Phylogeny of snake trypanosomes inferred by SSU rDNA sequences, their possible transmission by phlebotomines, and taxonomic appraisal by molecular, cross-infection and morphological analysis

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SUMMARY

Blood examination by microhaematocrit and haemoculture of 459 snakes belonging to 37 species revealed 2·4% trypanosome prevalence in species of Viperidae (*Crotalus durissus* and *Bothrops jararaca*) and Colubridae (*Pseudoboa nigra*). Trypanosome cultures from *C. durissus* and *P. nigra* were behaviourally and morphologically indistinguishable. In addition, the growth and morphological features of a trypanosome from the sand fly *Viannamyia tuberculata* were similar to those of snake isolates. Cross-infection experiments revealed a lack of host restriction, as snakes of 3 species were infected with the trypanosome from *C. durissus*. Phylogeny based on ribosomal sequences revealed that snake trypanosomes clustered together with the sand fly trypanosome, forming a new phylogenetic lineage within *Trypanosoma* closest to a clade of lizard trypanosomes transmitted by sand flies†. The clade of trypanosomes from snakes and lizards suggests an association between the evolutionary histories of these trypanosomes and their squamate hosts. Moreover, data strongly indicated that these trypanosomes are transmitted by sand flies. The flaws of the current taxonomy of snake trypanosomes are discussed, and the need for molecular parameters to be adopted is emphasized. To our knowledge, this is the first molecular phylogenetic study of snake trypanosomes.

Key words: *Trypanosoma*, snake, Squamata, sand fly, lizard, evolution, phylogeny, taxonomy, ribosomal gene, morphology.

INTRODUCTION

Trypanosomes are ubiquitous parasites of a large number of vertebrates and are usually transmitted by bloodsucking invertebrates. Arthropods (insects and ticks) are vectors of trypanosomes in mammals and birds, whereas leeches are vectors of trypanosomes in aquatic vertebrates. Leeches, flies and mosquitoes are implicated in the transmission of trypanosomes among amphibians and reptiles (Telford, 1995; Stevens *et al.* 2001; Hamilton *et al.* 2004, 2007). Among the reptilian trypanosomes, more species have been reported in lizards than in snakes, chelonians or crocodilians. In general, trypanosomes of snakes appear to be rare, despite reports of infection in snakes from distinct families inhabiting terrestrial and aquatic environments around the world. Pathogenicity of snake trypanosomes is virtually unknown (Telford, 1995).

The first references to the occurrence of trypanosomes in snakes appeared at the beginning of the 20th century in various continents (Wenyon, 1909; Brumpt, 1914), later followed by a few papers about snake trypanosomes in Brazil (Pessôa, 1928; Arantes and Fonseca, 1931*a*; Fonseca, 1935), Africa (Fantham and Porter, 1950, 1953) and North America (Ayala *et al.* 1983; Chia and Miller, 1984). Most papers on snake trypanosomes are only occurrence reports or morphological descriptions. Growth of these trypanosomes could not be sustained for more than a few passages in culture. Culturing was only successful with the following trypanosomes: *T. hydrae* and *T. yaegeri* from the aquatic snakes *Nerodia fasciata confluens* and *Agkistrodon piscivorus leucostoma*, respectively (Ayala *et al.* 1983; Chia and Miller, 1984) and *T. butantanense* from *Ophis merremii* (Arantes and Fonseca, 1931*a*) but no

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† Nucleotide sequence data reported in this paper are available in the GenBank database under the Accession numbers listed in Table 1.

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further studies of these trypanosomes were carried out.

Telford (1995) registered 21 species of snake trypanosomes. Several species should be synonymies and at least 2 species of trypanosomes from Brazilian snakes (*T. merremii* and *T. manguinhensis*) (Arantes and Fonseca, 1931*b*) were left out of his revision list. Snake trypanosomes have only been found in the blood of their hosts, and tissue forms have never been described. Morphology of the blood forms allows distinction of 2 major groups. One is represented by large and wide trypomastigotes with few dividing forms in peripheral blood (50–100 μm long and up to 3–5 μm wide) and includes *T. najae*; *T. clozeli* and *T. primeti* (cited by Telford, 1995); *T. brazili* (Brumpt, 1914; Brygoo, 1965*a*); *T. phylodriasi* (Pessôa, 1928); *T. constrictor* and *T. salamantae* (Pessôa and Fleury, 1969); *T. merremii* (Arantes and Fonseca, 1931*b*); *T. cascavelli* (Pessôa and De Biasi, 1972) and *T. yaegeri* (Ayala *et al*. 1983). The other group includes slender trypanosomes, shorter than 50 μm, with frequent dividing blood forms: *T. erythrolampi* (Wenyon, 1909), *T. butantanense* (Arantes and Fonseca, 1931*a*), *T. mattogrossense* (Fonseca, 1935) and *T. hydrae* (Ayala *et al*. 1983; Chia and Miller, 1984). Between the two groups lies *T. haranti*, which is short (31 μm) and wide (8–10 μm) (Brygoo, 1965*b*).

Aquatic leeches have been implicated as trypanosome vectors for hosts that live all or part of their lives in an aquatic environment, such as fishes and species of aquatic snakes, chelonians and anurans (Telford, 1995; Stevens *et al*. 2001; Hamilton *et al*. 2004; Ferreira *et al*. 2007). While the role of leeches as vectors of species that parasitize aquatic snakes is well documented, the transmission of trypanosomes to terrestrial snakes is poorly understood. Brumpt (1914) and Brygoo (1965*a*) reported on the development of *T. brazili* in the leech *Placobdella brasiliensis* fed on the aquatic snake *Helicops modestus*. Chia and Miller (1984) reported the development of *T. hydrae* in the leech *Placobdella parasitica*. Pessôa (1968) described the development of *T. hogei* in the leech *Haementeria lutzi* when it fed upon an infected snake *Rachidelus brazili*. These infected leeches failed to transmit their trypanosomes to *Helicops modestus*, suggesting host-restriction (Pessôa, 1968). Attempts to transmit snake trypanosomes through mosquitoes failed in all cases (Pessôa and De Biasi, 1972; De Biasi *et al*. 1975; Ayala *et al*. 1983). Trypanosomes of anurans and lizards can be transmitted by sand flies (Anderson and Ayala, 1968; Ayala and McKay, 1971; Ayala, 1971; Telford, 1995). However, there are no data about the role of sand flies as vectors of snake trypanosomes.

The evolutionary history of reptilian trypanosomes is far from understood. Although only 6 species of reptilian trypanosomes have been included in phylogenetic analyses to date, the results revealed that these trypanosomes are highly divergent and polyphyletic. Trypanosomes from major reptilian groups (lizards, chelonians and crocodilians) were separated in distant clades. *T. chelodina* from tortoise nested in the fish–trypanosome clade (species transmitted by aquatic leeches), whereas *T. grayi* that infects crocodiles and is transmitted by the tsetse fly, was positioned closest to avian trypanosomes. Lizard (Order Squamata) trypanosomes of Varanidae (*T. varani*) and Phrynosomatidae (*T. scelopori*) clustered with a trypanosome from Gekkonidae, forming the lizard clade. *T. therezieni* from Chamaeleonidae clustered with anuran trypanosomes (Haag *et al*. 1998; Stevens *et al*. 2001; Jakes *et al*. 2001; Hamilton *et al*. 2004, 2007). According to data from these studies, positioning of lizard trypanosomes within *Trypanosoma* is still unresolved, varying according to genes, taxa and methods used for phylogenetic inferences.

Altogether, biological and molecular data on reptilian trypanosomes suggest complex, distinct relationships between these parasites and their vertebrate and invertebrate hosts. Moreover, the data indicate a complex evolutionary history of trypanosomes even when isolated from the same order (Squamata). Analysis of a large number of trypanosomes and their putative vectors is crucial to an understanding of the evolutionary history of reptilian trypanosomes. The taxonomy of snake trypanosomes also lacks consistency, and the validity of species assigned to these trypanosomes has long been questioned (Brygoo, 1965*b*; Telford, 1995). In fact, all the existing species have been created according to morphology and host origin, which are insufficient taxonomic criteria for trypanosomatids in general (Wallace *et al*. 1983).

In this study, we isolated and characterized snake trypanosomes with the aim of establishing a molecular basis for the identification of species using methods other than morphological and host restriction analysis. We also characterized a trypanosome from sand flies that is closely related to snake trypanosomes. SSU rDNA sequences were used to infer phylogenetic relationships among snake, lizard and sand fly trypanosomes and between these trypanosomes and species from other reptiles and vertebrates.

MATERIALS AND METHODS

Handling of snakes

In this paper we report on the presence of trypanosomes in snakes sent to the Butantan Institute, São Paulo, Brazil, the major centre for the production of snake anti-venom sera. Snakes were captured in various regions of Brazil between 2000 and 2005. After being anaesthetized with CO_2, they were bled

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by tail or heart puncture using sodium citrate as anticoagulant. Infection of snakes was carried out by intravenous inoculation through the caudal vein. Prior to use, snakes for experimental infections were examined for trypanosome infection by repeated examination of blood samples by haemoculture and kept in separate cages. All procedures were performed according to the recommendations of Butantan Institute Ethics Committee.

Isolation and culture of snake trypanosomes

Blood samples from snakes were examined for the presence of trypanosomes by microhaematocrit centrifugation (MH). Regardless of the MH results, the blood samples were inoculated in tubes containing biphasic medium (BAB-LIT) consisting of 15% rabbit red blood cells mixed with 4% Blood Agar Base (BAB, DIFCO) overlaid with LIT (Liver Infusion Tryptose) medium supplemented with 10% FBS (Ferreira *et al*. 2007). Because this medium does not support continuous trypanosome growth, primary cultures were transferred to a monolayer of Hi-5 insect cells (*Trichoplusia ni*) overlaid with Grace's medium supplemented with 10% FBS.

For morphological analysis, glass-slide smears were prepared from the snakes' blood using either whole blood samples, buffy coats from MH capillary tubes or culture supernatants. All smears were fixed with methanol, stained with Giemsa and photographed with a digital camera.

Isolation and culture of trypanosomes from sand flies

The trypanosomes from sand flies used in the present study were isolated as previously described (Freitas *et al*. 2002). The sand flies were captured using CDC light traps suspended at ~1 m from the ground and from the bases of trees by aspiration of the trunk in the northern region of Rondonia State, Brazilian Amazonia. The isolate from the sand fly *Viannamyia tuberculata* was cultured as described above for snake isolates.

Experimental infection of snakes with trypanosomes

For experimental infections of snakes, cultured trypanosomes of *Crotalus durissus* were inoculated into the caudal vein of either the same species of snake or snakes of different species, namely, *Bothrops moojeni* and *Oxyrhopus guibei*. The trypanosome forms and number of inoculated parasites varied according to the experiment. Infection was assessed by MH analysis of blood samples collected by tail bleeding from day 15 to day 60–120 at intervals of 20–30 days. Parasitaemias were assessed by determining the number of flagellates in MH buffy coats smeared on glass slides and stained by Giemsa.

PCR amplification of SSU rDNA, sequencing and data analysis

Genomic DNA of cultured trypanosomes was extracted by the classical phenol-chloroform method. PCR amplifications of the V7–V8 SSU rDNA regions or whole SSU rDNA were carried out using the oligonucleotides and reaction conditions as described previously (Rodrigues *et al*. 2006; Ferreira *et al*. 2007). The PCR products were automatically sequenced, and the sequences obtained were aligned using ClustalX (Thompson, 1997). The resulting alignment was manually refined. Phylogenetic inferences were assessed by Parsimony (P) and Bayesian (B) methods. Parsimony analyses performed in PAUP* v4b10 via 100 replicates of random addition sequence followed by branch swap (RAS-TBR) and bootstrap analysis (100 replicates) was carried out as previously described (Ferreira *et al*. 2007). Distance matrices were generated using uncorrected *p*-distance. Bayesian analysis was performed in MrBayes v3.1.2 (Huelsenbeck *et al*. 2001). Tree searches employed GTR plus gamma and a proportion of invariable sites. To infer phylogenetic relationships, sequences of snake trypanosomes determined in this study (Table 1) were aligned with sequences of several other trypanosomes (Fig. 3A), which were generated by previous studies (Lukes *et al*. 1997; Haag *et al*. 1998; Stevens *et al*. 2001; Hamilton *et al*. 2004), recovered from GenBank (Accession number): (a) Trypanosomes from lizards, *T. therezieni* (AJ223571); *T.* sp Gecko (AJ620548); *T. scelopori* (U67182); *T. varani* V54 (AJ005279); crocodile, *T. grayi* (AJ005278), and tortoise, *T. chelodina* (AF297086); (b) trypanosomes from anurans, *T. rotatorium* (B2-II) (AJ009161); *T. mega* (AJ223567); *T. fallisi* (AF119806); *T. chattoni* (AF119807) plus Brazilian isolates from toads and frogs, *T.* sp 444 (EU021225); *T.* sp 406 (EU021236); *T.* sp 362 (EU021232); *T.* sp 316 (EU021226); *T.* sp 364 (EU021229); (c) Trypanosomes from fish, *T. triglae* (U39584); *T. boissoni* (U39580); *T. cobitis* (AJ009143); Sequences of trypanosomes from mammals and birds were also included: (a) Mammals, *T. vivax* (U22316); *T. brucei rhodesiense* (AJ009142); *T. congolense* (savannah) (AJ009146); *T. simiae* (AJ009162); *T. theileri* (AJ009164); *T. cyclops* (AJ131958); *T.* sp (ABF) of wallaby (AJ620564); *T. microti* (AJ009158); *T. lewisi* (AB242273); *T. dionisii* (AJ009152); *T. cruzi* (VINCH 89) (AJ009149); *T. cruzi marinkellei* (AJ009150); *T. conorhini* (AJ012411); *T. rangeli* (AJ009160); (b) Birds, *T. bennetti* (AJ223562); *T. corvi* (AY461665); *T. avium* (APO1) (AF416559); *T. avium* (SIM3) (AF416563). Sequences of trypanosomes from sand flies were also added to the aligned sequences: *T.* sp 103 (EU021237), *T.* sp 887 (EU021245) and *T.* sp 888 (EU021241). Other sequences included in the alignment were *T. binneyi*

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Table 1. Trypanosomes from snakes, lizards and sand fly nested in the clade lizard/snake positioned in the phylogenetic tree inferred in this study (Fig. 2)

(TryCC, Trypanosomatid Culture Collection, Department of Parasitology, ICB, USP, Brazil.)

TryCC code	Trypanosome		Host origin	Geographical origin	Vector	Accession number GenBank SSU rDNA
425	*T. cascavelli*[1]	Snake	*Crotalus durissus*	Brazil	Sand flies[5]	EUO95837
621	*T. cascavelli*[1]	Snake	*Crotalus durissus*	Brazil	Sand flies[5]	EUO95840
629	*T. cascavelli*[1]	Snake	*Crotalus durissus*	Brazil	Sand flies[5]	EUO95841
630	*T.* sp[1]	Snake	*Crotalus durissus*	Brazil	Sand flies[5]	EUO95842
631	*T. cascavelli*[1]	Snake	*Crotalus durissus*	Brazil	Sand flies[5]	EUO95843
632	*T.* sp[1]	Snake	*Crotalus durissus*	Brazil	Sand flies[5]	EUO95844
693	*T. cascavelli*[1]	Snake	*Crotalus durissus*	Brazil	Sand flies[5]	EUO95845
1052	*T.* sp[1]	Snake	*Pseudoboa nigra*	Brazil	Sand flies[5]	EUO95839
910	*T.* sp[1]	Sand fly	*Viannamyia tuberculata*	Brazil	—	EUO95838
—	*T. scelopori*[2]	Lizard	*Sceloporus occidentalis*	USA	Sand flies[6]	U67182
—	*T. varani* V54[3]	Lizard	*Varanus niloticus*	Senegal	Sand flies[6]	AJ005279
—	*T.* sp (gecko)[3,4]	Lizard	*Tarentola annularis*	Senegal	Sand flies[6]	AJ620548

[1] Trypanosomes isolated in this study.
[2] Ayala (1970).
[3] Minter-Goedbloed *et al*. (1993)
[3,4] Hamilton *et al*. (2004).
[5] Putative vectors based on molecular and/or field evidence.
[6] Experimentally confirmed vectors.

from platypus (AJ132351) and *T.* sp (K&A) from aquatic leech (AJ009167). The alignment used in this study is available from the authors upon request.

RESULTS

Occurrence and morphology of trypanosomes in the blood of the snakes investigated

We examined the blood of 459 snakes belonging to 37 different species in 4 families by microhaematocrit (MH) and haemoculture methods. The species were (a) Aniilidae – *Anilius scytale* (6 individuals examined); (b) Boidae – *Boa constrictor* (7), *Corallus hortulanus* (1); (c) Colubridae – *Chironius bicarinatus* (4), *C. exoletus* (12), *C. fuscus* (2), *C. quadricarinatus* (3), *C. scurrulus* (1), *Dipsas catesbyi* (1), *Drymarchon corais* (2), *Echinanthera* sp. (2), *Leptodeira annulata* (8), *Leptophis ahaetulla* (3), *Liophis almadensis* (2), *L. atraventer* (1), *L. miliaris* (1), *L. poecilogyrus* (4), *L. reginae* (3), *L. typhlus* (1), *Mastigodryas boddaerti* (1), *Oxyrhopus trigeminus* (1), *O. clathratus* (1), *O. guibei* (12), *Philodryas olfersii* (3), *P. patagoniensis* (4), *Pseudoboa nigra* (1), *Rachidelus brazili* (2), *Sibynomorphus mikanii* (1), *Spilotes pullatus* (9), *Thamnodynastes rutilus* (4), *T. strigatus* (1), *Tomodon dorsatus* (12), *Tropidodryas* sp. (2), *Xenopholis undulatus* (1); (d) Viperidae – *Bothrops* spp. (202), *Crotalus durissus* ssp. (134), *Lachesis muta* (4).

Microhaematocrit examination was positive for 7 *Crotalus durissus* (rattlesnake, cascavel) and for 4 *Bothrops jararaca* (lancehead, jararaca). Blood smears from rattlesnakes showed large and wide trypomastigotes (average 55 μm long by 8 μm wide) pointed at the extremities, with a large undulating membrane and usually a short free flagellum. The kinetoplast was very small and positioned adjacent to the outer body margin, distant from the large, ellipsoid and nearly central nucleus. Trypomastigotes were usually coiled in a circle with long overlapping extremities (Fig. 1A1) and dividing forms were not observed. No trypanosomes were found in lancehead blood smears, but microscopy analysis directly of the MH buffy coats revealed trypanosomes similar to those in the rattlesnake blood smears. Blood trypomastigotes from rattlesnakes examined in this study were indistinguishable from those of *T. cascavelli* first described by Pessôa and De Biasi (1972) in the same snake species, but could easily be distinguished from those of *T. butantanense* from *Ophis merremii* shown in microphotographs of blood smears used for its first description in an earlier study (Arantes and Fonseca, 1931*a*) (Fig. 1A2).

Isolation, growth and morphology of culture forms of snake trypanosomes

Snake blood samples examined for the presence of trypanosomes by haemocultures revealed 7 positive cultures from *C. durissus* (Viperidae) from southeastern Brazil and 1 from *Pseudoboa nigra* (Colubridae) from central Brazil (Table 1). Positive cultures from *C. durissus* came from snakes with positive MH whereas the culture from *P. nigra* came from a specimen that was not examined by MH.

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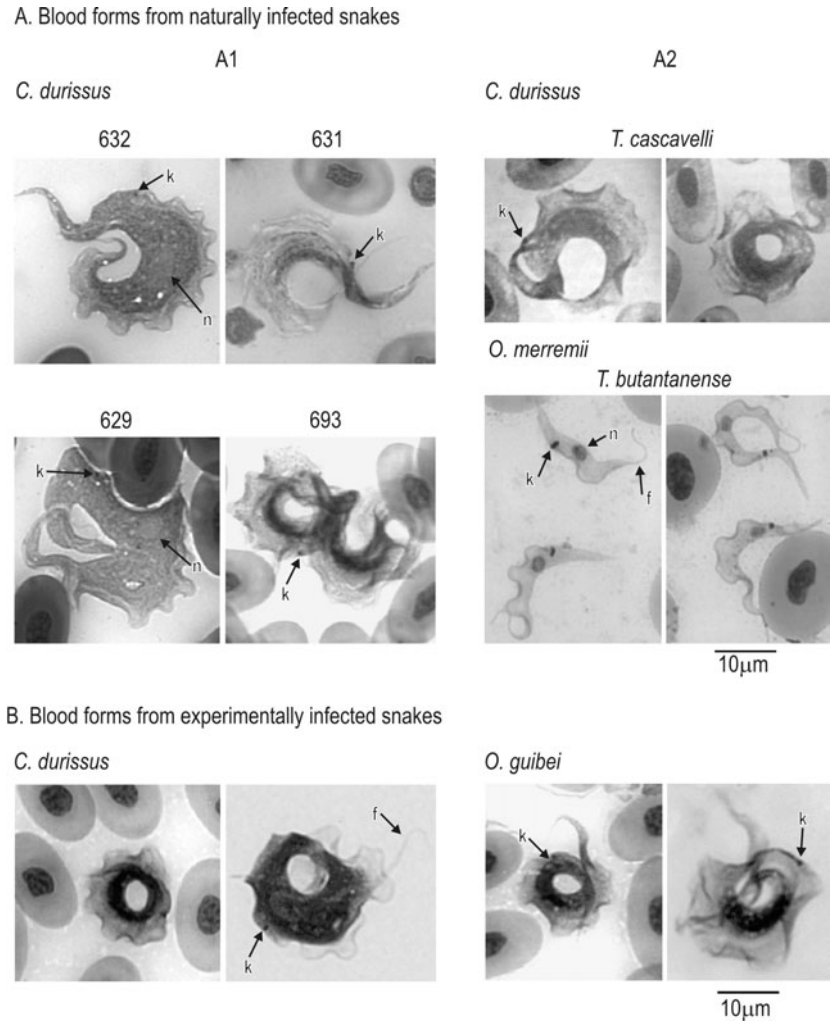

Fig. 1. Selected microphotographs (Giemsa-stained) of blood trypanosomes from naturally and experimentally infected snakes. (A1) Blood trypomastigotes of specimens of *Crotallus durissus* from which isolates 632, 631, 629 and 693 were obtained; (A2) blood trypomastigotes from archived slides used for the first description of *Trypanosoma cascavelli* from *C. durissus* and *Trypanosoma butantanense* from *Ophis merremii*. (B) Blood trypomastigotes of *C. durissus* and *Oxyrhopus guibei* snakes experimentally infected with cultured forms of isolate 425 from *C. durissus*. k, Kinetoplast; f, flagellum; n, nucleus.

Low parasitaemias may be responsible for negative results of blood smears and MH. On the other hand, haemocultures can be negative for MH-positive snakes. This was the case with *B. jararaca* (Viperidae), which although having a positive MH consistently failed to yield positive haemocultures suggesting different growth requirements. All cultures were stored in liquid nitrogen in our culture collection.

Cultures of trypanosomes from *C. durissus* (isolate 425) and *P. nigra* (isolate 1052) yielded abundant growth when co-cultivated over a monolayer of Hi-5 insect feeder cells. In culture, isolates from both snakes showed similar morphological features and growth behaviour. The morphology and developmental stages varied according to the culture phase (Fig. 2). After an almost quiescent phase of ~7 days, the number of flagellates increased markedly (log phase). In this phase there was a predominance of epimastigotes of different sizes and shapes, most of which were slender and had a sharp posterior extremity, a small dot-like kinetoplast positioned very close to the nucleus, a small undulating membrane and a long flagellum (Fig. 2). At the end of the log-phase, after ~10 days, the cultures displayed large epimastigotes with an enlarged anterior end, central nucleus and kinetoplast, conspicuous undulant membrane and a long free flagellum. Large trypomastigotes began to appear in this phase and predominated in the end of stationary phase (~15 days), when 2 trypomastigote forms were observed (Fig. 2). One form displayed a wide and roll-shaped body with a conspicuous and many-folded undulating membrane and the kinetoplast closer to the nucleus than to the posterior end. These wide forms were similar in shape but smaller than the blood trypomastigotes (Fig. 1A). The other trypomastigote form was small, slender, with a narrow undulating membrane and a small kinetoplast situated between the nucleus and the posterior

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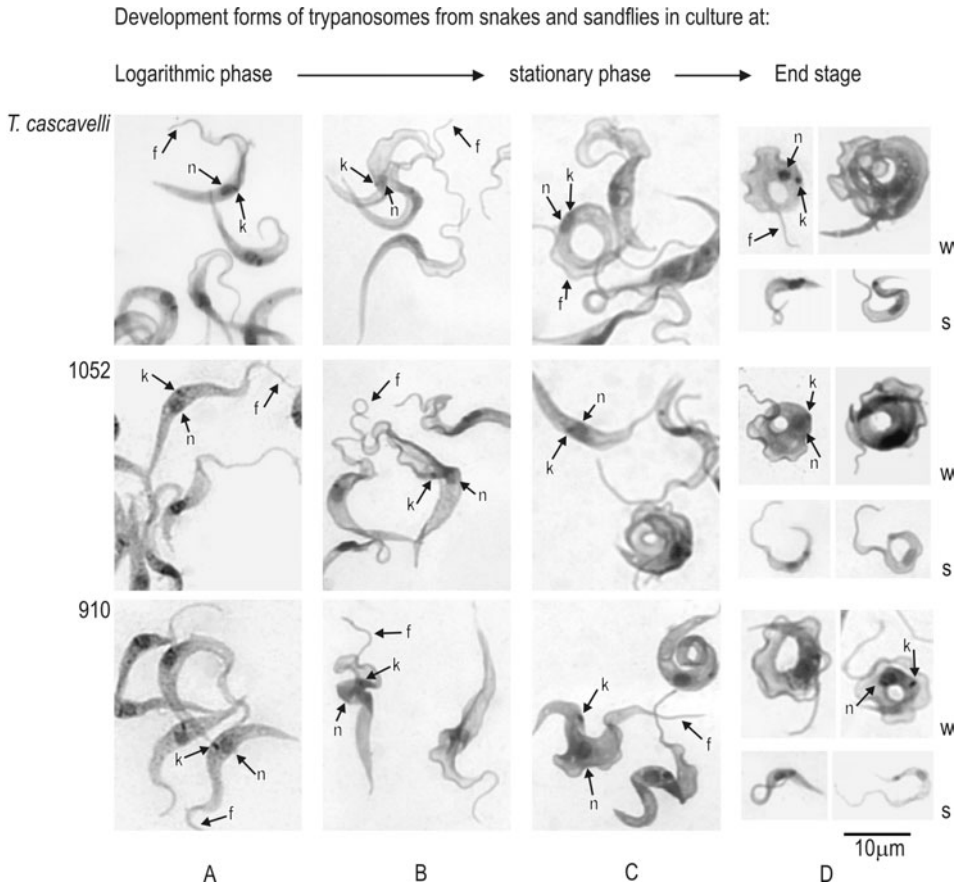

Fig. 2. Microphotographs (Giemsa-stained) selected to illustrate the development in culture of trypanosomes from snakes. Isolate 621 (*T. cascavelli*) from *C. durissus*; isolate 1052 from *Pseudoboa nigra*; and isolate 910 from sand fly. Sequential photographs represent the developmental forms observed in cultures of flagellates co-cultivated with Hi-5 insect feeder cells. (A) Epimastigotes (logarithmic phase, ~7 days); (B) enlarged epimastigotes (~10 days); (C) trypomastigotes and epimastigotes (stationary phase, ~15 days); (D) wide (w) and slender (s) trypomastigotes in the end stage of cultures (after 15 days). k, Kinetoplast; f, flagellum; n, nucleus.

end. These smallest forms, probably the metacyclic trypomastigotes, predominated in the end stage of cultures, and their number could be considerably increased when flagellates were transferred to Hi-5 cultures in Grace's medium containing only 2% FBS (Fig. 2).

Growth and morphology of culture forms of trypanosomes from sand flies

Trypanosome isolate 910 was recovered from the gut of a sand fly (*Viannamyia tuberculata*) captured in the Brazilian Amazonia that had a small number of flagellates in its anterior and posterior stomach. However, no red blood cells, which would indicate a recent blood meal, were observed in the fly's gut. This isolate exhibited the same growth and morphological features described above for snake trypanosomes (Fig. 2). This isolate was selected for this study because its V7–V8 SSU rDNA sequence was shown to be high, similar to corresponding sequences from snake trypanosomes. Other trypanosomes from sand flies included in this study differed in growth behaviour and morphology and clustered together with anuran trypanosomes in phylogenetic trees (Ferreira *et al.*, manuscript in preparation).

Experimental infection of snakes and cross-infectivity of snake trypanosomes

Culture forms (4×10^7) of rattlesnake trypanosomes (isolate 621) were inoculated into specimens of laboratory-raised *C. durissus*, which still had trypanosomes in their blood 120 days later. The course of infection differed according to the percentage of trypomastigotes (wide or slender) inoculated. Parasitaemia was significantly higher (maximum 4×10^3 flagellates/ml) in 2 snakes inoculated with ~70% slender and 30% wide forms than in 2 snakes inoculated with ~30% slender and 70% wide forms (maximum parasitaemia $3{\cdot}3 \times 10^2$ flagellates/ml). Snakes inoculated with epimastigotes did not become infected. Parasitaemias reached their highest levels in 2 weeks, remained high for 1 month and then gradually declined. The rattlesnakes with the highest parasitaemias became apathetic, asthenic and anorexic and died within 4 months. Giemsa-stained imprints of heart, spleen, bone marrow, brain

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and liver of these animals failed to reveal tissue trypanosomes.

For cross-infection experiments, cultured trypanosomes from rattlesnakes (isolate 425) were also inoculated (1×10^5 trypomastigotes, ~70% slender forms) in 1 snake of each of the following species: *Bothrops moojeni* (Viperidae), *Oxyrhopus guibei* (Colubridae) and *C. durissus* (Viperidae). All the infected snakes had large blood trypanosomes detectable by MH. The parasitaemias and course of infection varied according to the snake species. The number of blood parasites was always higher in rattlesnakes (maximum ~10^3 parasites/ml) than in *O. guibei* (maximum ~4×10^2 flagellates/ml). *B. moojeni* also became infected, but parasites were scarce and only detected by MH (maximum ~50 parasites/ml). Trypomastigotes found in blood from experimentally infected *C. durissus* and *O. guibei* were indistinguishable (Fig. 1B), but were clearly different from both wide and slender cultured trypomastigotes used for snake infections (Fig. 2). In addition, these forms were indistinguishable from those detected in the blood of naturally infected *C. durissus* (Fig. 1A).

Genetic diversity among snake trypanosomes assessed by sequence polymorphisms of the variable V7–V8 region of SSU rDNA

Sequences of the V7–V8 region of SSU rDNA were determined for the 8 snake-trypanosome isolates studied here. Comparison of aligned sequences revealed high sequence similarity among the 7 isolates from *C. durissus* (average of ~98% shared similarity). Only isolates 630 and 632 showed slight differences. In contrast, the *P. nigra* isolate diverged significantly from those from *C. durissus* (~93% similarity), and the resulting dendrogram clearly separated the cluster formed by the isolates from *C. durissus* from *P. nigra* isolate 1052 (Fig. 3B). The sequence of the trypanosome isolated from the sand fly *V. tuberculata* had an average of ~94% similarity with *C. durissus* trypanosome sequences, and smaller similarity (90%) with the *P. nigra* isolate. The highest similarities with sequences from snake trypanosomes were shared by 3 lizard trypanosomes, namely, *T. varani*, *T. scelopori* and *T.* sp gecko (average ~70%). *T. varani* was included in the dendrograms as an outgroup of snake trypanosomes (Fig. 3B).

Phylogenetic relationships among trypanosomes from snakes, other reptilians, sand flies and other vertebrates inferred from whole sequences of SSU rDNA

The phylogenetic relationships among trypanosomes from snakes and several species of *Trypanosoma* from other hosts was inferred using whole sequences of SSU rDNA from 1 isolate from *C. durissus* (isolate 425) and from the isolate 1052 from *P. nigra*. These sequences were aligned with all available sequences of reptilian trypanosomes and with selected sequences of trypanosomes from mammals, amphibians, fishes and birds. Phylogenies inferred using Parsimony (Fig. 3A) and Bayesian (data not shown) methods generated trees with very similar branching patterns, showing all major clades of trypanosomes. In both analyses, a clade was formed by snake and sand fly trypanosomes (snake clade, 100% bootstrap), which was always positioned closest to a clade formed by the lizard trypanosomes *T. varani*, *T. scelopori* and *T.* sp gecko (lizard clade, 100% bootstrap) (Fig. 3A).

The sand fly trypanosome (isolate 910) characterized in this study was tightly positioned together with the snake trypanosomes and analysis of whole SSU rDNA sequences confirmed that it is closest to *C. durissus* isolates (~98% similarity) than to the isolate 1052 from *P. nigra* (~93%). Three trypanosome isolates from sand flies (103, 887 and 888) clustered with anuran trypanosomes. Despite significant internal divergence, the clade formed by lizard and snake trypanosomes (lizard/snake clade) was well supported (100% bootstrap) and separated by a large genetic distance from all other clades. *T. therezieni* from chameleon clustered with anuran trypanosomes, and was the only squamate trypanosome positioned outside the lizard clade. The aquatic clade, constituted by leech-transmitted trypanosomes of fishes, tortoises and platypuses, was separated by a high genetic distance from the lizard/snake clade (Fig. 3A). Considering that previous phylogenetic analysis demonstrated that SSU rDNA gene sequences alone were not reliable markers for inferring deep level trypanosome phylogeny (Hamilton *et al.* 2004, 2007), we constructed an unrooted phylogeny to reveal the relationships among trypanosomes from snakes, other reptilians, sand flies and other vertebrates (Fig. 3A).

DISCUSSION

Despite several reports of trypanosomes in terrestrial and aquatic snakes not a single snake trypanosome has been molecularly characterized and, with the exception of the present study, snake trypanosomes have not been included in any phylogenetic studies.

The results of our surveys of snake blood showed a trypanosome prevalence of 5·6% for *C. durissus* and 2% for *B. jararaca*, with an overall prevalence of 2·33% in a total of 459 snakes from 37 species examined. In general, previous studies revealed low prevalence and parasitaemias of snake trypanosomes: De Biasi *et al.* (1975) had to examine 146 *Ophis merremii* in order to find an infected one; Pessôa and Fleury (1969) described 1 snake infected by *T. constrictor* and 2 infected by *T. salamantae*

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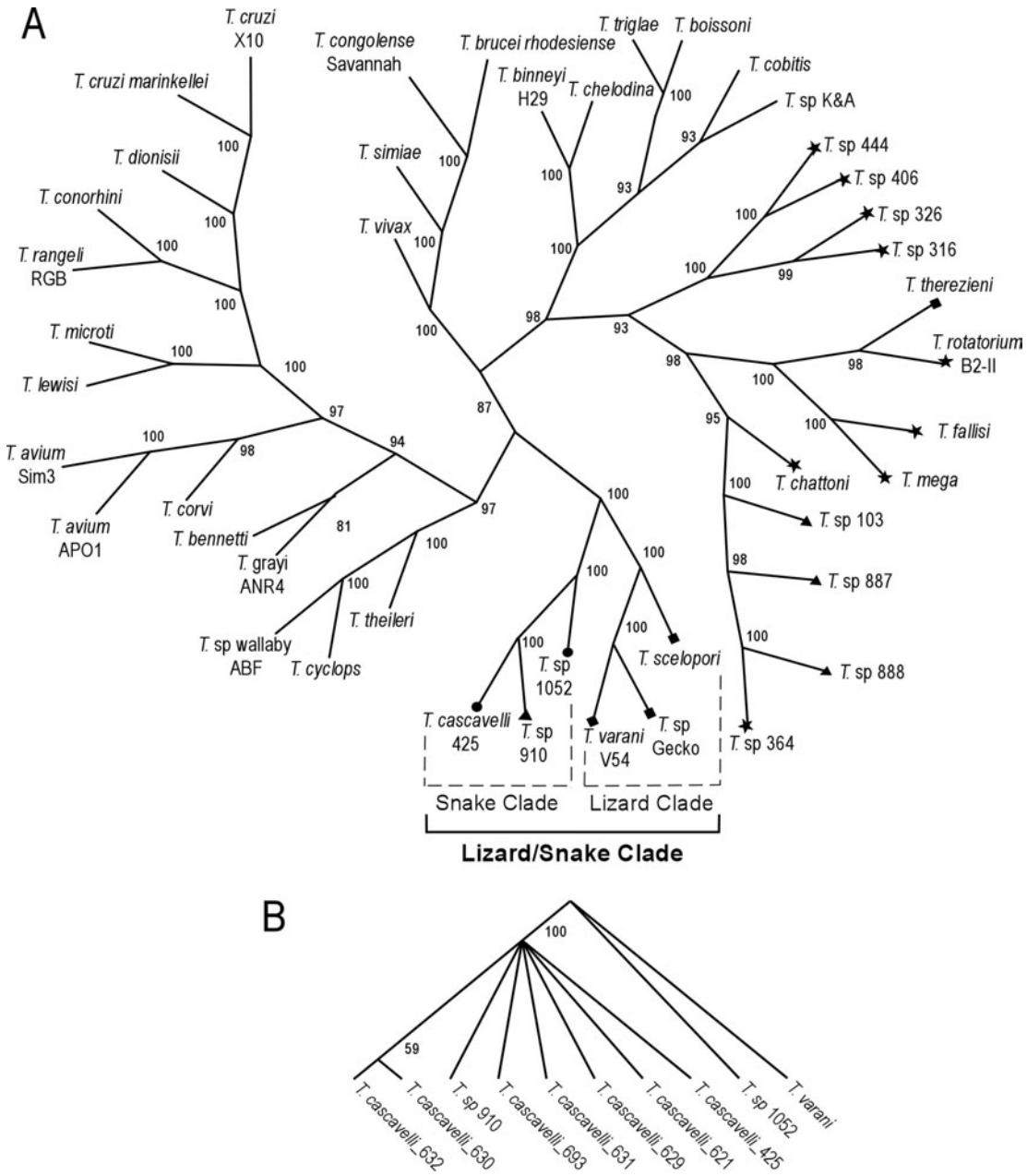

Fig. 3. Phylogenetic analyses based on ribosomal sequences inferred by Parsimony. (A) Unrooted phylogeny inferred using SSU rDNA sequences of 44 trypanosomes, including sequences of 2 isolates from snakes (●), 4 from lizards (■), 4 from sand flies (▲) and 8 from anurans (★). (B) Dendrogram based on V7-V8 SSU rDNA sequences from the following isolates nested in the snake clade: 5 isolates of *T. cascavelli* from *C. durissus;* *T.* sp 630 and *T.* sp 632 from *C. durissus;* *T.* sp 1052 from *P. nigra*; and *T.* sp 910 from sand fly. *T. varani* from lizard was used as outgroup for the trypanosomes of the snake clade. The numbers at the nodes (n) correspond to Parsimony percentage bootstrap values derived from 100 replicates.

among more than 700 specimens of Boidae examined; Pessôa and De Biasi (1972) had to examine 300 rattlesnakes to find 4 infected with *T. cascavelli*. In contrast, 30% of *Rachidelus brazili* were found infected with *T. hogei* (Pessôa, 1968). Also exceptional was the finding of snakes displaying moderate and high parasitaemias, such as *Ophis merremii* infected with *T. butantanense* (Arantes and Fonseca, 1931*a*) and the aquatic snakes *Nerodia fasciata confluens* and *Agkistrodon piscivorus leucostoma* infected with *T. hydrae* and *T. yaegeri*, respectively (Ayala *et al.* 1983).

Very little is known about host specificity in snake trypanosomes. In the few attempts at cross-infection that have been carried out, *T. constrictor* of *Boa constrictor* failed to infect *Helicops modestus* and *T. salamantae* of *Epicatres cenchria* failed to infect *Boa constrictor* (Pessôa and Fleury, 1969). In contrast, *T. butantanense* infected 9 snake species (Arantes and Fonseca, 1931*a*), and *T. hydrae*

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infected many species of colubrids (Ayala *et al*. 1983). Our results show that the trypanosome from the viperid *C. durissus* is able to infect the viperid *B. jararaca* and the colubrid *O. guibei*. These observations, although suggesting a lack of host restriction, do not allow any generalizations about what happens in field conditions. The fact that the 7 isolates from *C. durissus* captured in different locations at different times are similar to each other favours host-specificity. Cross-infection data from this and previous studies (Arantes and Fonseca, 1931*a*; Ayala *et al*. 1983) indicate that classification of snake trypanosomes based on the criterion of host origin is unacceptable.

Our results showed that the trypanosomes recovered either from the blood of *C. durissus* or *O. guibei* experimentally infected with a trypanosome isolate from *C. durissus* displayed identical morphology. In addition, the trypomastigotes from *C. durissus* examined in the present study are identical to those described for *T. cascavelli* (Pessôa and De Biasi, 1972). The fact that these trypanosomes come from the same host species does not prove that they are of the same species, as neither host origin nor morphology provides sufficient taxonomic criteria. Nevertheless, based on the morphological similarity of blood trypomastigotes from field-captured *C. durissus* in this study and those in the study of Pessôa and De Biasi (1972) and on the lack of significant morphological and genetic polymorphism among the *C. durissus* isolates, we are keeping the name *T. cascavelli* for the isolates from this snake characterized in this study. The sand fly isolate (910) remained as *Trypanosoma* sp. Although the trypanosome from *P. nigra* (*Trypanosoma* sp 1052) characterized in this study is the first described from this species and was clearly separated by morphological and molecular data from *C. durissus* isolates, further data are required before it can be described as a new species.

Like trypanosomes from fishes, tortoises and some species of anurans, trypanosomes from aquatic snakes can be transmitted by leeches (Pessôa and Fleury, 1969; Chia and Miller, 1984). Nevertheless, although also found in the Amazon region, the snakes *C. durissus* and *P. nigra*, which naturally harbour the trypanosomes characterized in this study, inhabit mainly savannah-like habitats, where leeches are not commonly found. The supposed role of ticks in the transmission of snake trypanosomes has never been investigated. Despite reports of the tick *Amblyoma rotundatum* in *C. durissus* (Dantas-Torres *et al*. 2005), we were unable to make ticks feed on rattlesnakes and trypanosomes did not develop in blood-ingurgitated *A. rotundatum* fed on the colubrid *O. guibei* infected with *T. cascavelli* (data not shown).

If leeches and ticks are excluded, haematophagous insects remain the only candidate vectors for terrestrial snake trypanosomes. Among them, sand flies stand out because they are vectors of trypanosomes among terrestrial lizards and anurans (Anderson and Ayala, 1968; Ayala and McKay, 1971; Minter-Goedbloed *et al*. 1993; Telford, 1995; Ferreira *et al*., manuscript in preparation). Accordingly, we characterized a new trypanosome from the sand fly *V. tuberculata* that was positioned closest to snake trypanosomes, suggesting that it is a trypanosome of another snake species. *V. tuberculata* has a widespread distribution and is commonly found in distinct ecotopes at the lower level of tree trunks (Freitas *et al*. 2002; Lainson *et al*. 2002). This is the first evidence of the role of sand flies in the transmission of trypanosomes among snakes.

The phylogenetic analyses of reptilian trypanosomes done in this study confirmed both their polyphyly and distribution in distant branches, with some clades suggesting a long association of trypanosomes with their vertebrate hosts, such as those related to Squamata and Crocodylia, which are separated by a large genetic distance, compatible with the Early Jurassic split among these major reptilian clades. The lizard/snake clade was formed only by trypanosomes from hosts of Squamata, including species of infraorders Serpentes, Iguania, Gekkota and Anguimorpha (Douglas *et al*. 2006). The trypanosomes from snakes here examined appear to be transmitted by sand flies, as indicated by the tight positioning of a trypanosome from a sand fly within the clade of snake trypanosomes. Corroborating the role of sand flies as vectors, all the lizard trypanosomes nested in the lizard/snake clade are sand fly-transmitted (Ayala, 1970; Ayala and McKay, 1971; Christensen and Telford, 1972; Minter-Goedbloed *et al*. 1993). However, sand flies also are proven vectors for anuran trypanosomes (Ayala, 1971; Ferreira *et al*., manuscript in preparation).

Herein, we provide biological, morphological and molecular phylogenetic evidence supporting a new lineage of trypanosomes from snakes and sand flies closely related to the lizard clade forming an assemblage of squamate trypanosomes, which we called the lizard/snake clade. This clade includes 2 trypanosomes from Brazilian snakes: *T. cascavelli* from *C. durissus* and a new unnamed species (*T.* sp 1052) from *P. nigra*. The lizard trypanosomes clustered into this clade were *T. scelopori* from the USA, and *T. varani* and *T.* sp gecko from Senegal, West Africa (Ayala, 1970; Hamilton *et al*. 2004). *T. scelopori* is from Phrynosomatidae and *T. varani* from Varanidae. These lizard families are respectively from the closely related Iguania and Anguimorpha infraorders, which are phylogenetically distant from the Serpentes (Douglas *et al*. 2006). Therefore, the considerable genetic distance separating snake and lizard clades suggests some patterns of host-parasite association in the evolutionary

history of squamate trypanosomes. However, all data corroborate more complex evolutionary histories for these trypanosomes. Accordingly, *T. therezieni* from the African chameleon, despite also belonging to the Iguania infraorder (Douglas *et al*. 2006), was distantly positioned within the clade of anuran trypanosomes in this and in previous studies (Haag *et al*. 1998; Stevens *et al*. 2001; Hamilton *et al*. 2004, 2007).

Clades harbouring reptilian trypanosomes also suggested an association with vectors and ecotopes. Although from the distant infraorder Gekkota, *T.* sp gecko nested within the lizard clade closest to *T. varani*. Interestingly, these trypanosomes were isolated from hosts of the same African region that share the same ecotope and sand fly vectors. There is clear evidence that sand flies are vectors of trypanosomes for lizards (Ayala, 1970; Ayala and McKay, 1971; Christensen and Telford, 1972; Minter-Goedbloed *et al*. 1993). Data from this study suggested that sand flies are vectors of trypanosomes for terrestrial snakes of savannah-like habitats. *T. chelodina* from aquatic turtle was nested in the clade that comprises fish trypanosomes transmitted by aquatic leeches (Siddal and Desser, 1992; Jakes *et al*. 2001). Taken together, results presented in this and in previous studies suggest that host switching, probably mediated by vectors, appears to play an important role in the evolutionary history of reptilian trypanosomes in general (Maia da Silva *et al*. 2007; Ferreira *et al*. 2007; Hamilton *et al*. 2007).

Trypanosomes of terrestrial snakes, which appear to be transmitted by sand flies, are morphologically distinguishable from species infecting aquatic snakes transmitted by aquatic leeches (Brygoo, 1965*a*, *b*; Chia and Miller, 1984). Phylogenetic analysis of trypanosomes from aquatic and terrestrial snakes can contribute to a better understanding of the evolutionary relationships of these trypanosomes with their vertebrate and invertebrate hosts. Furthermore, an understanding of the relationships among snake trypanosomes and those from other squamates could provide information helpful to evaluate the evolutionary scenarios hypothesized for the Serpentes, namely, that snakes evolved on land directly from burrowing lizards or that they evolved from extinct marine reptiles (Vidal and Hedges, 2005; Douglas *et al*. 2006).

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