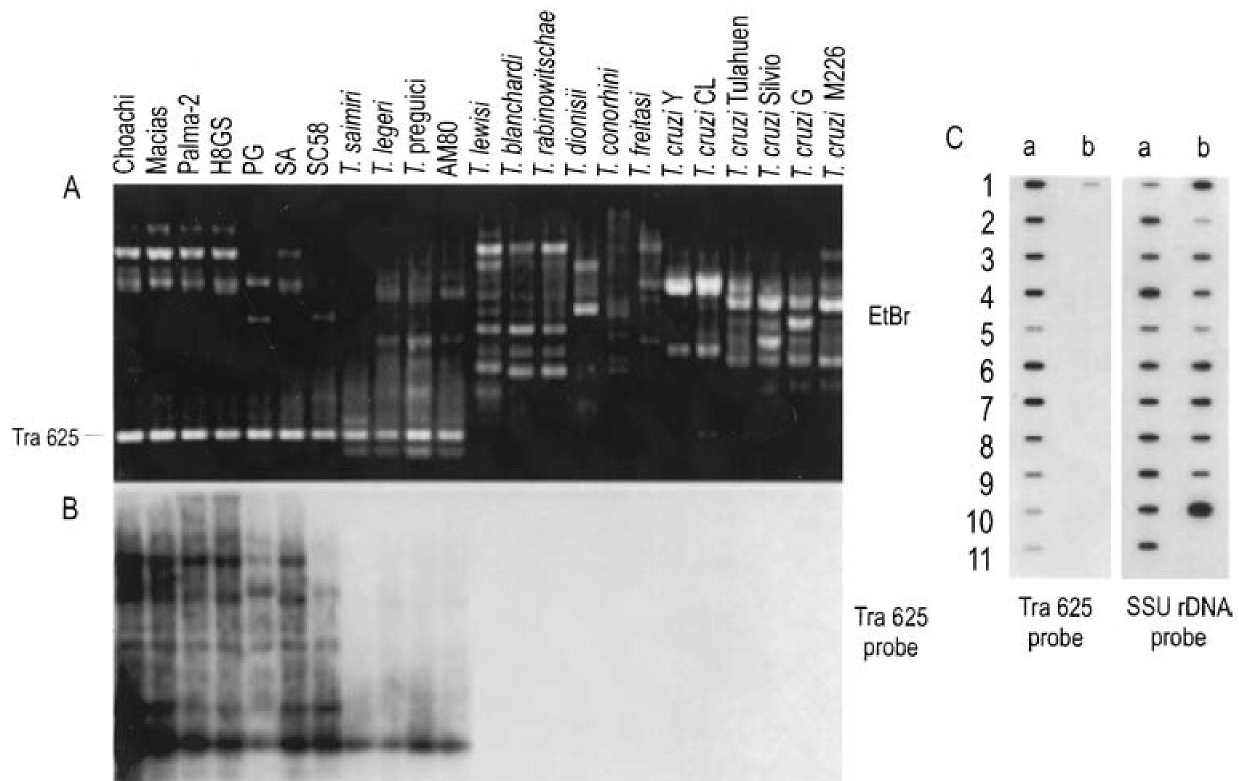

Fig. 4. (A) Agarose gels (2%) stained with ethidium bromide showing RAPD patterns generated by primer 625 using DNA of *Trypanosoma rangeli* isolates, related species (*T. saimiri*, *T. preguici* and *T. legeri*) and other trypanosomes. (B) Southern blot hybridization of the same gel (A) with Tra625 probe. (C) Slot blot of genomic DNA of trypanosomes hybridized with Tra625 probe and subsequently with SSU rDNA probe for DNA amount control. Tra = *T. rangeli*. 1a, *Tra* SA; 2a, *Tra* Macias; 3a, *Tra* H8GS; 4a, *Tra* 205; 5a, *Tra* PG; 6a, *Tra* Choachi; 7a, *Tra* Palma-2; 8a, *Tra* SC58; 9a, *T. saimiri*; 10a, *T. legeri*; 11a, *T. preguici*; 1b, *Tra* AM80; 2b, *T. lewisi*; 3b, *T. blanchardi*; 4b, *T. rabinowitschae*; 5b, *T. dionisii*; 6b, *T. conorhini*; 7b, *T. freitasi*; 8b, *T. cruzi* G; 9b, *T. cruzi* Y; 10b, DNA of Tra625 fragment from *T. rangeli* (SA) used as positive control; 11b, control without DNA.

developed a conventional PCR assay based on the Tra625 sequence for the diagnosis of *T. rangeli* (Fig. 5A). PCR-Tra625 amplified DNA from culture forms of all *T. rangeli* isolates and the related species (Fig. 5B). There was no amplification using DNA of the other trypanosome species previously tested by RAPD (Fig. 4 and data not shown) or *B. culicis*, a species of the genus *Blastocrithidia*, which is commonly harboured by triatomines (Fig. 5B).

The sensitivity of PCR-Tra625 was shown to be ~10 pg of DNA and could be enhanced to ~5·0 pg by post-hybridization of the amplified fragments with Tra625 probe (Fig. 5C), which correspond to ~50 cells. This assay is not sensitive enough for diagnosis of *T. rangeli* on blood of mammals. However, its suitability for field surveys of vectors was proved using as templates crude preparations from smears of DT, SG and H of triatomines infected with *T. rangeli* (Fig. 5D) or mixed-infected with both *T. rangeli* and *T. cruzi* (data not shown). Uninfected *R. prolixus* and *R. neglectus* DNA, mouse and human DNA and a tube without DNA were included as negative controls of PCR reactions. Identical results were obtained using either Tra625-PCR or the PCR method as described by Vargas *et al*. (2000) (data not shown).

DISCUSSION

Host and geographical ranges, genetic diversity, and the precise identification of the trypanosome species found in sylvatic animals is a fundamental problem in the epidemiology of American trypanosomiasis. In this study we have focused on elucidating the genetic diversity of *T. rangeli* populations from neotropical monkeys, opossums, sloth and anteaters of the Brazilian Amazon. Besides behaviours in mice and triatomines, all new isolates investigated in this study also showed morphological features compatible with *T. rangeli*. According to traditional taxonomical criteria, all new isolates from monkeys and opossums, and the previously classified *T. saimiri*, *T. preguici* and *T. legeri* can be classified as *T. rangeli*.

Cultures of isolates from wild mammals do not necessarily correspond to trypanosomes seen in blood, as these hosts can be infected by more than one species and only one can be isolated in culture.

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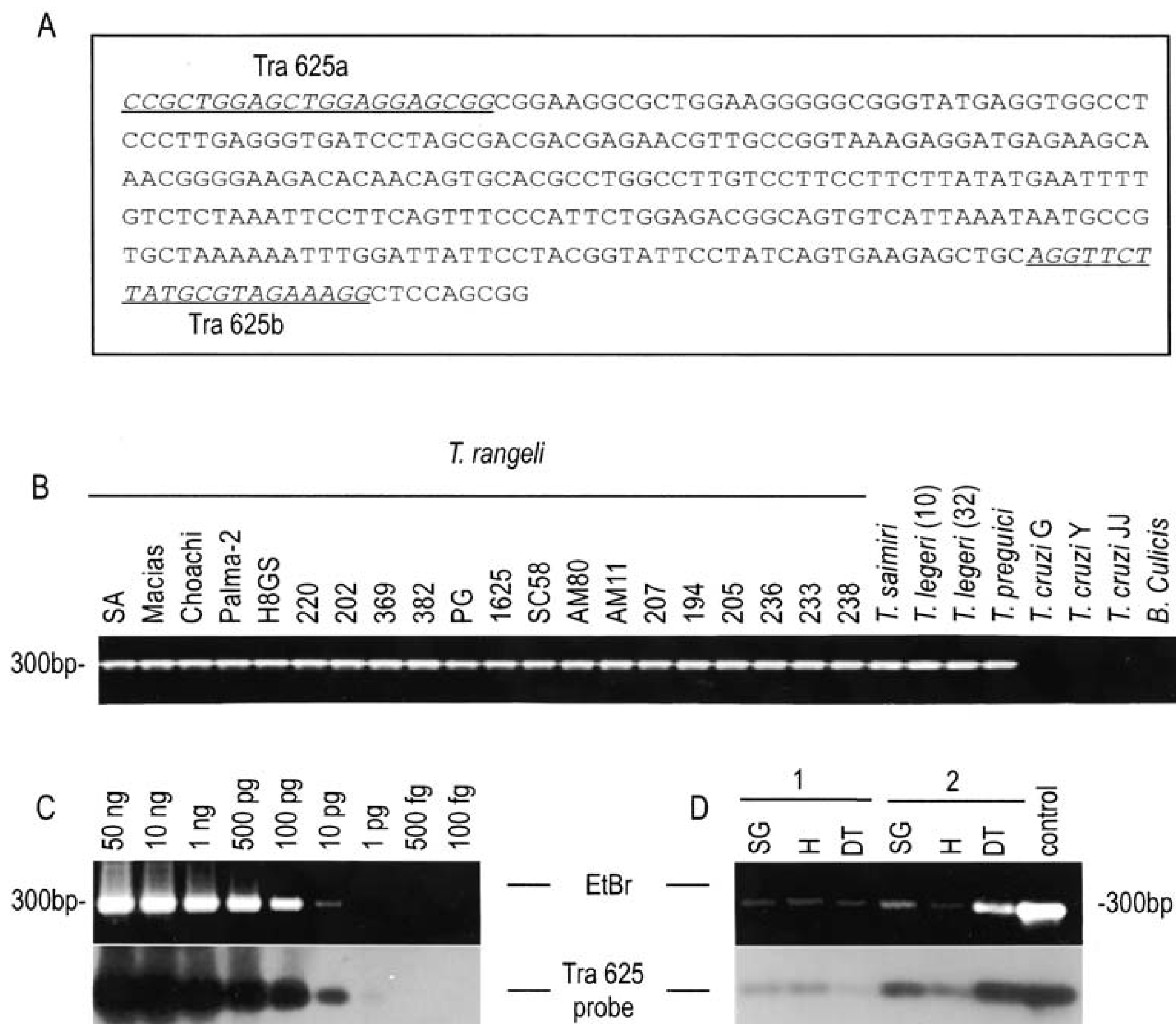

Fig. 5. (A) Nucleotide sequence of Tra625 fragment and localization of primers Tra625a and Tra625b (italicized and underlined, respectively). (B) Agarose gel (2%) stained with ethidium bromide (EtBr) showing DNA fragments generated by Tra625-PCR using genomic DNA from *Trypanosoma rangeli* isolates and related species and absence of DNA bands using DNA from *T. cruzi* isolates or from *Blastocrithidia culicis*. Sensitivity of the Tra625-PCR using purified DNA of *T. rangeli* stained with EtBr and after hybridization with Tra625 probe. (D) DNA bands generated by fTra625-PCR using as template crude preparations of DNA from smears of *Rhodnius prolixus* experimentally infected with *T. saimiri* (1) or *R. neglectus* infected with isolate 369 (2) in agarose gels (2%) stained with EtBr and hybridized with Tra625 probe.

This seems to be the case of the isolates classified as *T.* (*Megatrypanum*) *legeri* based on morphology of large blood trypomastigotes on anteaters, from which cultured flagellates were all revealed to be *T. rangeli*.

Traditionally, identifying trypanosomes as *T. rangeli* requires isolation by haemocultures, detection of metacyclic trypomastigotes in the SG of triatomines, and mice infection by bite or inoculation of metacyclic forms. Feeding directly on infected mammals may not be always successful for triatomine infection and even when inoculated directly into the haemocoel, infection of SG may not occur. This may be either due to specificity for sympatric vector species or caused by long periods in culture (D'Alessandro & Saravia, 1992). In addition, the ability to infect mice depends on the existence of large numbers of metacyclic forms, which is achieved only by recent cultures. Thus, identification of *T. rangeli* and descriptions of new species using exclusively this criteria must be avoided or done very carefully to avoid misclassification.

Based on RAPD analysis, all Brazilian populations from the Amazon segregated into two main groups. In Group A, originally described with isolates from Colombia and Venezuela (Grisard *et al.* 1999), we added Brazilian isolates from Pará (Marajó Island) and Rondônia. Group B, however, is a new assemblage of *T. rangeli* isolates composed exclusively of Brazilian isolates from Acre, Amazonas and Pará (Belém) States. The Panamanian and El Salvadorian isolates grouped together (Group C) and showed high genetic distances from all other isolates. The isolate from Southern Brazil (Group D), did not group with any other isolates examined either in this

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work or in a study based on mini-exon gene (Grisard *et al*. 1999). However, this isolate clustered with others from the same geographical origin, from the same rodent species and from *Panstrongylus megistus*, all sharing RAPD and isoenzyme patterns, thus constituting a homogeneous group from Southern Brazil (Steindel *et al*. 1994).

The high consistency of the two *T. rangeli* populations from Brazilian Amazon was ascertained by the highest genetic distances separating two groups of *T. rangeli*. Isolates from the same or neighbouring regions were distributed in both groups. Organisms from Pará (Belém), Amazonas and Acre exhibited marked similarity. However, other isolates from Pará (from Marajó Island, which is very close to Belém) and from Rondônia (close to Acre) were ascribed to group A, together with populations from Colombia and Venezuela. The partition of Brazilian Amazon isolates in this group had previously been demonstrated for a few isolates using SSU and ITS rDNA and mini-exon gene sequences (Silva *et al*. 1999), and is now being validated for all isolates (Maia da Silva *et al*., manuscript in preparation).

There are several *T. rangeli*-specific PCR assays based on several genes/sequences (Valejjo *et al*. 1999; Souto, Vargas & Zingales, 1999; Grisard *et al*. 1999; Vargas *et al*. 2000; Morales *et al*. 2002). However, most methods achieved remarkable separation of *T. rangeli* from *T. cruzi* ignoring other trypanosome species harboured by sylvatic mammals. Besides evaluation of few samples, not all genetic groups were tested by these methods, thus avoiding evaluation of suitability of these assays, despite the high intraspecific variability of *T. rangeli*. Because of the genetic complexity of *T. rangeli* populations, we decided to develop a PCR based on a RAPD-derived sequence. Besides high specificity to identify isolates from all genetic groups, suitability for testing crude DNA templates makes this method a reliable tool for detection of *T. rangeli* vectors.

According to our results, there are neither reliable differences in morphology or behaviour in mice and triatomines, nor sufficient degree of genetic polymorphism to classify any trypanosome from wild mammals examined in this study as *T. rangeli*-like. Moreover, we have no data to justify the status of separated species of *Herpetosoma* sp. allied to *T. rangeli*. Although *T. saimiri* differed from all isolates suggesting that this species perhaps deserves a separate name. *T. rangeli* isolates from South Brazilian and Central American sources were much more divergent. Moreover, it is believed that *T. saimiri* is restricted to the gut of triatomines and our *T. saimiri* behaviour in bugs was identical to *T. rangeli*. Host-restriction (Deane & Damasceno, 1961) was also not observed. This isolate infected mice as all *T. rangeli*, and two isolates from the same monkey species (*Saimiri sciureus*) differed from *T. saimiri* and clustered with typical *T. rangeli*. Morphology and triatomine behaviour of other *T. saimiri* isolates also indicated that this species is a synonym of *T. rangeli* (Ziccardi & Oliveira, 1998). Probably, the isolate here classified as *T. saimiri* belongs to an additional group of *T. rangeli*.

T. rangeli is a complex of isolates presenting particular genetic characteristics that permitted us to distribute the isolates into at least 4 distinct phylogenetic groups. The distribution of *T. rangeli* isolates in groups is independent of their host species and is not only determined by geographical isolation, despite some geographical segregation pattern. The same grouping defined by different and independent molecular markers suggested clonal evolution of *T. rangeli* populations. A recent study supported either clonal evolution or speciation of *T. rangeli* populations in their triatomine vectors (Valejjo *et al*. 2003). The isolate PG from Panama (Group C) can be distinguished by their inability to develop in vectors other than the sympatric species *R. pallescens* (Sousa & Dawson, 1976). Several studies on triatomine behaviours revealed vector restriction of some *T. rangeli* isolates to their local vector species (D'Alessandro, 1976; Machado *et al*. 2001; Guhl & Valejjo, 2003).

This is the first study to demonstrate that isolates from sylvatic mammals and man are highly genetically related, confirming the lack of host restriction of *T. rangeli* and suggesting that the same population can circulate among sylvatic mammals, humans and triatomines. We demonstrated that *T. rangeli* is more complex than previously described, with at least 4 genetic groups. More isolates from mammals and vectors of several geographical regions and new molecular markers must be investigated to make definitive statements concerning the determinant factors of this segregation as well as to ascribe some taxonomic status for each group.

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