Luciana Lima

Diversidade morfológica, biológica e genética, e relações filogenéticas de tripanossomas de morcegos do Brasil e Moçambique (África)

Tese apresentada ao Programa de Pós-Graduação em Biologia da Relação Patógeno-Hospedeiro do Instituto de Ciências Biomédicas da Universidade de São Paulo, para a obtenção do título de Doutor em Ciências.

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Área de concentração: Biologia da Relação Patógeno-Hospedeiro

Orientadora: Profa. Dra. Marta Maria Geraldes Teixeira

Versão Original

São Paulo

2011

DADOS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
Serviço de Biblioteca e Informação Biomédica do
Instituto de Ciências Biomédicas da Universidade de São Paulo

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Lima, Luciana.
Diversidade morfológica, biológica e genética, e relações filogenéticas de tripanossomas de morcegos do Brasil e Moçambique (África). / Luciana Lima. -- São Paulo, 2011.

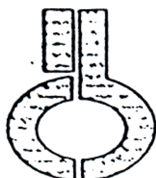
Orientador: Marta Maria Geraldes Teixeira.

Tese (Doutorado) – Universidade de São Paulo. Instituto de Ciências Biomédicas. Departamento de Parasitologia. Área de concentração: Biologia da Relação Patógeno-Hospedeiro. Linha de pesquisa: Biologia e filogenia de tripanossomatídeos.

Versão do título para o inglês: Morphological, biological and genetic diversity, and phylogenetic relationships of bat trypanosomes from Brazil and Mozambique (Africa)

Descritores: 1. *Trypanosoma* 2. Morcegos 3. Filogenia 4. Catepsina L 5. *Schizotrypanum* 6. Taxonomia I. Teixeira, Marta Maria Geraldes II. Universidade de São Paulo. Instituto de Ciências Biomédicas. Programa de Pós-Graduação em Biologia da Relação Patógeno-Hospedeiro III. Título.

ICB/SBIB067/2011



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Certificamos que o protocolo registrado sob nº **017** nas fls. **3** do livro **2** para uso de animais em experimentação, sob a responsabilidade da Profa.Dra. Marta Maria Geraldes Teixeira, Coordenadora da Linha de Pesquisa: "***Diversidade, taxonomia e filogenia molecular de tripanossomatídeos***" do qual participou(aram) o(s) aluno(s): **Manzélio Cavazzana Júnior, Angela Cristina Veríssimo Junqueira e Luciana Lima,** está de acordo com os Princípios Éticos de Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela *COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL* (CEEA) em **24.05.2004.**

São Paulo, 25 de maio de 2004

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Coordenadora da CEEA

Prof. Dr. Francisco Carlos Pereira
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UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS

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Título da Tese: Diversidade morfológica, biológica e genética, e relações filogenéticas de tripanossomas de morcegos do Brasil e Moçambique (África).

Orientador(a): Marta Maria Geraldes Teixeira.

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AGRADECIMENTOS

À Profa. Dra. Marta Maria Geraldes Teixeira pela oportunidade, incentivo e exemplo de profissionalismo.

Ao Prof. Dr. Erney Camargo pelo estímulo e colaboração dados a este trabalho.

À Marta Campaner pelos ensinamentos iniciais e pela dedicação no isolamento e manutenção dos parasitas utilizados neste estudo.

À Carmen Takata pelo auxílio constante no laboratório e grande ajuda na parte da morfologia deste trabalho.

À Profa. Gentilda K. Funayama Takeda por todo apoio prestado no meu ingresso ao laboratório.

Ao Prof. Dr. Fernando Paiva (UFMS) pela colaboração nas coletas do Pantanal.

Aos Profs: Dr. Luis Neves (Universidade Eduardo Mondlane – Maputo) e Carlos L. Pereira (Parque Nacional da Gorongosa) pela colaboração nas coletas em Moçambique.

Aos colaboradores: Dra. Vânia L.B. Nunes (UNIDERP), Dra. Ângela C.V. Junqueira (FIOCRUZ) pelas amostras cedidas.

Ao prof. Dr. Valdir Tadei *(in memoriam*) e Dra Caroline Cotrin Aires (Museu de Zoologia da USP) pela identificação dos morcegos brasileiros.

Aos amigos que já saíram do laboratório e aos que continuam – em especial à Flávia, que contribuiu diretamente em meu trabalho.

A todos os docentes e funcionários do Departamento de Parasitologia (ICB/USP).

Aos meus amigos do departamento.

A todos meu muito Obrigado.

Tente mover o mundo – o primeiro passo será mover a si mesmo.

Platão

Este trabalho contou com apoio financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Cordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

PREFÁCIO

"Esta tese foi elaborada de acordo as normas da CPG / ICB relativas a outras formas de elaboração de tese de doutorado que permite a inclusão, como Anexos, de resultados já publicados ou submetidos em periódicos internacionais indexados em língua inglesa. Permite ainda que detalhes metodológicos e resultados obtidos sejam aqueles contidos nos artigos anexados ao corpo da tese".

Artigos que compõem o corpo da tese:

Anexo 1- Phylogeographical, ecological and biological patterns shown by nuclear (ssrRNA and gGAPDH) and mitochondrial (Cyt b) genes of trypanosomes of the subgenus *Schizotrypanum* parasitic in Brazilian bats

Anexo 2- A new genotype of *Trypanosoma cruzi* associated with bats evidenced by phylogenetic analyses using SSU rRNA, cytochrome b and Histone H2B genes and genotyping based on ITS1 rDNA

Anexo 3- A *Trypanosoma cruzi*-like new species from African bats (Chiroptera: Molossidae) supported by morphological, developmental and multigene phylogenetic analyses

Anexo 4- *Trypanosoma rangeli* isolates of bats from Central Brazil: genotyping and phylogenetic analysis enable description of a new lineage using spliced-leader gene sequences

Anexo 5- A new trypanosome species infecting African bats (*Rhinolophidae* and *Hipposideridae*): taxonomic appraisal based on morphological and behavioral features and phylogenetic inferences using SSU rRNA and gGAPDH genes

Anexo 6- Genes of cathepsin L-like proteases in *Trypanosoma rangeli* isolates: Markers for diagnosis, genotyping and phylogenetic relationships

Anexo 7- Repertoire diversity, evolutionary relationships and genomic organization of cruzipain encoding genes in phylogenetic diverse *Trypanosoma cruzi* isolates and homologues from closely related *T. c. marinkellei* and *T. dionisii*

RESUMO

Lima L. Diversidade morfológica, biológica e genética, e relações filogenéticas de tripanossomas de morcegos do Brasil e Moçambique (África). Tese de Doutorado. Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2011.

Apesar de conhecido, há mais de 100 anos, que os morcegos albergam uma grande variedade de tripanossomas dos subgêneros *Schizotrypanum*, *Megatrypanum* e *Herpetosoma*, nosso conhecimento sobre a diversidade genética, variedade de hospedeiros e de vetores, ciclos de vida, distribuição e relações filogenéticas é restrito a poucas espécies de *Schizotrypanum*. A maioria dos tripanossomas de morcegos foi classificada exclusivamente com base em morfologia. A comparação de padrões eco-geográficos de tripanossomas e de morcegos permite avaliar possíveis cenários que expliquem a diversidade, relações filogenéticas e distribuição atual dos tripanossomas de morcegos.

Nessa tese, a diversidade genética e padrões filogeográficos de tripanossomas de morcegos brasileiros foi avaliada pelo exame de 1.043 morcegos, de 63 espécies pertencentes a 7 famílias, da Amazônia, Pantanal, Cerrado e Mata Atlântica. A prevalência de morcegos infectados por tripanossomas foi de 12,9% por hemocultura, resultando em 77 culturas. A maioria das culturas foi morfológica- e molecularmente identificada como *Schizotrypanum* spp. As análises filogenéticas dos genes SSUrRNA, gGAPDH e Cytb revelaram três clados, *T. dionisii*, *T. c. marinkellei* e *T. cruzi*, que juntos formam o clado *Schizotrypanum*. *T. dionisii* (32,4%) foi encontrado em morcegos pertencentes a 4 famílias, em todos os biomas; *T. c. marinkellei* (49,3%) apenas em filostomídeos da Amazônia e Pantanal. *T. cruzi* (18,2%) pricipalmente em morcegos vespertilionídeos e filostomídeos do Pantanal/Cerrado e da Mata Atlântica, com poucos isolados da Amazônia. Os clados de tripanossomas de morcegos foram relacionados com os vetores, história evolutiva, ecologia e filogeografia dos morcegos.

T. cruzi é um complexo de isolados geneticamente heterogêneo distribuídos em seis DTUs (TcI-VI), mais relacionadas com *T. c. marinkellei* do que com *T. dionisii*. Todos os isolados de morcegos da Amazônia foram genotipados como TcI, enquanto os das regiões Central e Sudeste revelaram um novo genótipo, que denominamos Tcbat, que não é virulento para camundongos. Tcbat foi encontrado em ambientes antrópicos, confirmando que morcegos são importantes como reservatórios e fontes de *T. cruzi* para o homem. A descoberta desse novo genótipo indica que a complexidade de *T. cruzi* é maior do que conhecemos.

Apenas *Schizotrypanum* spp. de morcegos da América do Sul e da Europa foram analisados molecularmente, espécies descritas em morcegos africanos necessitam de evidências filogenéticas. Caracterizamos 6 isolados de uma nova espécie de *T. cruzi*-like parasita de morcegos (Molossidae) de Moçambique (África), com morfologia típica de *T.*

cruzi-like. Esses isolados formaram um clado homogêneo no sugênero *Schizotrypanum* em inferências com SSUrRNA, gGAPDH, ITS1rDNA e SL, mais relacionado com *T. c. marinkellei* do que com *T. dionisii,* e separado por maiores distâncias de *T. cruzi*. Análises com todos esses genes mostraram que esses isolados constituem uma nova espécie, *T. erneyi*, a primeira espécie de *Schizotrypanum* de morcegos africanos molecularmente caracterizada. Os tripanossomas do subgênero *Schizotrypanum* e os morcegos estão intimamente unidos, sendo um exemplo impressionante de relação parasita-hospedeiro e longa história evolutiva compartilhada. A existência de *T. erneyi* na África lança nova luz sobre a diversidade, dispersão e evolução de *Schizotrypanum,* fornecendo novas pistas sobre a a origem de *T. cruzi,* que pode ter divergido de um tripanossoma exclusivo de morcego ou vice-versa.

T. rangeli é um parasita não patogênico do homem, animais domésticos e silvestres nas Américas Central e do Sul. Essa espécie compartilha vetores, hospedeiros e distribuição com *T. cruzi,* porém, nunca foi comprovada em morcegos. Estudos prévios demonstraram que *T. rangeli* é composto por 4 linhagens filogenéticas (A-D). Nessa tese, caracterizamos dois isolados em morcegos brasileiros, um da linhagem A e outro de uma nova linhagem (E). Estes são os primeiros isolados de *T. rangeli* de morcegos molecularmente caracterizados.

Dentre os isolados de morcegos de Moçambique, caracterizamos morfologica-, biologica- e filogeneticamente 12 isolados de rhinolophides, com tripomastigotas sanguíneos típicos do subgênero. Entretanto, filogenias baseadas em SSUrRNA e gGAPDH posicionaram esses isolados distantes de todos os demais tripanossomas, permitindo a descrição de uma nova espécie (*T. zambesiensis*) que não foi posicionada em nenhum subgênero, corroborando a polifilia de *Megatrypanum*, que tem sido revisto e revalidado filogeneticamente.

A enzima CATL tem um papel fundamental na invasão celular, diferenciação, imunidade e patogenicidade dos tripanossomas, sendo explorada como alvo para drogas e vacinas. A análise de genes CATL de 17 isolados de *T. rangeli,* representativos da diversidade genética e distribuição geográfica*,* comprovaram as linhagens previamente definidas por outros genes, corroborando ciclos de transmissão independentes associados com espécies simpátricas de *Rhodnius*. Esse gene se mostrou excelente para diagnóstico e genotipagem de *T. rangeli.* Análises de repertório e genealogia dos genes codificadores dessas enzimas podem ajudar a esclarecer o papel delas nos ciclos de vida e na patologia. Em um estudo bastante abrangente, comparamos genes codificadores de cruzipaína (CATL de *T. cruzi*) de isolados *T. cruzi* representantes da diversidade biológica, genética e patológica. Esses genes são conservados intra- e inter-isolados da mesma DTU, exceto nas DTUs TcV e VI que apresentam sequências polimórficas, concordante com origem por

hibridização entre TcII e TcIII. Comparando os genes de cruzipaína e homólogos de *T. cruzi*-like e *T. rangeli,* detectamos polimorfismos espécie e linhagem-específicos. As genealogias inferidas corroboraram as relações filogenéticas entre as diferentes espécies de tripanossomas.

Novas análises, com mais isolados e diversos genes, são ainda necessárias para se obter filogenias bem resolvidas, que são importantes para inferir as hipóteses prováveis da história evolutiva dos tripanossomas de morcegos.

Palavras-chave: *Trypanosoma*, Morcegos, *Schizotrypanum*, *Megatrypanum*, Filogenia, Taxonomia

ABSTRACT

Lima L. Morphological, biological and genetic diversity, and phylogenetic relationships of bat trypanosomes from Brazil and Mozambique (Africa). Tese de Doutorado. Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2011.

Although it has been known for more than 100 years that bats harbour a variety of trypanosomes from the subgenera *Schizotrypanum, Megatrypanum* and *Herpetosoma*, our knowledge regarding the genetic diversity, host range, vectors, life cycles, geographical distribution and phylogenetic relationships of these trypanosomes is restricted to a few species. Because the majority of bat trypanosomes were classified exclusively based on morphology, their taxonomy remains problematic and have been phylogenetically revised. Comparison of eco-geographical patterns of trypanosomes and bats can be helpful in evaluating scenarios that could account for the diversity, relationships and current distribution of bat trypanosomes.

In this study, the genetic diversity and phylogeographical patterns of trypanosomes that infect Brazilian bats were evaluated by examining 1043 bats from 63 species of 7 families captured in Amazonia, the Pantanal, Cerrado and the Atlantic Forest. The prevalence of trypanosome-infected bats as estimated by haemoculture was 12.9%, resulting in 77 cultures. Most cultures were morphologically and molecularly identified as *Schizotrypanum* spp. Phylogenetic analyses of SSUrRNA, gGAPDH and Cyt b genes revealed three subclades, *T. dionisii, T. c. marinkellei* and *T. cruzi*, all clustering together forming the clade *Schizotrypanum*. *T. dionisii* (32.4%) infected bats from 4 families captured in all biomes; *T. c. marinkellei* (49.3%) was restricted to phyllostomids from Amazonia to the Pantanal. *T. cruzi* (18.2%) was found mainly in vespertilionids and phyllostomids from the Pantanal/Cerrado and the Atlantic Forest, with a few isolates from Amazonia. Therefore, bat trypanosomes were related to theirs vectors, and the evolutionary history, ecology and phylogeography of the hosts.

T. cruzi is a complex of genetically heterogeneous isolates distributed in six discrete typing units (DTUs TcI-VI), more phylogenetically related to *T. c. marinkellei* than to *T. dionisii.* Phylogenetic relationships positioned all Amazonian bat isolates into TcI while isolates from Central and Southeast regions constituted the new genotype Tcbat, which lacked virulence and yielded low parasitaemias in mice. Tcbat was found only in bats from anthropic environments, confirming bats as important reservoirs and potential source of *T. cruzi* infections to humans. Our findings corroborated that the complexity of *T. cruzi* is larger than currently known.

Only bat *Schizotrypanum* spp. from South America and Europe have been characterised, alleged species from Australia and Africa still lack phylogenetic evidence. We

characterized 6 isolates from bats (Molossidae) captured in Mozambique. Morphological and behaviour features were all typical of *T. cruzi*-like. All the isolates constituted a homogenous clade within the *Schizotrypanum* by phylogenetic analysis of SSUrRNA, gGAPDH, ITS1 rDNA and SL sequences. Comparable distances separated this new species from their closest species, *T. c. marinkellei* and *T. dionisii,* while *T. cruzi* was separated by larger distances. These findings supported these trypanosomes as a new species, *T. erneyi,* the first molecularly characterised from African bats. *Schizotrypanum* trypanosomes and bats are tightly united in a striking example of an ancient and intimate host-parasite partnership. The existence of *T. erneyi* in Africa sheds new light on the diversity and evolutionary history of *Schizotrypanum*, providing new insights into the understanding of the origin and evolution of *T. cruzi,* which speculatively could have evolved from a bat-restricted trypanosome or vice versa

T. rangeli is a non-pathogenic parasite of man and domestic and wild animals in Central and South America transmitted by triatomines, which share hosts and overlapped distribution with *T. cruzi*. This species has never confidently been described in bats. Previous analyses demonstrated that *T. rangeli* comprised 4 phylogenetic lineages (A-D). Here, we characterized two isolates of *T. rangeli* from Brazilian bats, one assigned to lineage A and other to the new lineage E. These isolates are the earliest *T. rangeli* from bats molecularly characterized, and the reference-isolates of the lineage E.

We also characterized morphologically, biologically and phylogenetically 12 isolates from African rhinolophid microbats from Mozambique. The isolates showed blood trypomastigotes typical of the subgenus *Megatrypanum,* which have been questioned phylogenetically. However, culture and biological features separated the new bat isolates from *Schizotrypanum,* and SSUrRNA and gGAPDH phylogenies revealed that they are distant from all trypanosomes, prompting the description of a new species that could not be positioned within any subgenus.

Cathepsin L-like (CATL) enzymes are cysteine proteases that play a vital role in the metabolism, infectivity, cell differentiation, immunity and pathogenicity of trypanosomes, and have been exploited as potential targets for drugs, vaccines and diagnoses. An understanding of CATL evolutionary relationships can assist in clarifying the role of these enzymes in the parasite life cycles and pathogeneses. Phylogenetic analysis of CATL sequences from 17 isolates representative of the genetic diversity and geographical range of *T. rangeli* supported the lineages previously established, corroborating independent cycles and divergence associated with sympatric species of *Rhodnius*. In addition, CATL proved to be excellent targets for diagnosis and genotyping of *T. rangeli*. We also compared cruzipain (major CATL from *T. cruzi*) encoding genes from *T. cruzi* isolates representative of the overall biological, genetic and pathological diversity. Conserved genes are found within and

among isolates of the same DTU, excepting TcV-VI that showed polymorphic sequences supporting hybridization origin. Analyses of cruzipain from *T. cruzi* DTUs and homologues from *T. cruzi*-like disclosed species- and lineage-specific polymorphisms valuable to understand host-parasite interactions and crucial for evaluation of cruzipain as target for diagnostic, drugs and vaccine approaches. Genealogies of cruzipain encoding genes agree with the diversity and phylogenetic relationships of trypanosome species and *T. cruzi* DTUs.

Further analyses aiming better-resolved phylogenies and reliable molecular-clock model to estimate divergence times are required to infer the most likely hypothesis for the evolutionary history of bat trypanosomes.

Keywords: *Trypanosoma*, Bats, *Schizotrypanum*, *Megatrypanum*, Phylogeny, Taxonomy

LISTA DE ABREVIATURAS

B	Bayesiana
BAB	“Blood Agar Base”
CATB	Catepsina B
CATL	Catepsina L
cGAPDH	Gliceraldeído 3-fosfato dehidrogenase citosólica
CHCl3	Clorofórmio
COII	Citocromo oxidase II
Cytb	Citocromo b
DMSO	Dimetilsulfóxido
DNA	“Desoxiribonucleic acid”
DTU	“Discret Typing Units”
EDTA	Ácido etileno diamino tetracético
ETS	“External transcribed spacer”
g	grama
gGAPDH	Gliceraldeído 3-fosfato dehidrogenase glicossomal
GIPLs	Gliconositol fosfolipídeos
KCl	Cloreto de Potássio
kDNA	“Kinetoplast desoxiribonucleic acid”
KH_2PO_4	Fosfato de Potássio
h	Hora
HCl	Ácido clorídrico
IGS	“Intergenic spacer”
ITS	“Internal transcribed spacer”
Kb	Kilobases
LB	“Luria –Bertani Médium”
LIT	Meio de cultura Infuso de fígado
LSU	“Large Subunit”
M	Molar
mg	Miligrama
$MgCl_2$	Cloreto de Magnésio
MH	Microhematócrito
min	Minutos
ml	Mililitro
mM	Milimolar
mRNA	“Messenger ribonucleic acid”
MP	Máxima Parcimônia
MV	Máxima Verossimilhança
mya	“Millions years ago”
N_2	Nitrogênio
NaCl	Cloreto de sódio
NADH	Nicotinamida adenina dinocleótido hidreto
Na_2HPO_4	Fosfato disodium
NaOH	Hidróxido de sódio
ng	Nanograma
pb	Pares de bases
RAPD	“Random Amplified Polymorphic DNA”
rDNA	“Ribosomal desoxiribonucleic acid”
rRNA	“Ribosomal ribonucleic acid”
RNA	“Ribonucleic acid”
RNAse	Ribonuclease
SE	Solução salina Tris-EDTA
seg.	Segundos

SFB	Soro fetal bovino
SL	“Spliced leader”
SSU	“Small subunit”
TAE	Tampão Tris acetato-EDTA
TCC	Trypanosomatidae Culture Collection
TE	Tampão tris-EDTA
UV	ultravioleta
v	Volume
mg	micrograma
ml	microlitro
℃	Graus Celsius

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1. INTRODUÇÃO

1.1 O genêro *Trypanosoma*: história evolutiva

O gênero *Trypanosoma* pertence à Família Trypanosomatidade que compreende protozoários flagelados pertencentes à ordem Kinetoplastida, atualmente classe Kinetoplastea. Os cinetoplastídeos juntamente com os euglenóides e os diplonemídeos formam o filo Euglenozoa. Análises filogenéticas abrangentes do filo Euglenozoa apóiam a hipótese de que os cinetoplastídeos são mais relacionados com os diplonemídeos, e que os tripanossomatídeos formam um grupo monofilético que evoluiu de bodonídeos de vida livre (Busse e Preisfeld, 2002; 2003; Hughes e Piontkivska, 2003; Simpson et al., 2006; Roger e Simpson, 2009). A monofilia do filo Euglenozoa está bem estabelecida, porém, as análises filogenéticas ainda têm sido insuficientes para resolver a filogenia do filo, assim como sua relação com os demais filos de protozoários (Keeling et al., 2005; Moreira et al., 2007; Yoon et al., 2008; Hampl et al., 2009; Roger e Simpson, 2009).

A classe Kinetoplastea, que tradicionalmente compreende as subordens Trypanosomatina e Bodonina, se caracteriza pela presença do cinetoplasto, uma região especializada da mitocôndria constituída por moléculas de DNA circulares concatenadas (Simpson et al., 2006; Stevens, 2008). Um sistema de classificação recente propôs a separação da classe Kinetoplastea em duas subordens: Prokinetoplastina, que contêm ectoparasitas de peixes e endossimbiontes de protozoários amebóides e Metakinetoplastina que consiste em 4 grupos principais, três clados de bodonídeos (Neobodonida, Parabodonida e Eubodonida) e o clado dos tripanossomatídeos (Trypanosomatida) (Moreira et al., 2004). Os tripanossomatídeos formam um grupo monofilético que corresponde à família Trypanosomatidae. Esses organismos são parasitas obrigatórios de invertebrados, plantas e de, virtualmente, todas as ordens de vertebrados (Vickerman, 1976; Simpson et al., 2006, Stevens, 2008).

A família Trypanosomatidae apresenta uma grande diversidade de hospedeiros, desde plantas a animais invertebrados e vertebrados de praticamente todas as ordens, com ampla distribuição nos diferentes continentes. Essa família compreende parasitas monoxênicos, que apresentam apenas um hospedeiro vertebrado, ou heteroxênicos, quando participam do seu ciclo biológico dois hospedeiros, sendo um invertebrado e um vertebrado (Wallace, 1966, 1979; Vickerman, 1994; Simpson et al., 2006; Stevens, 2008).

De acordo com parâmetros morfológicos, hospedeiro de origem e ciclo de vida, os tripanossomatídeos estão atualmente distribuídos em 12 gêneros. Oito gêneros compreendem protozoários monoxênicos de insetos (*Herpetomonas*, *Crithidia*, *Blastocrithidia*, *Leptomonas*, *Wallaceina*, Sergeia, *Strigomonas* e *Angomonas*). Quatro

gêneros albergam espécies heteroxênicas em cujos ciclos há uma alternância entre hospedeiros invertebrados (artrópodes hematófagos) e vertebrados (*Trypanosoma*, *Leishmania* e *Endotrypanum*) ou entre insetos fitófagos e plantas (*Phytomonas*) (Wallace, 1966, 1979; Wallace et al., 1983; Hoare, 1972; Vickerman, 1976; Camargo, 1998; Merzlyak et al., 2001; Svobodová et al., 2007; Teixeira et al., 2011).

Hipóteses fundamentadas em reconstruções filogenéticas tentam explicar a origem dos tripanossomatídeos e dos ciclos heteroxênicos de *Trypanosoma*, *Leishmania*, *Endotrypanum* e *Phytomonas*. Esses estudos propõem diferentes histórias evolutivas, dependendo dos táxons e grupos externos utilizados, dos genes e dos métodos de inferências filogenéticas. As espécies de *Trypanosoma* do clado aquático, parasitas de peixes e anuros, não foram os primeiros tripanossomatídeos a divergir dos ancestrais de vida livre, contrariando a hipótese de que o ancestral teria sido um parasita de peixe transmitido por sanguessugas e relacionado com bodonídeos parasitas de peixes, como *Cryptobia* ou *Trypanoplasma* (Hamilton et al., 2004, 2007; Simpson et al., 2006).

Análises filogenéticas recentes de um grande número de espécies dos diferentes grupos de tripanossomatídeos têm gerado filogenias bem resolvidas que permitiram levantar a seguinte hipótese: Um bodonídeo de vida livre pode ter sido ingerido por insetos e se adaptado ao habitat intestinal originando os tripanossomatídeos monoxênicos. Com a aquisição da hematofagia, insetos passaram a inocular esses parasitas em vertebrados e alguns se adaptaram ao parasitismo passando, então, a circular entre insetos hematófagos e vertebrados terrestres. Essa hipótese sugere que as espécies heteroxênicas se originaram das monoxênicas, mais relacionadas com os bodonídeos (Stevens et al., 2001; Lukes et al., 2002; Hamilton et al., 2004, 2007; Simpson et al., 2006; Stevens, 2008).

Insetos hematófagos existentes há milhões de anos, como as moscas tsétsé e os flebotomíneos, podem ter participado desse processo dando origem, respectivamente, aos tripanossomas dos clados *T. brucei* e aos gêneros *Leishmania* e *Endotrypanum* (Stevens et al., 1999a, 2001; Poinar e Poinar 2004, 2008). A hipótese mais recente sugere que o gênero *Trypanosoma* evoluiu de um tripanossomatídeo (provavelmente ancestral de *Blastocrithidia triatomiae*) parasita de hemípteros (Stevens et al., 2001; Hamilton et al., 2004, 2007; Simpson et al., 2006; Stevens, 2008). Essas hipóteses poderão ser reforçadas, ou novas hipóteses poderão surgir, com a inclusão de novas espécies nos estudos filogenéticos assim como com análises combinadas de diversos genes e análises filogenômicas de tripanossomatídeos e outros membros do filo Euglenoza.

1.2 Filogenia e taxonomia do gênero *Trypanosoma*

As espécies do gênero *Trypanosoma* infectam diversas espécies de vertebrados de todas as classes, peixes, anfíbios, répteis, aves e mamíferos. Apenas *T. brucei gambiense* e *T. b. rhodesiense*, na África, e *T. cruzi* e *T. rangeli*, nas Américas Central e do Sul, infectam o homem e são considerados patogênicos, com exceção de *T. rangeli*. Estes tripanossomas não estão restritos a infecções humanas e se mantêm na natureza no ciclo silvestre, infectando diversas ordens de mamíferos (antropozoonoses). A maioria das espécies de tripanossomas circula apenas no ciclo silvestre (zoonoses) e não é patogênica para seus hospedeiros (Hoare, 1972; Simpson et al., 2006; Hamilton et al., 2007).

Durante os ciclos de vida com alternância entre vertebrados e invertebrados hematófagos, os tripanossomas apresentam várias formas presentes em diferentes combinações no sangue e/ou tecidos dos hospedeiros. As formas tripomastigotas são encontradas nos hospedeiros vertebrados (tripomastigotas sanguíneos) e invertebrados (tripomastigotas metacíclicos). As demais formas são espécies-dependentes, ocorrendo nos vertebrados (amastigotas intracelulares) e invertebrados (promastigotas e epimastigotas). Estas formas são definidas em função da posição do cinetoplasto em relação ao núcleo e da presença ou não de flagelo livre e membrana ondulante (Hoare, 1972; Wallace, 1979; Vickerman, 1994).

A maioria das espécies de tripanossomas se desenvolve ciclicamente nesses hospedeiros e é transmitida por artrópodes hematófagos incluindo moscas, hemípteros (triatomíneos e cimicídeos), mosquitos, pulgas e carrapatos. Algumas espécies são transmitidas por sanguessugas, entre elas os parasitas de peixes, anfíbios e répteis aquáticos. Sanguessugas terrestres também podem transmitir tripanossomas (Hoare, 1972; Stevens et al., 2001; Hamilton et al., 2005a, 2007).

A morfologia foi o primeiro parâmetro taxonômico dos tripanossomatídeos. Para as espécies heteroxênicas dos gêneros *Trypanosoma* e *Leishmania*, além da morfologia eram acrescentadas informações sobre ciclos biológicos e patogenia. O local de desenvolvimento e diferenciação das formas infectantes nos invertebrados determina a via de transmissão dos tripanossomas, que pode ser com as fezes (transmissão contaminativa) ou com saliva (transmissão inoculativa). De acordo com o desenvolvimento no hospedeiro invertebrado e, consequentemente, com a via de eliminação das formas infectantes pelo vetor, as espécies parasitas de mamíferos do gênero *Trypanosoma* foram divididas nas Secções Salivaria e Stercoraria (Hoare, 1964, 1972) (Figura 1).

Gênero Trypanosoma	
Secção Stercoraria (transmissão contaminativa)	**Secção Salivaria (transmissão inoculativa)**
Subgêneros (espécie-referência)	Subgêneros (espécie-referência)
Megatrypanum (*T. theileri*)	***Duttonella*** (*T. vivax*)
Schizotrypanum (*T. cruzi*)	***Nannomonas*** (*T. congolense*)
Herpetosoma (*T. lewisi*)	***Trypanozoon*** (*T. brucei*)
	Pycnomonas (*T. suis*)

Figura 1. Diagrama de classificação das espécies de tripanossomas de mamíferos. (Hoare,1972)

A Secção Salivaria compreende apenas tripanossomas de origem africana que se desenvolvem ciclicamente exclusivamente em moscas tsétsé (Glossina). Esse desenvolvimento pode ocorrer no tubo digestivo e glândulas salivares (*T. brucei*), apenas no tubo digestivo (*T. congolense*) ou no caso de *T. vivax* ser restrito à probóscide. *T. evansi* e *T. equiperdum* são apenas mecanicamente transmitidos, respectivamente, por insetos hematófagos ou pelo coito. Esta Secção compreende os subgêneros *Duttonella*, *Trypanozoon*, *Pycnomonas* e *Nannomonas*, que abrangem todos os tripanossomas africanos geralmente patogênicos para seus hospedeiros (Hoare, 1972; Stevens et al., 2001; Hamilton et al., 2007; Stevens, 2008). *T. vivax* (*Duttonella*), *T. evansi* e *T. equiperdum* (*Trypanozoon*) se adaptaram à transmissão mecânica e, por esta razão, são as únicas espécies deste grupo que ocorrem fora do continente Africano, inclusive na Ásia e nas Américas Central e do Sul (Hoare, 1972; Ventura et al., 2001; Cortez et al., 2006; Lai et al., 2008; Lun et al., 2010).

Na Secção Stercoraria, que compreende os subgêneros *Schizotrypanum* (espécie-tipo *T. cruzi*), *Herpetosoma* (*T. lewisi*) e *Megatrypanum* (*T. theileri*) (Hoare, 1972), estão classificadas as espécies que se desenvolvem exclusivamente no tubo digestivo do inseto vetor, sendo transmitidas pela contaminação com formas tripomastigotas metacíclicas eliminadas com as fezes dos vetores. Os tripanossomas desta Secção apresentam ampla distribuição geográfica.

Para as descrições de espécies de tripanossomas, os parâmetros tradicionais são morfologia e morfometria. Hoare (1972), na última revisão taxonômica do gênero *Trypanosoma*, criou subgêneros, identificou inúmeras sinonímias e propôs parâmetros para a identificação de espécies parasitas de mamíferos com base na morfologia das formas do sangue. Porém, as formas encontradas no sangue de diversas espécies, geralmente, não apresentam características que permitam identificar espécies ou mesmo subgêneros de tripanossomas. Esses organismos apresentam grande pleomorfismo e formas diferentes podem corresponder a espécies diferentes ou a estágios de diferenciação de uma mesma

espécie. Além da morfologia, os vetores, a origem geográfica e o hospedeiro de origem são outros parâmetros taxonômicos utilizados. Esses parâmetros partem do pressuposto de que cada espécie de hospedeiro alberga uma só espécie, e que a separação geográfica originou espécies diferentes de tripanossomas. Porém, apesar de associações entre algumas espécies e seus hospedeiros vertebrados e vetores, são raras as espécies restritas a um só hospedeiro. A distribuição da maioria das espécies não é conhecida, ainda assim, espécies cosmopolitas, assim como espécies restritas as Américas ou a África são bem conhecidas. Portanto, esses parâmetros foram totalmente invalidados (Stevens et al., 1999a,b, 2001; Maia da Silva et al., 2004a, 2010; Rodrigues et al., 2006, 2008; Hamilton et al., 2004, 2007, 2009; Ferreira et al., 2007, 2008; Viola et al., 2008, 2009).

A taxonomia molecular tem facilitado enormemente a análise da diversidade dos tripanosomatídeos. Atualmente, a adoção de parâmetros taxonômicos moleculares é obrigatória para os tripanossomatídeos em geral. Entretanto, ainda é difícil classificar os tripanossomatídeos em gêneros e espécies devido à falta de parâmetros confiáveis e de fácil utilização. Uma grande limitação tem sido a necessidade de aumentar o número de espécies e isolados analisados. O conhecimento abrangente da diversidade de espécies é uma condição indispensável para que sejam inferidas relações filogenéticas consistentes que, por sua vez, serão a base da nova taxonomia desses organismos. Um dos principais problemas nas revisões taxonômicas desses organismos tem sido a falta de critérios objetivos, estabelecidos com base em estudos filogenéticos e aceitos pelos pesquisadores da área.

Atualmente, análises filogenéticas são obrigatórias para o posicionamento taxonômico dos tripanossomatídeos. A criação de gêneros e descrição de uma nova espécie de tripanossomatídeo deve partir de uma hipótese gerada por análises filogenéticas. Os estudos filogenéticos vêm permitindo identificar novas espécies e sinonímias, e definir grupos que podem corresponder a novos gêneros (Stevens et al. 1999a,b, 2001; Maia da Silva et al. 2004a,b; Hamilton et al., 2004, 2005a,b, 2007, 2009; Rodrigues et al. 2006, 2008; Ferreira et al. 2007, 2008; Viola et al., 2008, 2009a,b; Teixeira et al., 2011).

As análises filogenéticas têm questionado a validade da maioria dos gêneros e subgêneros de tripanossomas definidos por parâmetros taxonômicos fenotípicos. Todos os estudos indicam gêneros e subgêneros artificiais, além de uma enorme sinonímia de espécies. Quase todos os gêneros são polifiléticos, exceto *Trypanosoma* (apesar de controvérsias recentes), *Leishmania* (apesar do relacionamento conflitante com *Endotrypanum*) e *Phytomonas* (Maslov et al. 1996; Hollar e Maslov 1997; Lukes et al. 1997; Stevens et al. 2001; Hamilton et al., 2004, 2005a, 2007; Simpson et al. 2006; Stevens, 2008).

Alguns estudos filogenéticos sugeriram que o gênero *Trypanosoma* era polifilético (Maslov et al., 1996; Hughes e Piontkivska, 2003; Piontkivska e Hughes 2005), porém, estudos posteriores com um número muito maior de taxa e novos “outgroups” demonstraram a monofilia desse gênero (Haag et al., 1998; Stevens et al., 1999a,b, 2001; Wright et al., 1999; Hamilton et al., 2004, 2007; Ferreira et al., 2007; Viola et al., 2009a,b). Recentemente, um estudo multigênico com mais de 50 genes corroborou, mais uma vez, com a monofilia dos tripanossomas (Leonard et al., 2011).

De acordo com as topologias das árvores filogenéticas baseadas nos genes SSU rRNA e gGAPDH (Hamilton et al., 2004, 2007; Simpson et al., 2006) foram definidos diversos clados que têm se mantido estáveis em todas as análises. Até o momento, foram estabelecidos pelo menos 9 clados principais: 1) clado aquático com tripanossomas de peixes e anuros; 2) clado de tripanossomas de crocodilianos; 3) clado *T. theileri* associado com ruminantes; 4) clado de tripanossomas de lagartos e cobras; 5) clado *T. brucei*, tripanossomas de mamíferos de origem africana transmitidos por tsétsé); 6) clado de tripanosomas de aves; 7) clado *T. lewisi* de tripanossomas, principalmente, de roedores; 8) clado *T. rangeli*; 9) clado *Schizotrypanum* (*T. cruzi* e *T. cruzi*-like) (Stevens et al., 1999a,b, 2001; Rodrigues et al., 2006, 2008; Hamilton et al., 2004, 2005a,b, 2007, 2008; Ferreira et al., 2007, 2008; Viola et al., 2008, 2009a,b) (Figura 2)

Apenas a ordem de divergência dos três clados principais, aquático, *T. brucei* e *T. cruzi/T. rangeli*, está bem estabelecida. Alguns desses clados compreendem subgêneros que estão sendo revalidados. Entretanto, diversos clados não correspondem a nenhum subgênero proposto, e diversas espécies não foram posicionadas em nenhum desses clados. Portanto, além da revisão das espécies, novos subgêneros deverão ser criados para acomodar a diversidade de tripanossomas que tem sido descoberta com os estudos filogenéticos de um grande número de tripanossomas de hospedeiros e regiões geográficas distintas. A maioria dos tripanossomas de mamíferos tem sido posicionada nas árvores filogenéticas junto com as espécies da Secção Stercoraria.

Nos últimos anos, diversas novas espécies, linhagens e genótipos de tripanossomas de mamíferos têm sido descritos em ruminantes (Adams et al., 2008, 2010; Rodrigues et al., 2006; Gibson et al., 2010), roedores (Sato et al., 2007; Jittapalapong et al., 2008; Maia da Silva et al., 2010; Guan et al., 2011) e marsupiais Autralianos (McInnes et al., 2009, 2011; Averis et al., 2009). Desde a última revisão dos tripanossomas de mamíferos, apenas uma nova espécie de tripanossoma de morcego foi descrita, *T. desterrensis* em Santa Catarina, Brasil (Grisard et al., 2003). Entretanto, os morcegos são conhecidos há mais de 110 anos como hospedeiros de dezenas de espécies de tripanossomas classificadas em diversos subgêneros (Hoare, 1972; Molyneux, 1991).

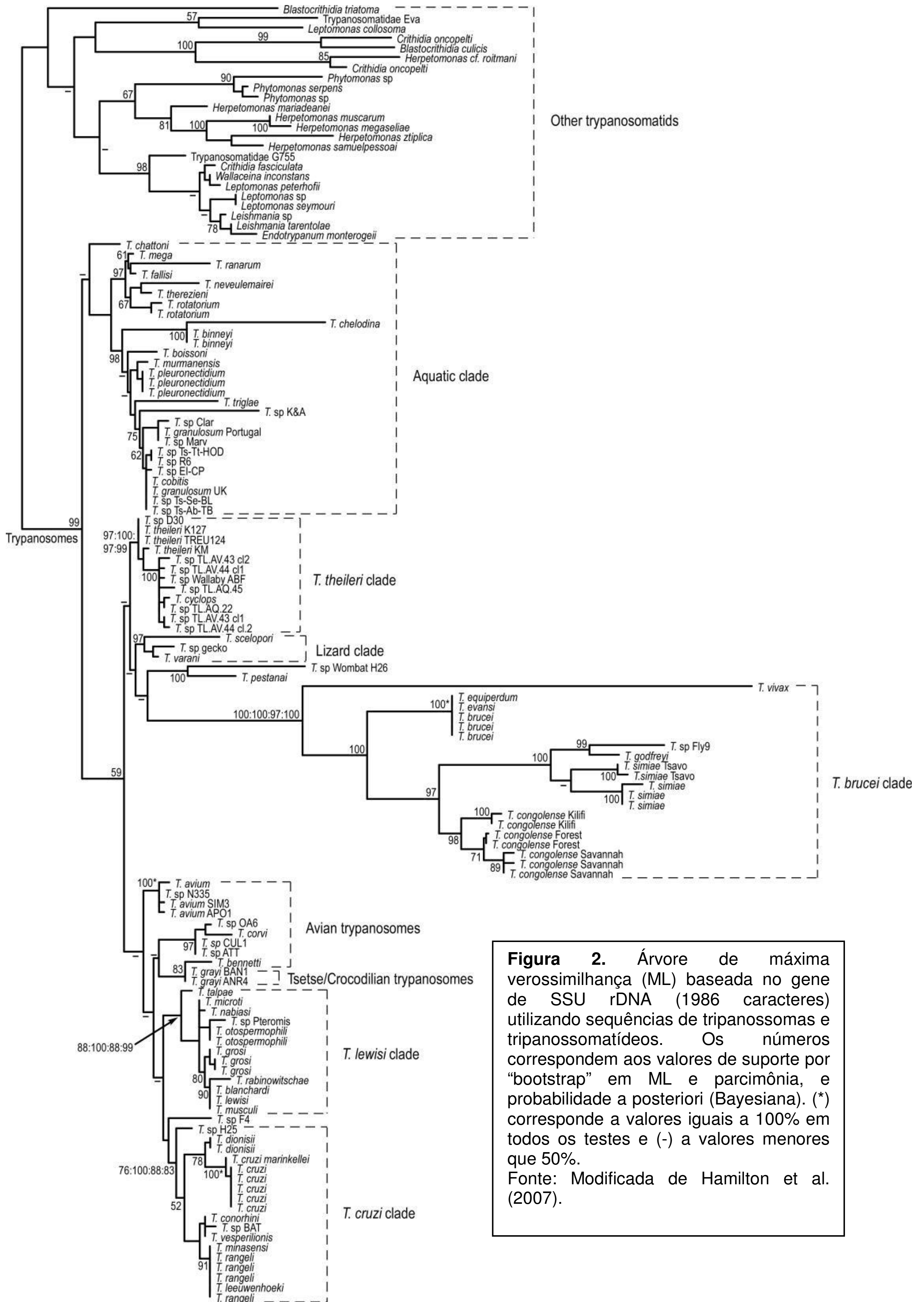


Figura 2. Árvore de máxima verossimilhança (ML) baseada no gene de SSU rDNA (1986 caracteres) utilizando sequências de tripanossomas e tripanossomatídeos. Os números correspondem aos valores de suporte por "bootstrap" em ML e parcimônia, e probabilidade a posteriori (Bayesiana). (*) corresponde a valores iguais a 100% em todos os testes e (-) a valores menores que 50%.
Fonte: Modificada de Hamilton et al. (2007).

1.3 Tripanossomas de morcegos

Pouco se sabe sobre o desenvolvimento de tripanossomas nos morcegos e em seus vetores naturais. Morcegos de diferentes famílias, espécies e hábitos alimentares têm sido encontrados infectados na África, Europa, Ásia, Austrália e Américas por tripanossomas da Secção Stercoraria (subgêneros *Herpetosoma*, *Schizotrypanum* e *Megatrypanum*) e Salivaria (*Trypanozoon*). Uma espécie de tripanossoma pode infectar mais de uma espécie de morcego, assim como são comuns morcegos com infecções mistas com mais de uma espécie de tripanossoma. Embora a ocorrência de tripanossomas em morcegos seja muito frequente, a maioria das espécies descritas não foi cultivada e os relatos são baseados apenas em dados morfológicos e nos hospedeiros de origem. As infecções mais frequentes são causadas por espécies dos subgêneros *Megatrypanum* e *Schizotrypanum* e os quirópteros insetívoros são os mais frequentemente infectados (Hoare, 1972; Marinkelle, 1976; Molyneux, 1991).

O número de tripanossomas isolados de morcegos caracterizados molecularmente é muito pequeno. Estudos filogenéticos sobre tripanossomas de morcegos são escassos e apenas *T. dionisii*, *T. vespertilionis*, *T.* sp bat (isolado na África) e *T. cruzi marinkellei* foram incluídos em árvores filogenéticas. As espécies deste subgênero validadas em filogenias moleculares são: *T. cruzi*, *T. cruzi marinkellei* e *T. dionisii*. Essas espécies formam um clado que corresponde ao subgênero *Schizotrypanum* (Stevens et al., 1999a, 2001; Hamilton et al., 2007). O posicionamento filogenético de *T. vespertilionis* sugere que esta espécie não pertence a este subgênero, ou que o isolado analisado foi classificado erroneamente (Stevens et al., 1999b, 2001). Os tripanossomas mais relacionados com *T. cruzi* formam um clado que compreende *T. rangeli*, *T. vespertilionis* (Europa), *T. conorhini* (Brasil) e os tripanossomas africanos *T.* sp HochNdi1 (macaco), *T.* sp NanDoum1 (carnívoro) e *T.* sp bat (morcego). Um tripanossoma isolado de canguru da Austrália (*T.* sp H25) foi posicionado como "outgroup" de todas essas espécies (Stevens et al., 1999b, 2001; Hamilton et al., 2007).

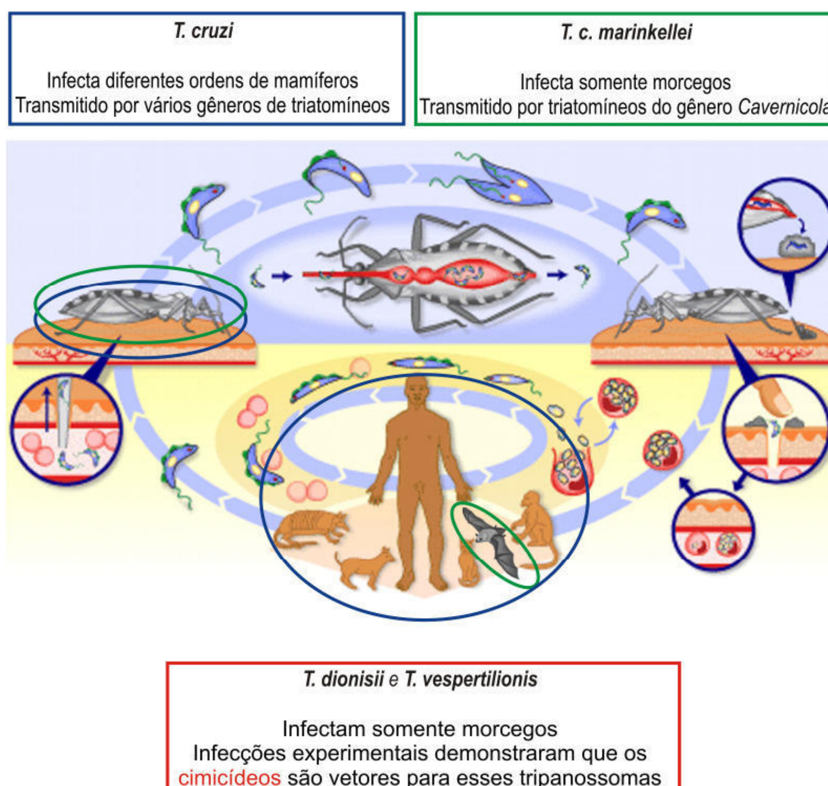
A ausência de parâmetros taxonômicos confiáveis, o pequeno número de espécies incluídas em estudos filogenéticos impede o conhecimento da diversidade e da história evolutiva dos tripanossomas em geral.

1.3.1 Subgênero *Schizotrypanum*

O subgênero *Schizotrypanum* compreende tripanossomas que infectam o homem e outros mamíferos. Com exceção de *T. cruzi*, as demais espécies desse subgênero são restritas aos quirópteros. Enquanto *T. dionisii* e *T. vespertilionis* foram descritos no Novo e

no Velho Mundo, *T. cruzi* é restrito a América Latina e *T. c. marinkellei* ocorre apenas nas Américas Central e do Sul (Hoare, 1972; Molyneux, 1991). Outras espécies deste subgênero foram descritas na América do Norte (*T. hedricki* e *T. myoti*), América Central (*T. phyllostomae*) e Austrália (*T. pteropi* e *T. hipposideri*) (Hoare, 1972; Marinkelle, 1976).

Os ciclos biológicos dos tripanossomas do subgênero *Schizotrypanum* são basicamente iguais, diferindo necessariamente nas espécies de hospedeiros mamíferos e vetores (Figura 3).



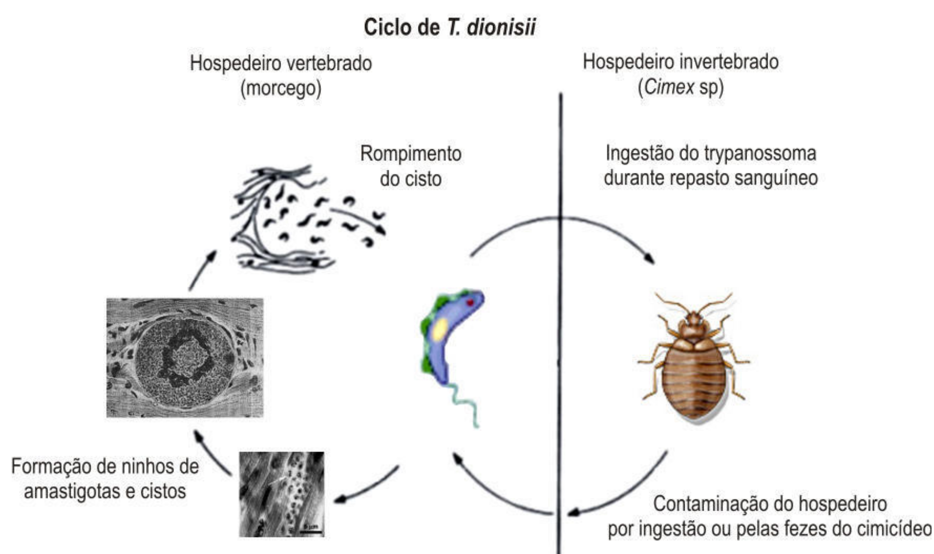


Figura 3. Representação do ciclo biológico de *T. cruzi* e de duas espécies exclusivas de morcegos: *T. c. marinkellei* e *T. dionisii*. Fonte: Modificado de Molyneux, 1991 e http://www.who.int/tdr/diseases/chagas/lifecycle.htm.

Os representantes do subgênero *Schizotrypanum* formam um grupo homogêneo, sendo as formas encontradas no sangue dos mamíferos praticamente indistinguíveis morfologicamente, todas muito semelhantes a *T. cruzi* (Figura 4). Por este motivo, todas as espécies desse subgênero são denominadas *T. cruzi*-like (Marinkelle, 1976; Molyneux, 1991).

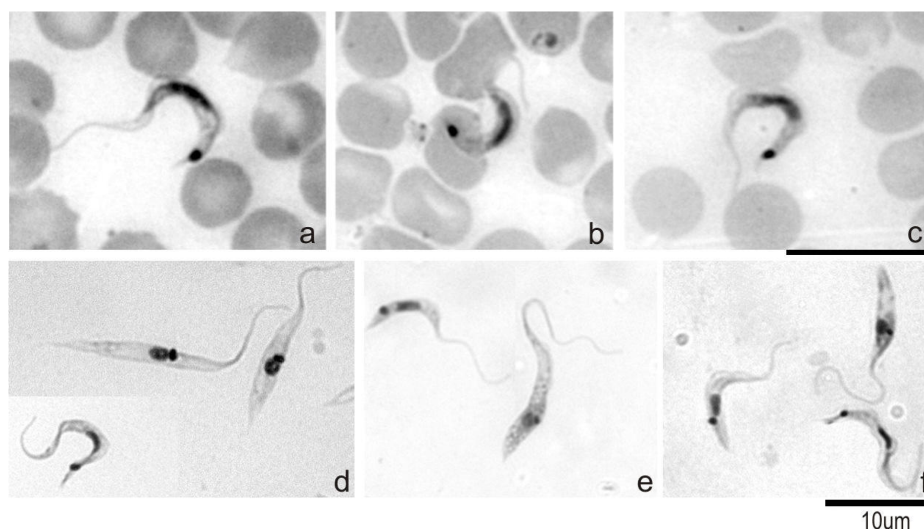


Figura 4. Morfologia por microscopia óptica, das espécies de tripanossomas do subgênero *Schizotrypanum* (*T. cruzi*-like): (a,b,c) formas tripomastigotas encontradas no sangue de morcegos naturalmente infectados; (d,e,f) formas epimastigotas e tripomastigotas metacíclicos em meio cultura (LIT). Ambos corados com Giemsa. Espécies: (a,d) *T. cruzi*; (b,e) *T. c. marinkellei* e (c,f) *T. dionisii.*

As espécies de *Schizotrypanum* são as únicas descritas até o momento que infectam células de mamíferos e se multiplicam no interior destas como amastigotas. A capacidade de infectar camundongos pode ser utilizada para separar *T. cruzi* das demais espécies desse subgênero, que são todas espécies exclusivas de morcegos. O desenvolvimento desses tripanossomas em vetores (triatomíneos e cimicídeos) pode ser utilizado para distinguir espécies (Hoare, 1972; Baker, 1985; Molyneux, 1991).

Análises de zimodemas foram utilizadas na identificação de espécies de *Schizotrypanum* (Miles et al., 1978, 1981a,b; Baker e Miles, 1979; Tibayrenc, 2003; Telleria et al., 2004). Glicoconjugados de superfície (aglutinação com lectinas), GIPLs (glicoinositol fosfolipídeos), perfís de polipeptídeos e reatividade com anticorpos monoclonais distinguiram tripanossomas de morcegos (Taylor et al., 1982; Schotellius et al., 1983; Petry et al., 1986, 1987; Barreto-Bergter et al., 1996; Branquinha et al., 1999).

Trypanosoma cruzi compreende populações bastante heterogêneas que diferem em características morfológicas, biológicas, patológicas, clínicas, imunológicas, bioquímicas e moleculares (Miles et al., 2009). Essa espécie apresenta uma estrutura populacional

complexa, com isolados distribuídos em seis grupos infragenéricos (DTUs - discrete typing units) denominados TcI-TcVI (Zingales et al., 2009). Os isolados pertencentes às DTUs TcI, III e IV predominam no ciclo silvestre, enquanto isolados das DTUs II, V e VI estão associados principalmente com o ciclo doméstico e peridoméstico de transmissão (Gaunt e Miles, 2000; Yeo et al., 2005; Westenberger et al., 2005; 2006; Llewellyn et al., 2009a,b; Marcili et al., 2009a,b; Lewis et al., 2009; Miles et al., 2009).

A maioria dos isolados de *T. cruzi* que foram caracterizados por diversos marcadores moleculares foi obtida de casos humanos de Doença de Chagas, de triatomíneos de áreas endêmicas e de animais do peridomicílio, especialmente de gambás (*D. marsupialis*). O ciclo silvestre do *T. cruzi* ainda é pouco estudado e apenas recentemente passou a ser alvo de estudos moleculares abrangendo diversas espécies de mamíferos, inclusive morcegos e vetores silvestres, com resultados que mostram uma grande complexidade (Miles et al, 1983; Lisboa et al., 2004, 2006, 2008, 2009; Herrera et al., 2008, 2009; Valente et al., 2009).

Tem sido sugerido que a história evolutiva do *T. cruzi* está historicamente associada com a de seus hospedeiros naturais e seus respectivos ecótopos e triatomíneos associados. Diversos eventos, aparentemente, participam dessas histórias evolutivas: associações de linhagens com ordens/espécies de mamíferos e de triatomíneos preferenciais, especiação simpátrica (nichos ecológicos) e alopátrica, transferência de isolados entre mamíferos que compartilham ecótopos mediado por vetores etc. Evidências filogenéticas e biogeográficas indicam que as linhagens TcI e TcIV circulam entre primatas na Amazônia e são transmitidas por triatomíneos do gênero *Rhodnius*. A associação das linhagens com hospedeiros e nichos ecológicos revelou uma sobreposição dos ciclos naturais de transmissão de TcI e TcIV no ecótopo arbóreo (Gaunt e Miles, 2000; Maia da Silva et al., 2008; Marcili et al., 2009a; Llewellyn et al., 2009a; Miles et al., 2009).

Estudos de isolados de *T. cruzi* da linhagem TcIII de mamíferos e de triatomíneos silvestres capturados do Norte a Sul do Brasil tem confirmado a ampla distribuição geográfica de TcIII, assim como a associação com ecótopos terrestres. Tatús, didelfídeos, roedores terrestres e cães domésticos foram encontrados infectados por TcIII, e triatomíneos terrestres dos gêneros *Panstrongylus* e *Triatoma* foram confirmados como vetores (Gaunt e Miles, 2000; Yeo et al., 2005; Martins et al., 2008; Marcili et al., 2009b; Llewellyn et al., 2009b).

Apesar do estudo de um número muito limitado de isolados de animais silvestres, os resultados dos estudos sobre isolados de *T. cruzi* dos ciclos silvestres corroboram a complexidade de *T. cruzi* (Yeo et al., 2005; Lewis et al., 2009; Llewellyn et al., 2009a; Miles et al., 2009). Todos os estudos apontam para a necessidade da utilização de análises filogenéticas, além da genotipagem, para compreender a história evolutiva de *T. cruzi* em associação com seus hospedeiros mamíferos e vetores, em diferentes ecótopos. Devido à

falta de estudos moleculares de isolados de *T. cruzi* de mamíferos e vetores de diferentes espécies, de origens geográficas distintas, a diversidade e as relações filogenéticas intraespecíficas de *T. cruzi* apenas começa a ser entendida.

Infecções causadas por *T. cruzi* em morcegos foram descritas por diversos pesquisadores (Dias, 1936; Funayama et al., 1970 a,b; Funayama, 1973; Barreto et al., 1974). Porém, a diversidade biológica e genética desses tripanossomas ainda é pouco conhecida (Steindel et al., 1998; Grisard et al., 2003; Barnabé et al., 2003; Lisboa et al., 2008). Triatomíneos que vivem em buracos de árvores e cavernas, telhados de folhas de palmeiras, tocas de animais silvestres, palmeiras, forros de residências e outros abrigos de morcegos podem ser vetores de *T. cruzi* entre os morcegos. A maioria dos morcegos infectados é insetívoro, devendo a infecção ocorrer principalmente por via oral com a ingestão dos vetores infectados (Marinkelle, 1976).

De todos os tripanossomas conhecidos até o momento, *T. c. marinkellei* é o mais filogeneticamente relacionado com *T. cruzi*. Apesar da semelhança morfológica e do compartilhamento de morcegos hospedeiros, esses tripanossomas diferem em vários aspectos, todos ainda muito pouco estudados. *T. c. marinkellei*, aparentemente, se restringe a morcegos da família Phyllostomidae e é transmitido apenas por triatomíneos do gênero *Cavernicola* (Marinkelle, 1982a). Entretanto, in vitro, *T. c. marinkellei* infecta e se desenvolve em células de diversos mamíferos semelhante a *T. cruzi*. *T. c. marinkellei* foi confirmado como uma espécie distinta por características biológicas, bioquímicas, imunológicas e moleculares (Baker et al., 1978; Mainkelle, 1982a; Ebert, 1983; Schotellius et al., 1983; Tibayrenc e Le Ray, 1984; Petry et al., 1986; Steindel et al., 1998; Barnabé et al., 2003; Telleria et al., 2010).

São poucos os isolados dessa espécie analisados, existem dúvidas sobre o ciclo de vida e não se conhece os mecanismos envolvidos na restrição de *T. c. marinkellei* aos morcegos nem aos triatomíneos do gênero *Cavernicola*. A grande proximidade de *T. c. marinkellei* com *T. cruzi* foi recentemente corroborada por estudos proteômicos que não permitiram distinguir essas duas espécies (Telleria et al., 2010). Apesar de compartilharem muitos antígenos, camundongos imunizados com *T. c. marinkellei*, ou outro *T. cruzi*-like, não foram protegidos de infecção por *T. cruzi* (Marinkelle; 1982a,b; Nascentes et al., 2008, 2010).

Trypanosoma dionisii é a espécie do subgênero *Schizotrypanum* mais distante filogeneticamente de *T. cruzi*. Formas amastigotas, similares as de *T. cruzi*, foram encontradas no músculo esquelético de morcegos infectados com essa espécie (Gardner e Molyneux, 1988). Entretanto, diferente das demais espécies de *Schizotrypanum*, além de “ninhos” de amastigotas nos músculos cardíaco, estriado e do estômago, *T. dionisii* produz “pseudocistos” contendo formas epimastigotas no coração, diafragma, músculos do esterno,

mucosa intestinal e ovário dos morcegos. O desenvolvimento de *T. dionisii* em cultura é semelhante ao de *T. cruzi*. Este parasita, cuja capacidade de invadir células não fagocíticas é inibida por tratamento dessas com citocalasina e pela imobilização dos parasitas por aquecimento, invade estas células sem serem interiorizados por pseudópodes, provavelmente, utilizando a mesma estratégia de *T. cruzi* (Baker et al., 1972; Glauert et al., 1982; Baker, 1985; Molyneux, 1991; Oliveira et al., 2009).

Como as demais espécies de *Schizotrypanum*, exceto *T. cruzi*, *T. dionisii* não infecta o homem, e alguns mecanismos de morte deste parasita foram propostos: morte extracelular de parasitas revestidos por anticorpos, citotoxicidade mediada por linfócitos e mediada por complemento. Foi demonstrado que *T. dionisii* é interiorizado e destruído no interior de fagossomas, sendo os neutrófilos mais eficientes que monócitos, que ocorre na presença ou não de anticorpos específicos (Mkwananzi et al., 1976; Thorne et al., 1979, 1981, Glauert et al., 1982; Molyneux, 1991).

Na Europa e no Canadá, *Cimex pipistrelli* é o vetor natural de *T. dionisii* e de *T. vespertilionis* e o desenvolvimento no vetor é semelhante ao de *T. cruzi* em triatomíneos (Bower e Woo, 1982; Gardner e Molyneux, 1988).

1.3.2 Subgênero *Herpetosoma*

As espécies de tripanossomas tradicionalmente classificadas no subgênero *Herpetosoma* com base na morfologia de formas do sangue (Figura 5) não são patogênicas para seus hospedeiros mamíferos. Essas espécies podem ser divididas em dois grupos: *T. lewisi* (parasitas principalmente de roedores) e *T. rangeli* (Hoare, 1972; D'Alessandro e Saraiva, 1999). Com base em filogenias moleculares, esse subgênero foi revisto uma vez que se mostrou polifilético em todas as análises inferidas (Stevens et al., 1999a,b; Maia da Silva et al., 2004b; 2007; Hamilton et al., 2004, 2007). Análises filogenéticas baseadas nos genes SSU rRNA e gGAPDH indicaram que *T. rangeli* é mais relacionado com *T. cruzi* do que com os tripanossomas africanos, apesar de ser transmitido de forma inoculativa como os membros do clado *T. brucei* (Salivaria) (Stevens et al., 1999a,b, 2001; Maia da Silva et al., 2004a,b, 2007). Todos esses estudos validam nesse subgênero apenas o grupo *T. lewisi*, excluindo *T. rangeli* que deverá ser revalidado no subgênero *Tejeraia* proposto há muitos anos para esse grupo (Añez, 1984; Maia da Silva et al., 2004b).

T. rangeli ocorre da América Central ao sul da América do Sul, compartilhando com *T. cruzi* a distribuição geográfica e a capacidade de infectar mamíferos de praticamente todas as ordens. Essa espécie parasita principalmente primatas, inclusive o homem, roedores, marsupiais e edentados (D`Alessandro e Saraiva, 1999; Guhl e Vallejo, 2003; Maia da Silva et al., 2007). As infecções humanas causadas por *T. rangeli* são comuns na

América Central, Colômbia e Venezuela, onde acarretam sérios problemas para o diagnóstico de *T. cruzi* (Guhl e Vallejo, 2003). No Brasil, *T. rangeli* foi descrito em diferentes mamíferos e triatomíneos (gênero *Rhodnius*), principalmente na Amazônia, onde foram descritos os únicos casos humanos brasileiros (Miles et al., 1983; Coura et al., 1996; Maia da Silva et al., 2004a,b, 2007).

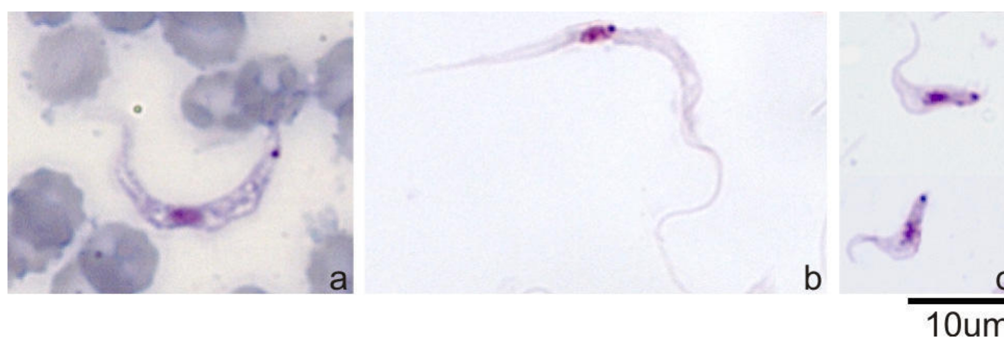


Figura 5. Morfologia, por microscopia óptica, de *T.rangeli*-like. (a) Forma tripomastigota no sangue de um morcego naturalmente infectado; (b,c) formas epimastigota e tripomastigotas metacíclicos em cultura. Coloração por Giemsa.

Diferente de *T. cruzi*, *T. rangeli* não é patogênico para mamíferos, mas sim para o inseto vetor, acarretando dificuldades no repasto sanguíneo e na ecdise, muitas vezes sendo letal para triatomíneos com grande número de parasitas. *T. cruzi* tem todo seu desenvolvimento restrito ao tubo digestivo, enquanto *T. rangeli* multiplica-se no tubo digestivo e completa seu desenvolvimento (metaciclogênese) nas glândulas salivares do inseto vetor; sua transmissão se dá por inoculação durante o repasto sanguíneo de triatomíneos do gênero *Rhodnius* (Añez, 1984; Guhl e Vallejo, 2003). No ciclo silvestre, a infecção por via oral pode ser um mecanismo muito importante, que deve acorrer com a ingestão de triatomíneos por diversos animais silvestres (Maia da Silva et al., 2008).

Estudos moleculares comparativos de isolados de diferentes hospedeiros mamíferos e espécies de *Rhodnius*, de regiões geográficas demonstraram que *T. rangeli* é um taxon complexo formado por diferentes linhagens. Trabalhos baseados em padrões de RAPD e análises filogenéticas do gene ribossômico (SSU e ITS) e de SL de isolados de *T. rangeli* de diferentes hospedeiros e regiões geográficas dividiram essa espécie em 4 linhagens (A-D) (Maia da Silva et al., 2004a, b, 2007). Estudos baseados no gene de mini-exon e em padrões de minicírculos de kDNA revelaram apenas duas linhagens (Grisard et al., 1999; Vallejo et al., 2003, 2009; Urrea et al., 2005).

A congruência filogeográfica das linhagens de *T. rangeli* com os complexos das espécies de *Rhodnius* corroborou a hipótese de uma extensa associação entre as linhagens de *T. rangeli* e espécies de *Rhodnius*, sugerindo uma longa história compartilhada do parasita com seu vetor. A segregação dos isolados de *T. rangeli* de vetores de distintos

complexos, independente do hospedeiro mamífero, sugere que a evolução das linhagens de *T. rangeli* está relacionada a ciclos de transmissão independentes, provavelmente ligados a ecótopos específicos de seus vetores (Maia da Silva et al., 2007).

De acordo com todos os marcadores analisados, a linhagem A é constituída por isolados da Venezuela, Colômbia, Honduras, Guatemala e Brasil (região oriental e ocidental da Amazônia). Esta linhagem foi associada ao complexo *R. prolixus* (*R. robustus* e *R. neglectus*), e encontrada em humanos, cães, macacos e gambás. A linhagem B, que contém isolados humanos e de macacos da Amazônia brasileira, está relacionada com o complexo *R. brethesi*. A linhagem C, compreende isolados de *R. pallescens* da Colômbia e Panamá e isolados humanos de diversos países da América Central. O isolado SC-58, de um roedor da região Sul do Brasil é o único representante da linhagem D, cujo vetor é deconhecido. Portanto, *T. rangeli* é um complexo de linhagens distintas bastante relacionadas com os complexos de espécies da tribo *Rhodinii* que, por sua vez, apresentam uma acentuada estrutura geográfica (Maia da Silva et al., 2004b, 2007; Vallejo et al., 2009).

A primeira descrição de *Herpetosoma* em morcegos foi na Colômbia: *T. rangeli*-like em *Artibeus lituratus* e *Glossophaga soricina*. Entretanto, o xenodiagnóstico de morcegos infectados com esse tripanossoma com *Rhodnius prolixus* e *Cavernicola pilosa* revelou a presença de formas epimastigotas somente na ampola retal dos triatomíneos (Marinkelle, 1966). Recentemente, estudos moleculares de parasitas em morcegos do Brasil (Lisboa et al., 2008) e Panamá (Cottontail et al., 2009) revelaram a presença de *T. rangeli* em infecções mistas com *T. cruzi* e *T. c. marinkellei* (Cottontail et al., 2009).

Infecções experimentais com *Rhonius prolixus* previamente infectados com um isolado de *T. rangeli* e que, posteriormente, se alimentaram em morcegos confirmaram que as formas infectantes encontradas nas glândulas salivares do triatomíneo eram infectantes para morcegos: *Carollia perpicillata* se mostrou mais suscetível a infecção por esse tripanossoma do que *Glossophaga soricina* (Thomas et al., 2007).

Existem apenas alguns relatos não confirmados de formas *T. lewisi*-like descritas em morcegos (Marinkelle, 1966; Molyneux, 1991). Outras espécies classificadas no subgênero *Herpetosoma* foram descritas em morcegos: *T. longiflagellum* em *Thaphozous nudiventris* no Iraque (Marinkelle, 1977); *T. lineatum* em *Vampyrops lineatum* na Venezuela (Hoare, 1972) e *T. aunawa*, descrito em *Miniopterus tristis* da Nova Guiné (Ewers, 1974). Essas descrições são baseadas na morfologia das formas tripomastigotas sanguíneas e pouco se sabe sobre os possíveis vetores para esse tripanossomas. Ewers (1974) encontrou sanguessugas terrestres (*Philaemon*) infectadas por tripanossomas na mesma caverna de onde foi capturado o morcego infectado com *T. aunawa*. Porém, não foi possível concluir se os tripanossomas encontrados nas sanguessugas eram os mesmos do morcego.

1.3.3 Subgênero *Megatrypanum*

O subgênero *Megatrypanum* foi definido por Hoare (1972) apenas com base em parâmetros morfológicos. De acordo com essa classificação tradicional, esse subgênero se caracteriza pela grande diversidade de espécies de tripanossomas e de hospedeiros mamíferos e vetores. Estes tripanossomas infectam mamíferos domésticos e silvestres, de praticamente todas as ordens, especialmente ruminantes, morcegos, carnívoros, roedores e marsupias. A espécie-tipo é *T. theileri*, encontrada em bovinos no mundo todo. As espécies classificadas nesse subgênero foram agrupadas com base exclusivamente na presença das maiores formas tripomastigotas observadas no sangue de mamíferos. Devido a aparente restrição pela espécie-hospedeira, as espécies desse subgênero são nomeadas de acordo com o hospedeiro de origem. Nenhuma espécie desse grupo foi encontrada no homem (Hoare, 1972).

As espécies do subgênero *Megatrypanum* não são patogênicas para seus hospedeiros, que apresentam baixas parasitemias e infecções crônicas. *T. theileri* se multiplica na corrente sanguínea sob formas epimastigotas que se diferenciam em tripomastigotas e seus vetores são principalmente tabanídeos (Wells, 1976; Rodrigues et al., 2003).

As análises filogenéticas realizadas utilizando sequências de SSU rRNA mostraram que este é um taxon artificial (Stevens et al, 1999a,b, 2001). Esse táxon foi recentemente revisado pelo nosso grupo e passou a compreender exclusivamente tripanossomas de animais silvestres e domésticos da ordem Artiodactyla que se posicionam em um clado muito homogêneo junto com *T. theileri* (Rodrigues et al., 2003, 2006, 2010a). Com o estudo de isolados de bovinos de diversas regiões do Brasil demonstramos a existência de duas grandes linhagens e diversos genótipos associados com a origem geográfica e espécie do hospedeiro, definidas com sequências de SSU rRNA, ITS rDNA e dos genes SL e Catepsina L (Rodrigues et al., 2006, 2010a,b). Todas as espécies posicionadas nesse subgênero isoladas de hospedeiros das ordens Rodentia, Marsupialia, Chiroptera, Edentata e Primata devem ser taxonomicamente revistas (Stevens et al., 1999b, 2001; Maia da Silva et al., 2004b; Rodrigues et al., 2006; Hamilton et al., 2005b, 2007, 2009).

Até o momento, apenas uma espécie de tripanossoma de morcego (*T.* sp bat) classificada morfologicamente no subgênero *Megatrypanum* foi posicionada na árvore filogenética do gênero *Trypanosoma*, o que mostrou que essa espécie não pode ser classificada em nenhum dos subgêneros tradicionalmente estabelecidos (Stevens et al., 1999b). Análises filogenéticas baseadas em sequências dos genes SSU rRNA e gGAPDH mostraram que o subgênero *Megatrypanum* é artificial e que apenas as espécies parasitas

de ruminantes (Artiodactyla) devem ser mantidas neste táxon (Stevens et al, 1999a; Rodrigues et al., 2006).

A maioria dos tripanossomas descritos em morcegos pertence ao subgênero *Megatrypanum*. Tripanossomas classificados neste subgênero têm sido descritos em morcegos na África, Europa, Ásia e Américas Central e do Sul. Porém, as informações sobre esses tripanossomas se restringem ao encontro de grandes formas tripomastigotas no sangue do hospedeiro (Figura 6). Embora a parasitemia das infecções causadas por essas espécies possa ser relativamente alta, comparada às espécies de *Schizotrypanum*, esses tripanossomas dificilmente são isolados em cultura. Consequentemente, a classificação das espécies de tripanossomas neste subgênero foi baseada apenas em critérios morfológicos e hospedeiro de origem, portanto, precisa ser analisada com marcadores moleculares.

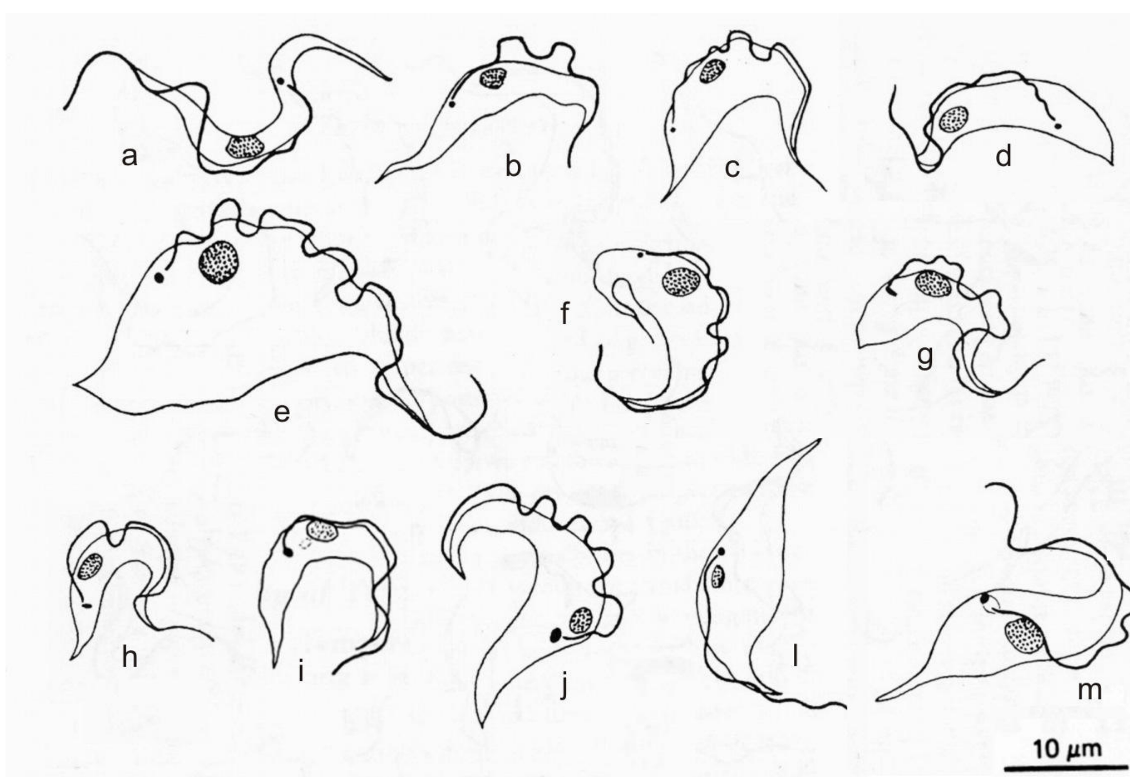


Figura 6. Espécies de tripanossomas do Subgênero *Megatrypanum*, encontradas no sangue dos morcegos. (a) *Trypanosoma megadermae* de *Lavia frons*; (b,c) *T. heybergi* de *Nycteris hispida*; (d) *T. heybergi*-like de um morcego frugívoro; (e) *T. heybergi* de *Nycteris capensis*; (f) *T. thomasi* de *Nycteris macrotis*; (g,h) *T. pessoai* de *Desmodus rotundus;* (i) *T. incertum* de *Pipistrellus pipistrellus*; (j) *T. mpapuense* de *Nycteris aethiopica*; (l) *T morinorum* de *Asellia tridens*; (m) *T. leleupi* de *Hipposideros caffer*. Fonte: Modificado de Hoare, 1972 e Gardner e Molyneux, 1988.

Nas Américas foram descritas as seguintes espécies: *T. pessoai* em *Desmodus rotundus* e *Carollia perspicillata* no Brasil e em outros dois morcegos do gênero *Artibeus* na Costa Rica; *T. leonidasdeanei* em *Saccopteryx bilineata* (Costa Rica); *T. pifanoi* em *Artibeus lituratus* e *Phyllostomus hastatus* (Colômbia) e *T. megadermae-like* em *Myotis nigricans* e

Glossophaga soricina na Venezuela e no Brasil, respectivamente (Deane e Sugay, 1963; Esquivel et al., 1967; Zeledon e Rosabal, 1969; Marinkelle e Duarte, 1968; Dias e Pifano, 1941; Dias, 1942).

Na Ásia foram descritas quatro espécies de tripanossomas: *T. scotophili* em *Scotophilus heati* na China (Liao, 1982); *T. megadermae* em *Rhinolophus hipposideros* no Irã (Edrissian et al., 1976); *T. magnusi* encontrado em *Pipistrellus kuhli* e *Taphozus nudiventri* do Iraque (Shamsuddin e Mohammed, 1978) e *T. rhinopoma* em *Rhinopoma harwickei* na Índia (Bandyopadhyay et al., 1980).

Apenas uma espécie, *T. incertum* foi descrita em *Pipistrellus pipistrellus* na Europa (Gardner e Molyneux, 1988). Por outro lado, o continente africano possui a maior parte das descrições de tripanossomas deste subgênero: *T. megadermae* em *Lavia frons* do Sudão (Wenyon, 1909); *T. mpapuense* em *Nycteris aeothipica* da Tanzânia (Reichenow, 1940); *T. lizae* em *Hipposideros cyclops* do Gabão (Miltgen e Landau, 1979); *T. morinorum* (Senegal e Congo) e *T. leleupi* (Congo e Burundi) são descritos em morcegos do gênero *Hipposideros* (Rhodhain, 1951; Hoare, 1972); *T. heybergi* encontrado em morcegos do gênero *Nycteris* no Congo, Quênia e Liberia (Rodhain, 1923; Heisch e Garnham, 1953; Bray, 1964) e descrito também em *Pipistrellus kuhli* e *Rhinolophus spp* do norte da África e na Zâmbia, respectivamente (Sergent e Sergent, 1905; Keymer, 1971) e *T. thomasi* descrito em *Nycteris macrotis* no Congo (Lips e Rodhain, 1956).

Apenas duas espécies foram descritas, na África, em morcegos da subordem Megachiroptera: *T. megachiropterum* descrito em *Pteropus tonganus* de Toga e *Trypanosoma* sp bat (*T.* sp bat) em *Rousettus aegyptiacus* do Gabão (Marinkelle, 1979; Stevens et al., 1999b).

Os vetores responsáveis pela transmissão das espécies de *Megatrypanum* entre os morcegos e os ciclos de vida nos seus vetores ainda não foram totalmente esclarecidos (Hoare, 1972; Marinkelle, 1976). Alguns trabalhos descrevem os cimicídeos como vetores, uma vez que foi demonstrado que *T. incertum* se desenvolve em *C. lectularius* e *C. pipistrelli* experimentalmente infectados e que a transmissão desse tripanossoma ocorre através da contaminação com as fezes dos cimicídeos (Gardner e Molyneux, 1988).

Em 1963, Berge e colaboradores encontraram diferentes formas de *T. leleupi* no estômago (tripomastigotas sanguíneos) e no intestino (formas metacíclicas) do cimicídeo *Stricticimex brevispinosus.* Anciaux de Faveaux (1965) encontrou formas epimastigotas desse mesmo tripanossoma em cimícideo do gênero *Afrocimex*. Além desses cimicídeos específicos de morcegos, ácaros aderidos em um morcego infectado com *T. heybergi*, apresentaram flagelados no intestino. Esta mesma espécie de tripanossoma também foi encontrada no intestino de ácaros do gênero *Ornithonyssus*. Entretanto, carrapatos, ácaros

e moscas (Strebilidae) coletadas de um morcego infectado com *T. heybergi*-like não apresentaram tripanossomas (Rodhain, 1923; Heisch e Garnham, 1953).

T. incertum não se desenvolveu em triatomíneos (Gardner e Molyneux, 1988). Tentativas de xenodiagnóstico com *T. pifanoi* e *T. pessoai* não obtiveram sucesso com triatomíneos como *Rhodnius prolixus*, *Triatoma dimidiata*, *T. phyllosoma* e *T. pallidipennis* (Marinkelle e Duarte, 1968; Deane e Sugay, 1963). Apenas *T. leonidasdeanei* apresentou temporariamente algumas formas epimastigotes em triatomíneos do gênero *Rhodnius* (Zeledon e Rosabal, 1969). Flebotomíneos foram incriminados como vetores de *T. pessoai* e *T. leonidasdeanei* (Deane et al., 1978; Zeledon e Rosabal, 1969).

1.3.4 Subgênero *Trypanozoon*

O subgênero *Trypanozoon* compreende espécies patogênicas para o homem (*T. brucei gambiense* e *T. b rhodesiense*) e para animais de importância econômica (*T. b. brucei*, *T. evansi* e *T. equiperdum*). Apenas *T. evansi* e *T. equiperdum*, que podem ser mecanicamente transmitidos, ocorrem fora da África. Morcegos não foram encontrados naturalmente infectados por *T. brucei*. Experimentalmente, *T. brucei* induziu infecções muito mais crônicas em morcegos insetívoros (*Tadarida condylura*) do que em camundongos. Já em morcegos frugívoros, como *Epomophorus anurus*, essas infecções são agudas, matando os morcegos em aproximadamente três dias (Woo e Hawkins, 1975).

T. evansi é muito comum no Pantanal do Brasil onde infecta cavalos e animais silvestres como capivaras e quatis (Ventura et al., 2001; Herrera et al., 2004, 2008). *Desmodus rotundus*, morcego hematófago comum nesta região, além de um importante reservatório desta infecção, pode ser também transmissor do parasita durante o seu repasto sanguíneo (Hoare, 1965, 1972). Um levantamento realizado em morcegos do Pantanal brasileiro detectou *T. evansi* em morcegos frugívoros e insetívoros apenas por PCR, indicando que a parasitemia é muito baixa nestes animais (Herrera et al, 2004).

1.4 Ordem Chiroptera

A ordem Chiroptera possui 18 famílias, 200 gêneros e cerca de 1.100 espécies, aproximadamente um quarto de toda a diversidade de mamíferos do mundo (Simmons, 2005). Se considerássemos as espécies já extintas, acrescentaríamos mais seis famílias, aumentando o número de gêneros para 250 (McKenna e Bell, 1997). Essa ordem apresenta uma distribuição mundial, ausente somente nas regiões polares e algumas ilhas oceânicas isoladas. Embora sejam encontrados em regiões de clima temperado, grande parte das espécies de morcegos habitam regiões tropicais e subtropicais. Estes animais têm vida

longa e vivem em colônias que variam muito de tamanho, de poucos indivíduos a milhares, dependendo da espécie e raramente são solitários (Nowak, 1991).

De acordo com caracteres morfológicos, a ordem Chiroptera foi tradicionalmente, com base em características morfológicas, dividida em duas subordens: Megachiroptera (morcegos sem sistema de ecolocalização) e Microchiroptera (apresentam ecolocalização). A subordem Megachiroptera é representada apenas pela família Pteropodidae, cujas espécies são frugívoras e utilizam visão e o olfato para localizar seu alimento. Esses morcegos são encontrados apenas no Velho Mundo, geralmente vivendo em grandes colônias em árvores, na África, Ásia e Oceania (Simmons e Geisler, 1998).

A subordem Microchiroptera está distribuída no Novo e Velho Mundo, com centenas de espécies de 17 famílias organizadas em 4 superfamílias: Rhinolophoidea (Rhinolophidae, Rhinopomatidae, Megadermatidae e Craseonycteridae); Emballonuroidea (Emballonuridae e Nycteridae); Noctilionoidea (Phyllostomidae, Noctilionidae, Mormoopidae, Thyropteridae, Furipteridae, Mystacinidae e Myzopodidae) e Vespertilionoidea (Vespertilionidae, Molossidae e Natalidae) (Jones et al., 2002; Teeling et al., 2005). Os morcegos dessa subordem são preferencialmente insetívoros, porém existem espécies que se alimentam de frutas, néctar, pólen, brotos de plantas, pequenos vertebrados (peixes, sapos, lagartos), morcegos e sangue. Existem apenas três espécies de morcegos hematófagos no mundo (*Desmodus rotundus*, *Diaemus youngi* e *Diphylla ecaudata*), todas da família Phyllostomidae que é restrita das Américas Central e do Sul. Seus abrigos incluem cavernas, cavidades em rochas e árvores, pontes, folhagens, telhados, etc. (Kunz, 1982; Lewis, 1995).

Durante muito tempo existiram muitas controvérsias sobre a monofilia da ordem Chiroptera. Um estudo baseado em caracteres morfológicos visuais sugeriu que megaquirópteros eram mais relacionados com primatas (lêmures) do que com os microquirópteros (Pettigrew, 1986). Contudo, estudos moleculares rejeitaram esta hipótese e apoiaram a monofilia da ordem Chiroptera (Simmons e Geisler, 1998; Jones et al., 2002; Teeling et al., 2000, 2002, 2005).

A classificação atual da ordem Chiroptera, baseada em inferências filogenéticas, não validou a separação dos morcegos em Micro- e Megachiroptera. Estudos paleontológicos e filogenéticos baseados em sequências de genes nucleares e mitocondriais demonstraram que a subordem Microchiroptera não é monofilética, pois alguns morcegos com capacidade de ecolocalização são mais relacionados com morcegos da subordem Megachiroptera (Teeling et al., 2002, 2005; Van Den Bussche e Hoofer, 2004). Com a confirmação desse relacionamento por diversos estudos, foram criadas duas novas subordens: Yinpterochiroptera e Yangochiroptera. A subordem Yinpterochiroptera é composta pelos morcegos da família Pteropodidae (Megachiroptera) e Rhinolophidea, Megadermatidae e Rhinopomatidae (Microchiroptera). A subordem Yangochiroptera é compreendida por todas

as outras famílias de morcegos da antiga subordem Microchiroptera (Teeling et al., 2005; Teeling, 2009) (Figura 7).

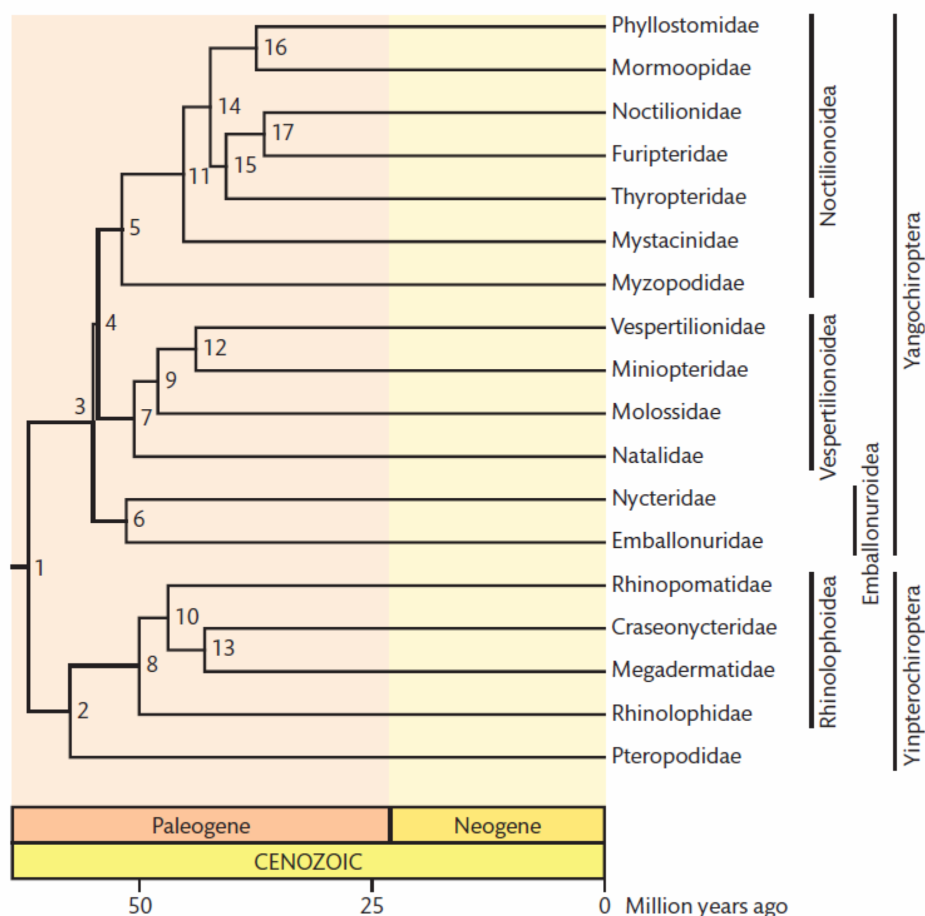


Figura 7. Árvore evolutiva dos morcegos (Chiroptera). Fonte: Teeling, 2009

Diversos estudos tentam esclarecer a origem dos morcegos, com evidências de que surgiram na América do Norte ou na África no Cretáceo (~65mya) (Telling, 2009). Análises filogenéticas e biogeográficas sugerem a África como o centro de origem e grande dispersão dos morcegos no Eoceno (~45mya). Existem evidências de que a rota principal foi da Eurásia para as Américas, via Beríngia, no Mioceno (~20mya) (Simmons, 2005; Eick et al., 2005). Endemismos são muito comuns em Chiroptera, sugerindo que a separação dos continentes criou uma barreira importante no movimento dos morcegos, sendo raras as famílias existentes nas Américas e no Velho Mundo (Stadelmann et al., 2007; Teeling, 2009)

Existem poucas famílias e gêneros com distribuição no Velho e Novo Mundos, como Emballonuridae, Vespertilionidae e Molossidae. Acredita-se que morcegos do gênero *Myotis* (Vespertiolionidae), que são distribuídos em todos os continentes, foram um dos últimos a colonizar o Novo Mundo quando ainda podiam atravessar o estreito de Beringer (~12mya) (Stadelmann et al., 2007). E muito difícil sugerir hipóteses evolutivas para esses mamíferos

uma vez que, raramente são encontrados fosseis antigos. (Eisenberg e Redford,1999; Teeling et al., 2005). Os registros fósseis mais antigos e preservados (*Icaronycteris index* e *Onichonycteris finneyi*) são da América do Norte (Green river em Wyoming) e foram datados no Eoceno (~52mya).

1.5 Genes utilizados para filogenia e taxonomia de tripanossomas

A ausência de critérios taxonômicos confiáveis têm levado a inúmeros erros de classificação dos tripanossomatídeos em geral. Embora exista hoje um consenso que os critérios devem ter como base a filogenia molecular, essa conduta tem sido pouco adotada ou utilizada de forma incorreta. Desde as primeiras análises filogenéticas baseadas em sequências de genes ribossômicos (Sogin et al., 1986; Fernandes et al., 1993), o uso de sequências gênicas tem sido valioso na reconstrução da história evolutiva desses organismos.

Marcadores moleculares têm sido utilizados por diversos grupos, porém, muitas vezes os resultados não são facilmente comparáveis. Entretanto, esse é um requisito indispensável na utilização de marcadores moleculares em taxonomia. A análise de um número limitado de espécies, concentrado em poucos grupos de tripanossomas impediu, até recentemente, inferências de árvores filogenéticas bem resolvidas. Atualmente, diversos genes, sequências e marcadores vêm sendo utilizados para análises de polimorfismo genético e inferências filogenéticas, sendo os mais utilizados os genes ribossômico (SSU rRNA), gGAPDH, "spliced leader”, genes mitocondriais e, mais recentemente, genes codificadores de Catepsina L-like.

1.5.1 Gene ribossômico

Sequências do gene ribossômico têm sido amplamente utilizadas para inferir relações filogenéticas entre espécies do filo Euglenozoa. Os tripanossomatídeos possuem uma dos mais complexos padrões de moléculas maduras de RNA. Os genes de RNA ribossômico (rRNA) consistem de unidades de repetição compostas por unidades de transcrição (cistrons ribossômicos) e são intercalados por um espaçador intergênico (IGS), que se repete em "tandem" mais de 100 vezes no genoma. Estes genes são processados em uma única unidade de transcrição conhecida como pré-rRNA. Após várias etapas de processamento o pré-rRNA dá origem a três moléculas de RNA maduros: 18S (SSU ou subunidade menor), 5.8S e 24S (LSU ou subunidade maior), que nestes organismos é constituída por dois fragmentos de alto peso molecular, 24Sα e 24Sβ e quatro subunidades

de rRNAs de baixo peso molecular (S1, S2, S4 e S6). As subunidades SSU e LSU são constituídas por sequências altamente conservadas e intercaladas por espaçadores de conservação intermediária ITS (ITS 1 e 2, espaçadores internos transcritos) e ETS (espaçador externo transcrito) que são flanqueados pelo espaçador intergênico (IGS), que apresenta sequências altamente variáveis (Sogin et al., 1986; Hernández et al., 1990) (Figura 8).

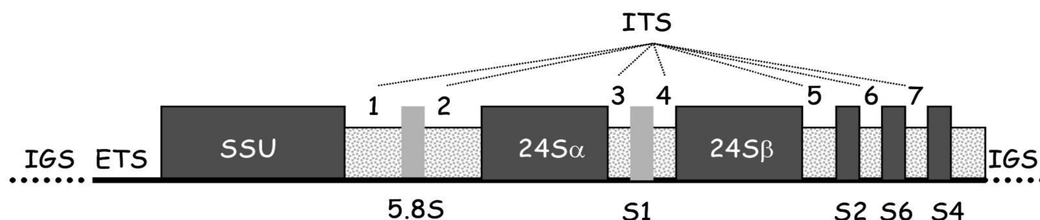


Figura 8. Representação esquemática do cistron ribossômico de rRNA precursores de tripanossomatídeos.

Esses genes são utilizados para inferências de relacionamentos filogenéticos porque ocorrem e são funcionalmente equivalentes em todos os organismos, e apresentam domínios com diferentes graus de conservação (Sogin et al., 1986; Hernández et al., 1990). A presença de diversas regiões, transcritas ou não, que exibem diferentes graus de conservação, faz desses genes excelentes alvos para identificação de gêneros, espécies, linhagens e genótipos (Souto et al., 1996, Zingales et al., 1998; Brisse et al., 2001; Stevens et al., 2001; Maia da Silva et al., 2004b; Hamilton et al., 2004, 2007; Rodrigues et al, 2006; Cortez et al., 2006; Ferreira et al., 2007, 2008; Viola et al., 2008, 2009a,b).

As sequências do gene SSU rRNA são as mais utilizadas devido a características importantes, tais como: a) o pequeno tamanho que permite fácil obtenção por amplificação por PCR; b) a presença de regiões variáveis flanqueadas por regiões conservadas que permitem alinhamentos altamente confiáveis, com oito regiões universalmente conservadas (U1-U8) e nove regiões variáveis (V1-V9) (Hernández et al., 1990). Além disso, existem dezenas de sequências de SSU rRNA de diferentes espécies e isolados do gênero *Trypanosoma* depositadas em bancos, permitindo identificar novas espécies e inferir com facilidade o relacionamento de novas espécies, linhagens e isolados.

Os espaçadores IGS e ITS são muito mais variáveis que as regiões SSU e LSU. As sequências do ITS rDNA contêm três regiões: ITS1, 5.8S (que é altamente conservado) e ITS2. As sequências de ITS1 e ITS2 diferem inter e intra-especificamente, sendo excelentes para análises de organismos filogeneticamente próximos assim como alvos para diagnóstico. Análises do tamanho e de sítios de restrição de ITS rDNA, especialmente ITS1,

diferenciaram linhagens de *T. cruzi* (Fernandes et al., 1999; Mendonça et al., 2002; Cuervo et al., 2002; Santos et al., 2002), *T. rangeli* (Maia da Silva et al., 2004b) e *T. theileri* (Rodrigues et al., 2006), além de espécies de tripanossomas Africanos (Desquenes et al., 2001; Njiru et al., 2005; Rodrigues et al., 2008) e tripanossomas de anuros (Ferreira et al., 2007).

1.5.2 Gene codificador da enzyma Gliceraldeído 3-fosfato desidrogenase glicossômica (gGAPDH)

As espécies da família Trypanosomatidae apresentam uma organela denominada glicossoma que contém enzimas envolvidas no metabolismo da glicose e glicerol (via glicolítica), sendo essa compartimentalização de enzimas da via glicolítica diferente de outros eucariotos cujas enzimas são citosólicas. A glicose é a principal fonte de energia utilizada pelos estágios dos tripanossomas no sangue dos mamíferos (Hannaert et al., 1992). Nos tripanossomas, foram encontrados dois genes que codificam a enzima glicossômica (gGAPDH), semelhante a dos eucariotos em geral, e um gene que codifica uma enzima citosólica (cGAPDH), mais relacionada com genes bacterianos. (Michels et al. 1986; Kendall et al. 1990). Divergências nas sequências desses genes permitiram o desenho de primers para a amplificação específica de gGAPDH (Hamilton et al., 2005a,b; 2007). (Figura 9)

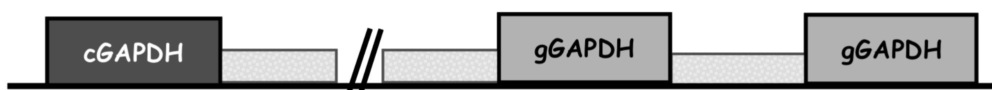


Figura 9. Representação esquemática dos genes de GAPDH.

Os genes de gGAPDH apresentam duas cópias praticamente idênticas e como são codificadores de proteínas estão sujeitos a diferentes pressões seletivas e apresentam taxas de evolução diferentes comparadas as dos genes ribossômicos. Esses genes são excelentes marcadores para estudos filogenéticos de tripanossomatídeos, permitindo alinhamentos confiáveis de sequências de organismos geneticamente distantes. Outra vantagem é a compatibilidade entre os genes gGAPDH e SSU rRNA para análises com sequências concatenadas (Hamilton et al., 2004, 2005a,b, 2007; Stevens, 2008).

Estudos filogenéticos de um grande número de espécies de tripanossomatídeos com sequências de gGAPDH e SSU rRNA geraram topologias congruentes e as análises independentes e combinadas desses genes têm sido recomendadas na descrição de gêneros, subgêneros e espécies de tripanossomatídeos (Hamilton et al., 2004, 2005a, 2009; Viola et al., 2009b; Maslov et al., 2010; Teixeira et al., 2011).

1.5.3. Gene "spliced leader" ou de mini-exon

A maioria dos genes dos cinetoplastídeos não apresentam introns e seus transcritos são RNAs policistrônicos sendo, em geral, o mecanismo pós-transcricional de "trans-splicing" responsável pela maturação dos mRNAs unitários. Esse processamento resulta na adição, na extremidade 5' dos mRNAs maduros, da sequência de 39 nucleotídeos denominada "spliced leader" RNA (SLRNA) ou "mini-exon derived RNA" (Agabian, 1990; Campbell e Sturm, 2000; Campbell et al., 2003; Liang et al., 2003; Mayer e Floeter-Winter, 2005; Hury et al., 2009).

Devido à presença de mais de 200 cópias do gene SL, repetidas em tandem no genoma dos tripanossomatídeos, e de regiões com diferentes graus de conservação, esses genes têm sido utilizados com finalidades taxonômicas e diagnósticas. Cada unidade de repetição do gene SL pode ser dividida basicamente em três partes: um exon altamente conservado de 39 nucleotídeos, um intron de 50-100 nucleotídeos moderadamente conservado, e uma região intergênica que varia de tamanho e de sequência entre espécies e linhagens de tripanossomatídeos e linhagens de tripanossomas (Figura 10).

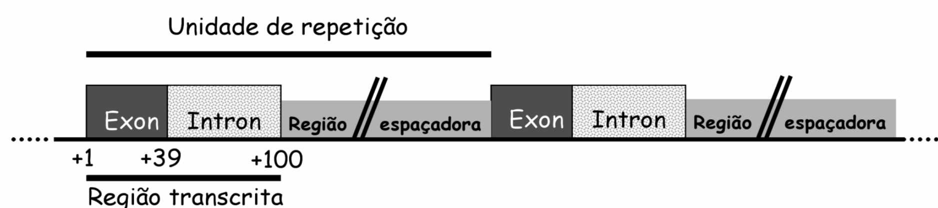


Figura 10. Representação esquemática da unidade de repetição do gene "Spliced leader"

Algumas espécies de tripanossomatídeos apresentam o rRNA 5S, que é composto por sequências altamente conservadas, inserido na região intergênica do gene SL (Gibson et al., 2000).

A comparação de sequências do gene SL na identificação de diferentes gêneros da família Trypanosomatidae revelou regiões com diferentes graus de conservação entre gêneros e espécies. Essa variabilidade tem sido favorável na identificação de marcadores úteis para espécies de praticamente todos os gêneros de tripanossomatídeos: *Leishmania* (Fernandes et al., 1994; Serin et al., 2007; Sukmee et al., 2008); *Endotrypanum* (Fernandes et al., 1993); *Phytomonas* (Serrano et al., 1999; Teixeira et al., 2000; Godoi et al., 2002); *Crithidia* (Fernandes et al., 1997; Yurchenko et al., 2009); tripanossomas africanos (Sturm et al., 1998; Ventura et al., 2001); *T. theileri* (Rodrigues et al, 2010a); linhagens de *T. rangeli* (Grisard et al., 1999; Maia da Silva et al., 2007) e linhagens e genótipos de *T. cruzi* (Souto et

al., 1996; Fernandes et al., 1998, 2001; Brisse et al., 2001, O´Connor et al., 2007; Herrera et al., 2007; Falla et al., 2009). Entretanto, o gene SL apresenta limitações que devem ser consideradas quando são utilizados: a) o polimorfismo das regiões espaçadores que impede que mesmo isolados de uma mesma espécie (exemplo *T. cruzi*) e espécies muito relacionadas sejam alinhadas (Gibson et al., 2000); b) o polimorfismo de cópias de um mesmo isolado/espécies, que exige análises de diversas sequências e pode gerar resultados difíceis de interpretar e que não representam as relações entre os organismos estudados (Tomasini et al., 2011).

1.5.4. Genes mitocondriais

Os genes mitocôndrias dos tripanossomatídeos estão organizados nas moléculas de DNA circular denominadas maxicírculos de kDNA, que fazem parte da rede enovelada de DNA (maxi- e minicírculos) que constituem o kDNA (DNA do cinetoplasto). A ausência de recombinação faz com que os genes mitocondriais sejam excelentes marcadores para estudos populacionais. As taxas de divergência dos genes mitocôndriais divergem bastante, mas em, em geral, são maiores do que as de gene nucleares de cópia única (cerca de 10 vezes superior). Alguns genes acumulam mais mutações, como os genes codificadores das subunidades da NADH desidrogenase e citocromo oxidase c (CO), enquanto os genes de citocromo b (Cyt b) são mais conservados (Meyer, 1993).

Sequências de Cyt b e COII têm sido as mais analisadas para estudos de tripanossomatídeos, principalmente *T. cruzi*, agrupando os isolados em clados congruentes com os gerados por sequências ribossômicas (Machado e Ayala, 2001; Brisse et al., 2003; Westenberger et al., 2006; Freitas et al., 2006; Baptista et al., 2006; D'Avila et al., 2009, Pena et al., 2009). Um estudo baseado no gene Cyt b permitiu separar *T. rangeli* das espécies do subgênero *Schizotrypanum* (*T. cruzi*, *T. c. marinkellei* e *T. dionisii*), confirmando a monofilia de *T. cruzi* e *T. c. marinkellei* e segregou os isolados de *T. c. marinkellei* em dois grupos (Barnabé et al., 2003).

Os maxicírculos de *T. cruzi* possuem cerca de 22 kb e 20 genes presentes em uma única cópia por maxicírculo (Figura 11) (Westenberger et al., 2006).

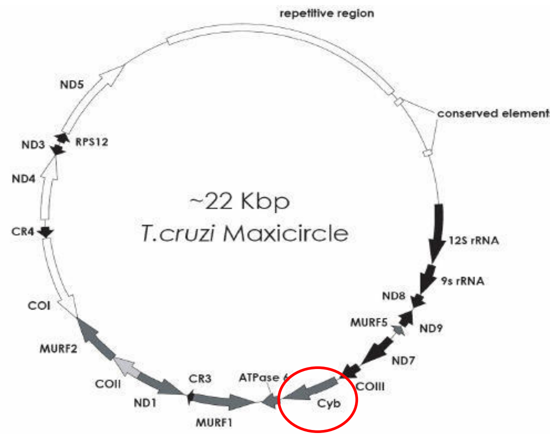


Figura 11. Representação esquemática do maxicírculo de kDNA com o gene de citocromo b (Cyb) em destaque.

1.5.5 Genes codificadores de enzimas Catepsina L- like

Durante o ciclo de vida dos tripanossomas diversas proteases são expressas e reguladas ao longo do desenvolvimento nos hospedeiros vertebrados e vetores, sendo as cisteíno-proteases Catepsina L-like (CATL-like), denominadas cruzipaínas em *T. cruzi*, as mais estudadas (Tomas et al., 1997; Aparício et al., 2004; Duschak et al., 2006; Mckerrow et al., 2006, 2009; Caffrey e Steverding, 2009).

Os genes codificadores de cruzipaína (~1404pb) fazem parte de uma família multigênica e estão organizados em repetições em tandem geradas por sucessivas duplicações gênicas. As unidades de repetição contêm uma região intergênica de ~500bp e regiões codificadoras constituídas por pré- e pró-domínios, domínio central e extensão C-terminal. A porção C-terminal, que sofre auto-hidrólise e não é encontrada na enzima madura, somente foi encontrada nos tripanossomatídeos (Cazzulo, 2001). (Figura 12).

Figura 12. Representação do gene da Catepsina L – like em tripanossomas.

Genes codificadores de CATL e CATB (genes de cópia única) têm sido utilizados em análises filogenéticas de parasitas. A maioria dos estudos tem sido baseados em sequências de CATB de helmintos, os estudos filogenéticos de genes codificadores de catepsinas, e proteases em geral, são mais escassos em protozoários (Robinson et al.,

2008; Dacks et al., 2008). Alguns estudos analisaram genes codificadores de catepsinas de espécies de *Leishmania* (Sakanari et al., 1997; Kuru et al., 2007). Genes homólogos ao gene codificador da cruzipaína foram caracterizados nos tripanossomas de mamíferos: *T. brucei*, *T.b. rhodesiense*, *T. congolense*, *T. rangeli* e *T. theileri*, e no tripanossoma de peixe *T. carassii*. Estudos recentes revelaram variantes de congopaina, a principal CATL-like de *T. congolense* (Mendoza-Palomares et al., 2008; Pillay et al., 2010) e polimorfismo de CATL entre genótipos de *T. vivax* (Cortez et al., 2009) e de *T. theileri* (Rodrigues et al., 2010b; Garcia et al., 2011).

Genes de bodonídeos, *Cryptobia salmositica* e *Trypanoplasma borreli*, também foram analisados filogeneticamente (Jesudhasan et al., 2007; Ruszczyk et al., 2008a,b; Cortez et al., 2009; Rodrigues et al., 2010b).

Apesar de múltiplas cópias, as filogenias inferidas têm gerado clados que refletem a filogenia das espécies de tripanossomas inferidas com base em seqüências de SSU rRNA e gGAPDH, sugerindo que esses genes evoluem em concerto e, assim, são bons marcadores para estudos evolutivos (Jackson, 2007).

A presença de múltiplas cópias de genes de CATL-like nos genomas dos tripanossomas, a facilidade de amplificação por PCR, e o polimorfismo entre genes de diferentes espécies fazem desses genes alvos interessantes para o desenvolvimento de métodos diagnósticos espécie-específicos (Tanaka, 1997; Cortez et al., 2009; Rodrigues et al., 2010b)

2. JUSTIFICATIVA E OBJETIVOS

Tripanossomas pertencentes às Secções Stercoraria (subgêneros *Schizotrypanum*, *Megatrypanum* e *Herpetosoma*) e Salivaria (*Trypanozoon*) têm sido descritos em morcegos de diversas famílias, hábitos alimentares e ecótopos em todos os continentes, exceto na Antártida. As espécies de tripanossomas mais prevalentes pertencem aos subgêneros *Megatrypanum* e *Schizotrypanum.* Os morcegos insetívoros são os mais frequentemente infectados, sugerindo assim, uma importante forma de transmissão por via oral pela ingestão de artrópodes infectados (Hoare, 1972; Marinkelle, 1976; Molyneux, 1991).

Embora os tripanosomas de morcegos sejam conhecidos há mais de 100 anos, e o número de espécies de tripanossomas descritas em morcegos seja muito grande, a maioria foi descrita com base apenas em dados morfológicos de formas do sangue e nos hospedeiros de origem. Muitas espécies de tripanossomas isolados de morcegos foram classificadas no subgênero *Schizotrypanum* e por apresentarem morfologia muito semelhante à de *T. cruzi* foram denominadas *T. cruzi*-like. Com exceção de *T. cruzi*, as demais espécies desse subgênero são restritas aos morcegos: *T. cruzi* e *T. c. marinkellei* são encontrados apenas nas Américas Central e do Sul, enquanto *T. dionisii e T. vespertilionis* ocorrem no Novo e no Velho Mundo. Outras espécies deste subgênero foram descritas na América do Norte (*T. hedricki* e *T. myoti*) e Central (*T. phyllostomae*) e Austrália (*T. pteropi* e *T. hipposideri*) (Hoare, 1972; Molyneux, 1991).

Embora sejam consideradas não patogênicas, pouco se sabe sobre os ciclos de vida e mecanismos de transmissão das espécies de *Schizotrypanum* entre os morcegos. Aparentemente, todas as espécies apresentam ciclos biológicos muito parecidos embora em diferentes mamíferos e vetores. *T. cruzi* pode ser transmitido entre morcegos por triatomíneos de vários gêneros, enquanto que *T. c. marinkellei* é transmitido apenas por triatomíneos do gênero *Cavernicola* (Marinkelle, 1976, 1982a; Molyneux, 1991). Infecções naturais e experimentais demonstraram que cimicídeos são vetores de *T. dionisii* e *T. vespertilionis* no Velho Mundo e, aparentemente, o desenvolvimento nesse vetor é semelhante ao de *T. cruzi* em triatomíneos (Peterson e Woo, 1984; Gardner e Molyneux, 1988).

Infecções causadas por *T. cruzi* em morcegos foram descritas por diversos pesquisadores. Porém, a diversidade biológica e genética dessa e das demais espécies de *Schizotrypanum* parasitas de morcegos ainda é pouco conhecida (Dias, 1942; Deanei, 1961; Barreto, 1968; Hoare, 1972; Marinkelle, 1976; Baker et al., 1978; Bower e Woo, 1981; Steindel et al., 1998; Grisard et al., 2003; Barnabé et al., 2003; Lisboa et al., 2008). A taxonomia tradicional dos tripanossomas de morcegos não é suficiente para classificar

subgêneros nem espécies como tem sido demonstrado nos estudos filogenéticos Barnabé et al., 2003; Stevens et al., 2001; Hamilton et al., 2007).

A forte associação entre o subgênero *Schizotrypanum* e morcegos de diversas famílias, com distribuição praticamente mundial, sugere uma história evolutiva antiga e compartilhada dessas espécies em associação com seus respectivos vetores (triatomíneos e cimicídeos). As espécies desse subgênero variam na capacidade de infectar mamíferos, de generalistas (apenas *T. cruzi*) à exclusivas de morcegos, são transmitidas por diferentes vetores, podem ser patogênicas ou não e diferem na distribuição geográfica, sendo algumas espécies cosmopolitas e outras americanas. O estudo abrangente de tripanossomas do subgênero *Schizotrypanum* pode ajudar a compreender as relações desses parasitas com seus hospedeiros e os processos evolutivos que resultaram nas diferenças entre as espécies.

Tripanossomas de morcegos classificados no subgênero *Megatrypanum* dificilmente são cultivados, sendo assim, muitas espécies são classificadas nesse subgênero apenas por apresentarem grandes formas tripomastigotas com membrana ondulante bem desenvolvida no sangue dos morcegos. Essa conduta tem agrupado tripanossomas de praticamente todas as ordens de mamíferos, dificultado o entendimento da real diversidade dessas espécies. Esses tripanossomas são muito frequentes na África, Europa e Ásia, com raras espécies descritas na América do Sul (Marinkelle, 1976; Molyneux, 1991). No Brasil, as espécies deste subgênero nunca foram cultivadas e foram descritas em morcegos da Amazônia (Deane e Sugay, 1963). As espécies de *Megatrypanum* parasitas de morcegos podem se transmitidas por cimídeos (África e Europa), flebotomíneos (America do Sul) e, provavelmente, por ectoparasitas específicos de morcegos (Molyneux, 1991).

A ocorrência de espécies do subgênero *Herpetosoma* é um aspecto controverso da diversidade de tripanossomas parasitas de morcegos, com relatos não confirmados de *T. lewisi*-like e *T. rangeli*-like (Marinkelle, 1966; Molyneux, 1991). Estudos recentes têm detectado *T. rangeli* no sangue de morcegos do Brasil e Panamá, porém, os isolados não foram obtidos e tampouco caracterizados filogeneticamente (Lisboa et al., 2008; Cottontail et al., 2009).

Estudos comparativos com um grande número de tripanossomas de morcegos, de diferentes espécies e regiões geográficas nunca foram realizados. Nos últimos anos foram acrescentadas diversas espécies na árvore filogenética do gênero *Trypanosoma,* porém*,* um pequeno número de tripanossomas de morcegos foi incluído: *T. dionisii* (dois isolados europeus); *T. c. marinkellei* (dois isolados do Brasil); *T. vespertilionis* (um isolado europeu) e *T.* sp bat de um morcego do Gabão, África. Esses estudos validaram *T. c. marinkellei* e *T. dionisii* no subgênero *Schizotrypanum* junto com *T. cruzi.* O posicionamento filogenético de *T. vespertilionis* sugere que esta espécie não pertence a este subgênero, ou que o isolado

analisado foi classificado erroneamente nesta espécie (Stevens et al., 2001). Apenas uma espécie de tripanossoma de morcego (*T.* sp bat) classificada morfologicamente no subgênero *Megatrypanum* foi cultivada e posicionada na árvore filogenética do gênero *Trypanosoma.* Esse estudo mostrou que essa espécie não pode ser classificada em nenhum dos subgêneros tradicionalmente estabelecidos (Stevens et al, 1999b). Análises filogenéticas baseadas em sequências dos genes SSU rRNA e gGAPDH mostraram que o subgênero *Megatrypanum* é artificial e que apenas os tripanossomas de ruminantes (Artiodactyla) devem ser mantidos neste taxon (Rodrigues et al., 2006; 2010a).

A falta de parâmetros taxonômicos confiáveis, o pequeno número de espécies disponíveis em cultura e os poucos isolados incluídos nos estudos filogenéticos têm dificultado o entendimento da diversidade e história evolutiva dos tripanossomas de morcegos. Consequentemente, a validade das espécies existentes, a diversidade genética inter- e intraespecífica e padrões biológicos, ecológicos e filogeográficos não são bem compreendidos.

Os morcegos são os únicos mamíferos com capacidade de vôo e que podem facilmente transpor barreiras geográficas sendo, assim, um modelo muito interessante para estudos filogeográficos. A distribuição geográfica, diversidade genética e relações filogenéticas de tripanossomas de morcegos são ainda muito limitadas para gerar hipóteses consistentes sobre a história evolutiva desses tripanossomas. Análises filogeográficas de tripanossomas de morcegos podem fornecer informações valiosas sobre a dispersão e evolução desses animais e de seus parasitas em geral. A adoção de critérios taxonômicos tradicionais e a falta de marcadores moleculares fazem com que a taxonomia dos tripanossomas de morcegos seja altamente controversa. Por esses motivos, esse estudo tem como objetivos:

1. Analisar a ocorrência e isolar tripanossomas de morcegos de diferentes espécies e famílias, de 4 Biomas brasileiros (Pantanal, Amazônia, Cerrado e Mata Atlântica) e de Moçambique na África.

2. Avaliar a diversidade e comparar os tripanossomas de morcegos quanto à morfologia (microscopia de luz e eletrônica) e comportamento biológico (desenvolvimento em cultura, capacidade de invadir e se multiplicar no interior de células e infectividade para triatomíneos e camundongos).

3. Definir o posicionamento inicial dos isolados de tripanossomas de morcegos através da região V7V8 do gene SSU rRNA (*DNA barcoding*).

4. Inferir relações filogenéticas e filogeográficas, baseadas em sequências do gene SSU rRNA, gGAPDH e Cytocromo b, dos tripanossomas de morcegos, entre tripanossomas

de morcegos brasileiros e africanos e espécies de outros mamíferos de diversos continentes.

5. Analisar a diversidade e o relacionamento intra-específico dos tripanossomas de morcegos, utilizando seqüências bastante polimórficas como ITS1 rDNA e “Spliced–leader” (SL)

6. Caracterizar genes codificadores de enzimas Catepsina L e inferir genealogias a fim de analisar a evolução dessas importantes moléculas nos ciclos de vida, invasão e diferenciação celular, assim como avaliar o potencial desses genes como marcadores filogenéticos e taxonômicos.

7. Classificar novos isolados de morcegos, e rever as espécies já descritas, com base em parâmetros taxonômicos tradicionais e filogenia molecular.

3. MATERIAIS E MÉTODOS

3.1 Captura e identificação dos morcegos

Os morcegos foram capturados em redes de neblina ("mist nest") com 3,0 x 6,0 m de comprimento, que foram armadas no início do entardecer e mantidas abertas por um período de 5–6 horas. Para algumas espécies de morcego foram realizadas buscas ativas em abrigos e os animais capturados foram transportados em sacos de pano para um laboratório quando disponível ou para uma área com maior assepsia e anestesiados com uma associação de Ketamina e Xilasina (1:2, 0,1ml/100g). No Brasil foram coletados morcegos dos seguintes biomas: Região Amazônica (RO e AM); Cerrado (GO, TO e MS); Pantanal (MS); Mata Atlântica (SP e PR) e alguns morcegos no Mato Grosso do Sul, localizados na área de transição do Pantanal/Cerrado. Na África foram coletados morcegos em Moçambique. A identificação dos morcegos brasileiros foi feita através de chaves de identificação pelo Dr. Valdir Tadei (in memoriam) e Dra Caroline Cotrin Aires (Museu de Zoologia da USP). Para a identificação dos morcegos africanos, foram selecionados um ou dois representantes de cada espécie, nos quais foi realizada uma biopsia de fígado. Os tecidos foram preservados em etanol 100% (v/v) e posteriormente processados para a extração de DNA com o kit Wizard DNA Clean-Up System (Promega). Após a extração, o DNA foi mantido a – 20ºC. Os DNAs obtidos foram utilizados para amplificação do gene de cytocromo b (~1140pb) utilizando "primers" e condições de PCR de acordo Cui et al. (2007). Os fragmentos amplificados foram sequenciados conforme descrito no item 3.14 e as sequências geradas foram submetidas ao banco de dados do GenBank.

Alguns exemplares de morcegos capturados foram sacrificados, fixados, identificados pelo Dr. Miguel T. U. Rodrigues (IB-USP), .depositados no Museu de Zoologia da Universidade de São Paulo.

3.2 Organismos

Os tripanossomas utilizados neste estudo e seus respectivos hospedeiros de origem e local de isolamento estão relacionados na Tabela 1. Todas as culturas de tripanossomas isoladas neste estudo ou cedidas por diversos pesquisadores do Brasil e do exterior, estão criopreservadas na coleção de tripanossomas do Departamento de Parasitologia, TCC/USP (Trypanosomatid Culture Collection). Os isolados são mantidos congelados em N_2 líquido (em meio LIT com 10% de SFB acrescido de 20% de DMSO).

Tabela 1. Isolados de tripanossomas de morcegos utilizados neste estudo.

TCC	Isolados	Hospedeiro		Origem geográfica	
		Família	Espécie		
T. cruzi					
507/640/642		Phyllostomidae	*C. perspicillata*	Porto Velho (RO)	- Brasil
204		Vespertilionidae	*M. albescens*	São Paulo (SP)	- Brasil
1122			*M. albescens*	Juquitiba (SP)	- Brasil
793/294			*M. levis*	São Paulo (SP)	- Brasil
499/597			*M. nigricans*	Miranda (MS)	- Brasil
947/949			*M. nigricans*	São Paulo (SP)	- Brasil
203			*M. ruber*	São Paulo (SP)	- Brasil
312/480			*N. albiventris*	Miranda (MS)	- Brasil
417		Thyropteridae	*T. tricolor*	Barcelos (AM)	- Brasil
T. c. marinkellei					
478/611/1089/ 1093		Phyllostomidae	*A. planirostris*	Miranda (MS)	- Brasil
626/627			*A. planirostris*	Aquidauana (MS)	- Brasil
1702/1703/1705/ 1708/1710/1713			*A. planirostris*	Bonito (MS)	- Brasil
344			*C. perspicillata*	Monte Negro (RO)	- Brasil
466/477/485/491/ 501/508			*C. perspicillata*	Porto Velho (RO)	- Brasil
1721			*C. perspicillata*	Bonito (MS)	- Brasil
420/708			*C. auritus*	Barcelos (AM)	- Brasil
456			*D. rotundus*	Miranda (MS)	- Brasil
426			*L. silvicolum*	Barcelos (AM)	- Brasil
332			*P. hastatus*	Barcelos (AM)	- Brasil
510			*P. hastatus*	Aquidauana (MS)	- Brasil
1701/1707/1714/ 1730			*P. hastatus*	Bonito (MS)	- Brasil
421/494/424			*P. discolor*	Barcelos (AM)	- Brasil
1067			*Phyllostomus sp*	(TO)	- Brasil
423			*T. bidens*	Barcelos (AM)	- Brasil
419			*T. cirrhosus*	Barcelos (AM)	- Brasil
502			*T. cirrhosus*	Porto Velho (RO)	- Brasil
-	B7		*P. discolor*	São Felipe (BA)	- Brasil
-	M1117		*P. hastatus*	Abaetetuba (PA)	- Brasil
T. dionisii					
403		Molossidae	*M. molossus*	Monte Negro (RO)	- Brasil
554			*Promops sp*	Miranda (MS)	- Brasil
464		Noctilionidae	*N. albiventris*	Miranda (MS)	- Brasil
383		Phyllostomidae	*Anoura sp*	São B. Campo (SP)	- Brasil
345/380			*C. perspicillata*	Monte Negro (RO)	- Brasil
495			*C. perspicillata*	Porto Velho (RO)	- Brasil
1110			*C. perspicillata*	Piedade (SP)	- Brasil
1147			*C. perspicillata*	Londrina (PR)	- Brasil
1700/1706			*C. perspicillata*	Bonito (MS)	- Brasil
454			*D. rotundus*	Miranda (MS)	- Brasil
483/493			*D. ecaudata*	Biritiba Mirim (SP)	- Brasil
633			*S. lillium*	Miranda (MS)	- Brasil
1087			*S. lillium*	Campo Limpo (GO)	- Brasil

1314			*S. lillium*	Adrianópolis (SP)	- Brasil
343			*U. bilobatum*	Monte Negro (RO)	- Brasil
211/289		Vespertilionidae	*E. brasiliensis*	São Paulo (SP)	- Brasil
293			*M. nigricans*	São Paulo (SP)	- Brasil
309			*M. nigricans*	Miranda (MS)	- Brasil
300/303			*M. albescens*	Miranda (MS)	- Brasil
	P3		*P. pipistrellus*		Inglaterra
	PJ		*P. pipistrellus*		Bélgica
T. erneyi					
1293		Molossidae	*Tadarida sp*	Marromeu	- Moçambique
1294			*Tadarida sp*	Marromeu	- Moçambique
1932			*M. condylurus*	Chupanga	- Moçambique
1934			*M. condylurus*	Chupanga	- Moçambique
1936			*M. condylurus*	Chupanga	- Moçambique
1946			*M. condylurus*	Chupanga	- Moçambique
T. rangeli					
643		Phyllostomidae	*P. lineatus*	Miranda (MS)	- Brasil
1719			*A. planirostris*	Bonito (MS)	- Brasil
T. zambesiensis					
1270			*R. landeri*	Chupanga	- Moçambique
1295			*R. landeri*	Chupanga	- Moçambique
1304			*R. landeri*	Chupanga	- Moçambique
1271			*R. landeri*	Chupanga	- Moçambique
1298			*R. landeri*	Chupanga	- Moçambique
1933			*R. landeri*	Chupanga	- Moçambique
1948			*R. landeri*	Chupanga	- Moçambique
1947			*R. landeri*	Chupanga	- Moçambique
1954			*R. landeri*	Chupanga	- Moçambique
1902			*R. landeri*	Chupanga	- Moçambique
1935			*R. landeri*	Chupanga	- Moçambique
1953			*H. caffer*	Gorongosa	- Moçambique
***T.* sp bat**					
60		Pteropodidae	*R. aegyptiacus*		Gabão
T. vespertilionis					
	P14	Vespertilionidae	*P. pipistrellus*		Inglaterra

3.3 Isolamento e cultivo dos tripanossomas de morcegos

As amostras de sangue dos morcegos foram obtidas por punção cardíaca e inoculadas em meio bifásico composto por uma fase sólida a 4% de “Blood Agar Base” (BAB) acrescido de 15% de sangue de coelho e a fase líquida composta por LIT (Liver Infusion Tryptose) (Camargo, 1964), acrescido de 15% de soro fetal bovino (SFB). Para evitar e eliminar a contaminação das amostras, foram adicionados 80μg/ml de gentamicina na fase líquida. Algumas amostras de sangue foram também preservadas em etanol 100% (v/v), para posterior análise e, em alguns casos, uma terceira amostra de sangue foi analisada por microhematócrito (MH). Foram confeccionados esfregassos em lâminas, de

todas as amostras de sangue obtidas para coloração por Giemsa e posterior análise em microscópio de luz de todas as amostras positivas por MH.

As hemoculturas foram incubadas a 28°C e analisadas após 7 dias de semeadas. As culturas positivas foram mantidas em meio BAB/LIT ou, quando necessário, co-cultivadas em meio TC-100 (Cultilab) acrescido de 5% de SFB em monocamadas de célula de inseto High five (*Trichopulsia ni*), incubadas a 28°C. Os meios de cultura utilizados estão descritos na Tabela 2.

Tabela 2. Meios de Cultura

Meio de cultura	Preparo e Armazenamento	Composição	Quantidade/ Litro
LIT[(1)]	Esterilizado em autoclave e armazenado a -20°C.	Triptose	5,00g
		Infuso de fígado	5,00g
		NaCl	4,00g
		KCl	0,40g
		Na_2HPO_4	8,00g
		Glicose	2,00g
		Hemina[a]	0,01g
		SFB (soro fetal bovino)[b]	100mL
BABb/LIT [(2)]	Armazenado a 4°C	Meio BAB (blood Agar base) a 4%	850mL
		Sangue de coelho estéril e desfibrinado	150mL

(1) Camargo (1964); (2) Rodrigues et al. (2003); a, hemina dissolvida em NaOH 0.1N; b, SFB (Soro Fetal Bovino), inativado à 56°C por 30 min e armazenado a -20°C.

3.4 Diferenciação celular

Para as análises de diferenciação celular (metaciclogênese), os tripanossomas representantes dos grupos de tripanossomas isolados de morcegos foram semeados em meio TCC-100 (acelular), acrescido de 3% de SFB e incubados a 28°C. Os ensaios de invasão celular foram realizados a partir de um inóculo de 10^5 parasitas/ml em monocamadas de células de mamíferos (LLC-MK2, Vero e HeLa) cultivadas em meio Dulbecco acrescido de 10% de SFB e incubadas a 37°C em atmosfera de 5% de CO_2.

3.5 Estudo morfológico por microscopia óptica

Para o estudo morfológico dos tripanossomas isolados de morcegos foram preparadas lâminas de esfregaços de sangue de morcegos naturalmente infectados, sangue de camundongos infectados experimentalmente, amostras de culturas em fase logarítmica e

estacionária e em co-cultivo de células. Os esfregaços foram fixados com metanol e corados com Giemsa.

3.6 Microscopia eletrônica de Transmissão e Varredura

Para análise de microscopia eletrônica de transmissão e varredura, os tripanossomas cultivados em meio acelular foram lavados com PBS 1X, fixados com glutaraldeído (2,5%) suplementado com paraformaldeido (1%) em tampão de cacodilato de sódio (0,1M e pH 7,3) por 2 horas a temperatura ambiente e posteriormente fixados em um tampão de tretróxido de ósmio por 1 hora. Após a fixacão, foi feita uma coloração com acetato de uranila por 16-18h, seguido de uma desitratação em etanol e incluído em resina Spurr. como previamente descrito (Ventura et al., 2000). Os cortes ultrafinos que foram corados com acetato de uranila e citrato de chumbo e examinados com um microscópio eletrônico Philips CM200.

3.7 Testes de infectividade dos tripanossomas para camundongos Balb/c

Culturas em meio BAB/LIT ou TC-100 com cerca de 8 a 10 dias, contendo $7x10^6$ – $1x10^7$ de formas tripomastigotas metacíclicas foram inoculadas, intraperitonealmente, em camundongos Balb/c com dois meses de idade. Foram utilizados 5 camundongos para cada isolado, imunossuprimidos com 20mg de ciclofosfamida (diluída em PBS estéril), a cada dois dias. De 6 a 30 dias após o inóculo, os camundongos foram examinados a cada três dias, através de microhematócrito (MH) de sangue colhido da cauda. Após 30 dias foram realizadas hemoculturas de sangue, obtido por punção cardíaca dos animais negativos no exame por MH, e confeccionadas lâminas com impressão de fígado, baço, rim e coração para pesquisa de formas amastigotas.

3.8 Testes de infectividade dos tripanossomas para triatomíneos

Foram inoculadas culturas de tripanossomas em fase estacionária de crescimento na hemocele de triatomíneos de diferentes gêneros e espécies (*Triatoma infestans*, *Panstrongylus megistus*, *Rhodnius prolixus*, *R. robustus* GII, *Rhodnius neglectus*), utilizando aproximadamente 20 insetos de cada gênero/espécie de triatomíneo para cada isolado analisado. As fezes dos triatomíneos foram examinadas após dez dias pós-inóculo (p.i.) e 50 a 60 dias p.i., os triatomíneos foram dissecados e foi pesquisada a presença de tripanossoma na hemolinfa, glândulas salivares e tubo digestivo.

3.9 Obtenção de DNA genômico de tripanossomas

Os tripanossomas obtidos em cultura foram lavados 2 vezes com PBS 1X, centrifugados por 10 min a 8.000g, e os "pellets" obtidos foram estocados a -20°C ou ressuspensos (1,0ml/10^9 tripanossomas) em SE e mantidos em banho de gelo para extração de DNA. Após a adição de 0,5% de Sarkosil, 100 µg/ml de Pronase e 10 µg/ml de RNAse, o material foi incubado em banho-maria, a 55-60°C, por 1 a 2 h. Após esta incubação, a mistura foi extraída uma vez com Fenol:Tris 1:1, duas vezes com Fenol:$CHCl_3$ 1:1, duas vezes com $CHCl_3$: isoamílico 24:1 e uma vez com $CHCl_3$. Após a última extração, o DNA foi precipitado com acetato de sódio a 0,3 M (pH 7,0) e 2 volumes de etanol gelado a 100%, por incubação, por 12 a 15 horas, a –20 °C. O DNA precipitado foi lavado com etanol a 70%; os "pellets" foram secos a 37°C e ressuspensos em TE. As soluções utilizadas estão descritas na Tabela 3.

Amostras diretas de sangue preservadas em etanol (v/v) foram processadas utilizando o com o kit Wizard DNA Clean-Up System (Promega). As amostras de DNA de cultura e de sangue foram mantidas a 4°C e -20°C, respectivamente. Ambas foram quantificadas em espectrofotômetro.

Tabela 3- Soluções utilizadas na obtenção de DNA genômico de tripanossomas.

Soluções	Preparação	Volume	Recomendações
PBS 1X	NaCl (8.0g); KCl (0.2g); Na_2HPO_4 (1.15g); KH_2PO_4 (0.2g); em H_2O bidestilada autoclavada.	1000 mL	Autoclavar 120°C / 30 min.
SE	99.5 ml NaCl (0.15M); 99.5 ml Tris (2.5mM) pH 8,0; 1ml EDTA (0.5M) pH 8,0.	200 mL	Autoclavar 120°C / 30 min.
Sarkosil 10%	N-laurylsarcosine (1.0g) em H_2O bidestilada autoclavada.	10 mL	Fundir em Banho-térmico 65°C.
Acetato de Sódio (3M)	CH_3COONa 3H2O mw:136.08 (20.41g) em H_2O bidestilada, acertar o pH para 7.0 com acido acético glacial diluído (1:10).	50 mL	Autoclavar 120°C / 30 min.
TE (Tris-EDTA)	Tris-HCl 2M pH 7.5 (1.25ml); EDTA 0.5M pH 8.0 (0.5ml) em H2O bidestilada autoclavada.	250 mL	Autoclavar 120°C / 30 min.
EDTA (0.5M)	EDTA (93.06g) em H2O bidestilada autoclavada, acertar o pH para 8.0 com NaOH (em lentilhas), aquecer para dissolver.	500 mL	Autoclavar 120°C / 30 min.

3.10 Amplificação de DNA por PCR (“Polymerase Chain Reaction”)

Para as reações de PCR foi utilizada a seguinte mistura de reação: 100 ng de DNA genômico; 100 ng de cada "primer"; 200mM de cada dNTP; 5µl de tampão (200mM Tris-HCl, pH 8,4, 500mM KCl e 1,5 mM $MgCl_2$); 2,5 u de Taq DNA polimerase e água bidestilada deionizada e autoclavada (qsp 50 µl). Os ciclos de amplificação e as temperaturas de anelamento foram definidos de acordo com os "primers" empregados (Tabela 4).

Tabela 4. Ciclos de amplificação e temperaturas utilizadas nas diferentes reações de PCR

Gene e oligonucletídeos	Condições de amplificação
SSU rDNA (completo) KDR3 (5´GAT CTG GTT GAT TCT GCC AGT AG 3´) KDR5 (5´GAT CCA GCT GCA GGT TCA CCT AC 3´)	1 ciclo: 3 min 94°C 29 ciclos: 1 min 94°C; 1 min 55°C; 1 min 72°C 1 ciclo: 10 min 72°C
SSU rDNA (V7-V8) 609F (5´ CAC CCG CGG TAA TTC CAG C 3´) 706R (5´ TTG AGG TTA CAG TCT CAG 3´)	1 ciclo: 3 min 94°C 29 ciclos: 1 min 94°C; 1 min 48°C; 1 min 72°C 1 ciclo: 10 min 72°C
oligonucleotídeos utilizados somente no sequenciamento: 1156F (5´CGT ACT GGT GCG TCA AGA GG 3´) 1156R (5´CCT CTG ACG CAC CAG TAC G 3´) 285F (5´GTG TTG ATT CAA TTC ATT C 3´) 285R (5´GAA TGA ATT GAA TCA ACA C 3´) 202F (5´ATG CTC CTC AAT GTT CTG 3´) 202R (5´CAG AAC ATT GAG GAG CAT3´)	
gGAPDH GAP-tryF (5´GGN CGC ATG GTS TTY CAG G 3´) GAP-tryR (5´CCC CAC TCG TTR TCR TAC C3´)	1 ciclo: 3 min 94°C 29 ciclos: 1 min 94°C; 1 min 55°C; 1 min 72°C 1 ciclo: 10 min 72°C
oligonucleotídeos utilizados somente no sequenciamento: GAP 4R (5´GTG CTG GGG ATG ATG TTC3´) GAP 3F (5´GTG AAG GCG CAG CGC AAC 3´)	
CATL (Catepsina L) inteira TDIO5-FOR (5' ATG ACG AGC TGG GCG CGT G 3') CATL3REV2 (5' TTA GCT TCA GGA GCG GCG ATG 3').	1 ciclo: 3 min 94°C 35 ciclos: 1 min 94°C; 1 min 55°C; 1 min 72°C 1 ciclo: 10 min 72°C
CATL (Catepsina L) domínio catalítico DTO154 (5´ACA GAA TTC CAG GGC CAA TGC GGC TCG TGC TGG 3´) DTO155 (5´TTA AAG CTT CCA CGA GTT CTT GAT GAT CCA GTA3´)	1 ciclo: 3 min 94°C 29 ciclos: 1 min 94°C; 1 min 54°C; 1 min 72°C 1 ciclo: 10 min 72°C
ITS 1 rDNA T-ITSF (5´ GAT ATT TCT TCA ATA GAG GAA GC 3´) 5.8SR (5´ GGA AGC CAA GTC ATC CAT C 3´)	1 ciclo: 3 min 94 °C 29 ciclos: 1 min 94°C; 1 min 55 °C; 1 min 72°C 1 ciclo: 10 min 72°C
SL (mini-exon) LSL1 (5´TTCTGTACTTCATTGGTATG3´) LSL2 (5´CCAATGAAGTACAGAAACTG3´)	1 ciclo: 3 min 94°C 29 ciclos : 1 min 94°C; 1 min 68°C; 1 min 72°C 1 ciclo: 10 min 72°C
Citocromo B (Tripanossomas) P18 (5´ GAC AGG ATT GAG AAG GGA GAG AG 3´) P20 (5´ CAA ACC TAT CAC AAA AAG CAT CTG 3´)	1 ciclo: 3 min 94°C 34 ciclos: 1 min 94°C; 30 seg 50 °C; 90 seg 72°C 1 ciclo: 10 min 72°C
Citocromo B (Morcegos) Bat_Cytb_1 (5'-TAG AAT ATC AGC TTT GGG TG-3') Bat_Cytb_2 (5'-AAA TCA CCG TTG TAC TTC AAC-3')	1 ciclo: 3 min 94°C 34 ciclos: 1 min 94°C; 30 seg 50 °C; 90 seg 72°C 1 ciclo: 10 min 72°C

3.11 Eletroforese de DNA em gel de agarose

As amostras de DNA genômico e fragmentos de DNA amplificado por PCR foram acrescidas de tampão de amostra (50% de glicerol; 0,4% de azul de bromofenol; 0,4% de xilenocianol) e submetidas à eletroforese em gel de agarose (1,5 a 2%) em tampão TAE (40mM de Tris-acetato; 2mM de EDTA, pH 8,0.) a 50 V/100 mA. Como marcador foi utilizado DNA Leader Mix (MBI Fermentas). Após a eletroforese, os géis foram corados com brometo de etídeo (0,5 ug/ml) ou GelRed® Nucleic Acid Gel Stain (Biotium) e fotografados em fotodocumentador ImageQuant 350 (GE Healthcare)

3.12 Purificação dos fragmentos de DNA amplificados por PCR

Fragmentos de DNA amplificados por PCR foram separados por eletroforese em gel de agarose a 1,5% e corados com brometo de etídeo ou GelRedR®Nucleic Acid Gel Stain (Biotium). Os fragmentos foram cortados dos géis e os DNAs foram extraído da agarose em coluna Spin X (Costar®). Os produtos purificados foram clonados ou submetidos diretamente à reação de seqüência.

3.13 Clonagem dos produtos amplificados por PCR

Fragmentos de DNA amplificados por PCR e purificados foram clonados em vetor pCR 2.1, utilizando os Kit TA Cloning (Invitrogen) e utilizados para a transformação em células dH10b. Os clones positivos foram ampliados por cultivo em meio LB contendo 100 µg/ml de ampicilina e purificados utilizando o sistema "Wizard Plus SV Minipreps DNA purification System" (Promega).

3.14 Sequenciamento de nucleotídeos

Fragmentos de DNA amplificados por PCR, clonados ou não, e purificados foram submetidos a reações de sequenciamento utilizando o kit Big Dye Terminator (Perkin Elmer), de acordo com especificações do fabricante, em sequenciador automático ABI PRISM 310 Genetic Analyzer (Perkin Elmer) e ABI PRISM 3100 Genetic Analizer (Perkin Elmer). As reações foram submetidas a 30 ciclos: 15 s 96 °C; 15 s 50 °C; 4 min 60 °C, com um ciclo inicial de 1 min 96 °C.

Nas reações de sequenciamento foram utilizados os oligonucleotídeos citados na tabela 4. Para sequenciamento de fragmentos de DNA clonados em vetor pCR 2.1 (Kit TA Cloning – Invitrogen) foram utilizados os oligonucleotídeos M13F e M13R.

3.15 Alinhamento de sequências de nucleotídeos e aminoácidos

Os cromatogramas das sequências foram analisados no programa Seqman do pacote de programas DNAstar (Nicholas et al., 1997). As sequências de nucleotídeos determinadas e as obtidas no GeneBank (http://www.ncbi.nlm.nih.gov/), foram alinhadas através do programa Clustal X (Thompson et al., 1997) alterando os parâmetros relativos à inserção de "indels" (peso de inserção=1, Extensão=1). As sequências de aminoácidos e o alinhamento de nucleotídeos foram manualmente ajustadas no programa GeneDoc v. 2.7.000 (Nicholas et al., 1997).

3.16 Construção da matriz de similaridade

A partir do alinhamento das sequências de nucleotídeos e/ou aminoácidos dos genes em estudo, foi determinada uma matriz de similaridade (baseadas em distância p não corrigida) utilizando-se o programa Point Replacer v.2.2. disponibilizado pelo autor (Alves, J. M.) no endereço http://reocities.com/CapeCanaveral/lab/3891/software.html.

3.17 Análises filogenéticas

Inferências filogenéticas foram determinadas pelos métodos de máxima parcimônia (MP), máxima verosimilhança (MV) ("maximum likelihood") e análise bayesiana (B). As árvores de MP foram construídas utilizando o programa PAUP* v. 4.0b10 (Swofford, 1998) via busca heurística com 100 replicatas de adição aleatória dos terminais seguida de troca de ramos ("RAS-TBR Branch-breaking"). As análises de suporte por "bootstrap" foram feitas em 100 replicatas com os mesmos parâmetros empregados na busca.

As análises de MV foram realizadas no programa RAxML v7.0.4 (Stamatakis, 2006). Foram empregadas 500 replicatas usando GTR como modelo de substituição e 4 categorias de gama e diagramas obtidos por parcimônia como árvores iniciais. Os parâmetros do modelo de substituição empregado foram estimados durante a busca. O suporte de ramos foi estimado com 500 replicatas de "bootstrap" no programa RAxML.

As análises bayesianas foram executadas no programa MrBayes v.3.1.2 (Ronquist e Huelsenbeck, 2003). Foram empregadas 500.000 gerações usando GTR como modelo de substituição e 4 categorias de gama mais proporção de sítios invariantes. Para a construção

do dendrograma final foram utilizados apenas os diagramas obtidos nas últimas 75 replicatas. Para a verificação de suporte de ramos nas análises bayesianas foram utilizados os valores de probabilidade a posteriori obtidos com o programa MrBayes.

3.18 Genealogias

As genealogias de sequências de nucleotídeos e de aminoácidos foram inferidas por análises de Network, utilizando o programa Splitstree v4.11.3 (Huson e Bryant, 2006). Foi utilizado o método de "Neighbor-net" e os valores de suporte foram estimados através da realização de 100 réplicas de "bootstrap", usando os mesmos parâmetros otimizados pelo método de "Neighbor-net".

4. Resultados e Discussão

Os resultados obtidos durante o trabalho desenvolvido para essa tese estão resumidamente apresentados e discutidos abaixo. Optamos por apresentar apenas resultados já publicados, ou em fase de publicação, que constituem os artigos e manuscritos anexados no final da tese e listados abaixo.

4.1. Tripanossomas do subgênero *Schizotrypanum* de morcegos brasileiros e africanos (Moçambique)

Anexo 1- Phylogeographical, ecological and biological patterns shown by nuclear (ssrRNA and gGAPDH) and mitochondrial (Cyt b) genes of trypanosomes of the subgenus *Schizotrypanum* parasitic in Brazilian bats

Anexo 2- A new genotype of *Trypanosoma cruzi* associated with bats evidenced by phylogenetic analyses using SSU rRNA, cytochrome b and Histone H2B genes and genotyping based on ITS1 rDNA

Anexo 3- A *Trypanosoma cruzi*-like new species from African bats (Chiroptera: Molossidae) supported by morphological, developmental and multigene phylogenetic analyses

Resumos dos trabalhos anexados

4.1.1 Padrões filogeográficos, ecológicos e biológicos demonstrados por genes nucleares (ssrRNA e gGAPDH) e mitocondriais (Cyt b) de tripanossomas do subgênero *Schizotrypanum* parasitas de morcegos brasileiros

Anexo 1. Phylogeographical, ecological and biological patterns shown by nuclear (ssrRNA and gGAPDH) and mitochondrial (Cyt b) genes of trypanosomes of the subgenus *Schizotrypanum* parasitic in Brazilian bats

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International Journal for Parasitology, 2010; 40(3):345-55.

A ordem Chiroptera é composta por diversas famílias de morcegos com diferentes hábitos alimentares e ecótopos encontrados em todos os continentes, exceto na Antártida. Esses mamíferos albergam tripanossomas das Secções Stercoraria (subgêneros

Schizotrypanum, Megatrypanum e Herpetosoma) e Salivaria (*Trypanozoon)*. Uma espécie de tripanossoma pode infectar mais de uma espécie de morcego, assim como são comuns morcegos com infecções mistas com duas ou mais espécies, inclusive espécies de diferentes subgêneros. Os morcegos insetívoros são os mais frequentemente infectados e as espécies mais prevalentes pertencem aos subgêneros *Megatrypanum* e *Schizotrypanum*.

A diversidade genética e os padrões filogeográficos e biogeográficos das espécies de tripanossomas do subgênero *Schizotrypanum,* que infectam morcegos brasileiros foram avaliados com o exame de amostras de sangue de 1043 morcegos, 63 espécies de sete famílias, capturados em quatro biomas brasileiros: Amazônia, Pantanal, Cerrado e Mata Atlântica. A prevalência dos tripanossomas que infectam morcegos foi estimada por hemocultura (12,9%) e resultou em 77 culturas. A maioria das culturas foi morfologicamente caracterizada como *T. cruzi*-like e identificadas por marcadores moleculares como *T. cruzi* (14), *T. c. marinkellei* (37) e *T. dionisii*-like (25). Análises filogenéticas baseadas na região V7-V8 do gene SSU rRNA, gGAPDH e cytocromo b demonstraram que os isolados obtidos formam um grupo monofilético que compreende apenas espécies do subgênero *Schizotrypanum.* Os isolados desse subgênero foram segregados em três clados correspondentes as três espécies de tripanossomas identificadas nos morcegos.

As diferentes espécies de tripanossomas puderam ser associadas com distintos padrões biogeográficos e filogeográficos. Morcegos de hábitos alimentares e ecótopos distintos foram encontrados infectados pela mesma espécie de tripanossoma. *T. dionisii*-like (32.4%) foi encontrado em 12 espécies pertencentes a quatro famílias de morcegos capturadas em todos os biomas estudados, de Norte a Sul do Brasil e ficaram agrupados com dois isolados de *T. dionisii* da Europa, apesar de separados por uma pequena distância genética. *T. c. marinkellei* (49.3%) foi encontrado apenas em morcegos da família Phyllostomidae, que apresentam diferentes hábitos alimentares, todos capturados da Amazônia ao Pantanal. Em menor prevalência (18.2%), os isolados de *T. cruzi* são provenientes de morcegos da família Vespertilionidae e Phyllostomidae capturados no Pantanal/Cerrado, Mata Atlântica e com poucos isolados da Amazônia.

4.1.2 Identificação de um novo genótipo de *Trypanosoma cruzi* associado à morcegos e caracterizado através de análises filogenéticas dos genes SSU rRNA, citocromo b, Histona H2B e genotipagem baseada em ITS1 rDNA

Anexo 2. A new genotype of *Trypanosoma cruzi* associated with bats evidenced by phylogenetic analyses using SSU rDNA, cytochrome b and Histone H2B genes and genotyping based on ITS1 rDNA

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Parasitology, 2009; 136(6):641-55.

T. cruzi é a unica espécie do subgênero *Schizotrypanum* capaz de infectar espécies de praticamente todas as ordens de mamíferos, inclusive o homem, as demais espécies pertencentes a este subgênero parasitam exclusivamente morcegos. O ciclo silvestre do *T. cruzi* ainda é pouco estudado e apenas recentemente passou a ser alvo de estudos abrangendo diversas espécies de mamíferos e vetores silvestres. Pouco se sabe sobre a diversidade biológica e genética, e as consequências das infecções causadas por *T. cruzi* em morcegos. Neste trabalho foram caracterizados 15 isolados de *T. cruzi* de morcegos capturados nas regiões Norte, Central e Sudeste do Brasil. Relações filogenéticas entre os isolados de *T. cruzi* inferidas com sequências dos genes SSU rRNA, citocromo b e Histona H2B posicionaram todos os isolados da Amazônia na linhagem *T. cruzi* I (TcI). Entretanto, isolados das outras regiões, genotipados como *T. cruzi* II com a utilização de marcadores tradicionais baseados nos genes ribossômico e de mini-exon, não foram agrupados com nenhuma linhagem de *T. cruzi*, e se posicionaram como um novo genótipo diferente de todos os estabelecidos até o momento para *T. cruzi.* Esses isolados formaram um novo genótipo que foi provisoriamente denominado TCbat até que sejam estabelecidos os critérios para descrição de novas linhagens (DTUs). Análises filogenéticas confirmaram que TCbat está posicionado no clado que contém todos os isolados de *T. cruzi,* que se apresenta sempre separado das espécies altamente relacionadas filogeneticamente e exclusivas de morcegos do subgênero *Schizotrypanum, T. c. marinkellei* e *T. dionisii*.

Nesse trabalho, foi padronizado um método de genotipagem baseado no polimorfismo de gene ITS1 rDNA capaz de distinguir TCbat dos outros genótipos de *T. cruzi,* assim como de outras espécies do subgênero *Schizotrypanum*. Em infecções experimentais em camundongos, TCbat apresentou baixas parasitemia e virulência. Os isolados do genótipo TCbat apresentaram características morfológicas e comportamento em triatomíneos distintos dos demais genótipos de *T. cruzi.* TCbat compreende somente isolados de morcegos capturados em ambientes antrópicos das regiões Central e Sudeste

do Brasil. Os resultados desse trabalho comprovam que a complexidade de *T. cruzi* é maior do que a que conhecemos e confirmam que os morcegos são importantes reservatórios e uma potencial fonte de infecção por *T. cruzi* para o homem.

4.1.3 Uma nova espécie de *Trypanosoma cruzi*-like de morcegos Africanos (Chiroptera: Molossidae), caracterização morfológica, desenvolvimento e análise filogenética multigênica

Anexo 3. A *Trypanosoma cruzi*-like new species from African bats (Chiroptera: Molossidae) supported by morphological, developmental and multigene phylogenetic analyses

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O subgênero *Schizotrypanum* compreende tripanossomas que infectam o homem e outros mamíferos. Com exceção de *T. cruzi*, as demais espécies desse subgênero são restritas aos morcegos. A maioria dos tripanossomas isolados de morcegos foi classificada neste subgênero e por apresentarem morfologia muito semelhante à de *T. cruzi* são conhecidos como *T. cruzi*-like. Neste trabalho, isolamos e caracterizamos uma nova espécie de *Trypanosoma cruzi*-like encontrada em morcegos da família Molossidae, capturados em Moçambique, sudeste da África. As amostras de sangue dos 25 morcegos capturados resultaram em 6 hemoculturas positivas para tripanossomas, revelando uma prevalência de 24%.

A análise morfológica dos novos isolados, assim como o comportamento em cultura com a diferenciação de formas epimastigotas para tripomastigotas metacíclicas, a capacidade de invadir e se desenvolver dentro de células de mamíferos, que é uma característica exclusiva das espécies do subgênero *Schizotrypanum,* indicaram que todos os isolados devem ser desse subgênero. Embora tenham sido originados de dois diferentes gêneros de morcegos da família Molossidae (*Tadarida* sp e *Mops* sp), esses seis isolados são morfologicamente indistinguíveis e análises de genes polimórficos como a região V7V8 do SSUrDNA e ITS1 rDNA demonstraram que esses isolados são intimamente relacionados.

Análises filogenéticas utilizando sequências de SSUrDNA, gGAPDH e cytb dos seis novos isolados de tripanossomas de morcegos revelaram um clado muito homogênio, posicionado no subgênero *Schizotrypanum*, apresentando divergências semelhantes quando comparado com *T. c. marinkellei,* a espécie mais relacionada, e *T. dionisii*, mais

distante de *T. cruzi.* O gene SL também apoiou a posicão desses novos isolados e a criação de uma nova espécie do subgênero *Schizotrypanum.*

A caracterização morfológica e biológica desses isolados, a utilização de sequências conservadas e polimórficas de genes nucleares e mitocondriais permitem classificar este tripanossoma, como uma nova espécie neste subgênero que denominamos *T. erneyi* n. sp. Além de ser o primeiro tripanossoma do subgênero *Schizotrypanum* isolado de morcegos africanos e caracterizado molecularmente, é a única espécie nova deste subgênero, apoiada por análises filogenéticas, descrita nos últimos 30 anos. Os resultados desse trabalho são importantes para a compreensão da origem e evolução de *T. cruzi* e do relacionamento entre os tripanossomas intimamente relacionados com essa espécie.

4.2. ***Trypanosoma rangeli*** **em morcegos brasileiros**

Anexo 4- *Trypanosoma rangeli* isolates of bats from Central Brazil: genotyping and phylogenetic analysis enable description of a new lineage using spliced-leader gene sequences

Resumo do trabalho anexado

4.2.1 ***Trypanosoma rangeli*** **em morcegos da região central do Brasil: genotipagem e análises filogenéticas baseadas em sequências do gene de mini-exon revelaram uma nova linhagem**

Anexo 4. *Trypanosoma rangeli* isolates of bats from Central Brazil: genotyping and phylogenetic analysis enable description of a new lineage using spliced-leader gene sequences

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Acta Tropica 2009; 109(3):199-207.

Trypanosoma rangeli é uma espécie de tripanossoma americano infectante para diversas ordens de mamíferos, inclusive o homem. Porém, apesar de alguns relatos, essa espécie nunca foi comprovada em morcegos, embora morcegos de diferentes espécies sejam comumente encontrados parasitados por várias espécies de tripanossomas. Neste trabalho foram caracterizados tripanossomas de morcegos capturados na região central do

Brasil (Mato Grosso do Sul), classificados como *T. rangeli, T. dionisii, T. cruzi marinkellei* e *T. cruzi*. Apenas dois isolados; Tra643 de *Platyrrhinus lineatus* e Tra1719 de *Artibeus planirostris* foram identificados como *T. rangeli* por parâmetros morfológicos, biológicos e moleculares, e confirmados nessa espécie por análises filogenéticas com diversas espécies de tripanossomas.

Análises filogenéticas baseadas em sequências do gene ribossômico (SSU rDNA) agruparam os dois isolados de morcegos no mesmo clado de isolados de *T. rangeli* de outros mamíferos silvestres, humanos e triatomínios. Esses isolados de *T. rangeli* formaram um clado separado dos demais tripanossomas de morcegos (*T. cruzi*, *T. c. marinkellei* e *T. dionisii*) capturados na mesma região. Genotipagem baseada no polimorfismo de tamanho e de sequência do gene de mini-exon demonstrou que o isolado Tra1719 pertence à linhagem A de *T. rangeli*. Por outro lado, Tra643 não se posicionou em nehuma das linhagens (A-D) previamente descritas para *T. rangeli*, portanto, pertence a um novo genótipo que nós denominamos linhagem E. Os dois isolados de *T. rangeli* deste estudo são os primeiros isolados de morcegos do Brasil Central caracterizados molecularmente. Os vetores de *T. rangeli* nessa região são desconhecidos. Entretanto nesse estudo, foram coletados na região de onde foram capturados os morcegos, triatomíneos da espécie *Rhodnius stali* que se revelaram infectados com *T. rangeli* e *T. cruzi*. Estes dados contribuem para o melhor entendimento dos isolados de *T. rangeli* e compreensão da alta complexidade que as populações dessa espécie apresentam e que podem ser melhor esclarecidas com a busca de novos isolados de hospedeiros mamíferos e espécies de triatomíneos do genêro *Rhodnius* de diferentes origens geográficas.

4.3. Tripanossomas do subgênero *Megatrypanum* em morcegos africanos (Moçambique)

Anexo 5- A new trypanosome species infecting African bats (*Rhinolophidae* and *Hipposideridae*): taxonomic appraisal based on morphological and behavioral features and phylogenetic inferences using SSU rRNA and gGAPDH genes

Resumo do trabalho anexado

4.3.1. Uma nova espécie de tripanossoma em morcegos africanos (*Rhinolophidae* and *Hipposideridae*): revisão taxonômica com base em características morfológicas e biológicas e inferências filogenéticas com os genes SSU rRNA and gGAPDH

Anexo 5. A new trypanosome species infecting African bats (Rhinolophidae and Hipposideridae): taxonomic appraisal based on morphological and behavioural features and phylogenetic inferences using SSU rRNA and gGAPDH genes

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Tripanossomas são tradicionalmente classificados no subgênero *Megatrypanum* de acordo com a morfologia das formas do sangue. Esses tripanossomas têm sido descritos em mamíferos de diversas ordens, inclusive morcegos da África, Europa, Ásia e Américas Central e do Sul. A maioria das informações sobre esses tripanossomas se restringe ao encontro de grandes formas tripomastigotas no sangue; a classificação dessas espécies é baseada em morfometria e hospedeiro de origem. Embora a parasitemia das infecções causadas por essas espécies possa ser relativamente alta comparada às espécies de *Schizotrypanum*, esses tripanossomas dificilmente são isolados em cultura.

Neste trabalho foram caracterizados morfologicamente, biologicamente e filogeneticamente 12 isolados obtidos de duas espécies de morcegos pertencentes à família Rhinolophidae, capturados em Moçambique, no sudeste da África. As amostras de sangue de 68 morcegos capturados geraram 12 hemoculturas positivas para tripanossomas, sendo 11 isoladas de *Rhinolophus landeri* e uma de *Hipposideros caffer.* A morfologia e o comportamento biológico desses isolados claramente os separa das espécies de tripanossomas do subgênero *Schizotrypanum,* que compreende a maioria dos tripanossomas que são cultivados e que foram caracterizados molecularmente. As formas tripomastigotas sanguíneas observadas no sangue desses morcegos apresentaram morfologia e tamanho compatíveis com espécies tradicionalmente classificadas no

subgênero *Megatrypanum,* que é um taxon controverso comprovadamente polifilético. Entretanto, análises filogenéticas baseadas nas sequências dos genes SSUrRNA e gGAPDH demonstraram que esses tripanossomas formam um grupo monofilético, muito distante de todas as espécies de tripanosomas de morcegos e de outros animais incluídos em árvores filogenéticas até o momento.

Os resultados obtidos permitiram classificar os novos isolados de morcegos africanos como uma nova espécie, que foi denominada *T. zambesiensis* n. sp.*,* e que não pode ser classificada em nenhum subgênero. Essa é única espécie de "*Megatrypanum"* de morcegos descrita com base em estudos morfológicos, biológicos e filogenéticos. De acordo com todas as análises, a morfologia não é um critério suficiente para classificar tripanossomas no subgênero *Megatrypanum* que, filogeneticamente validado, compreende apenas tripanossomas de ruminantes.

4.4. Genes codificadores de enzimas CatpesinaL-like: caracterização, genealogia e utilização como marcadores taxonômicos e filogenéticos

Anexo 6- Genes of cathepsin L-like proteases in *Trypanosoma rangeli* isolates: Markers for diagnosis, genotyping and phylogenetic relationships

Anexo 7- Repertoire diversity, evolutionary relationships and genomic organization of cruzipain encoding genes in phylogenetic diverse *Trypanosoma cruzi* isolates and homologues from closely related *T. c. marinkellei* and *T. dionisii*

Resumos dos trabalhos anexados

4.4.1. Genes de proteases como catepsina L-like em isolados de *Trypanosoma rangeli*: marcadores para diagnóstico, genotipagem e inferências filogenéticas

Anexo 6. Genes of cathepsin L-like proteases in *Trypanosoma rangeli* isolates: Markers for diagnosis, genotyping and phylogenetic relationships

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Acta Tropica, 2009; 112(3):249-59.

Nesse trabalho, foram sequenciados genes que codificam enzimas cisteíno-proteases do tipo catepsina L-like (CATL-like) de isolados de *T. rangeli* do homem, de

mamíferos silvestres e diversas espécies de triatomínios do gênero *Rhodnius.* Foram comparadas sequências de isolados da América Central e do Sul.

Análises filogenéticas de sequências codificadoras do domínio catalítico de CATL de *T. rangeli* e genes homólogos de outros tripanossomas, espécies de *Leishmania* e bodonídeos posicionaram as sequências de *T. rangeli* (rangelipaína) mais próximas da cruzipaína, CATL majoritária de *T. cruzi*.

Análises filogenéticas com sequências do domínio catalítico de CATL de 17 isolados representantes da diversidade filogenética e distribuição de *T. rangeli,* confirmaram as 5 linhagens (A-E) previamente definidas com os genes de mini-exon e ribossômico. A comparação da atividade proteolítica de isolados de *T. rangeli* em gel de gelatina, revelou diferentes perfis de bandas de cisteíno-proteases entre as linhagens de *T. rangeli* e entre isolados de uma mesma linhagem.

Sequências de CATL se mostraram excelentes como alvos para o diagnóstico e genotipagem de *T. rangeli* por PCR. Os dados de genes que codificam CATL-like concordam com os resultados de estudos anteriores com marcadores de kDNA, e os genes de mini-exon e ribossômicos. Os resultados corroboram a evolução clonal, ciclos de transmissão independentes e a divergência das linhagens de *T. rangeli* associados às espécies simpátricas de *Rhodnius*.

A topologia da árvore filogenética baseada em sequências do gene CATL apresentou total congruência com as topologias das árvores geradas pelos genes de SSU rDNA e gGAPDH para os tripanossomatídeos analisados. Portanto, esse trabalho demonstrou que esses genes podem ser utilizados como alvos para diagnóstico, taxonomia e estudos filogenéticos de espécies de tripanossomas, inclusive na genotipagem de isolados de uma mesma espécie.

4.4.2 Diversidade, relações evolutivas e organização genômica de genes codificadores de cruzipaína de *Trypanosoma cruzi* das diferentes e linhagens filogenética e genes homólogos de *T. c. marinkellei* e *T. dionisii.*

Anexo 6. Repertoire diversity, evolutionary relationships and genomic organization of cruzipain encoding genes in phylogenetic diverse *Trypanosoma cruzi* isolates and homologues from closely related *T. c. marinkellei* and *T. dionisii*

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A enzima cruzipaína, principal isoforma de catepsina L-like (CATL) descrita em *Trypanosoma cruzi*, é uma cisteíno protease que desempenha um papel fundamental na invasão celular, na diferenciação, na imunidade e na patogenicidade de *T. cruzi*, sendo muito explorada como alvos para drogas e vacinas. *T. cruzi* é um complexo de isolados geneticamente heterogêneos, distribuídos em seis DTUs (TcI-VI) com um novo genótipo, Tcbat, associado com morcegos. Filogeneticamente, *T. cruzi* é mais próximo de *T. cruzi marinkellei* e *T dionisii,* que são tripanossomas exclusivos de morcegos, apresentam morfologia muito semelhante a *T. cruzi* e por esse motivo, são conhecidos como *T. cruzi*-like. Essas três espécies pertencem ao subgênero *Schizotrypanum*, que compreende os tripanossomas que se desenvolvem em células de mamíferos.

Embora, *T. c. marinkellei* e *T. dionisii* apresentem características morfológicas, genômicas e proteômicas semelhantes a *T. cruzi*, diferem em hospedeiros, vetores e patogenicidade. Estudos anteriores sugerem que as variações nos níveis de expressão e a diversidade de genes codificadores de cruzipaína estão relacionadas com a capacidade de invasão e diferenciação celular, virulência e a patogenicidade de isolados de *T. cruzi.*

Uma análise de polimorfismo de genes codificadores de cruzipaína de 24 isolados de *T. cruzi*, com um total de 73 sequências, pertencentes as diferentes ao DTUs (TcI-VI) e ao novo genótipo Tcbat revelou que essa proteína é conservada em todos os domínios. No entanto, o domínio catalítico da cruzipaína apresentou um grande repertório de sequências que divergiram de acordo com as espécies de tripanossomas e os DTUs de *T. cruzi*. Observamos que as cópias do gene da cruzipaína eram muito conservadas dentro de um único isolado e entre isolados de uma mesma DTU, exceto para os isolados TcV-VI que apresentaram genes polimórficos, incluindo sequências únicas para esses híbridos ou sequências idênticas às de TcII e TcIII, apoiando a origem evolutiva dessas linhagens a partir de eventos de hibridização entre essas duas DTUs.

Genealogias de genes codificadores de CATL revelaram que as sequências desses genes de isolados de *T. cruzi* são mais relacionadas com sequências homólogas de *T. c.*

marinkellei do que com as de *T. dionisii.* Os genes dessas três espécies formam um clado que corresponde ao subgênero *Schizotrypanum*. Essas sequências se posicionaram mais distantes de *T. rangeli* e *T. brucei.* Portanto, os resultados estão em total concordância com a diversidade e as relações filogenéticas entre espécies de tripanossomas, e também entre isolados de *T. cruzi* representantes de todas as DTUs.

Apesar do alto grau de conservação entre os genes codificadores de cruzipaínas, a análise do polimorfismo revelou marcadores específicos para espécies e DTUs de *T. cruzi.* Análise comparativa de genes com funções tão importantes como as cruzipaínas entre isolados de *T. cruzi* representantes da sua grande diversidade biológica, genética e patológica, e a comparação com sequências homólogas de *T. cruzi*-like são muito importantes para a compreensão das interações desses parasitas com seus hospedeiros. Além disso, esses estudos são fundamentais para a avaliação da cruzipaína, e de outros genes, que vem sendo testados como alvos para quimioterápicos e vacinas contra *T. cruzi*.

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International Journal for Parasitology 40 (2010) 345–355

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Phylogeographical, ecological and biological patterns shown by nuclear (ssrRNA and gGAPDH) and mitochondrial (Cyt *b*) genes of trypanosomes of the subgenus *Schizotrypanum* parasitic in Brazilian bats ☆

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ARTICLE INFO

Article history:
Received 18 May 2009
Received in revised form 20 August 2009
Accepted 26 August 2009

Keywords:
Trypanosoma
Chiroptera
Phylogeny
Evolution
Biogeography
Brazilian biomes

ABSTRACT

The genetic diversity and phylogeographical patterns of *Trypanosoma* species that infect Brazilian bats were evaluated by examining 1043 bats from 63 species of seven families captured in Amazonia, the Pantanal, Cerrado and the Atlantic Forest biomes of Brazil. The prevalence of trypanosome-infected bats, as estimated by haemoculture, was 12.9%, resulting in 77 cultures of isolates, most morphologically identified as *Trypanosoma* cf. *cruzi*, classified by barcoding using partial sequences from ssrRNA gene into the subgenus *Schizotrypanum* and identified as *T. cruzi* (15), *T. cruzi marinkellei* (37) or *T.* cf. *dionisii* (25). Phylogenetic analyses using nuclear ssrRNA, glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) and mitochondrial cytochrome *b* (Cyt *b*) gene sequences generated three clades, which clustered together forming the subgenus *Schizotrypanum*. In addition to vector association, bat trypanosomes were related by the evolutionary history, ecology and phylogeography of the bats. *Trypanosoma* cf. *dionisii* trypanosomes (32.4%) infected 12 species from four bat families captured in all biomes, from North to South Brazil, and clustered with *T. dionisii* from Europe despite being separated by some genetic distance. *Trypanosoma cruzi marinkellei* (49.3%) was restricted to phyllostomid bats from Amazonia to the Pantanal (North to Central). *Trypanosoma cruzi* (18.2%) was found mainly in vespertilionid and phyllostomid bats from the Pantanal/Cerrado and the Atlantic Forest (Central to Southeast), with a few isolates from Amazonia.

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1. Introduction

Flagellates of the genus *Trypanosoma* (Euglenozoa, Kinetoplastida) are parasitic in virtually all mammalian species. They occur in species of Chiroptera throughout the world, with more than 30 trypanosome species recorded from more than 100 species of bats. Insectivorous bat species are more frequently infected and can harbour stercorarian (subgenera *Herpetosoma*, *Schizotrypanum* and *Megatrypanum*) and salivarian (*Trypanosoma evansi* of the subgenus *Trypanozoon*) trypanosomes (Marinkelle, 1977; Molyneux, 1991). The subgenus *Schizotrypanum* comprises species restricted to bats as well as *Trypanosoma cruzi*, which is the agent of human Chagas disease and is restricted to the American continent. *Trypanosoma cruzi* is the only species of this subgenus proven to be infective to virtually all mammals. There is no evidence that trypanosomes are pathogenic in bats, although bats are long-lived species and infections persist for years, with parasites localising in skeletal, cardiac and stomach muscle cells (Gardner and Molyneux, 1988; Molyneux, 1991).

Despite limited field and experimental evidence, haematophagous arthropods can act as vectors of trypanosomes among bats. *Trypanosoma cruzi* can be transmitted by triatomines that share their habitat with bats. Most trypanosome-infected bats are insectivorous and infection could also occur through the ingestion of infected arthropods. *Trypanosoma cruzi marinkellei* is, apparently, only transmitted by triatomines of the genus *Cavernicola*, which

☆ *Note:* Nucleotide sequences reported in this paper are available in the GenBank database under the following accession numbers: FJ001635–FJ001667; FJ900239–FJ900254; GQ140351–GQ140365.

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doi:10.1016/j.ijpara.2009.08.015

is found associated with bat colonies in caverns, hollow trees and palms (Marinkelle, 1976, 1982a; Molyneux, 1991). Experimental infections have demonstrated that ectoparasitic bugs of the family Cimicidae are vectors of *Trypanosoma dionisii* and *Trypanosoma vespertilionis* in bats in the Old World (Paterson and Woo, 1984). Co-phylogenetic relationships have been demonstrated between bat species and their ectoparasitic flies (Dittmar et al., 2006; Dick and Patterson, 2007) and mites (Bruyndonckx et al., 2009). However, the roles of these highly specialised ectoparasites in transmission, which depends on contact inside roosts, are unknown.

Variable prevalences of trypanosomes in bats have been reported in surveys conducted throughout the world using different approaches. Blood smear (BS) examination of 491 bats revealed that 20% were infected with *T. dionisii* and *T. vespertilionis* in England (Gardner and Molyneux, 1988). In Kenya (Africa), 21% of 427 bats were found to be infected with trypanosomes by the method of microhaematocrit (MH) centrifugation (Woo and Hawkins, 1975). In South American bats, prevalences varied widely according to detection methods. Of thousands of Colombian bats examined by BS and haemocultures (HE), approximately 9.0% were infected with *Schizotrypanum* spp. (Marinkelle, 1976, 1982b). Surveys also have been performed in Brazil. In Amazonia (Para State), of 588 bats examined by Dias et al. (1942) and 246 examined by Deane (1961), 4.6% and 2.4%, respectively, were positive (BS) for trypanosomes. Funayama and Barreto (1970, 1973) examined approximately 500 bats from Southeastern Brazil and found that 16% were infected with *T. cruzi* by xenodiagnosis (XE). Pinto and da Costa Bento (1986) examined 135 bats from the northeast and found that 32.6% were positive for *T.* cf. *cruzi* using a combination of XE and HE. Of 422 bats also from the northeast, 12% were positive for trypanosomes morphologically similar to *T. cruzi* by XE (Fabián, 1991). *Schizotrypanum* trypanosomes were also reported from small surveys conducted using HE in bats from South, Central and North Brazil (Steindel et al., 1998; Cavazzana et al., 2003; Lisboa et al., 2008; Maia da Silva et al., 2009; Marcili et al., 2009a).

Species of *Schizotrypanum* are: T. vespertilionis (widespread in the New and Old Worlds), *T. dionisii* (Europe), *Trypanosoma hedricki, Trypanosoma myoti* and *Trypanosoma phyllostomus* (North and Central America) *T. cruzi* and *T. cruzi marinkellei* (Central and South America). Despite the use of zymodemes and kinetoplast DNA fingerprinting (Baker et al., 1978; Teixeira et al., 1993; Steindel et al., 1998; Barnabé et al., 2003), molecular markers for the unambiguous separation of *Schizotrypanum* spp. remain unavailable.

The evolutionary processes that have led to the current phylogenetic structure of *Schizotrypanum* trypanosomes are poorly understood. The strong association between chiropterans and all *Schizotrypanum* spp. except *T. cruzi* suggests a long shared evolutionary history. However, association with different vectors (hematophagous insects) suggests independent histories for these species. Phylogeographical and ecological data have also provided valuable information for the understanding of host–parasite–vector interactions and for the reconstruction of the evolutionary histories of trypanosomes and their hosts (Page and Charleston, 1998; Gaunt and Miles, 2000; Stevens et al., 2001; Hamilton et al., 2007; Maia da Silva et al., 2007; Nieberding and Olivieri, 2007; Nieberding et al., 2008; Stevens, 2008; Viola et al., 2008, 2009).

A paucity of studies, a lack of reliable taxonomic parameters, and the few isolates included in phylogenetic studies have prevented a detailed understanding of the genetic diversity, and bio- and phylogeographical patterns of bat trypanosomes. To help clarify these issues, we carried out a large survey of trypanosomes in a range of bat species differing in ecological traits and captured in the Brazilian biomes of Amazonia, the Pantanal, Cerrado and the Atlantic Forest.

2. Materials and methods

2.1. Isolation, culture and morphology of bat trypanosomes

Trypanosomes were obtained by culturing peripheral blood samples of bats captured in the following Brazilian biomes: Amazonia, States of Amazonas (AM) and Rondonia (RO); Cerrado, Goias (GO), Tocantins (TO) and Mato Grosso do Sul (MS); the Pantanal (Mato Grosso do Sul); the Atlantic Forest, São Paulo (SP) and Parana (PR). Some of the bats from MS were captured in transition areas of the Pantanal and Cerrado and are referred to as Pantanal/Cerrado (Fig. 1, Table 1). Bats were captured, anesthetised and manipulated according to recommendations from the Brazilian Institute of the Environment (IBAMA). Haemocultures were prepared using biphasic medium and the isolates obtained were expanded in Liver Infusion Tryptose (LIT) medium for DNA preparation and cryopreservation in the Trypanosomatid Culture Collection (TCC) of the Department of Parasitology, University of São Paulo, São Paulo, Brazil, as described previously (Maia da Silva et al., 2004).

Stationary phase LIT cultures containing infective metacyclic forms (1×10^6 parasites/well) were used to infect monolayers of LLC-MK$_2$ (from monkey) and HeLa (human) cells cultivated on glass coverslips in 24-well plates (1×10^5 cells/well) as described previously (Marcili et al., 2009a). BSs from either naturally infected bats or mice experimentally infected with bat trypanosomes, smears of cultures, and infected HeLa cell monolayers on cover slips were fixed with methanol and stained with Giemsa.

2.2. PCR amplification and analysis of ssrDNA, glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) and cytochrome b (Cyt b) sequences

PCR amplification and sequencing of the V7-V8 region of ssrDNA (~900 bp) were performed for 55 isolates using primers and conditions described previously (Maia da Silva et al., 2004; Rodrigues et al., 2006). Complete sequences from ssrRNA and glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) genes were obtained, respectively, for eight and 15 selected bat isolates as described previously (Hamilton et al., 2007; Ferreira et al., 2007; Viola et al., 2009). Partial sequences (~500 bp) of Cyt *b* were amplified from nine bat isolates as described previously (Brisse et al., 2003).

Sequences of bat trypanosomes derived from this study were deposited in GenBank and aligned with sequences determined in our previous studies of bat isolates of *T. cruzi* (Marcili et al., 2009a) and *Trypanosoma rangeli* (Maia da Silva et al., 2009) (Table 2) and from other trypanosomes form bats and other hosts available in GenBank. Accession numbers of all sequences are on the trees (Figs. 1–3). Regions of ribosomal genes that could not be unambiguously aligned following Hamilton et al. (2007) were excluded from the analysis.

Phylogenetic trees were inferred by maximum likelihood (ML), maximum parsimony (MP) and Bayesian (B) methods. ML trees were inferred using RAxML v.7.0 (Stamatakis, 2006) using the GTRGAMMA model, gamma shape parameter and proportion of invariable sites, with MP starting trees. Model parameters were estimated in RAxML over the duration of tree search. Nodal supports were estimated with 500 replicates also in RAxML using the rapid bootstrapping algorithm. MP trees were inferred using PAUP* v. 4.0b10 (Swofford, 1998) with 500 replicates of random addition taxa and TBR branch swapping; all positions were of equal weight. B inferences were carried out with MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001) using the GTR model. The first 100 trees from 1,000,000 generations were discarded as burn in, and posterior probability values were calculated on the consensus of

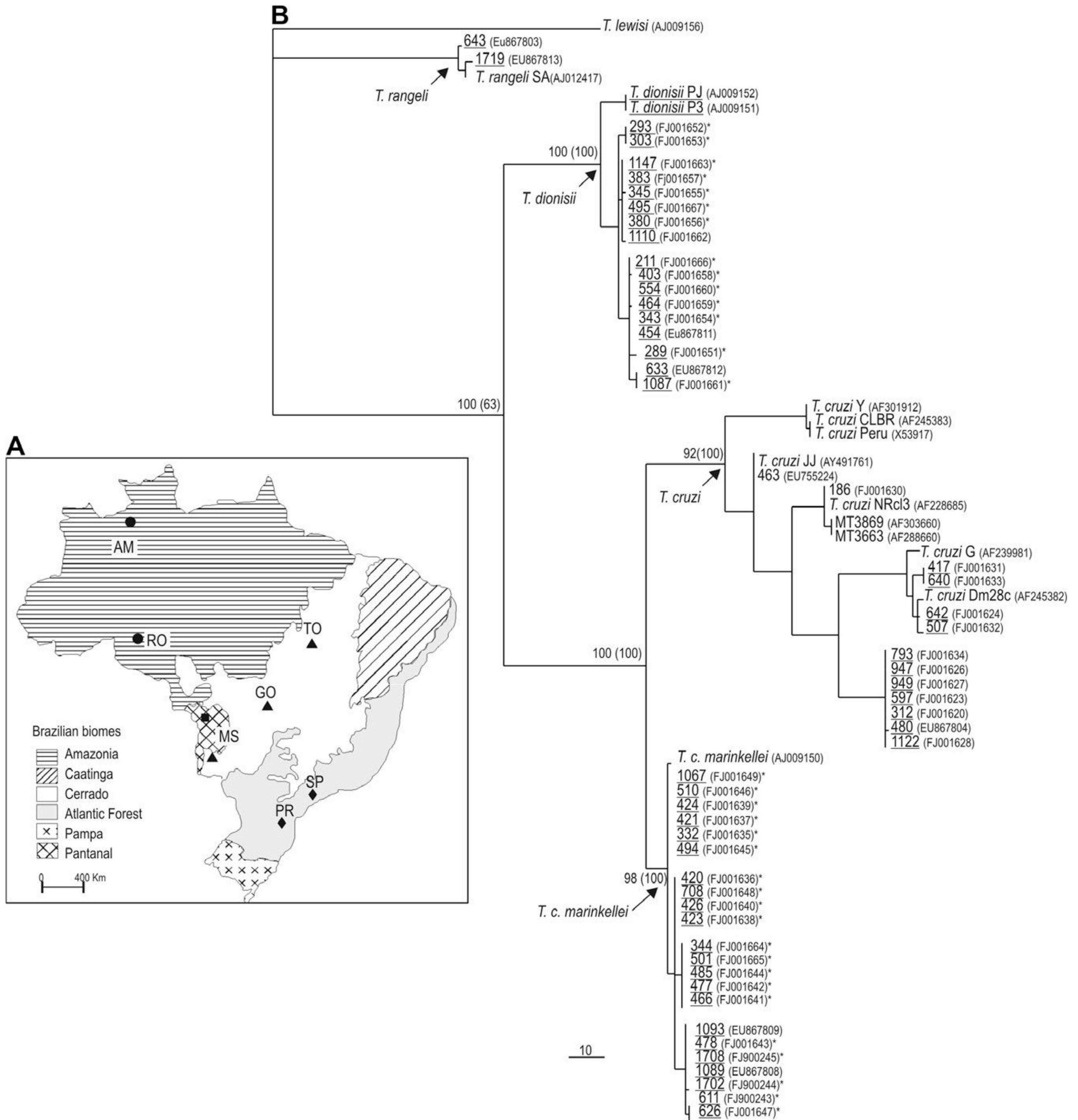

Fig. 1. Phylogeographical analysis of Brazilian isolates of trypanosomes from bats. (A) Geographical origin of bats captured in the following States of four Brazilian biomes: (●) States of Amazonas (AM) and Rondonia (RO) in Amazonia; (▲) States of Goias (GO), Tocantins (TO) and Mato Grosso do Sul (MS) in Cerrado; (■) State of Mato Grosso do Sul (MS) in the Pantanal; (♦) States of São Paulo (SP) and Paraná (PR) in the Atlantic Forest. B) Phylogeography of 55 bat trypanosomes (underlined) inferred by maximum likelihood (ML) and maximum parsimony (MP) trees using V7-V8 ssrDNA sequences (906 characters, 245 parsimony informative, −Ln = 2268.064452) of 68 isolates of the subgenus *Schizotrypanum* distributed in the clades *Trypanosoma cruzi*, *Trypanosoma cruzi marinkellei* and *Trypanosoma dionisii* using *Trypanosoma rangeli* and *Trypanosoma lewisi* as outgroups. Numbers at nodes are bootstrap values derived from 500 replicates, respectively, for ML/MP. The accession numbers of sequences in GenBank are in parenthesis on the trees, * indicate sequences determined in the present study.

last tree samples. Phylogenetic inferences carried out in this study were all performed according to our previous studies (Maia da Silva et al., 2007; Ferreira et al., 2008; Viola et al., 2009).

2.3. Infectivity analyses of bat trypanosomes to mice and triatomine bugs

Rhodnius prolixus, Rhodnius robustus (Amazonian genetic population II), *Triatoma infestans* and *Panstrongylus megistus* were used for behavioural analysis of selected bat trypanosomes in triatomine bugs. About 30 fifth instar nymphs of each species were inoculated intracoelomically with stationary cultures. The inoculated triatomines were fed on normal mice every 15 days, and ~10 triatomines of each species were examined for the presence of trypanosomes at 10, 20 and 30 days post inoculation as described previously (Maia da Silva et al., 2004; Marcili et al., 2009a).

BALB/c mice were inoculated (i.p.) with cultured metacyclic forms (~10^6/animal) of selected bat trypanosomes and examined

Table 1
Host species, preferential feeding habits, biome and geographical origins of Brazilian bats examined in this study.

Host species			Feeding habits[a]	Geographical origin[b] No. of individuals: examined positive HE[c]				
Family	Genus	Species		AF	AM	PA	CE	Total
Phyllostomidae	*Anoura*	sp.	I	1/1				1/1
		caudifer	I	6/0	1/0			7/0
		geoffroyi	I	7/0				7/0
	Artibeus	sp.	F	2/0	8/0	1/0	1/0	12/0
		fimbriatus	F	17/0	1/0			18/0
		lituratus	F	27/0	10/0		2/0	39/0
		obscurus	F	1/0	17/0			18/0
		planirostris	F		11/0	15/9	9/7	35/16
		cinereus	F		10/0		5/0	15/0
		gnomus	F		1/0			1/0
		glaucus	F		1/0			1/0
		hartii	F		1/0			1/0
	Carollia	sp.	F		21/1			21/1
		castanea	F		14/0			14/0
		perspicillata	F	42/5	134/12		10/2	186/19
	Chiroderma	*villosum*	I		3/0			3/0
	Choeroniscus	*minor*	N	1/0				1/0
	Chrotopterus	*auritus*	C	3/0	4/3			7/3
	Desmodus	*rotundus*	H	23/0	1/0	17/5	1/0	42/5
	Diphylla	*ecaudata*	H	2/2	1/0			3/2
	Glossophaga	*soricina*	N	12/1	31/0		4/0	47/1
	Histiotus	*velatus*	I	1/0				1/0
	Lichonycteris	*degener*	N			2/0		2/0
	Mimon	*bennetti*	I	4/0			1/0	5/0
	Platyrrhinus	*lineatus*	F	2/0	2/0	5/0	3/0	12/0
		helleri	F				1/0	1/0
	Phyllostomus	sp.	C		5/1			5/1
		discolor	F		5/3			5/3
		elongatus	F		9/1			9/1
		hastatus	C		3/0		8/8	11/8
	Pygoderma	*bilabiatum*	F	8/0				8/0
	Rhinophylla	*pumillo*	F		5/0		1/0	6/0
	Sturnira	*lilium*	I	82/2	1/0		5/1	88/3
		tildae	I	3/0				3/0
	Tonatia	*bidens*	I		3/3			3/3
	Lophostoma	*silvicolum*	I		3/2			3/2
	Trachops	*cirrhosus*	C		5/2			5/2
	Uroderma	sp.	F		1/0			1/0
		bilobatum	F		3/1		2/0	5/1
	Vampyressa	*pusilla*	F	1/0				1/0
Vespertilionidae	*Eptesicus*	sp.	I	2/0	1/0			3/0
		brasiliensis	I	3/2			1/1	4/3
	Micronycteris	sp.	I	2/0				2/0
	Myotis	sp.	I		1/0	28/2		29/2
		albescens	I	13/5		24/3		37/8
		levis	I	2/2				2/2
		nigricans	I	9/1	4/0	18/1	3/0	34/2
		ruber	I	3/1		3/0		6/1
Molossidae	*Eumops*	sp.	I			6/0		6/0
		perotis	I		3/0			3/0
	Molossops	*temminckii*	I				3/0	3/0
	Molossus	*molossus*	I		76/4	9/0		85/4
	Neoplatymops	*matogrossensis*	I		8/0			8/0
	Nyctinomops	*laticaudatus*	I		5/0			5/0
	Promops	sp.	I			1/1		1/1
	Tadarida	*brasiliensis*	I		6/2			6/2
		laticaudata	I		1/0			1/0
Emballonuridae	*Rhynchonycteris*	*naso*	I		3/0			3/0
Thyropteridae	*Thyroptera*	*tricolor*	I		1/1			1/1
	Saccopterix	*bilineata*	I				1/0	1/0
Noctilioridae	*Noctilio*	*albiventris*	I		6/0	66/12		72/12
		leporinus	P		13/0			13/0
Mormoopidae	*Pteronotus*	*parnelli*	I		65/25			65/25
Total	37	63		279/22	508/61	195/33	61/19	1043/135

[a] Preferential feeding habits of bats: I, insectivorous; F, frugivorous; N, nectarivorous; C, carnivorous; H, haematophagous; P, piscivorous.
[b] Biome of origin of bats: AF, Atlantic Forest; AM, Amazonia; PA, Pantanal; CE, Cerrado.
[c] Detection of trypanosomes by hemoculturing blood samples of bats.

Table 2
Isolates of *Trypanosoma cruzi marinkellei*, *Trypanosoma dionisii* and *Trypanosoma cruzi* from Brazilian bats characterized in this study.

Trypanosoma isolate TryCC[a]	Host origin		Geographic Origin		Biome[b]
	Family	Species			
T. c. marinkellei					
478[c]/611[c]/1089[c]/1093[c]	Phyllostomidae	*Artibeus planirostris*	Miranda	MS	PA
626[c]/627		*Artibeus planirostris*	Aquidauana	MS	PA/CE
1702[c]/1703/1705/1708[c]/1710/1713		*Artibeus planirostris*	Bonito	MS	PA/CE
344[c]		*Carollia perspicillata*	Monte Negro	RO	AM
466[c]/477[c]/485[c]/491/501[c]/508		*Carollia perspicillata*	Porto Velho	RO	AM
1721		*Carollia perspicillata*	Bonito	MS	PA/CE
420[c]/708[c]		*Chrotopterus auritus*	Barcelos	AM	AM
456		*Desmodus rotundus*	Miranda	MS	PA
426[c]		*Lophostoma silvicolum*	Barcelos	AM	AM
332[c]		*Phyllostomus hastatus*	Barcelos	AM	AM
510[c]		*Phyllostomus hastatus*	Aquidauana	MS	PA/CE
1701/1707/1714/1730		*Phyllostomus hastatus*	Bonito	MS	PA/CE
421[c]/494[c]/424[c]		*Phyllostomus discolor*	Barcelos	AM	AM
1067[c]		*Phyllostomus* sp.	–	TO	CE
423[c]		*Tonatia bidens*	Barcelos	AM	AM
419		*Trachops cirrhosus*	Barcelos	AM	AM
502		Trachops cirrhosus	Porto Velho	RO	AM
B7		*Phyllostomus discolor*	São Felipe	BA	AF
M1117		*Phyllostomus hastatus*	Abaetetuba	PA	AM
T. dionisii					
403[c]	Molossidae	*Molossus molossus*	Monte Negro	RO	AM
554[c]		*Promops* sp.	Miranda	MS	PA
464[c]	Noctilionidae	*Noctilio albiventris*	Miranda	MS	PA
383[c]	Phyllostomidae	*Anoura* sp.	São B. Campo	SP	AF
345[c]/380[c]		*Carollia perspicillata*	Monte Negro	RO	AM
495[c]		*Carollia perspicillata*	Porto Velho	RO	AM
1110		*Carollia perspicillata*	Piedade	SP	AF
1147[c]		*Carollia perspicillata*	Londrina	PR	AF
1700/1706		*Carollia perspicillata*	Bonito	MS	PA/CE
454[c]		*Desmodus rotundus*	Miranda	MS	PA
483/493		*Diphylla ecaudata*	Biritiba Mirim	SP	AF
633		*Sturnira lillium*	Miranda	MS	PA
1087[c]		*Sturnira lillium*	Campo Limpo	GO	CE
1314[c]		*Sturnira lillium*	Adrianópolis	PR	AF
343[c]		*Uroderma bilobatum*	Monte Negro	RO	AM
211[c]/289[c]	Vespertilionidae	*Eptesicus brasiliensis*	São Paulo	SP	AF
1059[c]		*Eptesicus brasiliensis*	–	TO	CE
293[c]		*Myotis nigricans*	São Paulo	SP	AF
309		*Myotis nigricans*	Miranda	MS	PA
300/303[c]		*Myotis albescens*	Miranda	MS	PA
P3		*Pipistrellus pipistrellus*	England	–	
PJ		*Pipistrellus pipistrellus*	Belgium	–	
T. cruzi					
507[c]/640/642	Phyllostomidae	*Carollia perspicillata*	Porto Velho	RO	AM
204	Vespertilionidae	*Myotis albescens*	São Paulo	SP	AF
1122		*Myotis albescens*	Juquitiba	SP	AF
793[c]/294		*Myotis levis*	São Paulo	SP	AF
499/597		*Myotis nigricans*	Miranda	MS	PA
947/949		*Myotis nigricans*	São Paulo	SP	AF
203		*Myotis ruber*	São Paulo	SP	AF
312/480		*Noctilio albiventris*	Miranda	MS	PA
417[c]	Thyropteridae	*Thyroptera tricolor*	Barcelos	AM	AM
T. rangeli					
643[c]	Phyllostomidae	*Platyrrhinus lineatus*	Miranda	MS	PA/CE
1719		*Artibeus planirostris*	Bonito	MS	PA/CE
Other bat trypanosomes					
T. sp bat (TryCC 60)	Pteropodidae	*Roussettus aegyptiacus*	Gabão–Africa	–	–
T. vespertilionis *P14*	Vespertilionidae	*Pipistrellus pipistrellus*	England	–	–

[a] Cultures of bat trypanosome isolates cryopreserved in the Trypanosomatid Culture Collection of the Department of Parasitology, University of São Paulo, São Paulo, Brazil. TryCC correspond to number codes of isolates cryopreserved in this collection.
[b] Brazilian biomes where bats were captured: AM, Amazonia; AF, Atlantic Forest; PA, Pantanal; CE, Cerrado; PA/CE, transition areas of the Pantanal and Cerrado.
[c] Isolates of bat trypanosomes from which sequences were determined in this study and deposited in GenBank.

by MH twice a week from 7 to 30 days p.i. and weekly from 30 to 60 days p.i. Blood samples from mice inoculated with bat trypanosomes were examined by HE as described previously (Marcili et al., 2009a). This work was approved by the Committee of Ethics of the Biomedical Institute of the University of São Paulo, permit N°. ICB017. During all research, mice were maintained and manipulated according to recommendations from this committee.

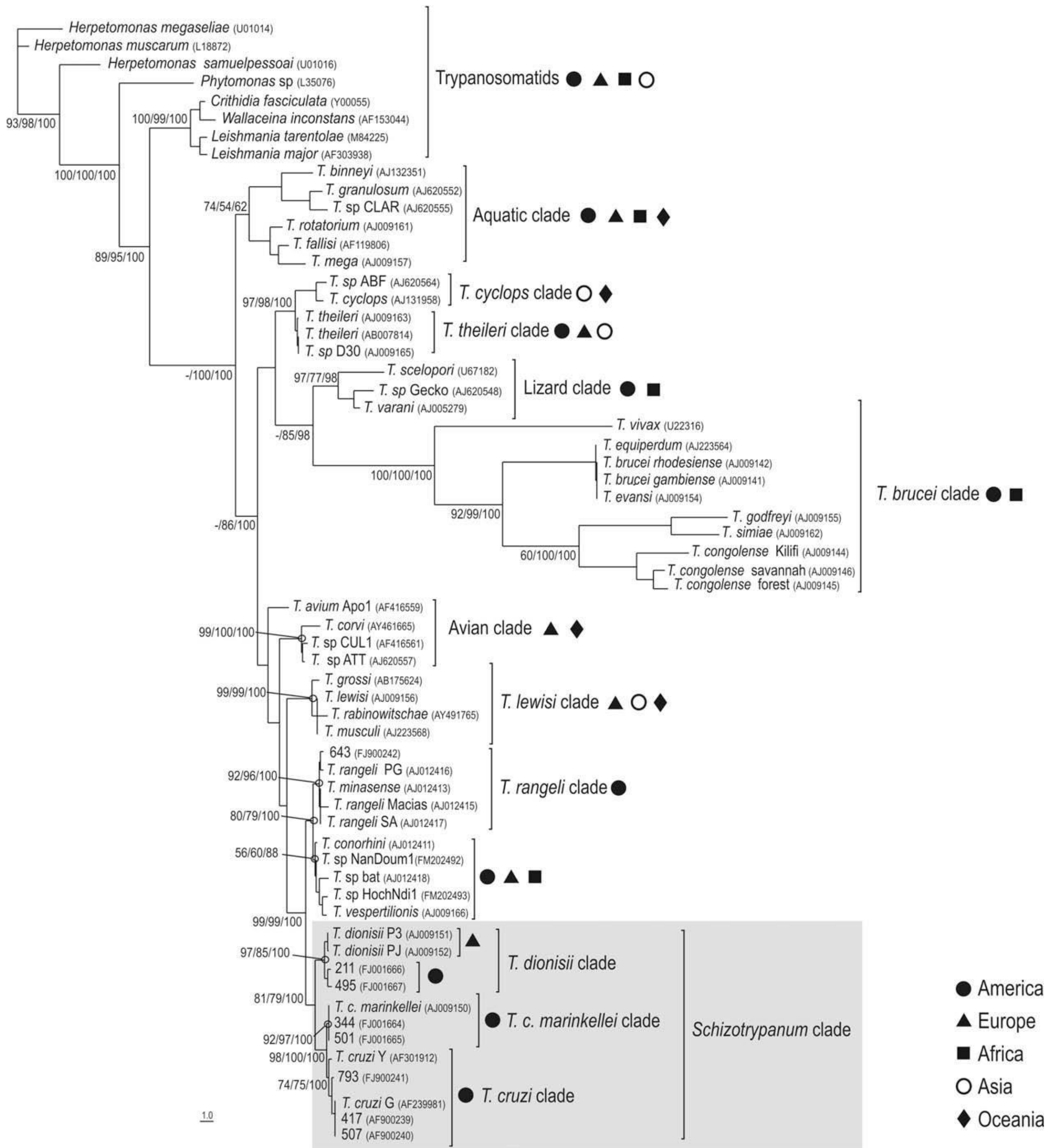

Fig. 2. Maximum likelihood phylogenetic trees of trypanosomes of the subgenus *Schizotrypanum* (grey box) based on ssrDNA (1816 characters, −Ln = 8428.724060). Trypanosomes from bats are underlined. Species of other subgenera of *Trypanosoma* were used as outgroups of *Schizotrypanum*. The geographic distribution of trypanosome species/isolates, as determined in this and in previous studies, clustered in the phylogenetic clades showed in this tree are: America (●), Europe (▲), Africa (■), Asia (○) and Oceania (♦). Numbers at nodes are the support values for the major branches (either the bootstrap or posterior probability) derived from 500 replicates respectively for maximum likelihood, maximum parsimony and Bayesian analyses. The accession numbers of sequences in GenBank are showed in parenthesis on the trees; ∗ indicate sequences determined in the present study.

3. Results

3.1. Isolation in culture and prevalence of bat trypanosomes from Brazilian biomes

HE of blood samples from 1043 specimens of bats, belonging to 63 species and seven families captured from 2002 to 2007, yielded a prevalence of 12.9% (Fig. 1A, Table 1). HE was a sensitive detection method, resulting in positive cultures even for some bat blood samples that were negative for trypanosomes using MH. MH was not always possible due to small blood sample volumes available from bats. However, approximately 20% of bats positive for MH did not generate positive HE, suggesting that, in addition to very low parasitaemias, trypanosomes differing in growth requirements were also present. Results indicated that a combination of MH and HE provides a more reliable estimate of prevalence, as shown for bats captured in the Pantanal (7.2% for MH and 20.3% by combination of MH and HE) and Rondonia, Western Amazonia (25% for MH and 35.5% for combined methods). Results from HE revealed variable overall prevalences for trypanosomes in the biomes

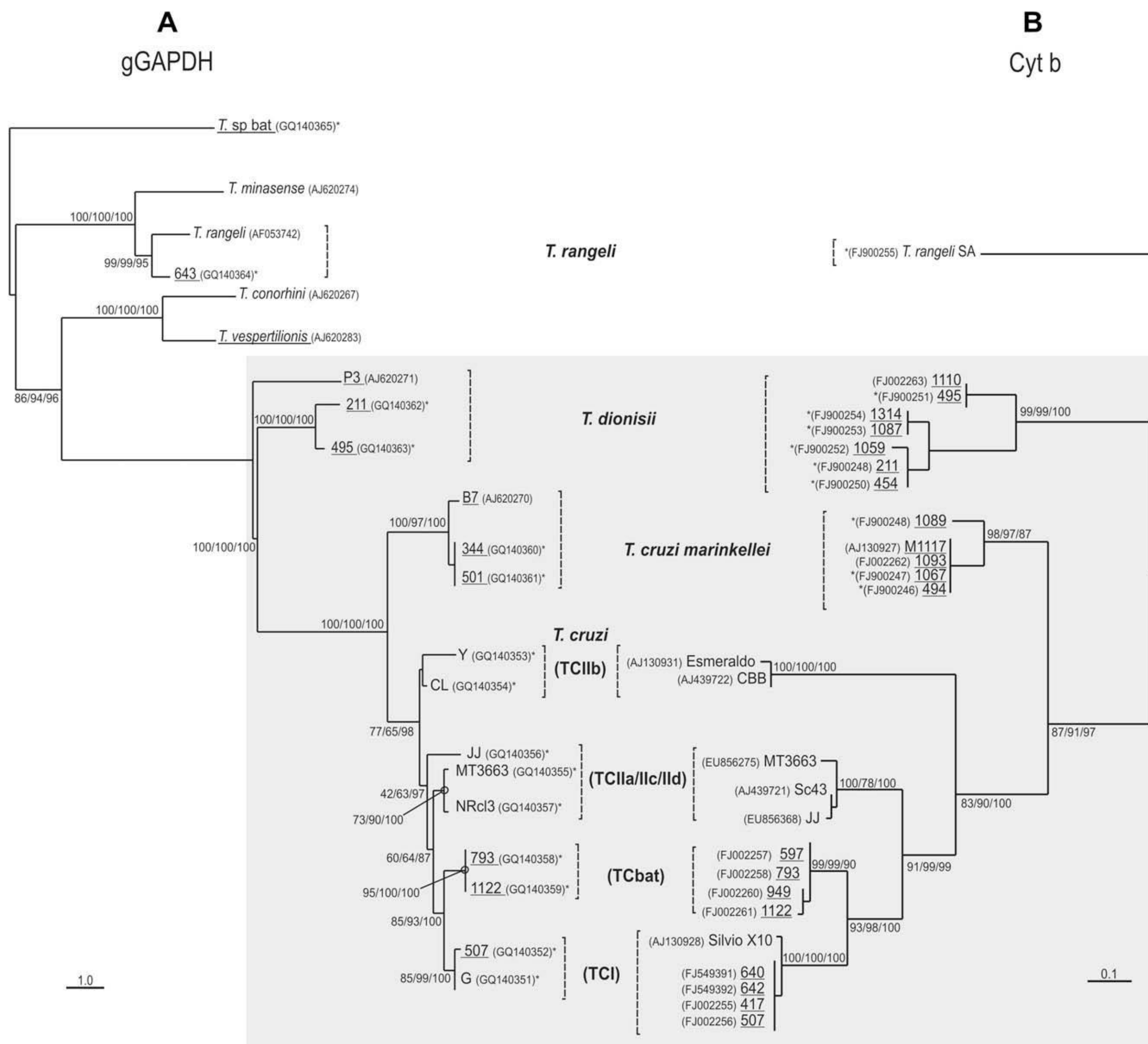

Fig. 3. Maximum likelihood phylogenetic trees of trypanosomes of the subgenus *Schizotrypanum* (grey box) based on glycosomal glyceraldehyde 3-phosphate dehydrogenase (853 characters, 166 parsimony informative/−Ln = 3127.747665) and Cyt *b* (490 characters, 129 parsimony informative/−Ln = 1719.922158) gene sequences. Trypanosomes from bats are underlined. Species of other *Trypanosoma* subgenera were used as outgroups. Numbers at nodes are the support values (either the bootstrap or posterior probability) derived from 500 replicates for maximum likelihood/maximum parsimony/Bayesian analyses. The accession numbers of sequences in GenBank are showed in parenthesis on the trees; ∗ indicate sequences determined in the present study.

investigated: 45.2% in Amazonia, 24.4% in the Pantanal, 16.3% in the Atlantic Forest, and 14.1% in Cerrado (Table 1). The prevalence of trypanosome-infected bats detected in this study by HE most likely reflects an underestimation due to low parasitaemias in bats, and the existence of species with different growth requirements. The highest prevalences were found in insectivorous bats from Amazonia and the Pantanal.

3.2. Growth and morphological analyses of bat trypanosomes of the subgenus Schizotrypanum

Analysis of the morphology of blood trypomastigotes of naturally infected bats revealed forms typical of the subgenus *Schizotrypanum* (Hoare, 1972; Marinkelle, 1976) in a small number of bat BSs. Morphological analyses allowed the separation of 77 cultures of isolates resembling *T. cruzi* (data not shown), which were designated *T.* cf. *cruzi*. In addition, we found 17 cultures that resembled species of the subgenus *Megatrypanum*, and two of *T. rangeli* of *Herpetosoma* (Maia da Silva et al., 2009). Cultures of *T. cruzi* were characterized by morphological, biological and molecular parameters (Marcili et al., 2009a).

3.3. Molecular identification of bat trypanosomes and phylogenies

The molecular identification of 50 morphologically identified *T.* cf. *cruzi* isolates of Brazilian bat trypanosomes selected to be representative of the host and geographical origins of isolates was performed by DNA barcoding based on the variable region (V7-V8) of ssrDNA, and phylogenetic relationships were inferred using ML and MP analyses (Fig. 1B, Table 2). The comparison of sequences from new bat isolates with those from reference species (GenBank) allowed us to classify the isolates into three species, including 11 isolates of *T. cruzi*, 22 of *T. c. marinkellei*, and 17 of *T. dionisii* trypanosomes. Sequencing of cloned amplified DNA disclosed several

mixed cultures of two or three species (data not shown), more often of *T. dionisii* with *T. c. marinkellei*, the species of higher prevalence in the biomes studied. Trypanosomes within the subgenus *Schizotrypanum* presented an overall 7.8% of V7-V8 ssrDNA sequence divergence. Despite polymorphisms within species, Brazilian *T. dionisii* trypanosomes were homogeneous (~0.47% divergence), and despite clustering with *T. dionisii* from Europe, they were separated by ~2.0% divergence. Isolates of *T. c. marinkellei* diverged only ~0.4% and were tightly clustered together with one isolate from northeast Brazil (Barnabé et al., 2003). *Trypanosoma cruzi* showed the highest intra-specific average divergence with ~4.6% separating bat isolates of genotypes TCI and TCbat (Marcili et al., 2009a).

Phylogenetic trees based on nuclear and mitochondrial gene sequences of selected bat trypanosomes representative of all clades disclosed using V7-V8 ssrDNA (Fig. 1B) were inferred using three alignments: (i) including whole sequences of ssrDNA of 13 isolates from bats and 49 from trypanosomes of other hosts (Fig. 2), (ii) containing gGAPDH sequences from 12 bat trypanosomes plus nine sequences from other species of trypanosomes (Fig. 3A), (iii) comprising Cyt *b* sequences from 20 bat isolates and seven from trypanosomes of other hosts (Fig. 3B). Phylogeny based on all genes demonstrated that isolates from Brazilian bats clustered (100% bootstrap) in a monophyletic assemblage that corresponded to the subgenus *Schizotrypanum* (internal divergence of ~7.3%, 3.7% and 9.3% for ssrDNA, gGAPDH and Cyt *b* sequences, respectively) (Figs. 2 and 3). In all analyses, independent of genes and analytical method, the clade *Schizotrypanum* was divided into the subclades *T. cruzi* (internal divergence of ~1.7%, 1.0% and 6.0% for ssrDNA, gGAPDH and Cyt *b*), *T. c. marinkellei* (~0.3%, 1.3% and 1.6%) and *T. dionisii* (~0.4%, 0.9% and 3.5%). Comparing ssrDNA, gGAPDH and Cyt *b* sequence divergences, *T. cruzi* was always closer to *T. c. marinkellei* (~0.3%, 2.6% and 10.0%) than to *T. dionisii* (~6.3%, 7.7% and 14.0%). The *T. c. marinkellei* clade was separated from the *T. dionisii* clade by ~4.8%, 7.4% and 13.0% divergence of these genes. The clade comprising Brazilian *T. dionisii* isolates was separated from European *T. dionisii* by ~1.8 and 5.4% sequences divergence of ssrDNA and gGAPDH sequences.

The closest relative to *Schizotrypanum* was the *T. rangeli* clade, although they were separated by large divergences (~19%, 14% and 15.5% for ssrDNA, gGAPDH and Cyt *b* sequences) and always clustered in a separated clade, which comprises trypanosomes infecting various mammals, including bats (Maia da Silva et al., 2007, 2009). The sequence of the isolate of *T. vespertilionis* from Europe included in this study, which is the only one available in GenBank, was clearly separated from the clade comprising all other *Schizotrypanum* species. "*Trypanosoma* sp. bat" from an African megabat (Stevens et al., 2001) was positioned as the outgroup for all other bat trypanosomes, which does not allow its inclusion in any established subgenus.

The concordance between phylogenetic relationships inferred using whole or partial ssrDNA, gGAPDH and Cyt *b* sequences (Figs. 1–3) supported a clonal populational structure and corroborated the applicability of V7-V8 rDNA for barcoding of *Schizotrypanum* trypanosomes. As shown previously for trypanosomes in general (Hamilton et al., 2004, 2007), phylogenetic analysis using a combined data set of ssrDNA and gGAPDH genes generated very similar phylogenetic trees (data not shown).

3.4. Growth and morphology of bat trypanosomes in LIT medium and cell cultures

Three selected bat trypanosomes from each of the species *T. cruzi*, *T. c. marinkellei* and *T. dionisii* were compared in cultures with or without mammalian cells. All LIT cultures from parasites cloned in agar plates displayed only epimastigotes and metacyclic trypomastigotes typical of *Schizotrypanum* (data not shown) (Marinkelle, 1976; Molyneux, 1991). Isolates from the three species developed within mammalian culture cells similar to *T. cruzi* (Marcili et al., 2009a). They invaded LLC-MK_2 (from monkey) and HeLa (human) cells and multiplied in the cytoplasm as amastigotes that differentiated to trypomastigotes, which leave the cells of origin and infect new cells (data not shown).

3.5. Behaviour of bat trypanosomes in experimental infections of mice and triatomines

Neither metacyclic trypomastigote forms nor trypomastigotes from cell cultures of *T. c. marinkellei* and *T. dionisii* infected mice and triatomine bugs. In contrast, both forms of bat *T. cruzi* assigned to TCI and TCbat genotypes infected mice (Marcili et al., 2009a). The behaviours of inoculated triatomines differed among the three species. Brazilian *T. dionisii* trypanosomes were incapable of surviving in triatomines for more than 10 days. Further studies are required to demonstrate that *T. dionisii* from Brazil possess the ability to develop and be transmitted by cimicid bugs, as shown by European *T. dionisii* (Molyneux, 1991). *Trypanosoma c. marinkellei* only transiently maintained an infection in the triatomine species employed in this study, with a few flagellates observed in the gut after ~20 days without multiplicative forms, and the absence of parasites after ~30 days. Bat *T. cruzi* isolates of TCI developed in triatomine bugs, whereas the behaviour of TCbat isolates was similar to that of *T. c. marinkellei* (Marcili et al., 2009a).

4. Discussion

Knowledge of the species of trypanosomes infecting bats, and their geographical distribution, genetic diversity and phylogeographical patterns is insufficient to generate any hypotheses about the evolutionary history of bat trypanosomes in New and Old Worlds. Prior to this and our recent studies of Brazilian bat trypanosomes assigned to *T. rangeli* (Maia da Silva et al., 2009) and *T. cruzi* (Marcili et al., 2009a), only a small number of bat trypanosomes had been included in phylogenetic trees: *T. dionisii* (two European isolates), *T. c marinkellei* (one isolate from Brazil), *T. vespertilionis* (one European isolate) and the unclassified "*T.* sp bat" from an African megabat (Stevens et al., 2001). *Trypanosoma c. marinkellei* and *T. dionisii* were clearly placed in the subgenus *Schizotrypanum* together with *T. cruzi.* In contrast, the position of *T. vespertilionis* indicated that this species either does not belong to *Schizotrypanum* or that the isolate examined was erroneously identified (Stevens et al., 1999, 2001). Comparison of 17 bat trypanosomes by zymodeme and randomly amplified polymorphic DNA (RAPD) patterns revealed homogeneity within European isolates of *T. dionisii* and *T. vespertilionis*, whereas Brazilian isolates of *T. c. marinkellei* were more heterogeneous (Barnabé et al., 2003). Thus the validity of the described species and their distribution in the New and Old Worlds are far from complete. Addressing these questions requires phylogenetic analyses of a large number of trypanosomes from a broad range of bat species and geographic origins.

In the present study, comprising a large survey for bat trypanosomes in four Brazilian biomes, the majority of cultures were morphologically assigned to *T. cruzi*. Bat trypanosomes were identified as *T. cruzi, T. c. marinkellei* and *T. dionisii* based on V7-V8 ssrDNA. The prevalence of *T. c. marinkellei* was higher (49.3%) than that of *T. dionisii* (32.4%) and *T. cruzi* (18.2%). The results strongly supported the suitability of these sequences for both barcoding and analysis of phylogenetic relationships among *Schizotrypanum*, as previously demonstrated for other clades of trypanosomes from mammals (Maia da Silva et al., 2004; Cortez et al., 2006; Rodrigues

et al., 2006), anurans (Ferreira et al., 2007, 2008), snakes (Viola et al., 2008) and crocodilians (Viola et al., 2009).

Phylogenetic relationships inferred using ssrRNA, gGAPDH and Cyt *b* generated trees with similar topologies and were also congruent with results based on V7-V8 ssrDNA sequences. Three major clades of bat trypanosomes within the subgenus *Schizotrypanum* were strongly supported in all phylogenies regardless of data sets and analytical methods in which the clade containing *T. cruzi* was closer to that containing *T. c. marinkellei* than to *T. dionisii.* No other species of *Schizotrypanum* besides these three species were isolated from bats in this study, suggesting that other species of this subgenus are rare in Brazil and/or difficult to cultivate. Closest to the *T. cruzi* clade was *T. rangeli*, another American trypanosome of wild mammals also transmitted by triatomine bugs but rarely found in bats. Only two cultures of *T. rangeli* from bats have been confirmed using morphological, biological and molecular parameters (Maia da Silva et al., 2009).

Phylogeographical, ecological and biological analyses of isolates classified as *Schizotrypanum* disclosed some patterns of association with bat species, biomes and geographic origin, as well as with their behaviour in culture, triatomine bugs and mice. Our results show overlapping geographic areas of the three *Schizotrypanum* species in the Pantanal/Cerrado and Amazonia whereas *T. c. marinkellei* was not found in bats from the Atlantic Forest region. All *T. c. marinkellei* isolates were from phyllostomid species (insectivorous, frugivorous, carnivorous and haematophagous bats), corroborating a strong association with this bat family, as suggested previously (Marinkelle, 1976). However, bats of this family were also infected by *T. cruzi* and *T. dionisii* as shown in this and other studies (Funayama and Barreto, 1973; Fabián, 1991; Cavazzana et al., 2003; Lisboa et al., 2008; Marcili et al., 2009a). The high prevalence of *T. c. marinkellei* (49.3%) may be explained by the abundance of phyllostomid bats, whereas its distribution may be determined by that of its triatomine vector, *Cavernicola pillosa*, which shares caves, holes in trees and palm leafs with bats. Nevertheless, scarcity of *Cavernicola* colonies in contrast to high prevalence of *T. c. marinkellei* suggests that other vectors may also transmit this species among bats. *Trypanosoma c. marinkellei* has previously been found in bats from Colombia, Venezuela and Northeast Brazil (Marinkelle, 1976, 1982a,b; Barnabé et al., 2003). In the present study, we found this species in bats captured in north and central regions of Brazil.

Isolates of *T. cruzi* from Pantanal/Cerrado and the Atlantic Forest areas were mainly from insectivorous bats of the families Vespertilionidae and Phyllostomidae, while a few isolates came from frugivorous and insectivorous bats from Amazonia. Infectivity to mice allowed us to classify these isolates as *T. cruzi*, and phylogenetic analyses disclosed two genotypes, TCI and TCbat thus far restricted to bats (Marcili et al., 2009a). Several studies have indicated that bats of varied feeding and roosting habits can be infected by distinct genotypes of *T. cruzi* (Funayama and Barreto, 1970, 1973; Fabián, 1991; Cavazzana et al., 2003; Lisboa et al., 2008; Añez et al., 2009; Marcili et al., 2009a). However, the prevalence of *T. cruzi*-infected bats (18.2%) was unexpectedly low compared with those of other *Schizotrypanum* spp., considering that these species are easy to cultivate and that *T. cruzi* is common in other wild mammals from all biomes investigated (Marcili et al., 2009a,b). This species can be transmitted by several triatomine species and bats are highly susceptible to *T. cruzi* infection by bite and ingestion of infected triatomines (Thomas et al., 2007) as well as by congenital transmission (Añez et al., 2009).

Results from this study demonstrated that bat trypanosomes clustering together with European isolates of *T. dionisii* have the widest distribution, occurring from northern to southern Brazil in all biomes investigated. Due to the genetic distances separating Brazilian and European isolates with three genes we examined, we considered the isolates from Brazil as *T.* cf. *dionisii*. These trypanosomes were found to infect 12 bat species from four families, including insectivorous, frugivorous and haematophagous species with roosts in tree holes, palm leaves, caverns and even house roofs. The worldwide distribution and high prevalence of *T. dionisii* trypanosomes are probably due to the lack of preferred bat hosts and transmission by cimicids, which are abundant and cosmopolitan ectoparasites of bats (Marinkelle, 1976; Molyneux, 1991). Lack of previous descriptions of *T. dionisii* trypanosomes in Brazil together with the absence in this study of *T. vespertilionis* or any closely related trypanosome, could be due to the controversial taxonomic status of bat trypanosomes (Molyneux, 1991). The taxonomic status of both Brazilian *T. dionisii* and *T. vespertilionis* still needs to be confirmed. Phylogenetic analyses of isolates from Europe, Africa and the Americas are required to understand geographical clustering within the *T. dionisii* clade. Supposition that *T. dionisii* is restricted to the Old World and that Brazilian *T. dionisii* trypanosomes corresponds to a separate taxon needs to be investigated by further biological characterization, transmission by cimicids and presence of pseudocysts in the tissues of bats.

We have demonstrated the existence in Brazil of *T. dionisii* trypanosomes, which are more closely related to European isolates of this species than to *T. rangeli*, another trypanosome found in neotropical bats. The phylogeographical patterns and genetic distances of bat trypanosomes provided by this study are better explained by the ability of bats to disperse over large areas, crossing oceans and continents, rather than by vicariance events. The reconstruction of the evolutionary histories of parasites has been linked to the comparable histories of their hosts (Page and Charleston, 1998; Paterson and Banks, 2001; Nieberding and Olivieri, 2007; Nieberding et al., 2008). Phylogenetic and biogeographic analyses have suggested that Africa is the centre of origin of modern-day bat families, with a Southern Hemisphere origin in the Cretaceous ~65 million years ago (mya). Two scenarios could account for the dispersal of bats from Africa in the Eocene ~45 mya: northwards dispersal to Eurasia and then via Beringia into America or transatlantic dispersal from Africa to America through island hopping or direct flight (Eick et al., 2005; Teeling et al., 2005). *Trypanosoma antiquus*, described from faecal droplets of the extinct *Triatoma dominicana* found in Dominican amber containing hair from bats, revealed metatrypanosomes similar to those of *T. cruzi*, supporting the hypothesis for bats as potential ancient hosts of trypanosomes transmitted by triatomines (Poinar, 2005). The estimates of divergence time based on nuclear and mitochondrial genes suggested that *T. cruzi* may have evolved from bat-restricted trypanosomes 10–20 mya (Machado and Ayala, 2001). Limited divergence among *Schizotrypanum* spp. is compatible with recent diversification, and their present day distribution is equally consistent with hypotheses that *T. cruzi* evolved from a bat-restricted trypanosome or vice versa (Stevens et al., 1999, 2001; Barnabé et al., 2003).

Patterns of association between bats and trypanosomes indicate a complex evolutionary history. Dispersion, vicariance and codivergence events, species diversity and roosting behaviour of bats, as well as "host switching" mediated by vectors and facilitated by intimate interactions with bats, could all have contributed to both the biological and ecological characteristics, as well as the phylogeographical patterns of bat trypanosomes. Patterns of association between host and parasite resulting from a combination of distinct evolutionary events have been suggested for other host-trypanosome phylogenetic histories. Phylogeographical and ecological analyses have suggested that preferential hosts and their ecotopes may be determining factors of trypanosome species and lineages divergence in sylvatic habitats. Nevertheless, association of trypanosomes with both vertebrate and invertebrate (vectors) hosts is, in general, far from absolute (Maia da Silva et al., 2007;

Hamilton et al., 2007; Ferreira et al., 2007, 2008; Viola et al., 2008, 2009; Marcili et al., 2009a,b).

To our knowledge, this is the first study characterizing a large number of *T. cruzi*, *T. c. marinkellei* and *T. dionisii* isolates by comparing molecular and biogeographical data. Here, for the first time, the same bat isolates previously classified by morphological methods were placed in phylogenetic trees and associated with their behaviour in culture, in mice and in triatomine bugs. In addition, clades of bat trypanosomes were evaluated with respect to bat species, feeding and roosting habits, and geographic origin.

Comparative analyses performed in this study showed that the morphology of blood and culture forms, and behaviour in cell culture should be considered as the preliminary parameters to assign trypanosomes to the subgenus *Schizotrypanum*. The "DNA barcoding" approach, validated in this study, distinguished all trypanosome species so far examined and can also reveal genotypes within species, dispensing the need to compare new isolates side by side with panels of reference cultures or DNA samples. Barcoding based on sequences of V7-V8 ssrDNA can assist species identification in surveys of bat trypanosomes, as well as screening species, isolates and genotypes to be included in phylogenetic studies. A broad phylogeographical analysis, including bat trypanosomes from Africa, Europe and America, is still required to understand the evolutionary history of *Schizotrypanum* and bat trypanosomes in general.

Acknowledgements

We are grateful to several students for their inestimable help with fieldwork. We are grateful to Dr. Valdir Tadei (in memoriam) and Caroline Cotrin Aires for help in the capture and identification of bats. Work in Rondonia was done in the ICB5-USP and UNIR. This work was supported by CNPq (UNIVERSAL) and FAPESP (PRONEX) to M.M.G. Teixeira, and Fiocruz and CNPq (UNIVERSAL) to J.R. Coura. F. Maia da Silva and L.B. Viola are postdoctoral fellows sponsored by CAPES (PRODOC) and CNPq, respectively. M. Cavazzana Jr., A. Marcili, L. Lima and A.C.V. Junqueira were recipients of scholarships from CNPq.

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A new genotype of *Trypanosoma cruzi* associated with bats evidenced by phylogenetic analyses using SSU rDNA, cytochrome b and Histone H2B genes and genotyping based on ITS1 rDNA

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(*Received 12 September 2008; revised 17 December 2008 and 30 January 2009; accepted 2 February 2009*)

SUMMARY

We characterized 15 *Trypanosoma cruzi* isolates from bats captured in the Amazon, Central and Southeast Brazilian regions. Phylogenetic relationships among *T. cruzi* lineages using SSU rDNA, cytochrome b, and Histone H2B genes positioned all Amazonian isolates into *T. cruzi* I (TCI). However, bat isolates from the other regions, which had been genotyped as *T. cruzi* II (TC II) by the traditional genotyping method based on mini-exon gene employed in this study, were not nested within any of the previously defined TCII sublineages, constituting a new genotype designated as TCbat. Phylogenetic analyses demonstrated that TCbat indeed belongs to *T. cruzi* and not to other closely related bat trypanosomes of the subgenus *Schizotrypanum*, and that although separated by large genetic distances TCbat is closest to lineage TCI. A genotyping method targeting ITS1 rDNA distinguished TCbat from established *T. cruzi* lineages, and from other *Schizotrypanum* species. In experimentally infected mice, TCbat lacked virulence and yielded low parasitaemias. Isolates of TCbat presented distinctive morphological features and behaviour in triatomines. To date, TCbat genotype was found only in bats from anthropic environments of Central and Southeast Brazil. Our findings indicate that the complexity of *T. cruzi* is larger than currently known, and confirmed bats as important reservoirs and potential source of *T. cruzi* infections to humans.

Key words: *Trypanosoma cruzi* lineages, Chagas disease, Chiroptera, genotyping, phylogeny, evolution, bat parasites, SSU rDNA, cytochrome b, Histone H2B.

INTRODUCTION

Trypanosoma cruzi is the only generalist species of the subgenus *Schizotrypanum;* all other species of this subgenus are restricted to Chiroptera. *T. cruzi* is transmitted by triatomine insects and is the agent of human Chagas disease, a major public health problem of the American continent. *Schizotrypanum* trypanosomes restricted to bats may occur exclusively in the Americas (*T. c. marinkellei*), or they may be widespread in the New and Old Worlds. Brazilian bats are commonly infected by *Schizotrypanum* spp., and the bat-restricted species are more prevalent than *T. cruzi* (Marinkelle, 1976; Pinto and da Costa Bento, 1986; Molyneux, 1991; Fabián, 1991; Cavazzana *et al*. 2003; Maia da Silva *et al*. 2009).

Different populations of *T. cruzi* circulate in enzootic cycles from the southern half of North America to southern South America, infecting species of virtually all mammalian orders (Gaunt and Miles, 2000). *T. cruzi* comprises highly phenotypic and genotypic heterogeneous populations classified as *T. cruzi* I (TCI) and *T. cruzi* II (TCIIa–e) lineages, which have been defined based on zymodemes, RAPD, ribosomal, mini-exon, and cytochrome b gene markers (Miles *et al*. 1981; Souto *et al*. 1996; Brisse *et al*. 2001; Marcili *et al*. 2008). In the southern cone of South America, isolates from

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Parasitology, Page 1 of 15. © 2009 Cambridge University Press
doi:10.1017/S0031182009005861 Printed in the United Kingdom

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humans and vectors of domestic and peridomestic transmission cycles are predominantly of lineages TCIIb and TCIId/e. TCI has been reported in the sylvatic cycle throughout Latin America, and predominantly infects humans in endemic areas northwest of the Amazon basin. TCIIa has been sporadically isolated from humans and occurs mainly in the Brazilian Amazonia. TCIIc is widespread in South America and also is mostly sylvatic and sporadically found in humans (Miles *et al.* 1981; Brisse *et al.* 2003; Yeo *et al.* 2005; Martins *et al.* 2008; Maia da Silva *et al.* 2008; Marcili *et al.* 2009).

Ecobiological and phylogenetic analyses have suggested that ecotopes and preferential mammalian hosts and vectors may be determining factors of *T. cruzi* lineages in sylvatic cycles. Although lineage association with both mammals and vectors is far from absolute some relevant correlations have been observed. TCI is strongly associated with opossums of *Didelphis* and with vectors of the genus *Rhodnius*. Nevertheless, isolates of this lineage also infect other didelphids, wild primates, rodents and carnivores, and can be found in other genera of triatomines. TCIIc is associated mainly with armadillos and few other terrestrial mammals, and is transmitted by triatomines of terrestrial ecotopes. Natural hosts and ecotopes of TCIIa are not clearly resolved, with recent reports of this lineage in wild primates and in *Rhodnius* spp. from the Brazilian Amazonia. TCIIa has been also reported in racoons and dogs in North America (Miles *et al* 1981; Gaunt and Miles, 2000; Yeo *et al.* 2005; Roellig *et al.* 2008; Marcili *et al.* 2009).

Several studies have reported Brazilian bats infected with *T. cruzi*, from the Amazonia rainforest to urban areas and including roofs of human dwellings in Central, Northeast and Southeast Brazil (Funayama and Barretto, 1970*a*,*b*, 1973; Barretto *et al.* 1974; Fabián, 1991; Cavazzana *et al.* 2003; Lisboa *et al.* 2008; Maia da Silva *et al.* 2009). Although the genetic diversity of *T. cruzi* isolates from bats is virtually unknown, recent studies showed that they can be infected by TCI, TCII and Z3 isolates (Lisboa *et al.* 2008; Maia da Silva *et al.* 2009; Añez *et al.* 2009). Furthermore, the phylogenetic relationships between *T. cruzi* isolates from bats and those from other mammals have not yet been addressed.

Several mammalian and triatomine species sustain domestic and sylvatic transmission cycles of *T. cruzi*, while domestic (dogs and cats) and peridomestic (opossums and rodents) animals are responsible for the interaction between these two cycles (Yeo *et al.* 2005; Gürtler *et al.* 2007; Marcili *et al.* 2009). Although poorly investigated, bats may play an important role as a risk factor for human Chagas disease. Bats harbouring *T. cruzi* have been observed in various sylvatic niches, as well as roosting in buildings, human dwelling lofts, and peridomiciliary environments, where they can attract triatomines from nearby ecotopes and serve as a source of infected blood for these vectors. Precipitin tests and experimental studies confirmed that triatomines feed on bats, which can be infected through contamination with feces and by ingestion of *T. cruzi*-infected bugs (Barreto *et al.* 1974; Thomas *et al.* 2007). It was recently reported that bats can be infected with *T. cruzi* by congenital transmission (Añez *et al.* 2009).

Identification of *T. cruzi* from bats requires careful analysis because *Schizotrypanum* species are morphologically indistinguishable and generically named as *T. cruzi*-like. In addition, more than one species of trypanosome can infect a given bat species, and mixed infections are common. Methods employed for distinguishing species of *Schizotrypanum* such as zymodemes (Baker *et al.* 1978; Barnabé *et al.* 2003) and restriction analysis of kDNA (Teixeira *et al.* 1993; Steindel *et al.* 1998) are time-consuming, require a large number of parasites, and cannot detect mixed infections. Moreover, these methods have not been evaluated for *T. cruzi* isolates from bats. Due to difficulties in the identification of *Schizotrypanum* species using methods employed for the diagnosis of *T. cruzi*, there are few unquestionable reports of *T. cruzi* in bats. *T. cruzi* can be convincingly confirmed by the ability to infect mice, a feature shared by all *T. cruzi* lineages but lacked by all *T. cruzi*-like trypanosomes (Funayama and Barretto, 1970*a*,*b*, 1973; Fabian, 1991; Maia da Silva *et al.* 2009).

A survey of trypanosomes infecting Brazilian bats disclosed several isolates of the subgenus *Schizotrypanum*. Analysis of SSU rDNA sequences from these isolates allowed separation of *T. cruzi* from other trypanosomes found infecting Brazilian bats: *T. c. marinkellei*, *T. dionisii*-like and *T. rangeli* (Cavazzana *et al.* 2003; Maia da Silva *et al.* 2009). In this study, we gathered a multigene data set from *T. cruzi* isolates from Brazilian bats to infer phylogenetic relationships among isolates from bats and other hosts representative of established lineages of *T. cruzi*. In addition, behavioural features of bat isolates were evaluated in culture and in experimentally infected mice and triatomines.

MATERIALS AND METHODS

Study areas and selection of T. cruzi *isolates*

T. cruzi isolates characterized in this study are from bats captured in the Brazilian States of Amazonia (North region, Amazonia), Mato Grosso do Sul (Center region, Cerrado/Pantanal) and São Paulo (Southeast region, the Atlantic Forest) (Fig. 1). Bats were captured using appropriated nets, anaesthetized and manipulated for blood-sample collection

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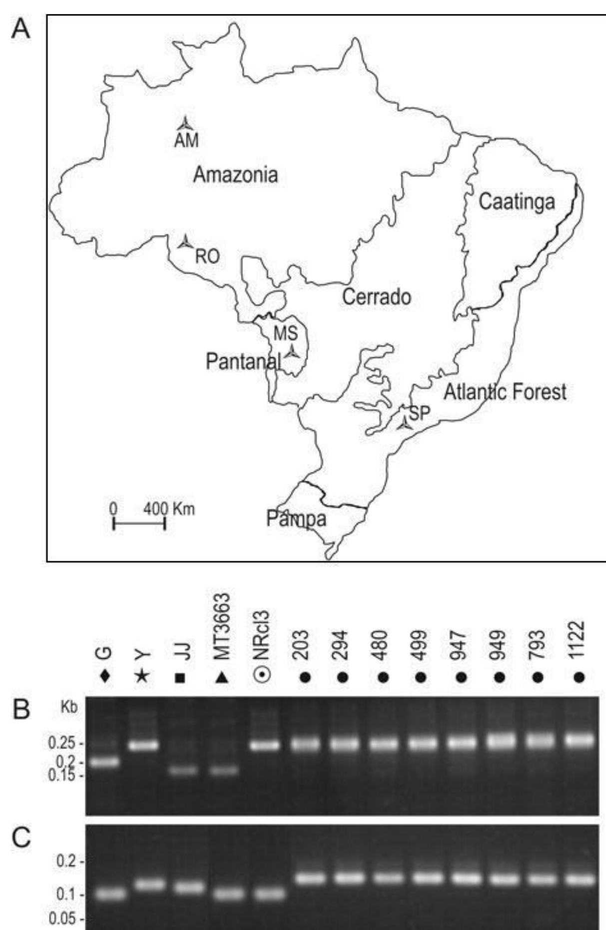

Fig. 1. (A) Geographical origin (⅄) of isolates of *Trypanosoma cruzi* from bats captured in the following States of different Brazilian biomes: Amazonas (AM) and Rondonia (RO) in Amazonia biome; Mato Grosso do Sul (MS) in the Pantanal/Cerrado; and São Paulo (SP) in the Atlantic Forest. DNA profiles generated by genotyping of isolates of *T. cruzi* using PCR assays based on mini-exon (B) and ribosomal (C) markers. Controls were performed using DNA from reference strains/isolates of *T. cruzi* lineages: TCI, G (◆); TCIIb, Y (★); TCIIa, JJ (■); TCIIc, MT3663 (▲); TCIId, NRcl3 (⊙) and TCbat (●). Isolates of TCbat: TryCC 203-1112.

according to permits of the IBAMA (Instituto Brasileiro do Meio Ambiente). Trypanosomes were isolated by haemoculture (HE), and purified cultures of *T. cruzi* from HE mixed with other *Schizotrypanum* spp. were obtained by HE of experimentally infected mice, approximately 30 days after inoculation of mixed cultures (Maia da Silva *et al.* 2004*a*). Isolates were cryopreserved in the Trypanosomatid Culture Collection of the Department of Parasitology, University of São Paulo. Brazilian *T. cruzi* isolates included in this study were from the following Brazilian States: PA, Pará; AC, Acre; AM, Amazonas; SP, São Paulo; BA, Bahia; RO, Rondônia; MG, Minas Gerais; RN, Rio Grande do Norte; MS, Mato Grosso do Sul; GO, Goiás.

Molecular diagnosis and genotyping of T. cruzi *isolates*

DNA from cultured trypanosomes was extracted using the traditional phenol/chloroform method. Diagnosis of *T. cruzi* isolates was done by PCR based on kDNA sequences and SSU rDNA sequence analysis as before (Maia da Silva *et al.* 2008, 2009). Genotyping of *T. cruzi* was done using PCRs based on mini-exon (Fernandes *et al.* 2001) and LSU 24Sα-rRNA (Souto *et al.* 1996) gene sequences. Reference strains of major *T. cruzi* lineages were used as controls: TCI (G), TCIIa (JJ), TCIIb (Y), TCIIc (MT3663) and TCIId (NRcl3).

PCR amplification, sequencing and data analysis of SSU rDNA, cytochrome b and Histone H2B genes

PCR amplification of a 900 bp DNA fragment corresponding to partial sequence of SSU rDNA (V7–V8 region) was performed using primers and PCR reactions previously described (Maia da Silva *et al.* 2004*b*). Amplification of 450 bp sequences of Histone H2B (H2B) gene was performed as described by Sturm *et al.* (2003). Sequences of mitochondrial cytochrome b (Cyb) were amplified (500 bp) using primers described by Brisse *et al.* (2003). New sequences from nuclear SSU rDNA (38 sequences), mitochondrial (Cyb) (33 sequences), and H2B (13 sequences) genes determined in this study were aligned with corresponding sequences from reference strains of *T. cruzi* and other bat trypanosomes from GenBank (Table 1). Alignments were made using ClustalW and manually refined. Phylogenetic inferences were done by parsimony (P) (PAUP*4.0b10, Swofford, 2002) and maximum likelihood (ML) (RAxML, Stamatakis, 2006), with bootstrap analyses performed with 100 replicates, as previously described (Rodrigues *et al.* 2006; Ferreira *et al.* 2008).

PCR-RFLP analysis of ITS1 rDNA from T. cruzi *and other* Schizotrypanum *species*

The primers and PCR conditions employed for amplification of ITS1 rDNA have been described previously (Maia da Silva *et al.* 2004*b*; Rodrigues *et al.* 2006). Amplified ITS1 rDNA was digested with several restriction enzymes. The enzyme *Bsh* 1236 was selected to standardize a PCR-RFLP assay able to separate lineages of *T. cruzi* and to distinguish *T. cruzi* from *T. c. marinkellei* and *T. dionisii*. Length and restriction profiles of amplified ITS1 rDNA were analysed by electrophoresis in 2·5 % agarose gels stained with ethidium bromide.

RAPD fingerprinting and karyotyping

RAPD profiles from *T. cruzi* isolates were assessed using 5 decameric oligonucleotide primers to amplify

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Table 1. *Trypanosoma cruzi* isolates, host and geographical origin, lineages and sequences of SSU rDNA, cytochrome b and Histone H2B genes employed in the phylogenetic analyses performed in this study

Trypanosome					GenBank Accession number[d] Sequences		
TryCC[a]	Isolate[b]	Host	Geographical origin	Lineage[c]	SSU rRNA	CytB	H2B
T. cruzi							
		Bats					
417	M2542	*Thyroptera tricolor*	AM/BR	TCI	FJ001631	FJ002255	FJ183404
507	MO115	*Carollia perspicillata*	RO/BR	TCI	FJ001632	FJ002256	
640	MO507	*Carollia perspicillata*	RO/BR	TCI	FJ001633	FJ549391	
642	MO92	*Carollia perspicillata*	RO/BR	TCI	FJ001624	FJ549392	
203	248	*Myotis ruber*	SP/BR	TCbat	FJ001617	FJ002253	FJ002264
204	519	*Myotis albescens*	SP/BR	TCbat	FJ001618		
294	998	*Myotis levis*	SP/BR	TCbat	FJ001619	FJ002254	
312	1296	*Noctilio albiventris*	MS/BR	TCbat	FJ001620		
480	PaMo122	*Noctilio albiventris*	MS/BR	TCbat	EU867804		
499	1336	*Myotis nigricans*	MS/BR	TCbat	FJ001622		FJ002265
597	1361	*Myotis nigricans*	MS/BR	TCbat	FJ001623	FJ002257	FJ002266
793	MO294	*Myotis levis*	SP/BR	TCbat	FJ001634	FJ002258	FJ002267
947	3681	*Myotis nigricans*	SP/BR	TCbat	FJ001626	FJ002259	
949	3679	*Myotis nigricans*	SP/BR	TCbat	FJ001627	FJ002260	FJ002268
1122	1122	*Myotis albescens*	SP/BR	TCbat	FJ001628	FJ002261	FJ002268
		Humans					
971	DRS	*Homo sapiens*	AP/BR	TCI	FJ549378	FJ599394	
978	XE6863/3	*Homo sapiens*	AP/BR	TCI	FJ549379	FJ549395	
1339	Silvio X10	*Homo sapiens*	PA/BR	TCI		AJ130928	AF545084
	CAN III	*Homo sapiens*	PA/BR	TCIIa			AY540669
85	José Julio	*Homo sapiens*	AM/BR	TCIIa	AY491761	EU856368	
	M6241 cl6	*Homo sapiens*	PA/BR	TCIIa		AJ130933	
34	Y	*Homo sapiens*	SP/BR	TCIIb	AF301912	FJ168768	AY540671
335	Sinésio	*Homo sapiens*	BR	TCIIb	FJ001621		
873	573LU	*Homo sapiens*	GO/BR	TCIIb	FJ001625		
1146	Basileu	*Homo sapiens*	MG/BR	TCIIb	FJ001629		
	Peru	*Homo sapiens*	Peru	TCIIb	X53917		
	Esmeraldo	*Homo sapiens*	BA/BR	TCIIb		AJ130931	AF545086
	CBB	*Homo sapiens*	Chile	TCIIb		AJ439722	AY540670
844	MT3869	*Homo sapiens*	AM/BR	TCIIc	AF303660		
	Tula14	*Homo sapiens*	Chile	TCIIc			DQ021895
967	NRcl3	*Homo sapiens*	Chile	TCIId	AF228685		
656	Tc656	*Homo sapiens*	Bolivia	TCIId	FJ183395	FJ183400	
	9280cl1	*Homo sapiens*	Bolivia	TCIId		AJ439725	
	MN11	*Homo sapiens*	Chile	TCIId			DQ021896
	MN12	*Homo sapiens*	Chile	TCIId			DQ021897
	Tula 12	*Homo sapiens*	Chile	TCIIe			DQ021894
		Triatomines					
	SC13	*Rhodnius pallescens*	Colombia	TCI		AJ130937	
884	884	*Panstrongylus megistus*	SP/BR	TCI	FJ549377		
1108	1108	*Rhodnius stali*	MS/BR	TCI	FJ549382	FJ549398	
1109	1109	*Rhodnius stali*	MS/BR	TCI	EU867806	FJ549399	
1116	1116	*Rhodnius stali*	MS/BR	TCI	EU867807	FJ549400	
	TEH	*Triatoma* sp.	Mexico	TCI		AJ130938	
82	RB X	*Rhodnius brethesi*	AM/BR	TCIIa	EU755217	EU856367	FJ183402
668	Rr 668	*Rhodnius robustus*	RO/BR	TCIIa	FJ183396	EU856372	
	M4167	*Rhodnius brethesi*	AM/BR	TCIIa			AY540668
698	Rr698	*Rhodnius robustus*	RO/BR	TCIIa	EU755228	EU856373	
	TU18	*Triatoma infestans*	Bolivia	TCIIb		AJ130932	
845	MT3663	*Panstrongylus geniculatus*	AM/BR	TCIIc	AF288660	EU856375	
1078	QJIII	*Triatoma rubrovaria*	RS/BR	TCIIc	FJ549380	FJ549396	
185	Tc185	*Triatoma infestans*	Bolivia	TCIId	FJ549373	FJ549388	
186	Tc186	*Triatoma infestans*	Bolívia	TCIId	FJ001630	FJ549389	
	SC43cl1	*Triatoma infestans*	Bolivia	TCIId	AF232214	AJ439721	AY540664
	CL Brener	*Triatoma infestans*	SP/BR	TCIIe	AF245383	AJ130935	AF545085
	P63cl1	*Triatoma infestans*	Paraguay	TCIIe			DQ021893

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Table 1. (*cont*.)

Trypanosome TryCC[a]	Isolate[b]	Host	Geographical origin	Lineage[c]	GenBank Accession number[d] Sequences SSU rRNA	CytB	H2B
		Wild primates					
262	AEAAB	*Cebuella pygmaea*	AC/BR	TCI	AY491763		
269	AV-AAF	*Saguinus midas*	AM/BR	TCI	EU755221	EU856369	
331	AM-ANV	*Cebus apella*	AC/BR	TCI	EU755222	EU856370	
463	MS2440	*Cebus albifrons*	AM/BR	TCIIa	EU755224	EU856371	
338	labiatus 17	*Saguinus labiatus*	AC/BR	TCIIa			
		Armadillo					
862	Tc862	*Euphractus sexcinctus*	RN/BR	TCIIc	FJ183397	FJ183401	
863	Tc863	*Euphractus sexcinctus*	RN/BR	TCIIc	FJ549376	FJ549393	
Arma 13 cl1		*Dasypus novemcinctus*	Paraguay	TCIIc	FJ549385	FJ549401	
	CM17	*Dasypus* sp.	Colombia	TCIIc			AY540667
		Didelphids					
30	G	*Didelphis marsupialis*	AM/BR	TCI	AF239981	FJ156759	FJ156760
45	AR5P	*Didelphis aurita*	SP/BR	TCI	FJ183394	FJ183398	
363	Roma 06	*Didelphis marsupialis*	RO/BR	TCI	FJ549375	FJ549390	
	Cuica cl1	*Philander opossum*	SP/BR	TCI		AJ439719	
1094	PAN ma1	*Philander frenata*	MS/BR	TCI	FJ549381	FJ549397	
125	EP24X	*Didelphis aurita*	SP/BR	TCI	FJ549371	FJ549386	
128	EP31P	*Didelphis aurita*	SP/BR	TCI	FJ549372	FJ549387	
	Dm28c	*Didelphis marsupialis*	Colombia	TCI			AF545083
139	IB42X	*Didelphis aurita*	SP/BR	TCIIb	FJ001616	FJ183399	FJ183403
712	MS2682	*Monodephis brevicaudata*	AM/BR	TCIIc	EU755230	EU856374	FJ183405
T. cruzi marinkellei							
1093	PanMo 67	*Artibeus planirostris*	MS/BR		EU867809	FJ002262	FJ002270
	M5631	*Dasypus novemcinctus*	PA/BR	TCIIc			AY540666
T. dionisii							
1110	35	*Carollia perspicillata*	SP/BR		FJ001662	FJ002263	FJ002271

[a] TryCC, Code number of the isolates/strains cryopreserved in the Trypanosomatid Culture Collection (TCC), Department of Parasitology, University of São Paulo, São Paulo, SP, Brazil.
[b] Original codes of isolates.
[c] Lineages determined based on mini-exon markers (Fernandes *et al*. 2001) and phylogenetic analyses inferred in this study.
[d] Sequences determined in this study and deposited in the GenBank are underlined.
Brazilian States: PA, Pará; AC, Acre; AM, Amazonas; SP, São Paulo; BA, Bahia; RO, Rondônia; MG, Minas Gerais; RN, Rio Grande do Norte; MS, Mato Grosso do Sul; GO, Goiás; BR, Brazil.

DNA from all *T. cruzi* isolates from bats and reference strains/isolates of all lineages (Maia da Silva *et al*. 2004*a*). The amplified DNA fragments were separated on 2·0% agarose gels and stained with ethidium bromide.

For comparison of karyotyping patterns, chromosome blocks prepared by embedding 10^7 epimastigotes of isolates belonging to several lineages in 1·2% low-melting agarose were submitted to Pulsed Field Gel Electrophoresis (PFGE) in a CHEF Mapper apparatus (Bio-Rad) as described previously (Cano *et al*. 1995).

Growth and morphology of T. cruzi *isolates from bats*

Isolates of *T. cruzi* from bats were cultivated in LIT medium supplemented with 10% or 3–5% fetal bovine serum (FBS), at 28 °C over a period of 15 days. Smears of logarithmic- (5 days) and stationary-phase (after 12 days) cultures were fixed in methanol and Giemsa stained. Cultures containing metacyclic trypomastigotes were transferred to 24-well plates containing glass cover-slips with monolayers of HeLa cells cultivated in RPMI medium with 5% FBS at 37 °C in a 5% CO_2 humid atmosphere (1×10^5 cells/well and 1×10^6 parasite/well). After 1 h, 24 h, 4 and 7 days, the cover-slips were washed 3 times in phosphate-buffered saline, fixed in methanol and Giemsa-stained for light microscopy.

Infectivity analysis of T. cruzi *isolates from bats to mice and triatomine bugs*

For evaluation of infectivity and virulence, 2 selected bat isolates of each lineage found in bats, TCI and TCII, were employed to infect Balb/c mice (6 for

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each isolate) by intra-peritoneal inoculation of cultures containing metacyclic forms (~10^6/animal). Mice blood samples were examined weekly from 7 to 30 days post-inoculation (p.i.) by the microhaematocrit method and chronic infection was confirmed by haemoculture after the 30th day p.i. Smears from the blood of experimentally infected mice were Giemsa-stained for light microscopy. Purified *T. cruzi* cultures were recovered from mice infected with trypanosomes from mixed cultures containing *T. c. marinkellei* or *T. dionisii*-like besides *T. cruzi* as described previously (Maia da Silva *et al.* 2004*a*, 2008*b*).

Eight species of triatomines were used for behavioural analysis of selected bat isolates: *Rhodnius prolixus, R. robustus* (genetic population II), *R. pictipes*, *R. domesticus*, *R. neglectus*, *Triatoma infestans*, *T. vitticeps* and *Panstrongylus megistus*. Mice infected with bat isolates of *T. cruzi* were used for xenodiagnosis with 20–30 fifth instar nymphs of each species. The infected triatomines were fed on normal mice every 15 days, dissected 15, 30, and 60 days p.i., and their guts examined for the presence of trypanosomes.

RESULTS

Genotypes of T. cruzi *isolates from bats*

The 4 bat isolates of *T. cruzi* from Amazonia were assigned to lineage TCI, and all isolates from Central and Southeast Brazil were ascribed to TCIIb using the traditional genotyping method based on mini-exon gene (Fernandes *et al.* 2001). Genotyping based on ribosomal (LSU 24Sα rRNA) markers (Souto *et al.* 1996) confirmed all the TCI isolates. However, using this method all the 11 bat isolates from Pantanal/Cerrado and the Atlantic Forest genotyped as TCIIb by mini-exon markers yielded DNA fragments slightly larger (~140 bp) than that generated for TCIIb isolates (125 bp), and different from those of other lineages (Fig. 1C, Table 1).

T. cruzi isolates from bats characterized in this study were recovered mainly from insectivorous bats of *Myotis* spp. (Vespertilionidae) (9 isolates), *Noctilio albiventris* (Noctylionidae) (2 isolates), and 1 was from *Thyroptera tricolor* (Tryropteridae). Three isolates from Amazonian bats were from frugivorous/insectivorous *Carollia perspicillata* (Phyllostomidae) (Table 1).

Phylogenetic relationships among T. cruzi *isolates from bats and other hosts based on SSU rDNA, cytochrome b and Histone H2B gene sequences*

Phylogenetic relationships and degrees of genetic relatedness were inferred by comparing sequences from genes V7–V8 SSU rDNA, Cyb and H2B of *T. cruzi* from bats aligned with corresponding sequences of isolates from humans, other wild mammals and triatomine bugs belonging to previously described lineages. Positioning of bat isolates in phylogenetic analysis of SSU rDNA and Cyb sequences corroborated 2 groups as previously evidenced by genotyping based on ribosomal and mini-exon markers. One clade comprised only TCI isolates from bats that cluster with TCI from other hosts forming a monophyletic assemblage showing a complex branching pattern. However, analysis based on SSU rDNA demonstrated that TCI from bats form a homogeneous subclade within clade TCI, differing from all other TCI isolates, which also formed subclades according to vertebrate host and/or geographical origin (Fig. 2).

The 11 bat isolates genotyped as TCII all nested into a well-supported assemblage composed exclusively of bat isolates from SP and MS, clearly separated from all previously established *T. cruzi* lineages in all analyses. Therefore, data provide evidence that these bat isolates belong to a new genotype that we designated TCbat. The clade TCbat was separated from TCIIb by the largest sequence divergences of all genes examined: ~5·7%, 11% and 12·3% for SSU rDNA, Cyb and H2B gene, respectively. Similar sequence divergences of these three genes separated TCIIb from TCI (~5·5%, 11·2% and 11%). Divergences between TCbat and TCI were also large for these genes (~6·2%, 4·2% and 8·4%), and similar to those separating this new genotype from TCIIa (~5·0%, 5·5% and 9·5%), TCIIc (~5·5%, 5·5%, 6·0%), and TCIId (~4·7%, 5·1% and 10·7%). TCIIe was represented in the phylogenetic analyses by the hybrid CL Brener strain, which showed sequences positioned within TCIIb (SSU rDNA) or closest to TCIIc/d (Cyb and H2B) (Fig. 2).

Therefore, the bat isolates of the new genotype clustered tightly together forming a clade well supported in all analyses and clearly different from all known lineages (Fig. 2). Despite being closest to TCI in analyses using SSU rDNA and Cyb sequences, positioning of TCbat was only well supported using Cyb sequences (bootstrap of 97%). In analysis using H2B sequences, placement of the new genotype was weakly supported close to clade comprising TCIIc/d/e. Therefore, analyses based on separated nuclear (SSU rDNA and H2B) and mitochondrial (Cyb) genes were unable to clearly resolve the relationships between TCbat and conventional *T. cruzi* lineages (Fig. 2). We found no evidence of hybrid characteristics in the new lineage by sequencing 5–8 clones of each SSU rDNA, Cyb and H2B genes from 2 bat isolates (TryCC 793 and 1122).

All phylogenetic analyses included *T. c. marinkelei* and *T. dionisii*-like, which enabled confirmation that isolates of TCbat genotype indeed belong to *T. cruzi* and not to other very closely related *Schizotrypanum* species from bats (*T. cruzi*-like) (Fig. 2).

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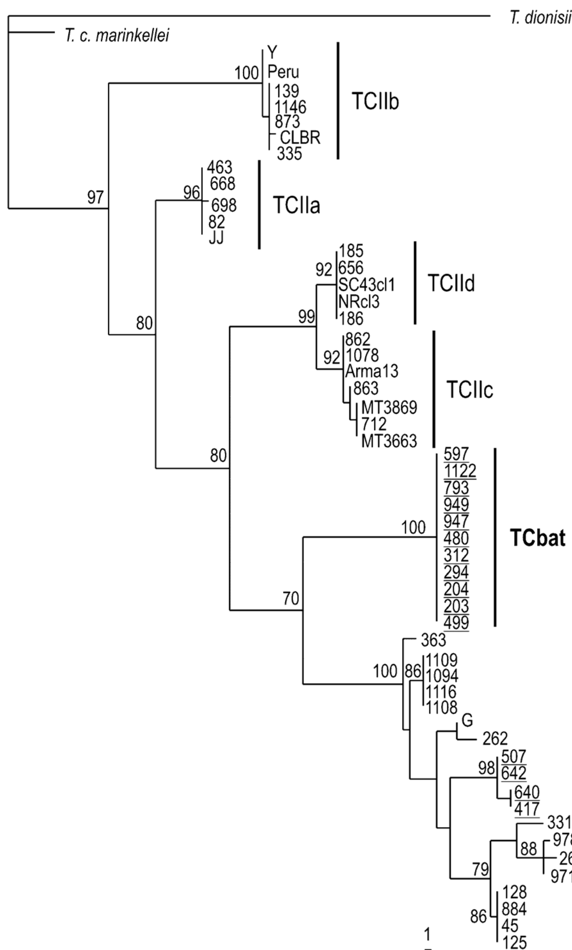

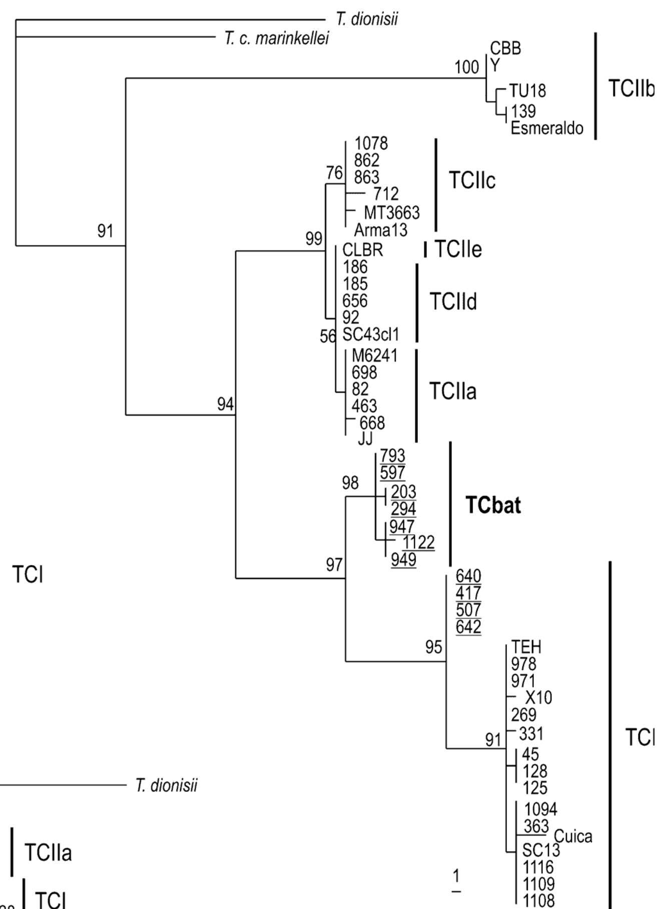

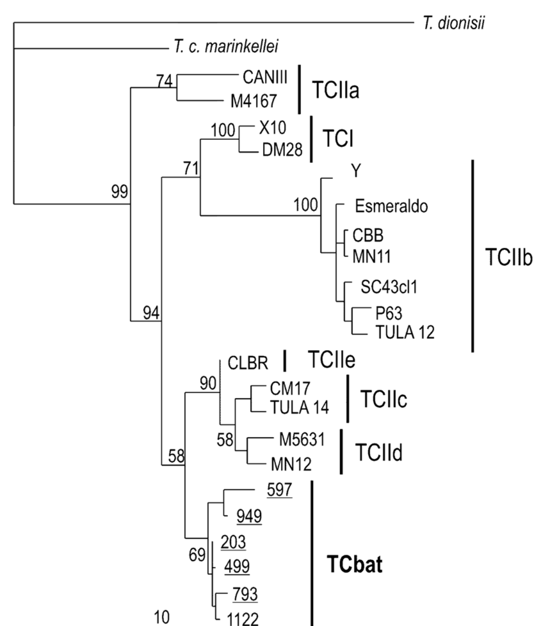

Fig. 2. Phylogenetic trees inferred by parsimony analyses based on (A) V7–V8 SSU rDNA sequences (825 characters, 108 parsimony informative) of 54 *Trypanosoma cruzi* isolates, (B) cytochrome b sequences (490 characters, 84 parsimony informative) of 52 isolates, and (C) Histone H2B partial sequence (457 characters, 104 parsimony informative) of 24 isolates. *T. cruzi* isolates from bats are underlined. The numbers at the nodes correspond to parsimony percentage bootstrap values derived from 100 replicates.

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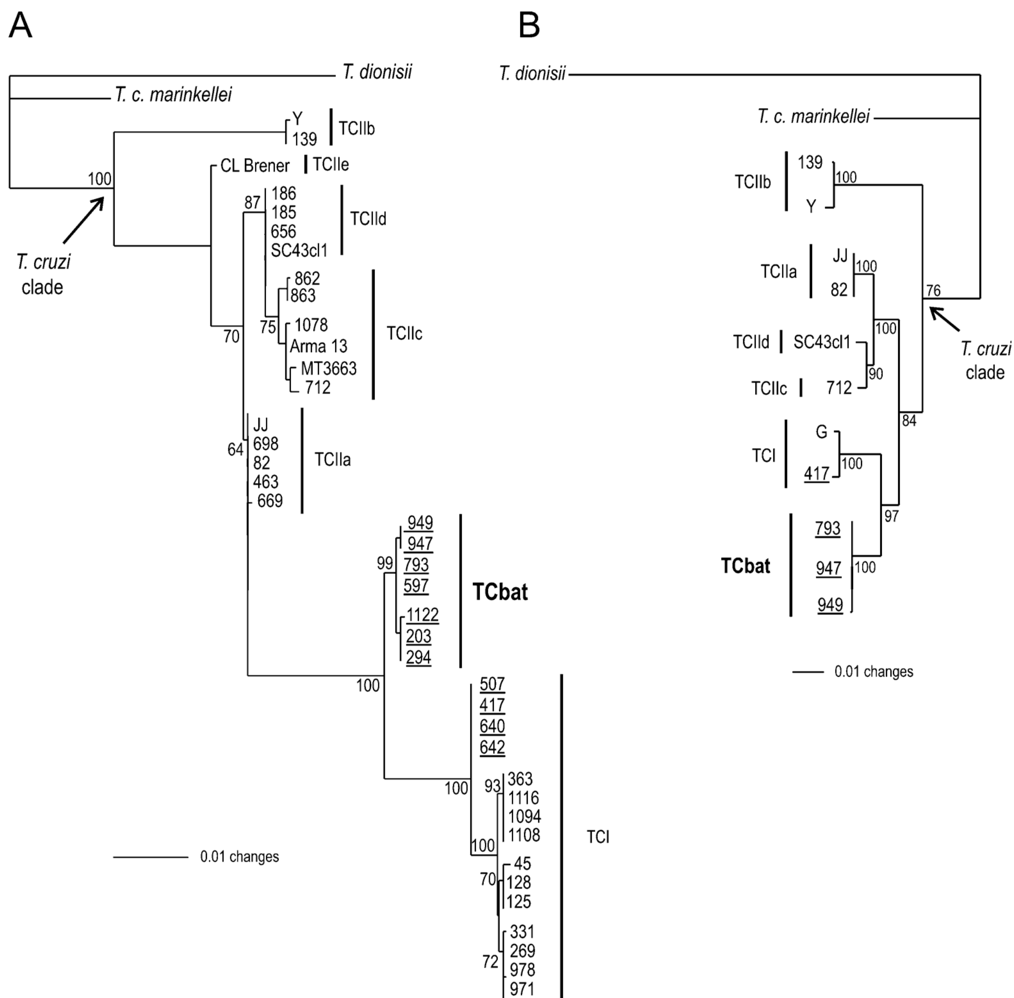

Fig. 3. Phylogenetic trees inferred by ML analyses using combined data sets: (A) SSU rDNA and cytochrome b sequences from 42 isolates (1315 characters, $-\text{Ln}=3044{\cdot}252544$), and (B) SSU rDNA, cytochrome b and Histone H2B sequences from 11 isolates (1791 characters, $-\text{Ln}=4761{\cdot}077323$) of *Trypanosoma cruzi* isolates. *T. cruzi* isolates from bats are underlined. Numbers at nodes are bootstrap values derived from 100 replicates.

Phylogenetic relationships among T. cruzi *and* T. cruzi*-like from bats* (Schizotrypanum) *inferred using a combined data set of SSU rDNA, cytochrome b and Histone H2B sequences*

Phylogenetic analyses were performed using 2 data sets of concatenated aligned sequences aiming to better resolve the phylogenetic relationships of the new *T. cruzi* genotype from bats in relation to previously established lineages of *T. cruzi* as well as of *T. cruzi*-like trypanosomes. Alignment 1 consists of SSU rDNA plus Cyb sequences from 40 *T. cruzi* isolates plus *T. c. marinkelei* and *T. dionisii*-like. Alignment 2 comprises sequences from SSU rDNA, Cyb and H2B from 11 *T. cruzi* isolates plus *T. c. marinkelei* and *T. dionisii*-like. Phylogenetic trees based on both alignments were inferred by P, Bayesian (data not shown) and ML methods (Fig. 3). In trees generated by the two alignments, the clade TCbat was always well supported and positioned closest to TCI. TCIIb was positioned as the basal lineage of the clade *T. cruzi*, while the other lineages were distributed in 5 major clades. Clade TCI and TCbat formed a monophyletic assemblage in all analyses using the two combined data sets (Fig. 3). TCbat was separated from the subclade formed by bat isolates assigned to TCI by large genetic distances. Independent of alignments and analytical methods, *T. cruzi*-like, *T. dionisii* and *T. c. marinkellei*, were always positioned as outgroups of the clade harbouring all *T. cruzi* isolates (Fig. 3).

Analyses using combined data sets positioned TCbat closer to TCI than to any other lineage, and unequivocally within *T. cruzi* (Fig. 3). The assemblage formed by TCI isolates corroborated the major subclades revealed by SSU rDNA analysis (Figs 2 and 3). The 4 TCI isolates from bats generated a subclade formed exclusively by isolates from Amazonia whereas isolates from opossum, monkeys and humans from this region clustered separately. However, these isolates were all genotyped as typical TCI by traditional PCR methods and tightly clustered together forming a clade separated from TCbat. Although TCbat isolates were all from bats

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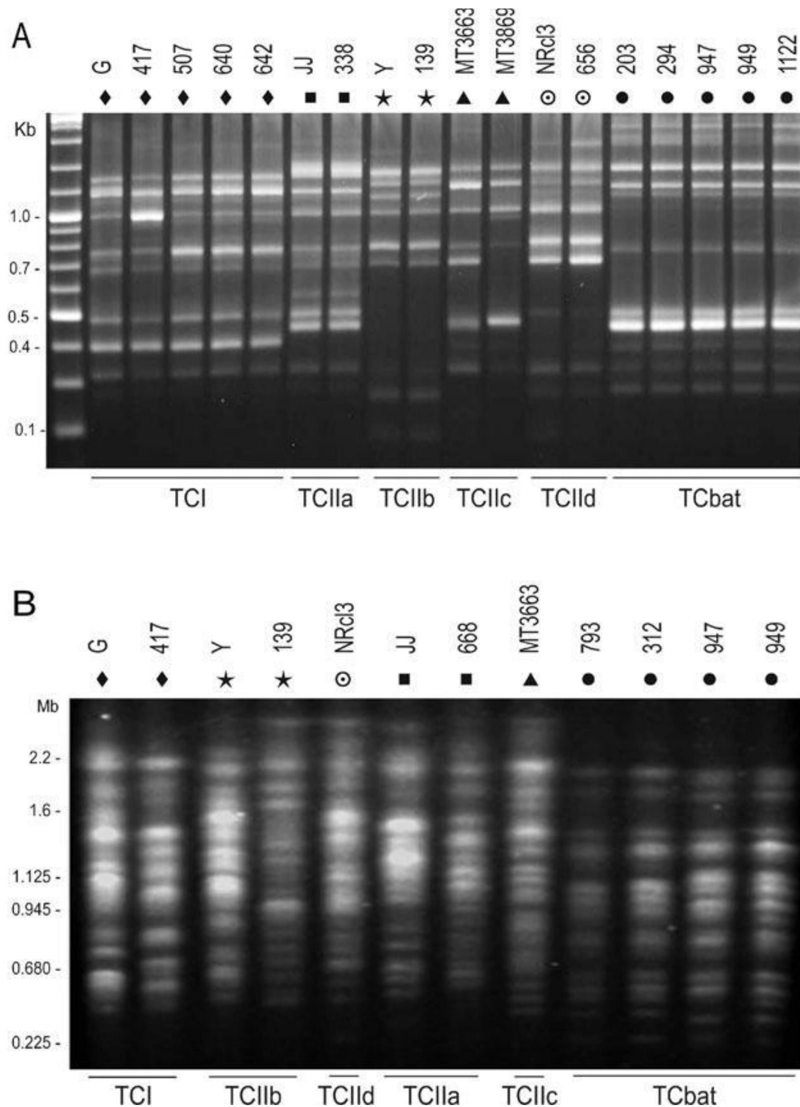

Fig. 4. Genetic polymorphism of *Trypanosoma cruzi* isolates analysed through RAPD and karyotype patterns selected to illustrate inter-lineage genetic variability, and small polymorphism of TCbat isolates. Agarose gel stained with ethidium bromide showing (A) RAPD profiles generated using primer 672, and (B) PFGE chromosome band profiles. Isolates included in these analyses were from TCbat (●) and lineages TCI (◆), TCIIa (■), TCIIb (★), TCIIc (▲) and TCIId (⊙).

captured in MS and SP states, isolates from didelphids and triatomines from these states never nested within clade TCbat while they were segregated in 2 clades according to their geographical origin (Figs 2 and 3).

Polymorphism analysis of bat isolates by RAPD and karyotype patterns

For additional detection of inter- and intralineage polymorphisms among bat trypanosomes, we compared RAPD patterns generated with 5 selected primers using DNA of all bat isolates assigned to both TCbat and TCI. RAPD profiles generated using all primers were shown to be almost identical for bat isolates within the same clade (data not shown). For illustrative purpose we selected 1 primer (672) that yielded a distinct pattern for TCbat genotype, allowing its separation from all *T. cruzi* lineages (Fig. 4A), and generating similar RAPD profiles shared by bat isolates assigned to the same clade (TCI and TCbat).

Despite relevant heterogeneity of karyotyping patterns generated by PFGE among isolates belonging to different lineages, and even within the same lineage, chromosome band profiles separate TCbat isolates from *T. cruzi* of established lineages. Similar patterns of chromosome bands are shared by the 4 isolates of TCbat examined, with small differences between isolates TryCC 793/312 and 947/949. Interestingly, karyotyping profiles of TCbat isolates apparently lack the largest chromosome bands and are less complex than those from isolates of the other lineages (Fig. 4B).

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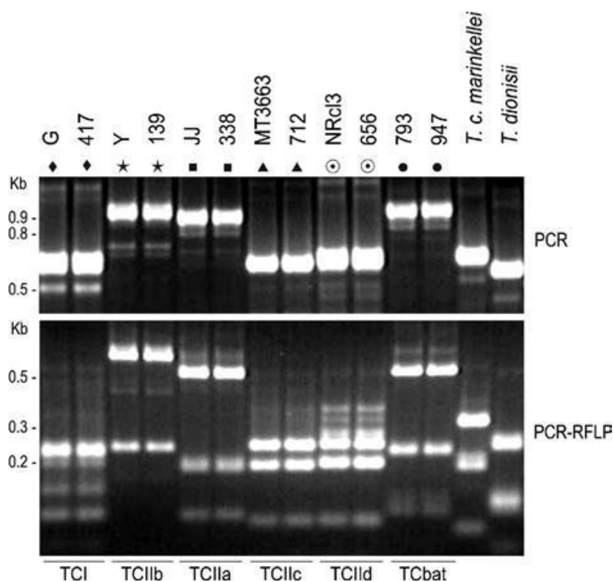

Fig. 5. (A) PCR-amplified DNA fragments corresponding to ITS1 rDNA. (B) Genotyping of *Trypanosoma cruzi* isolates based on restriction fragment length polymorphism (RFLP) of PCR amplified ITS1 rDNA digested with the restriction enzyme *Bsh* 1236. Lineages TCI (◆), TCIIa (■), TCIIb (★), TCIIc (▲), TCIId (⊙) and TCbat (●). Brazilian isolates of *T. dionisii*-like and *T. c. marinkeleii* were used as controls of *T. cruzi*-like trypanosomes from bats. Agarose gels (2·5%) stained with ethidium bromide.

Taken together, similar RAPD and karyotyping patterns, which are highly sensitive tools for polymorphism analyses, besides high conservation of SSU rDNA, Cyb and H2B sequences, indicated that TCbat isolates from SP and MS formed a highly homogeneous group different from other lineages of *T. cruzi*.

Development of a PCR-RFLP method targeting ITS1 rDNA sequences for T. cruzi *genotyping and identification of* Schizotrypanum *species*

Analyses of PCR-amplified DNA fragments corresponding to ITS1 rDNA revealed length polymorphism among *T. cruzi* isolates. However, this polymorphism did not allow either a clear separation between TCIIb and TCbat or identification of *T. c. marinkellei* and *T. dionisii*-like. However, restriction patterns generated by PCR-RFLP analysis of representative isolates of each *T. cruzi* lineage distinguished TCbat. Moreover, this method also allowed identification of *T. cruzi* lineages and separation of *T. cruzi* from *T. c. marinkellei* and *T. dionisii*-like (Fig. 5). In addition, these methods confirmed the existence of mixed-infected bats harbouring different combinations of TCI or TCbat together with *T. c. marinkellei* and/or trypanosomes closely related to European isolates of *T. dionisii* (data not shown).

Behavioural analyses of T. cruzi *isolates from bats in culture, in mice and in triatomines*

Analyses of culture behaviour of bat isolates in LIT medium supplemented with 10% FBS showed morphologically similar epimastigotes for isolates of TCI and TCbat (data not shown). However, in LIT containing 3–5% FBS, epimastigotes of TCbat isolates became very long, with several large dividing forms. Stationary cultures showed only forms with a long and pointed posterior extremity, in addition to metacyclic forms (Fig. 6). In contrast, in all culture media and growth conditions, TCI isolates showed epimastigotes that resembled isolates of other *T. cruzi* lineages. Metacyclic trypomastigotes from TCI and TCbat appeared similar (Fig. 6), despite lower numbers of these forms in TCbat compared to TCI. Bat isolates of TCI (TryCC 507 and 417) (data not shown) and TCbat (TryCC 204 and 793) developed inside HeLa cells (Fig. 6) and other mammalian cells similar to isolates of other *T. cruzi* lineages. Invasion by metacyclic trypomastigotes and multiplication inside mammalian cells as amastigotes, which differentiate to trypomastigotes that invade new cells, are features shared by all trypanosomes of *Schizotrypanum* (Molyneux, 1991).

Isolates assigned to TCI and TCbat were infective to mice. Despite the fact that bat isolates from these two lineages yielded low parasitaemias, blood trypomastigotes could be detected in blood smears (Fig. 6). The bat isolates lacked virulence to mice as assessed by mortality rates. Chronic infections in mice inoculated with these isolates were confirmed by haemocultures performed within approximately 120 days of infection.

Isolates of TCbat did not develop in 8 triatomine species investigated: *R. prolixus, R. neglectus, R. robustus* (genetic populations II); *R. pictipes; R. domesticus; T. vitticeps; T. infestans* and *P. megistus*. Results showed that TCbat isolates survived in the digestive tube of bugs from the triatomine for up to ~20 days, following complete elimination of the flagellates. In contrast, TCI isolates from bats developed in all these triatomines, which showed metacyclic trypomastigotes in their guts after 30 days of infection.

DISCUSSION

Data regarding prevalence rates, distribution, genetic diversity and phylogenetic relationships of *T. cruzi* isolates from bats are scarce. Comparative studies of *T. cruzi* with its very closely related bat-restricted trypanosomes may be helpful to the understanding of host-parasite relationships of the *Schizotrypanum* species. Moreover, a detailed understanding of the genetic diversity, ecobiology and phylogeography of *Schizotrypanum* trypanosomes is crucial for an understanding of the evolutionary

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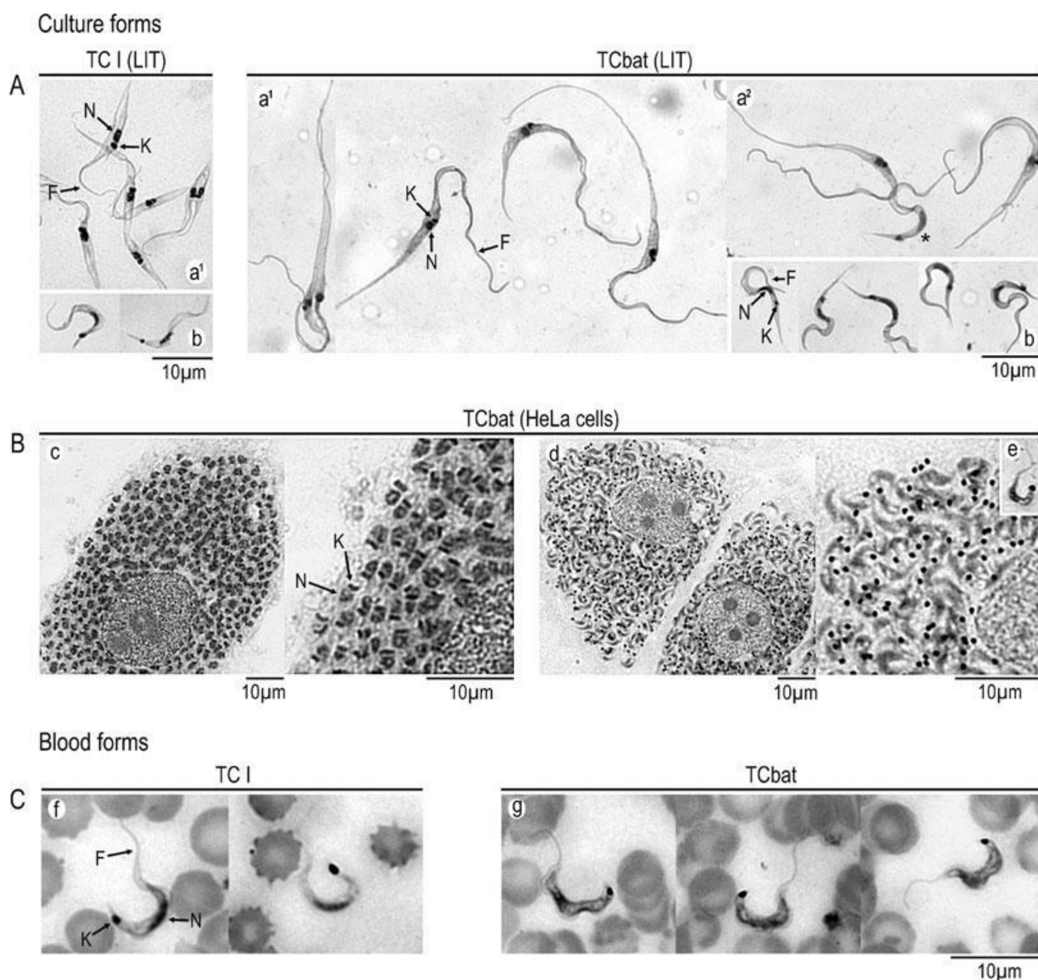

Fig. 6. Photomicrographs (Giemsa-staining) selected to illustrate morphological and growth features of bat trypanosomes. (A) Morphology of selected bat *Trypanosoma cruzi* isolates assigned to TCbat (TryCC 793) and TCI (TryCC 507) cultivated in LIT medium: epimastimastigotes of logarithmic (a^1) and stationary phase (a^2), and metacyclic trypomastigotes* (b). (B) Development within HeLa cells showing intracellular amastigotes (c) and trypomastigotes (d) 4 and 7 days post-infection, respectively, and trypomastigotes released from HeLa cells (e). (C) Trypomastigotes in blood smears from mice experimentally infected with bat *T. cruzi* isolates of TCI (f) and TCbat (g). N, nucleus; K, kinetoplast; F, flagellum.

history of *T. cruzi*. With these aims, we recently carried out a large survey of trypanosomes in Brazilian bats and obtained isolates of *T. cruzi, T. c. marinkellei* and *T. dionisii*-like (Cavazzana *et al.* 2003; Maia da Silva *et al.* 2009). *T. cruzi* was isolated mainly from Vespertilionidae and Phyllostomidae bats from Central and Southeast Brazil, besides some isolates from Amazonia, mostly from Phyllostomidae. Bats of Vespertilionidae (*Myotis*) and Phyllostomidae (*Phyllostomus*, *Artibeus*, *Desmodus* and *Anoura*) were implicated as important reservoirs of *T. cruzi*. These bats exhibit varied alimentary habits, including insectivorous, frugivorous, haematophagous and omnivorous species, and can be infected by *T. cruzi* in sylvatic and anthropic environments (Barreto *et al.* 1974; Funayama and Barretto, 1970*a,b*, 1973; Fabián 1991; Lisboa *et al.* 2008; Maia da Silva *et al.* 2009; Añez *et al.* 2009).

In this study, we characterized *T. cruzi* isolates from Brazilian bats genotyped as TCI and TCIIb using PCR based on mini-exon gene markers (Fernandes *et al.* 2001). However, genotyping of 15 bat isolates using ribosomal markers (Souto *et al.* 1996) confirmed 4 isolates as TCI, whilst amplified DNA fragments shown by isolates assigned to TCII were not compatible with any previously defined TCII sublineages (TCIIa–e), indicating that these isolates belong to a distinct genotype.

Analysis of phylogenetic relationships was carried out with the aim of understanding the phylogenetic relationships between the new isolates from bats and isolates from other hosts previously assigned to established *T. cruzi* lineages. For this purpose, we employed independent and concatenated SSU rDNA, Cyb and H2B sequences. Isolates from Amazonian bats always nested within TCI, as did the majority of isolates from this region (Miles *et al.*

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1981; Fernandes *et al.* 2001; Maia da Silva *et al.* 2008; Marcili *et al.* 2009). The 11 bat isolates previously genotyped as TCIIb clustered tightly together constituting a new clade separated by relevant genetic distances from all known lineages, thus confirming that they belong to a new genotype (TCbat). All analyses performed using separated or combined data sets separated isolates of genotype TCbat from isolates of lineage TCIIb. However, results did not indicate an unquestionable positioning of TCbat within any established lineages of *T. cruzi*. Phylogenetic analysis of *Schizotrypanum* trypanosomes using concatenated alignment of SSU rDNA, Cyb and H2B genes positioned all bat isolates characterized in this study within the clade *T. cruzi*, which is more closely related to *T. c. marinkellei* than to *T. dionisii* clades.

Most inferred phylogenetic analyses suggested that TCbat genotype is closer to TCI than to any other lineage, although separated by large genetic distances. In contrast to TCbat, isolates of TCI shared amplified DNA fragments of the same length when genotyped using traditional methods. In this study, we demonstrated that TCbat isolates differed from TCI isolates from Amazonian bats, and also from TCI from didelphids and triatomines from the same regions where bats infected with TCbat have been found. TCbat is clearly different from any isolate assigned to the TCI lineage, even from bat isolates assigned to TCI by all molecular markers investigated in this study.

The monophyletic assemblage formed by TCI from bats, humans, wild monkeys and didelphids provided evidence of geographical clustering within TCI. Recent studies have evidenced subclusters within TCI that could be associated with both mammalian hosts and geographical origin. Analysis of the highly polymorphic intergenic region of the mini-exon gene separated North American from South American isolates, and disclosed a subclade related to *Didelphis* sp. (O'Connor *et al.* 2007). Mini-exon markers revealed 4 haplotypes (Ia–Id) of Colombian TCI isolates related to distinct transmission cycles (Herrera *et al.* 2007). Analysis of Cyb sequences of TCI isolates from Chile revealed a new genotype (DTU1b) associated with caviomorph rodents (Spotorno *et al.* 2008).

Comparative analyses of TCbat and TCI isolates from a range of hosts and geographical origin may help to define recommendations for the description of new genotypes/lineages closely related to TCI. Recently, isolates of lineage TCI have been investigated using polymorphic molecular markers, and results showed high intralineage genetic diversity and a complex populational structure of TCI populations (O'Connor *et al.* 2007; Herrera *et al.* 2007; Spotorno *et al.* 2008). Our data do not support TCI and TCII as 2 major lineages within *T. cruzi* since TCIIa–e sublineages are not monophyletic and varied according to the gene analysed, as previously demonstrated (Machado and Ayala, 2001; Brisse *et al.* 2003; Sturm *et al.* 2003; Westenberger *et al.* 2005, 2006). Therefore, relationships among lineages of *T. cruzi* are far from understood. Further analysis from more isolates from several lineages may help to resolve the phylogeny of *T. cruzi*. Until more data can be gathered, we have designated the new genotype of *T. cruzi* from bats characterized in this study as TCbat.

Isolates of TCbat were shown to be distinguishable by morphological and biological features. These isolates showed very large and pointed epimastigotes, different from those of all other *T. cruzi* lineages. In addition, TCbat isolates were unable to yield established infection in triatomines of genera *Rhodnius*, *Triatoma* and *Panstrongylus*, a behaviour shared by *T. c. marinkellei* and *T. dionisii*. *T. c. marinkellei* seems to be transmitted only by triatomines of genus *Cavernicola*, which are usually associated with bat colonies, whereas *T. dionisii* is transmitted by cimicids (Marinkelle, 1976; Molyneux, 1991). Triatomines that live in tree holes and caves, palms and house roofs can transmit *T. cruzi* among bats. The majority of *T. cruzi*-infected bats are insectivorous and likely to be infected by ingestion of triatomines (Marinkelle, 1976; Thomas *et al.* 2007). Vector permissiveness to *T. cruzi* and association between lineages/strains and triatomine species depends on both vectors and parasite features, apparently, with superior vector competence of sympatric sylvatic species, as clearly demonstrated for *T. rangeli* (Maia da Silva *et al.* 2007). Unfortunately, triatomines of *Cavernicola* and other sylvatic species from regions where TCbat isolates originated were not available for this study. In Central Brazil, several triatomine species, mainly those inhabiting palms such as *R. neglectus, R. robustus* and *R. stali* could transmit *T. cruzi* among bats (Gurgel-Gonçalves *et al.* 2008). However, the genotypes of *T. cruzi* circulating in sylvatic triatomines from MS and SP were completely unknown. Isolates from *R. stali* (MS) and *P. megistus* (SP) included in this study were genotyped as TCI.

Altogether, sequence divergences and phylogenetic analysis of SSU rDNA, Cyb and H2B genes, morphology and behaviour in triatomines indicate that isolates of TCbat indeed belong to a new genotype of *T. cruzi* represented, so far, exclusively by bat isolates from antrophic areas of Central and Southeast Brazil. Both regions are endemic for Chagas disease. Distances separating these areas are easily crossed by bats found to be infected with TCbat. The limited data regarding *T. cruzi* genotypes in wild mammals are insufficient to rule out other mammals as hosts of TCbat, and also humans living in houses inhabited by bats.

The hypotheses that *T. cruzi* evolved from a trypanosome restricted to bats or *vice versa* remain

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to be elucidated. The present-day distribution of *Schizotrypanum* species and the ability of bats to disperse over long distances, including crossing oceans, are consistent with both hypotheses (Stevens *et al*. 1999; Barnabé *et al*. 2003). The evolutionary histories of *T. cruzi* lineages have been correlated with a long-standing association with vertebrate hosts. TCI and TCII have been associated respectively with marsupials of Didelphimorpha (opossums) and with placentals of Xenarthra (armadillos), the early mammals in South America (~65 mya). Primates and rodents entered South America from Africa during the Oligocene (~35 mya), whereas chiropterans dispersed from Africa in the Eocene (~45 mya), and arrived in the Americas via Beringia or by a transatlantic route (Eick *et al*. 2005). Bats are ancient hosts of *T. cruzi*-like species or lineages transmitted by triatomines in the Americas as indicated by the description of *Trypanosoma antiquus* in triatomine feces fossilized in Dominican amber, together with bat hairs (Poinar, 2005). The low genetic divergence showed in this and in previous studies is compatible with a recent split between *T. cruzi* and the bat-restricted *Schizotrypanum*, as well as with a recent diversification of all *T. cruzi* lineages (Machado and Ayala, 2001; Brisse *et al*. 2003; Barnabé *et al*. 2003).

Besides *T. cruzi*, other *Schizotrypanum* species such as *T. c. marinkellei* and *T. dionisii*-like, in addition to *T. rangeli* have also been found infecting bats in the same locations where bats infected with TCbat were captured (Cavazzana *et al*. 2003; Lisboa *et al*. 2008; Maia da Silva *et al*. 2009). Taking into account the ITS1 rDNA polymorphisms among *T. cruzi* lineages, we standardized a PCR-RFLP, targeting this gene that allowed separation of *T. cruzi* from other species of *Schizotrypanum* and to distinguish TCbat from other genotypes. This method was shown to be a sensitive tool for easy detection of new genotypes of *T. cruzi* and *T. cruzi*-like.

Data from this study corroborated the high complexity of *T. cruzi*, pointing towards the existence of distinct *T. cruzi* genotypes waiting to be described, and the method described in this study can be very helpful for this purpose. Our results provide evidence that the understanding of enzootic transmission cycles of *T. cruzi* can be improved with phylogenetic analysis of more isolates, especially from poorly investigated sylvatic vertebrate and invertebrate hosts of unexplored geographical regions and ecotopes.

We would like to thank all students and colleagues for their inestimable help in the fieldwork, and for providing us with blood samples of bats and reference strains of *T. cruzi*. We are grateful to Erney P. Camargo, Michel A. Miles, Martin Llewellyn and Michael Lewis for valuable comments on the manuscript. We are thankful to Valdir Tadei (in memoriam) and Caroline C. Aires for identification of bats. Work in Rondonia (Amazonia) was done in ICB5-USP. This work was supported by grants from the Brazilian agencies CNPq (UNIVERSAL) and FAPESP (PRONEX). F. M. da S. was sponsored by CAPES (PRODOC-PROTAX), and A. M., L. L. and A. C. V. J. were recipients of scholarships from CNPq.

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A *Trypanosoma cruzi*-like new species from African bats (Chiroptera: Molossidae) supported by morphological, developmental and multigene phylogenetic analyses

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ABSTRACT

We isolated and characterised a new *Trypanosoma cruzi*-like species from free-tailed bats (Molossidae: *Mops condylurus* and *Tadarida* sp.) captured in Mozambique in southeast Africa. Haemocultures from the blood samples of 25 bats resulted in 6 trypanosome isolates. Morphological analysis and culture behaviour showed epimastigotes that differentiated to trypomastigote metacyclics typical of *T. cruzi*-like as well as intracellular amastigotes that multiplied and differentiated to trypomastigotes within mammalian cells in culture, a characteristic exclusive to the subgenus *Schizotrypanum*. Although isolates came from bats belonging to two genera, they are very closely related phylogenetically; this relationship was inferred using polymorphic sequences from the V7V8 region of the SSU rRNA (small subunit of ribosomal RNA) and ITS1 rDNA (internal transcribed spacer of ribosomal DNA). Phylogenetic inferences using the SSU rRNA, gGAPDH (Glyceraldeyde 3-phosphate dehydrogenase glicosomal) and mitochondrial Cyt b (cytochrome b) of the new bat isolates revealed that they constituted a homogenous clade within the subgenus *Schizotrypanum*. Analysis of the spliced leader (SL) gene also supported the new isolates as a new species of this subgenus. Comparable genetic distances separated these isolates from their closest species, *T. c. marinkellei* and *T. dionisii,* while *T. cruzi* was separated by larger distances. Together, data from conserved and polymorphic sequences of both nuclear and mitochondrial genes allowed us to classify this trypanosome as a new species of the subgenus *Schizotrypanum.* Besides being the first molecularly characterised *Schizotrypanum* trypanosome from African bats, this trypanosome is the only new species of this subgenus supported by phylogenetic analysis described in the last 30 years. Findings from this study provide new insights into the evolutionary history of *T. cruzi* and its closely related trypanosomes.

Keywords: *Trypanosoma*, Chiroptera, bat parasites, *T. cruzi*, *Schizotrypanum*, taxonomy, phylogeny, evolution, Africa.

Introduction

Species of the genus *Trypanosoma* (Euglenozoa, Kinetoplastea) are obligate parasites of virtually all vertebrates. They have been reported in almost all mammalian orders and in more than 70 species of bats (Chiroptera) of different families and feeding habits. Bat trypanosomes are classified mainly in the subgenera *Schizotrypanum* and *Megatrypanum*, for which more than 30 trypanosome species have been recorded throughout the world, all apparently harmless for their proper hosts and most from insectivorous bats (Hoare, 1972; Marinkelle, 1976; Molyneux, 1991; Cavazanna et al., 2010).

Blood forms of *Schizotrypanum* trypanosomes are morphologically indistinguishable from *T. cruzi,* the agent of Chagas disease in Latin America. For this reason, these trypanosomes are often referred as *T. cruzi*-like. The known species of *Schizotrypanum* are

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exclusive to bats throughout the world, except in Latin America, where *T. cruzi* occurs in virtually all mammalian orders (Hoare, 1972; Marinkelle, 1976; Molyneux, 1991; Maia da Silva et al., 2009; Marcili et al., 2009; Cavazanna et al., 2010). The following species of *Schizotrypanum* have been reported in bats: *T. vespertilionis* in Europe, the Americas, Africa and Asia; *T. dionisii* and *T. pipistrelli* in the Old World, particularly in European countries; *T. pteropi* and *T. hipposideri* in Australia; *T. hedricki* and *T. myotis* in North America; *T. phyllostomae* and *T. c. marinkellei* in Central and South America; and *T. cruzi* in Latin America (Hoare, 1972; Marinkelle, 1976; Molyneux, 1991).

To date, phylogenetic analyses based on the SSU rRNA and gGAPDH genes have validated only three species of this subgenus: *T. cruzi, T. c. marinkellei* and *T. dionisii*. *T. cruzi* is a complex of genetically heterogeneous isolates distributed in six discrete typing units (DTUs TcI-TcVI) as well as the new genotype Tcbat. Phylogenetically, *T. cruzi* is closer to *T. c. marinkellei* than to *T. dionisii.* These three species share morphological, biological, genomic and proteomic features, but they differ in their hosts, vectors and pathogenicity (Stevens et al., 1999a,b; Barnabé et al., 2003; Hamilton et al., 2007; Maia da Silva et al., 2009; Cavazanna et al., 2010; Telleria et al., 2010). When phylogenetically analysed, an isolate of *T. vespertilionis* (P14) from a bat found in England was not confirmed as *Schizotrypanum,* indicating that either this isolate was erroneously classified or that *T. vespertillionis* is not a *Schizotrypanum* species. In phylogenetic trees, *T. vespertilionis* clusters with *T. rangeli*, *T. conorhini,* and African trypanosomes from monkey and civet. This clade, together with the clade *Schizotrypanum* and the marginally positioned *Trypanosoma* sp. from Australian kangaroo, constituted a monophyletic assemblage that had been previously designated as the *T. cruzi* clade (Stevens et al., 1999a,b; Hamilton et al., 2004; 2007; 2009). This large clade comprises trypanosomes transmitted by triatomine or cimicid bugs, but the vectors of some species are unknown.

The majority of bat trypanosomes classified as *T. cruzi*-like using morphological data were not validated as either a separate species or as a species of the subgenus *Schizotrypanum.* Only *Schizotrypanum* spp. from South America and Europe have been characterised by additional criteria, including culture behaviour, infectivity to other mammals and development in bat (Molyneux, 1991). Alleged species of *Schizotrypanum* from Africa (*T. vespertilionis* and *T. dionisii*), North America (*T. myotis* and *T. hedricki*) and Australia (*T. pteropi* and *T. hipposideri*) still need to be validated, but neither cultures nor DNA sequences are available for such analysis.

Although limited experimental infections have not provided sufficient evidence about host-parasite relationships, most *Schizotrypanum* species appear to be bat-restricted. *T. phyllostomae* and *T. cruzi* were infective to laboratory rodents, but only the latter species has been recovered from humans (Hoare, 1972; Marinkelle, 1976). Only a few studies on a limited number of trypanosome isolates have used biochemical (zymodemes) and molecular markers

able to distinguish *Schizotrypanum* species (Baker et al., 1972; 1978; Ebert, 1983; Tibayrenc & Le Ray, 1984; Barnabé et al., 2003; Steindel et al., 1998; Grisard et al., 2003; Stevens et al., 1999a,b, 2001; Hamilton et al., 2004; 2011; Lisboa et al., 2008; Cavazzana et al., 2010).

In the Old World, *T. cruzi* is absent, and bats are the only mammals in which autochthonous infections with *Schizotrypanum* spp. have been reported. To date, only trypanosomes from Brazil, Belgium and England have been phylogenetically confirmed as members of this subgenus (Barnabé et al., 2003; Stevens et al., 2001; Hamilton et al., 2004; 2007; Maia da Silva et al., 2009; Marcili et al., 2009; Cavazzana et al., 2010).

Consistent biological and molecular characterisation of *Schizotrypanum* trypanosomes from bats originating from a broad range of geographical origins are crucial to elucidate their relationships with hosts and vectors. Phylogenetic studies of bat trypanosomes are valuable to improving the understanding of the evolutionary history of *T. cruzi* as well as the whole subgenus *Schizotrypanum* and its allied trypanosomes. Reports about *Schizotrypanum* in African bats (Hoare, 1972) still lack phylogenetic evidence. In this study, trypanosomes from free-tailed bats captured in Mozambique, Southeast Africa, were classified as *Schizotrypanum* through analyses of their behaviour in culture, intracellular development, morphology by light and electron microscopy, and evaluation of infectivity to mice and triatomine bugs. Their phylogenetic relationships with other species of *Schizotrypanum* and with other trypanosomes from bats and other mammals from Brazil, Europe and Africa were inferred through multigene phylogenetic analyses.

MATERIALS AND METHODS

1. Capture and identification of bats and isolation of the trypanosomes in culture

Bats were captured in the districts of Marromeu (S18º17' E35º56'), Chupanga (S18º02' E35º34') and Parque Nacional da Gorongosa (S18º58' E34º21') from Sofala Province in Central Mozambique, Southeast Africa, in 2006 May and 2009 July. Bats captured with mist nets were anaesthetised, and blood samples were collected by heart puncture. The genera of the captured bats were determined by analysing morphological characteristics using conventional keys. For bat species identification, liver samples preserved in 100% ethanol were processed for the extraction of genomic DNA (Wizard DNA Clean-Up System, Promega) and sequencing of Cyt b (Cui et al., 2007). The sequences obtained were submitted to Blast analysis at Genbank.

To verify the presence of trypanosomes, blood samples from the bats were examined using the microhaematocrit (MH) and haemoculture (HE) methods. HEs were performed using a medium consisting of blood agar base (BAB), which contained 15% rabbit blood as a solid phase, and an overlay of LIT (Liver Infusion Tryptose) medium with 10% foetal bovine serum (FBS); the samples were incubated at 28ºC 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 University of São Paulo,Brazil. Collection (TCC) of the Department of Parasitology,

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

TCC[a] code	**Trypanosome isolate**	**Host species**		**Geographic origin**		**DTU**	**GenBank Acession number**				
							SSUrDNA V7V8	**gGAPDH**	**Cytb**	**ITS1**	**SL**
T. erneyi											
1293		bat	*Tadarida sp*	Marromeu	MZ	-	*	*	*	*	ND
1294		bat	*Tadarida sp*	Marromeu	MZ	-	*	*	*	*	ND
1932		bat	*M. condylurus*	Chupanga	MZ	-	*	*	*	*	ND
1934		bat	*M. condylurus*	Chupanga	MZ	-	*	*	*	ND	ND
1936		bat	*M. condylurus*	Chupanga	MZ	-	*	*	*	ND	ND
1946		bat	*M. condylurus*	Chupanga	MZ	-	*	*	*	*	*
T. dionisii					BR	-					
211		bat	*E. brasiliensis*	SP	BR	-	FJ001666	GQ140362	FJ900249	*	ND
1059		bat	*E. brasiliensis*	TO	BR	-	ND	ND	FJ900252	ND	ND
495		bat	*C. perspicillata*	RO	BR	-	ND	GQ140363	FJ900251	ND	ND
1110		bat	*C. perspicillata*	SP	BR	-	ND	ND	FJ002263	ND	ND
1314		bat	*S. lillium*	PR	BR	-	ND	ND	FJ900254	ND	ND
1087		bat	*S. lillium*	GO	BR	-	ND	ND	FJ900253	ND	ND
454		bat	*D. rotundus*	MS	BR	-	ND	ND	ND	ND	EU867796
403		bat	*M. molossus*	RO	BR	-	FJ001658	ND	ND	*	ND
554		bat	*Promops sp*	MS	BR	-	FJ001660	ND	ND	*	ND
-	PJ	bat	*P. pipistrellus*	-	BE	-	AJ009152	ND	ND	ND	ND
-	P3	bat	*P. pipistrellus*	-	UK	-	AJ009151	AJ620271	ND	ND	AJ250744
T. cruzi	***marinkellei***					-					
344		bat	*C. perspicillata*	RO	BR	-	FJ001664	GQ140360	ND	*	ND
501		bat	*C. perspicillata*	RO	BR	-	FJ001665	GQ140361	ND	ND	ND
478		bat	*A. planirostris*	MS	BR	-	ND	ND	ND	ND	EU867798
1093		bat	*A. planirostris*	MS	BR	-	ND	ND	FJ002262	ND	ND
1794		bat	*A. planirostris*	MS	BR	-	ND	ND	ND	*	*
1089		bat	*A. planirostris*	MS	BR	-	ND	ND	ND	ND	EU867797
494		bat	*P. discolor*	AM	BR	-	ND	ND	FJ900246	ND	ND
1067		bat	*Phyllostomus* sp	TO	BR	-	ND	ND	FJ900247	ND	ND
332		bat	*P. hastatus*	AM	BR	-	FJ001635	ND	ND	dep	ND
-	M-1909	bat	*P. discolor*	-	VE	-	ND	ND	ND	ND	AF116568
-	B3	bat	*P. discolor*	BA	BR	-	FJ649484	FJ649495	ND	ND	ND
-	B7	bat	*P. discolor*	BA	BR	-	ND	AJ620270	ND	ND	ND
-	M1117	bat	*P. hastatus*	PA	BR	-	ND	ND	AJ130927	ND	ND
T. cruzi											
793		bat	*M. levis*	SP	BR	Tcbat	FJ001634	GQ140358	FJ002258	ND	ND
1994	Clone from 793	bat	*M. levis*	SP	BR	Tcbat	ND	ND	ND	*	*
1122		bat	*M. albescens*	SP	BR	Tcbat	FJ001628	GQ140359	FJ002261	*	ND
507		bat	*C. perspicillata*	RO	BR	TcI	FJ001632	GQ140352	FJ002256	ND	ND
417		bat	*T. tricolor*	AM	BR	TcI	ND	ND	FJ002255	ND	ND
30	G	opossum	*D. marsupialis*	AM	BR	TcI	AF239981	GQ140351	FJ156759	*	*
	Sylvio x10	human	*H. sapiens*	PA	BR	TcI	ND	ND	AJ130928	ND	ND
-	SE	human	*H. sapiens*	AM	BR	TcI	ND	ND	ND	AF362825	ND

Table 1. Continues

T. cruzi											
34	Y	human	*H. sapiens*	SP	BR	TcII	AF301912	GQ140353	ND	ND	ND
2120	Esmeraldo cl3	human	*H. sapiens*	BA	BR	TcII	ND	*	AJ130931	ND	ND
-	Peru	human	*H. sapiens*	-	PE	TcII	X53917		ND	ND	ND
-	CBB	human	*H. sapiens*	-	CL	TcII	ND	ND	AJ439722	ND	ND
-	994	human	*H. sapiens*	PI	BR	TcII	ND	ND	ND	AF362829	ND
-	5894	human	*H. sapiens*	PI	BR	TcII	ND	ND	ND	AF362828	ND
863	Tc863	Armadillo	*E. sexcinctus*	RN	BR	TcIII	ND	ND	ND	FJ555660	ND
844	MT3869	human	*H. sapiens*	AM	BR	TcIII	AF303660	GQ140355	ND	ND	ND
845	MT3663	triatomine	*P. geniculatus*	AM	BR	TcIII	AF288660	*	EU856375	FJ555658	ND
337	Fuscicolis 15	primate	*S. fuscicolis*	AC	BR	TcIV	ND	*	EU856377	ND	ND
85	José Julio	human	*H. sapiens*	AM	BR	TcIV	ND	GQ140356	EU856368	ND	ND
967	NRcl3	human	*H. sapiens*	-	CL	TcV	ND	GQ140357	ND	ND	ND
186	Tc186	triatomine	*T. infestans*	-	BO	TcV	ND	*	ND	ND	ND
-	SC43	triatomine	*T. infestans*	-	BO	TcV	ND	ND	AJ439721	ND	ND
-	CL Brener Esmeraldo-like and	triatomine	*T. infestans*		BR	TcVI	ND	ND	ND	ND	•
	CL Brener Non-Esmeraldo-like										•
T. rangeli											
643		bat	*P. lineatus*	MS	BR	-	ND	GQ140364	*	ND	ND
031	SA	human	*H. sapiens*	-	CO	-	ND	ND	ND	ND	
086	AM80	human	*H. sapiens*	AM	BR	-	ND	*	*	ND	EF071547
	T. minascense	monkey	*S.boliviensis*	South America		-	ND	AJ620274	ND	ND	ND
other	**trypanosomes**										
	T. desterrensis	bat	*E. furinalis*	SC	BR	-	ND	ND	ND	ND	AF124146
	T.vespertilionis P14	bat	*P. pipistrellus*	-	UK	-	ND	AJ620283	ND	ND	ND
60	*T. sp* bat (60)	bat	*R. aegyptiacus*	-	GA	-	ND	GQ140365	ND	ND	ND
	HochNdi1	monkey	*C. nictitans*	-	CM	-	ND	FM164794	ND	ND	ND
	Nandoum1	carnivore	*N.binotata*	-	CM	-	ND	FM164793	ND	ND	ND
	T.conorhini	rodent	*R. rattus*	-	BR	-	ND	AJ620267	ND	ND	ND
	H25	kangaroo	*M. giganteus*	-	AU	-	ND	AJ620276	ND	ND	ND

[a] Cultures of bat trypanosome isolates cryopreserved in the Trypanosomatid Culture Collection of the Departament of Parasitology, University of São Paulo, São Paulo, Brazil. TCC correspond to number codes of isolates cryopreserved in -this collection. * Sequences determined in this study to be submitted to GenBank. • Sequences retrieved from TryTripDB database.

2. Growth behaviour and morphology of bat trypanosomes

For the morphological analysis, blood smears from either naturally infected bats or logarithmic and stationary phase cultures were fixed with methanol and Giemsa-stained for light microscopy. For the differentiation of epimastigotes into metacyclic trypomastigotes, flagellates were cultured in TC-100 medium (Grace medium) with 3.0% FBS and incubated at 28 °C. Metacyclic trypomastigotes (1×10^6/well) were employed to infect monolayers of HeLa 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 days post-inoculation (p.i.) the coverslips were removed, washed 3 times in phosphate-buffered saline, fixed in methanol and Giemsa-stained. *T. cruzi* TCC507 (TcI), *T. c. marinkellei* and *T. dionisii* were cultivated in parallel for comparative purposes.

***3. Sequencing and data analysis of SSU rDNA, gGAPDH and* Cyt b sequences.**

DNA obtained from cultured trypanosomes from bats using the phenol-chloroform extraction method was used as a template for PCR

amplification, cloning and sequencing. PCR amplifications of the variable V7-V8 SSU rRNA, whole SSU rRNA and ITS rDNA regions were carried out using the primers and reaction conditions described previously (Rodrigues et al. 2006; Ferreira et al. 2008). The amplifications of the gGAPDH sequences were carried out using the previously described primers and reaction conditions (Hamilton et al., 2007; Viola et al. 2009). The sequences (500 bp) of Cyt b were obtained as described previously (Brisse et al., 2003). The sequences from the SL gene transcript region (exon and intron) were obtained according to Maia da Silva et al. (2007).

The sequences were aligned using Clustal X (Thompson et al., 1997), and the resulting alignments were manually refined. We created several alignments for phylogenetic inferences: a) V7V8 SSU rRNA (~850bp) sequences from 23 *Schizotrypanum* spp.; b) SSU rRNA (~1722 bp) sequences of all available bat trypanosomes and trypanosomes from other hosts representing all major clades in the phylogenetic trees; c) gGAPDH sequences from *Schizotrypanum* using *T. rangeli, T. vespertilionis*, *T.* sp bat from African megabat, and *T.* sp H25 as outgroups for *Schizotrypanum;* d) Cyt b (~470bp) sequences from 28 *Schizotrypanum* spp. using *T. rangeli* as an outgroup; e) ITS1 rDNA sequences (approximately 900 bp) from 18 *Schizotrypanum* spp.; and f) SL sequences corresponding to transcript regions (118 to 128 bp) of *Schizotrypanum* spp. using *T. rangeli* as an outgroup. The concatenated SSU rDNA and gGAPDH sequences were also employed for phylogenetic inferences. The sequences from the new bat trypanosomes reported in this paper are available in GenBank under the accession numbers listed in Table 1.

Phylogenetic relationships were inferred using maximum likelihood (ML), Bayesian (BI) and maximum parsimony (MP) analyses. The parsimony and bootstrap analyses were carried out using PAUP* version 4.0b10 (Swofford, 2002); the ML analyses, using RAxML v.7.0.0 (Stamatakis, 2006); and the Bayesian inferences, in MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001). Nodal support was estimated with 500 bootstrap replicates in RAxML using GTRGAMMA as described previously (Ferreira et al. 2007).

4. Infectivity analysis of bat trypanosomes for triatomine bugs and mice.

Three species of triatomines were used for the behavioural analysis of selected trypanosomes isolated from bats: *R. robustus, R. neglectus* and *T. infestans*. Fifth instar nymphs, approximately 30 for each species, were inoculated intracoelomically with stationary phase cultures of bat trypanosomes containing metacyclic trypomastigotes. The inoculated triatomines were fed on normal mice every 15 days, and approximately 10 triatomines of each species were dissected 15, 30, and 60 days p.i. Their digestive tubes were examined for trypanosome development as described previously (Maia da Silva et al., 2004).

For the analysis of mice infectivity, Balb/c mice were inoculated with metacyclic forms (~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 MH and

by haemoculture 30 days p.i. as described previously (Maia da Silva et al., 2004).

5. Transmission Electron Microscope and Scanning Electron Microscope.

For the transmission electron microscopy (TEM) analysis, mid-log phase trypanosome cultures were fixed in glutaraldehyde, post-fixed with osmium tetroxide and embedded in Spurr resin; then, ultrathin sections stained with uranyl acetate and lead citrate as described before (Viola et. al. 2009), were examined in a JEOL 100CX transmission electron microscope. For scanning electron microscopy (SEM), flagellates fixed in glutaraldehyde were adhered to poly-L-lysine-coated coverslips and processed for observation in a ZEISS DSM940 digital scanning microscope as detailed previously (Teixeira et al., 2011).

RESULTS

1. Culture of bat trypanosomes and host identification

In this study, we captured bats in three areas of Mozambique and collected blood samples for analysis by MH and HE. Of the 25 blood samples examined, 6 yielded trypanosome cultures (24% prevalence). The bats all belonged to Molossidae (free-tailed bats) and were morphologically identified as *Mops* sp. and *Tadarida* sp. To confirm this morphological identification, Cyt b sequences from the DNA of bat liver tissues were obtained and 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 at the species-level was not possible because their closest relative in Genbank was *T. teniotis* with only 82% similarity (Table 1). These bats most likely belong to a species for which sequence information has not been obtained such as *T. ventralis,* which is closely related to *T. teniotis* from and reported in Mozambique and neighbouring countries (Mickleburgh et al., 2004a, b).

2. Growth behaviour, morphology and analyses of infectivity to mice and triatomine bugs

All six trypanosomes isolated from free-tailed bats grew easily in LIT and TC-100 media. Their culture epimastigotes were morphologically indistinguishable from those of *T. cruzi* and *T. c. marinkellei*, but *T. dionisii* was characterised by a short length. Metacyclic trypomastigotes made up ~ 40% of the flagellates in stationary cultures. Although these forms resembled those from *Schizotrypanum* spp., they are of two types varied in length and the positions of the kinetoplast: one, small and rounded with the kinetoplast close to the nucleus, and the other, slender and thin with the kinetoplast close to the posterior extremity (Fig. 1).

A feature exclusive to *Schizotrypanum* species is the ability to develop *in vitro* within mammalian cells. Therefore, all cultures were transferred to monolayers of HeLa and LLC-MK_2 cells to evaluate their ability to invade and develop inside cells. The isolates from molossids were able to develop inside cells similarly to all *Schizotrypanum* spp. The isolate TCC1946 was selected to illustrate the intracellular development

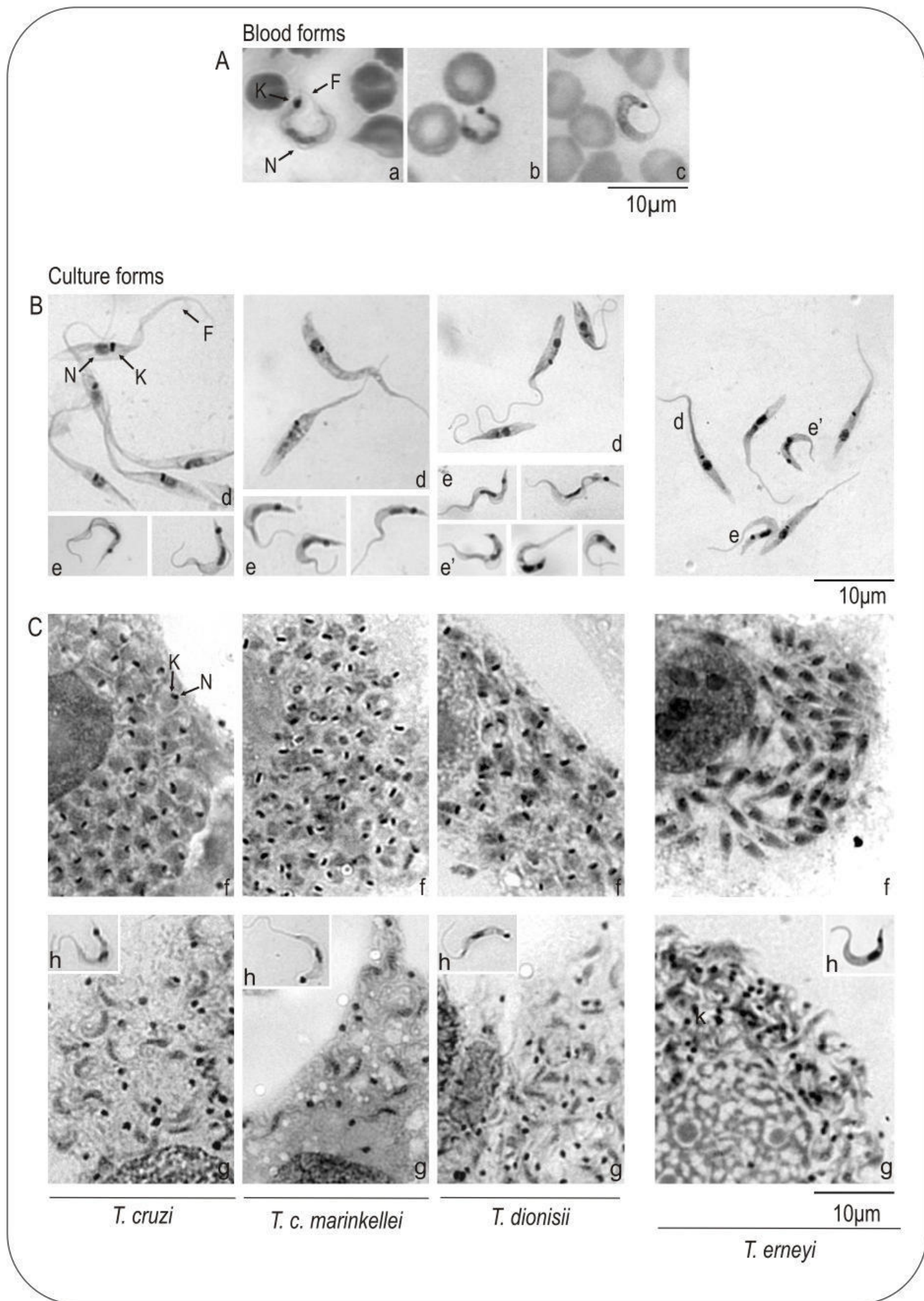

Fig. 1. Photomicrographs (Giemsa-staining) selected to illustrate morphological and growth features of bat trypanosomes. (A) Trypomastigotes in blood smears from bats naturally infected with *T. cruzi* (a); *T. c. marinkellei* (b) and *T. dionisii* (c). (B) Culture forms in LIT medium of *T. cruzi*, *T. c. marinkellei, T. dionisii* and *T. erneyi*: epimastigotes of logarithmic cultures (d); Metacyclic trypomastigotes from stationary cultures of long (e) and short (e') types. (C) Development within HeLa cells showing amastigotes (f) and trypomastigotes 4 and 7 days p.i. (g), and trypomastigotes released from cells (h). N, nucleus; K, kinetoplast, F, flagellum.

of these isolates compared to those of *T. cruzi*, *T. c marinkellei* and *T. dionisii* (Fig. 1). In all cases, metacyclic trypomastigotes invaded the cells and multiplied in the cytoplasm as amastigotes. After ~ 7 days of multiplication, amastigotes differentiated to trypomastigotes, which are released and can infect new cells. We could not find trypomastigotes in blood smears from the bats from which the isolates were obtained, which was indicative of low parasitaemia in the natural hosts, which is typical of *Schizotrypanum* spp. (Cavazanna et al., 2010). Blood trypomastigotes typical of these trypanosomes are shown in the Figure 1.

The only species of *Schizotrypanum* that proved infective to mice was *T. cruzi*, while all other species appeared to be restricted to bats. The new isolates TCC1946 and 1294 were not infective to Balb/c mice, similarly to *T. dionisii* and *T. c. marinkellei*. In addition, these isolates were unable to develop in triatomine bugs (*Rhodnius robustus, R. neglectus* and *Triatoma infestans*) like *T. dionisii* and *T. c. marinkellei,* but unlike *T. cruzi*, with the exception of the Tcbat genotype that also not develop in these bugs (Marcili et al., 2009).

3. Barcoding and phylogenetic analyses of the new trypanosomes from African molossids

Initially, we compared the V7V8 region of the SSU rRNA gene of all six new isolates from African molossids; we have been using this variable region for barcoding in the preliminary determination of new trypanosomatids (Maia da Silva et al., 2004; Ferreira et al., 2007; Cavazzana et al., 2010). Barcoding analysis showed that all the new isolates from African free-tailed bats shared high sequence similarity (~0.2% average divergence), and Blast analysis demonstrated that they were closest to *Schizotrypanum* trypanosomes (Fig. 2A). In the dendrogram inferred using the barcoded sequences from the six new isolates aligned with sequences described for *Schizotrypanum* spp., all new isolates formed a separate cluster, which diverged from the clusters formed by other *Schizotrypanum* spp. as follows: 11% from *T. dionisii* (~2.0% between Brazilian and European isolates), 9.5% from *T. c. marinkellei* and 14% from *T. cruzi* isolates of several DTUs (~4.0% internal divergence) (Fig. 2A).

Three isolates were positioned in the phylogenetic trees inferred from the SSU rRNA sequence data (Fig. 2B), and six were included in the gGAPDH sequence-derived tree (Fig. 3A). In all trees, when either independent or combined (not shown) data were used, the three trypanosomes from molossid bats nested together with *T. c. marinkellei, T. dionisii* and *T. cruzi,* and constituted a clade that corresponded to the subgenus *Schizotrypanum*. Within the new bat isolates, the 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 *Schizotrypanum* spp. for 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*. The clade harbouring all *Schizotrypanum* spp. was clearly separated from

its closest clade of *T. rangeli* isolates. Similar branching patterns were showed in the phylogenetic analyses using ML, MP and BI analyses(Figs.2,3).

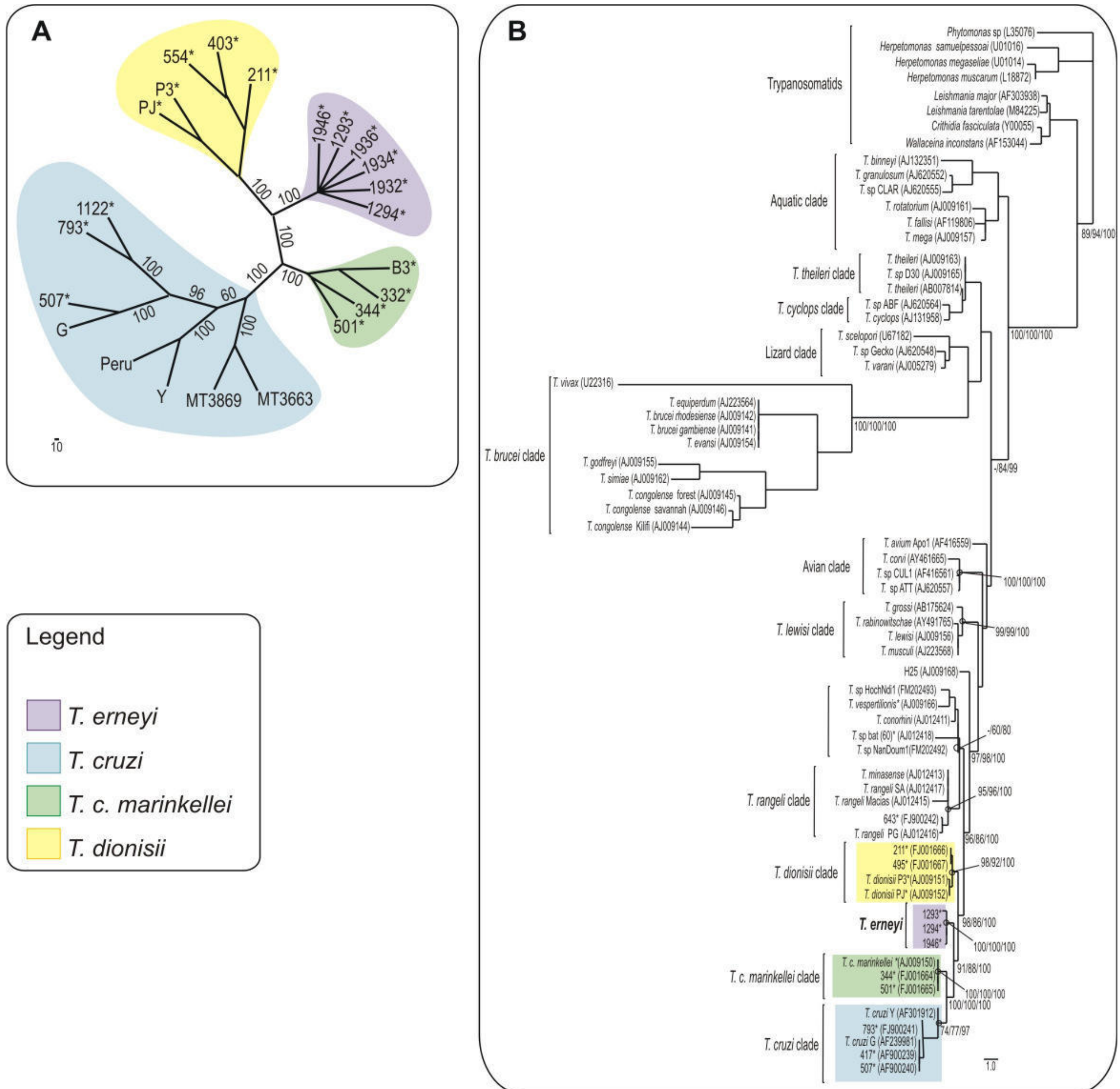

Fig. 2. Barcoding and phylogenetic relationships of the new isolates from African molossid bats inferred using ribosomal sequences. (A) Dendrogram inferred using the V7-V8 SSU rRNA sequences from all six molossid isolates and other isolates of the subgenus *Schizotrypanum*. Numbers at nodes are bootstrap values derived from 100 replicates. (B) Maximum likelihood analysis (ML) using aligned sequences of the SSU rRNA (1722 characters, –Ln = 8563.377778) from 15 trypanosomes of *Schizotrypanum* and 51 species of other subgenera. Numbers at nodes are bootstrap values derived from 500 replicates from the MP/ML/BI analyses. The trypanosomes from bats are indicated by (*).

Additional support for the phylogenetic positioning of the new bat isolates was inferred from phylogenetic trees based on Cyt b sequences (Fig. 3B); these trees showed the same topology as the SSU rRNA and gGAPDH-derived trees. The Cyt b sequences of the new isolates diverged by 12% from *T. dionisii,* 10% from *T. c. marinkellei* and 13% from *T. cruzi,* which corroborated the relationships with the species of *Schizotrypanum* that had been inferred using other genes.

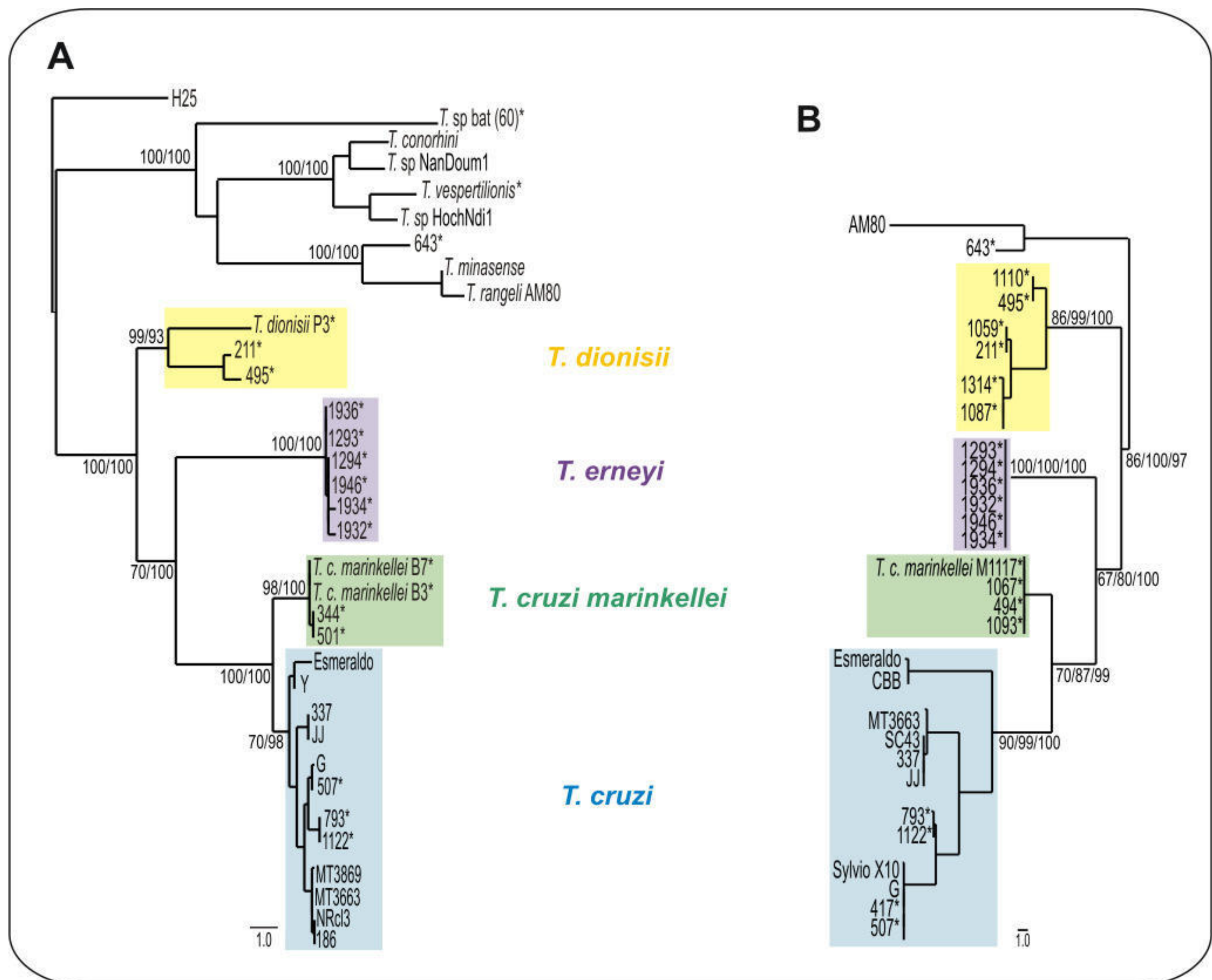

Fig. 3. Phylogenetic trees of trypanosomes from the subgenus *Schizotrypanum* inferred by ML analyses using: (A) gGAPDH sequences from 34 isolates (902 characters, -Ln = 3376.510575). (B) Cyt b sequences from 30 isolates (476 characters, -Ln = 1874.299574). Numbers at nodes are bootstrap values derived from 100 replicates from the MP/ML/BI analyses. The trypanosomes from bats are indicated by (*), and the different colours represent the different species of the subgenus *Schizotrypanum.*

4. Polymorphism analysis among new African isolates from bats using ITS1 rDNA and SL sequences

ITS1 rDNA sequences are highly polymorphic; thus, both the length and sequence polymorphisms have been used to identify species and to determine infraspecific genotypes and genetic relatedness among closely related species. These analyses have revealed polymorphisms undisclosed by the SSU rRNA and gGAPDH conserved genes for *T. cruzi*, *T. rangeli* and *T. theileri*, as well as anuran trypanosomes (Maia da Silva et al., 2004; Rodrigues et al., 2006; Ferreira et al., 2007; Marcili et al., 2009). Analysis of ITS1 rDNA length polymorphisms revealed unique lengths for each species; the lengths were as follows: ~350 bp for the new isolates from free-tailed bats, ~400 bp for *T. dionisii* and ~600 bp for *T. c. marinkellei*. Isolates of *T. cruzi* that had been classified into different DTUs showed ITS1 rDNA lengths that varied from ~500 to ~800bp for TcI and TcII, respectively (Marcili et al., 2009).

The dendrogram inferred using ITS1 rDNA sequences from the new isolates and other *Schizotrypanum* species (Fig. 4A) corroborated the clade formed by the new bat isolates that had been revealed by previous analyses. The divergences on ITS1 rDNA sequences were small (~2.0%) among the new isolates; isolates from different species, such as TCC1946 from *M. condylurus* and TCC1294 from *Tadarida* sp., were identical. The new bat isolates were separated by large divergences from *T. dionisii* (~89%), *T. c. marinkellei* (~65%) and *T. cruzi* of TcI, TcII, TcIII and Tcbat (average divergence of ~75%). The divergences of *T. cruzi* from *T.c. marinkellei* and *T. dionisii* were ~71% and 79%, respectively, and among *T. cruzi* isolates of different DTUs, the average divergence was ~44%. Together, these data corroborated the finding that all of these new bat isolates should be assigned to a single species and further confirmed that these isolates belonged to the subgenus *Schizotrypanum*.

The SL RNA gene of the trypanosomes consists of transcript sequences (exon and intron) and a highly polymorphic intergenic spacer. Transcript SL sequences are useful for identifying species and assessing genetic relatedness among closely related trypanosomes, whereas intergenic sequences are valuable for detecting infraspecific polymorphism (lineages and genotypes) (Gibson et al., 2000; Fernandes et al., 2001; Grisard et al., 2003; Maia da Silva et al., 2007; Rodrigues et al., 2010). Here, we determined SL transcript sequences from one isolate from a free-tailed bat to compare these sequences with sequences previously determined for other species of *Schizotrypanum*. In the dendrogram based on these SL sequences, the new isolates were separate from other bat trypanosomes, which corroborated the observation that these isolates were indeed different from all closely related *Schizotrypanum* spp. Ambiguous alignments hampered the use of SL sequences to compare more distant species outside this subgenus (Fig. 4B).

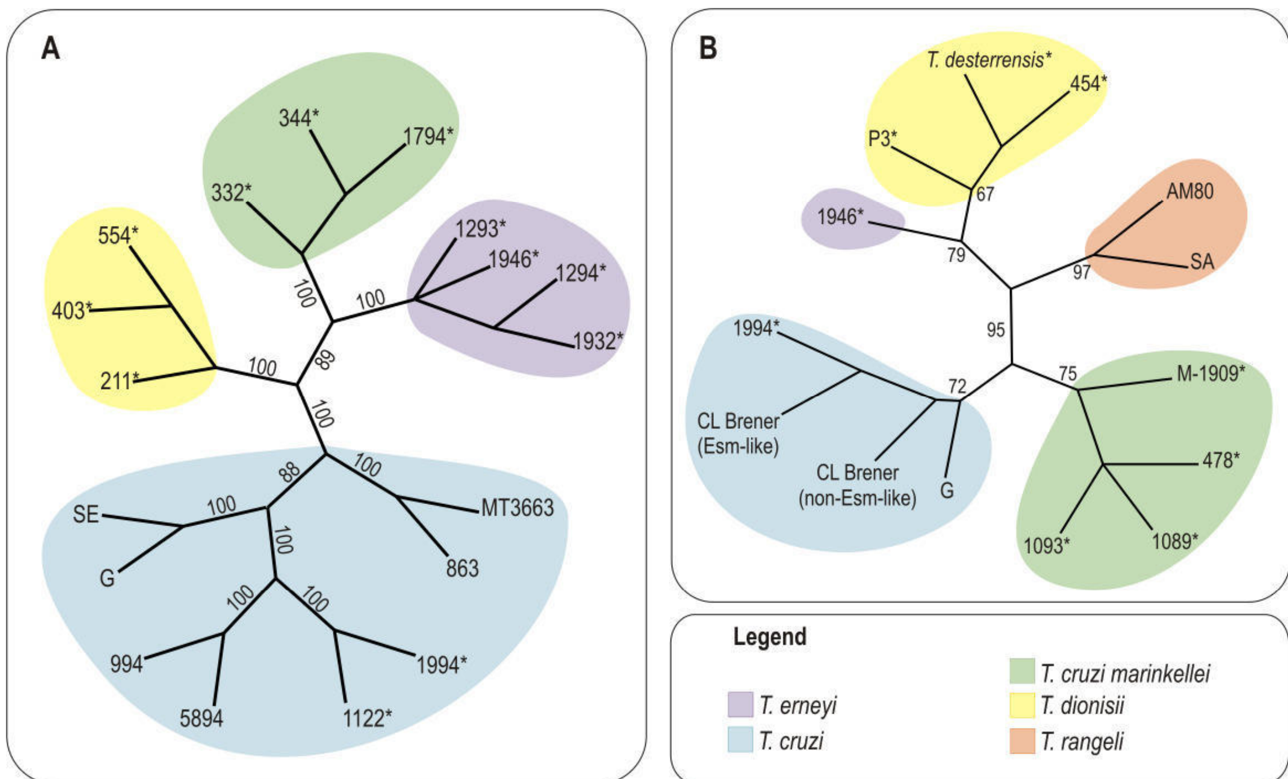

Fig. 4. Polymorphism analyses among isolates of *T. erneyi* and other *Schizotrypanum* spp. (A) Dendrogram inferred using the ITS1 rDNA sequences from four isolates of *T. erneyi,* as well as sequences from *T. c. marinkellei* and *T. dionisii.* (B) Dendrogram inferred using the transcript sequences of the SL gene from *T. erneyi*, *T. cruzi*, *T. c. marinkellei, T. dionisii, T. desterrensis* and *T. rangeli*. Numbers at nodes are bootstrap values derived from 100 replicates. The trypanosomes from bats are indicated by (*).

5. Ultrastructural comparison by SEM and TEM of Schizotrypanum trypanosomes

The SEM morphological analysis of culture forms of the isolates TCC1294 and 1946 showed elongated epimastigotes with long and thin flagella that were generally indistinguishable from those of *T. cruzi*, *T. c. marinkellei* and *T. dionisii.* These isolates had epimastigotes with varying lengths and shapes arranged in rosettes in the proliferative phase. A large number of twisted flagellates were found in this isolate compared to those found for other *Schizotrypanum* spp. The epimastigotes that detached from these rosettes were slender and less twisted. The metacyclic trypomastigotes varied in the lengths, shapes and positions of the kinetoplast, as described by light microscopy. In these forms, the flagellum attached to the body formed a small undulant membrane before becoming free at the anterior extremity of the body (Fig. 5).

Morphological analysis by TEM, which is usually quite informative for differentiating among trypanosome species, failed to unveil distinctive characteristics between the isolates TCC1294 and 1946, compared to *T. cruzi, T. c. marinkellei* and *T. dionisii.* The flagellates from all these species exhibited a well-developed cytostome and highly compacted kDNA fibril arrangements, resulting in kDNA disks with minimal thickness, which were both characteristic of *Schizotrypanum*. *T. erneyi*

also exhibited other features common to these trypanosomes, such as large number of acidocalcisomes and reservosomes and a reduced paraxial structure on the typical flagella array with one central and nine peripheral pairs of microtubules (Fig. 6).

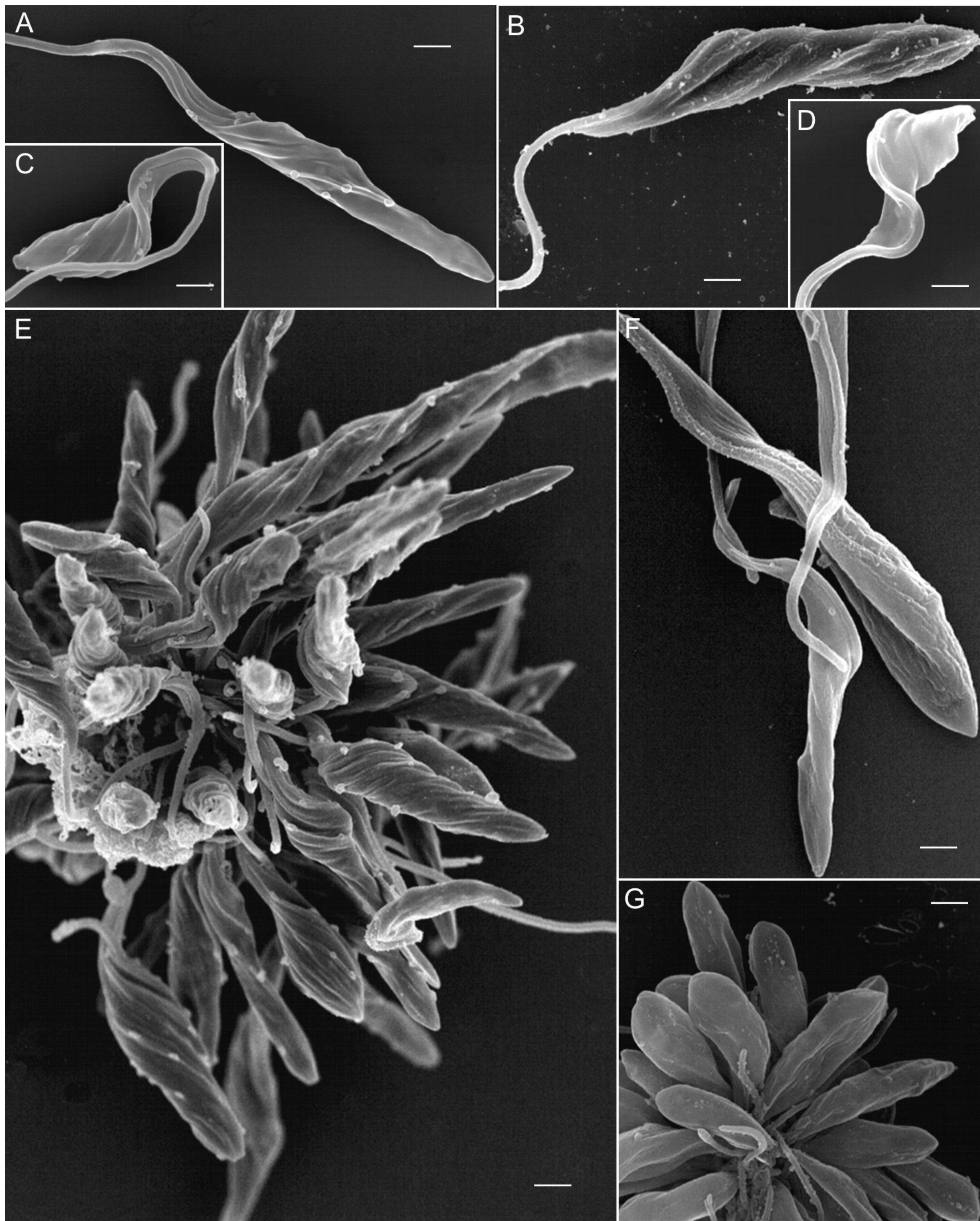

Fig. 5. : Scanning electron microscopy of trypanosomes from cultures: *Trypanosoma erneyi* (A-E), *T. c. marinkellei* (F) and *T. dionisii* (G). Elongated epimastigotes (A, B, F), metacyclic trypomastigotes from stationary culture (C, D), flagellates arranged in rosettes in the proliferative phase with large number of twisted epimastigotes (E). Rosettes of epimastigotes in culture of *T. dionisii.* Scale bars: 1μm

6. Uniqueness of the isolates from molossid bats

Their hosts, geographic origin, morphology, behaviour in culture, inability to infect mice and triatomine bugs, unique genetic polymorphisms on multiple genes, and phylogenetic positioning enabled the description of 4 new isolates from *Mopys condylurus* and 2 from *Tadarida* sp. as a new species of bat trypanosome within the subgenus *Schizotrypanum*. Accordingly, we are designating this isolates as *Trypanosoma* (*Schizotrypanum*) *erneyi* n. sp. (see Taxonomy).

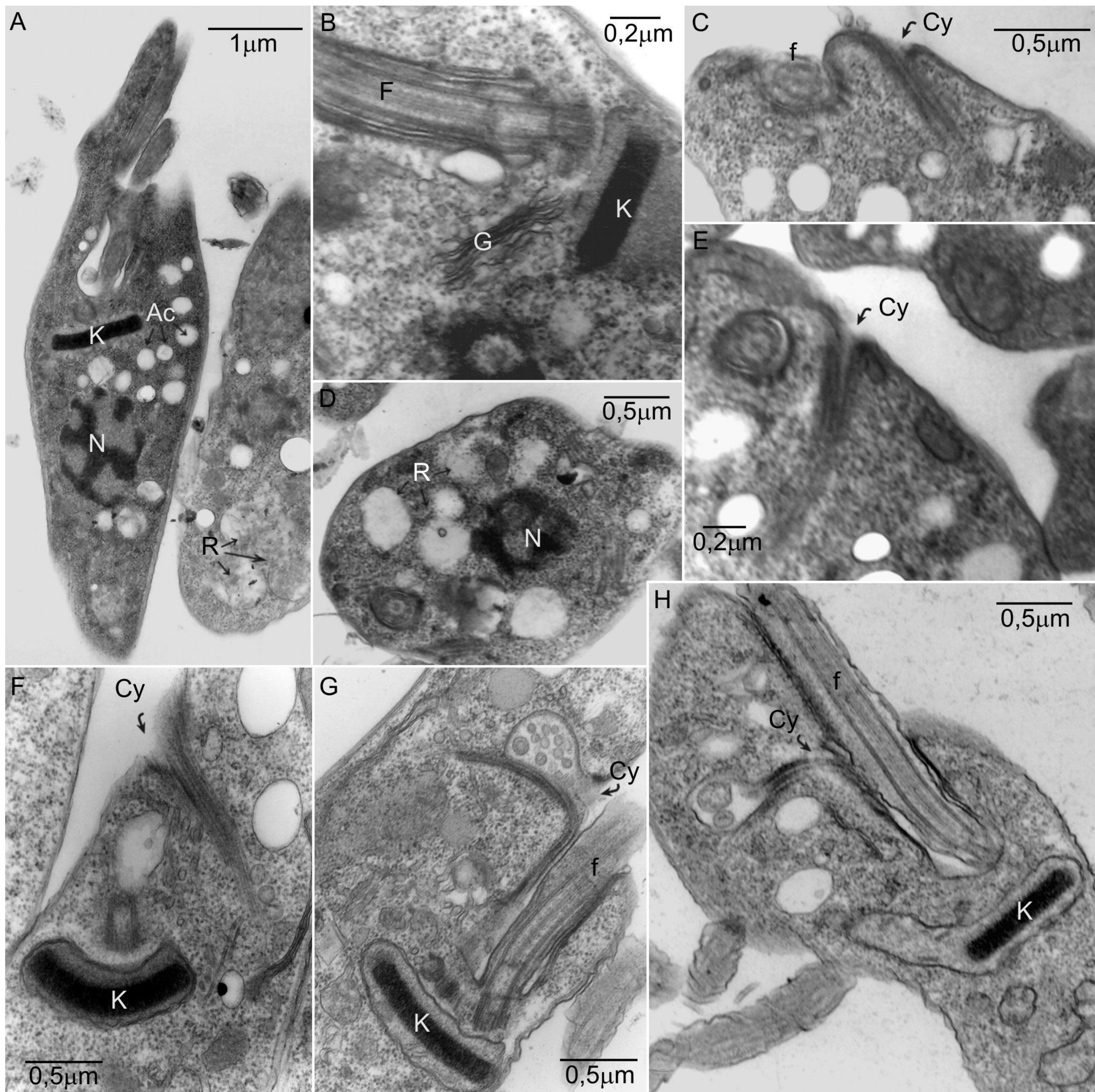

Fig 6: Transmission electron microscopy of *Trypanosoma erneyi* (A-E), *T. cruzi* (F), *T. dionisii* (G) and *T. c. marinkellei* (H). Longitudinal section showing the kinetoplast, nucleus and multiple acidocalcisomes and reservosomes (A); kinetoplast, flagellum and Golgi complex (B); transverse section showing various reservosomes (D); Subgenus *Schizotrypanum* characteristic arrangement of the compact kinetoplast and cytostome in *T. erneyi* (B, C, E), *T. cruzi* (F), *T. c. marinkellei* (H) and *T. dionisii* (G). Nucleus (N); kinetoplast (K), Golgi complex (G), reservosome (R), flagellum (f), acidocalcisomes (Ac), cytostome (Cy).

Taxonomical section

Description of a new species of the Phylum Euglenozoa Cavalier-Smith, 1981; class Kinetoplastea Honigberg, 1963; order Trypanosomatida (Kent, 1880) Hollande, 1952; family Trypanosomatidae Doflein, 1951: *Trypanosoma erneyi* Lima and Teixeira n. sp. Type material, hapantotype: culture TCC1946. Paratypes: cultures TCC1293, 1294, 1932, 1934 and 1936 whose hosts and collection sites are in Table 1. Cultures are deposited at the Trypanosomatid Culture Collection of the University of São Paulo, TCC-USP. Glass slides of the Giemsa-stained cultures are also kept at TCC-USP. Host: Chiroptera: Molossidae: *Mops condylurus*. Locality: Mozambique, province of Sofala, district of Chupanga (S18º02' E35º34'). Morphology: Epimastigotes and metacyclic trypanosomes from culture in TC-100 medium and amastigotes and trypomastigotes from HeLa cell cultures typical of *T. cruzi*-like (Fig.1). Diagnosis: Sequences deposited at Genbank under accession numbers listed in Table 1. 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 one of the common hosts of trypanosomes throughout the world and over 100 years have elapsed since the discovery of bat trypanosomes by Dionisii in 1909 (revised by Hoare, 1972), little is known about their genetic diversity and phylogenetic relationships, host ranges, vectors, life cycles and geographical distribution. Consequently, the taxonomy of these trypanosomes remains problematic. Phylogenetic studies of bat trypanosomes are fundamental for improving the taxonomy and understanding of the evolutionary history of *Schizotrypanum* and the origin of *T. cruzi,* which hypothetically could have evolved from a bat-restricted trypanosome (Stevens et al., 1999b; Hamilton et al., 2009; Cavazzana et al., 2010).

In this study, we isolated and characterised a new species of bat trypanosome from *Mops condylurus* and *Tadarida* sp. of Molossidae, captured in Mozambique in southeast Africa. Although the isolates were from two bat species captured in three distinct localities, they shared high similarity, even with respect to the highly polymorphic V7V8 SSU rRNA, ITS rDNA and SL sequences. Phylogenies based on the SSU rRNA, gGAPDH and Cyt b sequences showed similar topologies; the new isolates formed a new clade within the subgenus *Schizotrypanum,* which was closest to *T. c. marinkellei* followed by *T. dionisii*, with *T. cruzi* being the most divergent *Schizotrypanum* species. Together, conserved and polymorphic sequences of both nuclear and mitochondrial genes allowed the classification of these isolates as a new species of *Schizotrypanum,* which we designated *Trypanosoma erneyi* n. sp.

In a survey in Brazil, 1043 bats examined by haemoculture yielded an infectivity rate of ~13% resulting in 77 cultures of *Schizotrypanum* spp. from Vespertilionidae, Noctilionidae, Phyllostomidae and Molossidae; only two cultures were from molossids, and both were *T. dionisii* (Cavazzana et al., 2010). A survey of trypanosomes in blood smears from 427 bats in Kenya yielded a 21% rate of infectivity in bats, and *T. vespertilionis* was found in Emballonuridae and Vespertilionidae. Molossidae had the lowest overall prevalence of trypanosomes, even though it accounted for more than half of the bats captured (Woo & Hawkins, 1975). In the present study, we examined 25 molossids from Mozambique and found that 24% were infected by *Schizotrypanum* spp. using the sensitive method of haemoculture. The lack of trypomastigotes in blood of molossid bats examined in this study is indicative of low parasitemias typical of these species.

All isolates classified as *T. erneyi* n. sp. displayed morphological and growth characteristics compatible with *T. cruzi*-like. Metacyclic trypomastigotes of *T. erneyi* infected and developed as amastigotes within mammalian cells similarly to *T. cruzi*. However, these forms failed to experimentally infect Balb/c mice and were shown to be susceptible to human complement-mediated lysis, similarly to *T. dionisii* (Lima et al., in preparation). These findings suggested that *T. erneyi* n. sp. is a bat-restricted species. Comparative studies of *T. cruzi* and *T. cruzi*-like species that are non-infective to humans may improve the understanding of the evolution of the host range and virulence of *T. cruzi*. Knowledge on the host-parasite-vector interactions are crucial to interpreting the eco-biogeographical patterns of *Schizotrypanum* spp. However, we know nothing about the vector(s) of *T. erneyi* n. sp. Triatomines are absent in Africa, and similar to *T. dionisii* and *T. c. marinkellei*, *T. erneyi* was unable to experimentally infect these bugs, the vectors of *T. cruzi*. Cimicids, which are proven vectors of *T. dionisii* (Bower & Woo, 1981; Gardner & Molyneux, 1988) and usually associated to bats in Africa, can be vectors of *T. erneyi* n. sp.

In agreement with previous studies (Stevens et al., 1999a,b; Hamilton et al., 2007; Marcili et al., 2009; Cavazanna et al., 2010), our phylogenies evidenced a clade exclusive to *Schizotrypanum* spp., now constituted by *T. cruzi* (Brazil), *T. c. marinkellei* (Brazil), *T. dionisii* (Europe and Brazil) and *T. erneyi* (Africa). This clade clustered together with the clade harbouring *T. rangeli*, *T. vespertilionis,* and African trypanosomes from megabat, monkey and civet. The increasing number of trypanosomes in phylogenetic trees has changed the prevailing hypothesis regarding the origins and evolution of these trypanosomes. According to paleogeographical evidence, the divergence of the clades *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 on the periphery of the *T. cruzi* clade suggested an origin for *T. cruzi*, possibly in marsupials, on the southern super-continent comprising present day Antarctica, Australia and South America (~65 MYA) (Stevens et al., 1999a,b; 2001). However, the nesting of

trypanosomes from African mammals (monkey and civet) within the *T. cruzi* clade suggests that intercontinental transfer occurred after the continental separation, and bats most likely played a role in dispersion of these trypanosomes (Hamilton et al., 2009).

The understanding of the evolutionary history of bats is fundamental for evaluating the possible biogeographic scenarios that could account for the current distribution and phylogenetic relationships of trypanosomes infecting bats through the world. The evolutionary history of Chiroptera suffers from limited fossil record and incomplete molecular phylogenies. The suborder Yinpterochiroptera (megabats and rhinolophoid microbats) is exclusively of the Old World. Yangochiroptera comprises all other bats; three families of this suborder, Vespertilionidae, Molossidae and Emballonuridae have bats of the same genera, but not of the same species, in both the Old and New Worlds Earliest divergences among living bats, probably ~65 MYA in North America or Africa, was followed by a large radiation (~50 MYA) and diversification of bats in the Eocene. Despite the ability to fly long distances, bats are not capable of crossing the oceanic barriers nowadays separating the New and Old Words. Paleogeographical and phylogenetic evidence have suggested that the crossing of bats from the Old to the New World and vice-versa occurred through Beringia and was not possible after the middle Miocene (12 MYA) (Telling et al., 2005; Eick et al., 2005; Simmons et al., 2005; Stadelmann et al., 2007; Telling, 2009).

The new trypanosome species we describe here, *T. erneyi* n. sp, has only been isolated thus far from African molossid bats, although this may reflect insufficient sampling of molossids from other continents. Molossidae contains about 80 species of insectivorous bats and currently has a broad pantropical distribution: *Tadarida* is widespread throughout the world; *Mops* is present in Asia and Africa, but absent in the Americas. The oldest North American molossid fossil is from the middle Eocene, and both paleontological and phylogenetic data have suggested that molossids inhabited North and South America prior to the connection of these continents by the Panamanian Isthmus in the Pliocene (~3 MYA) (Czaplewski et al., 2003).

Regardless of their place of origin, trypanosomes are obligate parasites and must be carried by their bat hosts and/or by their large repertoire of ectoparasites (cimicids, hippoboscid 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. In this context, it is worth noting that a Caribbean Miocene amber contained, along with a fossil reduviid, *T. cruzi*-like trypanosomes and bat hairs (Poinar, 2005). The historical dispersion of bats also allowed to hypothesise that bats could have transferred trypanosomes from the New to Old World. Therefore, available data are consistent with both hypotheses *T. cruzi* could has evolved from bat trypanosomes or vice versa (Stevens et al., 1999a,b; 2001; Hamilton et al., 2007). Further analyses of the species diversity, host and

geographical range, phylogenetic relationships among the trypanosomes from bats and other mammals, and reliable molecular-clock model to estimate divergence times are required to infer the most likely hypothesis.

Undoubtedly, *Schizotrypanum* trypanosomes and bats are tightly united in a striking example of an ancient and intimate host-parasite partnership: there are no *Schizotrypanum* trypanosomes (except *T. cruzi*) in mammals other than bats. This relationship implies that the reconstruction of the shared history of bats and their trypanosomes may be useful for resolving the evolutionary history of bats. The existence of *T. erneyi* n. sp. in Africa, a continent known to harbour bats infected by *T. vespertilionis* and *T. dionisii*, sheds new light on the diversity, dispersion and evolutionary histories of the *Schizotrypanum* trypanosomes, providing new insights into the understanding of the origin and evolution of the human pathogen *T. cruzi*.

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Acta Tropica 109 (2009) 199–207

Contents lists available at ScienceDirect

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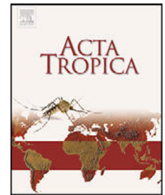
journal homepage: www.elsevier.com/locate/actatropica



Trypanosoma rangeli isolates of bats from Central Brazil: Genotyping and phylogenetic analysis enable description of a new lineage using spliced-leader gene sequences

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ARTICLE INFO

Article history:
Received 14 July 2008
Received in revised form
11 September 2008
Accepted 13 November 2008
Available online 20 November 2008

Keywords:
Trypanosoma rangeli
Bats
Rhodnius stali
Chiropteran parasites
Schizotrypanum
Phylogeny
Genotyping
Trypanosome diversity
Spliced leader gene

ABSTRACT

Trypanosoma rangeli infects several mammalian orders but has never confidently been described in Chiroptera, which are commonly parasitized by many trypanosome species. Here, we described trypanosomes from bats captured in Central Brazil identified as *T. rangeli*, *T. dionisii*, *T. cruzimarinkellei* and *T. cruzi*. Two isolates, Tra643 from *Platyrrhinus lineatus* and Tra1719 from *Artibeus planirostris* were identified as *T. rangeli* by morphological, biological and molecular methods, and confirmed by phylogenetic analyses. Analysis using SSU rDNA sequences clustered these bat trypanosomes together with *T. rangeli* from other hosts, and separated them from other trypanosomes from bats. Genotyping based on length and sequence polymorphism of PCR-amplified intergenic spliced-leader gene sequences assigned Tra1719 to the lineage A whereas Tra643 was shown to be a new genotype and was assigned to the new lineage E. To our knowledge, these two isolates are the earliest *T. rangeli* from bats and the first isolates from Central Brazil molecularly characterized. *Rhodnius stali* captured for this study was found infected by *T. rangeli* and *T. cruzi*.

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1. Introduction

Chiropterans of different families and genera are commonly infected by *Trypanosoma* species. Bats with different feeding habits are hosts of trypanosomes, although the insectivorous are the more commonly infected. Over 30 species of trypanosomes have been recorded in more than a hundred species of bats (Molyneux, 1991). In the American continent, trypanosomes classified as *T. cruzi*-like were reported in several species of Chiroptera from several countries including Costa Rica, Colombia, Venezuela, Argentina and Brazil (Hoare, 1972; Marinkelle, 1976; Molyneux, 1991). In Brazil, over 40 species of bats were found infected with more than 10 species of trypanosomes. Surveys of trypanosomes of bats in Brazil began in the Amazonian region (Dias, 1936; Deane, 1961) followed by studies in the Southeast (Funayama and Barretto, 1970, 1973; Teixeira et al., 1993), Northeast (Alencar et al., 1976; Pinto and Bento, 1986) and South (Steindel et al., 1998). Despite the vast knowledge regarding the occurrence of trypanosomes in bats, little is known about their natural vectors and development in both vertebrate and invertebrate hosts.

Most trypanosome species described in bats belong to the subgenera *Schizotrypanum* and *Megatrypanum*. Very few described species belong to the subgenus *Herpetosoma* to which *Trypanosoma rangeli* was traditionally classified (Hoare, 1972; D'Alessandro and Saravia, 1999; Marinkelle, 1976; Molyneux, 1991). However, phylogenetic analyses indicated that the subgenus *Herpetosoma* is polyphyletic and strongly supported division of this group into two monophyletic lineages related to *T. rangeli* and *T. lewisi* (Maia da Silva et al., 2004b, 2007). The existing reports on species of *Herpetosoma* in bats have described *T. lineatum* (*T. lewisi*-like) in Venezuela, *T. lewisi* in Puerto Rico, *T. longiflagellum* in Iraq and *T. aunauwa* in New Guinea (Fox and Thillet, 1962; Ewers, 1974; Marinkelle, 1977). Trypanosomes morphologically resembling *T. rangeli* infecting bats have only been described in Colombia (Marinkelle, 1976).

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doi:10.1016/j.actatropica.2008.11.005

The subgenus *Schizotrypanum*, which is closely related to *Herpetosoma*, comprises a monophyletic assemblage formed by *T. cruzi* and species exclusive of bats. These species, which are closely phylogenetically related and morphologically indistinguishable, are generically named *T. cruzi*-like (*T. cruzi marinkellei*, *T. dionisii* and *T. vespertilionis*) (Stevens et al., 1999, 2001; Barnabè et al., 2003). These species are the most studied due to their high prevalence and morphological, biochemical and phylogenetic affinities with *T. cruzi* (Baker et al., 1978; Schottelius et al., 1983; Petry et al., 1986; Barnabè et al., 2003).

T. rangeli infects humans, domestic and sylvatic mammals from Central to South America where it shares overlapping geographic areas, mammalian hosts and vectors with *T. cruzi*. A high prevalence of *T. rangeli* in humans has been reported in Central America and northwestern South America (D'Alessandro and Saravia, 1999; Guhl and Vallejo, 2003; Vallejo et al., 2003). In Brazil, only three human cases have been reported in the Amazon region (Coura et al., 1996), in contrast with the very high prevalence of *T. rangeli* in sylvatic mammals and triatomines of the genus *Rhodnius* (Miles et al., 1983; Maia da Silva et al., 2004a,b, 2007, 2008). In Brazil, outside the Amazonian region, *T. rangeli* was reported in rodents and *Panstrongylus megistus* in Southern region, in *Didelphis marsupialis* and *Rhodnius neglectus* in Central and Southeastern regions, and in *Rhodnius nasutus* in the Northeast (Steindel et al., 1991; Ramirez et al., 2002; Gurgel-Gonçalves et al., 2004; Dias et al., 2007).

Different from *T. cruzi*, whose development is restricted to the gut of its vector that are triatomines of several genera, *T. rangeli* multiplies in the gut and completes its development in the salivary glands of *Rhodnius* spp., apparently, the exclusive vectors of this species. Behaviour of *T. rangeli* from distinct geographical origin varied according *Rhodnius* species suggesting a tight evolutionary relationship between *T. rangeli* lineages and their sympatric vectors (Guhl and Vallejo, 2003; Vallejo et al., 2003; Maia da Silva et al., 2004b; Urrea et al., 2005). Comparison of *Rhodnius* and *T. rangeli* phylogenies revealed a total concordance between *T. rangeli* lineages and complexes of their vector species, suggesting a long history of these host–parasite assemblages (Maia da Silva et al., 2007).

Spliced-leader (SL) RNA sequences are useful as taxonomic and diagnostic tools for genera, species and lineages of trypanosomatids (Serrano et al., 1999; Teixeira et al., 2000), and are valuable tools for evaluating polymorphisms and genetic relatedness among closely related trypanosomes (Fernandes et al., 2001; Ventura et al., 2001) including lineages of *T. rangeli* (Grisard et al., 1999; Maia da Silva et al., 2007). *T. rangeli* has a highly conserved 5S rRNA gene inserted into the intergenic region as also observed in *T. conorhini*, *T. desterrensis* and *T. vivax* (Stevens et al., 1999; Grisard et al., 2003; Ventura et al., 2001).

In the present study, we investigated trypanosome infection in bats from the State of Mato Grosso do Sul, Central Brazil. Two isolates of *T. rangeli* were found among 18 trypanosome isolates from bats. These isolates were characterized by morphological analysis, behaviour in culture, mice and triatomines, and by molecular methods. Phylogenetic relationships among *T. rangeli* isolates from bats and other hosts representative of all previously described lineages and relationships with other bat trypanosomes were inferred using SSU rDNA and SL gene sequences.

2. Material and methods

2.1. Isolation, growth and identification of trypanosomes from bats in Central Brazil

Bats were captured in the State of Mato Grosso do Sul, Central Brazil in neighbouring areas of the Pantanal (Miranda) and Cerrado (Aquidauana and Bonito) biomes (Fig. 1), from 2003 to 2007, according to recommendations of the Brazilian Institute for the Environment (IBAMA). Bats were anesthetized and blood samples collected by heart puncture were inoculated in BAB-LIT medium as described previously (Maia da Silva et al., 2004a). Eighteen cultures of trypanosomes from bats captured in the two studied areas were characterized by morphological and molecular analysis (Table 1).

2.2. Light microscopy and molecular diagnosis of bat trypanosomes

For morphological analysis by light microscopy, smears of logarithmic and stationary phase cultures of trypanosomes from bats were fixed in methanol and Giemsa-stained as described previously (Maia da Silva et al., 2004a). Phenol-chloroform extracted DNA from cultured trypanosomes was used as templates for PCR amplifications. Molecular diagnosis of the bat isolates was performed using a PCR assay that distinguishes between *T. rangeli* and *T. cruzi* (Souto et al., 1999; Fernandes et al., 2001) and a PCR specific for *T. rangeli* (Tra625) (Maia da Silva et al., 2004a). Identification of bat trypanosomes of the subgenus *Schizotrypanum* (*T. c. marinkellei* and *T. dionisii*) was accomplished by V7-V8 SSU rDNA barcoding as described previously (Maia da Silva et al., 2004b; Rodrigues et al., 2006; Ferreira et al., 2007).

2.3. Behaviour and morphology of bat trypanosomes in mice and triatomines

The following species of *Rhodnius* were used for behaviour analysis of bat isolates of *T. rangeli*: *R. prolixus* (Venezuela), *R. robustus* (subpopulation II from West Brazillian Amazonia) and *R. neglectus* (Southeastern Brazil). Fifth instar nymphs, 20–30 of each species, were infecting by injecting stationary cultures from *T. rangeli* isolates into the coelomic cavity through the connexivum as describe before (Tobie, 1968), and fed on mice every 15 days. About 10 triatomines of each species were dissected 20, 30, and 60 days post-inoculation (p.i.). Balb/c mice were inoculated (i.p.) with stationary-phase cultures of bat trypanosomes containing metacyclic trypomastigote forms (10^5/animal). Mice blood samples were examined weekly up to 30 days using the microhematocrit method and by hemoculture after the 30th day post-inoculation. Mice infected with bat isolates of *T. rangeli* were used for oral infection of additional groups of 15-20 nymphs of *Rhodnius* spp., which were fed on mice and examined at 20 and 60 days p.i.

Fresh blood samples from infected mice and from the haemolymph (obtained by section of the triatomine legs), salivary glands and digestive tube of dissected triatomines were examined by phase contrast microscopy. Samples positive for trypanosome were smeared on a glass slide, fixed in methanol and Giemsa-stained for visualization by light microscopy.

2.4. Morphological and molecular diagnosis of *Rhodnius* spp. and isolation of trypanosomes from this species

Triatomines were collected in arboreal ecotopes in sylvatic and peridomestic environments in the area where bats were captured. Identification of triatomines was initially based on morphology according to Lent and Wygodzinsky (1979). Molecular diagnosis of triatomines was performed by sequencing the 16S rDNA gene fragments generated by PCR amplification using the following primers: TRIAT16S-F (5′CGT GCT AAG GTA GCA TAA T3′) and TRIAT16S-R (5′AAG GTC GAA CAG ACC TAG T3′). The reaction was cycled 30 times as follows: 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C with an initial cycle of 4 min at 94 °C and a final cycle of 10 min at 72 °C. Sequences obtained in this study were deposited in Gen-

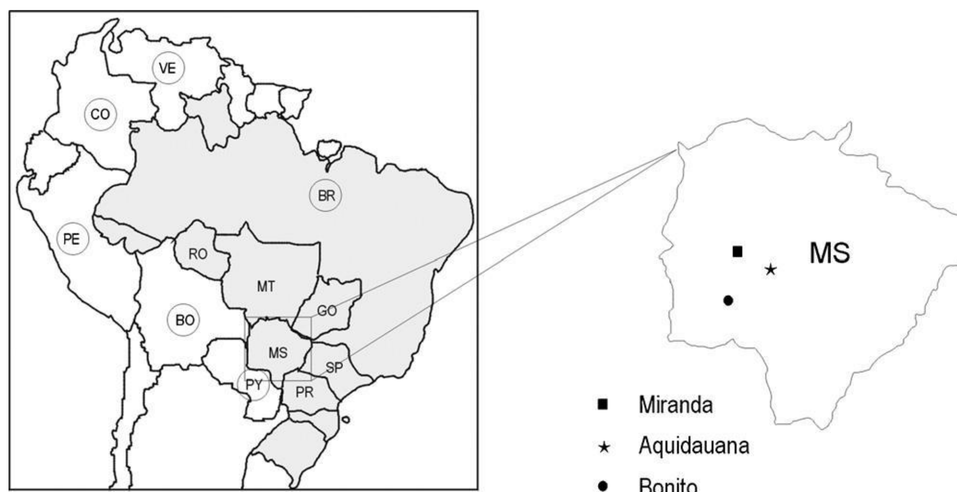

Fig. 1. Geographic origin of trypanosomes isolated from bats and from *R. stali* in this study. CO, Colombia; VE, Venezuela; PE, Peru; BO, Bolivia; PY, Paraguay; BR, Brazil. Brazilian states: RO, Rondônia; MT, Mato Grosso; MS, Mato Grosso do Sul; GO, Goiás; SP, São Paulo; PR, Paraná.

Bank (accession numbers) under the following accession numbers: *Rhodnius stali* 411 (EU867801) and *R. stali* 412 (EU867802). Field-collected triatomines were individually dissected and their gut and salivary glands removed, gently squeezed over glass slides and examined by direct microscopic observation. Samples positive for trypanosome using microscopy were inoculated in BAB-LIT medium for parasite isolation as described previously (Maia da Silva et al., 2004a).

2.5. Genotyping of T. rangeli and T. cruzi isolates

Genotyping of *T. rangeli* isolates was performed using primers TraSL1 and TraSL2 (TraSL-PCR) designed to amplify *T. rangeli*-specific SL intergenic spacer sequences that are variable in size according to lineages of *T. rangeli*, as described by Maia da Silva et al. (2007). Genotyping of *T. cruzi* isolates was performed using a PCR assay based on mini-exon gene sequences (Fernandes et al., 2001).

2.6. Sequencing, barcoding and phylogenetic inferences using SSU rDNA and spliced-leader gene sequences

The V7-V8 region of SSU rDNA from the bat isolates was amplified and used for species identification (barcoding) as performed previously (Maia da Silva et al., 2004b; Rodrigues et al., 2006; Ferreira et al., 2007). A phylogenetic tree was inferred using aligned sequences from the isolates identified as *T. rangeli* (Tra643 and Tra1719), and from selected bat isolates classified as *T. cruzi*, *T. c. marinkellei* and *T. dionisii*. SL repeats were amplified using primers RSL1 and RSL2, and sequences were determined for the whole SL repeat (987 bp) of *T. rangeli* Tra643, or only for the transcribed region for other bat isolates as described previously (Maia da Silva et al., 2007). Amplified DNA sequences from both SSU rDNA and SL genes were cloned and 3–5 clones from each isolate were sequenced and aligned with sequences from other *T. rangeli* isolates and other species of bat trypanosomes from GenBank (Table 1). Alignments were done using ClustalX and manually refined. Maximum parsimony (MP) and maximum likelihood (ML) analyses were carried out using a heuristic search strategy and the default options of PAUP. ML parameters were optimized using the hierarchical likelihood test in Modeltest and bootstrap analyses (100 replicates) done as described previously (Maia da Silva et al., 2007; Ferreira et al., 2007).

3. Results

3.1. Growth in culture, morphological and molecular diagnosis of bat trypanosomes

The majority of trypanosomes from bats characterized in this study presented growth and morphology characteristics of *Schizotrypanum* spp., except the isolates Tra643 and Tra1719, which presented long and slender epimastigotes (Fig. 2A) and short metacyclic trypomastigotes (Fig. 2B) typical of *T. rangeli*.

All bat isolates were tested using the PCR assay (Tra625-PCR) specific for *T. rangeli* (Maia da Silva et al., 2004a). This method generated fragments only for isolates Tra643 and Tra1719, and for *T. rangeli* used as positive control (Fig. 3A). The PCR developed to distinguish *T. rangeli* from *T. cruzi* (Souto et al., 1999) confirmed the identification of these two isolates as *T. rangeli*. However, this method generated DNA fragments of similar length for all *Schizotrypanum* species, failing to separate *T. cruzi*, *T. c. marinkellei* and *T. dionisii* (Fig. 3B). The PCR based on the mini-exon gene (Fernandes et al., 2001) also confirmed the two *T. rangeli* isolates from bats (Tra643 and Tra1719), and disclosed three *T. cruzi* isolates (Tc312, Tc480 and TC499). This PCR yielded negative results for the remaining bat isolates as well as for *T. c. marinkellei* and *T. dionisii* used as controls (Fig. 3C).

3.2. Behaviour of T. rangeli isolates from bats in experimentally infected Rhodnius spp. and in mice

The *T. rangeli* isolate Tra643 classified into the new lineage E was evaluated regarding its behaviour in triatomine bugs infected by intracoelomic and oral routes. After 30 days of infection by intracoelomical route, long and slender epimastigotes forming huge masses were present in the haemolymph of *R. robustus* and *R. neglectus* (Fig. 2C). Feeding *Rhodnius* spp. on mice infected with Tra643 did not produce flagellates in the haemolymph. In the digestive tube of *R. robustus* and *R. neglectus* this isolate yielded small number of epimastigotes typical of *T. rangeli* after (Fig. 2D). Flagellates were never observed in the salivary glands of triatomines infected with Tra643 by any of the above routes.

Balb/c mice infected with trypomastigotes from stationary cultures of bat isolates of *T. rangeli* presented low parasitemias, nevertheless detectable by microhaematocrit and hemoculture. The morphology of blood trypomastigotes of isolates Tra643 and Tra1719 was typical of *T. rangeli* (Fig. 2E) whereas blood trypo-

Table 1
Trypanosomes from bats isolated in this study and species/isolates used for analysis of SL and SSU rDNA gene sequence.

TryCC	*Trypanosoma* isolate	Host species		Geographic origin	GenBank accession number	
					SL	SSU rDNA
	T. rangeli lineage A					
024	H8GS	Human	*Homo sapiens*	Honduras	AF083351	AY491744
369	ROma 01	Opossum	*Didelphis marsupialis*	Brazil (RO)	EF071554	AY491748
021	Choachi	Triatomine	*Rhodnius prolixus*	Colombia	EF071557	AJ012414
701	ROR-62	Triatomine	*Rhodnius robustus* II	Brazil (RO)	EF071562	EF071578
1719	Tra1719	Bat	*Artibeus planirostris*	Brazil (MS) Bonito	[a]EU867799	[a]EU867813
	T. rangeli lineage B					
010	*T. legeri*	Ant eater	*Tamandua tetradactyla*	Brazil (PA)	EF071548	AY491769
086	AM80	Human	*Homo sapiens*	Brazil (AM)	EF071547	AY491766
207	AE-AAA	Monkey	*Cebuella pygmaea*	Brazil (AC)	EF071564	AY491752
416	2495	Monkey	*Alouatta stramineus*	Brazil (AM)	EF071566	AY491760
	T. rangeli lineage C					
014	PG	Human	*Homo sapiens*	Panama	EF071568	AJ012416
328	1625	Human	*Homo sapiens*	El Salvador	EF071569	AY491738
[b]	*T. leeuwenhoeki*	Sloth	*Choloepus didactylus*	Panama	AJ012420	AJ012412
[b]	RGB	Dog	*Canis familiaris*	Colombia	AJ01419	
	T. rangeli lineage D					
023	SC58	Rodent	*Echimys dasythrix*	Brazil (SC)	AF083350	AY491745
	T. rangeli lineage E					
643	Tra643	Bat	*Platyrrinus lineatus*	Brazil (MS) Miranda	[a]EU867800	[a]EU867803
	T. cruzi (lineage)					
30	G (TCI)	Marsupial	*Didelphys marsupialis*	Brazil (AM)		AF239981
[b]	Silvio (TCI)	Human	*Homo sapiens*	Brazil (AM)	X62674	
34	Y (TCII)	Human	*Homo sapiens*	Brazil (SP)		AF301912
[b]	Tulahuen (TCII)	Triatomine	*Triatoma infestans*	Chile	X00632	
312/480	(TCII)	Bat	*Noctilio albiventris*	Brazil (MS) Miranda		[a]EU867804
499	(TCII)	Bat	*Myotis nigrans*	Brazil (MS) Miranda		
1107–1109/1116	(TCI)	Triatomine	*R. stali*	Brazil (MS) Miranda		[a]EU867805/EU867806/ EU867807
	T. c. marinkellei					
[b]	B7	Bat	*Phyllostomus discolor*	Brazil (BA)		AJ009150
456		Bat	*Desmodus rotundus*	Brazil (MS) Miranda		
478		Bat	*Artibeus planirostris*	Brazil (MS) Miranda		
626/627		Bat	*Artibeus planirostris*	Brazil (MS) Aquidauana		
1089/1093		Bat	*Artibeus planirostris*	Brazil (MS) Miranda	[a]EU867797/ EU867798	[a]EU867808/EU867809
1702/1708		Bat	*Artibeus planirostris*	Brazil (MS) Bonito		
	T. dionisii					
[b]	P3	Bat	*Pipistrellus pipistrellus*	England	AJ250744	AJ009151
[b]	PJ	Bat	*Pipistrellus pipistrellus*	Belgian		AJ009152
309		Bat	*Myotis nigrans*	Brazil (MS) Miranda		[a]EU867810
633		Bat	*Sturnira lilliun*	Brazil (MS) Miranda		[a]EU867812
454		Bat	*Desmodus rotundos*	Brazil (MS) Miranda	[a]EU867796	[a]EU867811
1700/1706		Bat	*Carollia perspicilatta*	Brazil (MS) Bonito		
	Other bat trypanosomes					
[b]	*T. desterrensis*	Bat	*Eptesicus furinalis*	Brazil (SC)	AF124146	
[b]	*T. hastatus*	Bat	*Phyllostomus hastatus*	Brazil (SC)	AF116567	
[b]	*T. vespertilionis*	Bat	*Pipistrellus pipistrellus*	England		AF116564
[b]	*T sp 60*	Bat	*Rousettus aegyptiacus*	Gabon – Africa		AJ012418

TryCC, code number of cultures in the Trypanosomatid Culture Collection of the Department of Parasitology, University of São Paulo.
[a] Sequences determined in this study and deposited in the GenBank.
[b] Sequences retrieved from GenBank. Brazilian States: AC, Acre; AM, Amazonia; BA, Bahia; PA, Para; SC, Santa Catarina; SP, São Paulo; MS, Mato Grosso do Sul.

mastigotes of isolates 480 and 499 were typical of *T. cruzi* (data not shown). *T. c. marinkellei* and *T. dionisii* isolates were not infective for mice.

3.3. Identification of Rhodnius stali and characterization of trypanosomes infecting this species

Fourteen specimens of *Rhodnius* manually collected from arboreal ecotopes were initially classified as *R. pictipes* by morphological parameters. However, by comparing 16S rDNA gene sequences employed for molecular identification, three selected specimens appeared to be identical to each other and almost identical (∼98% sequence similarity) to a sequence from *R. stali* retrieved from GenBank (AY035437).

Seven specimens of *R. stali* were positive for trypanosomes by microscopic examination of gut contents. Most triatomines presented forms typical of *T. cruzi* yielding five cultures identified as *T. cruzi* by molecular diagnosis (Souto et al., 1999; Fernandes et al., 2001). The gut of one specimen of *R. stali* showed very long epimatigotes typical of *T. rangeli* (Fig. 2D). Unfortunately, its culture was lost due to the small number of flagellates and heavy bacterial and fungal contamination.

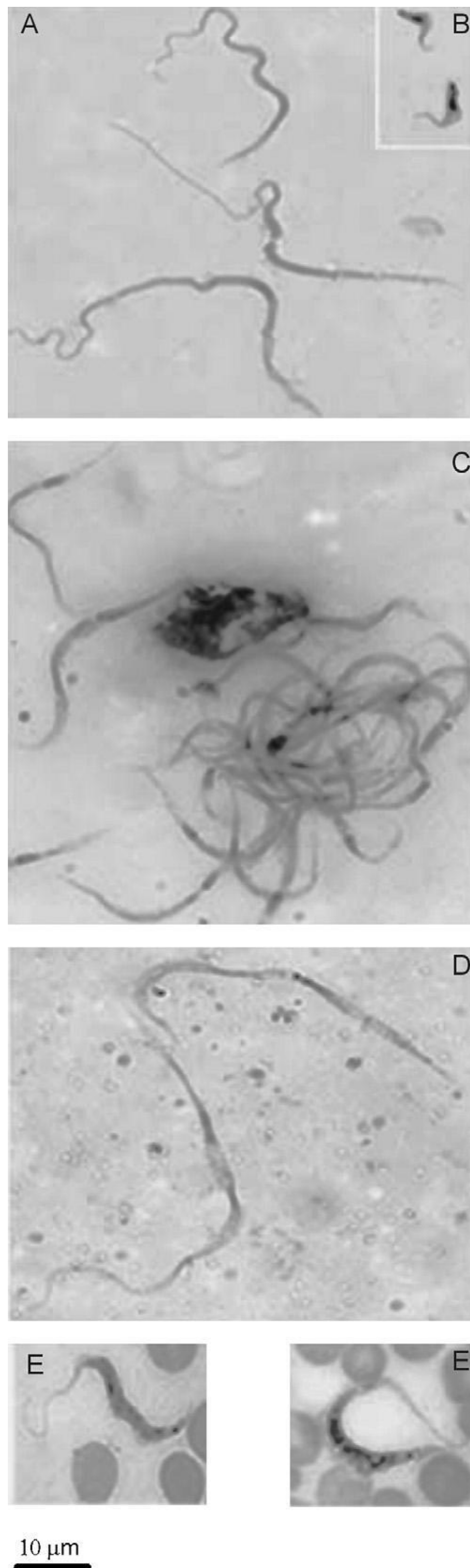

Fig. 2. Microscopy of Giemsa-stained forms of *T. rangeli*. Culture smears of the bat isolate Tra643 showing epimastigote (A) and metacyclic trypomastigote (B). Smears of *R. neglectus* experimentally infected with the isolate Tra643 showing a mass of epimastigotes in the haemolymph (C) and long epimastigotes in the digestive tube (D). Trypomastigote from blood of mice experimentally-infected with culture forms of *T. rangeli* Tra643 (E).

3.4. Different genotypes of T. rangeli and T. cruzi infect bats and R. stali in the State of Mato Grosso do Sul

High genetic diversity was found among trypanosome isolates from bats captured in the small studied area of the State of Mato Grosso do Sul (Fig. 1). Genotyping of *T. rangeli* isolates using TraSL-PCR (Maia da Silva et al., 2007) confirmed two genotypes present in bats. DNA from the isolate Tra1719 generated a fragment compatible to lineage A. The isolate Tra643 generated a fragment of different length that could not be associated to any previously defined lineages (A–D) of *T. rangeli* and was, thus, assigned to a new genotype (E) (Fig. 4A). Genotyping of *T. cruzi* isolates allowed to assign bat isolates to lineage TCII (*T. cruzi* II) while the isolates of *R. stali* were assigned to lineage TCI (*T. cruzi* I) (Fig. 3C).

3.5. Barcoding of bat trypanosomes and phylogenetic relationships of T. rangeli with other bat trypanosomes

For barcoding of bat trypanosomes we determined sequences from the V7-V8 region of SSU rDNA and compared with sequences from trypanosomes deposited in GenBank. This method allowed species identification of bat trypanosome isolates as follow: two isolates of *T. rangeli*, three of *T. cruzi*; eight of *T. c. marinkellei* and five of *T. dionisii* (Table 1). For phylogenetic inferences, sequences from the two *T. rangeli* isolates from bats (Tra643 and Tra1719) were aligned with sequences from isolates representative of all trypanosomes found to infect bats in the studied area: *T. dionisii* (isolates Tdi454, Tdi309 and Tdi633), *T. c. marinkellei* (Tcm1089 and Tcm1093) and *T. cruzi* (Tc480). *T. cruzi* isolates from *R. stali* (Tc1107, Tc1109 and Tc1116) were also included in the alignment (Table 1). Similar and strongly supported branching patterns were generated in phylogenetic trees using both ML and MP methods.

All bat isolates obtained in this study previously classified as *T. dionisii* clustered together with the European isolates P3 (England) and PJ (Belgium) of this species corroborating their classification. Similarly, isolates of *T. c. marinkellei* from the State of Mato Grosso do Sul clustered together with an isolate of this species from the State of Bahia, Northeastern Brazil (Barnabè et al., 2003). *T. cruzi* from bats and from *R. stali* obtained in this study clustered with reference isolates of this species belonging to distinct lineages (Fig. 5). The two bat isolates diagnosed as *T. rangeli* were nested within the clade *T. rangeli*, comprising closely related organisms (∼99% of sequence similarity) clearly separated from all trypanosomes of the subgenus *Schizotrypanum*: *T. dionisii* (∼83% of similarity), *T. c. marinkellei* (∼79%) and *T. cruzi* (∼78%). Closest to *T. rangeli* was the bat trypanosome *T. vespertilionis*, a species not confirmed within *Schizotrypanum* in this and in previous phylogenetic analysis (Stevens et al., 1999, 2001) (Fig. 5).

3.6. Genetic relatedness among T. rangeli isolates from bats and other hosts, and other bat trypanosomes inferred using SL gene sequences

Sequences of variable intergenic regions of the SL gene amplified by TraSL-PCR were determined for the two *T. rangeli* isolates from bats and compared with isolates from other hosts representative of all phylogenetic lineages. According to the generated dendrogram (Fig. 4B) and similarity matrix constructed using aligned intergenic sequences, the isolate Tra1719 was confirmed as belonging to lineage A whereas the isolate Tra643 represent a new genotype separated from all other by relevant divergences: C (∼11%); A (∼14%), D (∼17%) and B (∼36%). Sequence divergence between the previously defined lineages of *T. rangeli* ranged from ∼10 (A and D) to ∼33% (A and B). Taking into account polymorphisms on SL intergenic region evidenced by genotyping and by sequence

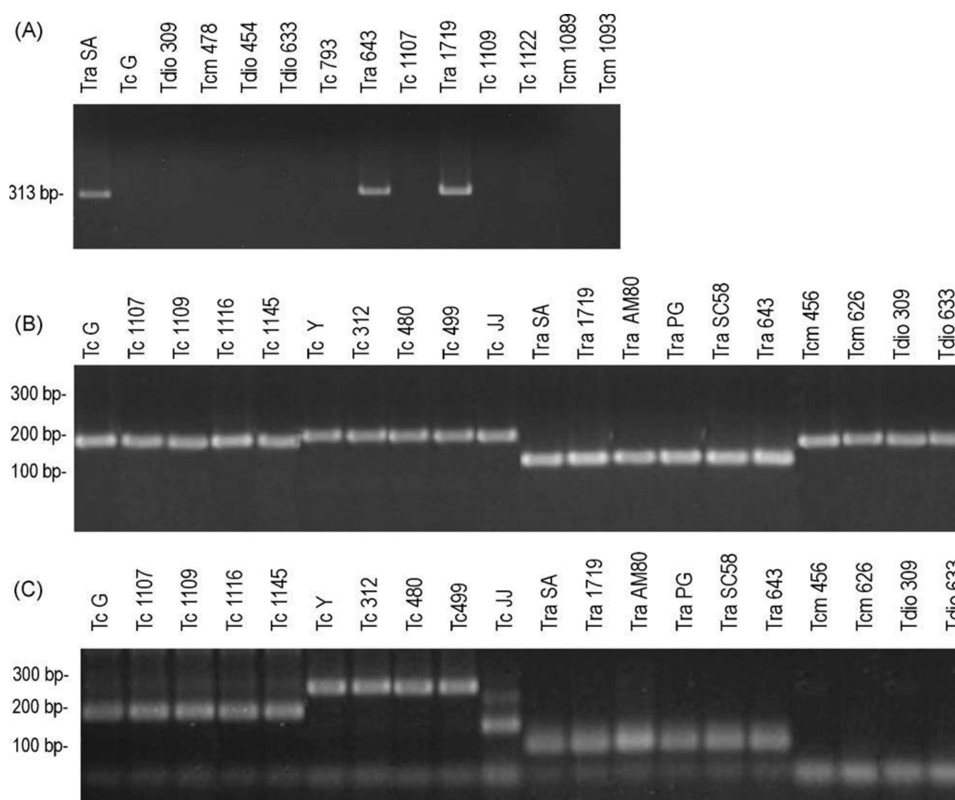

Fig. 3. Agarose gel (2%) showing amplified fragments stained with ethidium bromide generated by the following PCR assays: (A) *T. rangeli*-specific Tra625-PCR (Maia da Silva et al., 2004a), (B) PCR based on ribosomal sequences for simultaneous detection of *T. cruzi* and *T. rangeli* (Souto et al., 1999) and (C) PCR based on mini-exon gene markers for genotyping of *T. cruzi* lineages (Fernandes et al., 2001).

analysis, in addition to ecogeographical aspects, we propose this new genotype (Tra643) to be considered a new lineage (E) of *T. rangeli*.

Sequences of the transcript region of the SL gene were determined for the *T. rangeli* isolate Tra1719 from bat and for *T. c. marinkellei* and *T. dionisii* obtained in this study. These conserved sequences allowed accurate alignment with those from the bat isolate Tra643, *T. rangeli* isolates of other hosts, isolates of *T. cruzi*, *T. desterrensis* and *T. hastatus* from Brazilian bats, and *T. dionisii* (P3 and PJ) from Europe (Table 1). Conserved SL transcript sequences of the bat isolates Tra1719 and Tra643 shared ∼99% sequence similarity with isolates of lineages A, C and D, and diverged ∼2% from isolates of lineage B. In contrast, large divergences separated all *T. rangeli* isolates from other bat trypanosomes: *T. c. marinkellei* (∼35%); *T. dionisii* (∼30%); *T. desterrensis* (∼27%) and *T. hastatus* (∼44%). *T. dionisii* from Europe clustered with isolates of this species from Brazil although they were separated by significant divergence (∼23%). *T. desterrensis* from a Brazilian bat captured in Southern region nested within the clade *T. dionisii* separated by ∼8% of sequence divergence from isolates of Mato Grosso do Sul (Fig. 4C).

3.7. Characterization of the SL gene repeat of the isolate Tra643 and comparison with other T. rangeli isolates

For comparison of whole SL gene repeats of *T. rangeli* lineage E and lineages A–D previously characterized (Maia da Silva et al., 2007), full-length SL gene sequences (987 bp) from the bat isolate Tra643 was determined and aligned with sequences from isolates of all other lineages. The exon and intron sequences of Tra643 are identical to those of lineages A, C and D (the exon of lineage B differs in two nucleotides). The SL repeat unity of the isolate Tra643 assigned to lineage E possess the 5S rRNA gene (100% similarity among all lineages) inserted within the intergenic sequence, as previously noted for all other lineages of *T. rangeli* (Stevens et al., 1999; Grisard et al., 1999; Maia da Silva et al., 2007).

4. Discussion

In the present study, we isolated and characterized two cultures of *T. rangeli* from frugiverous/insectivorous bats; *Platyrrhinus lineatus* and *Artibeus planirostris*, both captured in the State of Mato Grosso do Sul, Central Brazil. These isolates (Tra643 and Tra1719) were identified as *T. rangeli* by PCR assays, morphology, and behaviour in culture, triatomines and mice. Phylogenetic analysis using SSU rDNA sequences positioned these bat trypanosomes together with isolates of *T. rangeli* from other wild mammals, man and triatomine bugs, clearly separated from *T. cruzi*, *T. c. marinkellei* and *T. dionisii* isolated from bats captured in the same geographic location. The phylogenetic relationships of bat trypanosomes are far from being understood due to the limited number of species studied (Stevens et al., 2001; Barnabè et al., 2003).

This is the first indisputable description of *T. rangeli* infecting bats. Previously reported *T. rangeli*-like found in bats in Colombia (Marinkelle, 1966) was neither cultured nor confirmed as *T. rangeli*. Frequent identification of *T. rangeli* infecting several mammalian orders, including primates, marsupials, rodents and xenartrans in contrast to lack of *T. rangeli* in chiropterans suggested that bats are not commonly infected by this species. This is very intriguing because trypanosomes of the subgenera *Schizotrypanum* and *Megatrypanum* are highly prevalent and widespread in bats from the New and Old Worlds (Molyneux, 1991). Moreover, it was experimentally demonstrated that captive bats became infected after being bitten by *T. rangeli* infected triatomines (Thomas et al., 2007). We are currently performing a survey of bat trypanosomes in several Brazilian biomes, including Amazonia where *T. rangeli* is highly prevalent in

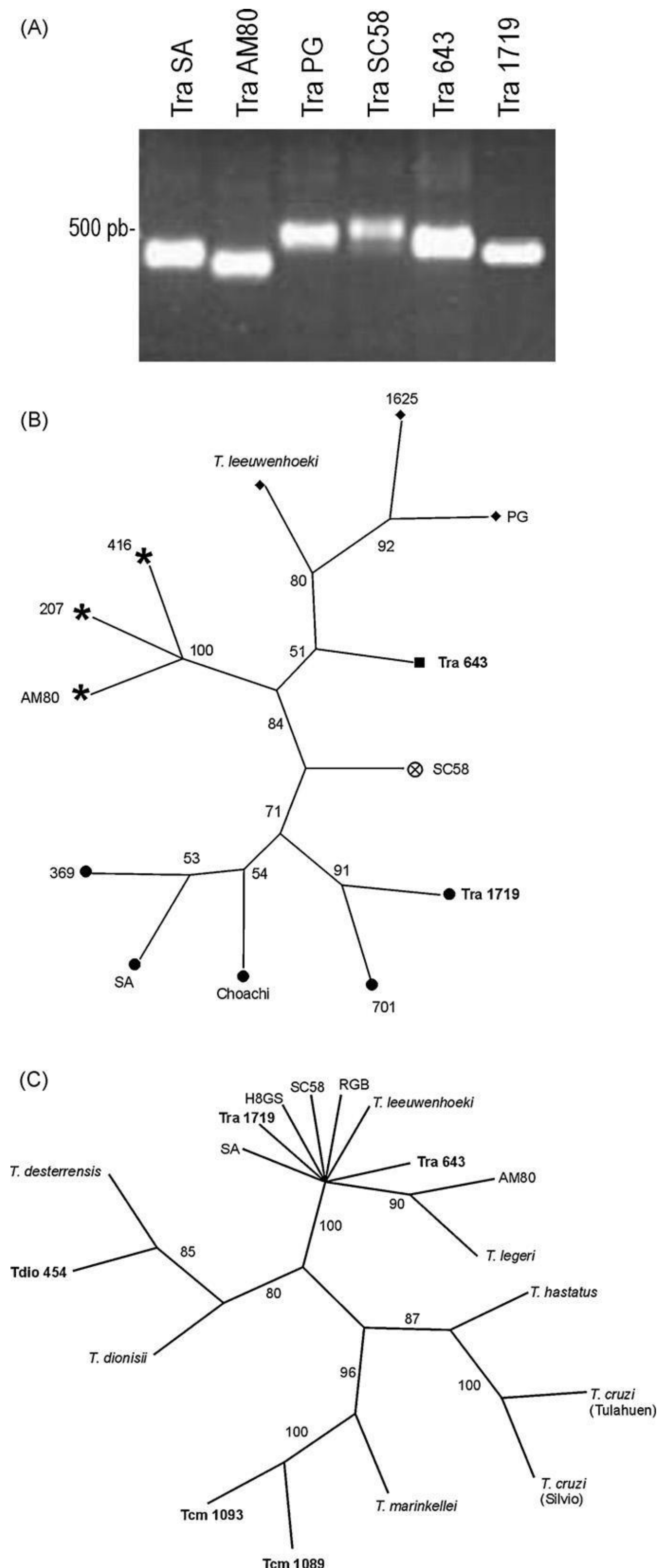

Fig. 4. (A) Length polymorphism of DNA fragments amplified from DNA of *T. rangeli* isolates of lineages A–E by TraSL-PCR (agarose gel stained with ethidium bromide), (B) phylogenetic relationships among *T. rangeli* from lineages A (●), B ((), C (♦), D (⊗) and E (■) based on SL intergenic spacer sequences and (C) phylogenetic relationships among bat *T. rangeli* isolates Tra643 and Tra1719 and isolates of *T. c. marinkellei* and *T. dionisii* obtained in this study, and other trypanosomes from bats (GenBank). The numbers in nodes refers to the bootstrap values of the clades in 100 replicates.

several mammals (Maia da Silva et al., 2008; Cavazzana et al., in preparation).

Phylogenies of the genus *Trypanosoma* based on SSU rDNA and *gGAPDH* gene sequences positioned *T. rangeli* close to *T. cruzi* and other *Schizotrypanum* species restricted to bats, and very distant from *T. brucei* (Stevens et al., 1999, 2001; Maia da Silva et al., 2004b, 2007). The V7-V8 variable region of SSU rDNA have been used for barcoding trypanosomes, allowing identification of all known species examined so far (Rodrigues et al., 2006, 2008; Ferreira et al., 2007) including *T. rangeli* (Maia da Silva et al., 2004b). Data from the present study reinforced the reliability and application of this method for identification of *T. rangeli* at specific and lineage levels and demonstrated its value for identification of bat *Schizotrypanum* trypanosomes.

The population structure of *T. rangeli* lineages concurs with the phylogeography of *Rhodnius* spp. and is compatible with the hypothesis of lineage divergence related to ecogeographical structure of *Rhodnius* spp., triatomines that inhabited palm trees in Central and South Americas (Urrea et al., 2005; Maia da Silva et al., 2004a,b, 2007). Analysis based on conserved sequences of transcript SL gene sequences revealed that bat isolates of *T. rangeli* Tra1719 and Tra643 nested within a major clade comprising lineages A, C and D. However, divergence of variable SL intergenic region sequences indicated that the isolate Tra1719 belong to the lineage A, while the isolate Tra643 could be assigned to a new genotype that we named lineage E.

The new lineage of *T. rangeli* (E), which is so far represented only by the isolate Tra643 from bat, was distinguished from all other previously defined lineages (A–D). In the search for the vector of *T. rangeli* from bats described in this study we have examined triatomines collected in arboreal ecotopes in the area of bat captures. By analysing 16S rDNA gene sequences we identified the local captured triatomines as *R. stali*, a species common in palms inhabited by bats and in nests of birds, found in the State of Mato Grosso do Sul, Brazil and in bordering areas of Bolivia (Lent et al., 1993). Of seven specimens of *R. stali* positive for trypanosomes, one showed in its digestive tube large epimastigotes typical of *T. rangeli* (Maia da Silva et al., 2004a). Unfortunately, we could neither isolate *T. rangeli* from *R. stali* nor amplify DNA of gut material from the *T. rangeli* infected *R. stali*.

Our collection effort for *Rhodnius* species was limited in the studied area. However, the finding of lineages A and E infecting bats captured in this area suggested the existence of more than one vector for *T. rangeli*. A possible local vector of *T. rangeli* lineage A (Tra1719), which was associated with *Rhodnius* species of complex *R. prolixus*, is *R. neglectus* that has been found infected with *T. rangeli* (Ramirez et al., 2002; Gurgel-Gonçalves et al., 2004; Marquez et al., 2007). Vectors of lineage E are unknown and the absence of trypanosome isolates obtained from triatomines assigned to this lineage does not allow its association with any *Rhodnius* species. The only triatomine species captured in the study area and found infected by *T. rangeli* was *R. stali*. However, due to flight mobility of bats, all *Rhodnius* species from this and from nearby areas can be vectors of *T. rangeli* for bats captured in the study area.

In this study, *R. robustus* and *R. neglectus* experimentally infected with the bat isolate Tra643 showed epimastigotes in both the digestive tract and haemolymph but not in the salivary glands. The ability of *T. rangeli* to reach salivary glands depend on both parasite isolates and vector species as suggested by the differential susceptibility of *Rhodnius* spp. to isolates of *T. rangeli* from different geographical origins (Tobie, 1968; D'Alessandro and Saravia, 1999; Machado et al., 2001; Guhl and Vallejo, 2003; Vallejo et al., 2003). It is possible that the lack of trypanosomes in the salivary glands of *Rhodnius* species investigated in the present study is due to a restriction of *T. rangeli* lineage E to its sympatric vector *R. stali*. Unfortunately,

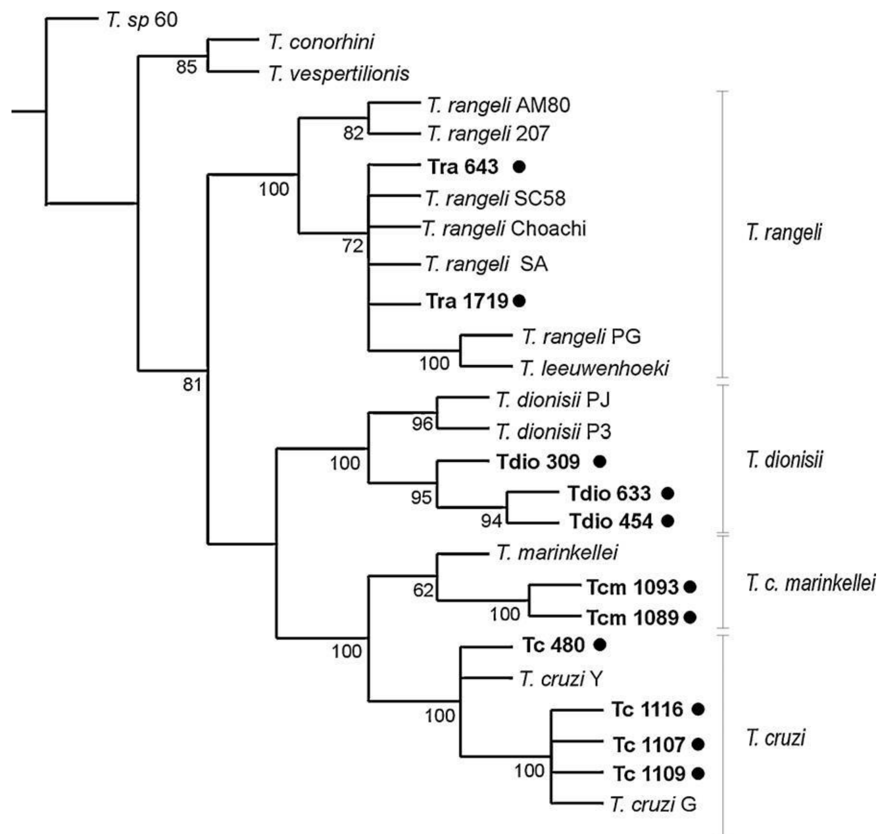

Fig. 5. Phylogenetic tree inferred using V7-V8 SSU rDNA sequences of nine isolates of *T. rangeli* from all phylogenetic lineages and 17 sequences from other trypanosomes include Brazilian bat isolates (●) by maximum parsimony (MP). The numbers in nodes are bootstrap values of the clades in 100 replicates.

R. stali and other sylvatic species from regions where bat isolates originated were not available for this study.

Despite the fact that *T. rangeli* had been previously described in *R. neglectus* from Central Brazil (Gurgel-Gonçalves et al., 2004), to our knowledge, this species was not confirmed previously in the State of Mato Grosso do Sul. Therefore, this is the first molecular study of either isolates from bats and from Central Brazil. Moreover, here we characterize for the first time *T. cruzi* from *R. stali*, which were molecularly assigned to the lineage TCI that is mainly associated to sylvatic mammals and *Rhodnius* species (Gaunt and Miles, 2000; Fernandes et al., 2001; Marcili et al., in preparation). In addition, this study described the isolation and molecular characterization of *T. dionisii* isolates from Brazilian bats and their comparison with European isolates. *T. c. marinkellei* from Central Brazil clustered together with isolates from Northeast Brazil (Barnabè et al., 2003). A broad analysis of bat *Schizotrypanum* trypanosomes are currently being performed in our laboratory to understand phylogeographical patterns in several Brazilian biomes.

Altogether, data from this study corroborated the high complexity of *T. rangeli* isolates, providing evidence that the understanding of population structure of this species will always be improved with analysis of isolates from more mammalian hosts and from different *Rhodnius* species, especially from not yet investigated geographical areas. More lineages of *T. rangeli* can be discovered using the molecular markers employed in this study, which allow simultaneous identification and genotyping of *T. rangeli*.

Acknowledgements

We are indebted to several students and collaborators for their inestimable help in the fieldwork. We are grateful to Dr. Valdir Tadei (in memoriam) and Caroline Cotrin Aires for identification of bats. This work was supported by grants from CNPq and UNIDERP. Flávia Maia da Silva is postdoctoral fellow of PRODOC-CAPES; and Arlei Marcili, Luciana Lima and Paola A. Ortiz are recipients of scholarships from CNPq.

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A new trypanosome species infecting African bats (Rhinolophidae and Hipposideridae): taxonomic appraisal based on morphological and behavioural features and phylogenetic inferences using SSU rRNA and gGAPDH genes

Lima, L.; Campaner, M.; Takata, C. S. A; Attias, M.; Pereira, C.; Neves, L.; de Souza W., Camargo, E. P. & Teixeira M. M. G.

Summary

In this study, we morphologically, biologically and phylogenetically characterised 12 trypanosome isolates from microbats captured in Mozambique, south-eastern Africa, including 11 from *Rhinolophus landeri* and 1 from *Hipposideros caffer,* both of the Rhinolophidae family. Culture characteristics and biological behaviour of these isolates clearly separated them from the species of *Schizotrypanum*, which comprises nearly all bat trypanosomes that have been maintained in culture and molecularly characterised. The trypanosomes in the blood of these bats morphologically resembled large trypanosomes traditionally assigned to the subgenus *Megatrypanum,* a controversial taxon. However, phylogenetic analyses based on SSU rRNA and gGAPDH genes revealed that the isolates from these bats are distant from all species of trypanosomes from bat and other hosts included thus far in phylogenetic trees. These isolates are the only *Megatrypanum* trypanosomes from African bats characterized using morphological, biological and phylogenetic analyses. Our findings prompted the description of a new species of trypanosome that could not be phylogenetically positioned within the newly revised subgenus *Megatrypanum.*

Keywords: *Trypanosoma*, Africa, Chiroptera, bat parasites, *Megatrypanum*, taxonomy, phylogeny, evolution.

Introduction

Trypanosomes (Euglenozoa: Kinetoplastea: *Trypanosoma*) are blood parasites adapted to all classes of vertebrates and are transmitted by a variety of bloodsucking arthropod and leech vectors widespread in all continents. Although bats are one of the most common hosts of trypanosomes, little is known about species diversity, vectors and the life cycles of the trypanosomes that infect bats. Chiroptera compose more than 20% of extant mammals and host numerous species and families of trypanosomes, with a worldwide distribution. Infected bats, apparently, remained asymptomatic for years (Hoare, 1972; Marinkelle, 1976; Molyneux, 1991).

Bats have been reported to be hosts of trypanosomes from the sections Salivaria (subgenus *Trypanozoon*) and Stercoraria (*Herpetosoma*, *Schizotrypanum* and *Megatrypanum*). *Megatrypanum,* followed by *Schizotrypanum,* comprise the majority of species described in bats (Hoare, 1972; Marinkelle, 1976; Molyneux, 1991). Only two species of *Megatrypanum* have been reported in macrobats: *T. (M.) megachiropterum* from *Pteropus tonganus* from Togo, Africa (Marinkelle, 1979), and *Trypanosoma* spp. from *Rousettus aegyptiacus* from Gabon, Africa (Stevens et al., 1999b). Several species of this subgenus have been reported from microbats throughout the world, with descriptions from South

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America, Asia, Europe and Africa. Most *Megatrypanum* trypanosomes have been isolated from South American and African microbats, with the largest number of species described from Africa: *T. (M.) megadermae* (Sudan); *T. (M.) heybergi* (Congo, Zambia and Kenya); *T. (M.) leleupi* (Congo and Burundi); *T. (M.) mpapuense* (Tanzania); *T. (M.) morinorum* (Senegal); and *T. (M.) thomasi* (Congo) (revised by Hoare, 1972 and Molyneux, 1991). A survey of trypanosomes in blood smears from African bats from Kenya reported a 21% prevalence of infection by trypanosomes among the 427 bats examined, with *Megatrypanum* spp. found mostly among Hipposideridae and Rhinolophidae (Woo & Hawkins, 1975).

Bats probably become infected with trypanosomes by licking contaminated vector faeces on their fur and by ingesting the infected vectors themselves. Species of *Megatrypanum* are thought to be transmitted by sand flies in South America (Zeledon & Rosabal., 1969). Cimicids have been shown experimentally to transmit the European *T. (M.) incertum* to bats. This species develops in *Cimex lectularius* and *C. pipistrelli*, and flagellates from these insects infect bats by the oral route (Gardner & Molyneux, 1988). In addition, ectoparasite cimicids collected from bats infected with the African *T. (M.) leleupi* exhibited epimastigote and metacyclic trypomastigotes in the intestine (Van der Berge, 1963; Anciaux de Faveaux, 1965).

The majority of trypanosomes reported from bats have not been cultivated, and the large number of species classified in different subgenera, mainly *Schizotrypanum* and *Megatrypanum*, is based on the morphology of blood trypomastigotes. The taxonomy of bat trypanosomes needs to be re-evaluated by analysing a broader sample that is representative of host-species diversity and worldwide distribution. The true diversity and evolutionary history of bat trypanosomes has only recently begun to be addressed phylogenetically. Few bat trypanosomes have a clearly defined taxonomic position in phylogenetic trees, and all belong to the subgenus *Schizotrypanum* (*T. cruzi*, *T. c. marinkellei, T. dionisii, T. erneyi*), forming a monophyletic assemblage (Stevens et al. 1999a,b; Hamilton et al., 2004; 2007; 2009; Marcili et al., 2009; Maia da Silva et al., 2009; Cavazzana et al., 2010; Lima et al., submitted). The other two bat trypanosomes included in phylogenies are *T. rangeli* from Brazil, *T. vespertilionis* from Europe (formerly classified as *Schizotrypanum*) and a species of *Megatrypanum* from an African megabat. These trypanosomes cluster together with *T. (M.) conorhini* and trypanosomes from African monkeys and civets; this clade, together with *Schizotrypanum,* form a large monophyletic assemblage designated the *T. cruzi* clade. A *Megatrypanum* trypanosome from an Australian kangaroo was positioned at the periphery of this major clade, which is distant from *T. (M.) theileri,* the type species of this subgenus. Therefore, phylogenies demonstrate the polyphyly of the subgenus *Megatrypanum,* which was originally defined, exclusively on a morphological basis, as being constituted of species from artiodactyls, bats, rodents and other mammals (Stevens et al., 1999a,b; Hamilton et al., 2004; 2007; 2009; Rodrigues et al., 2006; 2010). Recent studies of trypanosomes of ruminants have proposed that the subgenus *Megatrypanum* be limited to the clade in which *T. (M.) theileri* was placed (Rodrigues et al., 2006; 2010). Nevertheless, to validate this proposition, more *Megatrypanum* trypanosomes need to be included and phylogenetically characterised, specially isolates from bats that account for most of the species within this subgenus besides artiodactyls (Hoare, 1972).

In this study, we characterised trypanosomes isolated from bats of the Rhinolophidae family captured

in Mozambique, south-eastern Africa by morphological, biological and phylogenetic analysis. Rhinolophidae comprise microbats widespread throughout the Old World but absent in the New World. We obtained 12 isolates from these bats, including 11 from *Rhinolophus landeri* and 1 from *Hipposideros caffer*. The trypomastigotes in the blood of these bats were typical of the subgenus *Megatrypanum.* However, phylogenetic analyses revealed that these trypanosomes are distant from all trypanosome species included in phylogenetic trees so far, thus prompting the description of a new species and confirming the newly revised subgenus *Megatrypanum*.

MATERIALS AND METHODS

1. Collection sites, capture and identification of bats

Bats were captured in Mozambique, eastern Africa, in the district of Chupanga (S18º02' E35º34'), Zambezi valley, and the Parque Nacional de Gorongosa (S18º58' E34º21'), both of which are located in the Province of Sofala in central Mozambique. Bats captured with mist nets were anaesthetised, and blood samples were collected by cardiac puncture. The genera of captured bats were identified by morphological characteristics using conventional keys. For molecular identification, tissue samples from bat livers in 100% ethanol were processed for genomic DNA using Wizard DNA Clean-Up System (Promega) and were used for sequencing of the mitochondrial cytochrome b gene (Cyt b) as described previously (Cui et al.,2007). Sequences were analysed with BLAST on Genbank (Table 1).

2. Isolation in culture and growth behaviour and morphology of bat trypanosomes

Blood samples from bats were examined for the presence of trypanosomes using the microhaematocrit (MH) and haemoculture (HE) methods. For HE, bat blood samples were transferred to tubes containing a medium consisting of blood agar base (BAB) and 15% rabbit blood as a solid phase, with an overlay of LIT (liver infusion tryptose) medium and 10% fetal bovine serum (FBS), and were maintained at 25-28°C for approximately 15 days. Positive cultures were transferred to culture flasks containing a monolayer of insect cells (Hi-5 from *Trichoplusia ni)* in TC-100 medium (Grace's medium) containing 10% (v/v) FBS and were incubated at 25ºC as described previously (Viola et al., 2009). All isolates were expanded in Hi-5 cultures for DNA preparation and cryopreservation at the Trypanosomatid Culture Collection (TCC) of the Department of Parasitology, University of São Paulo, Brazil.

For morphological analysis, blood smears from both naturally infected bats and logarithmic and stationary phase cultures with Hi-5 cells were fixed with methanol and Giemsa-stained for light microscopy. Cultures at stationary phase containing metacyclic trypomastigotes were transferred to monolayers of HeLa cells to verify their ability to develop within cells (Marcili et al., 2009a). Epimastigote cultures were transferred to monolayers of mammalian cells (LLC-MK2) and incubated at 37ºC to assess the ability of the new trypanosomes to differentiate into large trypomastigotes resembling blood forms. For analysis of mouse infectivity, Balb/c mice were inoculated (i.p.) with cultures containing trypomastigote forms (~10^5/animal). Mouse blood samples were examined weekly from 7 to 30 days p.i. by MH and by HE after the 30th day p.i., as described previously (Maia da Silva et al., 2004).

Table 1. *Trypanosoma zambesiensis* isolates and other trypanosome species and their respective sequences from genes determined in this study or retrieved from GenBank.

TryCC[a]	samples	Host		Origin Geographic	GenBank Acession number			
			Genus/species		SSUrDNA V7V8	SSUrDNA	gGAPDH	ITS1
T. zambesiensis								
1270	Morcego 12	bat	*R. landeri*	Chupanga / Mz	*	*	*	*
1295	Morcego 29	bat	*R. landeri*	Chupanga / Mz	*	*	*	*
1304	Morcego 20	bat	*R. landeri*	Chupanga / Mz	*	*	*	*
1271	Morcego 17	bat	*R. landeri*	Chupanga / Mz	*	*	*	*
1298	Morcego 28	bat	*R. landeri*	Chupanga / Mz	*	*	*	*
1933	CHMO 30	bat	*R. landeri*	Chupanga / Mz	*	-	-	*
1948	CHMO 31	bat	*R. landeri*	Chupanga / Mz	*	-	-	*
1947	CHMO 32	bat	*R. landeri*	Chupanga / Mz	*	-	-	*
1954	CHMO 33	bat	*R. landeri*	Chupanga / Mz	*	-	-	*
1902	CHMO 34	bat	*R. landeri*	Chupanga / Mz	*	-	-	*
1935	MTR 16933	bat	*R. landeri*	Chupanga / Mz	*	-	-	*
1953	GOMO 28	bat	*H. caffer*	Gorongosa / Mz	*	*	*	*
Blood samples from *T. zambesiensis*								
-	Morcego 7	bat	*R. landeri*	Chupanga / Mz	-	-	-	*
-	Morcego 19	bat	*R. landeri*	Chupanga / Mz	*	-	-	*
-	Morcego 25	bat	*R. landeri*	Chupanga / Mz	*	-	-	*
Other trypanosomes								
-	H25	kangaroo	*M. giganteus*	Australia	AJ009168	AJ009168	AJ620276	-
60	*T. sp* bat	bat	*R. aegyptiacus*	Gabon	AJ012418	AJ012418	GQ140365	-
-	HochNdi1	monkey	*C. nictitans*	Cameroon	FM202493	FM202493	FM164794	-
-	Nandoum1	carnivore	*N.binotata*	Cameroon	FM202492	FM202492	FM164793	-
-	*T.conorhini*	rodent	*R. rattus*	Brazil	AJ012411	AJ012411	AJ620267	-
-	*T. vespertilionis*P14	bat	*P. pipistrellus*	England	AJ009116	AJ009116	AJ620283	-
T. rangeli								
643		bat	*P. lineatus*	Brazil	FJ900242	FJ900242	GQ140364	-
1719		bat	*A. planirostris*	Brazil	EU867813	-	-	-
031	SA	human	*H. sapiens*	Colombia	AJ012417	-	-	-
086	AM80	human	*H. sapiens*	Brazil	AY491766	AY491766	*	-
-	*T. minascense*	monkey	*S.boliviensis*	South America	-	AJ012413	AJ620274	-
020	Macias	human	*H. sapiens*	Venezuela	AJ012415	-	-	-
-	RGB	dog	*C. familiaris*	Colombia	AJ009160	-	-	-
***Schizotrypanum* species**								
T. dionisii								
211		bat	*E. brasiliensis*	Brazil	FJ001666	FJ001666	GQ140362	-
495		bat	*C. perspicillata*	Brazil	FJ001667	FJ001667	GQ140363	-
-	P3	bat	*P. pipistrellus*	England	AJ009151	AJ009151	AJ620271	-
T. erneyi								
1293		bat	*Tadarida sp*	Marromeu / MZ	*	*	*	-
1294		bat	*Tadarida sp*	Marromeu / Mz	*	*	*	-
1946		bat	*M. condylurus*	Chupanga / Mz	*	*	*	-
T. cruzi marinkellei								
344		bat	*C. perspicillata*	Brazil	FJ001664	FJ001664	GQ140360	
501		bat	*C. perspicillata*	Brazil	FJ001665	FJ001665	GQ140361	
-	B3	bat	*P. discolor*	Brazil	FJ649484	FJ649484	FJ649495	
T. cruzi								
30	G	opossum	*D. marsupialis*	Brazil	AF239981	AF239981	GQ140351	-
507		bat	*C. perspicillata*	Brazil	FJ001632	FJ900240	GQ140352	-
793		bat	*M. levis*	Brazil	FJ001634	FJ900241	GQ140358	-
34	Y	human	*H. sapiens*	Brazil	AF301912	AF301912	GQ140353	-
844	MT3869	human	*H. sapiens*	Brazil	-	AF303660	GQ140355	-
845	MT3663	triatomine	*P. geniculatus*	Brazil	-	AF288660	*	-
967	NRcl3	human	*H. sapiens*	Chile		AF228685	AJ620269	-

[a] Cultures of bat trypanosome isolates cryopreserved in the Trypanosomatid Culture Collection of the Departament of Parasitology, University of São Paulo, São Paulo, Brazil. TCC correspond to number codes of isolates cryopreserved in -this collection. ***** Sequences determined in this study to be submitted to GenBank.

3. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

To compare the morphology of bat trypanosomes by TEM and SEM, cultures at mid-log phase were fixed with 2,5% buffered glutaraldehyde supplemented with 1% paraformaldehyde in 0,1 M sodium cacodylate buffer, pH 7,3, for 2 hr at room temperature and post-fixed in 1% buffered osmium tetroxide for 1 hr. Following fixation, cells were incubated with 0,5% uranyl acetate, embedded in Spurr's resin, and ultrathin sections were stained with uranyl acetate as previously described (Teixeira et al., 2011). Sections were examined on a JEOL 100CX electron microscope. For scanning electron microscopy (SEM), a fraction of the flagellates fixed with glutaraldehyde were adhered to poly-L-lysine-coated coverslips and processed for observation on a ZEISS DSM 940 digital scanning microscope as detailed previously (Teixeira et al., 2011).

***4. Sequencing and data analysis of SSU rDNA, gGAPDH and ITS1 rDNA* sequences.**

DNA was isolated from cultured trypanosomes from bats by phenol-chloroform extraction and was used as a template for PCR amplification, cloning and sequencing. PCR amplification of the variable V7-V8 region of SSU rRNA, whole SSU rRNA and ITS rDNA genes was performed as described previously (Rodrigues et al.. 2006; Ferreira et al.. 2007). Amplification of gGAPDH sequences was conducted as described (Hamilton et al., 2005). Sequences (~180 bp) of ITS1 rDNA were obtained as described previously (Rodrigues et al., 2006).

Sequences were aligned using Clustal X (Thompson et al.., 1997), and the resulting alignments were manually refined. We created 3 alignments for phylogenetic inferences: a) concatenated sequences from SSU rRNA and gGAPDH (~ 2.900 bp) of trypanosomes representing all major clades in the phylogenetic trees of *Trypanosoma*. Accession GenBank numbers: *Phytomonas* sp. (AF016322/AF047496), *H. samuelpessoai* (U01016/AF047494), *H. megaseliae* (U01014/DQ092547), *H. muscarum* (L18872/DQ092548), *L. tarentolae* (M84225 /DQ092549), *C. fasciculata* (Y00055/AF053739), *W. brevicula* (AF153045/AF316620), *T. binneyi* (AJ132351/AJ620266),*T.* sp.CLAR (AJ620555/AJ620251), *T. granulosum* (AJ620551/AJ620246), *T. rotatorium* (AJ009161/AJ620256), *T. mega* (AJ009157/AJ620253), *T. fallisi* (AF119806/AJ620254), *T.* sp D30. (AJ009165/AJ620279), *T. theileri* (AJ009164/AJ620282), *T.* sp. TL.AQ.22 (AJ620574/AJ620280), *T. cyclops* (AJ131958/AJ620265), *T. sp* AAT (AJ620557/AJ620264), *T. avium* (AJ009140/AJ620263), *T. avium* (U39578/AJ620262), *T. vivax* (U22316/AF053744), *T. congolense* (savannah) (AJ009146/AJ620290), *T. congolense* (riverine forest) (AJ009145/AJ620289), *T. congolense* (kilifi) (AJ009144/AJ620288), *T. brucei* (M12676/X59955), *T. godfreyi* (AJ009155/AJ620292), *T. simiae* (AJ009162/AJ620293), *T. varani* (AJ005279 AJ620261), *T.* sp. Gecko (AJ620548/AJ620259), *T.* sp. R1 (AJ620568/AJ620281), *T. evansi* (AJ009154/AF053743), *T. lewisi* (AJ009156/AJ620272), *T. microti* (AJ009158/AJ620274); b) V7V8 SSU rRNA sequences (~ 880 bp) from bat trypanosomes and allied species; and c) ITS1 rDNA sequences (154 to 212 bp) exclusively from trypanosome isolates of horseshoe bats (Table1).

Phylogenies were inferred using maximum likelihood (ML), Bayesian inferences (BIs) and parsimony (P) analyses. Parsimony and bootstrap analyses were carried out using PAUP version 4.0b10 (Swofford 2002) with 500 replicates of random addition sequence followed by branch swapping (RAS-TBR), as previously described (Ferreira et al. 2007). ML analyses were performed using RAxML v.2.2.3 (Stamatakis 2006). Tree searches were performed with

GTRGAMMA, with 500 maximum parsimony starting trees. Model parameters were estimated in RAxML for the duration of the tree search. Nodal support was estimated with 500 bootstrap replicates (alignments 1 and 2) in RAxML using GTRGAMMA and maximum parsimony starting trees. BIs were performed in MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001).

RESULTS

1. Detection of trypanosomes in blood and haemocultures from bats

In this study, we evaluated trypanosome infection among 68 bats captured in Mozambique and identified as species of Rhinolophidae, genera *Rhinolophus* (47 bats captured in 2006 and 2009 in Chupanga) and *Hipposideros* (21 bats captured in 2009 in Gorongosa National Park). To confirm the morphological identification and ascertain the species of the bats, we determined the sequences of Cyt b genes from bat liver DNA. According to BLAST analysis on GenBank, bats harbouring the trypanosomes characterised in this study were classified as *Rhinolophus landeri* and *Hipposideros caffer*.

The examination of blood samples from 37 of the 47 *R. landeri* by MH revealed the presence of trypanosomes in 15 bats, yielding a high prevalence of ~ 40.5%. *H. caffer* could not be examined by MH due to fieldwork difficulties. Blood samples of all 68 bats were examined by HE, and we isolated 11 cultures from *R. landeri* and one from *H. caffer*. Interestingly, some blood samples that were positive by MH did not result in positive cultures, suggesting that these bats can be infected with other trypanosome species. The new trypanosomes from bats required Hi-5 insect cells to grow in culture. They were not able to invade mammalian cells, and when transferred to monolayers of LLC-MK2 at 37º C, epimastigotes differentiated into broad and dividing trypomastigotes. However, these trypomastigotes could not be maintained through successive passages in culture. In addition, the new isolates from bats were incapable of infecting Balb/c mice. Nothing is known about their vectors; no bat bugs were found attached to the bats examined in this study, and the isolates that we obtained from these bats were unable to experimentally infect triatomine bugs.

2. Morphology by light, transmission electron and scanning electron microscopy

Blood trypomastigotes of the bat isolates characterised in this study resemble those of *T.* (*M.*) *heybergi* (Hoare, 1972). These large trypomastigotes forms have a broad body with a pointed posterior end (average 32,4 µm long by 7,8 µm wide), a markedly frilled undulating membrane and a free flagellum averaging 9,95 µm long. The kinetoplast was very small and occupied a marginal position adjacent to the rounded and nearly central nucleus. In addition, these trypomastigotes have several surface striations (Fig. 1). Dividing forms were not observed in blood smears. Flagellates in log-phase cultures are long and slender epimastigotes (average 16 to 29µm long by 1,1 to 2µm wide) with the kinetoplast adjacent to the central nucleus or distant when the nucleus is dislocated toward the posterior end. Large epimastigotes show both ends of the body finely tapered, well-developed undulant membrane and large free flagellum (15,5 µm long). Flagellates in stationary cultures are large and slender epimastigotes, trypomastigotes ranging from very small forms with a terminal kinetoplast resembling metacyclic forms to larger forms with a long flagellum (Fig. 1). Wide trypomastigote forms found by co-cultivation of the bat trypanosomes with a monolayer of mammalian cells at 37ºC resembled blood forms found in bat hosts with large undulant membrane, but are smaller and without striations, and the kinetoplast is

punctual and close to the posterior end. These trypomastigotes are dividing forms (Fig. 1).

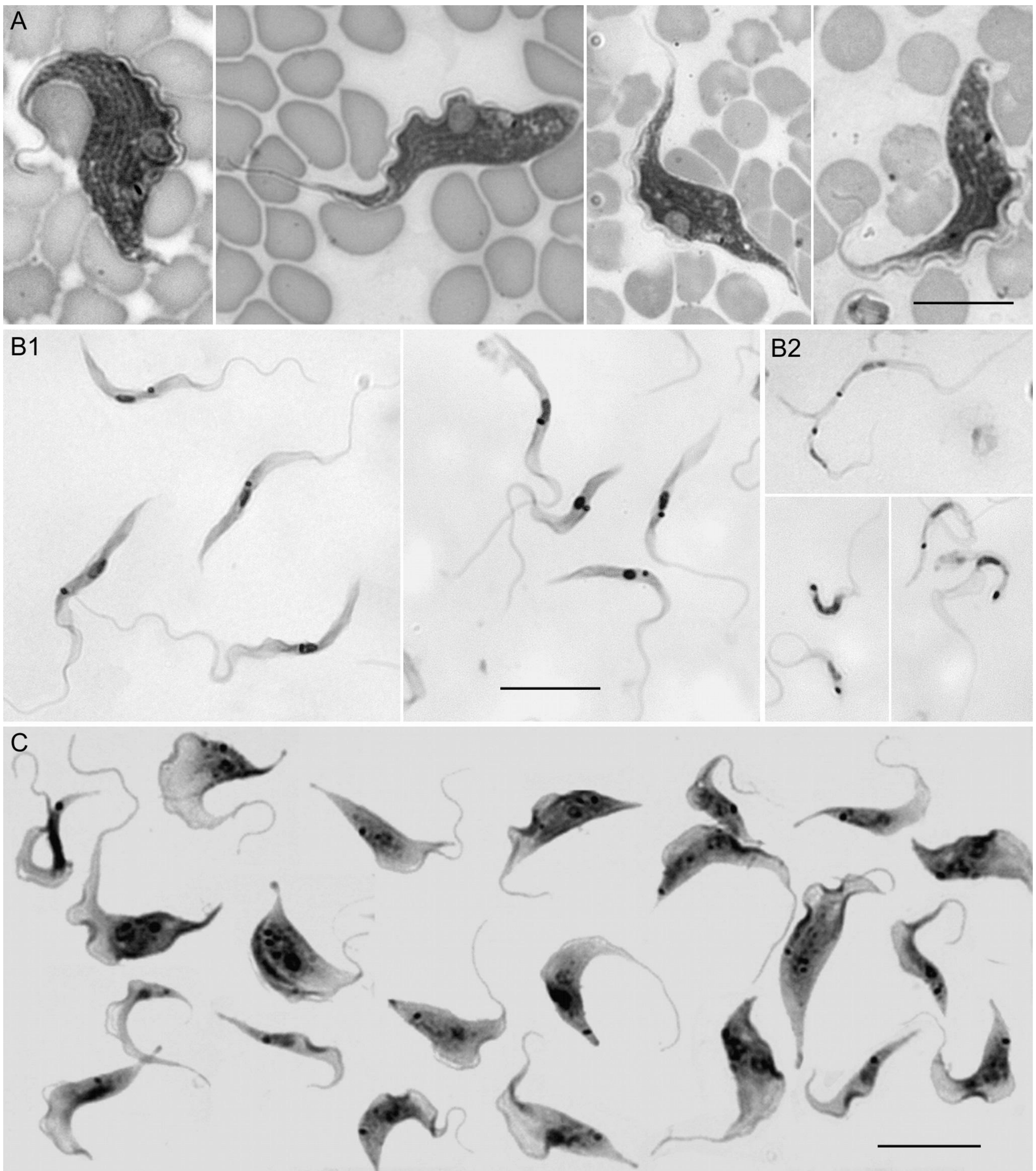

Fig 1: Light microscopy of Giemsa-stained forms of *Trypanosoma zambesiensis* n. sp. (A) blood trypomastigotes in naturally infected bats; (B1) Epimastigotes in log-phase cultures, (B2) trypomastigotes in stationary cultures; (C) trypomastigote forms in monolayer of LLC-MK2 mammalian cells at 37^{0}C. Scale bars: 10μm.

In this study, we characterised for the first time the ultrastructural features of a *Megatrypanum* trypanosome isolated from a bat. The SEM analysis of culture cells showed large rosettes of pointed and

twisted epimastigotes united by the flagella, detached long and slender epimastigotes with noticeable undulant membrane, rounded flagellates and small trypomastigotes (Fig. 2). Morphological analysis by TEM revealed the following characteristics: kDNA fibrils organised in a compacted and thin kinetoplast, presence of a cytostome and large amount of reservosomes(Fig.2).

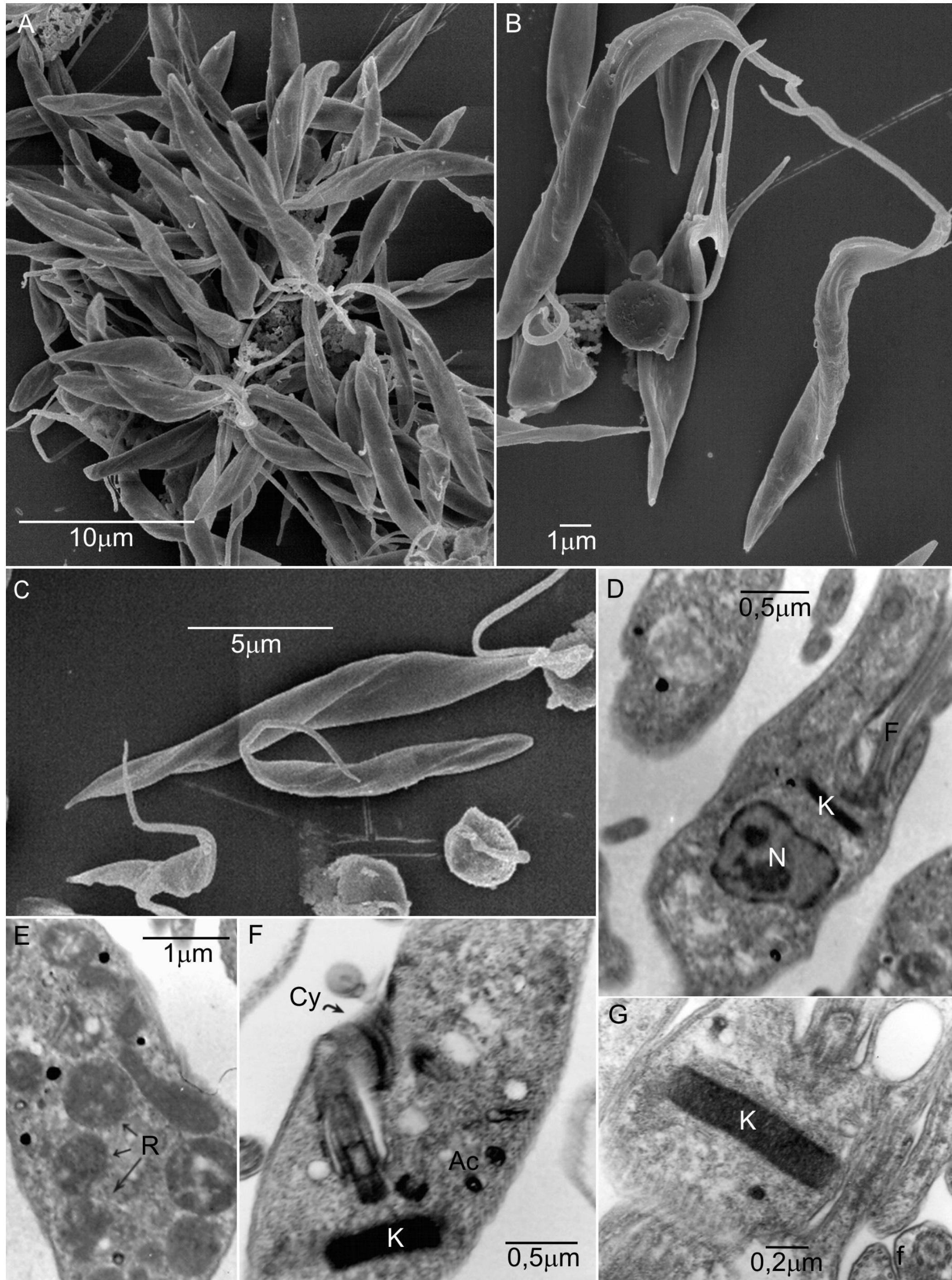

Fig 2: Scanning (A-C) and transmission electron microscopy (D-G) of *Trypanosoma zambesiensis* n. sp. Epimastigotes in rosettes (A), long and slender epimastigotes (B); epimastigotes, rounded flagellate and small trypomastigote (C). Longitudinal section showing kinetoplast, nucleus and flagellum (D); Reservosomes (E); the cytostome near of the flagellar pocket (F); and detail of the compacted kinetoplast (G). Nucleus (N); kinetoplast (K), reservosome (R), flagellum (f), acidocalcisomes (Ac), cytostome (Cy).

3. Barcoding and phylogenetic analyses of the new African bat trypanosomes

The first step in characterising new bat trypanosomes was the barcoding of the V7V8 region of the SSU rRNA gene; this polymorphic region has routinely been used for bar-coding to preliminarily distinguish any new trypanosomatid taxa (Rodrigues et al., 2006; Ferreira et al., 2007; Viola et al., 2008; Cavazzana et al., 2010). Barcoding analysis revealed that all new isolates from African bats shared high sequence similarity (average ~ 0.2% divergence), and BLAST analysis demonstrated that they are not highly related to any previously reported species. Despite a large sequence divergence, they share the greatest similarity with a *Trypanosoma* sp. from an Australian kangaroo (~ 8.7%), followed by a *Trypanosoma* sp. from a Gabon megabat (~ 15%). In the dendrogram inferred from the barcoded sequences of the 12 new isolates aligned to those of other bat trypanosomes, all of the new isolates formed a separate cluster, which largely diverged from all trypanosome species selected by similarity using BLAST (Fig. 3A). Blood samples that were positive by MH that did not result in positive cultures were also subjected to barcoding analysis, and these sequences were highly similar to those obtained from the cultures.

We selected 6 of the 12 new bat isolates to be positioned in phylogenetic trees using SSU rRNA and gGAPDH sequences. Results have demonstrated that the selected bat isolates are highly homogeneous, with relatively small divergences: ~0.2% for both SSU rRNA and gGAPDH genes. Phylogenetic analyses confirmed that these isolates are distant from all trypanosome species included in phylogenetic trees, thus enabling their classification as a new trypanosome species. This new species was more closely related to the trypanosomes from the Australian kangaroo (divergences of ~ 4.8% for SSU rRNA and ~10% for gGAPDH) than that of the Gabon megabat (~ 5.7% and 14%), which are both also traditionally classified in the subgenus *Megatrypanum* (Stevens et al., 1999a,b). However, these trypanosomes never clustered together; they were instead positioned in distinct branches separated by large genetic distances and were not well-supported in any phylogenetic analysis. The phylogenetic trees based on independent (data not shown) and combined SSU rRNA and gGAPDH gene sequences resulted in similar topologies; however, better support values were obtained by analysing the combined data set (Fig. 3B)

4. Polymorphism analysis among the new trypanosome isolates from African bats

Polymorphism analysis was performed by comparing ITS1 rDNA sequences, which have proven valuable for the detection of infraspecific variability of trypanosomes, such as *T. rangeli* (Maia da Silva et al., 2004), *T. theileri* (Rodrigues et al., 2006) and *T. cruzi* (Marcili et al., 2009b). The divergence of ITS1 rDNA sequences from 12 isolates and 3 blood samples from bats positive by MH (a total of 36 sequences, 2 to 4 sequences per isolate) was high among and within the isolates. Sequences of 5 isolates from *R. landeri* (TCC1271, 1298, 1902, 1933, 1947) were segregated in two main groups diverging by ~18%, Rhi1 (11% internal sequence divergence) and Rhi2 (5.0%), both comprising variable sequences distributed in several branches. The two sequences from *H. caffer* isolates clustered separated but closer to the group Rhi2 (12% divergence) than to group Rhi1 (20%) (Fig. 4A). Although some isolates showed sequences positioned in only one group this may be due to limited sequences The polymorphic copies of ITS1 rDNA sequences within the same isolate, specially very homogeneous isolates

like the new bat isolates characterized in this study, was unexpected compared to previous studies with isolates of other trypanosome species (Maia da Silva et al., 2004; Rodrigues et al., 2006; Marcili et al., 2009b). Similar results were found for sequences obtained directly from blood samples of bats. Although the new cultures derived from bats could not be cloned, the possibility that these polymorphic sequences represented a mixture of isolates is unlikely because the number of variant sequences is very large (~20 different sequences) and large insertions and a variable number of AT repeats accounted for almost all of the sequence divergence (Fig. 4B). In addition, sequencing of variable region of V7V8 SSU rRNA sequences without cloning the PCR-amplified genes did not reveal polymorphisms(Fig.4B)

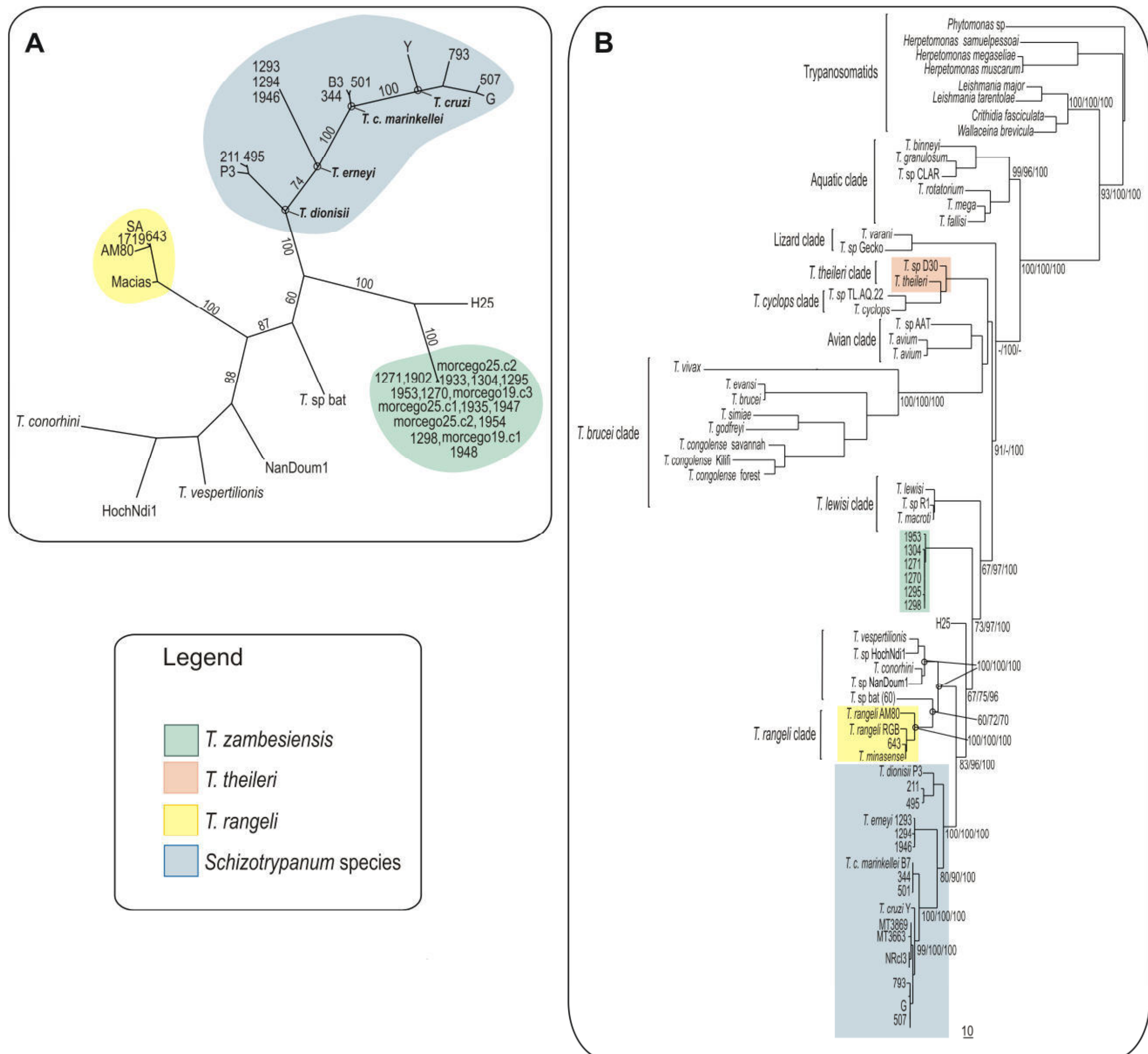

Fig 3. Barcoding and phylogenetic relationships of the new isolates from African rhinolophis bats inferred using ribosomal sequences. (A) Dendrogram inferred using the V7-V8 SSU rRNA sequences from 12 isolates from rhinolophis bats plus two blood samples that did not result in positive cultures, isolates of the subgenus *Schizotrypanum* and other species related. Numbers at nodes are bootstrap values derived from 100 replicates. (B) Maximum likelihood analysis (ML) using aligned of concatenated sequences of the SSU rRNA and gGAPDH (2.918 characters, –Ln = 24236.163647) from 6 new trypanosomes from rhinolophis bats and 68 isolates of other subgenera. Numbers at nodes are bootstrap values derived from 500 replicates from the MP/ML/BI analyses.

Fig. 4. Polymorphism analyses among isolates of *T. zambesiensis*. (A) Dendrogram inferred by parsimony, using the ITS1 rDNA sequences from 12 isolates of *T. zambesiensis* plus three blood samples that did not result in positive cultures. The numbers at the nodes correspond to bootstrap values derived from 100 replicates. (B) Partial alignment from ITS1 rDNA.

DISCUSSION

For a better appraisal of the genetic diversity and evolutionary history of trypanosomes, studies must include trypanosomes from all vertebrate classes and several orders using molecular approaches for their reliable identification and classification. Although mammals are the most studied hosts of trypanosomes, studies concentrate on pathogenic species infecting humans and livestock. Although it has been known for more than 100 years that bats are hosts to a large diversity of trypanosomes in Africa, Asia, South America and Europe, our knowledge regarding the genetic diversity, host range, vectors, life cycles, geographical distribution and phylogenetic relationships of these trypanosomes is restricted to a few species, primarily of the subgenus *Schizotrypanum.* However, most bats around the world are hosts of species classified in the subgenus *Megatrypanum* (Hoare, 1972, Molyneux, 1991).

The polyphyly of species classified according to traditional taxonomic parameters as *Megatrypanum* has been demonstrated since the first broad phylogenetic analysis of *Trypanosoma* (Stevens et al., 1999a,b; 2001). To date, studies revising this subgenus have focused on trypanosomes from artiodactyls, with one only species from bat (*Trypanosoma sp.* from megabat). Because artiodactyls and bats are the main hosts of the of *Megatrypanum* spp., studies of bat trypanosomes are required to validate the revision of this subgenus as being only constituted of trypanosomes from ruminants (Rodrigues et al., 2006; 2010).

In this study, we surveyed 68 bats, from *R. landeri* and *H. caffer* bats from Mozambique for trypanosome infection. The examination of blood samples by MH revealed that 40.5% of *R. landeri* were infected with large trypanosomes; *H. caffer* could not be examined by this method. According to morphological comparisons of trypanosomes described in blood of bats, large trypomastigotes of *Megatrypanum* spp. are of two main types: one related to *T.* (*M.*) *megadermae* of slender forms, and the other related to *T.* (*M.*) *heybergi* of very broad forms (Hoare, 1972). Blood trypomastigotes of the bat isolates characterised in this study resemble those of *T.* (*M.*) *heybergi*, a type common to African (*T.* (*M.*) *leleupi, T.* (*M.*) *mpapuense, T.* (*M.*) *morinorum* and *T.* (*M.*) *thomasi)* and South American (*T.* (*M.*) *pessoai* and *T.* (*M.*) *leonidasdeanei)* trypanosomes (Hoare, 1972; Molyneux, 1991). Despite being classified into the subgenus *Megatrypanum,* blood trypomastigotes of these species did not resemble *T. theileri* found in cattle. In fact, these forms have morphological features in common with the trypanosomes of anurans (Hoare, 1972; Ferreira et al., 2007). Blood forms of *Megatrypanum* spp. are noticeably larger than the forms typical of the subgenus *Schizotrypanum.* Whereas replication of *Schizotrypanum* spp. is intracellular, nothing is known about the multiplication of *Megatrypanum* spp. in bats (Hoare, 1972; Molyneux 1991).

We obtained 11 cultures from the blood samples of 68 *R. landeri* and one *H. caffer* bats examined by HE. The cultured epimastigote forms are very large compared to those from *T. cruzi*-like species. Culture forms of the new bat trypanosomes were examined using TEM and SEM. While a previous TEM study followed the development of *T.* (*M.) incertum* in cimicids (Gardner & Molyneux, 1988), to our knowledge, this is the first ultrastructural study on the culture forms of bat *Megatrypanum*. Besides some features such as the cytostome and compacted kinetoplast quite similar to *Schizotrypanum* spp.; there are no unambiguous differences that would be useful to

differentiate these trypanosome species from *T. (M.) theileri* (Rodrigues et al., 2003). Prior to this study, only four *Megatrypanum* trypanosomes from bats had been successfully grown in culture: *T. pifanoi*, *T. mpapuense* and *T. leleupi* (Hoare 1972) and the *Trypanosoma* sp. from a megabat (Stevens et al., 1999b). To our knowledge, the last trypanosome is the only isolate still available in culture.

As has been reported for *Megatrypanum* spp. in general, the new isolates from bats characterised here were not able to invade and develop within mammalian cells in vitro, and the epimastigotes differentiated to trypomastigotes resembling those found in blood smears when co-cultivated with mammalian cells at 37ºC. The new isolates were incapable of infecting Balb/c mice in agreement with previous failed attempts to infect mice and rats with other species of *Megatrypanum* from bats. In contrast, bats could be experimentally infected with *Megatrypanum* spp (Hoare, 1972; Marinkelle, 1976).

The phylogenetic positioning of the new bat trypanosome, which is morphologically and biologically compatible with species traditionally classified as *Megatrypanum,* separated from all other trypanosomes classified in this subgenus, confirmed the polyphyly of both *Megatrypanum* trypanosomes from bats and the entire subgenus (Stevens et al., 1999a,b; Hamilton et al., 2004; 2007; Rodrigues et al., 2006). In reality, only trypanosomes from ruminants clustered with *T. theileri,* justifying the subgenus *Megatrypanum* as a taxon exclusive of these trypanosomes (Rodrigues et al., 2006). The bat trypanosomes originally described as *Megatrypanum* cannot be positioned in any phylogenetically supported subgenus. Despite the urgent need for an extensive taxonomic revision of *Trypanosoma* on a phylogenetic basis, further amendment of the entire genus with the creation of new subgenera, which must represent well-supported clades comprising well-characterised trypanosomes, should be postponed because this revision will certainly benefit from the increasing number of new trypanosomes being included in phylogenies. For now, we believe that the assessment of synonymies and the naming of all species supported by a deep phylogenetic analysis is recommended to facilitate comparative studies and the selection of species that must be included in a further reappraisal of the whole *Trypanosoma* taxonomy.

We are designating the new African bat isolates as *Trypanosoma zambesiensis* n. sp. (see Taxonomy). The host and geographic origin of the isolates, their morphology and behaviour in culture, their inability to infect mice and their position in phylogenetic trees indicate that they constitute only one new species not closely related to any trypanosome species thus far included in phylogenetic trees. An unexpected characteristic of this new species was the polymorphic copies of ITS1 rDNA within the isolates. This finding contrast with the slight differences that we found previously by comparing ITS1 rDNA from isolates of *T. rangeli*, *T. cruzi*, *T. theileri* and *T. lewisi,* whose sequences diverged slightly within the same isolates while divergences among the isolates allowed the genotyping of isolates (Maia da Silva et al., 2004; 2010; Rodrigues et al., 2006; Marcili et al., 2009b). Although the divergence separating the major groups ranged from ~5% to 20%, distances are very small even compared to ITS1 rDNA divergences found among closely related species such as *T. cruzi* and *T. c. marinkellei* (~71%) or *T. dionisii* (79%).

Prior to this study, morphologically characterised *T. heybergi*-like trypanosomes were reported from *Nycteris, Rhinolophus* and *Hipposideros* from the central and east African countries of Congo, Burundi, Kenya, Zambia and Tanzania (Keymer, 1971; Hoare, 1972). Our findings confirm that the last two genera harbour this type of trypanosome, perhaps all

synomies. It is possible that the new species of trypanosome that we describe in this study corresponds to a *T. heybergi*-like species, which were previously described from *Rhinolophus* and *Hipposideros* from neighbouring countries of Mozambique. Unfortunately, cultures or DNA sequences from all previously described *T. heybergi*-like trypanosomes are not available for comparison. Besides *T. zambesiensis*, we also found in the same areas of Mozambique bats of the family Molossidae infected by *T. erneyi,* a new species of the subgenus *Schizotrypanum* (Lima et al., submitted). The molossid hosts of *T. erneyi* and the rhinolophid hosts of *T. zambesiensis* live in sympatry in Chupanga, Zambezi Valley, though roosting in separated refuges. Interestingly, our findings showed that *Schizotrypanum* or *Megatrypanum* were highly prevalent among molossids and rhinolophids, respectively, but cross- or mixed infections were not observed. Experimental infections strongly suggest that *Megatrypanum* species infecting bats are host-restricted or at least have a high degree of host-specificity (Hoare, 1972).

Comparative studies between trypanosome and bat phylogenies can be helpful in interpreting the phylogenetic relationships between trypanosomes infecting bats around the world. For this purpose, an understanding of bat evolutionary history is crucial for evaluating possible bio-geographic scenarios that could account for the genetic diversity, phylogenetic relationships and current distribution of bat trypanosomes. The evolutionary history of bats has been revised by recent phylogenetic studies. The order Chiroptera is one of the most diverse groups of mammals, with more than 1,000 species, originating in North America or Africa approximately 65 million years ago (MYA). Africa was reconstructed as the origin of modern-day bat families, with large radiations occurring during the Eocene (50 MYA). Rhinolophids and Hipposiderids are related microbats that were placed together with Old World megabats (Pteropodidae) in the suborder Yinpterochiroptera. *Rhinolophus* and *Hipposideros* are widespread throughout Asia, Oceania, Europe and Africa, while no species of this family inhabits the New World (Teeling et al., 2002; 2005; Eick et al., 2005; Simmons, 2005; Telling, 2009). The species found harbouring *T. zambesiensis* n. sp. are *R. landeri*, which are endemic to sub-Saharan Africa, and *H. caffer,* indigenous to an area spanning most of sub-Saharan Africa (except for the forested regions) to the south-western Arabian Peninsula (Simmons 2005).

Prior to this study, no species of *Megatrypanum* had been reported in bats from Mozambique, and no species of this subgenus from microbats had been included in phylogenetic analyses. Altogether, results from this study provide new insights into the genetic diversity of bat trypanosomes. The phylogenetic evidence produced by this study underscores the great diversity of trypanosomes infecting bats around the world and reinforces the need for a thorough reappraisal of the subgenus *Megatrypanum*.

Taxonomic section.

Description of a new species of the phylum Euglenozoa, Cavalier-Smith, 1981; class Kinetoplastea, Honigberg, 1963; order Trypanosomatida (Kent, 1880) Hollande, 1952; family Trypanosomatidae, Doflein, 1951.

Trypanosoma zambesiensis Lima and Teixeira n. sp. **Type material**, hapantotype: culture TCC1270. Paratypes: cultures TCC1270, 1271, 1295, 1298, 1304, 1902, 1933, 1935, 1947, 1948, 1953 and 1954, whose hosts and locality of collection are in Table 1. Cultures are deposited at the Trypanosomatid Culture Collection of the University of São Paulo, TCC-USP. Glass slides

of Giemsa-stained smears from cultures are also kept at TCC-USP. **Host**: Chiroptera, Rhinolophidae, *Rhinolophus landeri*. Additional host: *Hipposideros caffer.* **Locality***:* Mozambique, province of Sofala, district of Chupanga (S18º02' E35º34'), Zambezi valley. **Morphology**: Epimastigotes and trypomastigotes from cultures and bat blood trypomastigotes typical of *Megatrypanum* (Fig.1). **Diagnosis**: Sequences are deposited at Genbank (Table 1). **Etymology***:* The name in apposition was given because the first isolates of *Trypanosoma zambesiensis* n. sp. were obtained from bats captured in Chupanga, a small village bordering the Zambezi River, in Mozambique.

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Acta Tropica 112 (2009) 249–259

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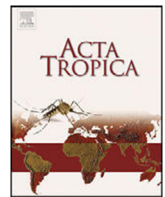

Genes of cathepsin L-like proteases in *Trypanosoma rangeli* isolates: Markers for diagnosis, genotyping and phylogenetic relationships

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ARTICLE INFO

Article history:
Received 22 May 2009
Received in revised form 27 July 2009
Accepted 28 July 2009
Available online 14 August 2009

Keywords:
Trypanosoma rangeli
Cathepsin L-like
Rangelipain
Cruzipain
Phylogeny
Genotyping
Lineages
Diagnosis

ABSTRACT

We have sequenced genes encoding cathepsin L-like (CatL-like) cysteine proteases from isolates of *Trypanosoma rangeli* from humans, wild mammals and *Rhodnius* species of Central and South America. Phylogenetic trees of sequences encoding mature CatL-like enzymes of *T. rangeli* and homologous genes from other trypanosomes, *Leishmania* spp. and bodonids positioned sequences of *T. rangeli* (rangelipain) closest to *T. cruzi* (cruzipain). Phylogenetic tree of kinetoplastids based on sequences of CatL-like was totally congruent with those derived from SSU rRNA and gGAPDH genes. Analysis of sequences from the CatL-like catalytic domains of 17 isolates representative of the overall phylogenetic diversity and geographical range of *T. rangeli* supported all the lineages (A–D) previously defined using ribosomal and spliced leader genes. Comparison of the proteolytic activities of *T. rangeli* isolates revealed heterogeneous banding profiles of cysteine proteases in gelatin gels, with differences even among isolates of the same lineage. CatL-like sequences proved to be excellent targets for diagnosis and genotyping of *T. rangeli* by PCR. Data from CatL-like encoding genes agreed with results from previous studies of kDNA markers, and ribosomal and spliced leader genes, thereby corroborating clonal evolution, independent transmission cycles and the divergence of *T. rangeli* lineages associated with sympatric species of *Rhodnius*.

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1. Introduction

Trypanosoma rangeli is a non-pathogenic parasite of man and domestic and wild animals in Central and South America. This species and *T. cruzi,* the agent of Chagas disease, are the only agents of human trypanosomiasis in the Americas; the two parasites share hosts and vectors and possess overlapping geographic distributions. *T. rangeli* is transmitted by triatomines of *Rhodnius* species and is considered harmful for these insects, whereas *T. cruzi* is transmitted by triatomines of various genera and is not harmful for its vectors (Guhl and Vallejo, 2003; Vallejo et al., 2009). In the triatomines, blood trypomastigotes of *T. rangeli* differentiate to epimastigotes, which multiply in the gut and invade the haemolymph. There, the parasites multiply freely or inside haemocytes until they reach the salivary glands, where metacyclogenesis takes place. Infective metacyclic trypomastigotes are inoculated into a new mammalian host by a salivary route. *T. rangeli* is unique in its development within vectors, differing even from *T. brucei*, which reach the salivary glands of tsetse from the proboscid and not through the haemolymph (Azambuja et al., 2005).

Cathepsin L-like (CatL-like) cysteine proteases (CP) are involved in numerous events in the complex life cycles of trypanosomes, playing vital functions in metabolism, infectivity, and multiplication and cell differentiation. They have also been implicated in parasite pathogenesis and in the modulation of host immune response (Authié et al., 2001; Lalmanach et al., 2002; Kosec et al., 2006; Mckerrow et al., 2006). CatL-like enzymes are involved in the pathogenicity and immune response of fish infected with the bodonids *Trypanoplasma borreli* and *Cryptobia salmositica* (Ruszczyk et al., 2008a,b). However, the role of these enzymes in trypanosomes that are non-pathogenic to their vertebrate hosts remains unknown.

CatL-like proteases of all trypanosomatids are encoded by a multigene family organised as multiple, tandemly repeated copies expanded by gene duplications. Genes homologous to the major CatL-like isoform of *T. cruzi* (cruzipain), which is the archetype of a multigene family of related isoforms, have been described in *T. b. brucei* (brucipain), *T. b. rhodesiense* (rhodesain), *T. congolense* (congopain), *T. rangeli* (rangelipain), *T. vivax* and *T. carassii.* Analyses restricted to a small number of sequences from a few kinetoplastids indicated congruent CatL-like and SSU rDNA phylogenies suggesting that CatL-like genes are valuable markers for phylogenetic studies of these organisms (Sakanari et al., 1997; Jesudhasan et al., 2007; Kuru et al., 2007; Ruszczyk et al., 2008a,b). It has been suggested that all parasitic CatL-like CP diverged following speciation (Sajid and Mckerrow, 2002). We recently

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doi:10.1016/j.actatropica.2009.07.036

described a complex repertoire of CatL-like genes in *T. vivax* isolates and confirmed that these sequences are excellent markers for diagnosis, genotyping and phylogenetic analysis (Cortez et al., 2009).

As in other trypanosomes, CatL-like genes in *T. rangeli* are organised in head-to-tail tandem repeats, divided in a pre–pro region, a catalytic domain with cysteine, histidine and asparagine residues that form the catalytic triad, and a C-terminal region (Martinez et al., 1995; Labriola and Cazzulo, 1995). The overall identities of predicted amino acids of whole CatL-like genes from *T. rangeli* (isolate LDG) were ∼80% and 62% compared with *T. cruzi* and *T. brucei*, respectively. However, in analysis restricted to catalytic domains (cd), sequences of the isolate LDG shared identities of 71% and 74%, respectively, with *T. brucei* and *T. cruzi* (Martinez et al., 1995), whereas *T. rangeli* isolate V showed higher identity with *T. brucei* (73%) than with *T. cruzi* (69%) (Tanaka et al., 1994).

The close relationship between *T. rangeli* and *T. brucei* suggested by their salivary transmission was reinforced by evidence from the affinities of CatL-like and β-tubulin genes, and similarities of karyotypes. These molecular markers and biological data generated a controversy and some authors proposed that *T. rangeli* should be transferred to a new subgenus in the Salivaria Section of trypanosomes (Añez, 1982; Amorim et al., 1993; Tanaka et al., 1994; Henriksson et al., 1996; Grisard, 2002). However, phylogenies inferred using SSU rRNA and gGAPDH genes positioned *T. rangeli* closest to *T. cruzi* and very distant from *T. brucei* (Stevens et al., 1999; Maia da Silva et al., 2004b, 2007; Hamilton et al., 2007). Phylogenies inferred using sequences from ITS rDNA and spliced leader genes identified at least five lineages (TrA–E) within *T. rangeli*. Lineages TrA, TrB and TrC, which are tightly associated with sympatric triatomine species from the complexes *R. prolixus, R. brethesi* and *R. pallecens,* respectively, constitute the three major evolutionary lines of this species (Maia da Silva et al., 2004a,b, 2007, 2008). In agreement, behaviour of *T. rangeli* differs in *Rhodnius* species from distinct geographical origins, with natural infection of salivary glands and thus transmission by bite restricted to local vectors (Guhl and Vallejo, 2003). According to kDNA organisation, *T. rangeli* isolates were assigned to KP1(−) or KP1(+) groups, related to *R. pallescens*/*R. colombiensis* and *R. prolixus*, respectively (Vallejo et al., 2003, 2009). Comparison of genotyping based on kDNA, ribosomal, Histone H2B and SL markers indicate that TrA corresponds to KP1(+), and TrC and TrD to KP1(−) (Vallejo et al., 2009; Puerta et al., 2009). The kDNA profile of TrB was not determined.

While diagnosis of *T. rangeli* in the haemolymph or salivary glands of triatomines is straightforward, diagnosis in the triatomine gut, where this species is commonly found mixed with *T. cruzi*, depends on problematic morphological identification and should be confirmed by molecular markers. Very low parasitaemia and serological cross-reactivity with *T. cruzi* complicates the diagnosis in humans and reservoirs (Vallejo et al., 2003). *T. rangeli* has been diagnosed using PCRs targeting kDNA (Vallejo et al., 1999), RAPD (Maia da Silva et al., 2004b), repetitive DNA (Vargas et al., 2000), and ribosomal (Souto et al., 1999) and SL (Fernandes et al., 2001; Maia da Silva et al., 2007) genes. Nevertheless, species and lineage identification should be improved by targeting genes encoding proteins with putative roles in host–parasite relationships.

Here, we characterised genes encoding CatL-like enzymes and examined proteolytic activities in *T. rangeli* isolates. Sequences from 17 isolates representative of all known lineages were used to infer phylogenetic relationships among CatL-like genes from *T. rangeli* and homologous genes from other trypanosomes. We demonstrated that these sequences could be targets for sensitive and specific diagnostic markers and showed that they may be useful for genotyping lineages in population structure analysis.

2. Materials and methods

2.1. *T. rangeli isolates, PCR amplification, sequencing and phylogenetic analyses of CatL-like genes*

T. rangeli isolates representing all established lineages were used in this study (Table 1). Logarithmic phase cultures of *T. rangeli* in LIT medium with 10% of fetal bovine serum were used for classic phenol-chloroform DNA extraction as in previous studies (Maia da Silva et al., 2004a), and used for preparation of lysates for analyses of proteolytic activities.

Fragments of DNA corresponding to cdCatL-like (∼500 bp) were PCR-amplified using primers DTO154 (5′ ACA GAA TTC CAG GGC CAA TGC GGC TCG TGC TGG 3′) and DTO155 (5′ TTA AAG CTT CCA CGA GTT CTT GAT GAT CCA GTA 3′) (Lima et al., 1994) as described previously (Cortez et al., 2009). Four to six cloned sequences from each *T. rangeli* isolate were determined, analysed through BLASTN, aligned with homologous sequences of other kinetoplastids from GenBank, and employed for phylogenetic analyses. Entire CatL-like sequences were also recovered from GeneDB database (Table 1). Sequences were aligned and refined visually using the program GeneDoc 2.6.002 (Nicholas and Nicholas, 1997). Phylogenies were inferred with nucleotide and amino acid sequences by parsimony (P) and maximum likelihood (ML) methods using respectively the programs PAUP 4.0b10 (Swofford, 2002) and RAxML v7.0.0 (Stamatakis, 2006). Parsimony analyses were carried out with 300 random-sequence-addition replicates followed by branch swapping (RAS-TBR). Bootstrap analyses employed 100 (nucleotide alignment) or 500 (amino acid alignment) replicates following strategy of P search. ML tree searches employed GTR GAMMA with 300 maximum parsimony-starting trees as described previously (Ferreira et al., 2008). ML bootstrap support was estimated with 1000 replicates in RAxML using rapid bootstrapping algorithm and maximum parsimony as starting trees. The model parameter (WAG GAMMA) for ML amino acid analyses was estimated by ProtTest program (Abascal et al., 2005). The alignments used in this study are available from the authors upon request. Sequences determined in this study are available in the GenBank under the accession numbers listed in Table 1.

2.2. *Standardisation of PCRs for species-specific diagnoses and lineage genotyping of T. rangeli*

Species-specific primers for *T. rangeli* (Tra-CatL: 5′ ACA CCG GCC GTG TAG GAC ATG 3′) and *T. cruzi* (Tcr-CatL: 5′ GGT AAT CGT GGC AAC CAC CGT 3′) were designed based on aligned cdCatL-like sequences. PCRs were standardised in 50 μl reactions containing: 100 ng DNA template, 200 μM each dNTP, 100 ng primer DTO 154, 100 ng of each specific primer (Tra-CatL or Tcr-CatL), and 1.25U Taq DNA polymerase. The reactions were submitted to initial denaturation of 94 °C for 3 min, 35 cycles of amplification at 94 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min, and a final extension cycle of 10 min at 72 °C. Lineage-specific primers were designed based on cdCatL-like sequences of *T. rangeli* that differ in at least three nucleotides among isolates of lineages TrA (primer TraCatL-A: 5′ GT CGG ACA TAT TGC ACG TC 3′), TrB (TraCatL-B: 5′ TTC GGC AAG TCA ACA TGA CC 3′) and TrC (TraCatL-C: 5′TCG ACA ATC CAG TCA AAG GCG CT 3′). We could not design primers for separation between TrA/TrE and TrC/TrD. The primer DTO 154 was employed as the forward primer in all reactions. PCR amplifications were performed as described above for species-specific PCRs, except that we used a lower annealing temperature of 64 °C. Amplified fragments were separated in 2.0% agarose gels and stained with ethidium bromide.

Table 1
Species, hosts and geographic origin of kinetoplastids employed for analysis of cathepsin L-like gene sequences and *T. rangeli* diagnosis and genotyping.

Species	Isolate	TryCC	Lineage	Host species		Geographic origin	GenBank and GeneDB accession number	
							Amino acid	Nucleotide
T. rangeli								
	S. Augustin (SA)	031	TrA	Human	*H. sapiens*	Colombia		FJ997556[a]
	AT-AEI	220	TrA	Monkey	*S. sciureus*	Brazil		
	MHOM/VE/99/D-99	530	TrA		*H. sapiens*	Venezuela		
	SMH-79	594	TrA	Human	*H. sapiens*	Guatemala		
	ROR-62	701	TrA	Triatomine	*R. robustus* II	Brazil		FJ997558[a]
	VE/9	775	TrA	Triatomine	*R. prolixus*	Venezuela		FJ997557[a]
	IM5051	1257	TrA	Monkey	*S. bicolor*	Brazil		
	Tra643	643	TrE	Bat	*P. lineatus*	Brazil		FJ997568[a]
	IM5038	901	TrE	Triatomine	*R. pictipes*	Brazil		FJ997570[a]
	IM5134	1182	TrE	Triatomine	*R. pictipes*	Brazil		FJ99757[a]
	IM5040	1224	TrE	Triatomine	*R. pictipes*	Brazil		
	IM5039	1301	TrE	Triatomine	*R. pictipes*	Brazil		FJ997569[a]
	PG	014	TrC	Human	*H. sapiens*	Panama		FJ997564[a]
	Pa487 GS	1254	TrC	Triatomine	*R. pallescens*	Panama		
	Pa479 GS	1260	TrC	Triatomine	*R. pallescens*	Panama		
	Pa4874	1292	TrC	Triatomine	*R. pallescens*	Panama		FJ997565[a]
	G5	–	TrC	Triatomine	*R. pallescens*	Colombia		FJ997566[a]
	LDG	–	TrC	Human	*H. sapiens*	Colombia	2117247A/B/C	L38514
	SC58	023	TrD	Rodent	*E. dasythrix*	Brazil		FJ997567[a]
	Tra Saimiri	012	TrB	Monkey	*S. sciureus*	Brazil		FJ997561[a]
	Tra Legeri	010	TrB	Anteater	*T. tetradactyla*	Brazil		FJ997559[a]
	AM80	086	TrB	Human	*H. sapiens*	Brazil		FJ997560[a]
	AE-AAB	194	TrB	Monkey	*C. pygmaea*	Brazil		
	5–31	238	TrB	Monkey	*S. labiatus*	Brazil		
	IM5050	1302	TrB	Monkey	*S. bicolor*	Brazil		FJ997562[a]
	MF8	1355	TrB	Triatomine	*R. pictipes*	Brazil		FJ997563[a]
T. cruzi								
	G	030	TCI	Marsupial	*D. marsupialis*	Brazil		FJ997574[a]
	JJ	085	TCIIa	Human	*H. sapiens*	Brazil		FJ997573[a]
	Y	034	TCIIb	Human	*H. sapiens*	Brazil	AAG35357.1	FJ997572[a]
	Tulahuen	–	TCIIb	Triatomine	*T. infestans*	Chile	P25779.1	
	CL-Brener	–	TCIIe	Triatomine	*T. infestans*	Brazil	AAL96762.1	
African trypanosomes								
T. congolense	IL300	–	–	Cattle	*B. taurus*	Kenya	AAA18215.1 AAD24589.1	L25130
T. b. brucei	EATRO 164	–	–	Hartebeest	*A. lichtensteini*	–		X16465
T. b. brucei	TREU 927	–	–	Fly	*Glossina* sp.	Kenya	XP_845224.1	
T. b. brucei	TCC-148	–	–	Cattle	*B. taurus*	Kenya	CAC67416.1	
T. b. rodhesiense	WRATat1.1	–	–	Human	*H. sapiens*	Kenya		AJ297265
T. vivax	Y486	–	–	Cattle	*B. taurus*	Nigeria	Tviv290f05.q1k_12 Tviv521g07.p1k_2	Tviv534d01.q1k_7
Fish trypanosome								
T. carasii		–	–	Fish	*C. carpio*	Czech Republic	ABQ23397.1	EF538803
Leishmania spp								
L. major	Friedlin	–	–	Human	*H. sapiens*	–	XP_001681137	
L. tropica		–	–	Human	*H. sapiens*	–	ABB88697.1	
L. donovani		–	–	Human	*H. sapiens*	–	AAL09443.1	
L. aethiopica	1093/02	–	–	Human	*H. sapiens*	–	AAZ23596.1	
Non-trypanosomatid kinetoplastid								
Trypanoplasma borreli		–	–	Fish	*C. carpio*	Germany	ABQ23398.1 ABQ23399.1	
Cryptobia salmositica	T4	–	–	Fish	*Oncorhynchus* spp	Canada	AAU14993 AAM09951	

TryCC: code number of cultures in the Trypanosomatid Culture Collection of the Department of Parasitology, University of São Paulo.
[a] Sequences determined in this study and deposited in the GenBank.

2.3. Southern blotting and PFGE analyses of CatL-like genes of *T. rangeli*

Genomic DNA from *T. rangeli* was digested with *Sau* 3AI (partial and total digestion) and *Taq* I (total digestion), separated in 2.5% agarose gel and transferred to nylon membranes. The membranes were hybridised with the probe Tra-cdCatL, which consists of a PCR-amplified cdCatL-like gene from *T. rangeli* SA labelled with ^{32}P, as in previous studies (Maia da Silva et al., 2004a). Chromosome blocks were prepared by embedding 10^8 parasites in 1.2% low melting point agarose subjected to PFGE (CHEF Mapper – BioRad) using 1% agarose gel, at 14 °C and under the following conditions: first phase: 35.40 s to 1 min 19 s linear ramped times for 30 h 45 min, 6 V/cm^3; second phase: 1 min 25 s to 2 min 38 s linear ramped times for 14 h, 6 V/cm^3. Chromosome bands were stained by ethidium bromide, and blotted onto Hybond N$^+$ Nylon membranes (Amersham). Chromoblot was prehybridised at 60 °C for 90 min in 3× SSC, 5% SDS, 1% Ficoll, 0.1% PVP and 100 μg/ml denatured salmon sperm DNA, hybridised for ∼18 h at 60 °C with the probe Tra-cdCatL, and washed at 60 °C for

30 min each twice in 3× SSC, 0.1% SDS and once in 1× SSC, 0.1% SDS.

2.4. Detection of proteolytic activities in gelatin gels and hydrolysis of Z-Phe-Arg-AMC

To assess proteolytic activities in gelatin gels, parasites were washed twice by centrifugation (1600 *g*, 10 min) in Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (PBS), resuspended (at 6×10^8 organisms/ml) in lysis buffer (20 mM Pipes, pH 6.8; 100 mM NaCl; 1 mM EDTA; 0.1% (v/v) Chaps; 50 μM digitonin; 0.1% (v/v) Triton X-100), and incubated for 30 min in an ice bath. Prior to electrophoresis in 11% resolving SDS-acrylamide gels containing 500 μg/ml gelatin, lysates were treated with 20 μg/ml antipain, and mixed with 4× sample buffer as previously described (Alfieri et al., 1989). After electrophoresis (5 °C; constant 5 mA), gels were incubated (33 °C, under agitation) two times for 30 min in 2.5% (v/v) Triton X-100 in 0.1 M buffer (acetate: pH 4.0 and 5.0; Tris-HCl: pH 7.5) containing 5 mM DTT, and then overnight (∼18 h), with one change, in buffer-DTT. Gels were fixed with 10% TCA, and stained with Coomassie blue R-250. Bands associated to cysteine peptidases were identified by incubating gel halves with 10 μM E-64.

In assays with Z-Phe-Arg-AMC (*N*-α-benzyloxycarbonyl-phenylalanyl-arginine-7-amide-4-methyl-isocumarin), 3–5 μl of lysates (prepared as described above) were added in a 1.5 ml cuvette placed in a holder controlled at 37.5 °C, and containing pre-warmed 50 mM acetate buffer (pH 5.4), 100 mM NaCl, 1 mM EDTA, and 2 mM DTT. Following 5–10 min incubation (in presence or absence of 8 μM E-64), the substrate (10 μM) was added, and the reaction was recorded continuously for up to 10 min in a Hitachi F-4500 fluorescence spectrophotometer (excitation wavelength: 380 nm; emission wavelength: 460 nm). The activity was expressed in arbitrary fluorescence units (AFU)/min μg of protein.

3. Results

3.1. Evolutionary relationships of sequences from genes encoding CatL-like mature proteins from trypanosomes and congruence with phylogeny based on SSU rRNA gene

To understand the evolutionary relationship between *T. rangeli* CatL-like genes and homologous genes from other trypanosome species and, thus, to make reliable interpretations about the controversial evolutionary relationships of *T. rangeli, T. cruzi* and *T. brucei*, we aligned predicted amino acid sequences for two to three sequences of mature CatL-like enzymes (217 amino acids) from each of the following species: *T. rangeli*, *T. cruzi*, *T. b. brucei, T. b. rhodesiense*, *T. congolense*, and *T. vivax*. We also aligned one sequence of *T. carassii* from fish. Aligned CatL-like amino acid sequences from these five species of trypanosomes, four species of *Leishmania*, *Tr. borreli* and *C. salmositica* disclosed polymorphisms distributed over the entire gene. The pre-domains and C-terminal regions showed the highest divergence, whereas pro- and catalytic domains revealed sequences conserved among closely related species, aside from regions of identical sequences shared by CatL-like sequences within the same species (see Supplementary Material). The identity of predicted amino acid sequences of mature CatL-like from *T. rangeli* (LDG) was highest (∼74%) compared with cruzipain from *T. cruzi* isolates (∼99.7% similarity), and despite significant similarities was smaller compared with sequences from *T. b. brucei* (72%), *T. vivax* (71%) and *T. congolense* (69%). In contrast, large distances separated trypanosome CatL-like sequences from those of *Leishmania* (41%) and bodonids (47%).

Comparison of whole sequences demonstrated that rangelipain shares higher identity with cruzipain than with brucipain in all domains. All residues critical for constituting the catalytic domain and the substrate-specific sites are conserved, and the number of non-synonymous substitutions is smallest between rangelipain and cruzipain (Fig. 1). Average GC contents of the full-length CatL-like genes are higher in *T. rangeli* (65%) and *T. cruzi* (63%) isolates than in *T. brucei* (∼54%).

To infer phylogenetic trees, pre–pro and C-terminal regions, which are absent in the mature enzyme, were excluded due to questionable alignment. Phylogenetic analyses of amino acid data sets separated CatL-like sequences of trypanosomes in two major clades, one formed by sequences from *T. rangeli* and *T. cruzi* (93% bootstrap) and the other containing sequences from *T. brucei*, *T. congolense* and *T. vivax* (90% bootstrap). *T. carassii* from fish was basal to the clade formed by all mammalian trypanosomes. Sequences from *Leishmania* spp. formed a sister clade of the assemblage comprising all trypanosome sequences, and bodonids were positioned as outgroup of Trypanosomatidae. Congruent phylogenetic trees were obtained using ML and MP methods (data not shown).

Despite divergences, sequences from different CatL-like gene copies of the same trypanosome species always clustered together in all phylogenetic analyses (data not shown), suggesting that CatL-like genes of *T. rangeli* are subject to high constraint. Comparison of cdCatL-like sequences of several *T. rangeli* isolates from five lineages suggested the existence of only one major isoform homologous to cruzipain of *T. cruzi*. This finding agrees with the homogeneity of CatL-like sequences from *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*, and contrasts with the high heterogeneity within *T. vivax* (Cortez et al., 2009) and between the two main isoforms of *T. cruzi* (Lima et al., 1994, 2001). Further studies are required to assess the existence of minor CatL-like isoforms in *T. rangeli*.

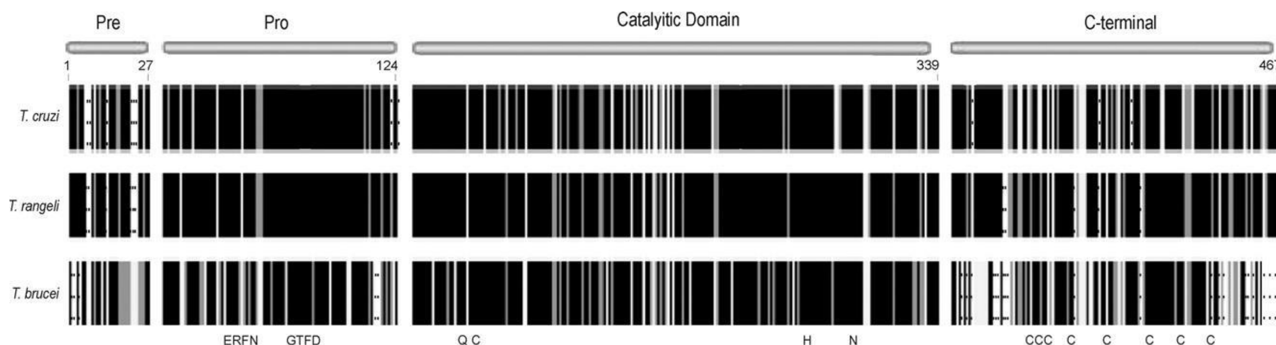

Fig. 1. Schematic alignment of amino acid sequences of cathepsin L-like genes from *T. rangeli* and homologous sequences from *T. cruzi* and *T. brucei*. Black blocks indicate identical or synonymous amino acids. Dark gray and light gray blocks represent sites showing different amino acids, polar and non-polar, respectively. Bars indicate the cleavage sites of the pre- and pro-domains, and the border between the catalytic domain and the C-terminal extension. The positions of aminoacids indicated above the alignment refer to rangelipain. The cathepsin L family signatures are indicated at the bottom: ERFININ (ERFN) and GNFD (GTFD) pro-domain motifs, glutamine [Q] of the oxyanion hole, cysteine [C], histidine [H] and asparagine [N] of catalytic triad in the catalytic domain as well as 8 cysteines in the C-terminal extension this sequences.

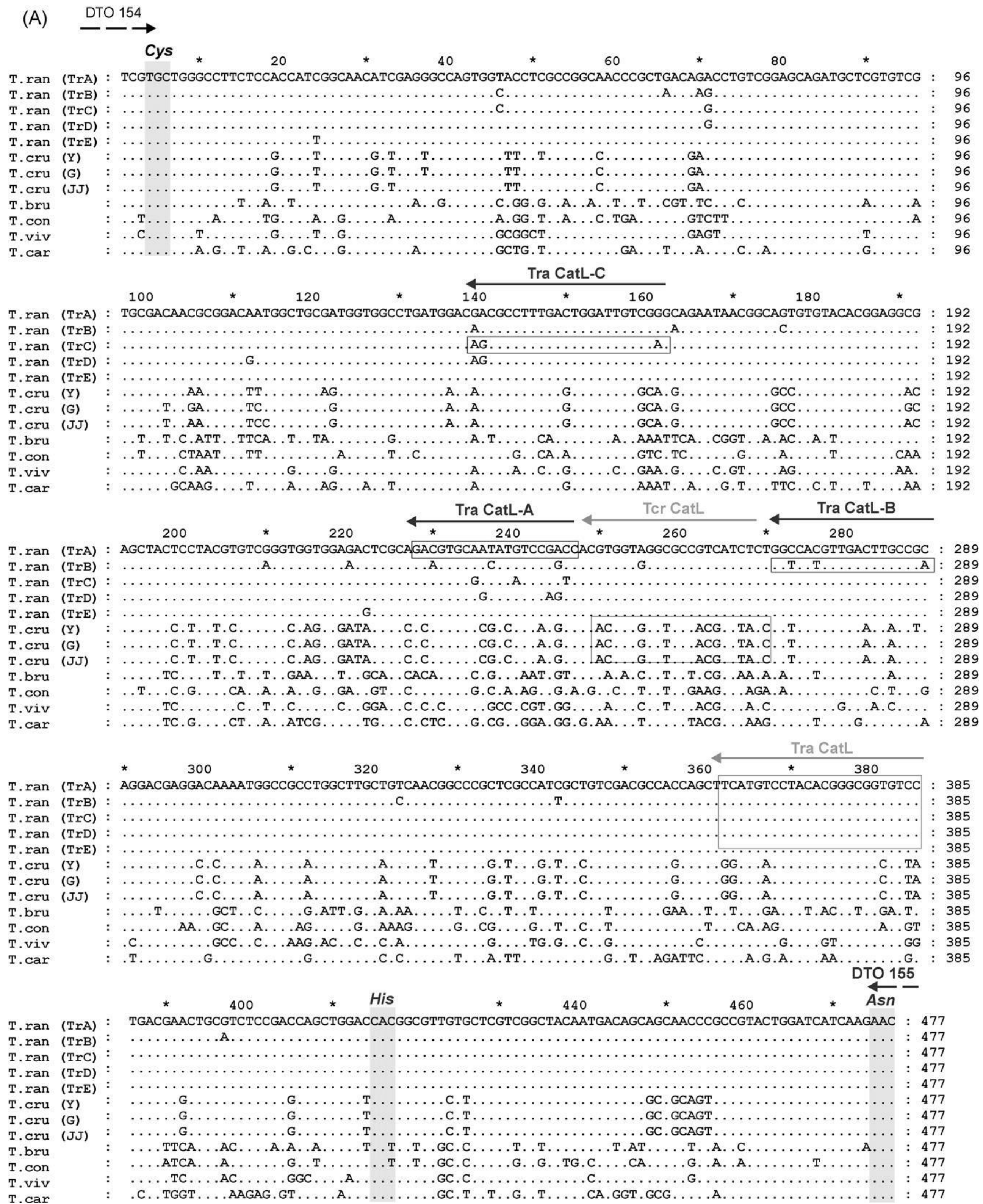

Fig. 2. (A) Aligned nucleotide sequences corresponding to catalytic domain of cathepsin L-like proteases from *T. rangeli* and other trypanosomes. Dashed black arrows indicate position of primers used for PCR-amplification of catalytic domains. Gray arrows indicate primers designed for the diagnostic PCRs of *T. cruzi* (PCR-DTcrCatL) and *T. rangeli* (PCR-DTraCatL). Black arrows indicate positions of primers employed for lineage genotyping. The codons of the catalytic triad are shaded. (B) Phylogenetic tree (ML) of nucleotide sequences of partial catalytic domain of cathepsin L-like genes from *T. rangeli* isolates and other trypanosomes. The numbers in nodes correspond to bootstrap values from 100 replicates respectively from ML/MP analyses (values above 50% are omitted).

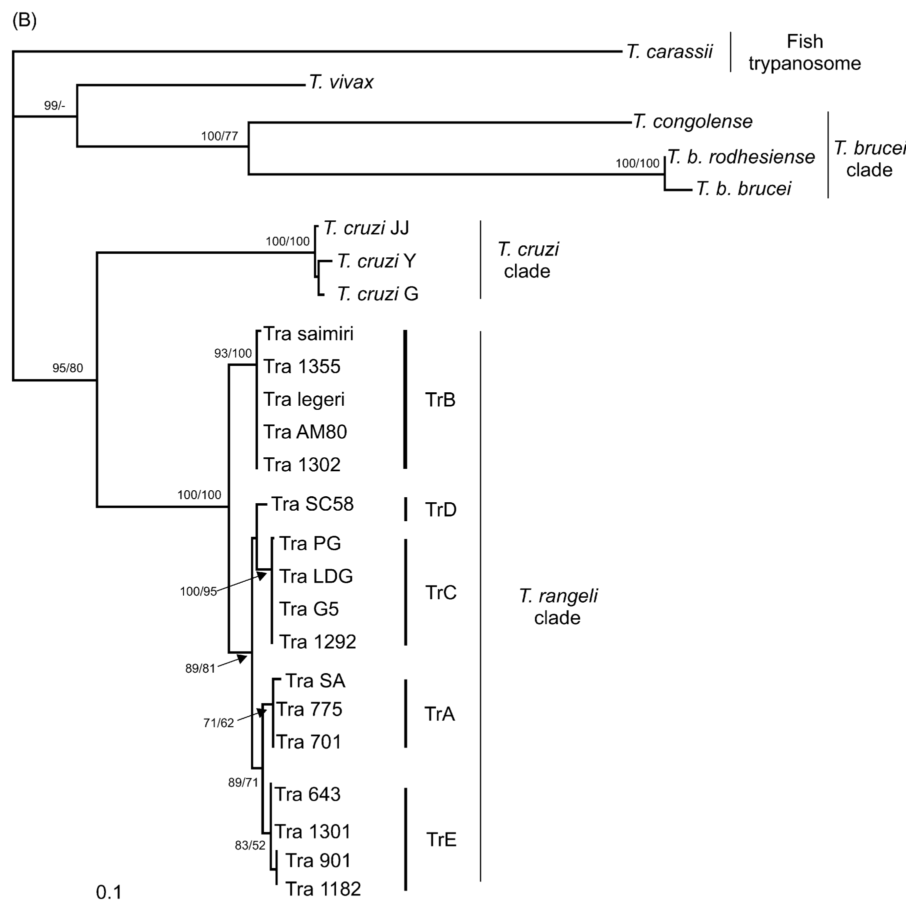

Fig. 2. (*Continued*).

3.2. *Population structure analysis of T. rangeli isolates by phylogenetic analysis of CatL-like genes*

We evaluated the suitability of CatL-like genes for analysis of the genetic structure of *T. rangeli* populations by comparing sequences (∼500 bp) of the cdCatL-like genes from 17 isolates from Central and South America, belonging to all previously defined lineages (TrA–E) (Table 1). The catalytic domain is important for enzymatic activity and is structurally and functionally conserved, thus allowing high-confidence alignments. About 4–6 cloned cdCatL-like sequences were determined from each isolate. Nineteen different sequences from a total of 74 sequences from 16 *T. rangeli* and 3 *T. cruzi* isolates were aligned with sequences of *T. rangeli* LDG and other trypanosomes from GenBank or GeneDB (Table 1). Genes from the same isolate or from isolates of the same *T. rangeli* lineage were identical or highly similar and always clustered tightly together. Nucleotide sequences from CatL-like genes of *T. rangeli* showed ∼97% similarity (Fig. 2A), and independently of lineages were all nested (100% bootstrap) in the clade *T. rangeli* (Fig. 2B).

Despite high homogeneity, the cdCatL-like-derived tree inferred using ML from the nucleotide data sets always revealed two major clades (89% and 93% bootstrap). The largest clade (89% bootstrap) comprised two subclades, one formed by sequences from isolates of lineage TrC (∼99.9% internal similarity and 100% bootstrap), and the other constituted by sequences from isolates of the TrA lineage (∼99.5% similarity and 71% bootstrap). A small distance (∼0.6%) separated TrA from isolates of TrE (∼99.8% similarity and 83% bootstrap). Positioning of the isolate SC58 representative of TrD was not resolved, alternating between TrC (∼1.7%) and TrA (∼1.8% divergence) depending on phylogenetic analysis. The other clade comprised homogeneous sequences from TrB isolates (∼99.9% similarity and 93% bootstrap), and was separated from all other lineages by large distances varying from ∼4.2% (TrA) to ∼4.4% (TrC) (Fig. 2B). Large divergences of ∼19.5% and ∼30% separated *T. rangeli* from *T. cruzi* or *T. brucei*, respectively. Nucleotide polymorphisms of cdCatL-like sequences were more relevant among lineages of *T. rangeli* (∼2.6% divergence) compared to those of *T. cruzi* lineages (∼1.0%).

The trees inferred using nucleotide sequences encoding cdCatL-like or mature CatL-like enzymes were totally congruent. Phylogenies of cdCatL-like nucleotide (Fig. 2B) or amino acid sequences also generated concordant topologies, with all lineages supported in all analyses. Nevertheless, relationships among *T. rangeli*, *T. cruzi* and *T. brucei* were not well resolved in analyses using amino acid sequence from small sequences restricted to cdCatL-like of CP (data not shown).

3.3. *Development of T. rangeli and T. cruzi-specific PCR targeting cdCatL-like sequences*

We standardised a *T. rangeli*-specific PCR (PCR-DTraCatL) based on cdCatL-like genes that detected all isolates from Central and South America, and ascribed isolates to five phylogenetic lineages (TrA–E). DNA fragments of similar lengths (∼385 bp) were generated for all *T. rangeli* isolates regardless their lineages (Fig. 3A). Amplification products were not generated for *T. cruzi* (Y, G, JJ), *T. dionisii*, *T. lewisi*, *T. conorhini* or *T. brucei*, thus indicating that this method should be specific to *T. rangeli*. *T. cruzi*-specific PCR-DTcrCatL was developed to amplify a DNA band of ∼270 bp for all *T. cruzi* isolates examined, from the phylogenetic lineages TCI, TCIIa and TCIIb (Fig. 3B) as well as TCIIc and TCIId (data not

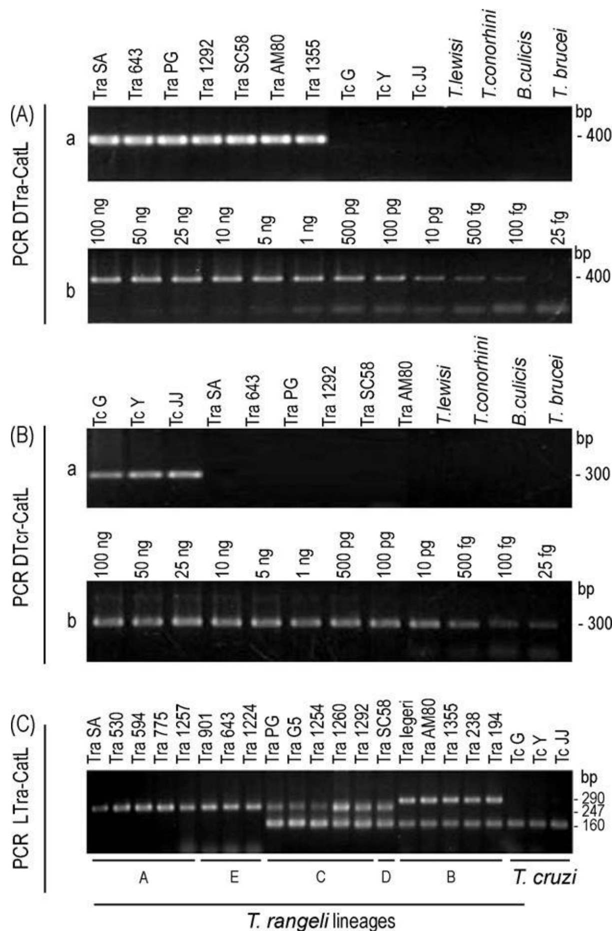

Fig. 3. Agarose gels stained with ethidium bromide showing results from PCR reactions targeting cathepsin L-like genes of *T. rangeli* and *T. cruzi*. (A) Specificity (a) and sensitivity (b) analyses of PCR-DTraCatL; (B) specificity (a) and sensitivity (b) analyses of PCR-DTcrCatL; (C) variable patterns of amplified products generated by multiplex PCR-LTraCatL using DNA of *T. rangeli* isolates from distinct lineages.

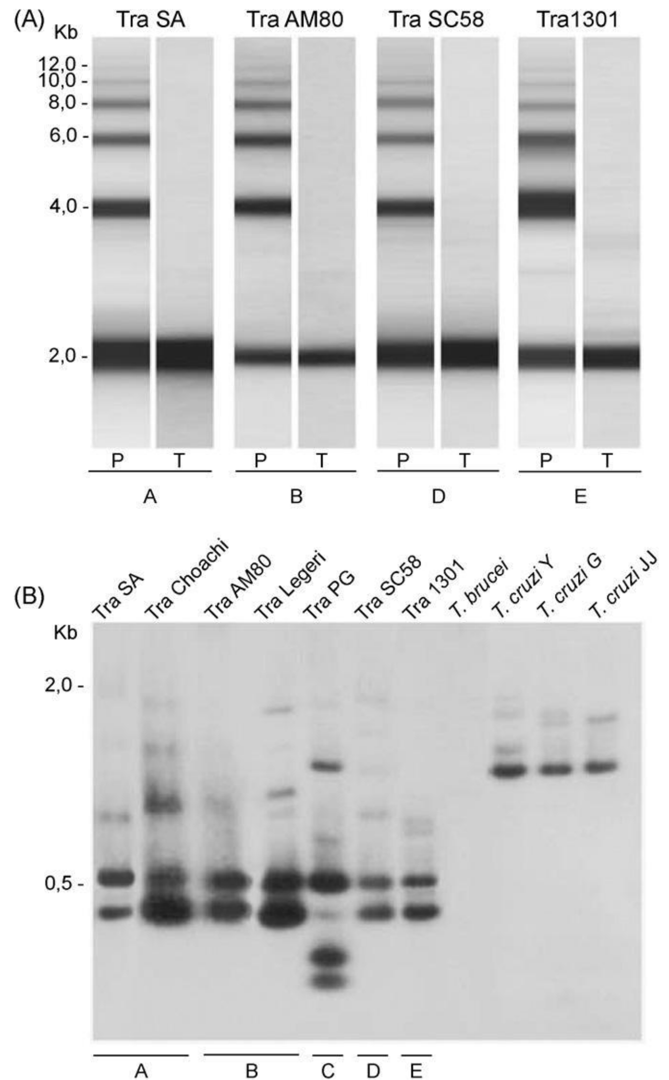

Fig. 4. Southern blot analyses of cathepsin L-like gene digested with restriction enzymes and hybridised with the probe Tra-cdCatL. (A) partial (P) and total (T) digestion of *T. rangeli* genomic DNA with *Sau* 3AI enzyme showing tandem arrays of CatL-like gene repeats and monomers of ~1.9 kb. Restriction profiles of DNA from *T. rangeli* isolates of all lineages (A–E), *T. brucei* and isolates of distinct *T. cruzi* lineages digested with *Taq* I enzyme (B).

shown). TraCatL-PCR and TcrCatL-PCR were highly sensitive detecting respectively DNA starting from 500 fg (~5 cells) and 25 fg (~1 cell). The difference in sensitivity was probably due to the smaller number of CatL-like gene copies in *T. rangeli* (~75) compared to *T. cruzi* (more than 100 copies) (Campetella et al., 1992; Eakin et al., 1992). A duplex PCR using mixed DNA of these two species substantially decreased detection of *T. rangeli* (data not shown).

3.4. Development of multiplex PCR based on cdCatL-like sequences for genotyping of T. rangeli lineages

A multiplex PCR assay was developed to amplify DNA fragments of different lengths for TrA, TrB and TrC. According to the results, a combination of bands generated three patterns: (1) DNA fragment of 247 bp for TrA and TrE; (2) a doublet of bands, the expected fragment of 160 bp plus a 247 bp band shared by TrC and TrD; (3) a doublet consisting of the expected 290 bp band plus a 160 bp band showed for TrB isolates. In addition, PCRs using DNA from *T. cruzi* exclusively showed a 160 bp band, a pattern not observed for any *T. rangeli* isolate (Fig. 3C). Despite attempts to use other primers and PCR conditions, we were unable to develop a multiplex PCR generating only one DNA band for each *T. rangeli* lineage, and to abolish the 160 bp band of *T. cruzi* due to high conservation of the target sequences. PCRs using separated primers and higher annealing temperatures generated only the expected DNA band for each *T. rangeli* lineage (data not shown).

3.5. Genomic organisation and chromosomal localisation of CatL-like genes

Southern blot of *T. rangeli* genomic DNA digested with *Sau* 3AI enzyme and hybridised with the probe Tra-cdCatL confirmed that CatL-like genes are present in multiple copies and organised in tandem monomers of the same length (~1.9 kb) (Fig. 4A) as previously reported for isolates of TrA (V) and TrC (LDG) (Tanaka et al., 1994; Martinez et al., 1995). Analyses using *Sau* 3AI revealed repeats of the same length and homogeneous genomic organisation of CatL-like genes for other isolates of all lineages, except for isolates of TrC, which possess a restriction site for this enzyme within the cdCatL-like (data not shown), and also displayed different restriction patterns with *Taq* I enzyme (Fig. 4B). In agreement with degree of CatL-like sequence divergence, the probe Tra-cdCatL of *T. rangeli* SA (TrA) strongly hybridised with all isolates of this species, and showed relevant hybridisation signals for all *T. cruzi* isolates but not for *T. b. brucei* (Fig. 4B).

Karyotypes of *T. rangeli* isolates showed individual PFGE profiles of chromosomal bands stained with Ethidium bromide for the

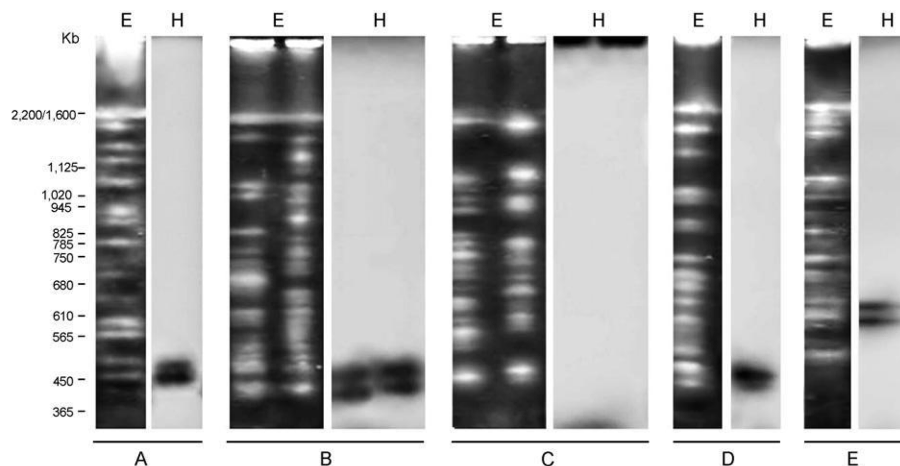

Fig. 5. PFGE karyotype patterns of *T. rangeli* isolates separated in the same gel stained with ethidium bromide (E), and cromoblots of the same gel hybridised with Tra-cdCatL probe (H) showing CatL-like genes in different chromosomal bands from each *T. rangeli* isolates (lineages A–E), excepting TrC isolates that showed hybridisation signal only in gel slots. *Saccharomyces cerevisiae* chromosomes were used as band size markers.

seven isolates analysed, even those belonging to the same lineage (Fig. 5). In chromoblots hybridised with the probe Tra-cdCatL, CatL-like genes were located in two chromosomal bands ranging from ~400 to 680 kb, disclosing two bands of variable size for isolates of lineages TrA (Tra220), TrB (AM80 and saimiri), TrD (SC58) and TrE (Tra1301) (Fig. 5). Exceptions were isolates PG and Tra1292 (TrC), which displayed hybridisation signals only in the gel slots where agarose plugs were spotted, a region without hybridisation signals for other isolates. This result indicates that CatL-like genes in TrC isolates are restricted to very large chromosomal bands not resolved under conditions standardised here for karyotyping of all other lineages (Fig. 5). Our data agree with previous studies (Tanaka et al., 1994; Martinez et al., 1995; Toaldo et al., 2001) locating CatL-like genes of *T. rangeli* LDG and PG (TrC) in very large chromosomal bands, in contrast to genes of isolates of TrA (V and UB66) and TrD (SC58), which were also located by these authors in bands of similar range, which were here detected as doublets in all isolates examined, including isolates of TrB and TrE lineages, which were never examined by PFGE before this study.

3.6. Activity of cysteine proteases in isolates of different T. rangeli lineages

Analysis of CP activities in gelatin gels of *T. rangeli* epimastigote lysates of lineages TrA, TrB, and TrC showed two or three bands of activity resolving in the region of 35–50 kDa in all isolates examined (Fig. 6A). These CP proteolytic profiles agreed with those from other *T. rangeli* isolates (De Santa-Izabel et al., 2004). Data are compatible with deduced amino acid sequence of the full-length CatL-like gene of *T. rangeli* (LDG), which encodes a protein of 472 amino acids with a predicted molecular mass of ~40 kDa (Martinez et al., 1995). Here, all bands were stimulated by 5 mM DTT, optimally detected at acidic pH (4.0 and 5.0) (Fig. 6A), and visualized with low intensity in gelatin gels incubated at pH 7.5 (data not shown). The potent inhibition by E-64 (10 μM) (Fig. 6B) confirmed all activities of *T. rangeli* as CP. *T. rangeli* isolates differed remarkably with regard to the banding profile of their CP. Differences were noted even among the isolates legeri, saimiri and AM80 of TrB. Compared to *T. cruzi*, results indicated low CP activity in *T. rangeli* epimastigotes (Fig. 6A).

Quantitative assays performed at pH 5.4 with Z-Phe-Arg-AMC confirmed high hydrolytic activity of *T. cruzi* lysates, in contrast to relatively low rates of substrate hydrolysis by lysates of *T. rangeli* isolates of TrA (SA) and TrB (saimiri and AM80). *T. rangeli* PG (TrC) displayed the lowest activity (Fig. 6C). Involvement of CP in the hydrolysis of Z-Phe-Arg-AMC was indicated by the effect of E-64 (8 μM). In 5 replicates, inhibition by E-64 in assays with lysates of *T. cruzi* was 99.1 ± 0.8%, and with lysates of *T. rangeli* were 93.9 ± 1.7%, 96.9 ± 0.8%, and 86.2 ± 3.5% (mean ± standard deviation of the mean), respectively for saimiri, AM80 and SA isolates. In 10 assays performed with lysates of *T. rangeli* PG, E-64 was significantly less effective (66.9 ± 5.2%).

4. Discussion

In the present study, we performed a comprehensive characterisation of sequences from genes encoding CatL-like enzymes from Central and South American isolates of *T. rangeli*. Phylogenetic analysis of genes encoding the mature CatL-like enzymes from *T. rangeli* (rangelipain) and homologous enzymes from other trypanosomes, *Leishmania* spp. and bodonids, positioned sequences from these organisms, in concordance with their placement in the phylogenetic trees of kinetoplastids based on SSU rDNA and gGAPDH genes (Hamilton et al., 2007). Genes encoding CatL-like enzymes could be suitable for phylogenetic studies of trypanosomatids since their multiple paralogous sequences are thought to be subject to concerted evolution (i.e., interdependent evolution that results in greater similarity of sequences within species than between species) (Jackson, 2007). This fact was evident with our analysis of CatL-like genes homologous to cruzipain in *T. rangeli*. Paralogous sequences from several isolates were much more similar within *T. rangeli*, and within each lineage, than with homologous genes from other species. We demonstrated similar evolutionary patterns of CatL-like sequences in *T. vivax* and *T. brucei* (Cortez et al., 2009).

Despite the fact that all CatL-like gene sequences from *T. rangeli* isolates characterised in this study always clustered together, the branching pattern within this monophyletic assemblage revealed clades of highly similar sequences, which can be correlated with previously established phylogeographical structuring of *T. rangeli* lineages (Maia da Silva et al., 2004b, 2007). We previously inferred phylogenies using ITS rDNA and SL sequences of isolates representative of the overall phylogenetic diversity of *T. rangeli*, and results revealed three main evolutionary lines within this species: TrA, TrB and TrC lineages. Lineages TrE and TrD were closely related to TrA and TrC, respectively, and were separated by large genetic distance from TrB (Maia da Silva et al., 2007, 2008). In contrast to the relevant divergence (~3.5%) among cdCatL-like sequences from *T. rangeli* of different lineages, small divergences (~1.0%) separated

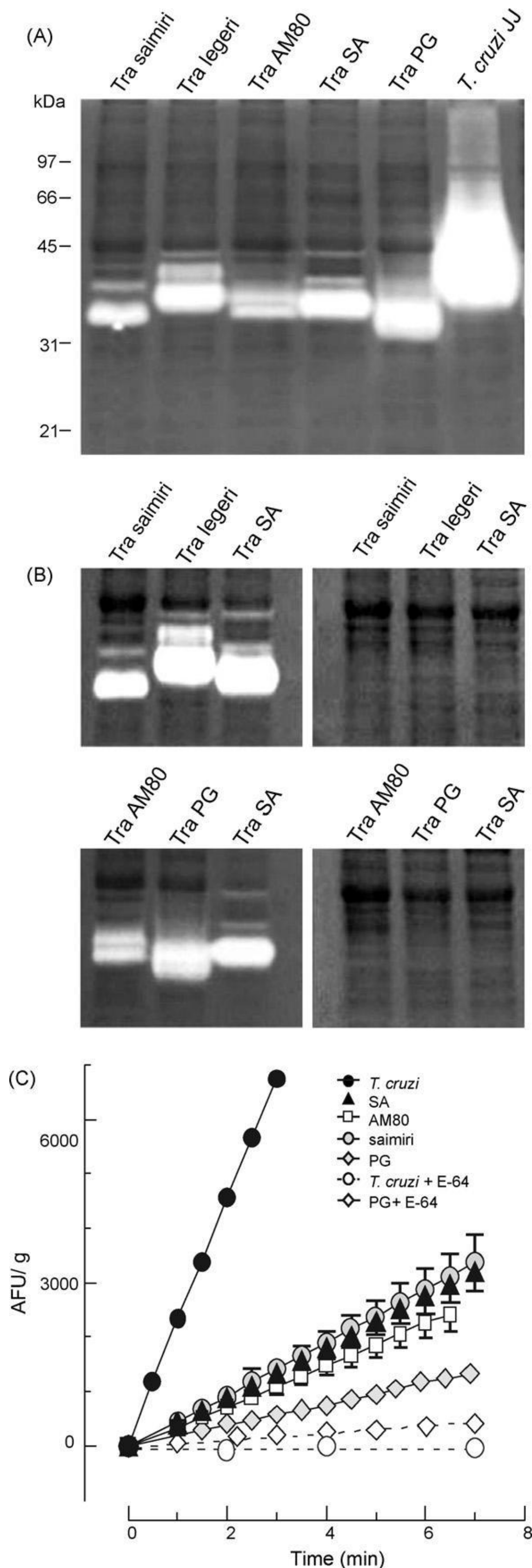

Fig. 6. Proteolytic activities of epimastigote lysates of *T. rangeli* and *T. cruzi*. (A) Activity banding profiles in gelatin gels, pH 5.0 and 5 mM DTT. (B) Similar experiments in which gel halves were incubated with 10 μM E-64. Gels were loaded with 10 μg of protein of *T. rangeli* from lineages TrB (saimiri, legeri, AM80), TrA (SA) and TrC (PG) and 7 μg of protein from *T. cruzi* JJ. (C) Hydrolysis of Z-Phe-Arg-AMC at pH 5.4 (37.5 °C) by lysates of *T. rangeli* isolates saimiri (○), SA (▲), AM80 (□) and PG (◇), and *T. cruzi* (●). The means ± SD (bars) of at least five experiments are indicated. The dashed lines indicate the inhibitory effect of 8 μM E-64 on hydrolytic activity of *T. cruzi* (○) and *T. rangeli* PG (◇).

CatL-like genes from *T. cruzi* isolates of TCI, TCIIb and TCIIa lineages, which are themselves separated by large phylogenetic distances. These data contrast with data from ribosomal sequences, which show high homogeneity among *T. rangeli* lineages (Maia da Silva et al., 2004b), and large divergences separating the lineages of *T. cruzi* (Marcili et al., 2009).

This is the most comprehensive study using sequences from protein-encoding genes to compare isolates of *T. rangeli* from all established phylogenetic lineages. All the five lineages were supported by phylogenies of CatL-like sequences. A study based on Histone H2A genes of isolates from Colombia and Southern Brazil supported two groups, KP1(+) and KP1(−) of *T. rangeli* isolates (Puerta et al., 2009). New evidence from CatL-like genes provided by the present study, together with our studies based on ribosomal and SL gene sequences support at least five lineages found so far within *T. rangeli*. All data corroborate independent genotypes of *T. rangeli* circulating as clonal populations in separated transmission cycles, with lineage divergence linked to sympatric species of *Rhodnius* (Vallejo et al., 2003, 2009; Urrea et al., 2005). Congruence between phylogeographical patterns of parasite lineages and vectors and transmission restricted to local vectors suggest a long coexistence of *T. rangeli* lineages and their vectors (Maia da Silva et al., 2007).

There is evidence that *T. rangeli* modulates its survival by overcoming the immune defences of the insect vector (Azambuja et al., 2005). Differential susceptibility of *Rhodnius* species was established for *T. rangeli* from *R. pallescens*, which does not develop in *R. prolixus* salivary glands. On the other hand, *T. rangeli* from *R. prolixus* does not develop in salivary glands of *R. pallescens* (Guhl and Vallejo, 2003; Vallejo et al., 2009). A recent study reported a trypanolytic protein in the haemolymph of *R. prolixus* that might be important in the differential behaviour of *T. rangeli* isolates (Pulido et al., 2008). It has been speculated that proteases are involved in the invasion of haemolymph and salivary glands, processes that require the ability of *T. rangeli* to cross membranes of the digestive tract and the salivary glands of triatomines (Azambuja et al., 2005). Although the basis of this differential susceptibility is unknown, restriction of *T. rangeli* to the salivary glands of sympatric *Rhodnius* species and parasite lineages vector-associated might be linked to putative roles of CP enzymes.

Trypanosomes share homologous genes encoding functionally different CatL-like enzymes. This functional diversification may have contributed to the adaptation of the trypanosomes to their different life cycles in vertebrate and invertebrate hosts. Here, we demonstrate that *T. rangeli* isolates from all lineages share CatL-like genes homologous to cruzipain, known to be critically involved in parasite invasion and differentiation in vertebrate hosts and in the gut of triatomine vectors (Lima et al., 2001; Lalmanach et al., 2002; Sajid and Mckerrow, 2002; Mckerrow et al., 2006). However, the functions of rangelipain, the major CP of *T. rangeli* expressed by epimastigotes (trypomastigotes were not investigated), remain unknown. The low expression in northern blot and proteolytic assays from epimastigotes of *T. rangeli* LDG (TrC) was hypothetically related to lack of pathogenicity for vertebrate hosts (Labriola and Cazzulo, 1995). Corroborating differences among isolates, northern blot analysis revealed relevant CatL-like enzyme expression in epimastigotes of *T. rangeli* V (TrA), and these enzymes were hypothesized to be involved in the escape of these forms from the gut and invasion of the hemolymph and salivary glands of triatomines, contributing to the pathogenicity of *T. rangeli* for its vectors (Tanaka et al., 1994).

The analysis performed here with epimastigote lysates of *T. cruzi* and *T. rangeli*, all compared under the same experimental conditions, confirmed lower expression of CP in all isolates of *T. rangeli* examined compared to *T. cruzi*. While rather comparable levels of activity hydrolyzing Z-Phe-Arg-AMC were found in lysates of *T. rangeli* SA (TrA), AM80 and saimiri (TrB), the lowest activity was displayed by isolate PG (TrC). In contrast to TrA and TrB isolates, hydrolyzis by PG isolate was only partially inhibited (~66%) by E-64. A previous study of *T. rangeli* LDG described a proteolytic activity not depicted in gelatin gels, unaffected by E-64 and, apparently, related to an alkaline peptidase (Labriola and Cazzulo, 1995). Since we have been unable to detect an activity resistant to E-64 in gelatin gels, we hypothesize that the activity hydrolyzing Z-Phe-Arg-AMC detected in *T. rangeli* PG involves either a CP non-inhibited by E-64 or other class of proteases, as suggested for LDG, which interestingly also belong to TrC lineage (Labriola and Cazzulo, 1995).

A previous comparative study of CP enzymes of *T. rangeli* isolates showed differences in the profiles in gelatin gels, and suggested that this method could be useful for lineage taxonomic purposes (De Santa-Izabel et al., 2004). However, in the experiments shown here, where lysates of *T. rangeli* from TrA, TrB and TrC were loaded into a same gel, different profiles were disclosed by all isolates, even those of the same lineage (TrB), thus precluding the use of gelatinolytic profiles as markers of *T. rangeli* lineages. However, the general profiles of *T. rangeli* isolates were more similar to each other than to cruzipain of *T. cruzi* that is highly expressed as a major band of ~50–60 kDa (Martinez and Cazzulo, 1992; Eakin et al., 1992). Together, characterisation of *T. rangeli* CP gelatinolytic profiles and hydrolysis of Z-Phe-Arg-AMC disclosed differences of enzymes within *T. rangeli*, strongly evident between TrC and TrA/TrB. This finding contrasts with closer phylogenetic relationships of TrA with TrC than with TrB. It is tempting to speculate that these differences would at least partly account for the restriction of TrC to species of complex *R. pallescens* (Vallejo et al., 2009), and for the ability of TrA and TrB to share vectors of complexes *R. prolixus* and *R. brethesi*, as we demonstrated by cross experimental infections and field data from the Brazilian Amazonia (Maia da Silva et al., 2004a, 2007).

In *T. rangeli*, the number of CatL-like gene copies was estimated to be ~75 (Martinez et al., 1995). Multiple copies and sequences shared exclusively by *T. rangeli* lineages allowed standardisation of a highly specific and sensitive diagnostic PCR to diagnose isolates of all lineages. Moreover, variable CatL-like sequences among *T. rangeli* revealed excellent targets for population genotyping. A multiplex PCR targeting these sequences proved useful to assess the population structure of *T. rangeli*. We recently described highly specific and sensitive PCR targeting CatL-like sequences for diagnosis, genotyping and population structure of *T. vivax* (Cortez et al., 2009). CatL-like gene polymorphisms also have been proved to be valuable markers to investigate biogeographical patterns of *T. cruzi* isolates (Higo et al., 2007; Rozas et al., 2008).

Results from this study provide the first insights towards understanding the evolutionary relationships of CatL-like genes homologous to cruzipain in *T. rangeli*. Knowledge of the repertoire and phylogenetic relationships of CatL-like genes of *T. rangeli* are helpful as the first step for further investigation of the functional roles of these molecules in interactions of this species with its vertebrate and invertebrate hosts. Taken together, data from this and previous studies in *T. vivax* (Cortez et al., 2009) demonstrated that CatL-like gene sequences are excellent targets for phylogenetic analysis, diagnosis, genotyping and population structure analyses.

Acknowledgements

We thank several collaborators from Brazil, Venezuela and Panama for *T. rangeli* isolates, and E.P. Camargo for revision of the manuscript. We are grateful to The Wellcome Trust for making sequences from completed and draft genomes of several trypanosome species freely available for analysis. This work was supported by CNPq and FAPESP within the UNIVERSAL and PRONEX programs. P.A. Ortiz and L. Lima are recipients of scholarships from CNPq; F. Maia da Silva is postdoctoral fellow of PNPD (CAPES).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2009.07.036.

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Repertoire diversity, evolutionary relationships and genomic organization of cruzipain encoding genes in phylogenetic diverse *Trypanosoma cruzi* isolates and homologues from closely related *T. c. marinkellei* and *T. dionisii*

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Abstract

Cruzipain, the major Cathepsin L-like isoform of *Trypanosoma cruzi*, is widely studied as targets for drugs and vaccines. *T. cruzi,* the agent of Chagas disease, is a complex of isolates (TcI-TcVI and Tcbat) highly phylogenetically related to the bat-restricted *Trypanosoma cruzi marinkellei* and *Trypanosoma dionisii.* These three species belong to the subgenus *Schizotrypanum*, which comprises the trypanosomes that develop within mammalian cells. Although *T. cruzi marinkellei* and *T. dionisii* share morphological, genomic and proteomic features with *T. cruzi,* they differ in hosts, vectors and pathogenicity. Studies suggest that variations in expression levels and diversity of cruzipain genes correlate with levels of cellular invasion and differentiation, virulence and pathology of *T. cruzi* isolates. Sequences of genes encoding cruzipain from *T. cruzi* isolates of all DTUs (TcI-VI) and Tcbat (73 sequences from 24 isolates) are conserved in all domains. However, cruzipain catalytic domain repertoires diverge according to trypanosome species and *T. cruzi* DTUs. Very conserved cruzipain copies are found within and among isolates of the same DTU, excepting TcV-VI, which show polymorphic genes, including sequences unique to these hybrids or identical to those of TcII-III, supporting their origin from hybridization between these two DTUs. In network genealogies, sequences from *T. cruzi* clustered tightly together and closer to *T. c. marinkellei* than to *T. dionisii,* and distant from *T. rangeli* and *T. brucei*. Genealogies of cruzipain encoding genes agree with the diversity and phylogenetic relationships of trypanosome species and *T. cruzi* DTUs. Despite high conserved cruzipain genes, analyses of *T. cruzi* isolates representative of the overall biological and genetic diversity and homologues from *T. cruzi*-like disclosed species- and DTU-specific polymorphisms valuable to understand host-parasite interactions and crucial for evaluation of cruzipain as target for diagnostic, drugs and vaccine approaches.

***Keywords*:** *Trypanosoma cruzi*; *T. cruzi marinkellei*; *T. dionisii*; cruzipain, cathepsin L-like; genotyping; phylogeny; sinteny; evolution; bat trypanosomes.

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Author Summary

Chagas disease, an important health problem in Latin America, is a life-long chronic infection caused by the protozoan *Trypanosoma cruzi*. Cruzipain, a cysteine protease that play fundamental roles in cell invasion, differentiation, immunity and pathogenicity of *T. cruzi* and other trypanosomes, has been exploited as targets for drugs and vaccines. *T. cruzi* is a complex of genetically heterogeneous isolates distributed in six discrete typing units (DTUs TcI-VI) plus the new genotype Tcbat. Phylogenetically, *T. cruzi* is closest to *T. cruzi*-like bat trypanosomes, *T. c. marinkellei* and *T. dionisii.* These three species share similar morphological, biological, genomic and proteomic features but differ in hosts, vectors and pathogenicity. Comparison of cruzipain encoding genes from *T. cruzi* isolates representative of the overall biological, genetic and pathological diversity, and homologues from *T. cruzi*-like, disclosed species and DTU-specific polymorphism valuable to understand host-parasite interactions and crucial for evaluation of cruzipain as target for diagnostic, drug and vaccine development.

Introduction

Cysteine proteases are among the molecules most widely explored on several aspects of the trypanosomosis caused by *T. cruzi*, Chagas disease, one of the most important public health problem in Latin America. Cathepsin L-like (CATL) are cysteine proteases that play central roles in cell invasion, differentiation, immunity, immune-modulation and pathogenicity of trypanosomes and have been exploited as potential targets for the development of drugs and vaccines. Different isoforms of CATL perform distinct roles in trypanosome interactions with mammalian hosts and vectors, differing in stage, cellular localization, and expression level during the life cycle. This functional diversification may have contributed to the adaptation of the trypanosome species to their different life cycles in both the vertebrate and vector hosts (Mckerrow et al., 2006; 2008; Atkinson et al., 2009; Caffrey and Steverding, 2009).

Trypanosoma cruzi is a complex of genetically heterogeneous isolates distributed in six infraspecific subdivisions, the discrete typing units (DTUs) TcI-TcVI (Zingales et al., 2009; Miles et al., 2009), and one new genotype (Tcbat) recently identified in isolates from Brazilian bats (Marcili et al., 2009a). Closest relatives of *T. cruzi* are *T. c. marinkellei* and *T. dionisii,* trypanosomes exclusive of bats that because share morphologically indistinguishable blood forms are referred as *T. cruzi*-like (Hoare, 1972; Molyneux, 1991). Blood forms of *T. cruzi*-like species differ from those of all other species infecting bats such as *T. rangeli* (Maia da Silva, 2009). Together, *T. cruzi*, *T. c. marinkellei* and *T. dionisii* form the subgenus *Schizotrypanum*; of them, only *T. cruzi* has been proven to infect mammals other than Chiroptera. The mechanisms underpinning bat and vector specificities are unknown; inability of *T. cruzi*-like species to infect hosts other than bats has been associated to complement mediated lysis, phagocytosis and killing by human neutrophils and monocytes. Survival and development within mammalian cells *in vitro* is a feature shared by all the *Schizotrypanum* species (Baker and Linston, 1978; Thorne et al., 1979; Glauert et al., 1982; Baker, 1985; Oliveira et al., 2009; Marcili et al., 2009a).

Bats infected by *T. cruzi*-like species show nests of amastigotes in cardiac, skeletal and stomach muscle cells likewise *T. cruzi* in a range of hosts including man. Previous studies showed that *T. cruzi* and *T. dionisii* share similar molecules with important roles in host-parasite interactions such as phospholipids and cysteine proteases (Branquinha et al., 1994, 1999; Barreto-Bergter et al., 1996) as well as epitopes associated to autoimmunity in Chagas disease (Petry et

al., 1987). Similar to *T. cruzi*, all *T. cruzi*-like isolates differentiate from epimastigotes to infective metacyclic trypomastigotes in cultures and in the vector guts, and are transmitted by feces contamination and oral routes. Differently from *T. cruzi* that can be transmitted by several genera of triatomine bugs, transmission of *T. c. marinkellei* seems to involve only triatomines of the genus *Cavernicola,* while cimicids are vectors of *T. dionisii* (Hoare, 1972; Marinkelle, 1976). Therefore, besides morphology and *in vitro* and *in vivo* behavior, *T. cruzi*-like species share celullar, biochemical and immunological features with *T. cruzi* and, hence, can be valuable as human non-infective models for studies of *T. cruzi*, and as targets for diagnosis, drugs and vaccines (Thorne et al., 1979; Glauert et al., 1982; Bower and Woo, 1982; Baker, 1985).

Cruzipain is the archetype of a large multigene family organized in tandemly repeated gene copies, and the major CATL isoform of *T. cruzi* expressed in all life cycle stages: intracelular amastigotes, insect epimastigotes, and blood trypomastigotes (Scharfstein et al., 1986; Eakin et al., 1992; Cazzulo et al., 2001). Cruzipain play fundamental roles in *T. cruzi*-host interactions, in the establishing, maintaining, exacerbating and controlling infections. *T. cruzi* relies on cruzipain to infect and multiply within nonprofessional phagocytic cells (Lima et al., 2001; Aparicio et al., 2004; Santos et al., 2005; Scharfstein and Lima, 2008,). Cruzipain is an immunodominant antigen that elicits potent humoral and cellular immune responses during infection in humans and mice. Cruzipain is expressed on cell surface and secreted, suggesting that it could be a target for immunity throughout all phases of *T. cruzi* infection and a target to develop an effective anti-*T. cruzi* vaccine. In fact, vaccination with cruzipain have controlled the *T. cruzi* load and restricted the inflammatory response; the response to the catalytic domain but not of the C-terminal extension of cruzipain induces protective immunity (Schnapp et al., 2002; Cazorla et al., 2008; 2010). Addition of cruzipain inhibitor to cultures of mammalian cells infected with *T. cruzi* blocks parasite replication and differentiation, thus arresting the parasite life cycle. Synthetic inhibitors of cruzipain are among the most promising new drugs for the treatment of Chagas disease (Cazzulo et al., 2001; McKerrow et al., 2008; 2009).

Several studies suggest that the variable levels of cruzipain expression in *T. cruzi* strains correlate to differential degrees of cellular invasion, metacyclogenesis and virulence. Different levels of cruzipain secreted by trypomastigotes were correlated to differential cell infectivity of G and Dm28 strains (TcI), both non-virulent to mice (Aparicio et al., 2004). Variable levels of cruzipain expressed by epimastigotes were described within both TcI and TcII, and the highest levels detected in TcI isolates were associated with enhanced metacyclogenesis and *in vitro* cell infectivity (Fampa et al., 2010a,b). A proteomic analysis revealed differences among isolates of the different DTUs, with the lowest cruzipain expression reported for TcIV (M4167) associated to low infectivity to culture cells (Kikuch et al., 2010). To complicate any link between cruzipain levels and virulence or DTU, proteolytic activity was lower in the virulent *T. cruzi* CL strain compared to the non-virulent CL-14 clone (Atayde et al., 2004).

Cruzipain expressed by strains of *T. cruzi* is encoded by multiple polymorphic genes (Campetella et al., 1992; Lima et al., 1994). However, cruzipain encoding genes were not yet comparatively examined in isolates of DTUs displaying different levels of cellular invasion and differentiation, and different degrees of virulence and pathogenicity. Although data on genetic diversity of cruzipain genes is very limited, previous studies suggested polymorphisms among and between *T. cruzi* DTUs: single-stranded conformation DNA

polymorphism differentiate TcI and TcII isolates (Higo et al., 2007), and restriction patterns of PCR-amplified genes differentiate TcI infrapopulations (Rozas et al., 2008).

Studies of bat trypanosomes are important to improve the understanding of the evolutionary history of *T. cruzi*, and its relationships with hosts and vectors. Comparative studies between pathogenic and non-pathogenic closely related trypanosomes can be helpful for understanding the evolution of the pathogenicity and virulence of *T. cruzi*. Comparison of cruzipain genes from isolates of all DTUs, from closely related *T. cruzi*-like species, and *T. rangeli* and *T. brucei,* which are species phylogeneticaly distant from *T. cruzi* with life cycles differing in vertebrate and invertebrate hosts, can assist in clarifying the potential role of these enzymes in the host-parasite relationships, virulence and pathogenicity. An understanding of the genetic diversity and evolutionary relationships among genes encoding cruzipain in different *T. cruzi* DTUs are crucial for successful design of new drugs, vaccination and diagnostic strategies targeting these enzymes.

In this study, we characterized genes encoding cruzipain from isolates of all *T. cruzi* DTUs, and compared them with homologues from *T. c. marinkellei, T. dionisii, T. rangeli* and *T. brucei.* The genetic diversity and phylogenetic relationships of cruzipain and homologous genes were analyzed using nucleotide and aminoacid sequences. Expression was evaluated by northen-blotting and proteolytic assays, and genome organization was assessed by comparative analysis of loci containing cruzipain gene copies surrounded by syntenic genes in *T. cruzi* (CL Brener and G strains), *T. dionisii*, *T. rangeli* and *T. brucei* genomes.

Materials and Methods

PCR amplification, sequencing and phylogenetic analysis of CATL sequences

The whole sequences of cruzipain genes (~1.400 pb) of *T. cruzi* strains Esmeraldo cl3 (assembly of three sequences) and CL Brener were retrieved from TriTrypDB. Homologous genes in *T. cruzi* G, Tcbat, *T. dionisii* (TCC211) and *T. rangeli* (AM80) were obtained from genome drafts that we are currently performing using standard pyrosequencing shotgun methodology according to Roche 454 protocols. Resulting reads were submitted to Roche's Newbler software (version 2.3) for assembly, and contigs containing genes described in this study were assembled in scaffolds (Table 1). Sequences of these genes were aligned with homologues from *T. cruzi* Sylvio X10.6, *T. rangeli* LDG and *T. b. brucei* TREU 927 from GenBank and *T. c. marinkellei* determined in this study. The whole sequence from *T. c. marinkellei* was obtained by PCR-amplification using the primers TDIO5-FOR (5' ATG ACG AGC TGG GCG CGT G 3') and CATL3REV2 (5' TTA GCT TCA GGA GCG GCG ATG 3') and the conditions described previously (Cortez et al., 2009). Whole cruzipain sequences employed in this study are availabe in GenBank (Table 1).

PCR products were cloned, and 3 to 8 clones from each species/isolates were sequenced. Sequences reflecting the spectrum of genetic polymorphism were deposited in GenBank (Table 1). Alignment of cd-cruzipain genes includes sequences from isolates of TcI-VI, Tcbat and homologues from *T. c. marinkellei* and *T. dionisii* from Brazil and England (Cavazzana et al., 2010). Sequences from two non-*Schizotrypanum* trypanosomes, *T.* sp Pte and *T.* sp bat (60) from Brazilian and African bats, respectively, were also added in the alignment as well as sequences of *T. rangeli* (SA, AM80, PG, 643, 1719 and LDG) representatives of major lineages (Maia da Silva et al., 2009; Ortiz et al., 2009), and sequences from *T. b. brucei*.

Sequences were aligned using Clustal X

Table 1. *Trypanosoma cruzi* strains and isolates, other trypanosome species, and their respective sequences encoding for cruzipain and homologous genes determined in this study or retrieved from data banks.

TCC[a] code	Trypanosome isolate	Host species		Geographic origin	DTU	Accession number Sequences of Cathepsin L-like genes
T. cruzi						
1321	Dm28	opossum	*D. marsupialis*	Colombia	TcI	JF421288[b]/JF421289[b]
-	Sylvio X10.6	human	*H. sapiens*	Brazil	TcI	U41454.1[d]
	JRcl4	human	*H. sapiens*	Venezuela	TcI	TJR4_1_c6805/TJR4_1_c12312
30	G	opossum	*D. marsupialis*	Brazil	TcI	JF421290[b]/JF421291[b]/JF421352[c]/JF825059[c]/JF825060[c]
417	M2542	bat	*T. tricolor*	Brazil	TcI	JF421292[b]/ JF421293[b]
507	MO115	bat	*C. perspicillata*	Brazil	TcI	JF421294[b]/ JF421295[b]
34	Y	human	*H. sapiens*	Brazil	TcII	AF314929.1[d]/ JF421310[b]/ JF421311[b]
2120	Esmeraldo cl3	human	*H. sapiens*	Brazil	TcII	JF421314[b]/ JF421315[b]/ scf7180000307932[e] scf7180000305060[e] / scf7180000304994[e]
844	MT3869	human	*H. sapiens*	Brazil	TcIII	JF421335[b]/ JF421336[b]
845	MT3663	triatomine	*P. geniculatus*	Brazil	TcIII	JF421337[b]/ JF421338[b]
1386	Unidero	dog	*C. familiaris*	Brazil	TcIII	JF421339[b]/ JF421340[b]
863	Tc863	armadillo	*E. sexcinctus*	Brazil	TcIII	JF421341[b]/ JF421342[b]
85	José Julio	human	*H. sapiens*	Brazil	TcIV	JF421304[b]/ JF421305[b]
337	Fuscicolis 15	monkey	*S. fuscicolis*	Brazil	TcIV	JF421306[b]/ JF421307[b]
778	Rb778	triatomine	*R. brethesi*	Brazil	TcIV	JF421308[b]/ JF421309[b]
187	Bertha	human	*H. sapiens*	Bolivia	TcV	JF421316[b] - JF421319[b]
186	Tc186	triatomine	*T. infestans*	Bolivia	TcV	JF421320[b] - JF421327[b]
967	NR cl3	human	*H. sapiens*	Chile	TcV	JF421328[b] - JF421334[b]
33	CL	triatomine	*T. infestans*	Brazil	TcVI	JF421312[b]/ JF421313[b]
	CL14	triatomine	*T. infestans*	Brazil	TcVI	JF825061[c] - JF825064[c]
-	CL Brener Esmeraldo-like and Non-Esmeraldo-like	triatomine	*T. infestans*	Brazil	TcVI	Tc00.1047053509429.320[e]/ Tc00.1047053507537.20[e] Tc00.1047053507603.270[e]/Tc00.1047053507603.260[e] Tc00.1047053507537.10[e]
	unassigned contigs					AAHK01021104[e]/ AAHK01015705[e]/ AAHK01014707[e] AAHK01010644[e]/ AAHK01012365[e]/ AAHK01018585[e] AAHK01019951[e]
294	998	bat	*M. levis*	Brazil	Tcbat	JF421296[b]/JF421297[b]
499	1336	bat	*M. nigricans*	Brazil	Tcbat	JF421298[b]/JF421299[b]
1994	MO294	bat	*M. levis*	Brazil	Tcbat	JF421300[b] / JF421301[b] / JF421353[c]
1122	1122	bat	*M. albescens*	Brazil	Tcbat	JF421302[c]/ JF421303[c]
T. cruzi	***marinkellei***					
344		bat	*C. perspicillata*	Brazil		JF421354[c]
501		bat	*C. perspicillata*	Brazil		JF421343[b]
611		bat	*A. planirostris*	Brazil		JF421344[b]
626		bat	*A. planirostris*	Brazil		-
T. dionisii						
-	P3	bat	*P. pipistrellus*	England		JF421345[b]
495		bat	*C. perspicillata*	Brazil		JF421346[b]
1098		bat	*Myotis* sp	Brazil		JF421347[b]
454		bat	*D. rotundus*	Brazil		JF421348[b]
211		bat	*E. brasiliensis*	Brazil		JF421355[c]
T. rangeli					Lineage	
643	Tra643	bat	*P. lineatus*	Brazil	E	FJ997568.1[d]
1719	Tra1719	bat	*A. planirostris*	Brazil	A	JF421351[b]
031	SA	human	*H. sapiens*	Colombia	A	FJ997556.1[d]
086	AM80	human	*H. sapiens*	Brazil	B	JF421356[c]
014	PG	human	*H. sapiens*	Panama	C	FJ997564.1[d]
-	LDG cl1	human	*H. sapiens*	Colombia	C	L38512.1[d]
other	**trypanosomes**					-
60	*T. sp* bat (60)	bat	*R. aegyptiacus*	Gabon		JF421350[b]
411	*T. sp* Pte	bat	*P. parneli*	Brazil		JF421349[b]
-	*T. brucei* TREU927	tsetse fly	*Glossina sp*	Kenya		XM_840125.1[d]

[a] TCC, Code number of the isolates/strains cryopreserved in the Trypanosomatid Culture Collection (TCC); Sequences from cruzipain and homologous genes: [b] whole genes, [c] catalytic domains determined in this study and deposited in GenBank; [d] sequences from GenBank; [e] whole or partial sequences retrieved from TriTrypDB.

(Thompson et al., 1997) and manually adjusted. The aminoacid sequence of cruzipain was used as a template to ensure codon-to-codon correspondence. Phylogenetic relationships were inferred using nucleotide and predicted amino acid sequences from the entire genes or restricted to pre-pro or cdCATL domains. Network genealogy was inferred in Splitstree v4.11.3 using neighbor-net method (Huson and Bryant, 2006). Internode supports were estimated by performing 100 bootstrap replicates using the same parameters optimized for network inferences.

Sinteny analysis

Syntenic genes flanking CATL genes were investigated in the genomes of *T. cruzi* CL Brener by comparing sequences from the two haplotypes, Non-Esmeraldo-like (TcIII) and Esmeraldo-like (TcII) and *T. brucei* 927 genomes available in TriTrypDB. Scaffolds comprising cruzipain from *T. cruzi* G and homologues from *T. dionisii* were from our genome drafts. Search on draft genome of Sylvio X10/1 (Franzén et al., 2011) disclosed partial cruzipain sequences in small reads that could not be positioned in homologous scaffolds.

Southern and Northern blot analyses of cruzipain genes

For Southern blot analysis of cruzipain genes, genomic DNA samples from isolates of *T. cruzi* (Y, G and JJ strains), *T. c. marinkellei* (TCC344), *T. dionisii* (TCC211) and *T. rangeli* (isolates AM80 and SA) were digested with *Taq* I enzyme, separated by 2.5% agarose gels, transferred to nylon membranes and hybridized for 14-16hr at 40˚C with a probe consisting of PCR-amplified cd-cruzipain of *T. cruzi* Y labeled with ^{32}P as described previously (Ortiz et al., 2009). For Northern blot analysis, total RNA from 10^9 epimastigotes was extracted using Trizol (Gibco BRL) and 10 pg from each species was electrophoresed in 1.0% agarose gel and blotted onto nylon membrane. The membrane was hybridized with the ^{32}P-labeled probe used for Southern blot analysis at 40ºC overnight and washed in 1X bufffer (0.3 M NaCl, 0.3 mM Na citrate, pH 7.0 containing 0.1% SDS) at 50ºC for 1 h as described previously (Ortiz et al., 2009).

Proteolytic activities in gelatin gels

To assess the proteolytic activities in gelatin gels, lysates of cultured epimastigotes were subjected to electrophoresis in 10% resolving SDS-acrylamide gels containing 500 µg/ml gelatin. Gels were incubated in 2.5% (v/v) Triton X-100 in 0.1 M buffer (acetate: pH 4.0 and 5.0; Tris-HCl: pH 7.5) containing 5 mM DTT, and then ~18 h in buffer-DTT. Bands associated to cysteine peptidases were identified by incubating gel halves with 10 µM E-64. Gels were fixed with 10% TCA, and stained with Coomassie blue R-250 as described before (Ortiz et al., 2009; Rodrigues et al., 2010).

Results

Characterization of CATL gene repeats in distinct *T. cruzi* DTUs and *T. dionisii*

Like mammalian enzymes, CATL of trypanosomatids are synthesized as inactive precursors, consisting of pre, pro and catalytic domains (cd) and a C-terminal extension. Proteolytic cleavage of the N-terminal prodomain generates the mature enzyme consisting of a cd domain and a C-terminal extension unique of trypanosomatids (Cazulo et al., 2001). To compare entire cruzipain sequences (~ 450 aminoacids), sequences from *T. cruzi* Sylvio X10.6 and G (TcI), Y and Esmeraldo cl3 (TcII), CL Brener (TcVI, one whole sequence typical from each Esmeraldo and non-Esmeraldo haplotypes) and from Tcbat were aligned with homologous sequences from *T. c. marinkellei* and *T. dionisii. S*equences from non-*Schizotrypanum* species *(T. rangeli* and *T. b. brucei)* were included in the alignments.

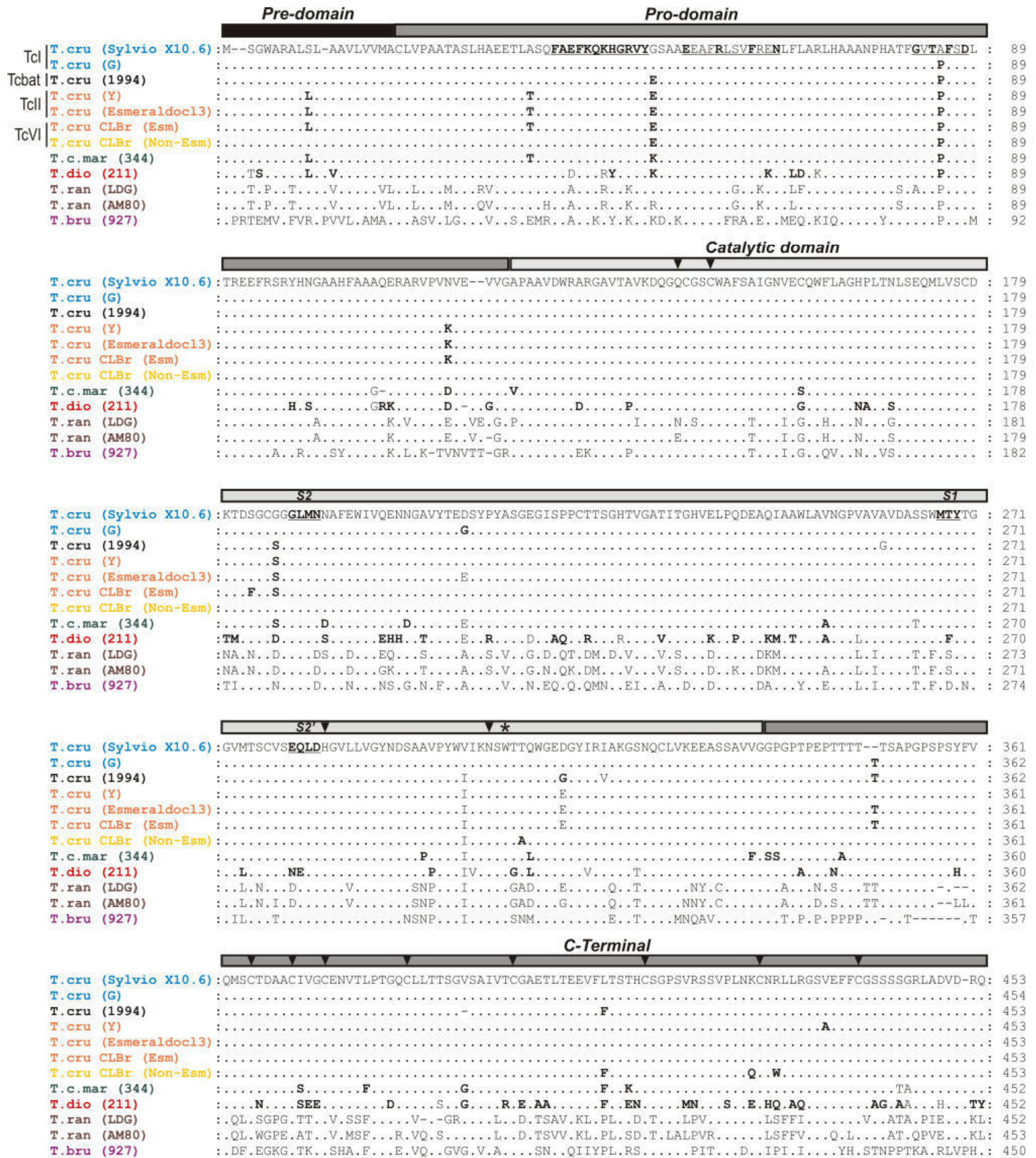


Fig. 1. Alignment of predicted aminoacid sequences from entire cruzipain and homologues genes. Pre, pro, catalytic domain and C-terminal extension amino acid sequences of cruzipain genes from *T. cruzi* Sylvio X10.6 and G of TcI, *T. cruzi* Y and Esmeraldo cl3 of TcII, *T. cruzi* CL Brener Non-Esmeraldo-like (TcIII) and Esmeraldo-like (TcII) and *T. cruzi* Tcbat (TCC1994), and homologues from *T. c. marinkellei* (344), *T. dionisii* (211), *T. rangeli* (LDG and AM80) and *T. brucei.* The CATL family signatures of pro-domain motifs ERFININ (ERFN) and GNFD (GTFD) are indicated in bold and underlined, the subsites S1, S2 and S2' are in bold, and the conserved Trp177 are indicated by (∗).The glutamine [Q] of the oxyanion hole, cysteine [C], histidine [H] and asparagine [N] of catalytic triad in the catalytic domain, and 8 cysteines in the C-terminal extension are indicated by arrow heads. The non-synonymous substitutions in sequences from *Schizotrypanum* species are in bold. *T. cruzi* (T. cru), *T. cruzi marinkellei* (T. c. mar); *T. dionisii* (T. dio); *T. rangeli* (T. ran) and *T. b. brucei* (T. bru).

Overall identities were high in N-terminal region, either in pre- and pro-domains (~94 and 91%, respectively) and catalytic domains (~90%), and most variable in the C-terminal regions (~85%) (Fig. 1). As typically found in the clan CA of peptidases, which are targeted to intracellular compartments and secreted, all cruzipain and homologous genes have a signal peptide at their N-terminal region, besides the catalytic triad of cysteine, histidine and asparagine residues (Cys25, His159 and Asn175) and the highly conserved Trp177. Important sites for autocatalytic cleavage, the motifs ERFNIN-like and GNFD of pro-domains, are conserved in all trypanosomes. The clan CA is characterized by having substrate specificity defined by the S2 pocket. In cruzipain genes from all *T. cruzi* DTUs and homologues from *T. c. marinkellei*, Glu (Q) and EQLD residues important for substrate specificity are conserved. However, in *T. dionisii* the residue EQLD changed to NELD and in *T. rangeli* to DEND, which probably implicate in important differences in substrate specificities (Fig. 1).

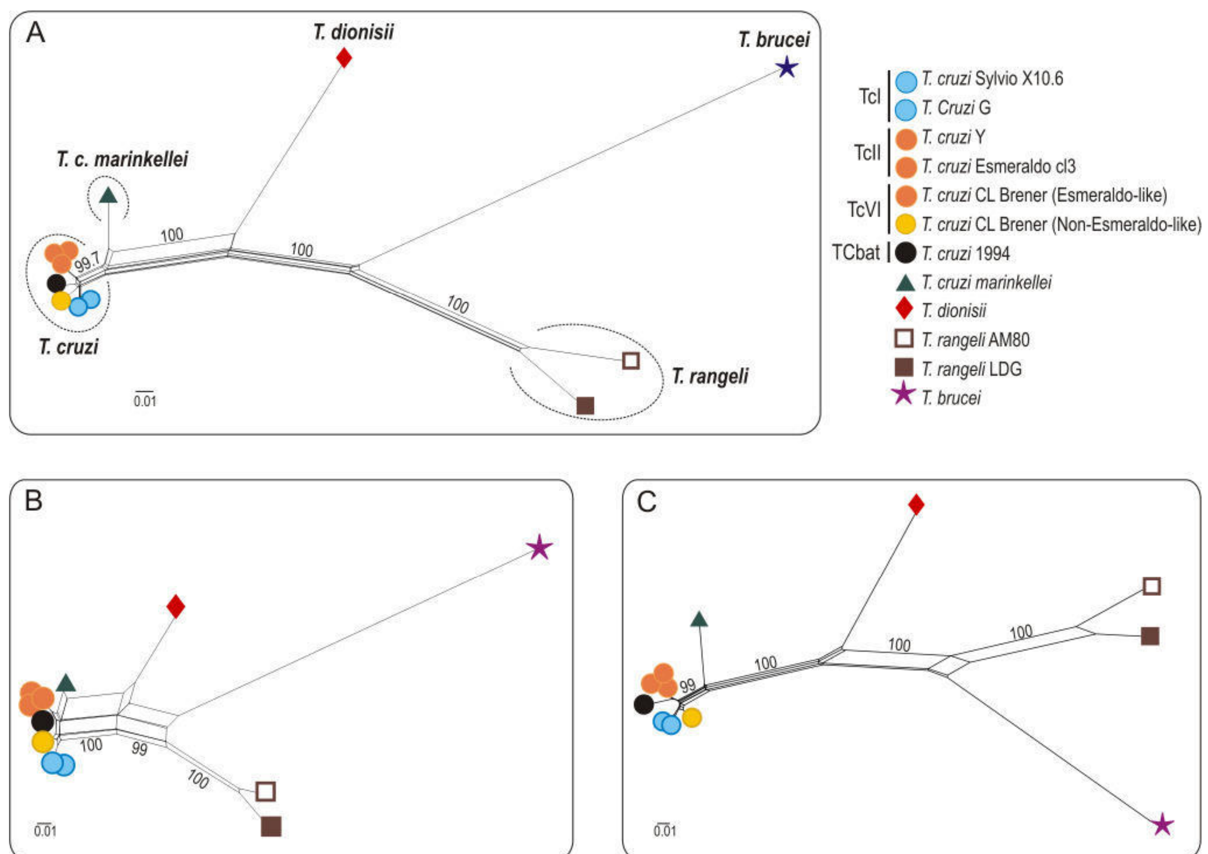


Fig. 2. Network genealogies of predicted amino acid sequences from genes encoding for cruzipain in *T. cruzi* and homologous genes of other trypanosome species. Networks produced using the Neighbour-Net algorithm in Splitstree v4.11.3, excluding all conserved sites and with Uncorrected p-distance. Networks were produced using entire sequences (A), pre- and pro-domains (B) or restricted to catalytic domains (C) of cruzipain encoding genes from the different trypanosomes are indicated by different colors according to the legend. Numbers in nodes correspond to support values estimated by performing 100 bootstrap replicates using the same parameter optimized for network inferences.

Divergences within cruzipain aminoacid sequences from *T. cruzi* isolates were small in all domains, and the number of non-synonymous (*Dn*) did not exceed synonymous (*Ds*) substitutions (*Dn/Ds*=1.0) in either the enzymatic and non-enzymatic C-terminal extension, indicating that these genes have been subjected to strong evolutionary constraining within *T. cruzi*. In contrast, *Dn*>*Ds* was detected in the catalytic domains between *T. cruzi* and *T. c. marinkellei (Dn/Ds=*2)*,* and especially between these species and *T. dionisii* (*Dn/Ds*=3,3) (Fig.1). The high ratio of *Dn/Ds* indicates that genes encoding cruzipain have been under positive selection pressure in these species. All findings suggest that these the trypanosome species examined can express enzymes with different substrate preferences and activities (Figs.1,2).

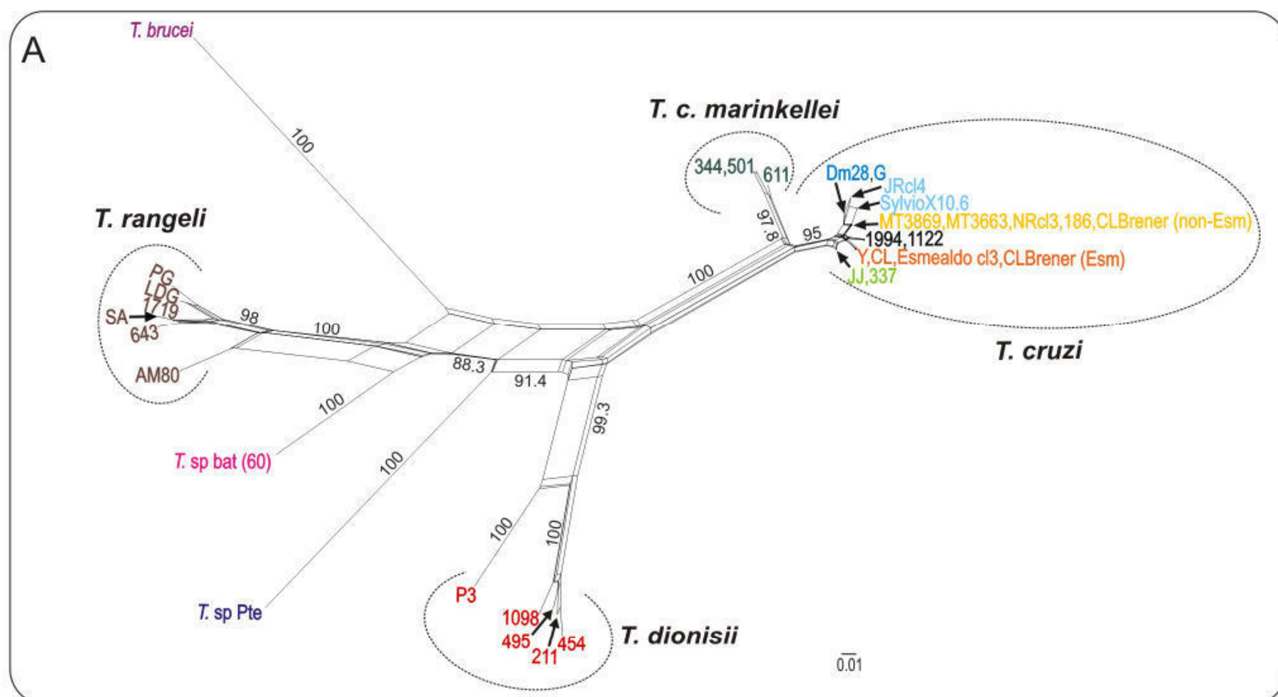
Sequences encoding homologous cruzipain of *T. c. marinkellei* were closely related to those of *T. cruzi* (~6.5% divergence) but the divergences were larger than those separating the *T. cruzi* DTUs (maximum of ~2.5%). Sequences from *T. cruzi* largely diverged from homologues of *T. dionisii* (~20%), *T. rangeli* (~33%) and above all of *T. b. brucei* (~43%) in all domains (Fig.1). Genealogies based on whole cruzipain genes, or restricted to pre-pro or to catalytic domains resulted in identical topologies (Fig. 2).

Genealogy of genes encoding *T. cruzi* cruzipain and homologues from *T. c. marinkellei*, *T. dionisii* and non-*Schizotrypanum* trypanosome species.

To study the relationships of cruzipain genes from all *T. cruzi* DTUs, *T. c. marinkellei* and *T. dionisii,* and homologues from *T.* sp Pte and *T.* sp bat (60), *T. rangeli* and *T. b. brucei* (Table 1), we aligned ~500 bp cdCATL sequences obtained in this study with the corresponding genes from *T. cruzi* CL Brener (Esmeraldo and non-Esmeraldo alleles), Esmeraldo cl3 (TriTrypDB), JRcl4 (WashU Project ID: 59941) and Sylvio X10.6 (GenBank). The analyses of either amino acid (Fig. 3) or nucleotide sequences (data not shown) generated networks of cruzipain genes with similar topologies. Sequences of cd-cruzipain from all *T. cruzi* DTUs always clustered together in a homogeneous assemblage (~1.2% internal sequence divergence) separated from *T. c marinkellei* (5.5% divergence), and largely divergent from the cluster formed by *T. dionisii* from Brazil (20%) and Europe (21%). Sequences of cruzipain genes of all *Schizotrypanum* species clustered together and separated (28%) from the homologous genes of *T. rangeli*, *T.* sp Pte and *T.* sp bat (60) (Fig. 3).

Distinguishing DTUs of *T. cruzi* according to polymorphisms on cruzipain genes

We evaluated the suitability of the small polymorphism on nucleotide sequences of cd-cruzipain genes in distinguishing among the *T. cruzi* DTUs by comparing 73 sequences from 24 isolates of all DTUs (59 sequences from 21 isolates were determined in this study). Sequences differ by 17 polymorphic nucleotide sites, all punctual nucleotide polymorphism, and were divided in 5 major sequence types besides hybrid sequences from TcV and VI (Fig. 4). Despite small polymorphisms, conservation of unique polymorphic sites distinguishes most DTUs. Cruzipain polymorphism also distinguished Tcbat from all established DTUs. Sequences from TcI, III, IV and Tcbat clustered together and separated from TcII (Fig. 4).



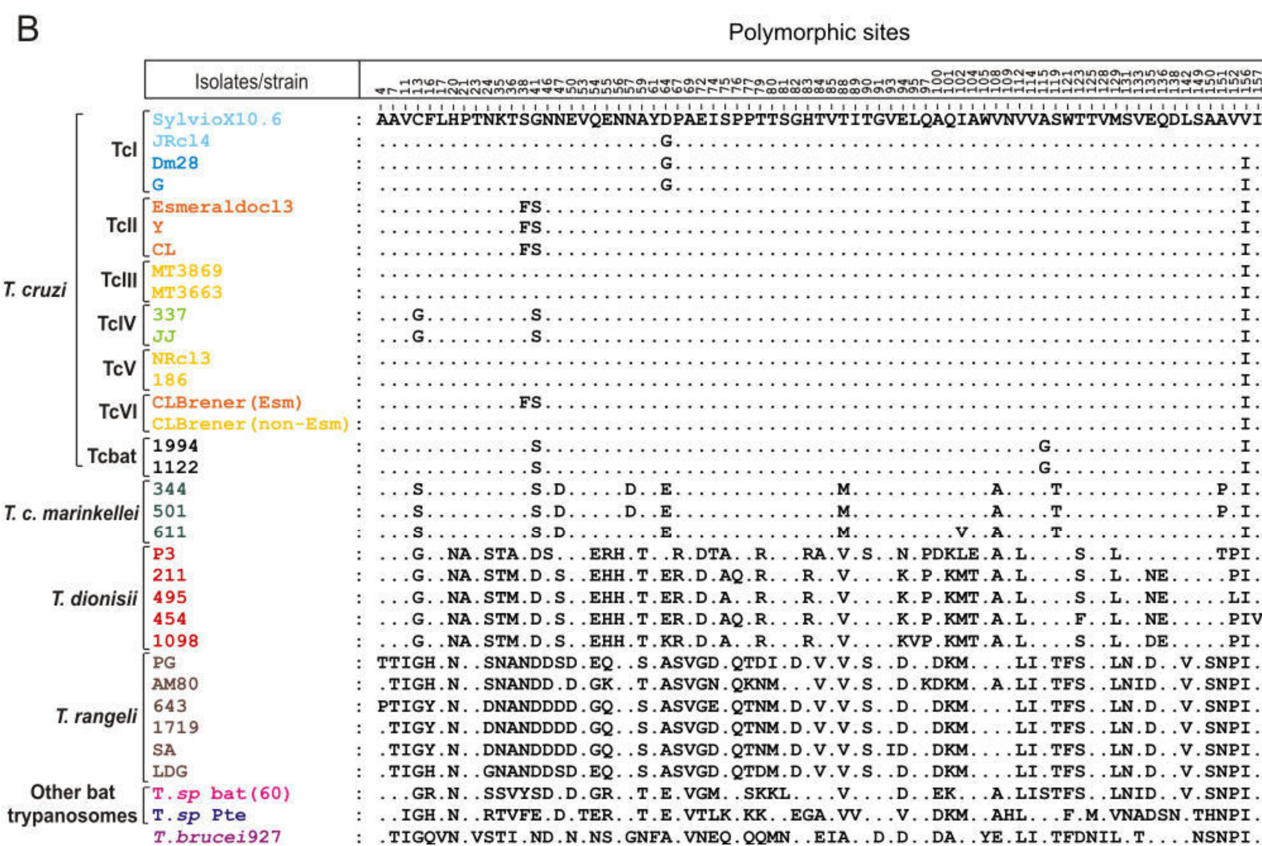


Fig. 3. Network and polymorphisms on catalytic domain of cruzipain genes from different trypanosome species. Genes from *Schizotrypanum* species (*T. cruzi, T. c. marinkellei* and *T. dionisii*) were compared with homologues from *T. rangeli* and other distantly related trypanosome species: *T.* sp Pte, *T.* sp bat (60) and *T. brucei.* (A) Network of aminoacid predicted sequences from 34 genes constructed with the Neighbour-Net algorithm excluding all conserved sites and with Uncorrected p-distance. The numbers in nodes correspond to bootstrap values from 100 replicates. (B) polymorphic aminoacid sites.

To validate the polymorphisms as DTU specific markers, we examined at least three isolates from each DTU, excepting TcVI for which the CL strain and its CL Brener and CL 14 clones were analyzed. Results supported high conservation of specific sites in distinct isolates of the same DTU supporting their utility as genotypic markers. Large sequence polymorphism detected in TcV and VI allowed to identify hybrids (Fig. 4). However, the need of evaluation several sequences can complicates the differentiation between TcV and VI.

Polymorphism of cruzipain gene copies within isolates and DTUs.

Any attempt to associate cruzipain polymorphisms with genetic or biological features of *T. cruzi* requires a good appraisal of the diversity of gene copies within both one isolate and each DTU. We have assessed the polymorphism on cruzipain gene copies by comparing 3 to 8 sequences from each isolate (Table 1). Cruzipain gene copies (homologues) from isolates of TcI, III, IV and Tcbat were identical or highly similar in their aminoacid sequences (Fig. 3) and slightly more polymorphic in their nucleotide sequences (Fig. 4). Very homogeneous but not identical copies were found by comparing sequences from six isolates of TcI. Similarly, TcII isolates showed almost identical copies. No polymorphic sites were found by comparing cd-cruzipain sequences from TcIII and IV isolates. Cruzipain sequences from Non-Esmeraldo-like haplotype of CL Brener, which have been considered the TcIII haplotype, were identical to those we have determined for TcIII (Fig. 4).

Despite the small number of cruzipain gene copies from each isolate compared these results strongly contrast with nucleotide polymorphism on gene copies of the heterozygous hybrids assigned to TcV (3 isolates, 4 to 8 sequences of each) and TcVI (CL Brener, 12 sequences). Different sequences were found in TcV (at least 6 sequence types) and TcVI (7 types), including sequences identical to those found in TcII or III, sequences found in both TcV and VI, and sequences so far detected in TcV or VI or in both DTUs (Fig. 3, 4).

Genomic organization and synteny of cruzipain gene copies and homologues in trypanosomes

Genome organization of cruzipain genes from *Schizotrypanum* species compared by Southern blot cross-hybridization (*T. cruzi* Y probe) of *T. cruzi* and *T. c marinkellei* genomic DNA showed similar profiles, suggesting conserved genome organization while differing from those of *T. dionisii* and *T. rangeli* (Fig. 5A). Previous studies showed that cruzipain genes are organized in the genomes of trypanosomes as tandem arrays of duplicated and polymorphic genes located in two or more chromosomes (Eakin et al., 1992; Campetella et al., 1992). Analysis of the genomic organization of cruzipain genes in *T. cruzi* CL Brener Esmeraldo-like and non-Esmeraldo-like haplotypes disclosed polymorphisms in number and organization of genes encoding cruzipain genes (cruzipain, cruzipain 2 and other putative isoforms). There was substantial variation in number, sequence and position of the duplicate genes of Esmeraldo-like and non-Esmeraldo haplotypes. Esmeraldo-like showed 1-4 cruzipain repeats dispersed in three loci, and non-Esmeraldo-like 3-5 copies in three loci (data from TriTrypDB).

A

DTU	Sequence type	Isolates/strain	Polymorphic sites -18 -27 -30 -37 -51 -57 -102 -105 -113 -114 -121 -183 -189 -191 -288 -344 -466
TcI		SylvioX10	: CCCTCCTAAGCTGCGAGCG
		JRcl4	: ....G...
		G	: ....G...
		G.c1	: ....A
		G.c3	: ....G..A
		G.c4	: ....G..A
		Dm28.c2	: ....G..A
		Dm28.c7	: ....G..A
		417.c1	: ....G..A
		417.c3	: ....G..A
		507.c1	: ....G..A
		507.c5	: ....G..A
TcII		Y.c3	: .T..TTC..AT.A...T.A
		Y.c5	: .T..TTC..AT.A...T.A
		Esmeraldo.c1	: .T..T.C..AT.A...T.A
		Esmeraldo.c3	: .T..T.C..AT.A...T.A
TcIII		MT3869.c1	: ....A........A
		MT3869.c4	: ....A........A
		MT3663.c1	: ....A........A
		MT3663.c6	: ....A........A
		1386.c1	: ....A........A
		1386.c2	: ....A........A
TcIV		JJ.c4	: ..TG.....A.CA.A...A
		JJ.c10	: ..TG.....A.CA.A...A
		377.c5	: ..TG.....A.CA.A...A
		377.c6	: ..TG.....A.CA.A...A
		778.c1	: ..TG.....A.CA.A...A
		778.c2	: ..TG.....A.CA.A...A
TcV		Bertha.c3	: .T..T.C..AT.A...T.A
		186.c5	: .T..T.C..A..A...T.A
		186.c10	: .T..T.C..A..A...T.A
		NRcl3.c1	: ....T.C..A..A...T.A
		NRcl3.c2	: ....T.C..A..A...T.A
		NRcl3.c3	: ....T.C..A..A...T.A
		NRcl3.c4	: ....T.C..A..A...T.A
		186.c1	: ....T.C..A..A...T.A
		186.c6	: ....T.C..A..A...T.A
		Bertha.c2	: ....TTC..A..A...T.A
		Bertha.c4	: ....TTC..A..A...T.A
		Bertha.c1	: .........A......T.A
		186.c9	: .........A......T.A
		186.c2	: ....A........A
		186.c7	: ....A........A
		186.c8	: ....A........A
		NRcl3.c5	: ....A........A
		NRcl3.c6	: ....A........A
		NRcl3.c7	: ....A........A
TcVI		Tc_14665*	: ....T.C..A..A...T.A
		Tc_14776*	: ....T.C..A..A...T.A
		CLbrener4(Esm)	: ....T.C..A..A...T.A
		CLbrener5(Esm)	: ....T.C..AT.A...T.A
		CLbrener2(Esm)	: .T..T.C..A..A...T.A
		Tc_18016*	: ....TTC..AT.A...T.A
		Tc_28863*	: ....TTC..AT.A...T.A
		Tc_16324*	: .T..TTC..A..A...T.A
		CL.c5	: .T..TTC..AT.A...T.A
		CL.c6	: .T..TTC..AT.A...T.A
		CLbrener3(Esm)	: .T..T.C..AT.A...T.A
		CL14.c7	: .T..T.C..AT.A...T.A
		Tc_18717 (non-Esm)	: ....A........A
		Tc_32449 (non-Esm)	: ....A........A
		CLBrener1(non-Esm)	: ....A........A
		CL14.c3	: ....A........A
		CL14.c5	: ....A........A
		CL14.c9	: ....A........A
Tcbat		294.c2	: T........A..AT...GA
		294.c4	: T........A..AT...GA
		499.c1	: T........A..AT...GA
		499.c3	: T........A..AT...GA
		1994.c1	: T........A..AT...GA
		1994.c3	: T........A..AT...GA
		1122.c4	: T........A..AT...GA
		1122.c6	: T........A..AT...GA

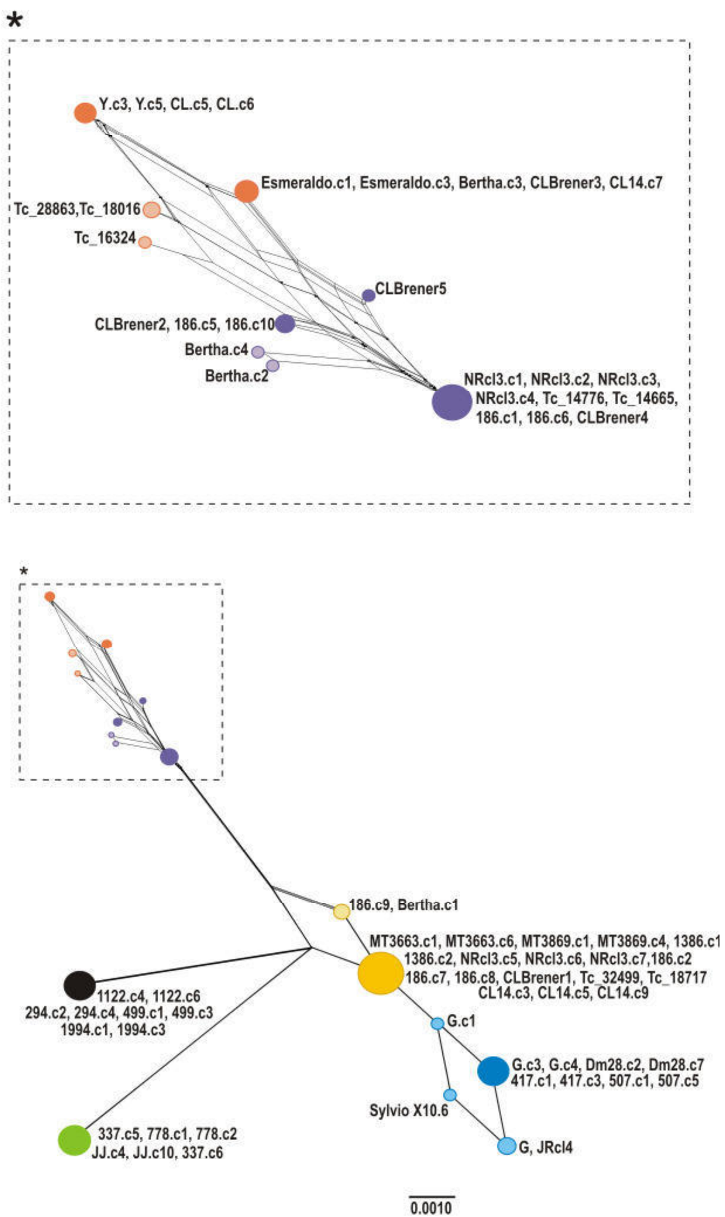


Fig. 4. Polymorphism and network of catalytic domain sequences of cruzipain genes from *T. cruzi* isolates of TcI-VI and Tcbat. (A) polymorphic nucleotide sites of cd-cruzipain encoding genes; (B) Network based on polymorphic nucleotides constructed with the Neighbour-Net algorithm excluding all conserved sites and with Uncorrected p-distance. The numbers in nodes correspond to bootstrap values from 100 replicates. CLBrener1 to CLBrener5 are sequences from TriTrypDB: Tc00.1047053509429.320, Tc00.1047053507537.20, Tc00.1047053507603.270, Tc00.1047053507603.260 and Tc00.1047053507537.10; *GenBank accession numbers are listed on Table1. Major types of sequences from the *T. cruzi* isolates of different DTUs are indicated by different colors according to the legend.

Homologous segments of the chromosome 6 (Fig. 6) containing 3 and 4 tandem copies of cruzipain in Esmeraldo-like and non-Esmeraldo-like, respectively, were compared with data from the genome drafts of *T. cruzi* G and *T. dionisii.* A syntenic block comprising 5 orthologous genes flanking cruzipain could be detected in all genomes (Fig. 6). From the genome draft of *T. dionisii,* two copies of homologous cruzipain genes could be placed in this syntenic segment. Genes arranged in the same order were also detected in *T. brucei* genome flanking 11 identical CATL gene copies in the chromosome 1 (Fig. 6), as well as in the genome draft of *T. vivax* and *T. congolense* (data not shown).

The cruzipain and homologous genes that we have compared represent only a fraction of the total CATL gene repeats existing in all trypanosome genomes. We have selected only sequences with the signatures of cruzipain (Fig. 1), thus removing cruzipain 2 and other isoforms. Assembly of repetitive sequences in tandem arrays is very problematic, and both the copy number and position are very difficult to be accurately determined. Mis-assembly from collapsed repeat sequence frequently arises during automated genome assembly when sequence reads originating from distinct repeat copies are incorrectly joined to generate a single unit. To avoid mis-assembled, we selected contigs containing sequences from both cruzipain and adjacent orthologous genes, which warrant the positioning of the genes encoding cruzipain in this syntenic region. The degrees of sequence identity between each syntenic gene (Fig. 6) agreed with the phylogenetic relationships among all trypanosomes we have compared (Hamilton et al., 2007; Cavazzana et al., 2010).

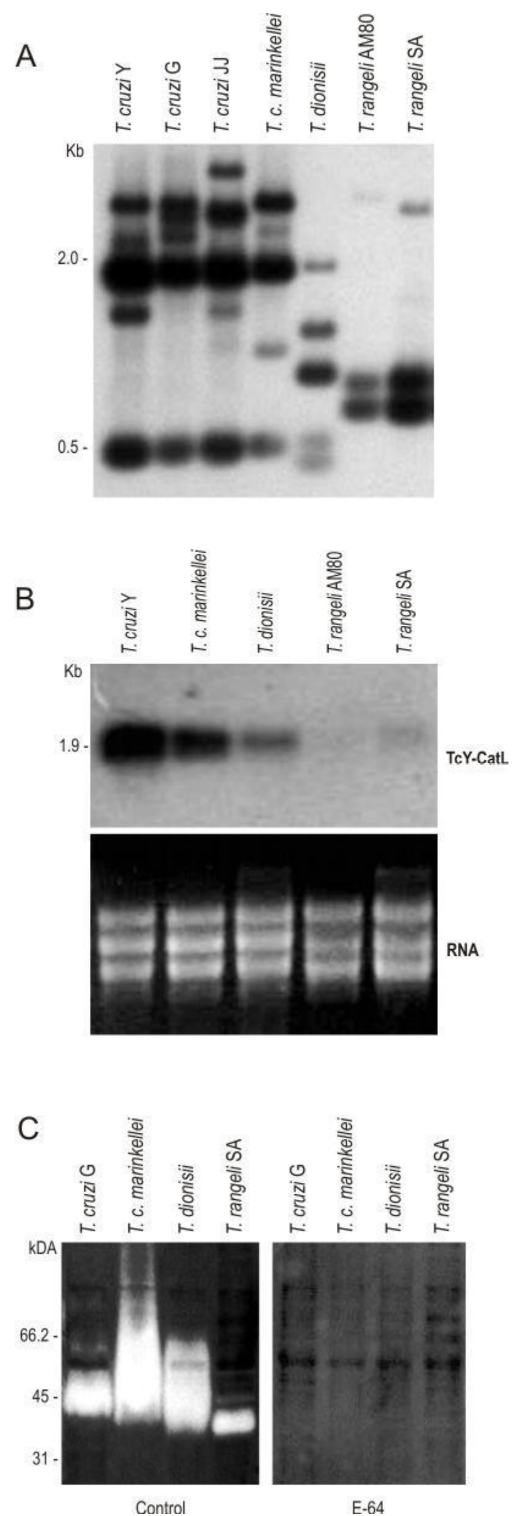


Fig. 5. Genomic and expression analysis of cruzipain and homologous genes in different trypanosome species (A) Genomic organization of cruzipain genes from *T. cruzi* isolates of different DTUs and homologues from *T. c. marinkellei, T. dionisii* and *T. rangeli* illustrated by southern blot analysis of genomic DNA digested with *Taq* I enzyme and hybridized with a probe consisting of ^{32}P labeled cd-cruzipain of *T. cruzi* Y. (B) Analysis of cruzipain transcripts from *T. cruzi* (Y) and cross-hybridization with other trypanosome species using the probe TcY-CatL; agarose gel used for this analysis stained with ethidium bromide (EtBr). (C) CATL proteolytic activities detected in epimastigote lysates of *T. cruzi* G, *T. c. marinkellei, T. dionisii* and *T. rangeli.* Activity banding profiles detected in gelatin gels, pH 5.0 and 5mM DTT, and inhibited in gel incubated with 10μM E-64.

Expression analyses of cruzipain homologues by Northern blot hybridization and detection of proteolytic activity in gelatin gels

To compare cruzipain transcripts in *Schizotrypanum* species, total RNA from epimastigotes of *T. cruzi* Y*, T. c. marinkellei* and *T. dionisii* was compared by northern-blot hybridization using *T. cruzi* Y derived cruzipain probe. Results showed high cross-hybridization with transcripts of *T. cruzi*, moderate hybridization with *T. c. marinkellei,* and a weak hybridization signal with transcripts of *T. dionisii.* This analysis allowed estimation of transcripts of similar size (~1.9 Kb) for these species, and lack of hybrization with *T. rangeli* RNA (Fig. 5B). In agreement, a probe consisting of *T. rangeli* cdCATL strongly hybridized with *T. rangeli* while hybridization with *Schizotrypanum* species was very weak (Ortiz et al., 2009).

Cruzipain is a glycoprotein of ~50-60 kDa post-transcriptionally regulated during the *T. cruzi* life cycle (Cazzulo et al., 2001; Aparicio et al., 2004). Here, activities related to CATL were detected in epimastigote lysates of all *Schizotrypanum* species examined: *T. cruzi* G (TcI), *T. c. marinkellei* and *T. dionisii* (Fig. 5C). In the *T. cruzi* isolates investigated (G: TcI; Y: TcII; JJ: TcIV) comparable activities were detected independently of DTUs (Fig. 5C and data not shown), whereas weaker activity was detected in *T. rangeli* (Ortiz et al., 2009). Remarkably, *T. c. marinkellei* and *T. dionisii* showed very high cruzipain-like activity, resolving in ~ 55 to 35 kDa region of gelatin gels (Fig. 5C). The activities of both *T. c. marinkellei* and *T. dionisii* were fully inhibited by the cysteine protease-specific inhibitor E-64, results suggesting the activity of CATL enzymes (Fig. 5).

Discussion

In this study, we compared cruzipain-encoding genes of *T. cruzi* isolates representatives of all known DTUs (TcI-VI) including the new *T. cruzi* genotype Tcbat, as well as homologous genes from *T. cruzi*-like isolates, *T. c. marinkellei* and *T. dionisii,* the closest relatives of *T. cruzi* (Baker et al., 1978; Baker, 1985; Barnabé et al., 2003; Maia da Silva et al., 2009; Marcili et al., 2009a; Cavazzana et al., 2010). Analyses of cruzipain genes from a large set of *T. cruzi* isolates representative of the overall biological and genetic diversity, and comparison with homologues from other species in a phylogenetic framework disclosed divergences strongly associated to phylogenetic diversity. Our findings revealed species-specific and DTU-specific variability valuable to understand the roles of cruzipain in host-parasite interactions.

Isolates of *T. cruzi* show a range of variation in important biological, immunological, pathological (morbidity and mortality) and clinical characteristics, and in preferences for mammalian and vector species, and susceptibility to drugs. Probably, a relevant part of this variation is due to genetic differences among isolates and can be related to specific DTUs. The genomes of the "low virulent" *T. cruzi* Sylvio X10/1 (TcI) and "virulent" CL Brener (TcVI) are highly similar in their gene-dense "core" coding regions, which show strongly conserved synteny interspersed with variable repetitive sequences (Franzén et al., 2011). Large differences among DTUs have emerged in repetitive content and sequences of several important gene families such as mucins, mucin-associated surface proteins (MASP), transsialidases, dispersed gene family 1 proteins

(DGF), surface protease gp63 and cysteine proteases and amastigote surface glycoprotein (amastins). These gene families are significantly larger in CL Brener compared to Sylvio and other TcI isolates, and this may have functional implications (Cerqueira et al., 2008; Minning et al., 2011; Franzén et al., 2011).

In this study, high conservation of genes encoding cruzipain was found among *T. cruzi* of all DTUs, Tcbat and *T. c. marinkellei* compared to *T. dionisii.* However, all sequences from these three species clustered together and distant from homologous sequences from *T. rangeli* and *T. brucei.* Sequences from the same species always clustered together. However, even with the high degree of conservation in cruzipain genes from all DTUs, polymorphisms on cd-cruzipain generated 5 branches within *T. cruzi*, each one comprising sequences from one DTU (TcI-VI) or Tcbat. In general, cruzipain gene copies within the same DTU showed very small or no variation at all, as we verified for isolates of TcI-IV.

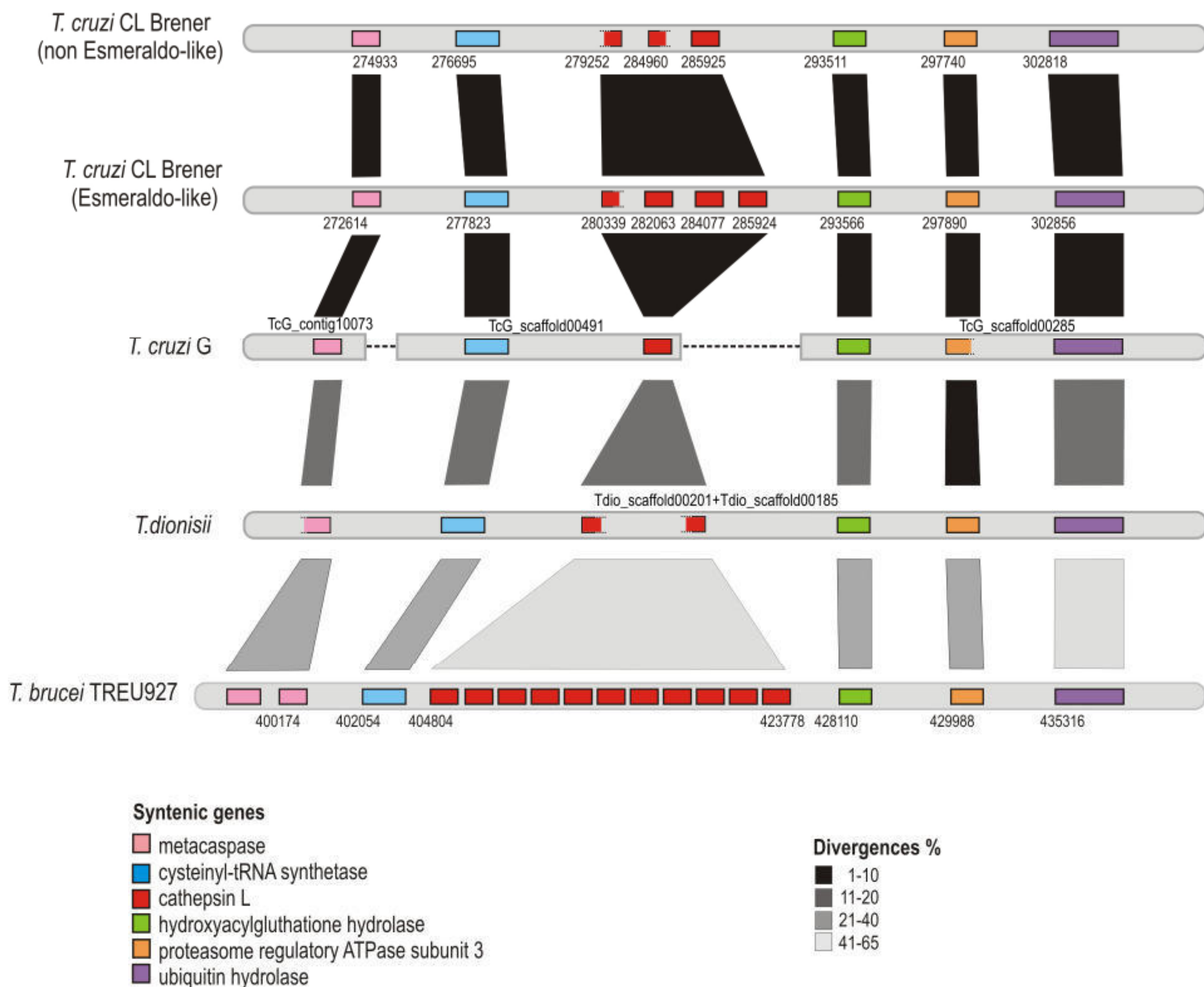


Fig. 6. Synteny of a loci containing cruzipain genes in *T. cruzi*, *T. dionisii* and *T. brucei.* Alignments of a segment from the chromosome 6 of *T. cruzi* CL Brener haplotypes non-Esmeraldo-like (TcIII) and Esmeraldo-like (TcII) showing three to four cruzipain copies (entire or partial genes). The flanking orthologous genes are marked with different colors according to legend; the shades of vertical gray bars indicate the variable degrees of divergences (%) between sequences according to legend. Data from the draft assembly of *T. cruzi* G and *T. dionisii* allowed to place one and two cruzipain gene copies, respectively, within the same syntenic region (figure do not reflect their actual position on chromosomes. Syntenic region from *T. brucei* genome comprising 11 copies in tandem of homologous genes (chromosome 1) was included in the alignment.

Data from these DTUs were consistent with their epidemiology and evolutionary histories, with distances among cruzipain genes suggesting that they have more time segregating from each other than more recently emerged TcV-VI. Isolates from each DTU appear to have long association with preferential mammals and vectors and, consequently, naturally circulate separated by biological, ecological and geographical barriers. Further studies need to investigate the real l polymorphism of cruzipain repeats in isolates of TcI and III representing the diversity of geographically structured (Llewellyn et al, 2009a,b; Marcili et al., 2009b; Miles et al., 2009).

In contrast to TcI-IV, TcV-VI isolates besides sequences identical to those of TcII (Esmeraldo-like) or TcIII (non-Esmeraldo-like) showed different cruzipain genes probably resulting from hybridization of TcII and TcIII. In agreement with their hybrid origin, sequences from TcV and VI clustered together with TcII and TcIII sequences as well as formed other groups of sequences, showing a reticulate pattern in the network genealogy of cruzipain genes. Tandem arrays of cruzipain genes in *T. cruzi* CL Brener showed sequences derived from TcII and III and hybrid sequences, with genes copies varying in the haplotypes, even within one single locus. All data corroborate that TcV and VI resulted from the hybridization between TcII and TcIII as hypothesized using other markers (Souto et al., 1996; Brisse et al., 2003; Westenberger et al., 2005; Sturm and Campbell, 2010). Due to unique molecular features, our findings confirmed Tcbat as a new genotype of *T. cruzi* not yet assigned to any DTU. Sequences from Tcbat clustered closer to TcI, III and IV than to TcII, but separated from all these DTUs as previously demonstrated with other markers (Marcili et al., 2009a; Hamilton et al., 2011). The greater phylogenetic affinities of TcI to TcIII-IV than to TcII, V-VI evidenced by cruzipain genealogies was also supported by genes encoding for the trypomastigote small surface antigen (TSSA) (Bhattacharyya et al., 2010).

Results from this study disclosed molecular markers able to identify the morphologically indistinguishable and closely phylogenetic related *T. cruzi*-like species. Reliable identification and knowledge on diversity and epidemiology of these species are crucial to understand the evolution of *T. cruzi* (Marcili et al., 2009a; Cavazzana et al., 2010). We have previously shown that *T. cruzi* and *T. rangeli* could be diagnosed by a sensitive and specific PCR targeting cruzipain sequences, and reported CATL markers for the diagnoses and genotyping of *T. vivax* and *T. theileri* (Cortez et al., 2009; Ortiz et al., 2009; Rodrigues et al., 2010).

Genealogy of cruzipain and homologous genes inferred in this study confirmed *T. c. marinkellei* as outgroup for *T. cruzi* isolates, as showed before by SSUrRNA, gGAPDH and Cytb genes (Hamilton et al., 2007; Cavazzana et al., 2010). In a comparative proteomics analysis, *T. c. marinkellei* was indistinguishable from *T. cruzi* reinforcing their very close relatedness (Telleria et al., 2010). Tcbat, *T. c. marinkellei* and *T. dionisii* were so far exclusively found in bats, but all develop inside a variety of mammalian cells *in vitro*. Only Tcbat were infective to mice likewise all isolates of *T. cruzi,* which are thought to be generalists despite having preferential mammalian hosts in nature. However, these three trypanosomes were incapable to develop in triatomine species commonly infected by *T. cruzi* (Marcili et al., 2009a; Cavazanna et al., 2010). Our findings evidenced differences on cruzipain homologues of *T. c. marinkellei* and *T. dionisii* that deserve further studies.

Evolutionary relationships of cruzipain and homologues from *T. brucei*, *T. congolense*, *T. vivax, T. theileri* and *T. rangeli* indicated that CATL family expanded by successive gene duplications followed by

divergences that gave rise to genes with greater similarity when originating from the same rather than distinct species/genotypes, hence, suggesting concerted evolution (Cortez et al., 2009, Ortiz et al., 2009; Rodrigues et al., 2010). Phylogenetic relationships of gene duplicates in *T. brucei* were consistent with concerted evolution being a widespread homogenising force of genes encoding CATL enzymes. However, evolutionary processes such as gene conversion and positive selection can generate diversity in sequence, quantity and order among tandemly repeated genes of trypanosomatids (Cerqueira et al., 2008; Jackson et al., 2007). In fact, CATL paralogous are virtually identical in *T. brucei ssp.* and *T. evansi* but show relevant divergence among lineages/genotypes of *T. congolense, T. vivax, T. rangeli* and *T. theileri* (Cortez et al., 2009; Ortiz et al., 2009; Pillay et al., 2010; Rodrigues et al., 2010; Garcia et al., 2011). Previous studies demonstrated that at least two isoforms are expressed by *T. cruzi,* cruzipain (>100 copies) and cruzipain 2 (~6 copies), and that the sequences and the number of duplicated cruzipain genes are not uniform among isolates (Lima et al., 1994; Campetella et al., 1992; Eakin et al., 1992). In this study, only cruzipain encoding genes were characterized.

Several studies correlated quantitative and qualitative differences on cruzipain expressed by different species, and among and within isolates of *T. cruzi* assigned to distinct DTUs, with variable levels of cellular invasion and differentiation, immune evasion, virulence and pathology (Lima et al., 2001; Aparicio et al., 2004; Atayde et al., 2004; Ortiz et al., 2009; Fampa et al., 2010a,b; Kikuch et al., 2010). Results from biological characterization of *T. cruzi* isolates here analyzed regarding their cruzipain repertoires showed highest levels of metacyclogenesis and cellular invasion (*in vitro*), and low virulence for mice of TcI isolates. Similar results were found in mice infected with TcIV and Tcbat, despite the lower metacyclogenesis and cell infectivity rates. In contrast, reduced metacyclogenesis and high virulence characterized TcII and TcIII isolates. Enhanced metacyclogenesis and cellular invasion *in vitro* are common features of *T. dionisii* isolates (Cavazzana et al., 2010; Lima et al., in preparation). Any correlation between cruzipain repertoire and genetic and biological features of *T. cruzi* and *T. cruzi*-like isolates requires more phylogenetic and functional evidence.

Since cruzipain plays fundamental roles in establishing, maintaining, exacerbating and controlling *T. cruzi* infection it has been exploited for vaccines. The non-enzymatic C-terminal domain is considered the immunodominant domain of cruzipain because induces strongest antibody and cellular response (Martinez et al., 1993; Cazorla et al., 2008; 2010). However, immunization of mice with the full-length recombinant cruzipain showed that high antibody titer against C-terminal neither reduced cell invasion nor the number of circulating parasites. When the C-terminal is removed, vaccination using N-terminal domains (pre-pro and cd) although induced low levels of antibodies, reduced parasitemia and limited tissue injury (Cazorla et al., 2008; 2010). The main goal of all vaccination strategy should be a polyvalent vaccine against infection by *T. cruzi* of any DTU. Despite high conservation, implications for a vaccine of DTU heterogeneity we have found in cd-cruzipain genes require investigations to warrant efficient cross-protection.

Drugs currently employed for the treatment of Chagas disease have significant limitations due to failure in chronic patients and serious side effects. Inhibitors targeting cruzipain have been explored for drug designs. The inhibitor for cruzain has focused on the S2 subsite (McKerrow, et al., 2006; 2008; 2009). In this study, we demonstrated that all substrate-binding subsites are conserved in all *T. cruzi* isolates, whereas

diverge in *T. dionisii* and other trypanosomes. *T. c. marinkellei* share identical subsites and, hence, is a valuable non-human infective model to test drugs against infective trypomastigotes and intracellular amastigotes.

The knowledge of the cruzipain gene repertoires and genetic variation among isolates can help to understand the evolutionary history that shape divergent *T. cruzi* DTUs and *T. cruzi*-like species. These isolates despite sharing genomic and proteomic features have many peculiarities that can potentially explain differences in host preference/restriction, virulence and pathogenesis. Characterization of genes encoding cruzipains, genealogies, genomic organization, expression analysis of transcripts and proteolytic activity were addressed in this study for the first time to compare *T. cruzi* DTUs and *T. cruzi*-like species. Results from this study are the initial steps toward understanding the roles played by genetic diversity of cruzipain and homologous enzymes in *T. cruzi* of all DTUs and in other trypanosome species. Although the results suggest conserved putative enzymes, we identified cruzipain polymorphisms indicating that, for vaccines and drugs targeting these enzymes, it is essential to demonstrate the effectiveness of inhibitors and immunogenicity of cruzipain variants from different *T. cruzi* DTUs.

Acknowledgements

This work was supported by grants from Brazilian agencies CNPq and PRONEX-FAPESP to M.M.G.Teixeira and ATOL-NSF Gto G.A.Buck.. Luciana Lima and Paola A. Ortiz are doctorate fellows sponsored by CNPq-PROTAX. F. Maia da Silva is postdoctoral fellow sponsored by PNPD-CAPES. We would like to thank Marta Campaner for culture of parasites, and the technical personnel of ATOL-Euglenoza project for their inestimable work in genome sequencing. We are grateful to The Wellcome Trust for making sequences from completed and draft genomes of trypanosomes freely available for analysis in TriTrypDB.

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