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

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ABSTRACT

Molecular phylogenetic studies have revealed the growing diversity of bat trypanosomes. Here, 14 isolates from blood samples of the vampire bat Desmodus rotundus (Phylllostomidae) 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 phyllodistom species. Trypanosomes of Neotropic 1, found in Triatoma ciriopharos and Artibeus jamaicensis (phyllodistomids), 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 dionisi, 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; Ramírez et al., 2014; Lima et al., 2015b; Dario et al., 2017a, b; Dos Santos et al., 2017; Bento et al., 2018). Molecular phylogenetic studies have revealed the growing diversity of bat trypanosomes. Here, 14 isolates from blood samples of the vampire bat Desmodus rotundus (Phylllostomidae) 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 phyllodistom species. Trypanosomes of Neotropic 1, found in Triatoma ciriopharos and Artibeus jamaicensis (phyllodistomids), 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 dionisi, Trypanosoma rangeli, Trypanosoma pessoai, and Trypanosoma madeirae.
Table 1

| Trypanosoma sp | Host Origin | Year | Geographic Origin | GenBank Accession number |
|---------------|-------------|------|-------------------|-------------------------|
| M1-Lajes      | bat         | 2008 | Laje do Muriaé/Rio de Janeiro | BR MK064212 MK064144 |
| M2-357        | bat         | 2006 | Miracá/Rio de Janeiro | BR MK064122 - |
| M2-1008       | bat         | 2008 | Paraty/Rio de Janeiro | BR MK064123 MK064145 |
| M3-209        | bat         | 2005 | Niterói/Rio de Janeiro | BR MK064124 MK064146 |
| M3-1185       | bat         | 2008 | Laje do Muriaé/Rio de Janeiro | BR MK064125 MK064146 |
| M4-Lajes      | bat         | 2008 | Laje do Muriaé/Rio de Janeiro | BR MK064126 MK064146 |
| M4-1012       | bat         | 2007 | Miracema/Rio de Janeiro | BR MK064127 MK064148 |
| M5-069        | bat         | 2004 | Miracema/Rio de Janeiro | BR MK064128 MK064149 |
| M5-1186       | bat         | 2008 | Laje do Muriaé/Rio de Janeiro | BR MK064129 MK064149 |
| M7-1013       | bat         | 2007 | Miracema/Rio de Janeiro | BR MK064130 MK064149 |
| M8-077        | bat         | 2007 | Miracema/Rio de Janeiro | BR MK064131 MK064149 |
| M9-066        | bat         | 2004 | Miracema/Rio de Janeiro | BR MK064132 MK064149 |
| M10-067       | bat         | 2004 | Miracema/Rio de Janeiro | BR MK064133 MK064151 |
| M10-1196      | bat         | 2008 | Laje do Muriaé/Rio de Janeiro | BR MK064134 MK064152 |
| AD245         | bat         |      | Ribeirão Grande/São Paulo | BR MK064135 - |
| AD1036        | bat         |      | Castelo/Espirito Santo | BR MK064136 MK064137 |
| SLA808        | bat         |      | Governador Celso Ramos/Santa Catarina | BR MK064137 MK064137 |
| AD720         | bat         |      | Município de Cunha/São Paulo | BR MK064138 - |
| ICC16         | bat         |      | arquipélago de Marajó/Pará | BR MK064139 MK064141 |
| ICC14         | bat         |      | arquipélago de Marajó/Pará | BR MK064141 MK064142 |
| PNP007        | bat         |      | Itacarambi/Minas Gerais | BR MK064142 MK064143 |
| ICC02         | bat         |      | arquipélago de Marajó/Pará | BR MK064143 MK064143 |
| T. lewisi     | Molteno E3 | rodent | Rattus rattus | -- -- UK AJ09156 AJ620272 |
| T. microti    | TRL 112    | vole | Microtis agrestis | -- -- UK AJ09158 AJ620273 |
| T. wauwau     |             |     |                   |                         |
| TCC410        | bat         | 2002 | Monte Negro/Rondonia | BR KT030809 KT030799 |
| TCC 411       | bat         | 2002 | Monte Negro/Rondonia | BR KT030810 KT030800 |
| TCC 986       | bat         | 2005 | Porto Velho/Rondonia | BR KT030821 KT030801 |
| TCC 988       | bat         | 2005 | Porto Velho/Rondonia | BR KT030823 KT030804 |
| TCC 1022      | bat         | 2005 | Porto Velho/Rondonia | BR KT030830 KT030805 |
| TCC 1878      | bat         | 2009 | Porto Velho/Rondonia | BR KT030835 KT030806 |
| T. livingstonei |             |     |                   |                         |
| TCC 1270      | bat         | 2006 | Chupanga | MZ KF192979 KF192958 |
| TCC 1271      | bat         | 2006 | Chupanga | MZ KF192980 KF192959 |
| TCC 1295      | bat         | 2006 | Chupanga | MZ KF192981 KF192960 |
| TCC 1298      | bat         | 2006 | Chupanga | MZ KF192982 KF192961 |
| TCC 1304      | bat         | 2006 | Chupanga | MZ KF192983 KF192962 |
| T. sp Neot 1  |             |     |                   |                         |
| 093_AJ_Bohio  | bat         | 2005 | Artibeus jamaicensis | PA KM406889 - |
| 134_AJ_Cacao  | bat         | 2005 | Artibeus jamaicensis | PA KM406888 - |
| 216_AJ_Guava  | bat         | 2005 | Artibeus jamaicensis | PA KM406898 - |
| 278_AJ_Leon   | bat         | 2005 | Artibeus jamaicensis | PA KM406897 - |
| 300_AJ_BCI    | bat         | 2005 | Artibeus jamaicensis | PA KM406866 - |
| 302_AJ_BCI    | bat         | 2005 | Artibeus jamaicensis | PA KM406884 - |
| RNM056        | bat         | 2012 | Trachops cirrhosus | Angicos/Rio Grande do Norte BR KT368795 - |
| RNM063        | bat         | 2012 | Trachops cirrhosus | Angicos/Rio Grande do Norte BR KT368796 - |
| T. sp Neot 2  |             |     |                   |                         |
| 082_AJ_Bohio  | bat         | 2005 | Artibeus jamaicensis | PA KM406907 - |
| 092_AJ_Bohio  | bat         | 2005 | Artibeus jamaicensis | PA KM406881 - |
| 173_AJ_Gigante | bat         | 2005 | Artibeus jamaicensis | PA KM406883 - |
| 196_AJ_PenaBlanca | bat | 2005 | Artibeus jamaicensis | PA KM406882 - |
| 275_AJ_Leon   | bat         | 2005 | Artibeus jamaicensis | PA KM406880 - |
| T. sp Neot 3  |             |     |                   |                         |
| 070_AJ_Guanaban | bat | Artibeus jamaicensis | 2005 PA KM406897 - |
| 109_AJ_Bohio  | bat         | 2005 | Artibeus jamaicensis | PA KM406879 - |
| 121_AJ_Cacao  | bat         | 2005 | Artibeus jamaicensis | PA KM406876 - |
| 240_AJ_Leon   | bat         | 2005 | Artibeus jamaicensis | PA KM406875 - |
| 268_AJ_Leon   | bat         | 2005 | Artibeus jamaicensis | PA KM406878 - |
| 269_AJ_Leon   | bat         | 2005 | Artibeus jamaicensis | PA KM406874 - |
| 282_AJ_Leon   | bat         | 2005 | Artibeus jamaicensis | PA KM406877 - |
| BAC044        | bat         | 2014 | Artibeus lituratus | Boyacá CO KT368797 KT368800 |
| BAC046        | bat         | 2014 | Artibeus lituratus | Boyacá CO KT368798 KT368801 |
| T. sp bat     |             |     |                   |                         |
| TCC 60        | bat         | 1997 | Rousettus aegyptiacus | GA AJ012418 GQ140365 |

(continued on next page)
| Trypanosoma sp | Host Origin | Year | Geographic Origin | GenBank Acession number |
|---------------|-------------|------|-------------------|-------------------------|
|               |             |      |                   | SSU rRNA | gGAPDH          |
| T. conorhini  | rodent      | 1947 | BR                | AJ012411 | AJ620267        |
| TCC25e        |             |      |                   |           |                 |
| T. vespertilionis | bat     | 1972 | UK                | AJ009166 | AJ620283        |
| F14           | pipistrellus|      |                   |           |                 |
| T. rangeli    | bat         | 2000 | São Paulo         | FJ001666 | GQ140362        |
| TCC 643       |             |      |                   |           |                 |
| TCC 1719      | bat         | 2005 | São Paulo         | FJ001667 | GQ140363        |
| RGB           |             |      |                   |           |                 |
| AM80          | human       | 1996 | Amazonas          | AY491766 | JN040973        |
| SC58          | rodent      | 1971 | –                 | AY230233 | KT368804        |
| PG            | human       | 1997 | –                 | AY230233 | KT368804        |
| San Agustin   | human       | 1994 | –                 | AY230233 | KT368804        |
| TCC 261       | bat         | 2006 | –                 | AY491738 | KT368808        |
| TCC 328       | human       | 2006 | –                 | AY491738 | KT368808        |
| 900           | bat         | 1974 | –                 | AY491738 | KT368808        |
| T. dionisii   | bat         | 2000 | São Paulo         | FJ001666 | GQ140362        |
| TCC 211       |             |      |                   |           |                 |
| TCC 495       | bat         | 2002 | Amazonas          | FJ001667 | GQ140363        |
| P3            | pipistrellus| 1971 | –                 | AY09151 | AJ620271        |
| x842          | nyctalus    | 2006 | –                 | FNS99058 | FNS99055        |
| T. erneyi     | bat         | 2006 | –                 | AY491738 | KT368808        |
| TCC 1293      |             |      |                   |           |                 |
| TCC 1946      | bat         | 2009 | Chupanga          | JN049887 | JN049864        |
| T. c. marinkellei | bat     | 1974 | Bahia             | A09150 | AJ620270        |
| B7            |             |      |                   |           |                 |
| TCC 344       | bat         | 2001 | Monte Negro/Rondonia | FJ001664 | GQ140360        |
| TCC 501       | bat         | 2002 | Porto Velho/Rondonia | FJ001665 | GQ140361        |
| T. cruzi      | bat         | 2004 | São Paulo         | FJ001628 | GQ140359        |
| TCC 1122      |             |      |                   |           |                 |
| TCC 1994      | bat         | 2004 | São Paulo         | FJ002241 | GQ140358        |
| TCC 507       | bat         | 2002 | Amazonas          | FJ002240 | GQ140352        |
| G             |             |      |                   |           |                 |
| opossum       |             |      |                   |           |                 |
| Y             | human       | 1953 | São Paulo         | AF301912 | GQ140353        |
| MT3663        | triatomine  | 2006 | –                 | AF288660 | JN049971        |
| MT3869        |             |      |                   |           |                 |
| Others Trypanosomes |   |      |                   |           |                 |
| T. sp HochNdi1 | monkey     | 2004 | –                 | FM202493 | FM164794        |
| T. sp NanDoum1 | palm civet | 2004 | –                 | FM202492 | FM164793        |
| T. sp H25     | kangaroo    | 1997 | –                 | AJ099168 | AJ620276        |
| T. sp G8      | woylie      | 2013 | –                 | KC753537 | KC012988        |
| T. sp BDA1    | woylie      | 2009 | –                 | FJ823108 | –               |
| T. sp D15     | possum      | 2009 | –                 | JN315381 | JN315395        |
| T. sp D17     | possum      | 2009 | –                 | JN315382 | JN315396        |
| T. sp D64     | possum      | 2009 | –                 | JN315383 | JN315397        |
| T. sp BRA2    | rodents     | 2007 | –                 | FJ823117 | –               |

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

BR, Brazil; GY, Guyana; GT, Guatemala; SR, Suriname; PA, Panama; 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 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 Suguoy, 1963; Marinkelle, 1976; Molyneux, 1991; Vilard et al., 2004). Recently, Leishmania infantum, L. amazonensis and L. braziliensis were detected by PCR in D. rotundus in Brazil (De Oliveira et al., 2017; 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 unvalued 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 were 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 (Borghesani 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.
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. 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.
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, \(-L_n = 8768.346166\)) 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.
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. madeira* 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 × 10⁵ 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% CO₂ atmosphere.

After 24 h, 6 × 10⁵ 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) predominant 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 fusiorm trypanostomites exhibiting discreet undulating membrane and terminal kinetoplast were detected in the end of stationary cultures (14th day) (Fig. 4 n). These trypanostomites 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 mammalian 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 soricina (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 Holland, 1952; Family Trypanosomatidae Dolfin, 1951; Genus Trypanosoma Gruby, 1843.

**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 Leishmaniose in the Instituto Nacional de Infectologia/FIOCRUZ.

**Mammalian host:** Chiroptera, Phyllostomidae, Desmodontinae, Demodous 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, polyphyletic infections 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 phyllostomid 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 Artibius jamacicensis (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. jansenii* from a Neotropical marsupial (Lopes et al., 2018), *T. nayessi* from Australian rodents and marsupials (Hamilton et al., 2012b; Botero et al., 2016), and one unnamed trypanosome of lemur 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. dionisi*, *T. rangeli*, and Trypanosoma 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. pessoui*, 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. dionisi*, 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 epimastigotes and trypomastigotes 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. dionisi*, 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 dexterensis* and *T. dionisi*, 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. dionisi* 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, Rhodinus* and *Panstrongylus* species with *T. c.
marinkellei, T. dionisi, T. erneyi, and T. cruzi of the genotype TcBat have all failed, despite the ability of all these species to survive for many years in the digestive tract of the triatomines (Cavazzana et al., 2010; Lima et al., 2012, 2015b). Recently, T. c. marinkellei and T. dionisi were detected by PCR surveys in the digestive tract of Triatominae vitiicipes (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 cyclically transmitted by a range of triatomine species, it was well-demonstrated that T. dionisi 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. pessasi and T. leonidasalanei to neotropic 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. madeireae 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. madeireae, 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. madeireae 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. madeireae in more than 60 species of bats examined, even though most species examined belonged to Phyllostomidae.

Taken together, our findings support T. madeireae 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. madeireae sharing virtually identical V77V 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. madeireae 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. dionisi (Cavazzana et al., 2010; Dario et al., 2017).

Supporting the link between D. rotundus and T. madeireae, other species of trypanosomes, T. dionisi 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. madeireae. 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. madeireae. 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). Successful dispersion of D. rotundus likely allowed for interchange and dispersion of their trypanosomes. The very interesting and apparent strong association of T. madeireae 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.

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