IJP: Parasites and Wildlife 8 (2019) 71–81

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

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

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

Juliana H.S. Barros[a,*], Luciana Lima[b], Armando O. Schubach[c], Marta M.G. Teixeira[b]

[a] Trypanosomatid Biology Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil
[b] Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[c] Surveillance and Clinical Research in Leishmaniasis Laboratory, National of Infectology Evandro Chagas Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil

ARTICLE INFO

*Keywords:*
Chiroptera
Vampire bats
Bat trypanosomes
Phylogeny
Taxonomy
*T. cruzi* clade

ABSTRACT

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

* Corresponding author.
*E-mail addresses:* juliana.barros@ioc.fiocruz.br, julianabiorj@hotmail.com (J.H.S. Barros).

https://doi.org/10.1016/j.ijppaw.2018.12.009
Received 12 November 2018; Received in revised form 29 December 2018; Accepted 31 December 2018
2213-2244/ © 2019 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

**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 | | Host Origin | Year | Geographic Origin | | GenBank Acession number | |
|---|---|---|---|---|---|---|---|
| isolates | | | | | | 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_PenaBlanca | bat | *Artibeus jamaicensis* | 2005 | – | PA | KM406882 | – |
| 275_AJ_Leon | bat | *Artibeus jamaicensis* | 2005 | – | PA | KM406880 | – |
| **T. sp Neot 3** | | | | | | | |
| 070_AJ_Guanabano | 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 |

(*continued on next page*)

**Table 1** (*continued*)

| Trypanosoma sp | | Host Origin | Year | Geographic Origin | | GenBank Acession number | |
|---|---|---|---|---|---|---|---|
| isolates | | | | | | 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.

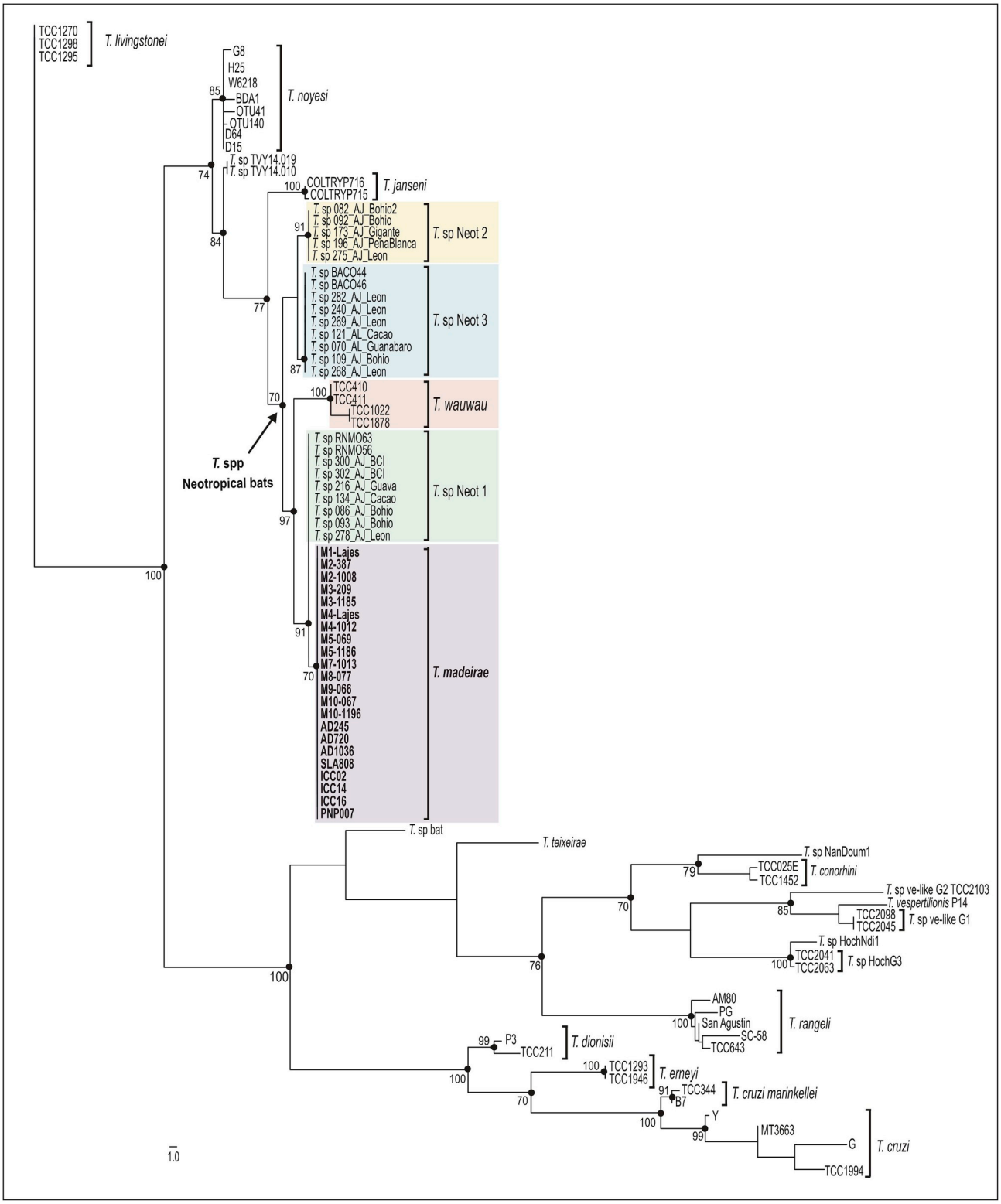


**Fig. 2.** Barcoding (V7-V8 SSU rRNA sequences) of *T. rotundus* from cultures and bat blood samples, and its related species of the clade *T. cruzi*. Phylogenetic tree inferred by Parsimony using 93 (~800 bp) of V7-V8 SSU rRNA sequences. The node numbers are bootstrap values derived from 500 replicates.

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.

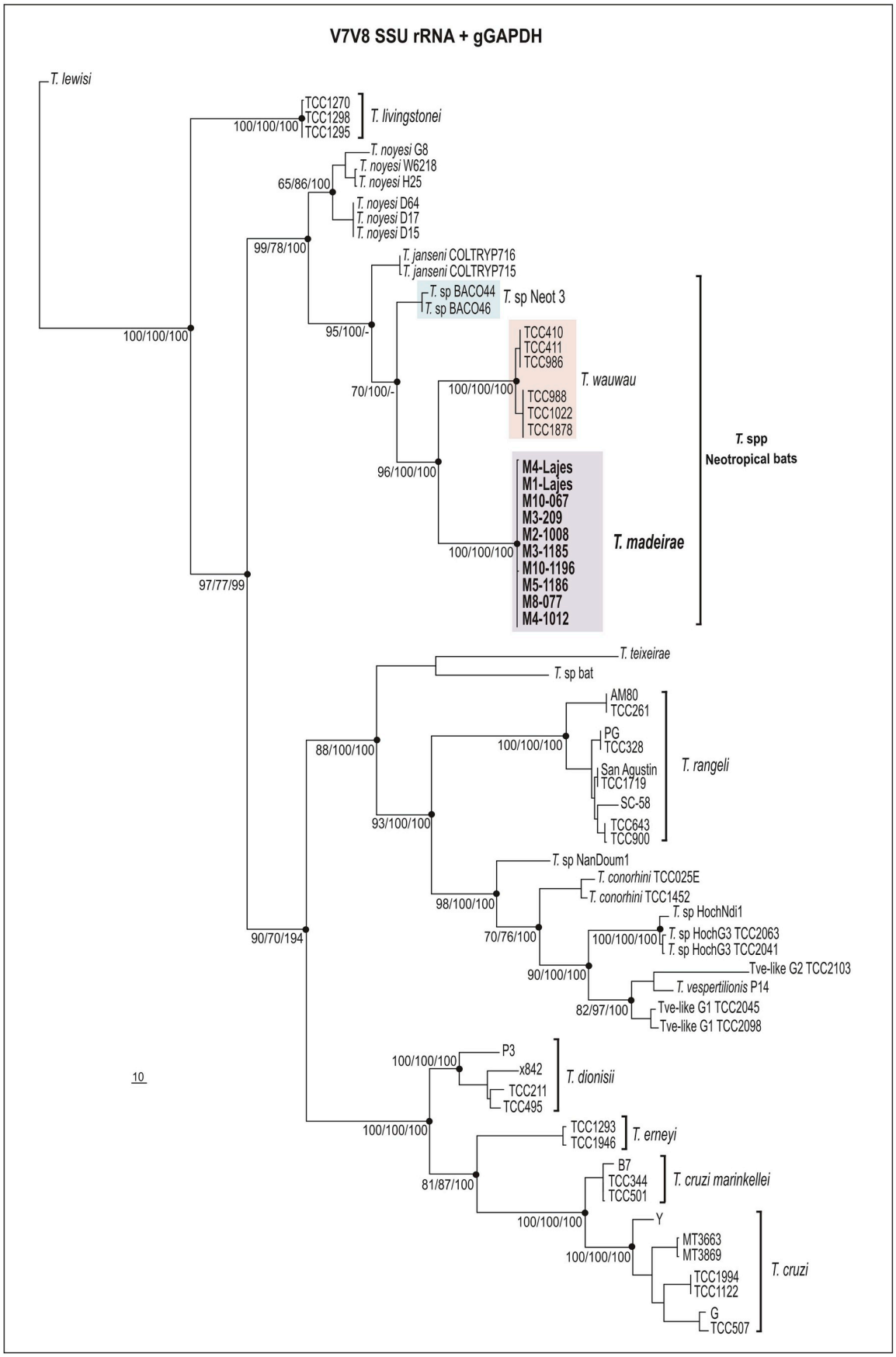


**Fig. 3.** Phylogenetic positioning of *T. rotundus* in the clade *T. cruzi*. ML phylogenetic analysis based on the concatenated sequences of V7V8 SSU rRNA and gGAPDH genes (1.690 characters, –Ln = 8768.346166) from ten isolates of *T. rotundus*, other 29 bat trypanosomes, and 21 trypanosomes from other mammals. *T. lewisi* was used as outgroup. The numbers at the nodes correspond respectively to P, ML (500 replicates) and BI support values.

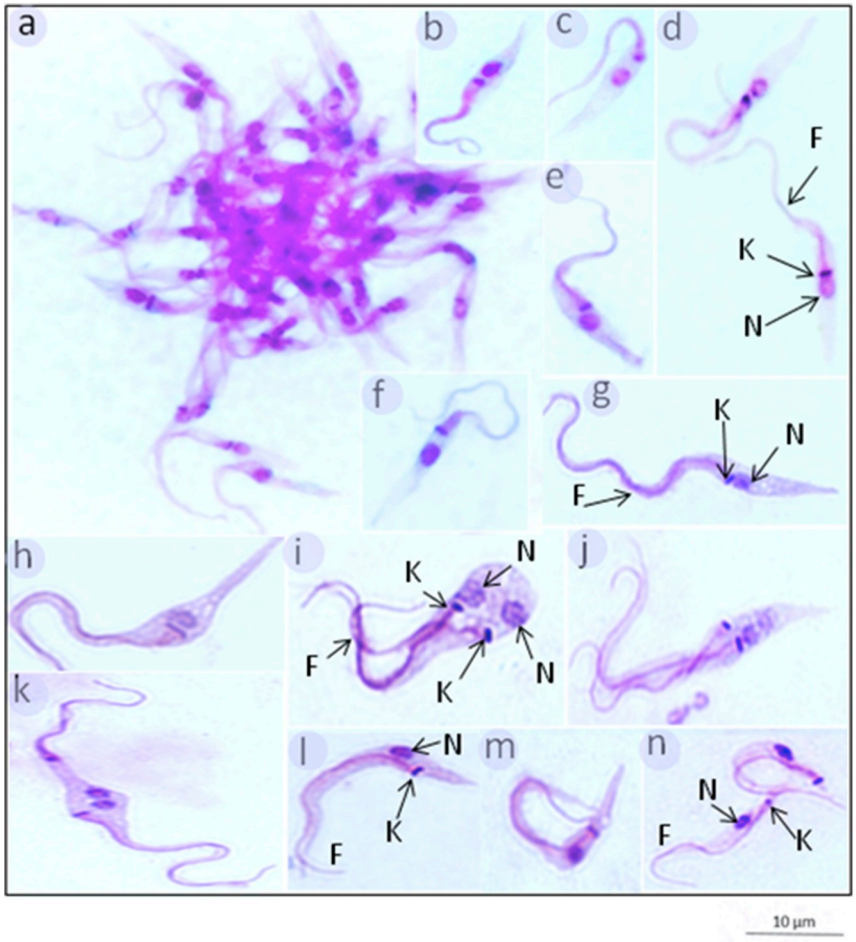


**Fig. 4.** Photomicrographs illustrative of the morphological diversity of culture forms of *T. madeirae* (isolate M3-209). (a) rosetes of epimastigotes, (b-d) flagellates resembling promastigotes forms, (d-h) epimastigotes (7 days), (i-k) large epimastigote forms under division, (l-m), large trypomastigotes, 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 ($3 \times 10^5$ 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, $6 \times 10^5$ 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 epimastigotes 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 dispersion 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

We thank the Genomic Platform-DNA Sequencing (PDTIS-Fiocruz) for DNA sequencing. The present study was partially supported by Program Research Support Foundation of the State of Rio de Janeiro (FAPERJ) INST E-26/111.637/2014. CAPES (LL is a PNPD postdoctoral fellow from the INCT EpiAmo) and FAPESP partially supported this study. MMGT and AOS are recipient of productivity grants from CNPq (National Council for Scientific and Technological Development), and AOS is grants Rio de Janeiro State Scientists Program (FAPERJ)

## References

Argibay, H.D., Orozco, M.M., Cardinal, M.V., Rinas, M.A., Arnaiz, M., Mena, Segura C., Gürtler, R.E., 2016. First finding of *Trypanosoma cruzi* II in vampire bats from a district free of domestic vector-borne transmission in Northeastern Argentina. Parasitology 143, 1358–1368.

Austen, J.M., O'Dea, M., Jackson, B., Ryan, U., 2015. High prevalence of *Trypanosoma vegrandis* in bats from Western Australia. Vet. Parasitol. 214, 342–347.

Baker, J.R., Chaloner, L.A., Green, S.M., 1971. Intracellular development in vitro of *Trypanosoma dionisii* of bats. Trans. R. Soc. Trop. Med. Hyg. 65, 427.

Barros, J.H., Romijn, P.C., Baptista, C., Pinto, A.G., Madeira, M.F., 2008. Report on natural infection of bats by trypanosomatid flagellates in different municipalities in the State of Rio de Janeiro. Rev. Soc. Bras. Med. Trop. 41, 683–685.

Barbosa, A.D., Mackie, J.T., Stenner, R., Gillett, A., Irwin, P., Ryan, U., 2016. *Trypanosoma teixeirae:* a new species belonging to the *T. cruzi* clade causing trypanosomosis in an Australian little red flying fox (*Pteropus scapulatus*). Vet. Parasitol. 223, 214–221.

Bento, E.C., Gómez-Hernández, C., Batista, L.R., Anversa, L., Pedrosa, A.L., Lages-Silva, E., Ramírez, J.D., Ramirez, L.E., 2018. Identification of bat trypanosomes from Minas Gerais state, Brazil, based on 18S rDNA and Cathepsin-L-like targets. Parasitol. Res. 117, 737–746.

Borghesan, T.C., Ferreira, R.C., Takata, C.S., Campaner, M., Borda, C.C., Paiva, F., Milder, R.V., Teixeira, M.M.G., Camargo, E.P., 2013. Molecular phylogenetic redefinition of *Herpetomonas* (Kinetoplastea, Trypanosomatidae), a genus of insect parasites associated with flies. Protist 164, 129–152.

Botero, A., Cooper, C., Thompson, C.K., Clode, P.L., Rose, K., Thompson, R.C., 2016. Morphological and phylogenetic description of *Trypanosoma noyesi* sp. nov.: an Australian wildlife trypanosome within the *T. cruzi* Clade. Protist 167, 425–439.

Bower, S.M., Woo, P.T., 1982. Immunological comparison of four *Trypanosoma* spp. (subgenus *Schizotrypanum*) from bats. Parasitology 85, 111–114.
Cottontail, V.M., Kalko, E.K., Cottontail, I., Wellinghausen, N., Tschapka, M., Perkins, S.L., Pinto, C.M., 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, e108603.
Cavazzana Jr., M., Marcili, A., Lima, L., Da Silva, F.M., Junqueira, A.C., Veludo, H.H., Viola, L.B., Campaner, M., Nunes, V.L., Paiva, F., Coura, J.R., Camargo, E.P., Teixeira, M.M.G., 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, 345–355.
Dario, M.A., Lisboa, C.V., Costa, L.M., Moratelli, R., Nascimento, M.P., Costa, L.P., Leite, Y.L.R., Llewellyn, M.S., Xavier, S.C.D.C., Roque, A.L.R., Jansen, A.M., 2017a. High *Trypanosoma* spp. diversity is maintained by bats and triatomines in Espírito Santo state, Brazil. PLoS One 12, e0188412.
Dario, M.A., Moratelli, R., Schwabl, P., Jansen, A.M., Llewellyn, M.S., 2017b. Small subunit ribosomal metabarcoding reveals extraordinary trypanosomatid diversity in Brazilian bats. PLoS Neglected Trop. Dis. 11, e0005790.
Da Costa, A.P., Nunes, P.H., Leite, B.H., Ferreira, J.I., Tonhosolo, R., Da Rosa, A.R., Da Rocha, P.A., Aires, C.C., Gennari, S.M., Marcili, A., 2016. Diversity of bats trypanosomes in hydroeletric area of Belo Monte in Brazilian Amazonia. Acta Trop. 164, 185–193.
De Oliveira, F.M., Costa, L.H., De Barros, T.L., Ito, P.K., Colombo, F.A., De Carvalho, C., Pedro, W.A., Queiroz, L.H., Nunes, C.M., 2015. First detection of *Leishmania* spp. DNA in Brazilian bats captured strictly in urban areas. Acta Trop. 150, 176–181.
Dos Santos, F.C.B., Lisboa, C.V., Xavier, S.C.C., Dario, M.A., Verde, R.S., Calouro, A.M., Roque, A.L.R., Jansen, A.M., 2017. *Trypanosoma* sp. diversity in Amazonian bats (Chiroptera; Mammalia) from Acre state, Brazil. Parasitology 16, 1–10.
Deane, L.M., Sarjeant, C.S., Fernandez, E., 1978. The finding of *trypanosoma (Megatrypanum) pessoai* Deane & Sugay, 1963 in bats of Venezuela. Bol. Dir. Malariol. Saneamiento Ambiental 18, 231–237.
Deane, L.M., Sugay, W., 1963. *Trypanosoma pessoai* n.sp., in vampire bats *Desmodus rotundus rotundus* from the state of São Paulo, Brazil. Rev. Inst. Med. Trop. Sao Paulo 5, 165.
Delpietro, H.A., Russo, R.G., Carter, G.G., Lord, R.D., Delpietro, G.L., 2017. Reproductive seasonality, sex ratio and philopatry in Argentina's common vampire bats. R. Soc. Open. Sci. 4, 160959.
Espinosa-Álvarez, O., Ortiz, P.A., Lima, L., Costa-Martins, A.G., Serrano, M.G., Herder, S., Buck, G.A., Camargo, E.P., Hamilton, P.B., Stevens, J.R., Teixeira, M.M.G., 2018. *Trypanosoma rangeli* is phylogenetically closer to Old World trypanosomes than to *Trypanosoma cruzi*. Int. J. Parasitol. 48, 569–584.
Garcia, E.S., Macarini, J.D., Garcia, M.C.N., Ubatuba, F.B., 1975. Alimentação de *Rhodnius prolixus* no laboratório. An Acad. Bras Ciências 47, 537–545.
Garcia, H.A., Rodrigues, C.M.F., Rodrigues, A.C., Pereira, D.L., Pereira, C.L., Camargo, E.P., Hamilton, P.B., Teixeira, M.M.G., 2017. Remarkable richness of trypanosomes in tsetse flies (*Glossina morsitans morsitans* and *Glossina pallidipes*) from the Gorongosa National Park and Niassa National Reserve of Mozambique revealed by fluorescent fragment length barcoding (FFLB). Infect. Genet. Evol. 5 pii: S1567-1348(17)30234-4.
Gardner, R.A., Molyneux, D.H., 1988. *Trypanosoma (Megatrypanum) incertum* from *Pipistrellus pipistrellus*: development and transmission by cimicid bugs. Parasitology 9, 433–447.
Gómez-Hernández, C., Bento, E.C., Rezende-Oliveira, K., Nascentes, G.A.N., Barbosa, C.G., Batista, L.R., Tiburcio, M.G.S., Pedrosa, A.L., Lages-Silva, E., Ramírez, J.D., Ramirez, L.E., 2017. *Leishmania* infection in bats from a non-endemic region of Leishmaniasis in Brazil. Parasitology 144, 1980–1986.
Hamilton, P.B., Teixeira, M.M.G., Stevens, J.R., 2012. The evolution of *Trypanosoma cruzi*: the 'bat seeding' hypothesis. Trends Parasitol. 28, 136–141.
Hayes, M.A., Piaggio, A.J., 2018. Assessing the potential impacts of a changing climate on the distribution of a rabies virus vector. PLoS One 21, e0192887.
Hoare, C.A., 1972. The Trypanosomes of Mammals: a Zoological Monograph. Blackwell Scientific Publishing, Oxford, England.
Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.
Johnson, N., Aréchiga-Ceballos, N., Aguilar-Setien, A., 2014. Vampire bat rabies: ecology, epidemiology and control. Viruses 6, 1911–1928.
Larsen, P.A., Hayes, C.E., Williams, C.V., Junge, R.E., Razafindramanana, J., Mass, V., Rakotondrainibe, H., Yoder, A.D., 2016. Blood transcriptomes reveal novel parasitic zoonoses circulating in Madagascar's lemurs. Biol. Lett. 12, 20150829.
Lidani, K.C.F., Bavia, L., Ambrosio, A.R., De Messias-Reason, I.J., 2017. The complement system: a prey of *trypanosoma cruzi*. Front. Microbiol. 20, 607.
Lima, L., Espinosa-Álvarez, O., Pinto, C.M., Cavazzana Jr., M., Pavan, A.C., Carranza, J.C., Lim, B.K., Campaner, M., Takata, C.S., Camargo, E.P., Hamilton, P.B., Teixeira, M.M.G., 2015a. 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. Parasites Vectors 8, 657.
Lima, L., Espinosa-Álvarez, O., Ortiz, P.A., Trejo-Varón, J.A., Carranza, J.C., Pinto, C.M., Serrano, M.G., Buck, G.A., Camargo, E.P., Teixeira, M.M.G., 2015b. Genetic diversity of *Trypanosoma cruzi* in bats, and multilocus phylogenetic and phylogeographical analyses supporting Tcbat as an independent DTU (discrete typing unit). Acta Trop. 151, 166–177.
Lima, L., Espinosa-Álvarez, O., Hamilton, P.B., Neves, L., Takata, C.S., Campaner, M., Attias, M., De Souza, W., Camargo, E.P., Teixeira, M.M.G., 2013. *Trypanosoma livingstonei:* a new species from African bats supports the bat seeding hypothesis for the *Trypanosoma cruzi* clade. Parasites Vectors 6, 221.
Lima, L., Silva, F.M., Neves, L., Attias, M., Takata, C.S., Campaner, M., De Souza, W., Hamilton, P.B., Teixeira, M.M.G., 2012. 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, 856–872.
Lopes, C.M.T., Menna-Barreto, R.F.S., Pavan, M.G., Pereira, M.C.S., Roque, A.L.R., 2018. *Trypanosoma janseni* n. sp. (Trypanosomatida: Trypanosomatidae) isolated from *Didelphis aurita* (Mammalia: Didelphidae) in the Atlantic rainforest of Rio de Janeiro, Brazil: integrative taxonomy and phylogeography within the *Trypanosoma cruzi* clade. Mem. Inst. Oswaldo Cruz 113, 45–55.
Lourenço, J.L.M., Minuzzi-Souza, T.T.C., Silva, L.R., Oliveira, A.C., Mendonça, V.J., Nitz, N., Aguiar, L.M.S., Gurgel-Gonçalves, R., 2018. High frequency of trypanosomatids in gallery forest bats of a Neotropical savanna. Acta Trop. 177, 200–206.
Marinkelle, C.J., 1976. The biology of the trypanosomes of bats. In: Lumsden, W.H.R., Evans, D.A. (Eds.), Biology of the Kinetoplastida. Academic, New York, pp. 175–216.
Martins, F.M., Templeton, A.R., Pavan, A.C., Kohlbach, B.C., Morgante, J.S., 2009. Phylogeography of the common vampire bat (*Desmodus rotundus*): marked population structure, Neotropical Pleistocene vicariance and incongruence between nuclear and mtDNA markers. BMC Evol. Biol. 9, 294.
Molyneux, D.H., 1991. Trypanosomes of bats. In: Kreier, J.P., Baker, J.R. (Eds.), Parasitic Protozoa. Academic Press, New York, pp. 195–223.
Madeira, M.F., Sousa, M.A., Barros, J.H., Figueiredo, F.B., Fagundes, A., Schubach, A., DE Paula, C.C., Faissal, B.N., Fonseca, T.S., Thoma, H.K., Marzochi, M.C., 2009. *Trypanosoma caninum* n. sp. (Protozoa: Kinetoplastida) isolated from intact skin of a domestic dog (*Canis familiaris*) captured in Rio de Janeiro, Brazil. Parasitology 136, 411–423.
Maeda, F.Y., Cortez, C., Alves, R.M., Yoshida, N., 2012. Mammalian cell invasion by closely related *Trypanosoma* species *T. dionisii* and *T. cruzi*. Acta Trop. 121, 141–147.
Maia da Silva, F., Marcili, A., Lima, L., Cavazzana Jr., M., Ortiz, P.A., Campaner, M., Takeda, G.F., Paiva, F., Nunes, V.L., Camargo, E.P., Teixeira, M.M.G., 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, 199–207.
Noyes, H.A., Stevens, J.R., Teixeira, M.M.G.T., Phelan, J., Holz, P., 1999. A nested PCR for the ssrRNA gene detects *Trypanosoma binneyi* in platypus and *Trypanosoma* sp. in wombats and kangaroos in Australia. Int. J. Parasitol. 29, 331–339.
Obame-Nkoghe, J., Leroy, E.M., Paupy, C., 2017. Diversity and role of cave-dwelling hematophagous insects in pathogen transmission in the Afrotropical region. Emerg. Microb. Infect. 6, e20.
Orozco, M.M., Enriquez, G.F., Cardinal, M.V., Piccinali, R.V., Gürtler, R.E., 2016. A comparative study of *Trypanosoma cruzi* infection in sylvatic mammals from a protected and a disturbed area in the Argentine Chaco. Acta Trop. 155, 34–42.
Pegorari, P.O., Goméz-Hernandéz, C., Barbosa, C.G., Oliveira, K.R., Pedrosa, A.L., Ramirez, J.D., Ramirez, L.E., 2018. Short Report Bat Trypanosomatids (First Report of Trypanosoma Wauwau) in Triângulo Mineiro. Pablo de bioRxiv, Brazil, pp. 347146.
Pinto, C.M., Kalko, E.K., Cottontail, I., Wellinghausen, N., Cottontail, V.M., 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, 1328–1332.
Pinto, C.M., Ocaña-Mayorga, S., Tapia, E.E., Lobos, S.E., Zurita, A.P., Aguirre-Villacís, F., et al., 2015. Trypanosomes, and triatomines in Ecuador: new insights into the diversity, transmisson, and origins of *Trypanosoma cruzi* and chagas disease. PLoS One 10, e0139999.
Ramírez, J.D., Tapia-Calle, G., Muñoz-Cruz, G., Poveda, C., Rendón, L.M., Hincapié, E., Guhl, F., 2014. Trypanosome species in neo-tropical bats: biological, evolutionary and epidemiological implications. Infect. Genet. Evol. 22, 250–256.
Schottelius, J., Marinkelle, C.J., Gomez-Leiva, M.A., 1986. Comparative investigations of Latin American trypanosomes with lectins and complement lysis test. Trop. Med. Parasitol. 37, 54–58.
Steindel, M., Grisard, E.C., Carvalho Pinto, C.J., Cordeiro, F.D., Ribeiro-Rodrigues, R., Romanha, A.J., 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, 601–607.
Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland MA.
Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Vilar, T.D., Bernardinelli, C., Valim, M.P., Freire, L.S., Desiderio, M.H.G., Freire, N.M.S., 2004. Registro de *Trypanosoma (Megatrypanum) pessoai* Deane& Sugay, 1963 em morcegos Desmo*dus rotundus* de Vargem Pequena, Rio de janeiro. Entomol. Vectores 11, 535–539.
Wilkinson, G.S., Carter, G.G., Bohn, K.M., Adams, D.M., 2016. Non-kin cooperation in bats. Philos. Trans. R. Soc. Lond. B Biol. Sci. 5, 37.
Zeledón, R., Rosabal, R., 1969. *Trypanosoma leonidasdeanei* sp. nov. in insectivorous bats of Costa Rica. Ann. Trop. Med. Parasitol. 63, 221–228.