Trypanosoma madeirae sp. n.: A species of the clade *T. cruzi* associated with the neotropical common vampire bat *Desmodus rotundus*

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A B S T R A C T

Molecular phylogenetic studies have revealed the growing diversity of bat trypanosomes. Here, 14 isolates from blood samples of the vampire bat *Desmodus rotundus* (Phyllostomidae) from Rio de Janeiro, Southeast Brazil, were cultivated, and morphologically and molecularly characterized. All isolates represent a novel species named *Trypanosoma madeirae* n. sp. positioned in the Neobat lineage of the clade *T. cruzi*. The Neobat lineage also comprises closely related trypanosomes of clades Neotropic 1, 2 and 3 from diverse phyllostomid species. Trypanosomes of Neotropic 1, found in *Trachops cirrhosus* and *Artibeus jamaicensis* (phyllostomids), likely represent a different species or genotype closely related to *T. madeirae*. Consistent with its phylogenetic positioning, *T. madeirae* differs from *Trypanosoma cruzi* in morphology of both epimastigote and trypomastigote culture forms and does not infect *Triatoma infestans*. Similar to its closest relatives of Neobat lineage, *T. madeirae* was unable to develop within mammalian cells. To date, PCR-surveys on archived blood/liver samples unveiled *T. madeirae* exclusively in *D. rotundus* from Southern to Northern Brazil. The description of a new species of bat trypanosome associated with vampire bats increases the repertoire of trypanosomes infecting *D. rotundus*, currently comprised of *Trypanosoma cruzi*, *T. cruzi marinkellei*, *Trypanosoma dionisii*, *Trypanosoma rangeli*, *Trypanosoma pessoai*, and *Trypanosoma madeirae*.

1. Introduction

Species of the genus *Trypanosoma* are parasites of a variety of vertebrate hosts and transmitted by diverse hematophagous arthropods and leeches. It has been known for more than a century that a large number of bat species are hosts for a wide assortment of trypanosome species throughout the world (Molyneux, 1991). Nevertheless, only recently, molecular studies have uncovered the real diversity of phylogenetic lineages, species, and genotypes of bat trypanosomes (Cavazzana et al., 2010; Lima et al., 2012, 2013, 2015a; Pinto et al., 2012, 2015; Ramírez et al., 2014; Dario et al., 2017a,b; Dos Santos et al., 2017; Lourenço et al., 2018). In addition to previously described species, molecular phylogenies have supported many candidates to new species (Cottontail et al., 2014; Lima et al., 2015b; Espinosa-Álvarez et al., 2018). However, the formal description of new species of bat trypanosomes has so far been restricted to a few species from Africa (Lima et al., 2012, 2013), Brazil (Lima et al., 2015a), and Australia (Barbosa et al., 2016).

In addition to bats of many families harboring *Trypanosoma cruzi*,

Trypanosoma cruzi marinkelei, *Trypanosoma dionisii*, and *Trypanosoma rangeli* (Maia da Silva et al., 2009; Cavazzana et al., 2010; Pinto et al., 2012, Ramírez et al., 2014; Lima et al., 2015b; Dario et al., 2017a,b; Dos Santos et al., 2017; Bento et al., 2018), Neotropical bats harbor *T. wauwau*, a species nested in the lineage Neobat that was linked to *Pteronotus* spp. (Mormoopidae) from Central and South America (Lima et al., 2015a; Da Costa et al., 2016), and a complex of unnamed trypanosomes distributed in clades Neotropic 1–3, which comprise mostly trypanosomes from phyllostomid bats (Pinto et al., 2012; Cottontail et al., 2014; Lima et al., 2015a). The diversity of trypanosome species infecting bats in the New and Old Words nested mostly in the clade *T. cruzi* (Hamilton et al., 2012; Pinto et al., 2012; Cottontail et al., 2014; Lima et al., 2012, 2013, 2015a,b; Barbosa et al., 2016; Espinosa-Álvarez et al., 2018); *T. evansi* and *T. vegrandis* are the only trypanosome species found in bats (and other mammals) that could not be nested into this clade (Ramírez et al., 2014; Austen et al., 2015). The clade *T. cruzi* also comprises trypanosomes from Australian (Hamilton et al., 2012; Botero et al., 2016) and Brazilian (Lopes et al., 2018) marsupials.

The order Chiroptera comprises more than thousand species of bats

Table 1

Trypanosomes, host and geographical origin, and GenBank accession numbers of the variable V7V8 region of ssrRNA and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) sequences.

Trypanosoma sp isolates		Host Origin	Year	Geographic Origin		GenBank Acession number	
						SSU rRNA	gGAPDH
M1-Lajes	bat	*Desmodus rotundus*	2008	Laje do Muriaé/Rio de Janeiro	BR	**MK064121**	**MK064144**
M2-387	bat	*Desmodus rotundus*	2006	Maricá/Rio de Janeiro	BR	**MK064122**	-
M2-1008	bat	*Desmodus rotundus*	2008	Paraty/Rio de Janeiro	BR	**MK064123**	**MK064145**
M3-209	bat	*Desmodus rotundus*	2005	Niterói/Rio de Janeiro	BR	**MK064124**	**MK064146**
M3-1185	bat	*Desmodus rotundus*	2008	Laje do Muriaé/Rio de Janeiro	BR	**MK064125**	**MK064146**
M4-Lajes	bat	*Desmodus rotundus*	2008	Laje do Muriaé/Rio de Janeiro	BR	**MK064126**	
M4-1012	bat	*Desmodus rotundus*	2007	Miracema/Rio de Janeiro	BR	**MK064127**	**MK064148**
M5-069	bat	*Desmodus rotundus*	2004	Miracema/Rio de Janeiro	BR	**MK064128**	-
M5-1186	bat	*Desmodus rotundus*	2008	Laje do Muriaé/Rio de Janeiro	BR	**MK064129**	**MK064149**
M7-1013	bat	*Desmodus rotundus*	2007	Miracema/Rio de Janeiro	BR	**MK064130**	-
M8-077	bat	*Desmodus rotundus*	2007	Miracema/Rio de Janeiro	BR	**MK064131**	**MK064149**
M9-066	bat	*Desmodus rotundus*	2004	Miracema/Rio de Janeiro	BR	**MK064132**	-
M10-067	bat	*Desmodus rotundus*	2004	Miracema/Rio de Janeiro	BR	**MK064133**	**MK064151**
M10-1196	bat	*Desmodus rotundus*	2008	Laje do Muriaé/Rio de Janeiro	BR	**MK064134**	**MK064152**
AD245	bat	*Desmodus rotundus*	–	Ribeirão Grande/São Paulo	BR	**MK064135**	-
AD1036	bat	*Desmodus rotundus*	–	Castelo/Espírito Santo	BR	**MK064136**	-
SLA808	bat	*Desmodus rotundus*	–	Governador Celso Ramos/Santa Catarina	BR	**MK064137**	-
AD720	bat	*Desmodus rotundus*	–	Município de Cunha/São Paulo	BR	**MK064138**	-
ICC16	bat	*Desmodus rotundus*	–	arquipélago de Marajó/Pará	BR	**MK064139**	-
ICC14	bat	*Desmodus rotundus*	–	arquipélago de Marajó/Pará	BR	**MK064141**	-
PNP007	bat	*Desmodus rotundus*	–	Itacarambi/Minas Gerais	BR	**MK064142**	-
ICC02	bat	*Desmodus rotundus*	–	arquipélago de Marajó/Pará	BR	**MK064143**	-
T. lewisi							
Molteno B3	rodent	*Rattus rattus*	–	–	UK	AJ009156	AJ620272
T. microti							
TRL 132	vole	Microtis agrestis	–	–	UK	AJ009158	AJ620273
T. wauwau							
TCC410	bat	*Pteronotus parnellii*	2002	Monte Negro/Rondonia	BR	KT030809	KT030799
TCC 411	bat	*Pteronotus parnellii*	2002	Monte Negro/Rondonia	BR	KT030810	KT030800
TCC 986	bat	*Pteronotus parnellii*	2005	Porto Velho/Rondonia	BR	KT030821	KT030801
TCC 988	bat	*Pteronotus parnellii*	2005	Porto Velho/Rondonia	BR	KT030823	KT030804
TCC 1022	bat	*Pteronotus parnellii*	2005	Porto Velho/Rondonia	BR	KT030830	KT030805
TCC 1878	bat	Pteronotus gymnonotus	2009	Porto Velho/Rondonia	BR	KT030835	KT030806
T. livingstonei							
TCC 1270	bat	Rhinolophus landeri	2006	Chupanga	MZ	KF192979	KF192958
TCC 1271	bat	Rhinolophus landeri	2006	Chupanga	MZ	KF192980	KF192959
TCC 1295	bat	Rhinolophus landeri	2006	Chupanga	MZ	KF192981	KF192960
TCC 1298	bat	Rhinolophus landeri	2006	Chupanga		KF192982	KF192961
TCC 1304	bat	Rhinolophus landeri	2006	Chupanga	MZ	KF192983	KF192962
T. sp Neot 1							
093_AJ_Bohio	bat	*Artibeus jamaicensis*	2005	–	PA	KM406889	–
134_AJ_Cacao	bat	*Artibeus jamaicensis*	2005	–	PA	KM406888	–
216_AJ_Guava	bat	*Artibeus jamaicensis*	2005	–	PA	KM406898	–
278_AJ_Leon	bat	*Artibeus jamaicensis*	2005	–	PA	KM406887	–
300_AJ_BCI	bat	*Artibeus jamaicensis*	2005	–	PA	KM406886	–
302_AJ_BCI	bat	*Artibeus jamaicensis*	2005	–	PA	KM406884	–
RNMO56	bat	*Trachops cirrhosus*	2012	Angicos/Rio Grande do Norte	BR	KT368795	–
RNMO63	bat	*Trachops cirrhosus*	2012	Angicos/Rio Grande do Norte	BR	KT368796	–
T. sp Neot 2							
082_AJ_Bohio_2	bat	*Artibeus jamaicensis*	2005	–	PA	KM406907	–
092_AJ_Bohio	bat	*Artibeus jamaicensis*	2005	–	PA	KM406881	–
173_AJ_Gigante	bat	*Artibeus jamaicensis*	2005	–	PA	KM406883	–
196_AJ_PenaBlanc-a	bat	*Artibeus jamaicensis*	2005	–	PA	KM406882	–
275_AJ_Leon	bat	*Artibeus jamaicensis*	2005	–	PA	KM406880	–
T. sp Neot 3							
070_AJ_Guanaban-o	bat	*Artibeus jamaicensis*	2005	–	PA	KM406897	–
109_AJ_Bohio	bat	*Artibeus jamaicensis*	2005	–	PA	KM406879	–
121_AJ_Cacao	bat	*Artibeus jamaicensis*	2005	–	PA	KM406876	–
240_AJ_Leon	bat	*Artibeus jamaicensis*	2005	–	PA	KM406875	–
268_AJ_Leon	bat	*Artibeus jamaicensis*	2005	–	PA	KM406878	–
269_AJ_Leon	bat	*Artibeus jamaicensis*	2005	–	PA	KM406874	–
282_AJ_Leon	bat	*Artibeus jamaicensis*	2005	–	PA	KM406877	–
BACO44	bat	*Artibeus lituratus*	2014	Boyacá	CO	KT368797	KT368800
BACO46	bat	*Artibeus lituratus*	2014	Boyacá	CO	KT368798	KT368801
T. sp bat							
TCC 60	bat	Rousettus aegyptiacus	1997	–	GA	AJ012418	GQ140365

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Table 1 (*continued*)

Trypanosoma sp isolates	Host Origin		Year	Geographic Origin		GenBank Acession number	
						SSU rRNA	gGAPDH
T. conorhini							
TCC25e	rodent	*Rattus rattus*	1947	–	BR	AJ012411	AJ620267
T. vespertilionis							
P14	bat	Pipistrellus pipistrellus	1972	–	UK	AJ009166	AJ620283
T. rangeli							
TCC 643	bat	Platyrrhinus lineatus	2003	Mato Grosso do Sul	BR	FJ900242	GQ140364
TCC 1719	bat	Artibeus planirostris	2005	Mato Grosso do Sul	BR	EU867813	KT368802
RGB	dog	*Canis familiaris*	1949	–	CO	AJ009160	AF053742
AM80	human	*Homo sapiens*	1996	Amazonas	BR	AY491766	JN040973
SC58	rodent	Echimys dasythrix	–	Santa Catarina	BR	AY230233	KT368804
PG	human	*Homo sapiens*	–	Panama	PA	AJ012416	KT368805
San Agustin	human	*Homo sapiens*	–	–	CO	AJ012417	KT368806
TCC 261	human	*Homo sapiens*	–	Rio Negro/Amazonas	BR	AY491758	KT368807
TCC 328	human	*Homo sapiens*	–	–	SV	AY491738	KT368808
900	triatomine	Rhodnius pictipes	–	Manaus/Amazonas	BR	KT368799	KT368803
T. dionisii							
TCC 211	bat	*Eptesicus brasiliensis*	2000	São Paulo	BR	FJ001666	GQ140362
TCC 495	bat	*Carollia perspicillata*	2002	Amazonas	BR	FJ001667	GQ140363
P3	bat	*Pipistrellus pipistrellus*	1971	–	UK	AJ009151	AJ620271
x842		Nyctalus noctula	2006	–	UK	FN599058	FN599055
T. erneyi							
TCC 1293	bat	Tadarida sp.	2006	Chupanga	MZ	JN040987	JN040964
TCC 1946	bat	Mopys condylurus	2009	Chupanga	MZ	JN040989	JN040969
T. c. marinkellei							
B7	bat	*Phyllostomus discolor*	1974	Bahia	BR	AJ009150	AJ620270
TCC 344	bat	*Carollia perspicillata*	2001	Monte Negro/Rondonia	BR	FJ001664	GQ140360
TCC 501	bat	*Carollia perspicillata*	2002	Porto Velho/Rondonia	BR	FJ001665	GQ140361
T. cruzi							
TCC 1122	bat	Myotis albescens	2004	São Paulo	BR	FJ001628	GQ140359
TCC 1994	bat	Myotis levis	2004	São Paulo	BR	FJ900241	GQ140358
TCC507	bat	*Carollia perspicillata*	2002	Amazonas	BR	FJ900240	GQ140352
G	opossum	*Didelphis marsupialis*	1983	Amazonas	BR	AF239981	GQ140351
Y	human	*Homo sapiens*	1953	São Paulo	BR	AF301912	GQ140353
MT3663	triatomine	Panstrongylus geniculatus	-	Amazonas	BR	AF288660	JN040971
MT3869	human	*Homo sapiens*	–	Amazonas	BR	AF303660	GQ140355
Others Trypanosomes							
T. sp HochNdi1	monkey	*Cercopithecus nictitans*	2004	–	CM	FM202493	FM164794
T. sp NanDoum1	palm civet	Nandinia binotata	2004	–	CM	FM202492	FM164793
T. sp H25	kangaroo	*Macropus giganteus*	1997	–	AU	AJ009168	AJ620276
T. sp G8	woylie	*Bettongia penicillata*	2013	–	AU	KC753537	KC812988
T. sp BDA1	woylie	Bettongia lesueur	2009	–	AU	FJ823108	–
T. sp D15	possum	*Trichosurus vulpecula*	2009	–	AU	JN315381	JN315395
T. sp D17	possum	*Trichosurus vulpecula*	2009	–	AU	JN315382	JN315396
T. sp D64	possum	*Trichosurus vulpecula*	2009	–	AU	JN315383	JN315397
T. sp BRA2	rodent	Rattus fuscipes	2007	–	AU	FJ823117	–

GenBank accession number of gene sequences characterized in this study are indicated in bold.

BR, Brazil; GY, Guyana; GT, Guatemala; SR, Suriname; PA, Panamá; MZ, Mozambique; CO, Colombia; UK, United Kingdom; GA, Gabon; BE, Belgium; CM, Cameroon; AU, Australia; VE, Venezuela; SV, El Salvador.

present all over the world excepting Antarctic and Arctic. However, only three species of bats, all belonging to the Neotropical Phyllostomidae family, are obligate blood-feeding bats: *Desmodus rotundus, Diphylla ecaudata,* and *Diaemus youngi.* While the two last species feed preferentially on birds, *D. rotundus* feeds primarily on mammalian blood, especially horses and cattle, and occasionally feed on humans. *D. rotundus* is known as a common vampire bat and is widespread in Latin America, from northern Mexico to Uruguay and Argentina (Hayes and Piaggio, 2018). This species inhabits burrows, moist caves, bridges, and many man-made structures, being commonly found in anthropogenic habitats. Bats of Phyllostomidae, Molossidae, and Vespertilionidae are reservoirs of rabies virus, an agent of lethal diseases for human and domestic animals. *D. rotundus* is extensively studied owing to its importance as a reservoir and source of rabies virus (Johnson et al., 2014).

There are many reports of trypanosomes infecting *D. rotundus.* Several microscopical surveys and experimental infection in mice (Hoare, 1972; Marinkelle, 1976), and molecular studies (Ramírez et al., 2014; Pinto et al., 2015; Argibay et al., 2016; Da Costa et al., 2016; Orozco et al., 2016) have detected *T. cruzi* in *D. rotundus* captured in Brazil, Colombia, Argentina, and Ecuador. *Trypanosoma cruzi marinkellei* and *T dionisii,* which are phylogenetically closely related to *T. cruzi,* were also identified in *D. rotundus* from Brazil (Cavazzana et al., 2010; Lima et al., 2015b; Lourenço et al., 2018; Pegorari et al., 2018), and Argentina (Argibay et al., 2016). In addition, *D. rotundus* also harbors *T. rangeli* in Brazil, Colombia, and Ecuador (Ramírez et al., 2014; Pinto et al., 2015; Lourenço et al., 2018). *Trypanosoma evansi* was detected in *D. rotundus* from Colombia, Panama, and Brazil (Hoare, 1972; Ramírez et al., 2014), and *Trypanosoma pessoai* was detected in *D. rotundus* captured in Brazil (Deane and Sugay, 1963; Marinkelle, 1976; Molyneux, 1991; Vilar et al., 2004). Recently, *Leishmania infantum, L. amazonensis* and *L. braziliensis* were detected by PCR in *D. rotundus* in Brazil (De Oliveira et al., 2015; Gómez-Hernández et al., 2017).

A range of hematophagous vectors can transmit trypanosomes among bats. The triatomines are vectors of *T. cruzi* and *T. rangeli,* cimicids transmit *T. dionisii* and *T. vespertilionis,* and sand flies were incriminated as vectors of *T. pessoai* (Zeledón and Rosabal, 1969; Deane et al., 1978; Bower and Woo, 1982; Gardner and Molyneux, 1988; Espinosa-Álvarez et al., 2018). However, vectors are unknown for many of the bat trypanosomes. The epidemiological and ecological data suggest that many arthropods cyclically or mechanically transmit trypanosomes to bats (Marinkelle, 1976; Molyneux, 1991; Cavazzana et al., 2010; Lima et al., 2012, 2013, 2015a; Barbosa et al., 2016; Dario et al., 2017a,b; Espinosa-Álvarez et al., 2018).

Therefore, Neotropical bats are hosts for a large and underestimated diversity of trypanosomes that have been unraveled using molecular phylogenetic approaches. The current knowledge on the genetic diversity of trypanosomes infecting hematophagous bats suggests a great diversity of trypanosomes in *D. rotundus* (Barros et al., 2008; Cavazzana et al., 2010; Ramírez et al., 2014; Pinto et al., 2015; Argibay et al., 2016; Orozco et al., 2016), and *Diphylla ecaudata* (Cavazzana et al., 2010; Lourenço et al., 2018). In a previous study, we surveyed for trypanosomes in 78 *D. rotundus* captured in southeastern Brazil (Rio de Janeiro) by hemoculturing (Barros et al., 2008).

In the present study, 14 cryopreserved cultures of trypanosomes from *D. rotundus* (Barros et al., 2008) were characterized based on their morphological and developmental features in culture, and their positioning in the *Trypanosoma* phylogenetic tree have enabled their description as a novel species of bat trypanosome.

2. Materials and methods

2.1. Culture and light microscopy of bat trypanosomes

Fourteen trypanosome cultures were characterized in the present study, all of which were obtained by hemoculturing from 78 specimens (21 positive for trypanosomes by hemoculture) of *D. rotundus* captured in the Municipal Districts of Miracema, Paraty, Maricá, Niterói, and Laje do Muriaé in the State of Rio de Janeiro (RJ) (Barros et al., 2008). Hemocultures were performed in tubes containing a biphasic medium (NNN/Schneider's) supplemented with 10% fetal calf serum (FCS), with incubation at 28 °C. All 21 cultures obtained are deposited at the cryobank of the Laboratório de Vigilância em Leishmanioses, Instituto Nacional de Infectologia, Fiocruz, RJ (Table 1). The study was approved by the Animal Ethics Committee User (CEUA-FIOCRUZ), approved protocol No: L-051/08.

Light microscopy was performed on trypanosomes at 3, 7, 10, and 14 days of culture. Smears in glass slides were stained by Giemsa, and photomicrographs obtained using the Motic Image Plus 2.0 Software. The measurements (µm) were taken from 20 epimastigote forms of log-phase culture.

2.2. PCR amplification and phylogenetic analyses of SSU rRNA and gGAPDH genes

The cultured epimastigotes of bat trypanosomes were harvested by centrifugation, washed in sterile PBS, and DNA was extracted using DNAzol (Invitrogen). The sequences of the variable V7V8 region (~800 bp) of small subunit of ribosomal gene - *SSU rRNA* (~800 bp from cultured trypanosomes and ~560 bp from archived blood samples), and glycosomal glyceraldehyde-3-phosphate dehydrogenase (*gGAPDH*) (~800 bp) gene were obtained and amplified using oligonucleotides and reaction conditions described previously (Borghesan et al., 2013; Noyes et al., 1999). All the amplified nucleotide sequences were determined using an automatic sequencer (3730 DNA Analyzer, Applied Biosystems), submitted to BLAST searches, aligned using Clustal X (Thompson et al., 1997), and the resulting alignments manually refined. We created two alignments for phylogenetic inferences, one including V7V8 *SSU* rDNA sequences, and a second alignment consisted of concatenated sequences of V7V8 *SSU rDNA* and *gGAPDH* (~1690 bp) from all trypanosomes of the *T. cruzi* clade available in Genbank using *Trypanosoma lewisi* as outgroup. The trypanosomes included in the phylogenetic analyses, their respective host species and geographical origin, and GenBank accession numbers are shown in Table 1.

Fig. 1. Geographical origin of *Trypanosoma rotundus* n. sp. isolates obtained by hemoculturing and archived blood samples from *Desmodus rotundus* captured in the following Brazilian states: PA, Pará; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo and SC, Santa Catarina.
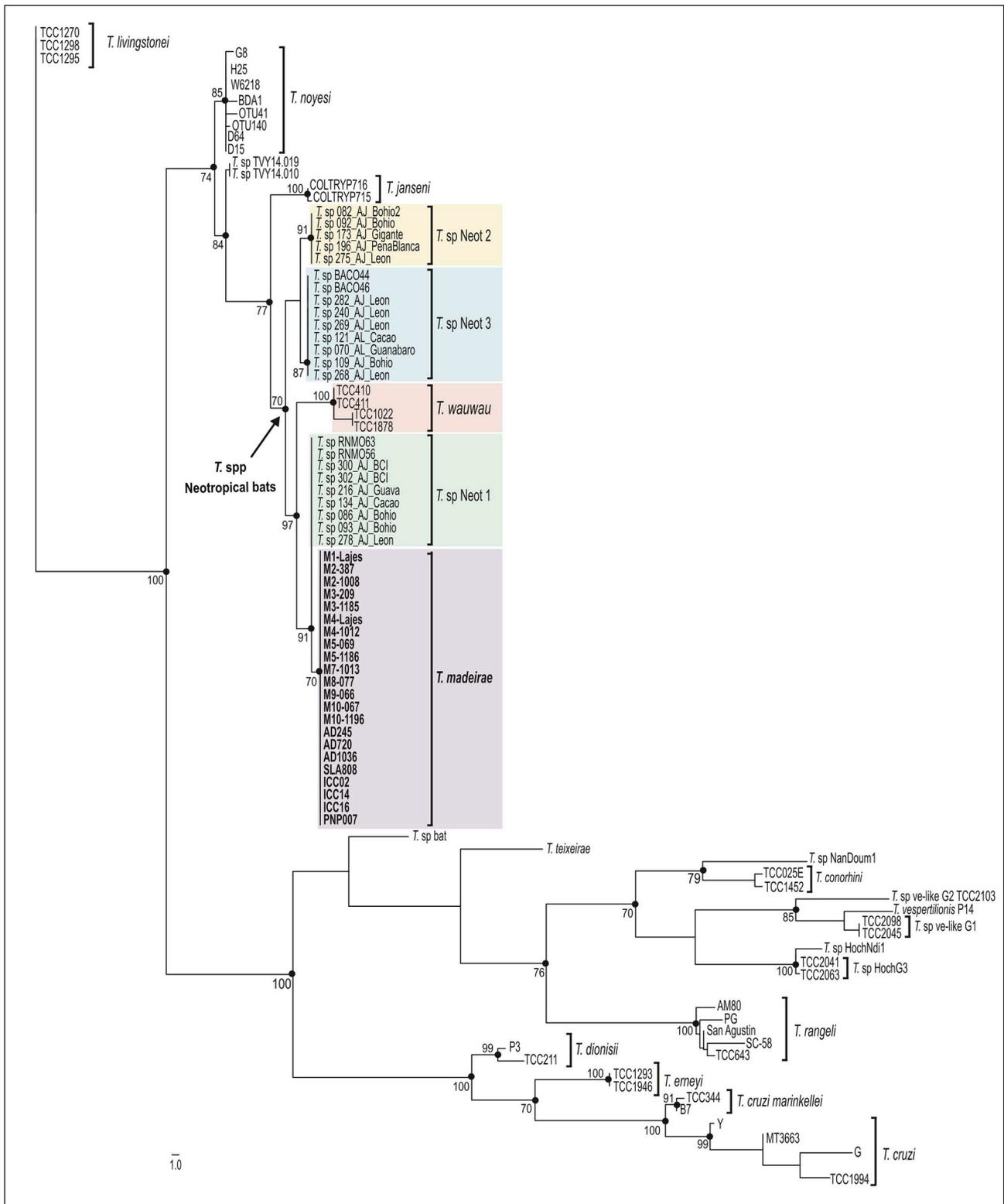
Fig. 2. Barcoding (V7-V8 SSU rRNA sequences) of *T. rotundus* from cultures and bat blood samples, and its related species of the clade *T. cruzi*. Phylogenetic tree inferred by Parsimony using 93 (~800 bp) of V7-V8 SSU rRNA sequences. The node numbers are bootstrap values derived from 500 replicates.

Phylogenies were inferred using parsimony (P), maximum likelihood (ML) and Bayesian (BI) inferences as previously described (Lima et al., 2012, 2013). 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). ML analyses were performed using RAxML v.2.2.3 (Stamatakis, 2006). The tree searches were performed with GTRGAMMA, with 500 maximum parsimony starting trees. The model parameters were estimated in RAxML for the duration of the tree search. The nodal support was estimated with 500 bootstrap replicates in RAxML using GTRGAMMA and maximum parsimony starting trees. MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) was used for BI inferences.
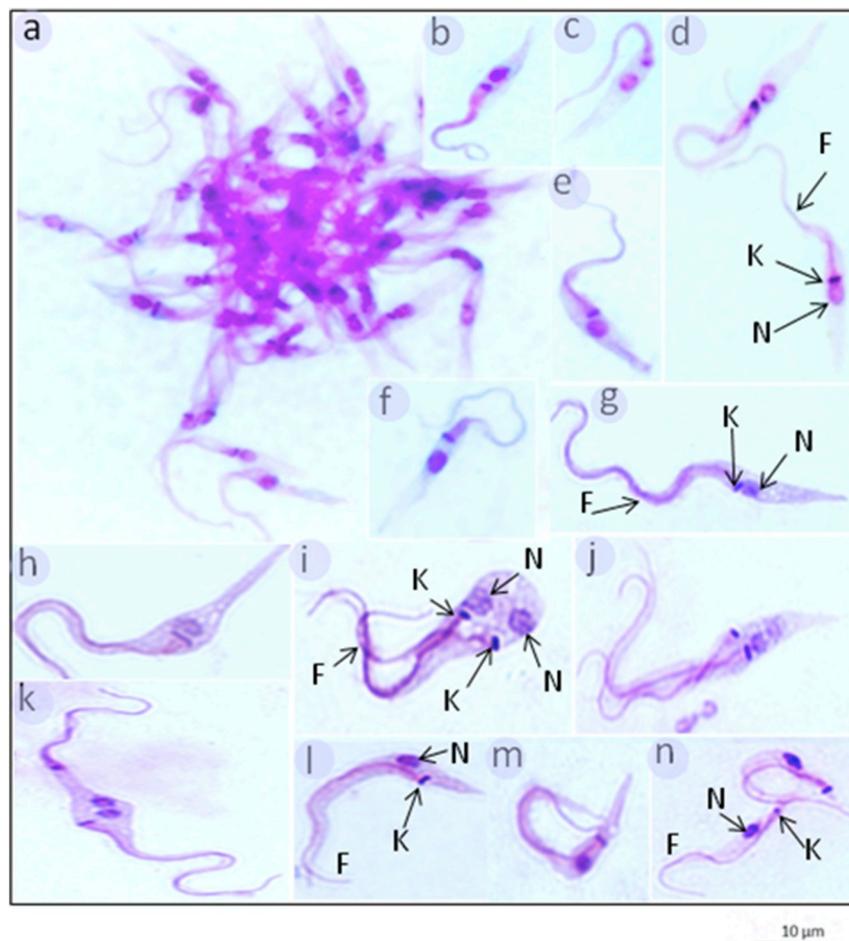
Fig. 3. Phylogenetic positioning of *T. rotundus* in the clade *T. cruzi*. ML phylogenetic analysis based on the concatenated sequences of V7V8 SSU rRNA and gGAPDH genes (1.690 characters, –Ln = 8768.346166) from ten isolates of *T. rotundus*, other 29 bat trypanosomes, and 21 trypanosomes from other mammals. *T. lewisi* was used as outgroup. The numbers at the nodes correspond respectively to P, ML (500 replicates) and BI support values.

Fig. 4. Photomicrographs illustrative of the morphological diversity of culture forms of *T. madeirae* (isolate M3-209). (a) rosetes of epimastigotes, (b-d) flagellates resembling promastigotes forms, (d-h) epimastigotes (7 days), (i-k) large epimastigote forms under division, (l-m), large trypomastigotes, and (n) slender trypomastigotes (10 days). Giemsa stained. 1000x. K, kinetoplast, N, nucleus, F, flagellum. The scale bar indicates 10 μm.

2.3. Survey of trypanosomes in bat blood samples by nested PCR of SSU rRNA and sequencing

Blood/liver samples of *D. rotundus* captured at the Brazilian states of São Paulo (05 bats), Minas Gerais (01), Espírito Santo (18), Santa Catarina (03) and Pará (25), all preserved in ethanol (v/v) at the Trypanosomatid Collection of the Department of Parasitology, University of São Paulo, Brazil, were used for DNA preparation as described previously (Garcia et al., 2017). DNA samples were used for nested PCR of *SSU rRNA* (~560 bp from archived blood samples) (Noyes et al., 1999), and amplified DNA fragments sequenced as described above. All sequences determined from these samples were included in the alignment of V7V8 *SSU rRNA* employed for the phylogenetic analysis described above.

2.4. Mouse macrophages and triatomine infection

Since all samples of *T. madeirae* shown up identical molecular profile, we selected four 4 samples (M066, M067, M069, M209) for biological characterization.

Macrophages were obtained by washing the peritoneal cavity of Swiss Webster mice, plated (3×10^5 macrophages/well) in chamber slides (Lab-Tec°, Nalgene Nunc International), with RPMI medium, supplemented with 10% of fetal calf serum and incubated at 37 °C in a humidified 5% CO_2 atmosphere.

After 24 h, 6×10^5 trypanosomes from stationary cultures (10th day) were seeded to macrophage monolayer. After 2 h, non-internalized parasites were removed by washing with phosphate buffered solution (PBS) pH 7.2 (37 °C) and the culture medium (RPMI) was renewed. The cell infection was assessed after 3, 24, 48 and 72 h of incubation. At each time point, the slides were washed with phosphate buffered solution (PBS) pH 7.2 (37 °C), fixed with methanol, and stained with Giemsa. A total of 100 random macrophages at each time point were examined under an optical microscope as reported previously (Madeira et al., 2009).

Nymphs of 4th and 5th instars of *Triatoma infestans,* kindly provided by the Triatomine Collection of Fiocruz, were employed to assess the ability of new bat trypanosomes for infecting triatomines. Infection was assessed by artificial xenodiagnoses method using rabbit erythrocytes mixed (v/v) with bat trypanosome cultures incubated at 37 °C to feed the triatomines according Garcia et al. (1975). About forty triatomines were fed with each culture, and at 15, 30, and 45 days post-feeding ten triatomines were dissected, and the presence of trypanosomes in the digestive tube, hemolymph and salivary glands were microscopically investigated at each time point.

2.5. Lyses of trypanosome mediated by human complement system

The epimastigote forms of selected isolates (M066, M067, M069, M209) were investigated for their resistance to lysis mediated by the components of the complement system present in fresh human sera following the protocol described in Steindel et al. (1998). For the negative control of complement mediated lysis, parasites were incubated with inactivated human serum. In addition, *T. cruzi* (Y strain) and *T.*

rangeli (SC-58) epimastigotes were respectively used as positive and negative controls. Parasite lysis was assessed in triplicates using a Neubauer chamber.

3. Results

3.1. Barcoding and phylogenetic positioning of new bat trypanosomes

The barcoding of trypanosomes using V7V8 *SSU rRNA* sequence has been enough to identify new species and many genotypes of bat trypanosomes, and valuable for preliminary inferences of taxonomic affiliations (Lima et al., 2012, 2013; 2015a; Espinosa-Álvarez et al., 2018). Here, we determined *SSU rRNA* sequences of trypanosomes from *D. rotundus* obtained from 14 cultures and directly from DNA obtained from blood samples of bats. The barcode sequences of these trypanosomes were submitted to BLAST analysis and aligned with sequences of trypanosomes of the clade *T. cruzi* available in Genbank, and then phylogenetic inferences were carried out by parsimony analysis. The branching pattern of the inferred dendrogram corroborated with previously known clades within the clade *T. cruzi* (Lima et al., 2012, 2013; 2015b; Hamilton et al., 2012; Pinto et al., 2012; Cottontail et al., 2014; Botero et al., 2016; Dario et al., 2017a; Espinosa-Álvarez et al., 2018; Lopes et al., 2018) and, in addition, revealed a new clade formed exclusively by sequences from trypanosomes isolated from *D. rotundus* (Fig. 2). This clade corresponds to a novel species herein named as *T. madeirae* n. sp., supported by its phylogenetic positioning and the degree of both *SSU rRNA* and gGAPDH sequence divergences from known species.

The analysis of V7V8 *SSU rRNA* sequences of the archived blood samples of *D. rotundus* corroborated the homogeneity of *T. madeirae* n. sp. isolates regardless of the wide geographical origin of vampire bats examined in this study (Fig. 1; Table 1). The sequences of V7V8 *SSU rRNA* of isolates of *T. rotundus* n. sp. were separated by smallest divergence (~0.4%) from those of trypanosomes nested in the clade *T.* sp Neot 1 (Fig. 2). This clade harbored trypanosomes from *Artibeus jamaicensis* from Panamá (Cottontail et al., 2014) and *Trachops cirrhosus* from Brazil (Lima et al., 2015a), but so far no isolate from *D. rotundus*. In contrast, the clades *T.* sp Neot 2 and *T.* sp Neot 3 diverged by large genetic distances from *T. madeirae* n. sp.: ~4.0% and ~3.0% of V7V8 *SSU rRNA* sequence divergence, respectively.

Phylogenetic analyses (ML, P and Bayes) of the clade *T. cruzi* using concatenated *SSU rRNA* and *gGAPDH* sequences are well-resolved (high support values). In these analyses, the isolates of *T. madeirae* n. sp. formed a strongly supported clade within the Neobat lineage (Fig. 3). Despite high *SSU rRNA* sequence conservation among species of trypanosomes of the clade *T. cruzi*, relevant degree of divergence of sequences of more polymorphic *gGAPDH* genes have been supported the identification of many species of trypanosomes within this major clade. In our analyses, *gGAPDH* sequence divergences of 10% separated *T. madeirae* n. sp. from *T. wauwau,* which was, until the present study, the only named species of trypanosome within the Neobat lineage (Fig. 3).

3.2. Morphological and biological features of bat trypanosomes

The morphology of trypanosomes cultivated in NNN overlaid by Schneider's medium was evaluated at both log- and stationary phase cultures. All the isolates examined exhibited highly similar morphological features. In early cultures (3-5th days) predominated rosettes of dividing flagellates (Fig. 4a) that progressively detached (7th day), and the free-swimming forms resembling both promastigotes (Fig. 4 b-d) and epimastigotes (Fig. 4 e-g). In these forms, the rod-shaped kinetoplast was located either very close (Fig. 4 d, e, g) or distant (Fig. 4 d, f) to the nucleus, in the anterior region of the parasites. Epimastigote (20 flagellates) of log-phase cultures (Fig. 4 e, f) were measured for taxonomical purpose: the body length average was 2.45 ± 0.32 μm (2.0–3.,0 μm), width was 1.94 ± 0.29 μm (1.48–2.22 μm), and the

length of free flagellum averaged 2.63 ± 0.70 μm (1.8–3.5 μm). These forms evolved to long and wide epimastigotes lacking noticeable undulating membrane (Fig. 4 g,h), which can multiply by binary fission (Fig. 4 h—k). After 10 days of culturing, a few transient forms with posterior kinetoplast could be observed (Fig. 4 l,m). Then, small and fusiform trypomastigotes exhibiting discreet undulating membrane and terminal kinetoplast were detected in the end of stationary cultures (14th day) (Fig. 4 n). These trypomastigote forms (20 flagellates) of stationary-phase cultures were also measured: the body length average was 18.75 ± 1.76 μm (17.5–20.0 μm), width was 1.75 ± 0.35 μm (1.5–2.5 μm), and the length of free flagellum averaged 6.25 ± 1.76 μm (5.0–7.5 μm).

3.3. Trypanosoma madeirae n. sp does not develop within mammalian cells in vitro, is lysed by complement of fresh human sera, and likely is not infective for triatomine bugs

The potential ability of trypanosomes in invading and developing within mammalian cells is valuable as a complementary information in the description of new species as showed previously for bat trypanosomes developing (Cavazzana et al., 2010; Lima et al., 2012, 2015b) or not (Lima et al., 2013, 2015a) inside mammal cells. Here, we evaluated the ability of *T. madeirae* to invade and develop in mouse macrophages. In general, after 3 h of incubation, the parasites adhered to the cell surface, and after 24–48 h rounded internalized forms resembling amastigotes could be observed. However, after 72 h, the macrophages did not exhibit intact parasites, and many vacuoles were present in the cytoplasm. Therefore, none of the four isolates of *T. madeirae* n. sp developed within mouse macrophages cultivated at 37 °C. Our finding is consistent with the phylogenetic positioning of this species closely related to other bat trypanosomes unable to survive and multiply inside cells (Lima et al., 2015a; Espinosa-Álvarez et al., 2018). Also, we demonstrated that epimastigotes of *T. madeirae* are lysed by the human complement system.

All nymphs of *T. infestans* fed with rabbit blood cells mixed with cultures of *T. madeirae* were totally free of flagellates in the digestive tube, hemolymph, and salivary glands. No flagellate was observed on the 15th day after feeding, indicating that *T. madeirae* was quickly destroyed in the digestive tube.

3.4. Biogeographical analysis supports relevant association between Trypanosoma madeirae n. sp and Desmodus rotundus

Trypanosoma madeirae was detected in bats captured in the same site (Miracema) in 2004 and 2007, suggesting that the infection is well established in bat colonies, and long-term infections in *D. rotundus*. However, *T. madeirae* was not found in any other bat species sharing capture sites with *D. rotundus* in Rio de Janeiro: *Lonchorhina aurita* (3 bats), *Artibeus cinereus* (2 bats), *Glossophaga sorcina* (1 bat), *Carollia perspicillata* (1 bat), and even the hematophagous *Diaemus youngii* (1 bat).

Aiming to assess some links between *T. madeirae* and *D. rotundus,* trypanosomes were surveyed by nested PCR (V7V8 SSU rRNA) in 52 archived blood samples from *D. rotundus* captured in five Brazilian states: Pará (PA), Minas Gerais (MG), São Paulo (SP), Santa Catarina (SC), and Espírito Santo (ES). The results revealed *T. madeirae* in 8 bats from all states indicating the presence of this species in Amazonia, Cerrado, and Atlantic Forest biomes, and thus its wide geographical distribution (Table 1, Fig. 1). Notably, despite huge geographical distance between the states of PA, northern Brazil (Amazonia) and SC (southern Brazil), identical *SSU rRNA* barcodes supported both the high genetic homogeneity of *T. madeirae,* and a putative link of this species of trypanosome with *D. rotundus* (Fig. 2).

4. Taxonomic section

Taxonomic summary: Phylum Euglenozoa Cavalier-Smith, 1981; Class Kinetoplastea Honigberg, 1963; Order Trypanosomatida Hollande, 1952; Family Trypanosomatidae Doflein, 1951; Genus *Trypanosoma* Gruby, 1843.

Species Name: *Trypanosoma madeirae* sp. n.

Type material: Hapantotype, the culture of the isolate M3-290. Paratypes, the cultures of the isolates M1-Lajes, M3-1185, M4-Lajes, M5-1186, M10–1196, M2-387, M2-1008, M3-209, M4-1012, M5-069, M7-1013, M8-077,M9-066 and M10-067. All cultures are cryopreserved at the Laboratório de Vigilância em Leishmanioses in the Instituto Nacional de Infectologia/FIOCRUZ.

Mammalian host: Chiroptera, Phyllostomidae, Desmodontinae, *Demodus rotundus*.

Additional host: Unknown.

Locality: state of Rio de Janeiro, Brazil.

Additional localities in Brazil: States of Pará, Minas Gerais, Espírito Santo, São Paulo and Santa Catarina.

Morphology: Epimastigotes averaged 5.0 μm of body length, 1.92 of body width, and 2.63 of free flagellum. Flagellates with a near central nucleus, small kinetoplast, and under-developed undulating membrane.

Species diagnosis: DNA sequences to *T. madeirae* deposited in GenBank accession numbers: *SSU rRNA* (MK064121-MK064143) and *gGAPDH* (MK064144-MK064152).

Etymology: The species is named *T. madeirae* sp. n. in honour of Dra. Maria de Fatima Madeira, from the Oswaldo Cruz Foundation, RJ, who greatly contributed to studies on trypanosomatid biology, including *Leishmania* spp. and *Trypanosoma caninum*.

5. Discussion

5.1. Phylogenetic positioning of Trypanosoma madeirae n. sp

In the present study, we described *Trypanosoma madeirae* n. sp isolated from the hematophagous bat *D. rotundus* captured in the Atlantic Forest biome, Rio de Janeiro, Southeast Brazil. To date, this species was identified only in *D. rotundus* species. Altogether, phylogenetic inferences and degrees of sequence divergences based on V7V8 *SSU rRNA* and *gGAPDH* sequences strongly support *T. madeirae* placed within the *T. cruzi* clade (Figs. 2 and 3), similar to most bat trypanosomes described to date. Thus, our findings provide additional support to the 'bat-seeding' hypothesis for the origin of the species of this clade (Hamilton et al., 2012). *Trypanosoma madeirae* n. sp. clustered with other trypanosomes from phylostomid bats in the Neobat phylogenetic lineage, which comprises closely related trypanosomes distributed in the clades Neotropic 1, 2, and 3. Each clade is formed by sequences obtained from bat blood samples representing a single species waiting for a formal taxonomic description. *Trypanosoma madeirae* is very closely related to *T.* sp Neotropic 1, so far detected in *Trachops cirrhosus* and *Artibeus jamaicensis* (phyllostomids) in Brazil and Panama. The high similarity of *SSU rRNA* sequences shared by both *T. madeirae* and *T.* sp Neot 1 suggests that these two trypanosomes likely represent very closely related species or different genotypes of *T. madeirae,* but an answer to this question requires comparative analyses using the more polymorphic *gGAPDH* sequences. However, *T. madeirae* n. sp. was clearly separated from *T. wauwau,* the only named species of trypanosome within the Neobat lineage (Figs. 2 and 3).

The Neobat lineage also harbors *T. wauwau* (Lima et al., 2015a), the only species of this lineage obtained in culture and formally described before *T. madeirae.* The trypanosomes positioned basal to this lineage were *T. janseni* from a Neotropical marsupial (Lopes et al., 2018), *T. noyesii* from Australian rodents and marsupials (Hamilton et al., 2012b; Botero et al., 2016), and one unnamed trypanosome of lemurs from Madagascar known just by a small DNA sequence (Larsen et al., 2016)

(Figs. 2 and 3).

In addition to *T. madeirae, T. cruzi, T. c. marinkellei, T. dionisii, T. rangeli,* and *Trypansoma* spp Neot 1, 2, and 3 have been molecularly identified in *D. rotundus* (Brazil and Venezuela). However, differing from *T. madeirae*, which was so far detected exclusively in *D. rotundus,* these trypanosomes have been detected in a range of bat species (Cavazzana et al., 2010; Cottontail et al., 2014; Ramírez et al., 2014; Pinto et al., 2015). Unfortunately, *T. pessoai,* a species previously reported in *D. rotundus* in Brazil (Deane and Sugay, 1963; Deane et al., 1978; Molyneux, 1991; Vilar et al., 2004), is not available for molecular comparison with *T. madeirae.*

5.2. Morphological and biological characterization of T. madeirae n. sp

The flagellates from log- and stationary-phase cultures were examined by light morphology. The typical epimastigote forms of *T. madeirae* at log-phase cultures were slender flagellates with a near central nucleus, small lateral kinetoplast, and an under-developed undulating membrane. Both epimatigotes and trypomastigotes of *T. madeirae* differ from those of *T. cruzi, T. dionisii,* and *T. rangeli* (Maia da Silva et al., 2009; Lima et al., 2012).

By taking into account its phylogenetic positioning in the Neobat lineage, we compared behavioral and morphological features of *T. madeirae* with those described for *T. wauwau,* the only closely related species that are available in culture and was previously isolated in culture and morphologically characterized. Both epi- and trypomastigote cultured forms of *T. wauwau* markedly differ from those observed in cultures of *T. madeirae* (Lima et al., 2015a). We demonstrated that *T. madeirae* can survive at 37 °C and enter murine macrophagic cells, probably internalized by phagocytosis, but it is unable to survive inside these cells. Similar behavior was observed in the closely related *T. wauwau* using monolayers of monkey LLC-MK2 cells (Lima et al., 2015a). In contrast, all bat trypanosomes of the subgenus *Schizotrypanum*, such as *T. cruzi, T. dionisii,* and *T. erneyi,* invade, differentiate and replicate within macrophages, LLC-MK2, and other mammalian cells (Baker et al., 1971; Cavazzana et al., 2010; Lima et al., 2012; Maeda et al., 2012; Espinosa-Álvarez et al., 2018).

The complement system, a key component of innate immunity, plays a very important role as the first line of defense against trypanosomes (Lidani et al., 2017). The epimastigotes of *T. madeirae* are susceptible to lysis by human complement system, similar to epimastigotes of *T. cruzi, Trypanosoma desterrensis* and *T. dionisii,* which are both species of the subgenus *Schizotrypanum,* whereas epimastigotes of *T. rangeli* are not lysed when incubated with fresh human sera (Schottelius et al., 1986; Steindel et al., 1998; Maeda et al., 2012). Is it well known that when describing new *Trypanosoma* species it is very challenging to find the right culture media to grow all stages, especially to induce transformation and growth of metacyclic trypomastigotes. Although we have been done some attempts to enhance metacyclic forms in cultures, *T. madeirae* always shows up low percentage of typical trypomastigote forms. These results may have influenced either macrophage infection rates and/or survival inside cell. It is important to consider that metacyclic trypomastigotes of *T. madeirae,* which were scarce even in stationary cultures, may exhibit differences regarding susceptibility to the human complement system. Differing from the complement-resistant metacyclic trypomastigotes of *T. cruzi,* metacyclic trypomastigotes of *T. dionisii* are susceptible to complement-mediated lysis (Maeda et al., 2012).

Previous studies showed that most cultured trypanosomes of the clade *T. cruzi* did not develop in triatomine bugs (Cavazzana et al., 2010; Lima et al., 2012, 2013, 2015b) *T. cruzi* and *T. rangeli* are so far the only species unquestionably cyclically transmitted by triatomines. Here, many attempts of infecting *T. infestans* with *T. madeirae* failed; the flagellates were completely destroyed in the digestive tract of the bugs after ~15 days. Previous efforts of obtaining established experimental infection of *Triatoma*, *Rhodnius* and *Panstrongylus* species with *T. c.*

marinkellei, T. dionisii, T. erneyi, and *T. cruzi* of the genotype TcBat have all failed, despite the ability of all these species to survive for many days in the digestive tract of the triatomines (Cavazzana et al., 2010; Lima et al., 2012, 2015b). Recently, *T. c. marinkellei* and *T. dionisii* were detected by PCR surveys in the digestive tract of *Triatoma viticipes* (Dario et al., 2017a,b), but colonization of the triatomine guts by these species was not demonstrated. In addition to the fact that *T. cruzi* is cyclicaly transmitted by a range of triatomines species, it was well-demonstrated that *T. dionisii* and *T. vespertilionis* are cyclically transmitted by cimicid bugs (Bower and Woo, 1982; Gardner e Molyneux, 1988; Espinosa-Álvarez et al., 2018). In addition, sand flies were incriminated as vectors of *T. pessoai* and *T. leonidasdeanei* to neotropical bats (Zeledón and Rosabal, 1969; Deane et al., 1978). The epidemiological and ecological data suggest that transmission of trypanosomes among bats should also occur through ingestion (during grooming) of their ectoparasites (flies, ticks, bugs, mites, and fleas) containing trypanosome infected blood meal (Cavazzana et al., 2010; Lima et al., 2012, 2013, 2015a; Barbosa et al., 2016; Dario et al., 2017a,b; Espinosa-Álvarez et al., 2018).

Therefore, vectors of *T. madeirae* and all other trypanosome species nested in the Neobat lineage are so far unknown. Many cave-dwelling hematophagous insects living together with *D. rotundus*, such as mosquitoes (Culicidae), sand flies (Phlebotominae), bat flies (Nycteribiidae and Streblidae), biting midges (Ceratopogonidae), bat bugs (cimicidae), fleas and ticks (Obame-Nkoghe et al., 2017), are all potential vector candidates. It is also tempting to speculate whether the transmission of *T. madeirae,* apparently specifically among *D. rotundus,* might be due to its social cooperative behavior of sharing blood meals that is regurgitated to feed starving bats (Wilkinson et al., 2016), thus allowing the transmission of this trypanosome specifically among bats of this species.

5.3. Phylogeography and host-parasite association

Notably, taking into account the large number of surveys of trypanosomes in Neotropical bats carried out using molecular methods, *T. madeirae* was exclusively found in *D. rotundus* (Phyllostomidae). This species was detected in 22 (14 cultures and 8 archived blood samples) out of 130 (78 captured in RJ and examined by hemoculturing and 52 from other regions screened by nested PCR) specimens of *D. rotundus* captured from Northern to Southern Brazil. The survey of trypanosomes in more than 1700 bats captured across South America (Cavazzana et al., 2010; Pinto et al., 2015; Lima et al., 2015a,b; Dario et al., 2017a,b; Dos Santos et al., 2017; Bento et al., 2018; Lourenço et al., 2018) did not reveal *T. madeirae* in more than 60 species of bats examined, even though most species examined belonged to Phyllostomidae.

Taken together, our findings support *T. madeirae* as a new species of trypanosome, so far exclusively found in *D. rotundus.* Vampire bats from wide geographical range (North to South) and distinct Brazilian biomes were found infected with isolates of *T. madeirae* sharing virtually identical V7V8 *SSU rRNA* barcodes. However, without experimental cross-infections, strict host-restriction of trypanosomes cannot be warranted to any trypanosome species, even though relevant data have suggested important degrees of association between some trypanosomes and their bat hosts. This study demonstrated that *T. madeirae* may be a species more linked to vampire bats among other trypanosome species that also infect *D. rotundus,* even those that also nested in the lineage Neobats. The Neobat lineage also harbors *T. wauwau,* a species linked to *Pteronotus* spp. (Mormoopidae) reported in large surveys of bats in many countries from Central and South America (from Amazon to the Atlantic Forest) (Lima et al., 2015b; Da Costa et al., 2016). Recently, *T. wauwau* was reported for the first time in one phyllostomid bat of the genus *Anoura* in Minas Gerais, Brazil (Pegorari et al., 2018). Previously, bats of *Anoura* captured in different biomes were found infected with *T. dionisii* (Cavazzana et al., 2010; Dario et al., 2017).

Supporting the link between *D. rotundus* and *T. madeirae,* other species of trypanosomes, *T. dionisii* and *T. wauwau,* were identified in bats sharing shelters with *D. rotundus* (Cavazzana et al., 2010; Lima et al., 2015). Interestingly, although vampire bats often shared shelters with other bat species, they generally hung separately (Delpietro et al., 2017). The frequent contact between blood meal of *D. rotundus* and wild and domestic animals (Johnson et al., 2014), and even humans may favor interspecific transmission of *T. madeirae.* Host switching appears to be a common process allowing for the expansion of host ranges of trypanosomes nested in *T. cruzi* clade, a process likely mediated by cimicid/triatomine vectors by which the generalist *T. cruzi* and *T. rangeli* most likely originated (Hamilton et al., 2012; Lima et al., 2012; Espinosa-Álvarez et al., 2018).

The lack of trypanosome geographical structure suggested a constant flow of bats carrying *T. madeirae.* This hypothesis is consistent with studies demonstrating that young males of *D. rotundus* systematically disperse to new colonies. Colonies of *D. rotundus,* with a longevity up to 16 years, can be large (> 300 bats) and in the absence of environmental disturbances adults spend most of their lifetime in the same or neighboring colonies, while young males migrate to more distant new colonies (Martins et al., 2009; Johnson et al., 2014). Successive dispersal of *D. rotundus* likely allowed for interchange and dispersion of their trypanosomes. The very interesting and apparent strong association of *T. madeirae* with *D. rotundus* must be further confirmed by more comprehensive surveys of trypanosomes from *D. rotundus,* other hematophagous bats, and bats of many other species and families, using more sensitive and effective methods suitable for unraveling the full repertoire of trypanosomes harbored by bats.

Declarations of interest

None.

Acknowledgments

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