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Evolutionary Insights from Bat Trypanosomes: Morphological, Developmental and Phylogenetic Evidence of a New Species, *Trypanosoma* (*Schizotrypanum*) *erneyi* sp. nov., in African Bats Closely Related to *Trypanosoma* (*Schizotrypanum*) *cruzi* and Allied Species

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Parasites of the genus *Trypanosoma* are common in bats and those of the subgenus *Schizotrypanum* are restricted to bats throughout the world, with the exception of *Trypanosoma* (*Schizotrypanum*) *cruzi* that also infects other mammals and is restricted to the American Continent. We have characterized trypanosome isolates from Molossidae bats captured in Mozambique, Africa. Morphology and behaviour in culture, supported by phylogenetic inferences using SSU (small subunit) rRNA, gGAPDH (glycosomal glyceraldehyde 3-phosphate dehydrogenase) and Cyt b (cytochrome b) genes, allowed to classify the isolates as a new *Schizotrypanum* species named *Trypanosoma* (*Schizotrypanum*) *erneyi* sp. nov. This is the first report of a *Schizotrypanum* species from African bats cultured, characterized morphologically and biologically, and positioned in phylogenetic trees. The unprecedented finding of a new species of the subgenus *Schizotrypanum* from Africa that is closest related to the America-restricted *Trypanosoma* (*Schizotrypanum*) *cruzi marinkellei* and *T. cruzi* provides new insights into the origin and evolutionary history of *T. cruzi* and closely related bat trypanosomes. Altogether, data from our study support the hypothesis of an ancestor trypanosome parasite of bats evolving to infect other mammals, even humans, and adapted to transmission by triatomine bugs in the evolutionary history of *T. cruzi* in the New World.

Key words: Africa; bats; Chiroptera; evolution; phylogeny; *Trypanosoma*.

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Introduction

Species of the genus *Trypanosoma* are obligate parasites that belong to the protistan family Trypanosomatidae (Euglenozoa, Kinetoplastea), which comprises flagellates found in all vertebrate classes and transmitted by haemathophagous arthropods and leeches (Hamilton et al. 2007; Hoare 1972; Stevens et al. 2001). Trypanosomes have been recorded in more than 70 species of bats (Chiroptera) from a wide range of families. Although found primarily in insectivorous species, bat hosts of trypanosomes encompass a range of feeding habits. Most species of bat trypanosomes are found within the subgenera *Schizotrypanum* and *Megatrypanum,* with more than 30 species described, based on morphology of blood trypomastigote forms and host of origin, throughout the world. There is no evidence that trypanosomes cause harm to bats (Cavazzana et al. 2010; Hoare 1972; Marinkelle 1976; Molyneux 1991).

The blood forms of all species of the subgenus *Schizotrypanum* are morphologically similar to those of *Trypanosoma cruzi,* the agent of Chagas disease; hence, these trypanosomes are often referred as *T. cruzi*-like. Trypanosomes of this subgenus are known only to infect bats, with the exception of *T. cruzi*, which infects almost all mammalian orders (Cavazzana et al. 2010; Hoare 1972; Maia da Silva et al. 2009; Marcili et al. 2009; Molyneux 1991). The following species of *T.* (*Schizotrypanum*) have been reported in bats: *Trypanosoma vespertilionis* in Europe, the Americas, Africa and Asia; *Trypanosoma dionisii* and *Trypanosoma pipistrelli* in the Old World, particularly European countries; *Trypanosoma pteropi* and *Trypanosoma hipposideri* in Australia; *Trypanosoma hedricki* and *Trypanosoma myotis* in North America; *Trypanosoma phyllostomae* and *Trypanosoma cruzi marinkellei* in Central and South America; and *T. cruzi* in all Latin America, as well as South and Southwest of the USA (Hoare 1972; Marinkelle 1976; Molyneux 1991).

To date, molecular phylogenetic analyses have validated only three species of the subgenus *Schizotrypanum*: *T. cruzi, T. c. marinkellei* and *T. dionisii*. *Trypanosoma cruzi* consists of a complex of genetically heterogeneous isolates distributed in six discrete typing units (DTUs TcI-TcVI) and the newly identified genotype Tcbat. *T. cruzi* is more closely related to *T. c. marinkellei* than to *T. dionisii.* These three species share morphological, biological, genomic and proteomic features, but they differ in hosts, vectors and pathogenicity (Barnabé et al. 2003; Cavazzana et al. 2010; Hamilton et al. 2007; Maia da Silva et al. 2009; Stevens et al. 1999, 2001; Telleria et al. 2010). Phylogenetic analyses of an isolate of *T. vespertilionis* (P14) from England did not support its classification into the subgenus *Schizotrypanum*, indicating that either this bat isolate was erroneously identified or that *T. vespertillionis* is not a species of this subgenus. This controversial isolate of *T. vespertilionis* clusters with *T. rangeli*, *T. conorhini* and African trypanosomes (from monkey, civet and megabat hosts) forming one clade closest to those corresponding to *T.* (*Schizotrypanum*). These trypanosomes, along with a marginally positioned *Trypanosoma* sp. from Australian kangaroo, constitute a large monophyletic assemblage that has been designated "*T. cruzi* clade" (Hamilton et al. 2007, 2009, 2012; Stevens et al. 1999, 2001).

Although limited experimental infections have provided insufficient evidence to fully understand host-parasite relationships, most species of the subgenus *Schizotrypanum* appear to be bat-restricted. *T. phyllostomae* and *T. cruzi* can be infective to laboratory rodents, but only the latter species has been recovered from humans (Hoare 1972; Marinkelle 1976). Only a few studies of a limited number of trypanosomes have used biochemical (zymodemes) and molecular markers to distinguish species of this subgenus (Baker et al. 1978; Barnabé et al. 2003; Cavazzana et al. 2010; Grisard et al. 2003; Lisboa et al. 2008; Stevens et al. 1999, 2001; Tibayrenc and Le Ray 1984).

Putative species of the subgenus *Schizotrypanum* from Africa, North America and Australia were described exclusively using morphological parameters and are therefore awaiting validation with molecular approaches. So far, only trypanosomes from Brazil (*T. cruzi*, *T. c. marinkellei*, *T. dionisii*), Belgium and England (*T. dionisii*) have been confirmed through phylogenetic analyses (Cavazzana et al. 2010; Hamilton et al. 2007, 2009, 2012; Maia da Silva et al. 2009; Marcili et al. 2009). Only species from Brazil have been characterized by a combination of molecular phylogenetic and additional criteria such as culture behaviour, infectivity to other mammals and intracellular development (Cavazzana et al. 2010; Marcili et al. 2009).

Phylogenetic studies of bat trypanosomes of the subgenus *Schizotrypanum* from a broad range of geographical locations are needed to understand the relationships between these species and their hosts and vectors, and the origin and evolutionary history of *T. cruzi* and bat-restricted trypanosomes. In this study, trypanosomes from bats captured in Mozambique, Africa, were classified as a new species of this subgenus based on their behaviour

Table 1. *Trypanosoma erneyi* isolates and other trypanosome species and their respective gene sequences determined in this study or retrieved from data banks.

TCC[a] code	Trypanosome isolate	Host species		Geographic origin	GenBank Accession number			
					V7-V8 SSU rRNA	gGAPDH	Cyt b	ITS1rDNA
T. erneyi								
1293		bat	*Tadarida* sp.	MZ	**JN040987**	**JN040964**	**JN040956**	**JN040974**
1294		bat	*Tadarida* sp.	MZ	**JN040988**	**JN040965**	**JN040957**	**JN040975**
1932		bat	*Mops condylurus*	MZ	**JN040990**	**JN040966**	**JN040958**	**JN040976**
1934		bat	*Mops condylurus*	MZ	**JN040991**	**JN040967**	**JN040959**	ND
1936		bat	*Mops condylurus*	MZ	**JN040992**	**JN040968**	**JN040960**	ND
1946		bat	*Mops condylurus*	MZ	**JN040989**	**JN040969**	**JN040961**	**JN040977**
T. dionisii								
211		bat	*Eptesicus brasiliensis*	BR	FJ001666	GQ140362	FJ900249	**JN040978**
1059		bat	*Eptesicus brasiliensis*	BR	ND	ND	FJ900252	ND
495		bat	*Carollia perspicillata*	BR	FJ001667	GQ140363	FJ900251	ND
1110		bat	*Carollia perspicillata*	BR	ND	ND	FJ002263	ND
1314		bat	*Sturnira lillium*	BR	ND	ND	FJ900254	ND
1087		bat	*Sturnira lillium*	BR	ND	ND	FJ900253	ND
403		bat	*Molossus molossus*	BR	FJ001658	ND	ND	**JN040979**
554		bat	*Promops* sp.	BR	FJ001660	ND	ND	**JN040980**
-	PJ	bat	*Pipistrellus pipistrellus*	BE	AJ009152	ND	ND	ND
-	P3	bat	*Pipistrellus pipistrellus*	UK	AJ009151	AJ620271	ND	ND
T. cruzi marinkellei								
344		bat	*Carollia perspicillata*	BR	FJ001664	GQ140360	**JN543701**	**JN040981**
501		bat	*Carollia perspicillata*	BR	FJ001665	GQ140361	**JN543702**	ND
1093		bat	*Artibeus planirostris*	BR	ND	ND	FJ002262	ND
1794		bat	*Artibeus planirostris*	BR	ND	ND	ND	**JN040983**
494		bat	*Phyllostomus discolor*	BR	ND	ND	FJ900246	ND
1067		bat	*Phyllostomus* sp.	BR	ND	ND	FJ900247	ND
332		bat	*Phyllostomus hastatus*	BR	FJ001635	ND	ND	**JN040982**
-	B3	bat	*Phyllostomus discolor*	BR	FJ649484	FJ649495	ND	ND
-	B7	bat	*Phyllostomus discolor*	BR	ND	AJ620270	ND	ND
-	M1117	bat	*Phyllostomus hastatus*	BR	ND	ND	AJ130927	ND
T. cruzi								
793		bat	*Myotis levis*	BR	FJ001634	GQ140358	FJ002258	ND
1994	Clone from 793	bat	*Myotis levis*	BR	ND	ND	ND	**JN040984**

Table 1 (*Continued*)

TCC[a] code	Trypanosome isolate	Host species		Geographic origin	GenBank Accession number			
					V7-V8 SSU rRNA	gGAPDH	Cyt b	ITS1rDNA
1122		bat	*Myotis albescens*	BR	FJ001628	GQ140359	FJ002261	**JN040985**
507		bat	*Carollia perspicillata*	BR	FJ001632	GQ140352	FJ002256	ND
417		bat	*Thyroptera tricolor*	BR	ND	ND	FJ002255	ND
30	G	opossum	*Didelphis marsupialis*	BR	AF239981	GQ140351	FJ156759	**JN040986**
-	Sylvio X10	human	*Homo sapiens*	BR	ND	ND	AJ130928	ND
-	SE	human	*Homo sapiens*	BR	ND	ND	ND	AF362825
34	Y	human	*Homo sapiens*	BR	AF301912	GQ140353	FJ168768	ND
-	Esmeraldo cl3	human	*Homo sapiens*	BR	AY785564	scf7180000306202[b]	AJ130931	ND
-	994	human	*Homo sapiens*	BR	ND	ND	ND	AF362829
-	5894	human	*Homo sapiens*	BR	ND	ND	ND	AF362828
863	Tc863	armadillo	*Euphractus sexcinctus*	BR	ND	ND	ND	FJ555660
844	MT3869	human	*Homo sapiens*	BR	AF303660	GQ140355	FJ555635	ND
845	MT3663	triatomine	*Panstrongylus geniculatus*	BR	AF288660	**JN040971**	EU856375	FJ555658
337	Fuscicolis 15	monkey	*Saguinus fuscicolis*	BR	ND	**JN040972**	EU856377	ND
85	JJ	human	*Homo sapiens*	BR	ND	GQ140356	EU856368	ND
967	NRcl3	human	*Homo sapiens*	CL	ND	GQ140357	ND	ND
186	Tc186	triatomine	*Triatoma infestans*	BO	FJ001630	**JN040970**	FJ549389	ND
T. rangeli								
643		bat	*Platyrrhinus lineatus*	BR	EU867803	GQ140364	**JN040963**	ND
031	SA	human	*Homo sapiens*	CO	AJ012417	ND	ND	ND
086	AM80	human	*Homo sapiens*	BR	AY491766	**JN040973**	**JN040962**	ND
other	**trypanosomes**							
P14	*T. vespertilionis*	bat	*Pipistrellus pipistrellus*	UK	ND	AJ620283	ND	ND
60	*T.* sp. bat	bat	*Rousettus aegyptiacus*	GA	ND	GQ140365	ND	ND
	HochNdi1	monkey	*Cercopithecus nictitans*	CM	ND	FM164794	ND	ND
	Nandoum1	carnivore	*Nandinia binotata*	CM	ND	FM164793	ND	ND
	T. conorhini	rodent	*Rattus rattus*	BR	ND	AJ620267	ND	ND
	H25	kangaroo	*Macropus giganteus*	AU	ND	AJ620276	ND	ND

[a]Codes of cultures of trypanosomes cryopreserved in the Trypanosomatid Culture Collection of the Departament of Parasitology, University of São Paulo, SP, Brazil; [b]sequence retrieved from TriTryp data bank; *Sequences determined in this study and deposited in GenBank were indicated in underlined and bold.

AU, Australia; BE, Belgium; BO, Bolivia; BR, Brazil; CM, Cameroon; CL, Chile; CO, Colombia; GA, Gabon; MZ, Mozambique; UK, United Kingdom.

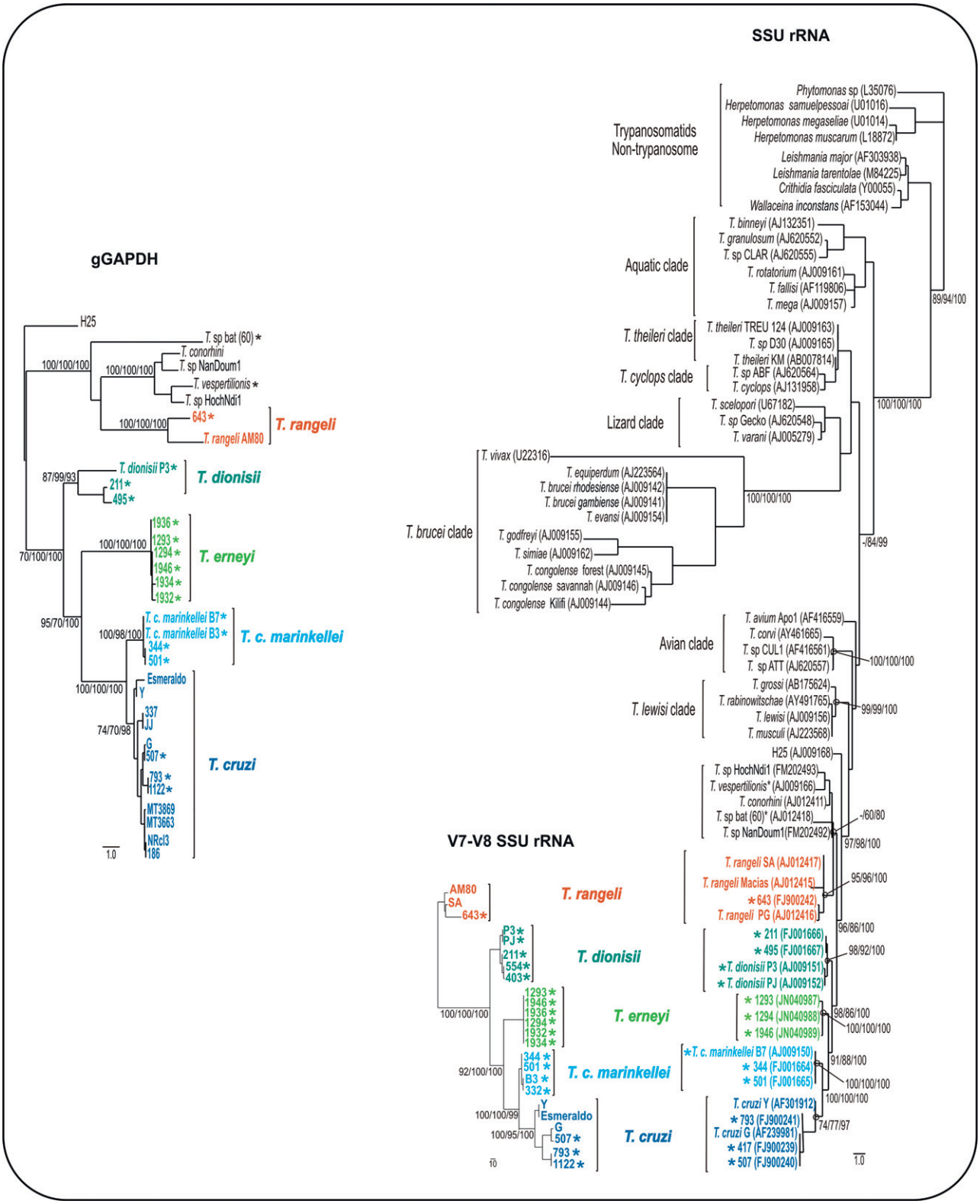


Figure 1. Barcoding and phylogenetic relationships of the new trypanosome isolates from African bats. **V7-V8 SSU rRNA**, Phylogram inferred by maximum parsimony (MP) using the V7-V8 SSU rRNA sequences from six isolates from bats aligned with the sequences of other species of the subgenus *Schizotrypanum*. **SSU rRNA**,

in culture, intracellular development, and morphology including ultrastructural features. The genetic diversity and phylogenetic relationships of these trypanosomes with other *T.* (*Schizotrypanum)* spp. and with other trypanosomes from bats and other mammals from Brazil, Europe and Africa were inferred using SSU rRNA, gGAPDH, Cyt b and ITS1 rDNA sequences.

Results

Culture of Bat Trypanosomes and Host Identification

In this study, we captured bats in two areas of Mozambique and collected blood samples for analysis of trypanosomes by microhaematocrit, blood smears examination and haemoculture methods. Of the 24 blood samples examined, 6 yielded trypanosome cultures (a prevalence of 25%). The cultures (coded TCC1946, 1293, 1294, 1932, 1934 and 1936) and their hosts and collection sites are listed in Table 1. All bats examined belonged to the family Molossidae and were identified to genera *Mops* or *Tadarida* based on morphological parameters. To verify bat species status using genetic data, Cyt b sequences from the DNA of liver tissue were submitted to Blast analysis at GenBank. Most of the bats were identified (97% sequence similarity) as *Mops condylurus,* a species widely distributed over much of sub-Saharan Africa. Two bats were classified as *Tadarida* sp.; identification to species level was not possible due the absence of highly similar sequence in GenBank (*Tadarida teniotis* was the closest match, with 82% similarity).

Barcoding and Phylogenetic Analysis of the New Trypanosomes from African Molossids

We initially compared the V7-V8 region of the SSU rRNA gene of all six new isolates from African molossid bats. We have been using this variable region for barcoding of trypanosomatids and results have demonstrated that this short sequence, which is very easy to amplify by PCR, to sequence and compare to a large number of sequences available in GenBank, is sufficiently polymorphic to distin-

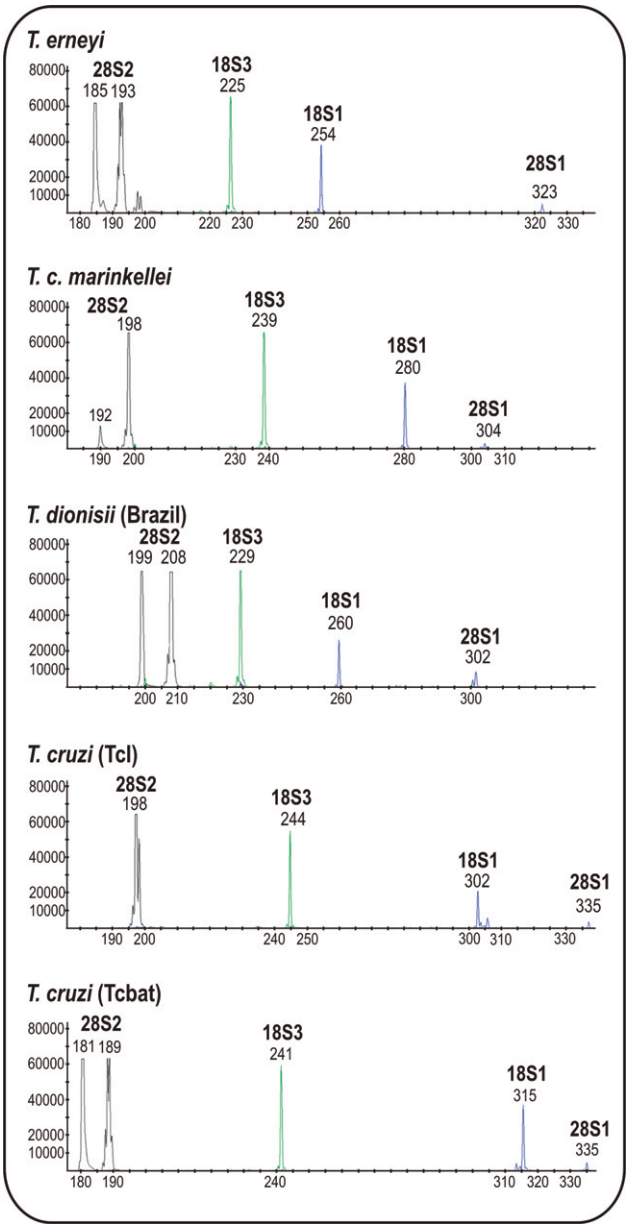
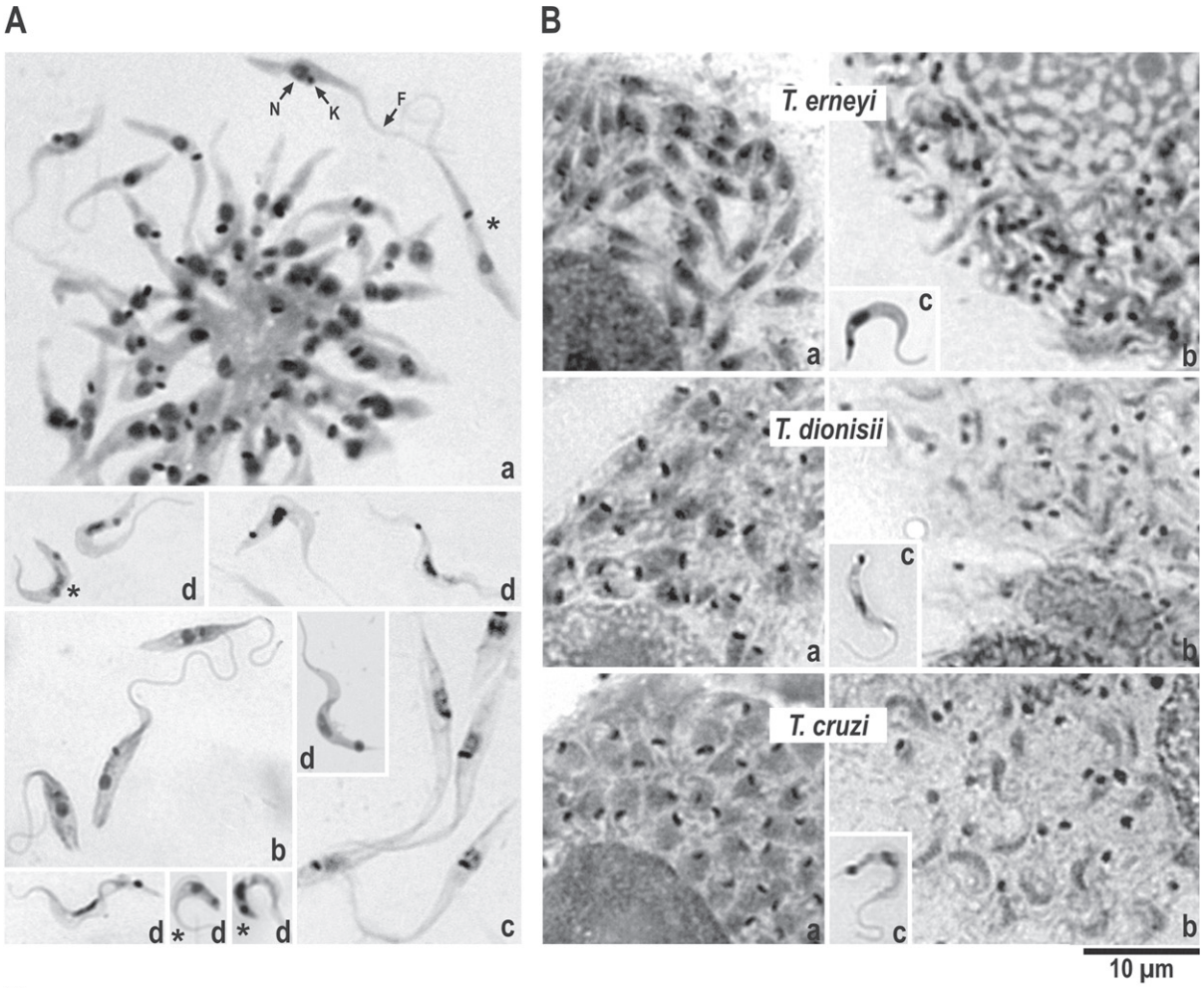


Figure 2. Fluorescent fragment length barcoding (FFLB) profiles of trypanosomes from the subgenus *Schizotrypanum*. Electropherograms of *Trypanosoma erneyi* sp. nov., compared with those from other species of this subgenus: *T. dionisii*; *T. c. marinkellei* and *T. cruzi* (TcI and Tcbat). x axis, size of fragment (bp); y axis, fluorescence intensity.

Phylogenetic tree inferred using maximum likelihood (ML) of SSU rRNA sequences from 15 species of the subgenus *Schizotrypanum,* including *Trypanosoma* (*Schizotrypanum) erneyi* sp. nov., and 42 species of other subgenera and other trypanosomatid genera (1722 characters, –Ln = 8563.377778). **gGAPDH,** Phylogenetic trees of trypanosomes from the subgenus *Schizotrypanum* inferred by ML based on gGAPDH gene sequences using trypanosomes of other subgenera as outgroups (902 characters, -Ln = 3376.510575). The trypanosomes from bats are indicated by (*). Numbers at nodes are bootstrap values derived from 500 replicates from the MP/ML/Bayesian analyses. Codes within parenthesis are GenBank accession numbers.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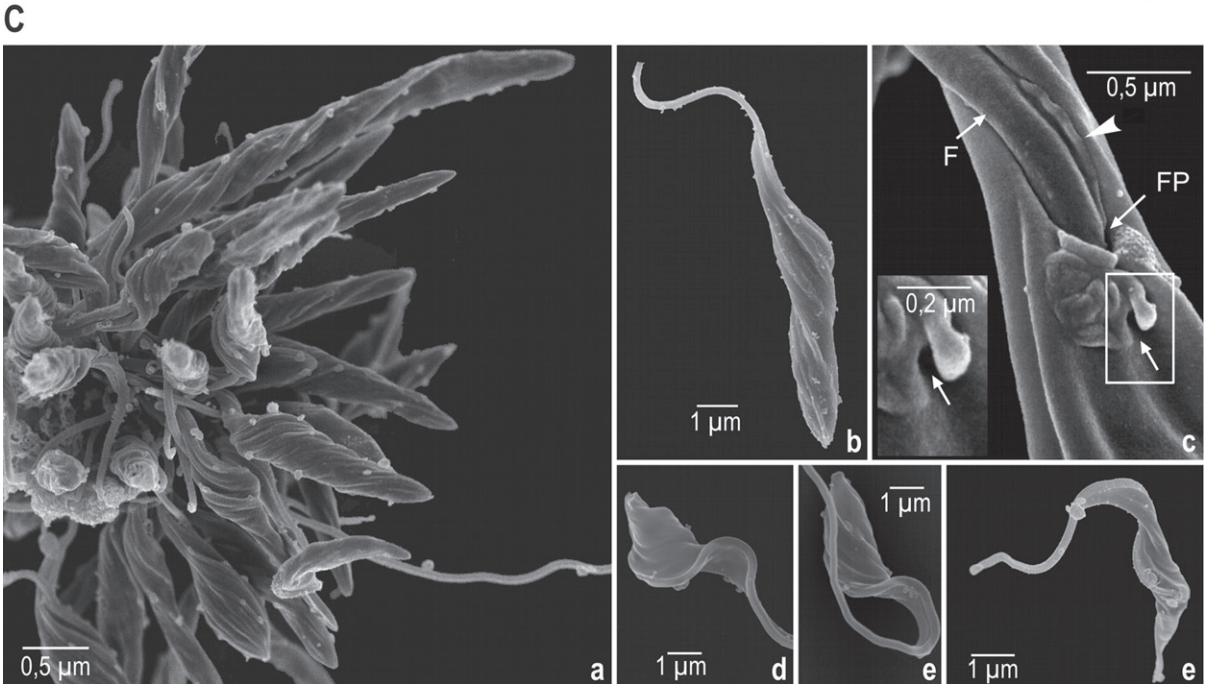
c
b
10 µm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guish all species examined to date (Cavazzana et al. 2010; Ferreira et al. 2008; Garcia et al. 2011; Maia da Silva et al. 2004; Rodrigues et al. 2006; Teixeira et al. 2011; Viola et al. 2009). All the six new isolates from African bats shared high V7-V8 SSU rRNA sequence similarity (~0.2% average divergence), and BLAST analysis demonstrated that they were closest to species of *T.* (*Schizotrypanum*) (Fig. 1). However, the new sequences diverged from all known species of this subgenus by relevant genetic distances: 11% from *T. dionisii* (~2.0% divergence between Brazilian and European isolates), 9.5% from *T. c. marinkellei,* and ~11 to 15% from *T. cruzi* isolates of distinct DTUs.

Phylogenies based on SSU rRNA and gGAPDH genes have been used to address evolutionary and taxonomic questions of trypanosomatids including genus and subgenus reappraisal and determination of species boundaries (Cavazzana et al. 2010; Ferreira et al. 2008; Garcia et al. 2011; Hamilton et al. 2004, 2007, 2009; Svobodova et al. 2007; Teixeira et al. 2011; Viola et al. 2009; Yurchenko et al. 2006). In the present study, three isolates were included in the phylogenetic trees inferred from SSU rRNA sequences and six were included in the gGAPDH sequence-derived tree (Fig. 1). Within the clade harbouring the new bat isolates, sequence divergences were rather small, averaging 0.1% for whole SSU rRNA and 0.25% for gGAPDH. The divergences between the clade formed by the new bat isolates and the other *T.* (*Schizotrypanum*) spp. in the SSU rRNA and gGAPDH sequences were 4.5 and 8.5% for *T. dionisii*, 4.0 and 8.2% for *T. c. marinkellei* and 5.7% and 8.3% for *T. cruzi,* respectively. The clade harbouring all species of the subgenus *Schizotrypanum* was clearly separated from its most closely related clade containing *T. rangeli*, including a bat isolate, *T. vespertilionis* from European bat and *Trypanosoma* sp. from African megabat.

Additional support for the phylogenetic positioning of the new bat isolates was provided from the phylogenies derived from Cyt b sequences (Supplementary Fig. S1), which showed the same topology as the SSU rRNA- and gGAPDH-derived trees (Fig. 1). The Cyt b sequences of the new isolates diverged by 12% from *T. dionisii,* 10% from *T. c. marinkellei* and 13% from *T. cruzi.* Similar branching patterns resulted from independent or combined SSU rRNA, gGAPDH and Cyt b sequences (Supplementary Fig. S1).

Results indicate that both nuclear (SSU rRNA and gGAPDH) and mitochondrial (Cyt b) sequences are useful for distinguishing species, hence, all can be used for barcoding of trypanosomes, and resulted in a well-resolved phylogeny of the subgenus *Schizotrypanum* with four strongly supported clades: *T. dionisii, T. c. marinkellei*, *T. cruzi* and the bat trypanosomes from Mozambique. All phylogenetic analyses confirmed that the new bat trypanosomes differed from all other species, which are representative of all major clades of *Trypanosoma,* included in our phylogenetic analyses. The positioning in the phylogenetic trees and distinguishing polymorphisms in SSU rRNA, gGAPDH and Cyt b sequences strongly support the classification of the new bat isolates (four isolates from *Mops condylurus* and two from *Tadarida* sp.) as a new species of the subgenus *Schizotrypanum*. Accordingly, we are designating these isolates as *Trypanosoma* (*Schizotrypanum*) *erneyi* sp. nov.

Fluorescent Fragment Length Barcoding (FFLB) of Trypanosomes in Bat Blood Samples

We previously demonstrated that the technique of FFLB, which relies on amplification of relatively small regions of rRNA genes and fluorescence

Figure 3. (A-B) Photomicrographs of Giemsa-stained culture forms (Liver Infusion Tryptose medium) of *Trypanosoma* (*Schizotrypanum*) *erneyi* sp. nov., and comparison with other species of the subgenus *Schizotrypanum.* **(A)** Epimastigotes of *T. erneyi* arranged in rosettes (a) and free epimastigotes showing long and slender bodies with long free flagella and the kinetoplast positioned either close or far (*) from the nucleus. The smaller epimastigotes of *T. dionisii* with the kinetoplast, in general, far from the nucleus (b) and epimastigotes of *T. cruzi* (c) with the kinetoplast most time positioned adjacent to the nucleus. Metacyclic trypomastigotes (d) of long and short (*) types. N, nucleus; K, kinetoplast; F, flagellum. **(B)** Development of *T. erneyi, T. cruzi and T. dionisii* within monolayers of HeLa cells showing amastigotes at 4-5 days p.i (a) and, at 7-8 days p.i., trypomastigotes inside the cells (b) or released from the cells and free in the supernatant (c). **(C)** Scanning electron microscopy of *T. erneyi*. Large and twisted epimastigotes of logarithmic culture arranged in rosettes attached by the flagella (a); free twisted epimastigotes with long free flagellum (b). Region of the emergence of the flagellum of an epimastigote (c) showing the flagellum (F), flagellar lamellae (arrowhead) and flagellar pocket (FP) with an enlarged area (rectangle) exhibiting the cytostome (Cy) opening (arrow) and the membrane ruffled around it (c). Metacyclic trypomastigotes of short (d) and long (e) types.

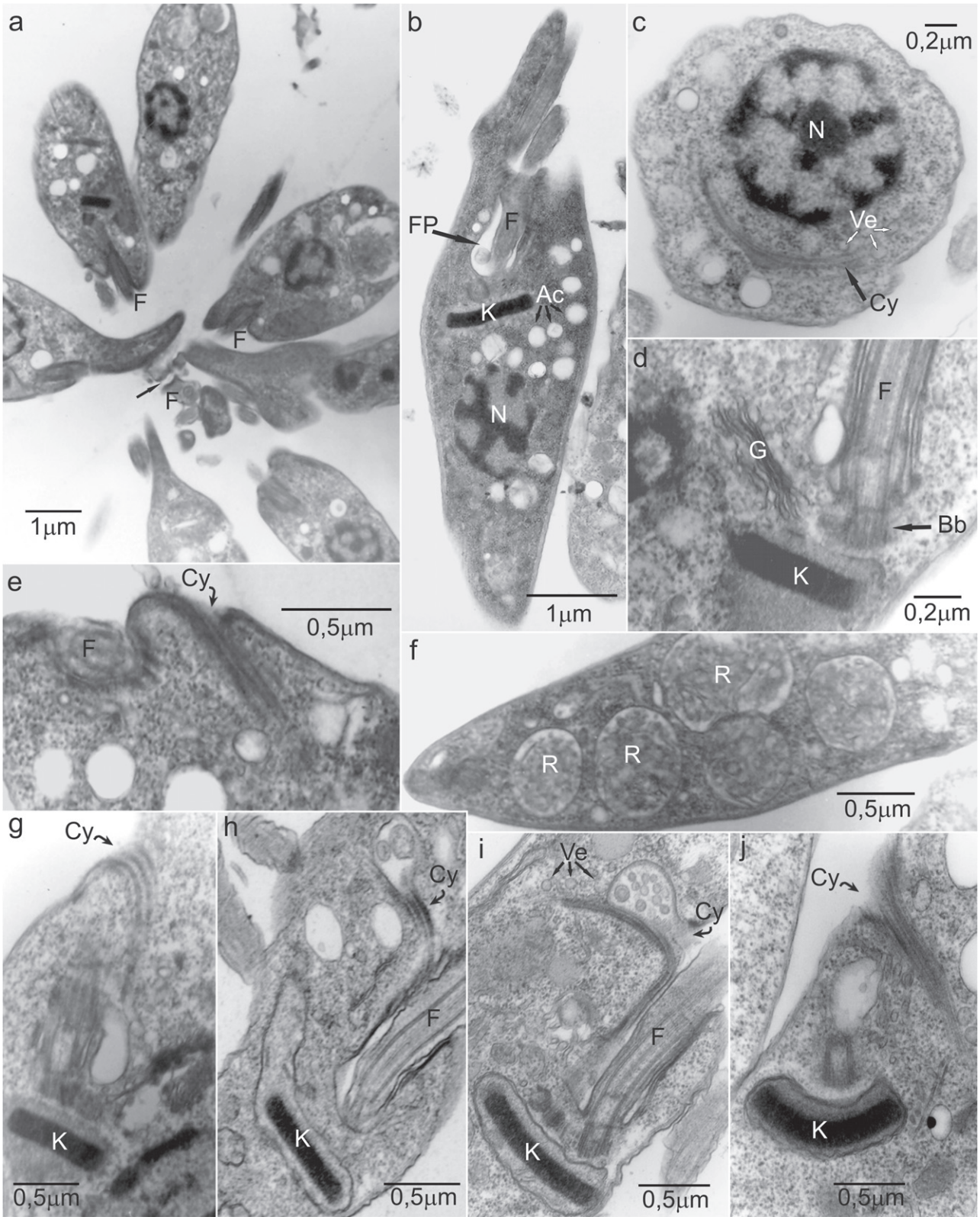

Figure 4. Ultrastructural organization by transmission electron microscopy of *Trypanosoma* (*Schizotrypanum*) *erneyi* sp. nov. and comparison with other species of the subgenus *Schizotrypanum*. Culture forms of *T. erneyi*

detection, is able to distinguish a wide range of trypanosomes, including species of the subgenus *Schizotrypanum* (Hamilton et al. 2011). Here, we initially compared the FFLB profiles of cultured trypanosomes found in bats: *T. cruzi*, *T. c. marinkellei, T. dionisii* and *T. erneyi*. One unique FFLB profile was generated for the 6 isolates of *T. erneyi* (Fig. 2).

Because the FFLB method can detect trypanosomes with high sensitivity (Hamilton et al. 2008), we surveyed for trypanosomes in DNA preparations from blood samples of 17 molossid bats preserved in ethanol all negative for trypanosomes by microscopic examination of blood smears and microhaematocrit, indicative of low parasitaemia. When examined by haemoculture, samples from 6 bats tested positive for trypanosomes and 11 samples tested negative. However, FFLB analysis revealed the same profile shown by cultures of *T. erneyi* in a total of 15 bat blood samples, indicating a high prevalence (88%) of this species in the African molossid bats, and demonstrating the high sensitivity and therefore the suitability of FFLB technique for the detection and identification of trypanosomes directly from blood samples.

Polymorphism Analysis of New Bat Isolates Using ITS1 rDNA Sequences

Both the length and sequence polymorphisms of ITS1 rDNA, highly divergent compared to the conserved SSU rRNA, gGAPDH and Cyt b genes, have been used to identify species and intraspecific genotypes of several trypanosomes of mammals such as *T. cruzi*, *T. rangeli* and *T. theileri*, and anuran and snake trypanosomes (Ferreira et al. 2008; Garcia et al. 2011; Maia da Silva et al. 2004, 2007; Marcili et al. 2009; Rodrigues et al. 2006; Viola et al. 2009). Lengths of ITS1 rDNA were different for each species: ~350 bp for *T. erneyi*, ~450 bp for *T. dionisii* and ~600 bp for *T. c. marinkellei*. ITS1 rDNA of *T. cruzi* isolates from different DTUs varied from ~500 bp (TcI and TcIII) to ~800 bp (Tcbat and TcII) (Marcili et al. 2009).

Divergences in ITS1 rDNA sequences were small (maximum ~2.5%) among the isolates of *T. erneyi*, even isolates from different bat species, *M. condylurus* and *Tadarida* sp. (~1.5%). Large divergences separated *T. erneyi* from *T. dionisii* (~72%), *T. c. marinkellei* (~60%) and *T. cruzi* (~76% to 80%), similar to divergences between *T. cruzi* and *T. c. marinkellei* (~63%) or *T. dionisii* (76%). The dendrogram based on ITS1 rDNA sequences supports the assignment of all the new bat isolates to a single new species (*T. erneyi*) of the subgenus *Schizotrypanum* (Supplementary Fig. S2).

Growth Behaviour, Morphology and Infectivity to Mice and Triatomine Bugs

All six isolates of *T. erneyi* grew easily in LIT and TC-100 media. Their behaviour in culture and their morphology were compatible to *T.* (*Schizotrypanum*). Epimastigotes were slender and exhibited a rounded kinetoplast positioned far from the nucleus; similar positioning of the kinetoplast is found in the small epimastigotes of *T. dionisii*. In contrast, *T. cruzi* and *T. c. marinkellei* (data not shown) had larger and indistinguishable epimastigotes with the kinetoplast adjacent to the nucleus (Fig. 3A). Metacyclic trypomastigotes of *T. erneyi* resembled those of the reference species of the subgenus *Schizotrypanum* (Molyneux 1991). These forms were quite homogeneous in *T. cruzi* and *T. c. marinkellei* (data no shown) or of two types in *T. erneyi* and *T. dionisii*: short, with an almost terminal kinetoplast, and long and thin, with the kinetoplast more distant to the posterior end (Fig. 3A).

A unique characteristic of all species of the subgenus *Schizotrypanum* is the development within mammalian cells in vivo and in vitro. All cultures from bat trypanosomes containing metacyclic trypomastigotes developed inside cells when transferred to monolayers of HeLa (Fig. 3B) and LLC-MK_2 (data not shown) culture cells. The isolate TCC1946 was selected to compare the intracellular development of *T. erneyi* to those of *T. cruzi* and *T. dionisii*. In all cases, metacyclic trypomastigotes invaded the cells and multiplied extensively in

(**a-g**): rosette of epimastigotes attached by the flagellar region (arrow) (**a**); longitudinal section of a epimastigote showing the kinetoplast, the nucleus, multiple acidocalcisomes and flagellar pocket with the emergence of the flagellum (**b**); transverse section showing small vesicles (arrows) close to the cytostome (**c**); epimastigote showing a Golgi complex, the kinetoplast, the flagellum inside the flagellar pocket and the basal body (**d**); invagination of the cytostome close to the flagellar pocket (**e**); posterior end of an epimastigote with several reservosomes (**f**). Comparison of the arrangement of the kDNA fibrils resulting in a very compact disk-shaped kinetoplast and the cytostome in epimastigotes of *T. erneyi* (**c**, **d**, **g**), *T. c. marinkellei* (**h**), *T. dionisii* (**i**) and *T. cruzi* (**j**). Nucleus (N); kinetoplast (K), Golgi complex (G), flagellum (F), acidocalcisomes (Ac), cytostome (Cy), vesicle (Ve), flagellar pocket (FP), basal body (Bb), reservosome (R).

the cytoplasm as amastigotes. After approximately seven days of multiplication, amastigotes differentiated into trypomastigotes (Fig. 3B). At this stage, trypomastigotes were released from the cells and can infect new cells.

Similarly to *T. dionisii* and *T. c. marinkellei*, trypomastigotes from LIT and HeLa cells of isolates TCC1946 and 1294 of *T. erneyi* were not infective to Balb/c mice. To date, the only species of *T.* (*Schizotrypanum*) that has proven infective to mice is *T. cruzi*; all other species appear restricted to bats (Cavazzana et al. 2010). Additionally, similarly to *T. dionisii* and *T. c. marinkellei*, the isolates of *T. erneyi* did not develop in the triatomine bugs *Rhodnius robustus, R. neglectus* and *Triatoma infestans*. In contrast, isolates of *T. cruzi* (except for the Tcbat genotype) can develop in these bugs (Marcili et al. 2009).

Ultrastructural Analyses of *T. erneyi* and other Species of the Subgenus *Schizotrypanum*

Morphological analysis by scanning electron microscopy of culture forms of *T. erneyi* (Fig. 3C) revealed slender epimastigotes with a long flagellum resembling those of *T. cruzi*, *T. c. marinkellei* and *T. dionisii.* In the proliferative phase, the twisted epimastigotes of *T. erneyi* were arranged in rosettes attached by their flagella (a) or free in the supernatant (b). The surface displayed many grooves and the cell bodies were highly twisted. These morphological characters are not common to *T. dionisii*, *T. c. marinkellei* and *T. cruzi* compared in this study (data not shown). The membrane around the cytostome, a small opening localized close to the flagellar pocket was ruffled, in contrast to the smoothness of the rest of the body and flagellar membranes (c) as described for *T. cruzi* (de Souza 2009). The metacyclic trypomastigotes of *T. erneyi* showed one or two body torsions and varied in length and shape (d, e), consistent with the appearance of two morphotypes in this species previously detected by Giemsa-staining (Fig. 3A).

Morphological analysis by transmission electron microscopy (Fig. 4) showed that the epimastigotes of *T. erneyi* (a-g) and the other bat trypanosomes examined (h-j) exhibited an ultrastructural organization similar to that described for *T. cruzi* (Cunha-e-Silva et al. 2006; de Souza 2009; Porto-Carreiro et al. 2000). The epimastigotes from all trypanosomes within the subgenus *Schizotrypanum* showed a typical cytostome, formed by an invagination of the cell surface in a region localized close to the flagellar pocket, which penetrates deeply into the cytoplasm towards the posterior end surrounded by microtubules and vesicles (c, e). The organization and function of the cytostome as the main structure involved in the endocytic process were thoroughly described in *T. cruzi* (Porto-Carreiro et al. 2000). Other features shared by all *T.* (*Schizotrypanum*) spp. include a large number of acidocalcisomes (b), cytoplasmic vacuoles of high Ca^{2+} concentration, and reservosomes (f), which are compartments found in the posterior region of epimastigotes that accumulate endocytosed macromolecules (Cunha-e-Silva et al. 2006; de Souza 2009).

Kinetoplast DNA (kDNA), which corresponds to the mitochondrial DNA of trypanosomatids, comprises thousands of interlocking DNA circles that form a complex network. In epimastigotes of *T. erneyi*, the compacted kDNA fibril arrangement resulted in disk-shaped kDNA (b, d, g) similar to the disk-shaped kDNA observed in this study in *T. c. marinkellei* (h), *T. dionisii* (i) and *T. cruzi* (j), corroborating previous studies of *T.* (*Schizotrypanum*) spp. (de Souza 2009; Mühlpfordt 1981).

Taxonomy Section

Taxonomic positioning: Phylum Euglenozoa Cavalier-Smith, 1981, class Kinetoplastea Honigberg, 1963, order Trypanosomatida (Kent, 1880) Hollande, 1952, family Trypanosomatidae Doflein, 1951

Genus *Trypanosoma* Gruby, 1843: This genus comprises flagellates infective to all vertebrate orders that in their vectors, haematophagous arthropods and leeches, multiply as epimastigotes that in general differentiate to trypomastigotes infective to vertebrate hosts. All members of this genus are characterized by the unique presence of trypomastigotes, which are forms absent in all other genera of Trypanosomatidae. Developmental stages appear in different combination during the life cycle, in both the invertebrate and vertebrate hosts, according to the subgenus/species of *Trypanosoma*.

Subgenus *Schizotrypanum* Chagas, 1909: The type-species of this subgenus is *Trypanosoma* (*Schizotrypanum*) cruzi Chagas, 1909. This subgenus comprises species parasitic in mammals only, mostly bats, in which they multiply intracellularly as amastigotes that differentiate to trypomastigotes; these forms are released by cells and invade new cells, and are infective to a new mammalian host. The intracellular development in vertebrate cells is unique of this subgenus. Species of of the subgenus *Schizotrypanum* are transmitted by haematophagous hemipterans (Triatominae and Cimicidae); the development in their vectors is restricted to digestive tracts where multiplication occurs as epimastigotes that differentiate to infective metacyclic trypomastigotes. Culture in LIT medium of all species of this subgenus presented epimastigotes in the logarithmic phase and metacyclic trypomastigotes in the stationary phase. General morphology is similar in all species of this subgenus. Epimastigotes are characterized by the following ultrastructural features: cytostome, disk-shaped

and compacted kinetoplast and large numbers of acidocalcisomes and reservosomes. This subgenus was phylogenetically validated in this study as a clade strongly supported using sequences from SSU rRNA, gGAPDH and Cyt b genes.

Trypanosoma (*Schizotrypanum*) *erneyi* Lima and Teixeira sp. nov.

Type material: hapantotype, culture of the isolate TCC1946; paratypes, cultures TCC1293, 1294, 1932, 1934 and 1936 whose hosts and collection sites are in Table 1. All cultures are deposited at the Trypanosomatid Culture Collection of the University of São Paulo, SP, Brazil (TCC-USP). Hapantotype slides (Giemsa-stained smears from LIT cultures of TCC1946) are deposited in the TCC-USP collection. **Host and locality:** Chiroptera: Molossidae: *Mops condylurus* and bats of the genus *Tadarida* collected in Mozambique, Province of Sofala, District of Chupanga (S18°02′ E35°34′). Additional localities: Mozambique, Province of Sofala, District of Marromeu (S18°17′ E35°56′). **Vector**: unknown. **Description:** Twisted epimastigotes with long flagellum in LIT cultures, intracellular development and ultrastructural features (cytostome and disk-shaped kinetoplast) typical of *T.* (*Schizotrypanum*) species (Figs 3, 4). Despite small morphological characteristics of epimastigote forms, morphology is not sufficient to distinguish the bat-restricted trypanosomes of the subgenus *Schizotrypanum*. The new species, *T. erneyi,* can be surely distinguished from the other species of this subgenus exclusively by its unique gene sequences, which are deposited in the GenBank (TCC1946): SSU rRNA (JN040989), gGAPDH (JN040969), Cyt b (JN040961) and ITS1 rDNA (JN040977). **Etymology:** The name is given in honour of Prof. Erney Plessmann Camargo, a distinguished Brazilian parasitologist and founder of our research group at the University of São Paulo.

Discussion

Although bats are commonly infected with trypanosomes throughout the world, and even though over 100 years have passed since the discovery of bat trypanosomes by Dionisi in 1898 (revised by Hoare 1972), little is known about their genetic diversity, phylogenetic relationships, host ranges, vectors, life cycles and geographical distributions. Consequently, the taxonomy of these trypanosomes remains problematic. Phylogenetic studies of bat trypanosomes are necessary to improve taxonomy and our understanding of the evolutionary history of the subgenus *Schizotrypanum*, particularly the origin of *T. cruzi*, which might have evolved from a bat-restricted trypanosome or vice–versa (Cavazzana et al. 2010; Hamilton et al. 2009).

In this study, we isolated and characterized a new species of trypanosome from Molossidae bats captured in Mozambique in southeast Africa. Although the isolates came from two different bat species from two different localities, they shared high sequence similarity, even in highly polymorphic ITS1 rDNA sequences. Phylogenies based on the SSU rRNA, gGAPDH and Cyt b sequences shared identical topologies. The new isolates formed a new clade within the subgenus *Schizotrypanum*, with *T. c. marinkellei* isolates as a sister clade. Together, sequences of nuclear and mitochondrial genes supported the classification of these isolates as a new species of *T.* (*Schizotrypanum*), which we designated *Trypanosoma* (*Schizotrypanum*) *erneyi* sp. nov. Until now, reports on species of this subgenus from African bats were restricted to the morphology of blood forms since no cultures or DNA sequences were available before this study.

A survey for trypanosomes by haemoculture in 1043 Brazilian bats (Cavazzana et al. 2010) yielded an infectivity rate of ∼13% with species of *T.* (*Schizotrypanum*), most in Vespertilionidae, Noctilionidae and Phyllostomidae bat families. Only two cultures, both of *T. dionisii*, were from bats of Molossidae, which was poorly represented in this previous study. A survey of trypanosomes in blood smears from 427 bats in Kenya, East Africa, yielded a rate of 21% infectivity in bats of Emballonuridae and Vespertilionidae, whereas Molossidae had the lowest prevalence (Woo and Hawkins 1975). This finding agrees with our negative results in examination of blood smears of molossids from Mozambique, which is indicative of the low parasitemias common in chronic infections caused by species of *T.* (*Schizotrypanum*). We detected trypanosomes of the subgenus *Schizotrypanum* in 25% of 24 bats examined by haemoculture, whereas the FFLB method detected 88% of the 17 bats examined, thereby confirming its high sensitivity. This method revealed to be excellent for surveys and diversity analysis of trypanosomes directly in blood samples, as previously shown using samples from digestive tract of tsetse flies (Hamilton et al. 2008).

All isolates classified as *T. erneyi* displayed morphological (including ultrastructural organization), developmental and growth characteristics consistent with other species of the subgenus *Schizotrypanum*. Similar to the metacyclic trypomastigotes of all species of this subgenus, those of *T. erneyi* multiply within mammalian cells as amastigotes, which later develop into trypomastigotes and are released from the cells. However, metacyclic trypomastigotes of *T. erneyi* failed to infect Balb/c mice and have been shown to be susceptible to human complement-mediated lysis, similarly to *T. c. marinkellei* and *T. dionisii* (Lima et al. unpubl. observ.). These findings suggest that *T. erneyi* may be a bat-restricted species.

Comparative studies of *T. cruzi* and *T. cruzi*-like species that are non-infective to humans may improve our understanding of the evolution of the diversity of host species (host range) and virulence of *T. cruzi*. Knowledge of host-parasite-vector interactions is crucial to interpreting the eco-biogeographical patterns of trypanosomes. However, we know nothing about the vector(s) of *T. erneyi.* Triatomine insects are absent in Africa, and similar to *T. dionisii*, *T. erneyi* was unable to infect these bugs, which are vectors of *T. cruzi*. Species of Cimicidae (*Cimex*), which are proven vectors of *T. dionisii* in Europe (Bower and Woo 1981; Gardner and Molyneux 1988), and bat bugs of Polyctenidae (closely related to Cimicidae) are commonly associated with African bats and could potentially be vectors of *T. erneyi*. In addition, bats are parasitized by dipterans, ticks, mites and fleas, all potential vectors of trypanosomes (Hoare 1972; Molyneux 1991).

In agreement with previous studies (Cavazzana et al. 2010; Hamilton et al. 2004, 2007; Marcili et al. 2009; Stevens et al. 1999), our phylogenies revealed a monophyletic assemblage constituted by species of the subgenus *Schizotrypanum.* The clade formed exclusively by species of the subgenus *Schizotrypanum, T. cruzi, T. c. marinkellei, T. dionisii* and *T. erneyi,* is closely related to the clade formed by *T. rangeli, T. vespertilionis, T. conorhini* and the African trypanosomes from megabat, monkey and civet. Together with the *Trypanosoma* sp from Australian kangaroo, these clades constituted the large previously defined "*T. cruzi* clade" (Hamilton et al. 2004, 2007, 2009, 2012; Stevens et al. 1999, 2001).

The increasing number of trypanosomes included in recent phylogenetic analyses, and the strong association of bat trypanosomes with the "*T. cruzi* clade", have changed the prevailing hypothesis regarding the origin and evolution of trypanosomes. It was initially speculated that the divergence of *T. brucei* and *T. cruzi* followed the separation of Africa and South America 100 million years ago (MYA). The placement of the trypanosome from kangaroo at the periphery of the "*T. cruzi* clade" suggested an origin for *T. cruzi*, possibly in marsupials, on the southern supercontinent (~65 MYA) comprising present day Antarctica, Australia and South America (Stevens et al. 1999, 2001). More recently, the nesting of trypanosomes from African terrestrial mammals (monkey and civet) within the "*T. cruzi* clade", close to *T. vespertilionis* from bats, suggested that intercontinental transfer occurred after the continental separation, and bats likely played a role in the spreading of these trypanosomes (Hamilton et al. 2009, 2012).

To date, the new trypanosome species described here, *T. erneyi*, has only been isolated from African molossid bats (genera *Tadarida* and *Mops*), although this may reflect insufficient sampling of molossids from other continents. The family Molossidae contains about 80 species of insectivorous bats and has a broad pantropical distribution with the genus *Tadarida* spreading widely throughout the world whereas *Mops* occurs only in Asia and Africa. The oldest North American molossid fossil is from the middle Eocene, and paleontological and phylogenetic data suggest that molossids inhabited North and South America prior to the connection of these continents by the Panamanian Isthmus in the Pliocene (Czaplewski et al. 2003).

The putative origin of bats in Laurasia during the early Paleocene (~65 MYA) and changes in global climate that appear to have facilitated the large radiation of bats from Africa in the Eocene suggests some possible biogeographic scenarios that could account for the current distribution and phylogenetic relationships of bat trypanosomes throughout the world. The suborder Yinpterochiroptera (megabats and rhinolophoid microbats) is exclusive to the Old World whereas the Yangochiroptera comprises all other bat species, and includes only three families (Vespertilionidae, Molossidae and Emballonuridae) with genera (but not single species) that span the Old and New Worlds (Bisson et al. 2009; Eick et al. 2005; Simmons 2005; Teeling 2009; Teeling et al. 2005). Bats may have crossed through the Bering land bridge at various times during the Miocene and Pleistocene ice ages. Migrations of bats between the New and Old Worlds could help to explain the present day distribution of closely related and, apparently, recently diverged bat trypanosomes of the subgenus *Schizotrypanum* (Hamilton et al. 2012). Although paleo- and phylogeographical evidence of past migratory behaviour of bats is scarce, recent movements of bat families are suggested by the close relationships among bats of Vespertilionidae from the Old and New World. Bats of the Vespertilionidae, which are commonly infected by species of the subgenus *Schizotrypanum* around the word, like those of the Molossidae from which *T. erneyi* was isolated, dispersed widely in the past and are presently capable of long distance migrations (Bisson et al. 2009; Czaplewski et al. 2003; Stadelmann et al. 2007).

Regardless of their place of origin, trypanosomes have been carried through various environments by their bat hosts and/or by their large repertoire of ectoparasites (cimicids, flies, fleas, mites and ticks).

It is reasonable to suppose that trypanosome-infected bats entered North America in the Eocene and then entered South America in the Miocene and Pliocene (Bisson et al. 2009; Stadelmann et al. 2007). However, given the historical dispersal of bats, we can hypothesise that bats may have transferred trypanosomes from the Old to New World and vice versa. Evolutionary insights from the present study are consistent with the hypothesis that *T. cruzi* evolved from ancestral bat trypanosomes, although other scenarios in which trypanosomes infective to other mammals evolved to become bat-restricted cannot be ruled out. Future analyses of the diversity, host species, geographical range, and phylogenetic relationships of trypanosomes from bats and other mammals are required to infer the most likely hypothesis.

Trypanosomes of the subgenus *Schizotrypanum* and bats are tightly united in a striking example of an ancient and intimate host-parasite partnership: there are no species of this subgenus (except *T. cruzi*) in mammals other than bats. The existence of *T. erneyi* in Africa and its close relationship with America-restricted *T. cruzi* and *T. c. marinkellei* sheds new light on the diversity, origin, dispersion and evolutionary history of these trypanosomes providing new insights into the understanding of the origin and evolution of the human pathogen *T. cruzi*. Data from the present study favour an ancestor trypanosome of bats, probably transmitted by cimicids, evolving to infect other mammals, including humans, and adapted to transmission by triatomines in the New World.

Methods

Capture and identification of bats, and isolation of trypanosomes in culture: Bats were captured in the districts of Marromeu (S18°17′ E35°56′) and Chupanga (S18°02′ E35°34′) in the Province of Sofala in Central Mozambique, Southeast Africa, in May 2006 and July 2009. The bats were captured with mist nets and were anaesthetised, and blood samples collected by heart puncture. The bats were identified using conventional morphological keys and sequencing of Cyt b (Cui et al. 2007) using DNA from liver samples preserved in ethanol and processed using the Wizard DNA Clean-Up System (Promega). Sequences were submitted to BLAST analysis at GenBank.

To detect the presence of trypanosomes, blood samples were analysed using microhaematocrit, Giemsa-stained blood smears and haemoculture methods. Haemocultures were performed using a medium consisting of blood agar base (BAB) containing 15% rabbit blood as a solid phase and an overlay of LIT (Liver Infusion Tryptose) medium with 10% FBS with incubation at 28 °C as described previously (Maia da Silva et al. 2004). All isolates were expanded in LIT with 5% FBS for DNA preparation and cryopreservation in the Trypanosomatid Culture Collection (TCC) of the Department of Parasitology, University of São Paulo, Brazil. Blood samples were preserved in ethanol (v/v) and processed using the Wizard DNA Clean-Up System (Promega) and analysed using FFLB methodology, following Hamilton et al. (2008, 2011).

Growth behaviour, development and morphology of bat trypanosomes: For morphological analysis, smears from logarithmic and stationary cultures from the isolates TCC1294 and 1946 were fixed with methanol and Giemsa-stained. For the differentiation of epimastigotes into metacyclic trypomastigotes, flagellates were cultured in LIT medium with 3.0% FBS at 28 °C. Metacyclic trypomastigotes (1×10^6/well) were employed to infect monolayers of HeLa and LLC-MK_2 cells cultivated in RPMI medium with 5% FBS, at 37 °C and 5% CO_2, on glass coverslips in 24-well plates (1×10^5 cells/well). On the 1st, 4th and 7th day p.i., the coverslips were removed, washed in phosphate-buffered saline, fixed in methanol and Giemsa-stained. *T. cruzi* (TCC507), *T. c. marinkellei* (TCC344) and *T. dionisii* (TCC495) were cultivated in parallel for comparison.

For transmission electron microscopy (TEM) analysis, log and mid-log phase cultures (LIT medium) of the isolates TCC1294 and 1946, *T. c. marinkellei* (TCC344) and *T. dionisii* (TCC495) were fixed in glutaraldehyde, post-fixed with osmium tetroxide and embedded in Spurr resin. Ultrathin sections were then stained with uranyl acetate and examined using a JEOL 100CX electron microscope following Teixeira et al. (2011). For scanning electron microscopy (SEM), flagellates fixed in glutaraldehyde were adhered to poly-L-lysine-coated coverslips and processed for observation in a ZEISS 900EM digital scanning microscope as described previously (Viola et al. 2009).

Fluorescent fragment length barcoding: FFLB analysis was carried out using four primer sets and PCR conditions described previously (Hamilton et al. 2008, 2009). Initially, we tested DNA from several reference species of trypanosomes aiming standardization of species-specific profiles, which can be slightly different, in our laboratory at the University of São Paulo, Brazil using an ABI PRISM™ 3500 DNA Sequencer. Then, DNA preparations from cultured bat isolates and from bat blood samples were tested by FFLB. The barcode consists of profiles formed by four amplified DNA fragments variable in length according to species/isolates.

Phylogenetic analyses: DNA extracted from cultured trypanosomes using the phenol-chloroform extraction method was used for PCR amplification, cloning and sequencing. PCR amplifications of the V7-V8 SSU rRNA, whole SSU rRNA, and ITS1 rDNA were carried out as described previously (Ferreira et al. 2008; Rodrigues et al. 2006). Amplification of the gGAPDH and Cyt b (500 bp) genes was performed using the previously described primers and reaction conditions (Hamilton et al. 2004; Marcili et al. 2009; Viola et al. 2009).

Sequences were aligned using Clustal X (Thompson et al. 1997), and the alignments were manually refined. We created several alignments for phylogenetic inference: a) V7-V8 SSU rRNA (~850 bp) sequences from 21 species of the subgenus *Schizotrypanum*; b) SSU rRNA (~1722 bp) sequences of all available trypanosomes from bats and other hosts, representing all major clades of the *Trypanosoma* phylogeny; c) gGAPDH sequences from *T.* (*Schizotrypanum*) spp. using *T. rangeli, T. vespertilionis*, *Trypanosoma* sp. bat from African megabat, and *Trypanosoma* sp. (H25) from Australian kangaroo as outgroups; d) Cyt b (~470 bp) sequences from 29 species of the subgenus *Schizotrypanum* using *T. rangeli* as an outgroup; and e) ITS1 rDNA sequences (~900 bp) from 18 species of this subgenus. The concatenated V7-V8 SSU rRNA, gGAPDH and Cyt b sequences were employed for phylogenetic inferences. All sequences are available in GenBank under the accession numbers listed in Figure 1 and Table 1.

Phylogenetic relationships were inferred using maximum likelihood (ML), Bayesian (BI) and maximum parsimony (MP) analyses. Parsimony and bootstrap analyses were carried out using PAUP* (v. 4.0b10; Swofford 2002), with ML analyses conducted using RaxML (v.7.0.0; Stamatakis 2006) and BI analyses conducted using MrBayes (v3.1.2; Huelsenbeck and Ronquist 2001). Nodal support was estimated with 500 bootstrap replicates in RAxML using GTRGAMMA, following Ferreira et al. (2008) and Viola et al. (2009).

Infectivity analysis of new bat trypanosomes for triatomine insects and mice: Three species of triatomines, *R. robustus, R. neglectus* and *Triatoma infestans*, were used to analyse the behaviour of selected new bat isolates. Thirty 5th instar nymphs of each triatomine species were inoculated with stationary phase cultures of bat trypanosomes containing metacyclic trypomastigotes. The inoculated triatomines were fed in non-infected mice every 15 days. Approximately 10 triatomines of each species were dissected at 15, 30, and 60 days p.i., and their digestive tubes were examined for trypanosomes.

To analyse mice infectivity, Balb/c mice were inoculated with metacyclic forms ($\sim 10^6$/animal) from stationary-phase cultures in TC-100 medium. Mice blood samples were examined weekly from 7 to 30 days p.i. by microhaematocrit and by haemoculture 30 days p.i. as described previously (Maia da Silva et al. 2004).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2011.12.003.

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