Parasitology Research (2018) 117:737–746
https://doi.org/10.1007/s00436-017-5744-z

ORIGINAL PAPER

Identification of bat trypanosomes from Minas Gerais state, Brazil, based on 18S rDNA and Cathepsin-L-like targets

Elaine Cristina Bento[1] · César Gómez-Hernández[2] · Lara Rocha Batista[2] · Laís Anversa[3] · André Luiz Pedrosa[2] · Eliane Lages-Silva[2] · Juan David Ramírez[4] · Luis Eduardo Ramirez[2]

Received: 26 June 2017 / Accepted: 29 December 2017 / Published online: 16 January 2018

Abstract

Several bat species can be infected by trypanosomes, but there is not much information about which of these parasites infect bats from Triângulo Mineiro and Alto Paranaíba, Minas Gerais state, Brazil, a formerly endemic region for *Trypanosoma cruzi*, the causative agent of Chagas disease. The aim of this study was to describe, characterize, and identify the presence of trypanosomes in bats. The captured bats (448) belong to four families and to 19 different species. Of those, 37 bats were found to be positive for trypanosomes by microhematocrit, (infection rate 8.3%) and 27 were positive after hemoculture analysis. Initially, the isolates were identified by PCR (18S rDNA, 24Sα rDNA, spliced leader, COII RFLP-PCR) using primers originally designed for *T. cruzi*. PCRs (18S rDNA, 24Sα rDNA) showed compatible bands for TcI, whereas COII RFLP-PCR showed a similar pattern associated to TcII. However, there was no DNA amplification using spliced leader as a target, revealing a discrepancy between the results. Phylogenetic analysis of Cathepsin L-like and 18S rDNA sequences proved that 15 of the isolates corresponded to *Trypanosoma cruzi marinkellei* and one to *Trypanosoma dionisii*. These results revealed that the diversity of trypanosome species in a region considered endemic for Chagas disease is greater than previous descriptions. All this can confirm the necessity of using DNA sequencing approaches in order to determinate trypanosomes species isolated from bats.

Keywords Bats · *Trypanosoma cruzi marinkellei* · *Trypanosoma dionisii* · Cathepsin L-like · 18S rDNA

Introduction

Bats influence the ecosystem dynamics where they are found, acting as pollinators, seed dispersers, animal population regulators, or even as hosts of different virus, fungi, nematodes, and trypanosomatid species (Brener and Andrade 1979; Jansen et al. 2015). *Trypanosoma cruzi*, the causative agent of Chagas disease, is divided into two subspecies: *T. c. cruzi* and *T. c. marinkellei* (Baker et al. 1978). Phylogenetic studies have demonstrated that *T. cruzi* clade presents a group of approximately 18 species, of these, only *T. c. cruzi* species is not restricted to bats (Molyneux 1991; Cottontail et al. 2014; Lima et al. 2013). It has been suggested that this parasite's adaptation to a wide variety of vertebrate hosts was a characteristic acquired from an ancestral trypanosome restricted to bats (Barnabé et al. 2003; Hamilton et al. 2012; Lima et al. 2015). *T. cruzi* is a genotypically heterogeneous species that has been divided into six discrete typing units (DTUs) (TcI-TcVI), besides Tcbat, a *T. cruzi* genotype associated to bats in South and Central America (Zingales et al. 2012; Marcili et al. 2009; Lima et al. 2015).

T. c. marinkellei which is restricted to bats does not infect mice and is transmitted by triatomines of the genus *Cavernicola* (Marinkelle 1976; Marinkelle 1982). Meanwhile, Tcbat that was initially detected in the Brazilian Amazon region (Marcili et al. 2009) infects mice and was

Elaine Cristina Bento and César Gómez-Hernández contributed equally to this work.

✉ Elaine Cristina Bento
elaineuftm@gmail.com

[1] Centro de Educação Profissional (CEFORES), Universidade Federal do Triângulo Mineiro, Praça Manoel Terra, 330, Bairro Abadia, Uberaba, Minas Gerais CEP 38015-050, Brazil

[2] Instituto de Ciências Biológicas e Naturais, Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais, Brazil

[3] Instituto Adolfo Lutz, Centro de Laboratório Regional de Bauru (IAL-CLR Bauru), Bauru, São Paulo, Brazil

[4] Facultad de Ciencias Naturales y Matemáticas, Programa de Biología, Grupo de Investigaciones Microbiológicas–UR (GIMUR), Universidad del Rosario, Bogotá, Colombia

found in a Colombian child and in pre-Columbian mummies in Chile (Guhl et al. 2014; Ramírez et al. 2014). Moreover, other trypanosomes as *T. dionisii*, *T. rangeli*, *T. conorhini*, *T. evansi*, and *T. theileri* have been described in bats (Cottontail et al. 2009; García et al. 2012; Marcili et al. 2009; Maia da Silva et al. 2009; Pinto et al. 2012; Ramírez et al. 2014; Lima et al. 2015; Dario et al. 2017).

Recent studies regarding the genetic diversity of *T. cruzi* isolated from bats based on molecular techniques (miniexon, 18S rDNA, 24Sα rDNA, histone genes, cytochrome b, and glycosomal GAPDH) have demonstrated the presence of the genetic groups TcI, TcII, and TcIII (Lisboa et al. 2008; Añez et al. 2009; Maia da Silva et al. 2009; Cavazzana et al. 2010; Lima et al. 2015; Argibay et al. 2016; Dario et al., 2017) and Tcbat (Marcili et al. 2009; Pinto et al. 2012; Lima et al. 2015). Besides *T. cruzi*, there are around nine species described in Europe, America, and Australia that were found infecting bats (Ewers 1974; Gardner and Molyneux 1988; Marinkelle 1976; Cavazzana et al. 2010; Lima et al. 2015; Dario et al. 2017). These species are especially important due to their high prevalence and their morphological, biochemical, and phylogenetical similarities with *T. cruzi* (Marinkelle 1976; Baker et al. 1978; Steindel et al. 1998; Barnabé et al. 2003, Lima et al. 2012a). In Latin America, trypanosomes species described in bats are *T. cruzi*, Tcbat, *T. cruzi marinkellei*, *T. rangeli*, *T. dionisii*, *T. desterrensis*, and *T. hastatus* in Brazil (Teixeira et al. 1993; Steindel et al. 1998; Grisard et al. 2003; Lisboa et al. 2008; Maia da Silva et al. 2009; Cavazzana et al. 2010; Marcili et al. 2013; Costa et al. 2016, Lima et al. 2015; Dario et al. 2017); *T. cruzi* in Argentina (Argibay et al. 2016); *T. cruzi marinkellei* and *T. dionisii* in Bolivia (Garcia et al. 2012); *T. cruzi*, Tcbat, *T. cruzi marinkellei*, and *T. rangeli* in Panama (Cottontail et al. 2009; Pinto et al. 2012); and *T. cruzi*, Tcbat, *T. c. marinkellei*, *T. dionisii*, *T. rangeli*, *T. evansi*, and *T. theileri* in Colombia (Ramírez et al. 2014).

Several targets are used to discriminate trypanosomes species detected in bats, such as SSU rRNA (18S or small subunit), ITS rDNA, gGAPDH (glycosomal glyceraldehyde 3-phosphate dehydrogenase), spliced leader, mitochondrial genes (kDNA, cythocrome oxidase, cytochrome b), and Cathepsin L-like genes (Barnabé et al. 2003; Maia da Silva et al. 2009; Marcili et al. 2009, Cortez et al. 2009; Cavazzana et al. 2010; Lima et al. 2012b; García et al. 2012; Pinto et al. 2012; Ramírez et al. 2014). However, most of them require isolation of parasites and direct sequencing for species discrimination.

The Triângulo Mineiro and Alto Paranaíba regions located at the Minas Gerais state in Brazil were considered endemic areas for Chagas disease. In these regions, there are no reports of bats naturally infected by trypanosomes. Based on this information, the aim of this study was to describe, characterize, and identify the presence of trypanosomes in bats captured in those areas.

Materials and methods

Study area and capture of bats

The bats were captured around different rural regions from Triangulo Mineiro (Água Comprida; Conceição das Alagoas, Conquista; Uberaba; Uberlândia) and Alto Paranaíba (Araxá; Nova Ponte and Perdizes). These regions belong to the Brazilian savanna known as cerrado areas (Rizzini 1997). Its climate is described as tropical savannah, with two defined seasons, dry (from April to September) and rainy (from October to March). The region's annual precipitation varies between 1400 and 17,000 mm, and it is higher between December and February (Motta 1993). The bat capture sessions took place at night. Four mist nets of 7 m in length by 2 m in height were used and armed for 6 h/night (6 pm to 0 am) close to bat feeding sources and day shelters. Surveys were made at short time intervals. Taxonomic keys were used for bat classification (Vizotto and Taddei 1973; Reis et al. 2007). The animals were sacrificed by intramuscular administration of xylazine (Rompun® Bayer, Brazil) 3 mg/kg associated with ketamine (Ketalar® Pfizer, Brazil) 30 mg/kg. In cases of uncertainty regarding identification, some specimens were collected, prepared by wet route and sent for identification, and deposited, as specimen-testimony, in the Collection of the Department of Zoology of the Department of Biology at the Universidade Federal de Lavras (UFLA). The determination of the preferential food habit will be carried out in the future.

Blood collection and *Trypanosoma* spp. isolation

Depending on the weight of each captured bat, 0.5 to 3 mL of blood was collected in aseptic conditions by cardiac puncture. Blood samples were evaluated by microhematocrit and hemoculture in LIT medium (liver infusion tryptose—NaCl 0.4%; KCl 0.04%; Na2HPO4 0.8%; glucose 0.2%; tryptose 0.5%; liver infusion 0.5%; hemoglobin 2.0%; gentamicin 0.024 mg/mL; penicillin G 500 UI/mL) (Camargo 1964; Woo 1970).

DNA extraction and PCR

DNA extraction from parasite pellets was performed by traditional phenol-chloroform method (Gomes et al. 1998). Initially, *T. c. cruzi* DTUs and *T. rangeli* were assessed by five different PCR protocols (Table 1). We analyzed the 24α rDNA locus in order to accomplish two goals, first, determine *T. c. cruzi* and *T. rangeli* by a multiplex PCR (Souto et al. 1999) and second, by the amplification of the D7 divergent domain (Souto and Zingales 1993) in order to characterize the different *T. c. cruzi* DTUs. Also, the amplification of the variable site domain of rDNA 18s gene (Yeo et al.

Table 1 Sequence targets and primers used to identify *Trypanosoma* spp. isolated from bats by PCR

Target	PCR	Primer names	Primer sequences (5′ → 3′)	Product size[a] (in bp)	Reference
24Sα rDNA	24Sα—multiplex	D72	TTTTCAGAATGGCCGAACAGT	Tr—210; TcI—250; TcII—265	Souto et al.1999
		D75	GCAGATCTTGGTTGGCGTAG	TcI/TcIII—110; TcIV—120; TcII/TcVI—125; TcV—110/125	
		RG3	GGCCAAAGGGTAAGGCTC		
24Sα rDNA	Divergent domain	D71	AAGGTGCGTCGACAGTGTGG	Tc IV—155; TcI—160; TcII/TcIII/TcV/TcVI—165	Souto and Zingales 1993
		D72	TTTTCAGAATGGCCGAACAGT		
18S rDNA	Variable domain	V1	CAAGCGGCTGGGTGGTTATTCCA		Yeo et al. 2005
		V2	TTGAGGGAAGGCATGACACATGT		
18S rDNA	Tcm-18S	EB18SF	TGTGGTCCGTGAACACATTC	*T. c. marinkellei*—568	This study
		EB18SR	TTCATGGGTGTCATCGTTTG		
Spliced leader	SL-IR ac	UTCC	CGTACCAATATAGTACAGAAACTG	TcI—150; TcII/TcV/TcVI—157; TcIII/TcIV—200	Burgos et al.2007
		TCac	CTCCCCAGTGTGGCCTGGG		
	SL-IR I	UTCC	CGTACCAATATAGTACAGAAACTG	TcI—475	
		TC2	CCTGCAGGCACACGTGTGTG		
	SL-IR II	UTCC	CGTACCAATATAGTACAGAAACTG	TcII/TcV/TcVI—425	
		TC1	TCCGCCACCTCCTTCGGGCC		
Cytochrome oxidase subunit 2	COII[b]	Tcmit-10	CCATATATTGTTGCATTATT	TcI—212; TcII—263; TcIII to TcVI—293	Freitas et al. 2006
		Tcmit-21	TTGTAATAGGAGTCATGTTT		
Cathepsin L-like	CAT-L	DTO154	ACAGAATTCCAGGGCCAATGCGGCTCGTGCTGG	500	Cortez et al. 2009
		DTO155	TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA		

[a] TcI to TcVI, *Trypanosoma cruzi* I a VI; *T. c. marinkellei*, *Trypanosoma cruzi marinkellei*; Tr, *Trypanosoma rangeli*

[b] PCR-RFLP with the restriction enzyme *Alu*I

2005), the spliced leader sequence (Burgos et al. 2007), and the mitochondrial cytochrome oxidase subunit II gene (COII RFLP-PCR) (Freitas et al. 2006) were used for *T. c cruzi* incrimination. All sequence and primer names are detailed in Table 1.

For Cathepsin L-like enzyme catalytic domain (Cat-L), amplifications of a 500-bp fragment by DTO154 and DTO155 primers were used (Table 1) (Cortez et al. 2009). For 18S rDNA gene, a 568-bp fragment was amplified by specifically designed primers for this work (EB18SF and EB18SR), based on a *T. cruzi marinkellei* sequence available at GenBank (accession number FJ001665) (Table 1). PCR was performed in a final volume of 30 μL containing 3.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Recombinant, Fermentas Life Sciences), 0.4 mM of each dNTP, 1.5 ρmol/μL of each primer, and 30 ηg de DNA. The amplification program had an initial denaturation of 4 min at 95 °C, 30 cycles (95, 50, e 72 °C/1 min), followed by a final extension of 10 min at 72 °C. The thermocycler used was the MJ Research PTC-100 (Inc. Watertown, MA, USA). PCR products were visualized in 0.8% agarose gel in TBE 1× (89 mM Tris-borate, 2 mM EDTA pH 8.0) and stained with GelRed™ 1× (Biotium, Inc. CA). The bands were visualized in UV transilluminator, and the DNA purification was conducted by extracting the specific bands from agarose gels, using the Corning® Costar® Spin-X® (Sigma-Aldrich, USA) method.

Trypanosoma species identification

PCR products of Cathepsin L-like (500 bp) and 18S rDNA (568 bp) genes were sequenced using the BigDye Terminator Cycle Sequencing kit, version 3.1 (ABI PRISM™) and processed with the 3130 XL Genetic Analyzer (Applied Biosystems), according to manufacturer instructions. The chromatograms were analyzed using *ChromasPro* 2.1.4 (Technelysium, Pty, Ltd.) and a consensus sequence was generated. The Phred value considered was > 20. Sequences were aligned using muscle algorithm included in SeaView 4.5.2 software (Gouy et al., 2010). Neighbor-joining and maximum-likelihood phylogenetic methods were performed on MEGA 7 with 1000 bootstrap iterations (Kumar et al. 2016). The sequences are available under KX833232 to KX833246, KX826485 to KX826500 and KY287667 accession numbers.

Table 2 Preferential feeding habits and positivity for *Trypanosoma* spp. (cultures and blood samples) of bats captured in different cities of Triângulo Mineiro and Alto Paranaíba regions, Minas Gerais state, Brazil

Family	Species	*N* (%)	Preferential feeding habits	Local of capture	Positivity for *Trypanosoma* spp.	
					Blood samples (%)	Cultures (%)
Phyllostomidae	*Anoura caudifera*	16 (3.57%)	Nectarivorous	AC; CO; PE	4/16 (25%)	0
	Glossophaga soricina	212 (47.31%)	Nectarivorous	AC; AR; CO; NP; UB; PE	5/212 (2.4%)	1/212 (0.5%)
	Carollia perspicillata	36 (8.04%)	Frugivorous	AC; CO; PE	9/36 (25%)	3/36 (8.3%)
	Artibeus cinereus	2 (0.45%)	Frugivorous	UR	0	0
	Artibeus obscurus	3 (0.67%)	Frugivorous	PE	0	1/3 (33.3%)
	Artibeus planirostris	6 (1.34%)	Frugivorous	AC; PE	1/6 (16.7%)	4/6 (66.7%)
	Platyrrhinus lineatus	11 (2.46%)	Frugivorous	AC; CO; PE; UR	0	0
	Sturnira lilium	8 (1.79%)	Frugivorous	AC; PE	0	0
	Desmodus rotundus	31 (6.92%)	Hematophagous	AC; PE; UB;UR	0	0
	Phyllostomus hastatus	34 (7.59%)	Omnivorous	PE; UB	16/34 (47.1%)	16/34 (47.1%)
	Phyllostomus discolor	2 (0.45%)	Omnivorous	UR	2/2 (100%)	2/2 (100%)
Molossidae	*Eumops perotis*	18 (4.02%)	Insectivorous	AC	0	0
	Molossops neglectus	2 (0.45%)	Insectivorous	CO	0	0
	Molossops temminckii	1 (0.22%)	Insectivorous	UR	0	0
	Molossus molossus	61 (13.61%)	Insectivorous	UB	0	0
	Nyctinomops laticaudatus	2 (0.45%)	Insectivorous	AR; UB	0	0
	Tadarida brasiliensis	1 (0.22%)	Insectivorous	UB	0	0
Noctilionidae	*Noctilio albiventris*	1 (0.22%)	Insectivorous	CA	0	0
Vespertilionidae	*Eptesicus furinalis*	1 (0.22%)	Insectivorous	AC	0	0
Total	–	448 (100)	–	–	37	27

AC Água Comprida, *Ar* Araxá, *CA* Conceição das Alagoas, *Co* Conquista, *NP* Nova Ponte, *Pe* Perdizes, *Ub* Uberaba, *Ur* Uberlândia

Ethical considerations

All procedures were taken in accord with the Ethics Committee for Animals Use (CEUA), Universidade Federal do Triângulo Mineiro (approved protocols no 51), following the recommendations of the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA: license number: 076/2003, 121/2004, 143/06 and 13206-1).

Results

Diversity of capture bats and positivity for *Trypanosoma* spp. infection

A total of 448 bats were captured representing four families: Phyllostomidae ($n = 361$, 80.6%), Molossidae ($n = 85$, 19%), Noctilionidae ($n = 1$, 0.2%), and Vespertilionidae ($n = 1$, 0.2%) of 15 different species (Table 2). Trypanosomes were detected in 8.3% (37/448) of blood samples analyzed by microhematocrit and 6% (27/448) of hemoculture in LIT medium (Table 2). However, from these 27 strains, only 16 survived in LIT culture during the time required for DNA extraction. Bats found parasitized by *Trypanosoma* spp. belong to the following species: *Anoura caudifera* (4/16), *Glossophaga soricina* (5/212), *Carollia perspicillata* (9/36), *Artibeus planirostris* (1/6), *Phyllostomus hastatus* (16/34), and *Phyllostomus discolor* (2/2) (Table 3).

Identification of *Trypanosoma* species

The DNA samples were analyzed by PCRs with two targets of 24Sα rDNA (Souto and Zingales, 1993; Souto et al. 1999) and one target of 18S rDNA (Yeo et al. 2005). The multiplex PCR directed to the D7 divergent domain of the 24Sα rDNA gene amplified a 250-bp fragment in all samples (Table 3, Fig. 1a). This size is compatible with the 250-bp fragment found in the TcI control sample. We did not detect amplification products with sizes compatible with *T. rangeli* control. The PCR directed to the D7 divergent domain of the 24Sα rDNA amplified a fragment of 110 bp in all samples (Table 3, Fig. 1b). The size of the detected fragment is compatible with the 110 bp fragment of TcI. On COII RFLP-PCR, 14 isolates (EM001, EM051, EM239, EM242, EM243, EM245, EM362, EM364, EM367, EM369, EM370, EM441, EM465, EM466) showed a similar pattern with TcII (212 bp) and only one (EM437) exhibited a band of > 300 bp (Table 3, Fig. 1c). PCR analysis of the 18S rDNA locus revealed the amplification of a 160-bp fragment in all samples (Table 3, Fig. 1d). Again, the fragment detected is compatible with the one detected in the TcI control. There was not *T. cruzi* spliced leader fragment amplification in any of the samples.

Table 3 Isolates of trypanosome bats, host, and geographic origin

Isolate name	Bat species	Result by genetic marker (PCR band sizes in bp)							Conclusion
		24Sα—multiplex	24Sα—divergent domain	18S—variable domain	*Tcm*-18S[a]	Spliced leader	COII	CAT-L[a]	
EM001, EM239, EM242, EM243, EM245, EM362, EM364, EM367, EM369, EM370, EM441	*Phyllostomus hastatus*	250	110	160	568	Neg.	212	500	*T. cruzi marinkellei*
EM465, EM466	*Phyllostomus discolor*	250	110	160	568	Neg.	212	500	*T. cruzi marinkellei*
EM051	*Glossophaga soricina*	250	110	160	568	Neg.	212	500	*T. cruzi marinkellei*
EM437	*Artibeus planirostris*	250	110	160	568	Neg.	> 300	500	*T. cruzi marinkellei*
EM030	*Carollia perspicillata*	240	Neg.	Neg.	Neg.	Neg.	Neg.	500	*T. dionisii*

[a] PCR products were sequenced and sequences were analyzed by BLASTN

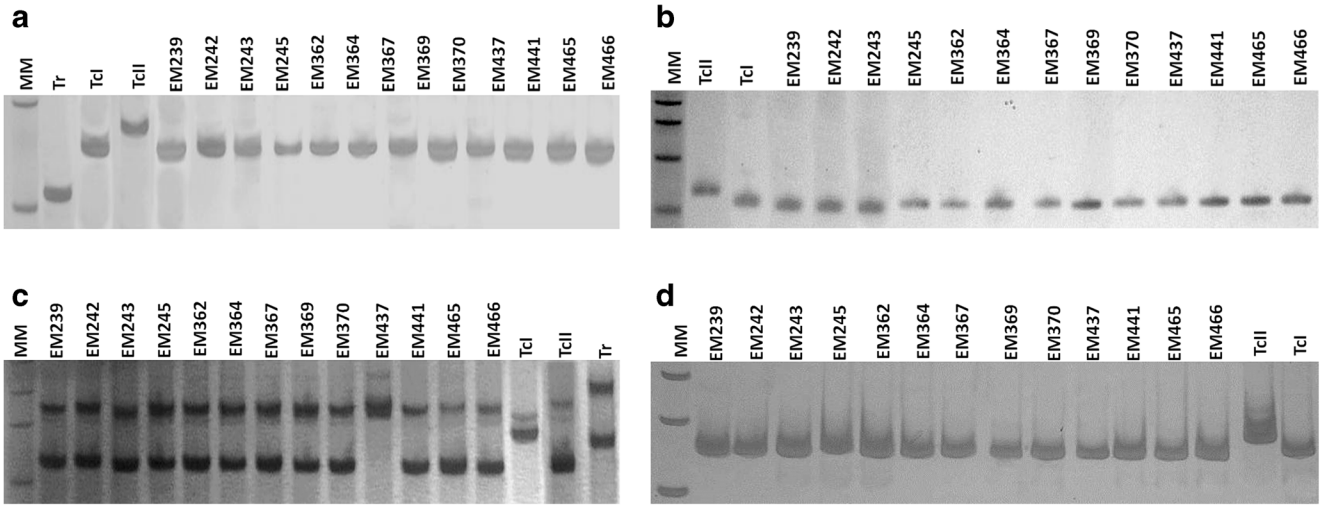

Fig. 1 Six percent polyacrylamide gels representing PCR conducted for trypanosome samples from bats. **a** Multiplex PCR for divergent domain D7 of 24Sα rDNA gene (Souto et al.1999). **b** PCR for divergent domain D7 of 24Sα rDNA gene (Souto and Zingales, 1993). **c** RFLP-PCR of subunit II from oxidase cytochrome mitochondrial gene (Freitas et al. 2006). **d** Variable size domain of 18S rDNA PCR (Yeo et al. 2005). (MM = molecular marker of 100 pb; TcI = positive control *T. cruzi* I, Alv strain; TcII = positive control *T. cruzi* II, JG strain; TR = positive control *T. rangeli*, P19 strain)

In order to confirm parasite species, we have determined the sequences of the products of 18S rDNA (V1 and V2 primers) and cathepsin L-like (DTO154 and DTO155) genes. However, products of primers V1 and V2 were too small (155–165 bp) to generate high quality sequences. Therefore, there were used new primers that amplify a fragment of 568 bp of the 18S rDNA locus of *T. cruzi marinkellei*. Accordingly, sequencing of these PCR products generated high quality sequences that were adequate for phylogenetic analysis.

Phylogenetic analyses

Primers directed to the cathepsin L-like genes allowed the amplification of a 500-bp fragment in all samples. All fragments were sequenced and used for parasite identification by comparison with trypanosomatid sequences available in GenBank. Phylogenetic analysis of these sequences proved that 15 of the bat isolates corresponded to *T. cruzi marinkellei* (EM001, EM051, EM239, EM242, EM243, EM245, EM362, EM364, EM367, EM369, EM370, EM437, EM441, EM465, and EM466) and one to *Trypanosoma dionisii* (EM030) (Figs 2 and 3).

Furthermore, we used *T. cruzi*-specific primers to amplify and in sequencing a 568-bp fragment of the 18S rDNA locus. Phylogenetic analysis using 18S rDNA sequences also showed that they correspond to *T. cruzi marinkellei*, confirming the results of cathepsin L-like sequences (Fig. 3). As expected, there was no amplification of *T. dionisii* (EM030) since the primers EB18SF and EB18SR were specifically designed to amplify *T. cruzi marinkellei* fragments.

Discussion

The sequencing analysis of 18S rDNA and cathepsin L-like genes allows an identification related to the trypanosomatid species (Cortez et al. 2009; Lima et al. 2012b; Dario et al., 2017). In this study, phylogenetic analysis classified 15 samples as *T. cruzi marinkellei* and one as *T. dionisii*. The *T. cruzi marinkellei* was identified in *Phyllostomus hastatus* (EM001, EM232, EM243, EM245, EM362, EM364, EM367, EM367, EM370, EM441), *Phyllostomus discolor* (EM465, EM466), *Artibeus planirostris* (EM437), and *Glossophaga soricina* (EM051) and meanwhile, *T. dionisii* was identified in *Carollia perspicillata* (EM030). The fact that we found greater genetic variability with the cathepsin-L like target than with 18S rDNA target is expected, considering that the regions of 18S rDNA locus are highly conserved in eukaryotes (Torres-Machorro et al. 2010). All bats that were found naturally infected by trypanosomes belong to the Phyllostomidae family. Some authors suggest that *T. cruzi marinkellei* has a strong association with Phyllostomidae bats (Marinkelle 1976; Cavazzana et al. 2010); however, they also were found infected with other trypanosomatids, such as *T. cruzi*, *T. rangeli*, and *T. dionisii* (Maia da Silva et al. 2009; Marcili et al. 2009; Cavazzana et al. 2010).

Despite the knowledge about the occurrence of trypanosomes in bats, little is known about their natural vectors. The participation of triatomines in the transmission of trypanosomes between bats is not clear, and *Cavernicola pilosa* is the only vector species reported (Marinkelle, 1982). Experimentally, it has been demonstrated that bats can be infected with *T. rangeli* and *T. cruzi* after blood feeding with triatomines of the species *Rhodnius prolixus* and that the ingestion of triatomines infected with these trypanosomes can

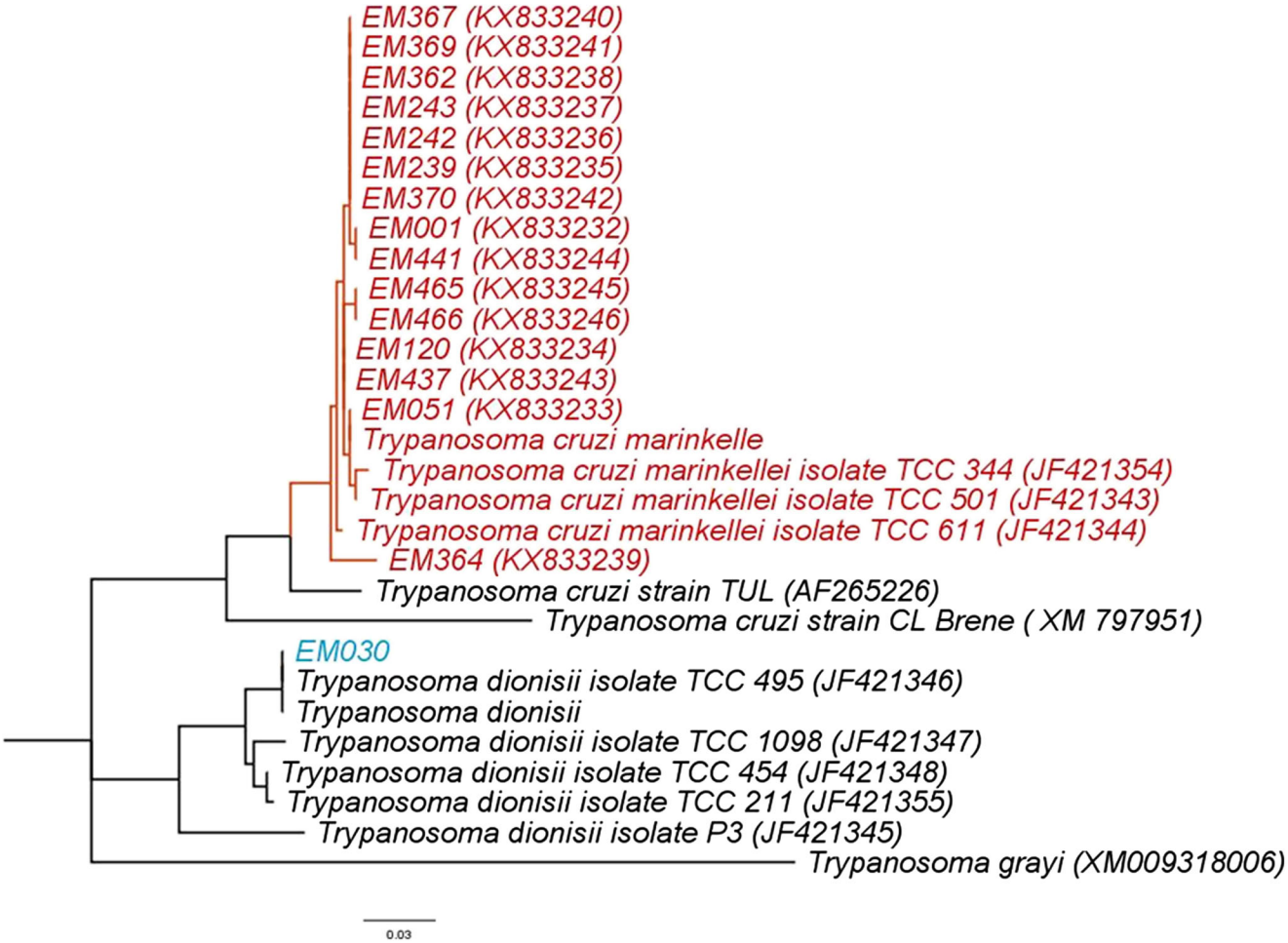

Fig. 2 Maximum composite likelihood phylogenetic reconstruction of cathepsin L-like fragment based on *neighbor-joining* method. The replicate percentage of trees where taxons were grouped by bootstrap test (1000 replicates) is presented by black dots with values over 80%. Isolate EM030 is highlighted in green

lead to the infection of mice and bats (Thomas et al. 2007). In the present study, *T. c marinkellei* and *T. dionisii* strains were isolated from bats that may also consume insects at some stage of life, but which have preferentially frugivorous (*Artibeus planirostris* and *Carollia perspicillata*), omnivorous (*Phyllostomus discolor* and *Phyllostomus hastatus*), and nectarivorous eating habits (*Glossophaga soricina*) (Reis et al. 2007). These data may suggest that other vectors besides that of the *Cavernicola* genus (Marinkelle, 1982) may act in the transmission cycle of these bat trypanosomes in the studied area. In addition, the possibility of direct transmission of trypanosomes among bats should be considered because of their social behavior, which tend to live in colonies (Molyneux, 1991).

In this study, the prevalence of *T. cruzi marinkellei* was much higher than that of *T. dionisii*. Besides these, it is possible that other species also occur in the studied region, such as *T. cruzi*, Tcbat, and *T. rangeli*; however, they appear to be less frequent.

The results provided by multiplex PCR of D7 divergent domain of the 24Sα rDNA gene have discarded any *T. rangeli* presence in the analyzed bats. As previously described, the frequency of this parasite in bats is low (Maia da Silva et al. 2009; Dario et al. 2017). However, in a previous work conducted in the same region, we have found *T. rangeli* in *Didelphis albiventris* and *Rhodnius neglectus* (Ramirez et al. 2002). PCR for both targets of 24Sα rDNA gene and 18S rDNA gene resulted in a product of 110 and 160 bp, respectively, very similar to the fragment expected for TcI. These results suggest that *T. cruzi marinkellei* and *T. cruzi* share a conserved primer region for ribosomal genes. Thus, PCR with ribosomal targets as the ones used in this study and kDNA (primers 121–122) (Garcia et al. 2012) are not ideal to be used for trypanosome identification of bats, because they could wrongly suggest that *T. cruzi* was found instead of *T. cruzi marinkellei*, underestimating the real prevalence of this last one.

Primers with spliced leader as target are specific for *T. cruzi* (Souto et al. 1996; Burgos et al. 2007) and not amplify *T. cruzi marinkellei* samples, as observed in other studies conducted in Brazil, Colombia, Panama, and Venezuela (Barnabé et al.

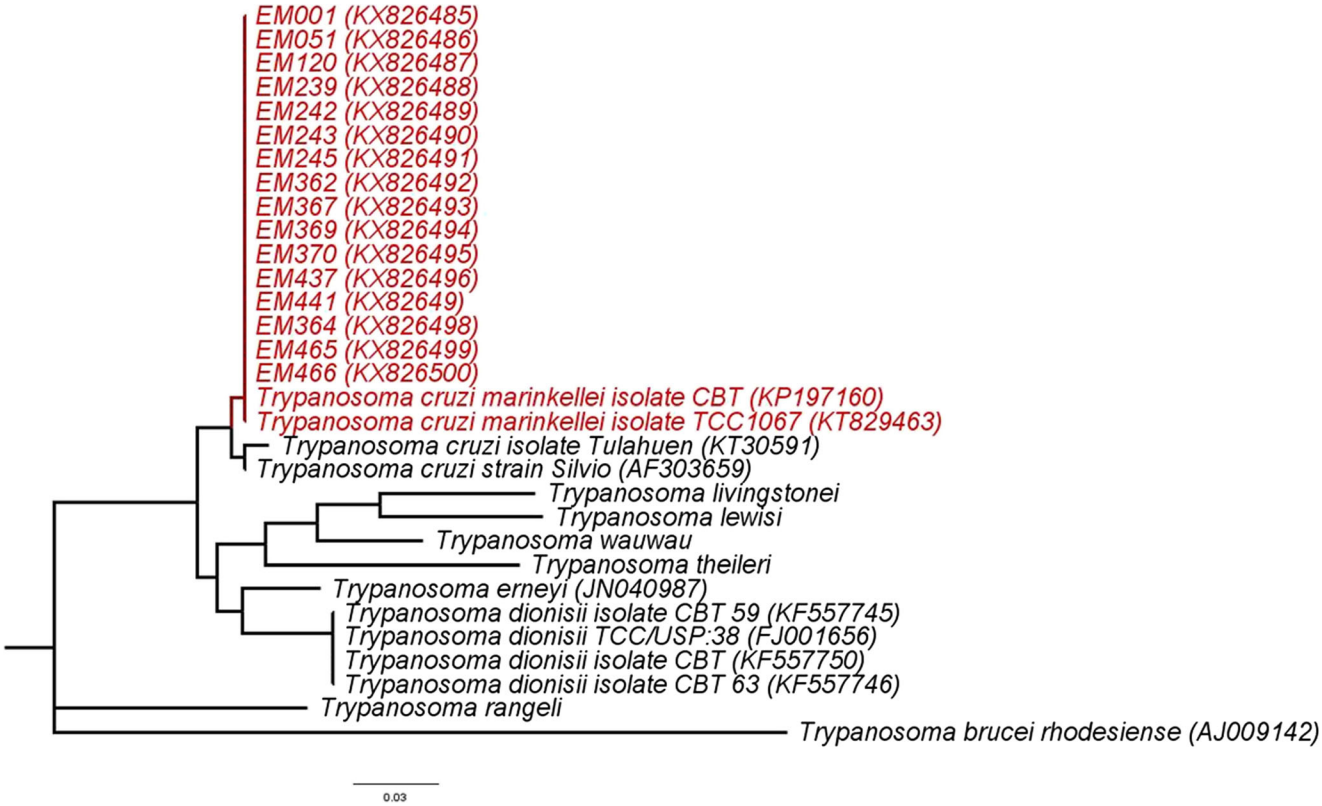

Fig. 3 Maximum composite likelihood phylogenetic reconstruction of 18S rDNA gene fragment based on *neighbor-joining* method. The replicate percentage of trees where taxons were grouped by bootstrap test (1000 replicates) is presented with black dots (over 80%)

2003; Maia da Silva et al. 2009; Cottontail et al. 2009; Cavazzana et al. 2010).

COII RFLP-PCR can differentiate *T. cruzi* DTUs and *T. rangeli* KP1+ of KP1− and co-infection between these parasites (de Sá et al. 2013). In this study 14 samples amplified fragments with sizes compatible with the fragment obtained from TcII and EM437 strain amplified non-specific band. Considering this result, COII RFLP-PCR is not a good target for trypanosome identification from bat samples.

Considering that our previous attempts to identify by PCR the *Trypanosoma* species isolated from bats has failed since some primers amplified sequences with identical sizes from different species of trypanosomes, we decided to sequence the fragment generated by PCR with primers V1 and V2 (Yeo et al. 2005). However, the small sizes of the fragments amplified vary (155 to 165 bp) in impaired sequence determination. Accordingly, we designed new primer pairs (EB18SF and EB18SR) that amplify a region spanning the sequences of the product of primers V1 and V2. With this approach, we were able to determine sequences of the 18S rDNA locus in the phylogenetic analyses. Primers EB18SF and EB18SR were designed from a *T. c. marinkellei* sequence (Cavazzana et al. 2010) and their use for characterization of field isolates may be limited, since they did not allow detection of *T. dionisii*.

In conclusion, these results show how other trypanosome species such as *T. cruzi marinkellei* and *T. dionisii* are present in this region that was considered an endemic area for Chagas disease. It was also demonstrated that the *T. cruzi marinkellei* has presented cross-reactivity in four molecular techniques used as specific of *T. cruzi* (targets 18S rDNA, COII and two target by 24Sα rDNA) and reinforced the necessity of using DNA sequencing to accurately determine the trypanosome species isolated from bats.

Funding This work was supported by the National Incentive Program for Research in Parasitology Basic/2010, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) process 307284/2014-0 (research productivity fellowship).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

This article does not contain any studies with human participants performed by any of the authors.

References

Añez N, Crisante G, Soriano PJ (2009) *Trypanosoma cruzi* congenital transmission in wild bats. Acta Trop 109(1):78–80. https://doi.org/10.1016/j.actatropica.2008.08.009

Argibay HD, Orozco MM, Cardinal MV, Rinas MA, Arnaiz M, Segura CM, Gürtler RE (2016) First finding of *Trypanosoma cruzi* II in vampire bats from a district free of domestic vector-borne transmission in Northeastern Argentina. Parasitology 143(11):1358–1368. https://doi.org/10.1017/S0031182016000925

Baker JR, Miles MA, Godfrey DG, Barrett TV (1978) Biochemical characterization of some species of *Trypanosoma (Schizotrypanum)* from bats (Microchiroptera). Am J Trop Med Hyg 27(3):483–491. https://doi.org/10.4269/ajtmh.1978.27.483

Barnabé C, Brisse S, Tibayrenc M (2003) Phylogenetic diversity of bat trypanosomes of subgenus *Schizotrypanum* based on multilocus enzyme electrophoresis, random amplified polymorphic DNA, and cytochrome b nucleotide sequence analyses. Infect Genet Evol 2: 201–208. https://doi.org/10.1016/S1567-1348(02)00130-2

Brener Z and Andrade ZA (1979) *Trypanosoma cruzi* e doença de Chagas, 1ª ed. Guanabara Koogan, Rio de Janeiro. 463 p

Burgos JM, Altcheh J, Bisio M, Duffy T, Valadares HMS, Seidenstein ME, Piccinali R, Freitas JM, Levin MJ, Macchi L, Macedo AM, Freilij H, Schijman AG (2007) Direct molecular profiling of minicircle signatures and lineages of *Trypanosoma cruzi* bloodstream populations causing congenital Chagas disease. Int J Parasitol 37(12):1319–1327. https://doi.org/10.1016/j.ijpara.2007.04.015

Camargo EP (1964) Growth and differentiation in *Trypanosoma cruzi*. I Origin of metacyclic trypanosomes in liquid media. Rev Inst Med Trop Sao Paulo 6:93–100

Cavazzana JRM, Marcili A, Lima L, Da Silva FM, Junqueira ACV, Veludo HH, Viola LB, Campaner M, Nunes VLB, Paiva F, Coura JR, Camargo EP, Teixeira MMG (2010) 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. Int J Parasitol 40(3):345–355. https://doi.org/10.1016/j.ijpara.2009.08.015

Cortez AP, Rodrigues AC, Garcia HA, Neves L, Batista JS, Bengaly Z, Paiva F, Teixeira MM (2009) Cathepsin L-like genes of *Trypanosoma vivax* from Africa and South America—characterisation, relationships and diagnostic implications. Mol Cell Probes 23(1):44–51. https://doi.org/10.1016/j.mcp.2008.11.003

Costa AP, Nunes PH, Leite BH, Ferreira JI, Tonhosolo R, da Rosa AR, da Rocha PA, Aires CC, Gennari SM, Marcili A (2016) Diversity of bats trypanosomes in hydroeletric área of Belo Monte in Brazilian Amazonia. Acta Trop 164:185–193. https://doi.org/10.1016/j.actatropica.2016.08.033

Cottontail VM, Wellinghausen N, Kalko EKV (2009) Habitat fragmentation and haemoparasites in the common fruit bat, *Artibeusjamaicensis* (Phyllostomidae) in a tropical lowland forest in Panamá. Parasitology 136(10):1133–1145. https://doi.org/10.1017/S0031182009990485

Cottontail VM, Kalko EKV, Cottontail I, Wellinghausen N, Tschapka M, Perkins SL, Pinto CM (2014) High local diversity of *Trypanosoma* in a common bat species, and implications for the biogeography and taxonomy of the *T. cruzi* clade. PLoS One 9(9):e108603. https://doi.org/10.1371/journal.pone.0108603

Dario MA, Moratelli R, Schwabl P, Jansen AM, Llewellyn MS (2017) Small subunit ribosomal metabarcoding reveals extraordinary trypanosomatid diversity in Brazilian bats. PLoS Negl Trop Dis 11(7):e0005790. https://doi.org/10.1371/journal.pntd.0005790

de Sá ARN, Steindel M, Demeu LMK, Lückemeyer DD, Grisard EC, Neto QAL, de Araújo SM, Toledo MJO, Gomes ML (2013) Cytochrome oxidase subunit 2 gene allows simultaneous detection and typing of *Trypanosoma rangeli* and *Trypanosoma cruzi*. Parasit Vectors 6(1):363. https://doi.org/10.1186/1756-3305-6-363

Ewers WH (1974) *Trypanosoma aunawa* sp. n. from an insectivorous bat, *Miniopterustristris*, in New Guinea, which may be transmitted by a leech. J Parasitol 60(1):172–178

Freitas JM, Augusto-Pinto L, Pimenta JR, Bastos-Rodrigues L, Gonçalves VF, Teixeira SMR, Chiari E, Junqueira ACV, Fernandes O, Macedo AM, Machado CR, Pena SDJ (2006) Ancestral genomes, sex and the population structure of *Trypanosoma cruzi*. PLoS Pathog 2(3):24. https://doi.org/10.1371/journal.ppat.0020024

García L, Ortiz S, Osorio G, Torrico MC, Torrico F, Solari A (2012) Phylogenetic analysis of Bolivian bat trypanosomes of the subgenus *Schizotrypanum* based on cytochrome b sequence and minicircle analyses. PLoS One 7(5):e36578. https://doi.org/10.1371/journal.pone.0036578

Gardner RA, Molyneux DH (1988) *Trypanosoma (Megatrypanum) incertum* from *Pipistrellus pipistrellus*: development and transmission by cimicid bugs. Parasitology v 96(03):433–447. https://doi.org/10.1017/S0031182000080082

Gomes ML, Macedo AM, Vago AR, Pena SD, Galvao LM, Chiari E (1998) *Trypanosoma cruzi*: optimization of polymerase chain reaction for detection in human blood. Exp Parasitol 88(1):28–33. https://doi.org/10.1006/expr.1998.4191

Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol and Evol 27(2):221–224. https://doi.org/10.1093/molbev/msp259

Grisard EC, Sturm NRE, Campbell DA (2003) A new species of trypanosome, *Trypanosoma desterrensis* sp. n., isolated from South American bats. Parasitology 127(3):265–271. https://doi.org/10.1017/S0031182003003536

Guhl F, Auderheide A, Ramírez JD (2014) From ancient to contemporary molecular eco-epidemiology of Chagas disease in the Americas. Int J Parasitol 44(9):605–612. https://doi.org/10.1016/j.ijpara.2014.02.005

Hamilton PB, Teixeira MMG, Stevens JR (2012) The evolution of *Trypanosoma cruzi*: the 'bat seeding' hypothesis. Trends Parasitol 28(4):136–141. https://doi.org/10.1016/j.pt.2012.01.006

Jansen AM, Xavier SCC, Roque ALR (2015) The multiple and complex and changeable scenarios of the *Trypanosoma cruzi* transmission cycle in the sylvatic environment. Acta Trop 151:1–15. https://doi.org/10.1016/j.actatropica.2015.07.018

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–1874. https://doi.org/10.1093/molbev/msw054

Lima L, Ortiz PA, da Silva FM, Alves JMP, Serrano MG, Cortez AP, Alfieri SC, Buck GA, Teixeira MMG (2012a) Repertoire, genealogy and genomic organization of cruzipain and homologous genes in *Trypanosoma cruzi*, *T. cruzi*-like and other trypanosome species. PLoS One 7(6):e38385. https://doi.org/10.1371/journal.pone.0038385

Lima L, da Silva FM, Neves L, Attias M, Takata CSA, Campaner M et al (2012b) Evolutionary insights from bat trypanosomes: morphological, developmental and phylogenetic evidence of a new species, *Trypanosoma (Schizotrypanum) erneyi* sp. nov., in African bats closely related to *Trypanosoma (Schizotrypanum) cruzi* and allied species. Protist 163(6):856–872. https://doi.org/10.1016/j.protis.2011.12.003

Lima L, Espinosa-Álvarez O, Hamilton PB, Neves L, Takata CS, Campaner M, Attias M, de Souza W, Camargo EP, Teixeira MM (2013) *Trypanosoma livingstonei*: a new species from African bats supports the bat seeding hypothesis for the *Trypanosoma cruzi* clade. Parasit Vectors 6(1):221. https://doi.org/10.1186/1756-3305-6-221

Lima L, Espinosa-Álvarez O, Pinto CM, Cavazzana M Jr, Pavan AC, Carranza JC, Lim BK, Campaner M, Takata CS, Camargo EP, Hamilton PB, Teixeira MM (2015) New insights into the evolution of the *Trypanosoma cruzi* clade provided by a new trypanosome species tightly linked to Neotropical *Pteronotus* bats and related to an Australian lineage of trypanosomes. Parasit Vectors 8:657. https://doi.org/10.1186/s13071-015-1255-x

Lisboa CV, Pinho APS, Herrera H, Gerhard M, Cupolillo E, Jansen AM (2008) *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae) genotypes in neotropical bats in Brazil. Vet Parasitol 156(3-4):314–318. https://doi.org/10.1016/j.vetpar.2008.06.004

Maia Da Silva F, Marcili A, Lima L, Cavazzana JRM, Ortiz PA, Campaner M, Takeda GF, Paiva F, Nunes VL, Camargo EP, Teixeira MMG (2009) *Trypanosoma rangeli* isolates of bats from Central Brazil: genotyping and phylogenetic analysis enable description of a new lineage using spliced-leader gene sequences. Acta Trop 109(3):199–207. https://doi.org/10.1016/j.actatropica.2008.11.005

Marcili A, Lima L, Cavazzana JRM, Junqueira ACV, Veludo HH, Maia Da Silva F, Nunes VLB, Paiva F, Camargo EP, Teixeira MMG (2009) 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. Parasitology 136(06):641–655. https://doi.org/10.1017/S0031182009005861

Marcili A, da Costa AP, Soares HS, Acosta I, de Lima JT, Minervino AH, Melo AT, Aguiar DM, Pacheco RC, Gennari SM (2013) Isolation and phylogenetic relationships of bat trypanosomes from different biomes in MatoGrosso, Brazil. J Parasitol 99(6):1071–1076. https://doi.org/10.1645/12-156.1

Marinkelle CJ (1976) The biology of the trypanosomes of bats. In: Lumdsen WHR, Evans DA (eds) Biology of the Kinetoplastida. Academic Press, New York, pp 175–216

Marinkelle CJ (1982) Developmental stages of *Trypanosoma cruzi*-like flagellates in *Cavernicola pilosa*. Revista de Biologia Tropical 30(2):107–111

Molyneux AJ (1991) Trypanosomes of bats. In: Kreier JP, Baker JR (eds) Parasitic protozoa. Academic Press, New York, pp 95–223

Motta PEF (1993) Os solos do Triângulo Mineiro e sua aptidão agrícola. Horizonte, Belo

Pinto CM, Kalko EK, Cottontail I, Wellinghausen N, Cottontail VM (2012) Tcbat a bat-exclusive lineage of *Trypanosoma cruzi* in the Panama Canal Zone, with comments on its classification and the use of the 18S rRNA gene for lineage identification. Infect Genet Evol 12(6):1328–1332. https://doi.org/10.1016/j.meegid.2012.04.013

Ramirez LE, Lages-Silva E, Alvarenga-Franco F, Matos A, Vargas N, Fernandes O, Zingales B (2002) High prevalence of *Trypanosoma rangeli* and *Trypanosoma cruzi* in opossums and triatomidas in a formely-endecic area of Chagas disease in Southeast Brazil. Acta Trop 84(3):189–198. https://doi.org/10.1016/S0001-706X(02)00185-7

Ramírez JD, Tapia G, Muñoz G, Poveda C, Rendón LM, Hincapié E, Guhl F (2014) Trypanosome species in neotropical bats: biological, evolutionary and epidemiological implications. Infect Genet Evol 22:250–256. https://doi.org/10.1016/j.meegid.2013.06.022

Reis NR, Peracchi AL, Pedro WA, Lima IP (2007) Morcegos do Brasil. Londrina, 253p

Rizzini CT (1997). Tratado de Fitogeografia do Brasil., Rio de Janeiro

Souto RP, Zingales B (1993) Sensitive detection and strain classification of *Trypanosoma cruzi* by amplification of a ribosomal RNA sequence. MolBiochemParasitol 62(1):45–52. https://doi.org/10.1016/0166-6851(93)90176-X

Souto RP, Fernandes O, Macedo AM, Campbell DA, Zingales B (1996) DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. Mol Biochem Parasitol 83(2):141–152. https://doi.org/10.1016/S0166-6851(96)02755-7

Souto RP, Vargas N, Zingales B (1999) *Trypanosoma rangeli*: discrimination from *Trypanosoma cruzi* based on a variable domain from the large subunit ribosomal RNA gene. Exp Parasitol 91(4):306–314. https://doi.org/10.1006/expr.1998.4380

Steindel M, Grisard EC, Carvalho Pinto CJ, Cordeiro FD, Ribeiro-Rodrigues R, Romanha AJ (1998) Characterization of trypanosomes from the subgenus *Schizotrypanum* isolated from bats *Eptesicus* sp. (Chiroptera: Vespertilionidae) captured in Florianópolis, Santa Catarina state, Brazil. J Parasitol 84(3):601–607. https://doi.org/10.2307/3284730

Teixeira LF, Gonçalves AM, Romanha AJ, Steindel M, Pinto AS (1993) Schizodeme and zymodeme analysis of trypanosomes of the subgenus *Schizotrypanum* from the bat. Parasitol Res 79(6):497–500. https://doi.org/10.1007/BF00931591

Thomas ME, Rasweiler JJ IV, D'alessandro A (2007) Experimental transmission of the parasitic flagellates *Trypanosoma cruzi* and *Trypanosoma rangeli* between triatomine bugs or mice and captive neotropical bats. Mem Inst Oswaldo Cruz 102(5):559–565. https://doi.org/10.1590/S0074-02762007005000068

Torres-Machorro AL, Hernández R, Cevallos AM, López-Villaseñor I (2010) Ribosomal RNA genes in eukaryotic microorganisms: witnesses of phylogeny? FEMS Microbiol Rev 34(1):59–86. https://doi.org/10.1111/j.1574-6976.2009.00196.x

Vizotto LD, Taddei VA (1973) Chave para determinação de quirópteros brasileiros. São José do Rio Preto, Gráfica Francal, 72p

Woo PTK (1970) The haematocrit centrifuge techinique for the diagnosis of African tripanosomiasis. Acta Trop 27(4):384–386

Yeo M, Acosta N, Llewellyn M, Sanchez H, Adamson S, Miles GA, Lopez E, Gonzalez N, Patterson JS, Gaunt MW, De Arias AR, Miles MA (2005) Origins of Chagas disease: *Didelphis* species are natural hosts of *Trypanosoma cruzi* I and armadillos hosts of *Trypanosoma cruzi* II, including hybrids. Int J Parasitol 35(2):225–233. https://doi.org/10.1016/j.ijpara.2004.10.024

Zingales B, Miles MA, Campbell DA, Tibayrenc M, Macedo AM, Teixeira MMG, Schijman AG, Llewellyn MS, Lages-Silva E, Machado CR, Andrade SG, Sturm NR (2012) The revised *Trypanosoma cruzi* subspecific nomenclature: rationale, epidemiological relevance and research applications. Infect Genet Evol 12(2):240–253. https://doi.org/10.1016/j.meegid.2011.12.009