Molecular characterization and phylogenetic analysis of *Trypanosoma* spp. detected from striped leaf-nosed bats (*Hipposideros vittatus*) in Zambia

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- *Trypanosoma dionisii*

**Abstract**

Bat trypanosomes consist of more than 30 trypanosome species from over 70 species of bats. Recent studies suggest that bats play a role in disseminating trypanosomes from African continent to the terrestrial mammals both in the Afrotropic-Palearctic Ecozones and Nearctic Ecozone. However, the diversity, distribution, and evolution of bat trypanosomes are still unclear. To better understand their evolution, more genetic data of bat trypanosomes from a variety of locations are required. During a survey of Borella spp. of bats inhabiting a cave in Zambia, we observed flagellate parasites from 5 of 43 hemocultures. Sequence and phylogenetic analyses of the glycosomal glyceraldehyde 3-phosphate dehydrogenase gene (*ggAPDH*; 572 bp) and the 18S ribosomal RNA gene (185 rRNA gene; 1,079–1,091 bp) revealed that all were *Trypanosoma spp.* belonged to the *Trypanosoma cruzi* clade. Three and two of them exhibited the similarity with *T. conorhini* and *T. dionisii*, respectively. The present study provides the first genetic data on *Trypanosoma* spp. of bats inhabiting Zambia.

**1. Introduction**

Approximately 1,240 bat species are recognized in the world, representing about 20% of all classified mammalian species worldwide. They play a role as a natural reservoir of wide variety of pathogens including virus, bacteria, and protozoa (Calisher et al., 2006). Trypanosomes, which are blood parasites widespread in all continents and commonly transmitted by blood sucking arthropods and leeches (Hoare, 1972; Hamilton et al., 2007), have been reported in over 70 bat species in the world. These bat trypanosome species belong to three subgenera, namely *Herpetosoa*, *Megatrypanum*, and *Schizotrypanum*. *T. cruzi*, which is a causative agent of human Chagas disease in South America (Bern, 2015), has been detected in a variety of terrestrial animals including bats. Many species in the subgenus *Schizotrypanum* constitute a large monophyletic assemblage that has been designated as *T. cruzi* clade. The members of bat trypanosomes in the *T. cruzi* clade
include *T. vespertilionis* in European bats and *T. vespertilionis*-like *Trypanosoma* spp. in West African bats, *T. cruzi marinkellei* in South American bats, and *T. dionisii* in European, Asian, and South American bats (Hoare, 1972; Mafie et al., 2018). Recent molecular evidence from studies on bat trypanosomes suggest that the *T. cruzi* clade evolved from a broader clade of bat trypanosomes, and that bat trypanosomes had successfully made the host switch to other terrestrial mammalian species in both the Nearctic Ecozone and Afrotropic-Palaearctic Ecozones (Hamilton et al., 2012a). Therefore, molecular investigation of the genus *Trypanosoma* in bats all over the world is important for better understanding of evolution and diversity of pathogenic trypanosomes.

In Sub-Saharan Africa, sestre-transmitted trypanosomes, including *T. brucei* sensu lato, *T. congolense*, and *T. vivax*, threaten human and animal health (Bücher et al., 2017; Morrison et al., 2016). Non-teste-transmitted trypanosomes such as *T. lewisi* are also known to be prevalent in rodents in the region (Hoare, 1972; Keymer, 1971). In bats, *Livingstonei* and *T. erneyi* belonging to the *T. cruzi* clade were detected in Mozambique (Lima et al., 2012, 2013). The ancestral species of the parasites in the *T. cruzi* clade in bats is considered as the origin of trypanosomes in land mammals. The phylogenetic position of *T. li-
vingstonei* is peripheral to *T. cruzi* clade; this fact supports the hypothesis that *T. cruzi* originated from bats (Lima et al., 2013). Thus, genetic investigation of trypanosomes in bats is important to understand whole picture of evolution and distribution of trypanosomes and their genetic diversity.

During the survey on *Borrelia* spp. in bats captured in a cave in Zambia, where a patient suffering from relapsing fever got a tick bite (Qi et al., in press), we unexpectedly observed flagellate protozoa in the five hemocultures. We found that these parasites were phylogenetically divided into two distinct subclades within the *T. cruzi* clade. This study provides the first genetic characterization of bat trypanosomes in Zambia.

2. Materials and methods

2.1. Culture

In December 2017, a total of 43 bats (32 *Roussettus aegyptiacus* and 11 *Hipposideros vittatus*) were captured at the cave (15.44 S, 28.51 E) in Zambia as part of a surveillance program of filovirus infection and *Borrelia* spp. in accordance with the ethical standards approved by the Department of National Parks and Wildlife (formerly Zambia Wildlife Authority), Ministry of Tourism and Arts of the Republic of Zambia (Act No. 12 of 1998) (Changula et al., in press). Uncoagulated whole blood samples were collected from bats. For the purpose of *Borrelia* spp. isolation, 100 μl of the peripheral blood were added into the Babour-Stoenner-Kelly (BSK)-M medium and incubated for 4 weeks at 34 °C, with 5.0% CO2 as previously described (Takano et al., 2014). The culture was observed microscopically every 2–3 days.

2.2. Morphological investigation

Giemsa staining was carried out for visualizing flagellate protozoa from one of the bat samples in the BSK-M medium (ZB17-105). Microscopy (Shimadzu Motic BA210E) was used for observation and image acquisition of the parasites.

2.3. DNA extraction and polymerase chain reaction (PCR)

The culture media that contained the parasites were harvested at 2 weeks after culture initiation. After centrifugation at 1,600 g for 10 min, DNA was extracted from the pellet using DNAzol (Invitrogen, MA, USA) according to the manufacturer’s instructions.

PCR amplification of the glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH), and 18S ribosomal RNA genes was carried out using previously described primers (Table 1) (Hamilton et al., 2004; da Silva et al., 2004; Lemos et al., 2015). All PCR reactions were conducted in a 20 μl-reaction mixture containing 2 μl of 10 × Ex Taq Buffer (TaKaRa Bio Inc., Shiga, Japan), 0.1 μl of Ex Taq Hot Start Version (TaKaRa Bio Inc.), 1.6 μl of 2.5 mM dNTPs mixture, 200 nM of each primer, and 2 μl of template DNA. The reaction conditions were 98 °C for 1 min and 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. The PCR products were subjected to electrophoresis in a 1.2% agarose gel and stained with ethidium bromide.

2.4. Sequencing and phylogenetic analyses

Cycle sequencing was performed using the BigDye Terminator version 3.1 chemistry (Applied Biosystems, MA, USA). Sequencing products were run on a 3130xl Genetic Analyzer (Applied Biosystems). Sanger sequencing data were analyzed using GENETYX version 9.1 (GENETYX Corporation, Tokyo, Japan). In order to obtain a longer sequence of the 18S rRNA gene, sequences of each sample from 2 PCRs targeting 18S rRNA gene were combined. Approximately 1,080-bp fragments of the 18S rRNA gene sequence were obtained.

The obtained gGAPDH and 18S rRNA gene sequences of trypanosomes from bats were aligned with closely related parasite sequences deposited in the database (DDBJ/EMBL/GenBank) using ClustalW multiple alignment program. Phylogenetic trees were constructed by using three methods; the maximum likelihood, neighbor joining, and minimum evolution methods embedded in the MEGA version 6.06 (Tamura et al., 2013). Phylograms were constructed with concatenated gGAPDH and 18S rRNA gene sequences, as previously described (Cottonati et al., 2014; Espinosa-Alvarez et al., 2018). The DDBJ/EMBL/GenBank accession numbers obtained in this study are as follows: gGAPDH: LC415422 and LC415423, 18S rRNA gene: LC415424 and LC415425.

2.5. Species delimitation

Poisson tree processes (PTP) model for species delimitation was used for inferring the relationship between *Trypanosoma* spp. detected in this study and their closely related species. PTP species delimitation analysis was carried out via the bPTP webserver (Zhang et al., 2013). We employed a maximum likelihood phylogeny of the concatenated sequences of 18S rDNA and gGAPDH with default parameters as reported elsewhere (Cottonati et al., 2014).

3. Results

3.1. Detection of flagellate protozoa

Of the 43 bat blood samples that were added to the BSK-M medium, flagellate protozoa were observed at about 1 week after culture initiation in 5 samples from *H. vittatus*. During the 4 weeks of observation period, the number of parasites decreased gradually. Though the parasites were co-cultured with primary cells derived from *H. vittatus*, the number of parasites did not increase (data not shown). Microscopic observation of the slides prepared from one culture (ZB17-105) clearly showed flagellate protozoa with stumpy and slender forms of variable sizes (13.0–36.0 μm from the anterior to the posterior) (Fig. 1). Unfortunately, the morphological data from other cultures were not recorded.

3.2. Sequence analysis of *Trypanosoma* spp.

Sequence analysis of gGAPDH revealed the presence of two distinct sequence types. The gGAPDH sequence type 1 from the samples ZB17-105, −111, and −115 (GenBank no. LC415422) showed 97.0% (555/572 bp) identity with the *T. conorhini* isolate TCC2156, which was detected from a triatomine (*Triatoma rubrofasciata*) of Hawaii.
the African palm civet (*N. binotata* (1074/1099 bp) identity with FM202492) (Hamilton et al., 2009). The sequence type 2 from the samples ZB17-108 and −109 (GenBank no. LC415423) showed 94.2% (539/572 bp) identity with the *TryCC495* from a bat (*GQ140363*) (Cavazzana et al., 2010).

−ZB17–105, sp. sequence type 2 and *panosoma* both of which formed a distinct monophyletic group (Fig. 3).

The same grouping was obtained by the sequence analysis of 18S rRNA. The 18S rRNA gene of the sequence type 1 from the samples ZB17-105, −111, and −115 (GenBank no. LC415424) showed 97.7% (1074/1099 bp) identity with *Trypanosoma* sp. isolate NanDoum1 from the African palm civet (*N. binotata*) in Cameroon (GenBank no. FM202492) (Hamilton et al., 2009). The sequence type 2 from the samples ZB17-108 and −109 (GenBank no. LC415425) showed 95.2% (1033/1084 bp) identity with the *T. dionisii* isolate P3 from a bat (*Pipistrellus pipistrellus*) captured in the United Kingdom (GenBank no. AJ009151) (Stevens et al., 1998).

### 3.3. Phylogenetic analysis of *Trypanosoma* spp.

In the phylogenetic trees based on the concatenated 18S rRNA and *gGAPDH* gene sequences, the sequence type 1 (ZB17-105, −111, and −115) and type 2 (ZB17-108 and −109) belonged to the clades clustering respectively with *T. conorhini* and *T. dionisii* (Fig. 2). The topologies of the trees generated with three different methods are in good agreement, except for the position of parasites in the Australian clade (*T. noyesi* and *Trypanosoma* sp. isolate H25) and the Neobats clade (*T. conorhini*). The topologies differ slightly from those reported by Malo et al. (2018). The phylogenetic trees and PTP analysis based on the concatenated 18S rRNA and *gGAPDH* gene sequences, the detected trypanosome infection in *H. vittatus* bats and the first molecular study of bat trypanosomes in Zambia.

The nucleotide sequences of both *gGAPDH* and 18S rRNA genes of the samples ZB17–105, −111, and −115 (sequence type 1) showed high similarity with that of a trypanosome member of the *T. conorhini* clade. In the phylogenetic trees, *Trypanosoma* spp. detected in this study support the hypothesis that a distinct monophyletic group (Fig. 3).

### 4. Discussion

We observed two distinct sequence types of *Trypanosoma* spp. in BSK-M medium inoculated with blood samples of striped leaf-nosed bats (*H. vittatus*). BSK medium and its derivatives are generally used for the isolation of *Borrelia* spp., however, Mafie and colleagues also incidentally isolated *T. dionisii* from an Eastern bent-winged bat (*Miniopterus fuliginosus*) using the BSK medium (Mafie et al., 2018). To the best of our knowledge, this study is the first record of trypanosome infection in *H. vittatus* bats and the first molecular study of bat trypanosomes in Zambia.

The nucleotide sequences of both *gGAPDH* and 18S rRNA genes of the samples ZB17–105, −111, and −115 (sequence type 1) showed high similarity with that of a trypanosome member of the *T. conorhini* clade. In the phylogenetic trees, *Trypanosoma* spp. detected in this study support the hypothesis that
the ancestor species of the *T. conorhini* clade might have originated in the Afrotropic Ecozones.

Both gGAPDH and 18S rRNA gene sequences of *Trypanosoma* sp. sequence type 2 (ZB17-108 and −109) showed 95.2% and 94.2% identities with those of *T. dionisii*, respectively. In the phylogenetic trees based on the concatenated gGAPDH and 18S rRNA gene sequences, the detected trypanosome formed a monophyletic group with *T. dionisii* distributed in the European and American continents (Hoare, 1972;...
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