A new species of mammalian trypanosome, *Trypanosoma (Megatrypanum) bubalisi* sp. nov., found in the freshwater leech *Hirudinaria manillensis*

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Abstract

Leeches have long been considered potential vectors for the aquatic lineage of trypanosomes, while bloodsucking insects are generally considered as the vectors for the terrestrial lineage of trypanosomes. The freshwater leech, *Hirudinaria manillensis*, is a widely distributed species in Southern China and could potentially act as the vector for trypanosomes. Prior to this study, no trypanosomes have been reported from this leech. However, in this study, leeches were collected from three different places in Guangdong province, China, and a large number of flagellates were isolated and successfully cultured *in vitro*. Based on morphology, these flagellates looked like a typical trypanosome species. Analysis was carried out on the molecular sequences of the *18S rRNA* gene and the glycosomal glyceraldehyde-3-phosphate dehydrogenase (*gGAPDH*) gene. To our surprise, these flagellates were identified as likely to be a mammalian trypanosome belonging to the clade containing *Trypanosoma (Megatrypanum) theileri* but they are significantly different from the typical TthI and TthII stocks. Analyses of blood composition indicated that the source of the blood meal in these leeches was from the water buffalo (*Bubalus bubalis*). To further test if this flagellate from the freshwater leech was indeed a mammalian trypanosome, we transferred the trypanosomes cultured at 27°C to 37°C and they could successfully adapt to this mammalian body temperature, providing further supporting evidence. Due to the significant genetic differences from other related trypanosomes in the subgenus *Megatrypanum*, we propose that this flagellate, isolated from *H. manillensis*, is a new species and have named it *Trypanosoma bubalisi*. Our results indicate that freshwater leeches may be a potential vector of this new mammalian trypanosome.

Keywords: *Trypanosoma theileri*-like; *Hirudinaria manillensis*; Morphology; Cultivation; Phylogenetic analysis; Leech; new species

1. Introduction

Trypanosomes are parasitic haemoflagellates of vertebrates including humans. Some trypanosomes can cause severe diseases in hosts resulting in high morbidity and high mortality, leading to disastrous public health problems and dramatic economic losses worldwide (Hoare, 1972; Maslov et al., 2018; Lukeš et al., 2018). The spread of these deadly diseases requires transmission vectors which are, generally, blood-sucking invertebrates. For instance, blood-sucking insects, such as tsetse flies of the genus *Glossina*, Tabanidae flies and Triatomine bugs are generally considered the main vectors of trypanosomes from the terrestrial lineage which encompasses trypanosomes of mammals (including humans), birds, snakes, lizards and crocodiles (Solano and Delafosse, 1995; Simpson et al., 2006; Hamilton et al., 2007; Krafsur, 2009; Baldacchino et al., 2013). On the other hand, blood-sucking leeches have long been known as the main vectors of trypanosomes from the aquatic lineage (Chia and Miller, 1984).

There is a sustained interest in the relationship between leeches and trypanosomes. Leeches in the Glossiphoniidae and Piscicolidae families inhabit both marine and freshwater environments and may serve as parasites for aquatic vertebrates such as fish, amphibians, reptiles (turtle, crocodiles) as well as mammals (platypus) (Yang, 1996; Apakupakul et al., 1999). In fact, these leeches have long been considered the main vectors of trypanosomes in aquatic vertebrates infected with these parasites (Qudri, 1962; Pessoa, 1968; Chia and Miller 1984; Paparini et al., 2014; Fermino et al., 2020; Smit et al., 2020). For example, *Trypanosoma danilewskyi*, a trypanosome of carp, is transmitted by a Glossiphoniidae leech, *Hemiclepsis matginata,* in which the trypanosome undergoes a developmental changes and migrates from the leech crop to the proboscis sheath where they are transmitted to a new vertebrate host in the blood feed (Qudri, 1962). However, the members in the Order Arhynchobdellida, a group of common blood sucking leeches, do not have a sheath and proboscis. Whether the leeches in this order, such as the medicinal leech, belonging to the Family Hirudinidae, and the terrestrial Haemadipsidae, can also be vectors for vertebrate trypanosomes remains unclear.

There have been numerous reports of trypanosomes found in the terrestrial Haemadipsidae leeches in the last century. For example, Tubangui (1932) found trypanosomes in *Haemadipsa zeylanica* from the Philippines, while Richardson (1968) reported a trypanosome in *Chtonobdella* sp. leeches caught in Australia. Ewers (1974) described a bat trypanosome *Trypanosoma (Herpetosoma) aunawa* in a terrestrial leech, *Philaemon* sp. from Guinea, which fed on bats in their cave habitat. Using molecular methods, Hamilton et al., (2005) confirmed the presence of frog and marsupial trypanosomes in four Haemadipsidae leech species from Australia, while Siddall et al., (2019) reported that they could amplify unknown trypanosome DNA from 56.7% (25/44) terrestrial leeches collected from Australia and New Guinea. Recently, Ellis et al., (2021) isolated a new subspecies of *Trypanosoma cyclops* from the terrestrial leech *Chtonobdella bilineata* in Australia. In some of the above cases, terrestrial leeches have been suggested as the potential vectors of trypanosomes based on different analyses (Tubangui, 1932; Richardson, 1968; Hamilton et al., 2005). However, the development of these trypanosomes within the Haemadipsidae leeches has not yet been well understood.

In the present study, we identified a new species of mammalian trypanosome in a freshwater leech, *Hirudinaria manillensis*, collected from Southern China. Based on the morphology, *in vitro* cultivation and genetic analysis, it belongs to the subgenus of *Megatrypanum* and blood meal analysis suggests that the water buffalo is the host of this trypanosome.

2. Materials and methods

2.1. Sampling sites and morphological identification of leeches

Leech specimens were collected from April to July 2019 in three locations in Guangdong province, China, including Shalang town (SL, 21°46′53.25″N, 111°16′0.83″E), Nanlang town (NL, 22°29′12.14″N, 113°32′53.68″E) and Xintong town (XT, 21°55′22.27″N, 111°02′56.69″E) (Fig. 1A). Leeches were caught from natural water bodies (Fig. 1B, C) and transferred to the laboratory within 3-4 days. They were then maintained in the laboratory for 1-6 weeks after arrival and subsequently used for further studies. Leeches were identified, morphologically, according to the descriptions by Yang (1996) and by gene analysis (Medlin et al., 1988).

2.2. Detection of flagellates in the leech crop contents and tissues

The leeches, collected from each site, were randomly divided into four groups on the date of arrival (Groups: 0, 2, 4 and 6 weeks after arrival), and were examined at the indicated time points, respectively. For detection, leeches from each location (SL, n=99; NL, n=55; XT, n=44) were individually kept in a clean petri dish and opened from the dorsal surface of the body to let the ingested crop contents flow out.

One drop of the crop contents was mixed with 10 μl phosphate-buffered saline (PBS) and mounted on a slide for microscopical examination (DM500; Leica Microsystems, Switzerland) and/or video recording (Vert. A1; Carl Zeiss, Germany). Smears of leech crop contents were made and air dried, and then stained with Giemsa after fixation with methanol. Smears were finally mounted with neutral balsam.

The intestinal tract contents were also prepared as wet smears for microscopical examination. The leech tissues from the mouth region, such as the jaw and pharynx, were sampled either for histopathological analyses or for DNA extraction. Briefly, leech tissues were isolated and immediately fixed in 4% paraformaldehyde with PBS buffer for 24 h at 4°C. Then the fixed samples were embedded in paraffin and cut into 4 μm-thin sections. Subsequently, the sections were deparaffinized in xylene and rehydrated in water, stained with hematoxylin and eosin (H & E) (Tajima et al., 2020) and observed by light microscopy (Nikon Eclipse Ni-U; Nikon Corporation, Japan).

2.3. Morphological analysis of flagellates

Morphological analyses of flagellates were conducted using well-stained Giemsa smears. For each group, about 80-200 randomly selected flagellates were measured using Image J (Schneider et al., 2012) and displayed using MATLAB (R2019a), which included the following parameters: body length (BL), total length with flagellum (L), posterior end to mid-nucleus (PN), posterior end to kinetoplast (PK), kinetoplast to mid-nucleus (KN), free flagellum length (FF), anterior end to mid-nucleus (NA), cell maximum body width (BW), nucleus width (NW), nucleus length (NL). In addition, nuclear index (NI = PN/NA), kinetoplast index (KI = PN/KN) representing the position of nucleus/kinetoplast in the body and flagellar index (FI = FF/BL) representing the proportion of the free flagellum to the length of body were calculated (Hoare, 1972; Gu et al., 2007).

2.4. DNA extraction and PCR amplification of leech tissue and crop contents

For extraction of genomic DNA, 5-8 individual leeches from each collection sites were used. Tissue samples were obtained from the posterior sucker of the leech and DNA extraction was carried out as described elsewhere (Green and Sambrook, 2017) with DNA samples being subsequently stored at -20°C until use. The *18S rRNA* gene was amplified with a set of eukaryotic *18S rRNA* gene primers A and B (Medlin et al., 1988) (Supplementary Table 1). PCR reactions were conducted in a 50 μl reaction mixture with 5 units of EasyTaq DNA polymerase (TaKaRa, Japan) in 1× Easy Taq DNA polymerase buffer with 10 nM of dNTP-mix, 10 pM of each primer and 80 ng of template DNA. The cycling conditions included 94°C for 4 min, then 35 cycles of 94°C (20 s), 47°C (20 s), and 68°C (105 s), and with a final extension at 70°C for 7 min (Medlin et al., 1988).

DNA samples were extracted from the crop contents of leeches collected from three sites (SL, n=35; NL, n=26; XT, n=22) and stored as above. Specific primers for the vertebrate mitochondrial 16S ribosomal RNA (*mt16S rRNA*) gene (Bo et al., 2016) and mammalian mitochondrial cytochrome b (*Cyt b*) gene (Rádrová et al., 2013) (Supplementary Table 1) were used to identify the blood that leeches had fed on. Briefly, amplicons were separated on 1% agarose gels, stained with ethidium bromide (EtBr), and visualized under UV light. The bands were purified using MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, Japan), and were cloned using the pMD™19-T vector Cloning Kit (Promega, Wisconsin, United States) according to the manufacturer's instructions.

Sequences were aligned and identified using MEGA software (Kumar et al., 2016) and BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.5. In vitro cultivation and cloning of flagellates

For cultivation at 27°C, about 200 µl of leech crop material, containing flagellates, were washed with 10 ml PBS buffer and pelleted at 1000 ×g for 5 min. After two additional washes, the pellet was resuspended with 3 ml culture medium and split into various different culture media to maximise the cultivation success, including SDM79 (Brun and Jenni, 1977), HMI-11 (Hirumi and Hirumi, 1989), RPMI-1640 (Gibco, USA), Grace's (Gibco, USA) and L15 (HyClone, USA), in each case, supplemented with 10% fetal bovine serum (FBS) (ExCell Bio, China) and 1% penicillin-streptomycin (10000 U/mL penicillin- 10000 µg/L streptomycin) (Gibco, USA). The mixture with flagellates was incubated at 27°C and monitored daily.

In order to exclude a potential mixed infection of more than one flagellate species from the leech specimens, single flagellates were obtained by cloning using serial dilution from the continuous cultures. Briefly, flagellates were diluted to around 1000 parasites per ml and 1 µl of this medium containing flagellates was pipetted into the well of 96-well plate. After confirming the presence of only one parasite in the well by microscopic examination, 100 µl medium was added and cultured at 27°C. After a month of cultivation, all clones were collected for DNA extraction and for subculture and cryopreservation.

2.6. Adaptation of flagellates from 27°C to a 37°C

To evaluate the possibility that the flagellates can survive at the mammalian body temperature, flagellates cultured at 27°C were transferred to a 37°C culture system supported with Madin-Darby Bovine Kidney (MDBK) cells as feeder cells in RPMI-1640 medium (GIBCO, USA) with 10% FBS. A marine fish trypanosome *Trypanosoma epinepheli* isolated from barramundi (*Lates calcarifer*) and a freshwater fish trypanosome *Trypanosoma* sp. from *Micropterus salmoides* in our laboratory were incubated in the same conditions and acted as the control groups. Growth curves of these flagellates were monitored daily using a haemocytometer and proliferative flagellates were observed following Giemsa-staining.

2.7. Phylogenetic and genotyping analysis

The genetic diversity of the isolated flagellates from the leech crop contents of three collected sites was initially assessed using Trypanosomatid *18S rRNA* gene analysis, which is a very valuable marker for resolving the phylogenetic position of trypanosomes isolated from various kinds of hosts (Maslov et al., 1996; Kostygov et al., 2021). To clarify the taxonomical position of the isolated flagellates, the glycosomal glyceraldehyde-3-phosphate dehydrogenase (*gGAPDH*) gene also used. The primers and conditions for PCR amplification of the *18S rRNA* gene (S762 and S763) and *gGAPDH* gene (G3 and G5; G1 and G4a) were presented in Supplementary Table 1 and elsewhere (Maslov et al., 1996; Hamilton et al., 2007).

To construct the phylogenetic tree, 36 *18S rRNA* sequences of trypanosomes were obtained from GenBank and their accession numbers are described below. *Trypanoplasma borreli* (Accession no. L14840) was used as outgroup. The sequences were aligned using the MEGA 7 (Kumar et al., 2016) program, and corrected manually using BioEdit 7.2 (Hall, 1999). The Neighbor-Joining (NJ) tree was generated using Kimura’s 2-parameter (K2P) model with complete deletion option and a bootstrap value of 1,000 replicates, while Maximum Likelihood (ML) used the General Time

Reversible model and Maximum Parsimony (MP) phylogenies for character-based analyses to verify the tree topology.

Genotyping analysis used in the comparison with the *T. theileri* group was carried out as previously described (Rodrigues et al., 2006, 2010). With *Trypanoplasma borreli* (Accession no. X74535) as the outgroup and used a bootstrap value of 1,000 replicates, the *gGAPDH* gene ML tree was constructed by MEGA 7 for the 33 sequences of trypanosomes, including phylogenetic lineages of TthI, TthII and the *T. cyclops* clade trypanosomes (*T. cyclops* and one terrestrial leech trypanosome *Trypanosoma* sp. TL.AQ.22 from Australia (Hamilton et al., 2005)). NJ and MP phylogenies were used to verify the ML tree topology. The divergence in *gGAPDH* between our isolated trypanosomes and TthI, TthII and the *T. cyclops* clade trypanosomes were conducted using the DnaSP 6 softwore (Rozas et al., 2017).

The accession numbers of other *gGAPDH* and *18S rRNA* gene sequences obtained from GenBank are listed in Supplementary Table 4.

3. Results

3.1. Leech specimen identification

The leeches, collected from the three places (Fig. 1), were all identified morphologically as *Hirudinaria manillensis* (Fig. 1D, E) by the following characteristics: a flat body with length of 110-120 mm and width of 10-15 mm, dorsal color of yellowish brown or olive green with a light blue-grey longitudinal stripe in the middle, ventral side of yellowish brown, a line of pigment dots and an orange line presenting longitudinally on each side of the body, trivalve jaws monostichodont with salivary papillae, caudal sucker large but significantly smaller than the body width, gonopores separated by five annuli, five pairs of eyespots. The leech identification was also confirmed by *18S rRNA* PCR yielding 597 bp fragments (GenBank accession no. MZ520975) (Supplementary Fig.

1A), as the obtained sequences were all identical and 100% matched to the *H. manillensis* reference (GQ368789).

3.2. Trypanosomes from leech crop contents

The leech specimens collected from 3 different places were examined at the indicated time points of 0-week, 2-weeks, 4-weeks and 6-weeks after arrival. Some of the leeches in each group appeared to have fed recently, as the crops retained a dark red color, although no red blood cells were found. Flagellates were present in some crop contents of these leeches, which were identified morphologically as trypanosomes (Fig. 2A, B, C). In all leeches collected from the 3 places, 35 of 99 samples (35.4%) from Shalang town, 26 of 55 (47.3%) from Nanlang town and 22 of 44 (53.7%) from Xintong town were found trypanosome-positive, respectively. However, the intestinal and mouth regions of all leeches were trypanosome-negative by microscopic examination and by PCR/histological analysis.

When quantified, the trypanosomes from most of the positive leech crop contents numbered around 10^3/ml, but some could be as high as 5×10^6 /ml (Supplementary Video 1). Large numbers of active trypanosomes, including some that were dividing, were observed in the wet smears (Fig. 2E, F; Supplementary Video 1A). We could clearly see that the nuclei of the trypanosomes started dividing prior to the division of the kinetoplasts.

Trypanosomes were morphologically characterized as mainly trypomastigotes with a large nucleus and rounded kinetoplast located in the anterior part and near the posterior end of the body respectively, but with a short or even no free flagellum (Fig. 2A, B, C). It was noteworthy that these trypomastigotes could be observed in various lengths or shapes and sometimes occurred simultaneously in the crop contents of the leeches from the week 0, week 2, week 4 and week 6 groups (Fig. 2D; Supplementary Video 1). They could be divided into three main forms (Fig. 2G; Table 2). The first form (leech form 1, LF1) (Fig. 2A; Fig. 2G, the red dots) and the second form (leech form 2, LF2) (Fig. 2B; Fig. 2G, the blue dots) of trypanosomes were mainly found in the week 0, week 2 and week 4 groups of leeches with lengths of 4.5-9.3 µm (mean 6.2 µm) and 10.4-16.3 µm (mean 13.3 µm) respectively. The flagellates in LF2 show a tapered anterior end and the distance between nucleus and kinetoplast is greater than that in LF1. The third form (leech form 3, LF3) (Fig. 2C; Fig. 2G, the green dots) of trypanosomes was found as the majority component in the week 6 group of leeches and was only occasionally present in earlier groups (Fig. 2D; Supplementary Video 1B). These flagellates are further elongated and with a more tapering and narrower shape of length 18.7-29.2 µm (mean 23.1 µm).

3.3. Phylogenetic analyses of the newly isolated trypanosomes

For species identification, we amplified the *18S rRNA* fragment (2056 bp) (Supplementary Fig. 1B, GenBank accession no. MZ567219) and *gGAPDH* fragment (788 bp) (Supplementary Fig. 1C, GenBank accession no. MZ668450) by using trypanosomes specific primers from 28 leeches collected from SL (including 3 of the week 0 group, 10 of the week 2 group, 2 of the week 4 group and 13 of the week 6 group), 15 leeches from XT and 13 leeches from NL respectively. All of the sequences from trypanosomes from each of the crop samples from different leeches were identical. BLAST analysis identified that the closest *18S rRNA* sequence belongs to those found in a *Trypanosoma theileri*-like sample in a housefly from Northern Russia (GenBank accession no. MK156793). A Neighbor-Joining tree constructed based on the *18S rRNA* sequences also revealed that the trypanosomes isolated from the freshwater leeches belonged to the mammalian group of trypanosomes and are closely related to the subgenus of *Trypanosoma (Megatrypanum)* along with the *T. theileri*-like trypanosome MK156793 at the base (Fig. 3A), while trypanosomes of *Trypanosoma cyclops* clade (including *T. cyclops* from the *Macaca* monkey and *Trypanosoma* sp. TL.AQ.22 isolated from the Haemadipsid leech *Philaemon* sp. from Australia) are more distantly branched.

Results from phylogenetic analysis of *gGAPDH* sequences further supported the notion that the trypanosomes isolated from the leeches in the present study belong to the subgenus of *Trypanosoma (Megatrypanum)* (Fig. 3B). However, to our surprise, our data clearly showed that the trypanosomes we isolated from the freshwater leeches are clearly distinguishable from the typical phylogenetic lineages of the *T. theileri* TthI and TthII clades and also the *T. cyclops* clade. The divergence in *gGAPDH* within phylogenetic lineages of TthI, TthII is 7.8% (Table 1), and the one between our isolated trypanosomes and the above groups are 9.3% and 10%, respectively. Given that, we propose to assign the trypanosome isolated from the freshwater leech as a new *Trypanosoma (Megatrypanum)* species belonging to a "TthIII" phylogenetic lineage.

We have successfully established 13, 13 and 8 cloned populations from the flagellate cultures of leeches collected from SL, XT, and NL, respectively. All clones shared identical sequences in *18S rRNA* gene and *gGAPDH* gene (GenBank accession nos. MZ567220 and MZ668451), meaning that the flagellates from the three collection sites are the same species.

Being clustered alongside members of *T. theileri* group, we think the flagellate may be a trypanosome of a vertebrate or even have a mammalian host that the leeches have fed on. Therefore, we tried to determine the source of the blood from the leech crop contents. DNA of the crop contents was extracted and mammalian *Cyt b* gene was successfully amplified from 19 of them (SL=14; XT=3; NL=2) with specific mammalian *Cyt b* gene primers. BLAST analysis of these *Cyt b* gene sequences shows that all amplicons are identical (GenBank accession no. MZ668449) and were 100% matched with those of water buffalo (*Bubalus bubalis*, KX758330). These results are also supported by the sequences (about 200 bp) (GenBank accession no. MZ520977) of mitochondrial *16S rRNA* gene amplified with specific vertebrate primers. In addition, 76 of 80 TA cloned sequences of mitochondrial *16S rRNA* from eight flagellate positive-leech crop contents (SL=3; XT=3; NL=2) also matched the water buffalo sequence MT186740, while four matched those sequences reported from cattle (*Bos taurus*, MN714218) (Supplementary Table 2). Hereby, we have temporarily named this trypanosome as *Trypanosoma (Megatrypanum) bubalisi* sp. nov. based on the name of the predicted vertebrate host.

3.4. In vitro cultivation of flagellates at 27°C and at 37°C

The extracted mixture crop contents of leech specimens were split into various culture media at 27°C, including SDM79, HIM-11, RPMI-1640, Grace's and L15 media supplied with 10% FBS and 1% penicillin-streptomycin. Only medium L15 could support the growth of *Trypanosoma bubalisi* (Fig. 4A).

In the situation without changing the culture medium, the morphologies of the cloned cultured trypanosomes (continuously cultured) were observed over time and they consisted basically of trypomastigotes (Fig. 4B, C, D; Table 2). They display a tapered anterior end and a sharpened posterior end; the undulating membranes in most individuals are not well developed and are without an obvious free flagellum; the nuclei are located in the anterior part of the body; the kinetoplast is generally located in the posterior end of the body. However, a series of morphological changes were found as time proceeded and were mainly reflected in the body length. At the beginning of inoculation (cultured forms of the flagellates at 27°C for 3 days, Cf27-d3) (Fig. 4B; Fig. 4H, the red dots; Table 2), most trypanosomes looked stubby, being wider, and the body length ranged from 5.1-17.3 μm, with a mean of 8.2 μm. In most individuals, the kinetoplast was a certain distance from the nucleus, while in some individuals, the kinetoplasts were very close to the nuclei (Fig. 4B left; Fig. 4I, the blue dots). At 6 days post inoculation (cultured forms of the flagellates at 27°C for 6 days, Cf27-d6) (Fig. 4C; Fig. 4H, the blue dots; Table 2), these trypanosomes showed a logarithmic growth trait; their body shape was more slender with a length ranging between 3.6-14.8 μm, mean 7.1 μm. Accompanied by nutrient depletion of the inoculation medium, for 19 days post incubation (cultured forms of the flagellates at 27°C for 19 days, Cf27-d19) (Fig. 4D; Fig. 4H, the green dots; Table 2), the growth of the trypanosomes slowed down and presented a homogeneous morphology showing a monomorphic appearance of elongation with tapering. The body was slender and narrow with a body length ranging from 4.5-18.9 μm, mean 11.8 μm. In the dividing cells, we can clearly see that the nuclei divided prior to the kinetoplasts which coincided exactly with the trypanosomes' cell division when observed in leeches. In addition, we found an interesting phenomenon from the cultivation. No obvious morphological changes in the trypanosomes were observed and they could survive over 2 months in the culture if the subculturing process was stopped.

We were interested in the status of this trypanosome species at 37°C. Therefore, the trypanosomes, cultured at 27°C, were transferred into 37°C where they were cultured in a system, containing MDBK cells as supporting cells, with RPMI-1640 medium supplemented with 10% FBS. Figure 5 indicates the morphology and division of some trypanosomes in this system at 37°C. The *T. bubalisi* trypanosomes could clearly survive at 37°C (Fig. 5A). In contrast, complete death was found in a marine fish trypanosome species *T. epinepheli* and freshwater fish trypanosomes *Trypanosoma* sp. (from *Micropterus salmoides*) when they were cultured at 37°C for 3 and 72 hours respectively. After 48 hours of incubation at 37°C, typical trypomastigote forms (Fig. 5C, D; Table 2) were observed and the number of trypomastigotes increased (Fig. 5A). These trypomastigotes are the typical *Trypanosoma theileri*-like forms found in the mammalian host. They have a length (with free flagellum) ranging from 4.2-27.2 μm (mean 13.4 μm) and a larger body width from 1.1-6.1 μm (mean 2.6 μm), the posterior end of the body is extended and sharpened (Fig. 5C, D), the kinetoplast is large and located close to the posterior end of the body, while the undulating membrane is well-developed. After 10 days cultivation at 37°C, dividing cells could still be clearly observed in which the nuclei divided prior to the kinetoplasts (Fig. 5E) which is consistent with the pattern of division found in the leeches.

3.5. Morphological index analysis

In our study, we obsvered a series of morphological changes to the flagellates, isolated from leech crop contents, during *in vitro* culture. To investigate the pattern of these morphological changes and the relationship between the morphological index of flagellates isolated from leeches and those cultured *in vitro,* we measured and analyzed the relevant morphological indices of the flagellates from each stage. The analysis results revealed that the flagellates from the leech crop contents could be classified into three main groups (Fig. 2), with clear distinctions among the different groups based on BL, BW, KI and NI (Fig. 2G). These flagellates could be successfully cultivated at 27°C in L15 medium. The morphological characteristics of the flagellates from the leech crop contents were analysed, but showed that all flagellates cultivated at 27°C did not form distinct clusters (Fig. 4H). The majority of the flagellates were found in the form of trypomastigotes which were similar to those found in the leeches (LF1 and LF2) (Fig. 2A, B; Fig. 4B, C). A subset of cells (Fig. 4B left; Fig. 4I, the blue dots), showing very close proximity of nuclei and kinetoplasts (epimastigotes), were observed but these forms were not found in the leeches. Furthermore, no LF3 form flagellate was identified in the 27°C cultures. Comparing the morphologies of the flagellates cultured at 37°C in the present study and those of *T. theileri* and *T. theileri*-like trypanosomes cultured at 37°C (based on data collected from the literature) (Fig. 5F, the black triangular icon), we found that our flagellates are distinct from the others based, especially, on the body length (BL) ($p < 0.01$).

3.6. Taxonomic summary

The results from the morphological, genetic and culture investigations showed that this trypanosome represents a new species, the description of which is as follows:

Taxonomy

Phylum: Euglenozoa Cavalier-Smith, 1981 emend. Simpson, 1997

Class: Kinetoplastea Honigberg, 1963 emend. Vickerman, 1976

Order: Trypanosomatida Kent, 1880

Family: Trypanosomatidae Doflein, 1901

Genus: *Trypanosoma* Gruby, 1843

Trypanosoma bubalisi sp. nov. Su, 2021

Morphology

Flagellates are morphologically characterized as mainly trypomastigotes with a large elongated oval nucleus, parallel to the long axis and is located in the anterior part of the body; the kinetoplast is oval and is located near/at the posterior end of the body; the undulating membrane and flagellum are clearly visible with a short, or absent, free flagellum. It is noteworthy that the trypomastigotes manifest themselves in various lengths or shapes in the crop contents of *H. manillensis* leeches and can be divided into three main forms. The first form LF1 is a stumpy shape with length from 4.5-9.3 μm; the second form LF2 is much longer than LF1 with length from 10.4-16.3 μm and the third form LF3 is elongated and tapering in a slender and narrow shape with length from 18.7-29.2 μm, which are much longer than LF1 and LF2. The genetic analysis of the three forms demonstrates that each is genetically identical.

Suggested veterbrate host

Bubalus bubalis (Artiodactyla: Bovidae). Based on the gene analysis of the blood samples from the leeches, the source of the blood was from *Bubalus bubalis*.

Type invertebrate host

Hirudinaria manillensis (Arhynchobdellida: Hirudinidae).

Type locality

Town of Shalang (21° 46′ N, 111° 16′E), Maoming city; Nanlang (22° 29′ N, 113° 32′E), Zhongshan city and Xintong (21° 55′ N, 111° 02′E), Gaozhou city, Guangdong Province, China.

Type material

Hapantotype: the cultures of the isolated trypanosomes from *H. manillensis* leeches of Shalang/Xintong/Nanlang, *Trypanosoma bubalisi* CPO-SL (TBCPO-SL), *T. bubalisi* CPO-NL (TBCPO- NL) and *T. bubalisi* CPO-XT (TBCPO- XT); Giemsa-stained smears of leech crop contents and culture forms of *T. bubalisi* trypanosomes.

Paratypes: the cultures of the single clones of TBCPO-SL1, TBCPO-NL1, TBCPO-XT1; their respective collection locations are at the State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou, Guangdong Province, China.

Vector

Likely *Hirudinaria manillensis*.

Site in host: Crop of *Hirudinaria manillensis*, a freshwater leech.

Gene sequences

The 18*S rRNA* gene and partial *gGAPDH* gene of *Trypanosoma bubalisi* sp. nov. trypanosomes are 2056 bp (GenBank accession no. MZ567219) and 788 bp (GenBank accession no. MZ668450), respectively.

Etymology

This species was first discovered in *H. manillensis*, a freshwater leech, but it may be a parasite of water buffalo (*Bubalus bubalis*) based on the colocalization of this parasite with water buffalo blood in the blood meal of the leech, therefore, it is named as *Trypanosoma bubalisi* sp. nov.

4. Discussion

It has been known that the Glossiphoniidae and Piscicolidae leeches are the vectors of many trypanosomes of fish and other aquatic vertebrates (Qudri, 1962; Woo, 1969; Woo and Reilly, 1981; Woo and Jones, 1990; Paparini et al., 2014; Fermino et al., 2020; Smit et al., 2020), while the terrestrial Haemadipsidae leeches are the vector of trypanosomes of frogs, marsupials and mammals (Ewers, 1974; Hamilton et al., 2005; Ellis et al., 2021). However, due to a variety of reasons, the complete life cycles of some trypanosomes in their vectors and vertebrate hosts are not well understood. In addition, little is known about trypanosome infections in the freshwater blood-sucking leeches of the family Hirudinidae.

In the present study, we found some flagellates in the crop contents of a freshwater leech species *Hirudinaria manillensis* in three locations within the Guangdong province, China. These flagellates exhibit typical morphological characteristics of trypanosomes. Considering that co-infections are quite common for various aquatic vertebrate trypanosomes (Grybchuk-Ieremenko et al., 2014; Spodareva, et al., 2018), cloned flagellates were obtained for *18S rRNA* sequencing. The phylogenetic analyses revealed that all cloned flagellates shared an identical sequence and are the same species of *Trypanosoma* (*Megatrypanum*) *theileri*-like trypanosomes.

Trypanosoma (Megatrypanum) is a complex of phylogenetically related species of trypanosomes that apparently infect the species of Ruminantia (former Artiodactyla) (Garcia et al., 2011; Votýpka, et al., 2015; Kostygov et al., 2021), including cattle and water buffalo. Studies on the biological behavior of this leech (*H. manillensis*) showed that it often attacks people or livestock, especially wading animals such as water buffalo, that are working in the fields or swimming in water (Yang, 1996). We therefore suggested that it might be a mammalian trypanosome and, most likely, an unknown trypanosome from water buffalo because they are the livestock most frequently in contact with water. To identify the blood meals from the leeches, sequence analyses based on both *Cyt b* gene and mitochondrial *16S rRNA* amplicons from leech-derived ingested DNA (iDNA) clearly indicated that blood collected from the leeches originated from water buffalo (*Bubalus bubalis*). Because leech-derived ingested DNA (iDNA) has been successfully applied for surveys of vertebrate host biodiversity in recent years (Fahmy et al., 2020), we could thus conclude that the last blood meal of the tested leeches were taken from a water buffalo.

As further evidence to ascertain the host of this trypanosome, the demonstration of successful survival and replication of this trypanosome at 37°C, for weeks, provides direct evidence to demonstrate that this trypanosome is indeed a mammalian parasite. This was in strong contrast with the marine fish trypanosome, *Trypanosoma epinepheli* and the freshwater fish trypanosome, *Trypanosoma* sp. (from *Micropterus salmoides*), neither of which could survive at 37°C for more than 72 hours. Unfortunately, we haven’t yet successfully isolated this parasite from water buffalo and this is, probably, due to the extremely low parasitemia of *T. theileri*-like trypanosomes in these animals (Hoare, 1972; Rodrigues et al., 2003). Therefore, more studies are needed to clarify this point in the near future.

Trypanosoma theileri is a typical species in the subgenus *Megatrypanum* which is widely distributed around the world including China (Du and Li, 1982; Xue and Du, 1985; Wang and Hu, 1988; Pan, 1993; Shi, 2003; Lee et al., 2010). It is divided into two main phylogenetic lineages with at least 10 phylogenetically defined genotypes based on the following molecular markers: internal transcribed spacer (ITS) rDNA, spliced leader (*SL*), cathepsin L-like (*CATL*) and *gGAPDH* genes (Rodrigues et al., 2010; Garcia et al., 2011). Maximum likelihood phylogenetic tree analysis based on the *gGAPDH* gene showed that the trypanosome isolated from *H. manillensis* differed from the two known *T. (Megatrypanum) theileri* phylogenetic lineages. Considering the divergence between the *gGAPDH* sequences from the TthI and TthII isolates was 7.5% (Garcia et al., 2011), the divergence between the new trypanosome and the subtyping of TthI and TthII was even greater, at 9.3% and 10%, respectively. Therefore, it is obvious that the trypanosome isolated from *H. manillensis* certainly belongs to a new phylogenetic lineage (TthIII) of *Trypanosoma* (*Megatrypanum*).

Besides the genetic differences, the morphology of the new trypanosome is also different from the described species in the subgenus *Megatrypanum* in both the culture forms and body length (Du and Li, 1982; Rodrigues et al., 2003). *Trypanosoma* (*Megatrypanum*) contains a group of large mammalian blood trypanosomes with a small kinetoplast located very close to the nucleus and trypomastigotes forms in cell culture which vary in average size from 27-36 μm (Rodrigues et al., 2003). On the other hand, the new trypanosome varies in size from 4.2-27.2 μm and is mainly found with the kinetoplast situated in the middle of the region between the posterior end and the nucleus. In addition, it is a very interesting characteristic that no free flagellum could be observed in any of the forms of this new trypanosome whether they were collected from the leech crop or cultured at

27°C. Based on the obvious features of these trypanosomes, we consider that it is a new species in the subgenus of *Megatrypanum* and have named it *Trypanosoma bubalisi* sp. nov.

A close relationship between *T. theileri* and *Trypanosoma cyclops* has been suggested previously (Stevens et al., 1998; Hamilton et al., 2005). *T. cyclops* was isolated from two species of south-east Asian *Macaca* monkeys in Malaysia by Weinman (1972) and it was demonstrated to be an early-branched member of the *T. theileri* clade (Martinkovic et al., 2012; Rodrigues et al., 2006). Interestingly, based on their analyses, *T. bubalisi* sits between these two groups, therefore, it is suggested that it possibly represents an evolutionary intermediate position. By analysis of *gGAPDH* sequences divergence, however, we found that the *T. cyclops* clade was even further removed from the two main phylogenetic lineages of *T. theileri* and the new third lineage of *T. bubalisi*. Therefore, we consider that the *T. cyclops* clade should be considered as a fourth lineage (TthIV).

So far as we know, species within the *Trypanosoma (Megatrypanum)* group are principally transmitted by tabanid flies or by some specific blood sucking arthropods, such as sheep keds (Böse et al., 1987; Böse and Heister, 1993; Calzolari et al., 2018; Werszko et al., 2019). Therefore, previous studies on the transmission of *T. theileri* and *T. theileri*-like trypanosomes have primarily been focused on blood sucking arthropods with a much lesser consideration of other invertebrate vectors. In the present study, different morphological forms of trypanosomes (LF1, LF2 and LF3) were observed in the leech crop contents indicating a series of developmental stages of this trypanosome in the leech. The proportions of LF1 and LF2 predominated initially in the population and then declined with time, whilst LF3 appeared later and then increased in proportion. This led us to suggest that a differentiation process from LF1 and LF2 into LF3 is occurring. When we successfully established *in vitro* cultivation of these trypanosomes, the cloned population grew well at 27°C and was represented as LF1 and LF2 only (Cf27-d3 and Cf27-d6/ Cf27-d19) whilst LF3 was not found. It is suggested that LF3 is probably a specific form in the leech crop which is an outcome of differentiation. Although we did not find the metacyclic stage in the leech, our findings indicated that this new trypanosome could survive and undergo a series of morphological developmental stages in the leech crop. More studies on the relationship between *T. bubalisi* and *H. manillensi* are needed and will certainly provide evidence to gain a better understanding of the role of this leech on the transmission of this new mammalian trypanosome.

In conclusion, we have isolated a new trypanosome from the freshwater leech *H. manillensis.* It is suggested that the mammalian host of this trypanosome is the water buffalo. Based on morphological and molecular analysis, this trypanosome is named *Trypanosoma bubalisi* sp. nov. which is a new TthIII phylogenetic lineage to the *Trypanosoma* (*Megatrypanum*) clade. Our work also highlights the potential role of freshwater leeches on the transmission of mammalian trypanosomes.

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Legends to Figures

Fig. 1. Sampling sites and morphological identification of leeches (*Hirudinaria manillensis*). (A) Locations of sampling sites in the Guangdong province, P. R. China. (B, C) Pictures of the types of environments where *H. manillensis* were collected (Shalang town), the red arrow shows a leech in the natural water body. (D, E) The morphology of *H. manillensis*. The anterior end is to the left, (D) the dorsal view and (E) the ventral view.

Fig. 2. Photomicrographs and morphological indices of Giemsa-stained trypanosomes from leech crop contents. (A) leech form 1 (LF1); (B) leech form 2 (LF2); (C) leech form 3 (LF3) and dividing forms (E, F). N, nucleus; K, kinetoplast. (D) The percentages of each form of trypanosomes from the Shalang leeches are indicated at time points. (G) Morphological indices of each form. BW, maximum body width; BL, body length; PN, posterior end to mid-nucleus; NA, anterior end to mid-nucleus; KN, kinetoplast to mid-nucleus; NI, nuclear index; KI, kinetoplastic index. Bars correspond to 10 μm.

Fig. 3. Phylogenetic trees of trypanosomes based on *18S rRNA* and *gGAPDH* sequences. (A) Phylogenetic trees of trypanosomes based on *18S rRNA* sequences, including 36 trypanosome sequences of aquatic and terrestrial lineages. (B) Phylogenetic trees of trypanosomes based on *gGAPDH* sequences, including 33 trypanosome sequences of phylogenetic lineages of TthI, TthII and the *T. cyclops* clade trypanosomes. *Trypanoplasma borreli* was used as outgroup and the *Trypanosoma bubalisi* from our work is shaded. Numbers at nodes are the support values for the major branches derived from 1000 replicates respectively for Neighbor Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses; ns, not supported and the accession numbers of sequences in GenBank are showed in parenthesis.

Fig. 4. The growth curves, photomicrographs and morphological indices of trypanosomes cultured at 27°C *in vitro*. (A) The growth curve of trypanosomes in SDM79, HMI-11, RPMI-1640, Grace's and L15 medium at 27°C for 11 days. (B-D) Photomicrographs of Giemsa-stained main forms of trypanosomes in L15 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 27°C. (E-G) suggested progression of cell division. N, nucleus; K, kinetoplast; bars correspond to 10 µm. (H) Morphological indices of trypanosomes cultured at 27°C for 3 days (Cf27-d3), 6 days (Cf27-d6) and 19 days (Cf27-d19). (I) Morphological indices of different forms of trypanosomes. Try, trypomastigotes; Epi, epimastigotes. BW, maximum body width; BL, body length; PN, posterior end to mid-nucleus; NA, anterior end to mid-nucleus; KN, kinetoplast to mid-nucleus; NI, nuclear index; KI, kinetoplastic index.

Fig. 5. The growth curves, photomicrographs and morphological indices of trypanosomes cultured at 37°C *in vitro*. (A) The growth curve of *T. bubalisi* and *Trypanosoma* sp. from *Micropterus salmoides* in RPMI-1640 and SDM79 medium at 37°C for 96 h. Photomicrographs of Giemsa-stained trypanosomes at 37°C for 24 h (B), 48 h (C), 72 h (D) and the dividing form at 240 h (E), bars correspond to 10 µm. (F) Morphological indices of *T. bubalisi* and other reference *T. theileri* and *T. theileri*-like at 37°C (Kingston et al., 1992; Saisawa et al., 1993; Woo et al., 1970; Büscher and Friedhoff, 1984; Nalbantoğlu and Karaer, 2008; Kingston et al., 1982; Matthews et al., 1977; Kingston and Morton, 1975). BW, maximum body width; BL, body length; PN, posterior end to mid-nucleus; NA, anterior end to mid-nucleus; KN, kinetoplast to mid-nucleus.

Table 1. The *gGAPDH* sequence divergence (%) between *Trypanosoma bubalisi* and the phylogenetic lineages of the *T. theileri* TthI, TthII clades and the *T. cyclops* clade trypanosomes.

	TthI[a]	**TthII**[b]	***T. cyclops***[c]	***T. bubalisi***
TthI[a]		7.8	11.6	9.3
TthII[b]	7.8		10.5	10.0
T. cyclops[c]	11.6	10.5		10.3
T. bubalisi	9.3	10.0	10.3	

a		**b**		**c**	
Sequence	**Host**	**Sequence**	**Host origin**	**Sequence**	**Host origin**
HQ664791	Buffalo	HQ664801	Cattle	AJ620280	haemadipsid leech
HQ664784	Buffalo	HQ664802	Cattle		
HQ664785	Buffalo	HQ664803	Cattle	FJ649493	*Macaca* monkey
HQ664786	Buffalo	HQ664794	Cattle		
HQ664787	Buffalo	HQ664795	Cattle		
HQ664788	Buffalo	HQ664796	Cattle		
HQ664789	Buffalo	HQ664797	Cattle		
HQ664790	Buffalo	HQ664798	Cattle		
HQ664792	Cattle	HQ664799	Cattle		
HQ664793	Cattle	AJ620282	Cattle		
		HQ664800	Cattle		
		HQ664804	Duiker		
		HQ664805	Duiker		
		FM164792	Sitatunga		
		HQ664806	Deer		

Table 2. Comparisons of the morphological indices (in µm) of trypanosomes from the freshwater leech crop contents and *in vitro* cultivation in comparison to those reported from *T. theileri*, *T. theileri*-like trypanosomes and *T. cyclops*.

Group	N =		PK	KN	PN	NA	BL
LF1	131	m	2.0±0.6	2.1±0.6	4.1±1.0	2.1±0.6	6.2±1.4
		r	1.2-3.4	1.1-3.8	2.9-6.5	1.1-3.0	4.5-9.3
LF2	155	m	3.0±1.6	5.1±1.7	8.2±1.8	5.2±1.7	13.3±1.6
		r	0.5-9.7	1.2-9.9	2.5-11.6	2.0-12.1	10.4-16.3
LF3	81	m	6.5±1.3	9.4±1.3	15.9±2.1	7.2±1.2	23.1±2.4
		r	3.3-11.6	5.2-12.5	12.3-22.6	5.8-14.3	18.7-29.2
Cf27-d3	55	m	2.5±0.8	2.5±0.8	4.4±1.2	3.8±1.1	8.2±2.0
		r	0.7-5.4	0.7-4.0	1.7-9.0	2.0-8.3	5.1-17.3
Cf27-d6	149	m	2.4±0.8	2.3±0.7	4.0±1.3	3.1±1.2	7.1±2.0
		r	0.4-5.1	0.7-5.2	1.5-9.7	0.7-6.5	3.6-14.8
Cf27-d19	73	m	2.5±0.8	3.1±0.8	5.4±1.4	6.3±2.1	11.8±3.2
		r	0.8-4.5	1.0-5.5	2.1-9.4	1.9-11.1	4.5-18.9
C37	245	m	1.7±1.5	4.7±1.2	6.4±1.9	5.9±1.6	12.3±2.9
		r	0-10.7	1.3-10.0	2.1-15.5	1.9-11.9	4.2-26.5
Trypanosoma theileri [1]		m	7.4±3.3	8.9±2.6	16.2±5.1	20.2±6.3	36.4±10.5
		r	0-17	2-20	5-33	7-36	13-59
T. theileri [2]		m	11.6±2.9	5.1±1.3	16.6±3.6	16.6±3.3	33.9±5.3
T. theileri [2]		m	12.5±3.7	2.1±1.7	14.5±4.9	14.5±4.9	31.2±5.3
T. theileri [2]		m	26.6±3.8	-	-	6.7±1.4	33.6±7.3
T. theileri [3]		m	29.8	6.4	36.2	39.7	75.5
		r	17-43	4-13	21-56	30-53	61-96
T. theileri [3]		m	25.9	8.2	33.1	39.3	73.6
		r	13-37	5-12	17-49	32-55	64-82
T. theileri [4]		m	12.8	5.5	18.3	15.4	33.7
		r	9-18	3-12	13-22	10-20	23-41
Trypanosoma melophagium[5]	111	m	14.7±2.9	5.1±1.1	19.8±3.5	19.5±1.9	-
		r	-	-	-	-	-
T. melophagium [4]	304	m	14.7±2.9	5.1±1.1	19.8±3.5	19.5±1.9	39.3
T. melophagium [6]	50	m	9.4	1.9	10.9	11.5	23.6
		r	6-13.5	0.1-3.7	6-15.7	6-15.7	16.5-33
Trypanosoma cervi [7]	21	m	9.1±3.8	7.2±1.6	16.5±4.3	21.9±5.9	38.5±9.2
T. cervi [7]	14	m	12.8±5.0	7.0±1.8	19.2±5.8	25.0±2.6	45.4±8.1
T. cervi [7]	56	m	7.5±3.3	5.9±1.6	13.1±2.9	17.0±3.4	30.3±5.1
T. cervi [7]	41	m	13.5±4.8	8.3±2.5	22.1±5.0	26.3±6.3	48.2±9.1
T. cervi [7]	4	m	7.5±1.0	7.0±0	14.5±1.0	15.2±1.0	30.3±1.7
T. cervi [4]	27	m	5.7	6	11.5	15.5	27.4

		r	3-11	4-8	8-18	10-22	21-34
T. cervi [4]	174	m	11.5±5.6	7.0±2.1	18.5±6.3	23.3±7.3	42.0±12.4
		r	3-27	2-14	8-36	10-43	21-74
T. cervi [8]	28	m	9.2	5.4	14.6	18.4	33.1
		r	3-15	2-13	10-19	12-26	26-42
***T. cervi* sp. n.**[9]	14	m	12.2	7	19.4	24.8	45.4
		r	5-20	4-9	11-32	20-30	32-56
Trypanosoma stefanskii [4]	50	m	15.9±6.4	5.8±2.1	23.3±5.6	32.0±5.1	55.1±9.2
		r	0-28	0-10	13-33	20-40	37-71
T. stefanskii [4]	40	m	14.2±5.7	5.8±1.3	19.9±6.3	28.2±6.1	48.0±11.5
		r	-	-	-	-	26-70
T. stefanskii [4]	72	m	13.5±5.3	6.3±2.0	19.6±5.0	27.8±6.7	47.3±10.3
		r	2-24	3-15	11-30	11-42	26-68
***Trypanosoma* sp.**[4]	86	m	9.8±5.7	5.8±1.4	15.5±5.7	18.1±5.0	33.6±9.5
Trypanosoma cyclops [10]	3	m	9.1±0.5	9.7±1.8	18.8±2.4	7.8±1.6	26.1±3.4
		r	8.7-9.6	7.7-11.1	16.1-20.4	6.4-9.6	22.7-29.5

Table 2. Continued.

Group		L	BW	FF	NI	KI	FI
LF1	m	=BL	2.0±0.3	0	2.0±0.5	2.0±0.4	-
	r	-	1.4-2.5	-	1.3-3.1	1.5-3.1	-
LF2	m	=BL	1.5±0.4	0	1.8±0.9	1.8±1.3	-
	r	-	0.6-2.6	-	0.2-5.3	1.1-9.3	-
LF3	m	=BL	1.7±0.4	0	2.2±0.4	1.7±0.2	-
	r	-	1.0-3.6	-	1.0-3.4	1.3-2.8	-
Cf27-d3	m	9.9±2.1	2.3±0.4	1.7±0.8	1.2±0.4	1.9±0.6	0.2±0.1
	r	6.4-19.7	1.6-3.3	0-3.9	0.3-2.0	1.1-4.3	0-0.5
Cf27-d6	m	8.8±2.6	3.1±0.7	1.8±1.4	1.5±0.8	1.8±0.5	0.3±0.2
	r	4.2-16.8	1.5-5.5	0-9.3	0.4-5.1	0.8-3.4	0-1.4
Cf27-d19	m	12.3±3.2	2.1±0.4	0.5±0.6	0.9±0.3	1.7±0.3	0±0.1
	r	4.5-18.9	1.1-3.6	0-2.7	0.5-1.8	1.0-2.8	0-0.2
Cf37	m	13.4±3.5	2.6±0.7	1.1±1.5	1.1±0.4	1.4±0.3	0.1±0.1
	r	4.2-27.2	1.1-6.1	0-8.2	0.5-2.6	0.7-3.0	0-1.1
Trypanosoma theileri [1]	m	50.5±12.7	3.3±2.0	14.2±4.5	0.9±0.2	0.5±0.4	0.36
	r	16-90	1-13	1-37	0.4-1.7	1-4	-
T. theileri [2]	m	48.6±6.4	3.1±0.8	15.2±5.7	0.7±0.3	3.4±0.6	2.3±0.9
T. theileri [2]	m	46.4±8.1	2.8±0.7	15.2±5.5	0.9±0.9	7.1±2.3	2.3±0.9
T. theileri [2]	m	56.4±8.8	3.2±0.9	23.1±4.4	4.3±1.5	-	1.5±0.9
T. theileri [3]	m	84.4	8.1	8.9	0.9	5.7	0.1
	r	67-109	5.5-11	7-14	0.7-1.1	4.4-5	-
T. theileri [3]	m	82	4.4	8.4	0.84	4	0.1
	r	69-95	3-6	5-13	0.54-1.1	3.6-4.1	
T. theileri [4]	m	47.6	2.6	13.9	1.2	3.3	0.4
	r	31-65	1.4-4	8-24	1.1-1.3	1.8-4.5	-
Trypanosoma melophagium[5]	m	45.3±4.1	3.1	6±1.6	1.1	3.8	-
	r		2.1-4.6	--	0.9-1.2	3.3-4.9	-
T. melophagium [4]	m	45.3±4.1	3.1	6±1.6	1.1	0.26	0.2
T. melophagium [6]	m	31.1	2	6.8	0.9	4.2	-
	r	25.5-40.5	1.5-3	4.5-10.5	0.5-1.6	2.5-6.6	-
Trypanosoma cervi [7]	m	45.4±9.3	4.2±1.8	7.1±2.8	1.3±0.2	0.4±0.6	0.2±3.0
T. cervi [7]	m	51.9±7.9	4.4±1.6	6.5±2.5	1.3	0.4	0.1
T. cervi [7]	m	37.2±5.7	5.2±2.0	7.0±3.4	1.3	0.4	0.2
T. cervi [7]	m	57.7±10.4	5.6±2.4	9.5±3.3	1.1	0.4	0.2
T. cervi [7]	m	38.5±1.9	2.8±0.3	8.3±0.8	0.9±0.1	0.5	0.3
T. cervi [4]	m	35.5	4.2	8.2	0.7	1.9	0.3
	r	26-42	2-8	0-16	0.5-1.2	1.2-3.2	-
T. cervi [4]	m	50.1±13.6	5.5±2.5	8.2±3.2	0.8±0.2	0.4	0.2
	r	26-83	1-13	0-21	0.4-2.7	-	-

T. cervi **[8]**	m	38.8	6.1	5.7	0.8	2.9	0.1
	r	28-51	2-9	1-14	0.4-1.3	1.2-5.5	-
T. cervi **sp. n.[9]**	m	52	4.6	6.6	0.8	2.8	0.1
	r	40-61	3-8	3-11	0.5-1.3	2.8-3.6	-
Trypanosoma stefanskii **[4]**	m	55.1±9.2	5.7±1.7	0	0.7±0.2	0.3±1.4	0
	r	34-71	3-10	-	0.4-1.1	0-8	-
T. stefanskii **[4]**	m	48.0±11.5	5.8±1.9	0	0.7±0.2	0.3±0.9	0
	r	26-70	2-11	-	0.4-0.9	1.7-5.4	-
T. stefanskii **[4]**	m	55.0±10.3	6.5±2.5	7.7±2.7	0.7±0.3	0.3±1.1	0.1
	r	37-75	2-13	4-17	0.4-2.3	1-6	-
Trypanosoma **sp.[4]**	m	44.0±8.5	-	10.4±2.5	0.9±0.2	0.3±1.0	0.3
Trypanosoma cyclops **[10]**	m	34.1±5.9	2.9±0.2	8.0±2.6	2.5±0.4	2.0±0.1	0.3±0.1
	r	27.8-39.5	2.6-3.0	5.1-10.0	2.1-2.8	1.8-2.1	0.2-0.3

LF1, leech form 1; **LF2**, leech form 2; **LF3**, leech form 3; **Cf27-d3, -d6, and -d19**, refers to the cultured forms of the flagellates at 27°C for 3, 6 and 19 days, respectively; **Cf37**, refers to the morphological index of the flagellates cultured at 37°C. The numbers of 1-10 beside the names of trypanosomes are the morphological indices of *T. theileri* and *T. theileri*-like trypanosomes which were cited from references (**1**, Kingston et al., 1992; **2**, Pan, 1993; **3**, Saisawa et al., 1993; **4**, Woo et al., 1970; **5**, Büscher and Friedhoff, 1984; **6**, Nalbantoğlu and Karaer, 2008; **7**, Kingston et al., 1982; **8**, Matthews et al., 1977; **9***,* Kingston and Morton, 1975; **10**, Weinman, 1972). The m represents the mean and the standard deviation, while r represents the range of the length. PK, posterior end to kinetoplast; KN, kinetoplast to mid-nucleus; PN, posterior end to mid-nucleus; NA, anterior end to mid-nucleus; BL, body length; L, total length with flagellum; FF, free flagellum length; BW, maximum body width; and indices: nuclear index NI = PN/NA; kinetoplastic index KI = PN/KN; flagellar index FF:BL (=FF/BL).

Table 1. Comparisons of the morphological indices (in µm) of trypanosomes from the freshwater leech crop contents and *in vitro* cultivation in comparison to those reported from *T. theileri*, *T. theileri*-like trypanosomes and *T. cyclops*.

Group	N =		PK	KN	PN	NA	BL
LF1	131	m	2.04±0.69	2.00±0.63	4.15±1.02	2.14±0.63	6.29±1.50
		r	1.10-3.58	1.13-3.78	2.91-6.47	1.10-3.58	4.52-9.30
LF2	155	m	3.03±1.58	5.12±1.73	8.15±1.81	5.16±1.74	13.31±1.56
		r	0.45-9.74	1.16-9.89	2.46-11.63	1.95-12.11	10.38-16.33
LF3	81	m	6.47±1.26	9.41±1.30	15.88±2.07	7.19±1.88	23.07±2.42
		r	3.35-11.56	5.18-12.52	12.31-22.64	5.77-14.35	18.71-29.24
Cf27-d3	55	m	2.47±0.79	2.51±0.77	4.43±1.25	3.81±1.08	8.24±1.95
		r	0.69-5.42	0.73-4.04	1.68-9.01	2.03-8.27	5.15-17.28
Cf27-d6	149	m	2.40±0.75	2.3±0.7	3.99±1.29	3.06±1.24	7.05±1.99
		r	0.43-5.12	0.72-5.19	1.53-9.69	0.68-6.51	3.62-14.85
Cf27-d19	73	m	2.55±0.83	3.15±0.79	5.39±1.42	6.3±2.08	11.83±3.24
		r	0.75-4.48	1.03-5.47	2.11-9.35	1.94-11.07	4.51-18.88
C37	245	m	1.72±1. 5	4.68±1.23	6.37±1.94	5.92±1.55	12.31±2.93
		r	0-10.67	1.310.03	2.05-15.51	1.9-11.92	4.22-26.48
Trypanosoma theileri [1]		m	7.4±3.3	8.9±2.6	16.2±5.1	20.2±6.3	36.4±10.5
		r	0-17	2-20	5-33	7-36	13-59
T. theileri [2]		m	11.58±2.85	5.1±1.33	16.56±3.58	16.63±3.30	33.85±5.29
T. theileri [2]		m	12.46±3.69	2.06±1.68	14.52±4.94	14.52±4.94	31.21±5.31
T. theileri [2]		m	26.63±3.75			6.72±1.43	33.6±7.26
T. theileri [3]		m	29.8	6.4	36.2	39.7	75.5
		r	17-43	4-13	21-56	30-53	61-96
T. theileri [3]		m	25.9	8.2	33.1	39.3	73.6
		r	13-37	5-12	17-49	32-55	64-82
T. theileri [4]		m	12.8	5.5	18.3	15.4	33.7
		r	9-18	3-12	13-22	10-20	23-41
Trypanosoma melophagium[5]	111	m	14.7±2.9	5.1±1.1	19.8±3.5	19.5±1.9	
		r					
T. melophagium [4]	304	m	14.7±2.9	5.1±1.1	19.8±3.5	19.5±1.9	39.3
T. melophagium [6]	50	m	9.4	1.9	10.9	11.5	23.6
		r	6-13.5	0.1-3.7	6-15.7	6-15.7	16.5-33

Trypanosoma cervi[7]	21	m	9.14±3.83	7.19±1.57	16.52±4.26	21.9±5.87	38.48±9.23
T. cervi[7]	14	m	12.75±4.97	7±1.84	19.21±5.81	25±2.63	45.43±8.11
T. cervi[7]	56	m	7.5±3.25	5.77±1.6	13.11±2.93	17.02±3.35	30.29±5.08
T. cervi[7]	41	m	13.54±4.84	8.34±2.51	22.05±4.98	26.27±6.32	48.17±9.06
T. cervi[7]	4	m	7.5±0.96	7±0	14.5±0.96	15.2±1.03	30.3±1.7
T. cervi[4]	27	m	5.7	6	11.5	15.5	27.4
		r	3-11	4-8	8-18	10-22	21-34
T. cervi[4]	174	m	11.5±5.6	7±2.1	18.5±6.34	23.3±7.3	42±12.44
		r	3-27	2-14	8-36	10-43	21-74
T. cervi[8]	28	m	9.2	5.4	14.6	18.4	33.1
		r	3-15	2-13	10-19	12-26	26-42
***T. cervi* sp. n.**[9]	14	m	12.2	7	19.4	24.8	45.4
		r	5-20	4-9	11-32	20-30	32-56
Trypanosoma stefanskii[4]	50	m	15.9±6.39	5.8±2.07	23.3±5.59	32±5.1	55.1±9.23
		r	0-28	0-10	13-33	20-40	37-71
T. stefanskii[4]	40	m	14.2±5.66	5.8±1.32	19.88±6.3	28.2±6.07	48±11.5
		r					26-70
T. stefanskii[4]	72	m	13.5±5.32	6.3±1.96	19.57±4.94	27.8±6.71	47.3±10.3
		r	2-24	3-15	11-30	11-42	26-68
***Trypanosoma* sp.**[4]	86	m	9.8±5.7	5.8±1.4	15.5±5.7	18.1±5.0	33.6±9.5
Trypanosoma cyclops[10]	3	m	9.07±0.47	9.73±1.8	18.8±2.35	7.8±1.64	26.13±3.4
		r	8.7-9.6	7.7-11.1	16.1-20.4	6.4-9.6	22.7-29.5

Table 1. Continued

Group		L	BW	FF	NI	KI	FI
LF1	m	=BL	2.00±0.37	0	2.03±0.52	2.03±0.44	-
	r		1.43-2.78		1.26-3.11	1.46-3.09	
LF2	m	=BL	1.49±0.45	0	1.81±0.87	1.82±1.31	-
	r		0.56-2.60		0.24-5.34	1.06-9.26	
LF3	m	=BL	1.71±0.44	0	2.25±0.40	1.70±0.17	-
	r		1.03-3.55		1.00-3.43	1.29-2.77	
Cf27-d3	m	9.89±2.11	2.27±0.42	1.66±0.75	1.21±0.35	1.87±0.6	0.21±0.1
	r	6.42-19.65	1.59-3.26	0-3.89	0.3-2.04	1.05-4.23	0-0.49
Cf27-d6	m	8.83±2.55	3.05±0.73	1.78±1.37	1.54±0.83	1.8±0.5	0.26±0.21
	r	4.19-16.83	1.47-5.45	0-9.27	0.45-5.15	0.84-3.4	0-1.4
Cf27-d19	m	12.29±3.19	2.11±0.42	0.46±0.59	0.91±0.27	1.73±0.3	0.04±0.06
	r	4.51-18.9	1.1-3.59	0-2.73	0.48-1.76	1-2.83	0-0.23
Cf37	m	13.36±3.51	2.65±0.72	1.05±1.46	1.13±0.39	1.38±0.29	0.09-0.12
	r	4.22-27.2	1.08-6.14	0-8.22	0.52-2.46	0.71-2.97	0-1.05
Trypanosoma theileri [1]	m	50.5±12.7	3.3±2.02	14.2±4.5	0.88±0.22	0.54±0.42	0.36
	r	16-90	1-13	1-37	0.43-1.67	1-4	
T. theileri [2]	m	48.63±6.39	3.12±0.81	15.15±5.69	0.72±0.25	3.37±0.63	2.29±0.87
T. theileri [2]	m	46.36±8.06	2.76±0.68	15.15±5.52	0.88±0.92	7.05±2.31	2.33±0.91
T. theileri [2]	m	56.36±8.75	3.16±0.87	23.08±4.35	4.25±1.48		1.49±0.87
T. theileri [3]	m	84.4	8.1	8.9	0.91	5.68	0.12
	r	67-109	5.5-11	7-14	0.7-1.1	4.4-5	
T. theileri [3]	m	82	4.4	8.4	0.84	4	0.12
	r	69-95	3-6	5-13	0.54-1.1	3.6-4.1	
T. theileri [4]	m	47.6	2.6	13.9	1.2	3.3	0.42
	r	31-65	1.4-4	8-24	1.1-1.3	1.8-4.5	
Trypanosoma melophagium [5]	m	45.3±4.1	3.1	6±1.6	1.1	3.8	
	r		2.1-4.6		0.9-1.2	3.3-4.9	
T. melophagium [4]	m	45.3±4.1	3.1	6±1.6	1.1	0.26	0.15
T. melophagium [6]	m	31.1	2	6.8	0.9	4.2	
	r	25.5-40.5	1.5-3	4.5-10.5	0.5-1.6	2.5-6.6	
Trypanosoma cervi [7]	m	45.43±9.27	4.19±1.75	7.1±2.76	1.3±0.16	0.43±0.56	0.18±2.96
T. cervi [7]	m	51.93±7.85	4.43±1.56	6.5±2.47	1.3	0.35	0.12

T. cervi [7]	m	37.2±5.74	5.21±1.97	7.02±3.36	1.27	0.41	0.17
T. cervi [7]	m	57.66±10.4	5.59±2.4	9.46±3.28	1.13	0.35	0.18
T. cervi [7]	m	38.5±1.85	2.8±0.25	8.3±0.75	0.9±0.06	0.48	0.26
T. cervi [4]	m	35.5	4.2	8.2	0.7	1.9	0.26
	r	26-42	2-8	0-16	0.5-1.2	1.2-3.2	
T. cervi [4]	m	50.1±13.64	5.5±2.48	8.2±3.24	0.8±0.22	0.37	0.16
	r	26-83	1-13	0-21	0.42-2.67		
T. cervi [8]	m	38.8	6.1	5.7	0.8	2.9	0.12
	r	28-51	2-9	1-14	0.4-1.3	1.2-5.5	
***T. cervi* sp. n.**[9]	m	52	4.6	6.6	0.78	2.77	0.14
	r	40-61	3-8	3-11	0.5-1.3	2.8-3.6	
Trypanosoma stefanskii [4]	m	55.1±9.23	5.66±1.65	0	0.73±0.15	0.27±1.44	0
	r	34-71	3-10		0.43-1.1	0-8	
T. stefanskii [4]	m	48±11.5	5.8±1.94	0	0.71±0.15	0.29±0.89	0
	r	26-70	2-11		0.41-0.93	1.7-5.4	
T. stefanskii [4]	m	55±10.34	6.53±2.5	7.7±2.73	0.73±0.25	0.3±1.09	0.14
	r	37-75	2-13	4-17	0.41-2.27	1-6	
***Trypanosoma* sp.**[4]	m	44±8.5		10.4±2.5	0.87±0.16	0.34±0.98	0.34
Trypanosoma cyclops [10]	m	34.13±5.91	2.87±0.23	8±2.57	2.45±0.35	1.95±0.13	0.3±0.07
	r	27.8-39.5	2.6-3	5.1-10	2.07-2.76	1.84-2.09	0.22-0.34

LF1, leech form 1; **LF2**, leech form 2; **LF3**, leech form 3; **Cf27-d3, -d6, and -d19**, refers to the cultured forms of the flagellates at 27°C for 3, 6 and 19 days, respectively; **Cf37**, refers to the morphological index of the flagellates cultured at 37°C. The numbers of 1-10 beside the names of trypanosomes are the morphological indices of *T. theileri* and *T. theileri*-like trypanosomes which were cited from references (**1**, Kingston et al., 1992; **2**, Pan, 1993; **3**, Saisawa et al., 1993; **4**, Woo et al., 1970; **5**, Büscher and Friedhoff, 1984; **6**, Nalbantoğlu and Karaer, 2008; **7**, Kingston et al., 1982; **8**, Matthews et al., 1977; **9***,* Kingston and Morton, 1975; **10**, Weinman, 1972). The m represents the mean and the standard deviation, while r represents the range of the length. PK, posterior end to kinetoplast; KN, kinetoplast to mid-nucleus; PN, posterior end to mid-nucleus; NA, anterior end to mid-nucleus; BL, body length; L, total length with flagellum; FF, free flagellum length; BW, maximum body width; and indices: nuclear index NI = PN/NA; kinetoplastic index KI = PN/KN; flagellar index FF:BL (=FF/BL).

Table 2. The *gGAPDH* sequence divergence (%) between *Trypanosoma bubalisi* and the phylogenetic lineages of the *T. theileri* TthI, TthII clades and the *T. cyclops* clade trypanosomes.

	TthI[a]	**TthII**[b]	***T. cyclops***[c]	***T. bubalisi***
TthI[a]		7.8	11.6	9.3
TthII[b]	7.8		10.5	10.0
T. cyclops[c]	11.6	10.5		10.3
T. bubalisi	9.3	10.0	10.3	

a		**b**		**c**	
Sequence	**Host origin**	**Sequence**	**Host origin**	**Sequence**	**Host origin**
HQ664791	Buffalo	HQ664801	Cattle	AJ620280	haemadipsid leech
HQ664784	Buffalo	HQ664802	Cattle		
HQ664785	Buffalo	HQ664803	Cattle	FJ649493	*Macaca* monkey
HQ664786	Buffalo	HQ664794	Cattle		
HQ664787	Buffalo	HQ664795	Cattle		
HQ664788	Buffalo	HQ664796	Cattle		
HQ664789	Buffalo	HQ664797	Cattle		
HQ664790	Buffalo	HQ664798	Cattle		
HQ664792	Cattle	HQ664799	Cattle		
HQ664793	Cattle	AJ620282	Cattle		
		HQ664800	Cattle		
		HQ664804	Duiker		
		HQ664805	Duiker		
		FM164792	Sitatunga		
		HQ664806	Deer		

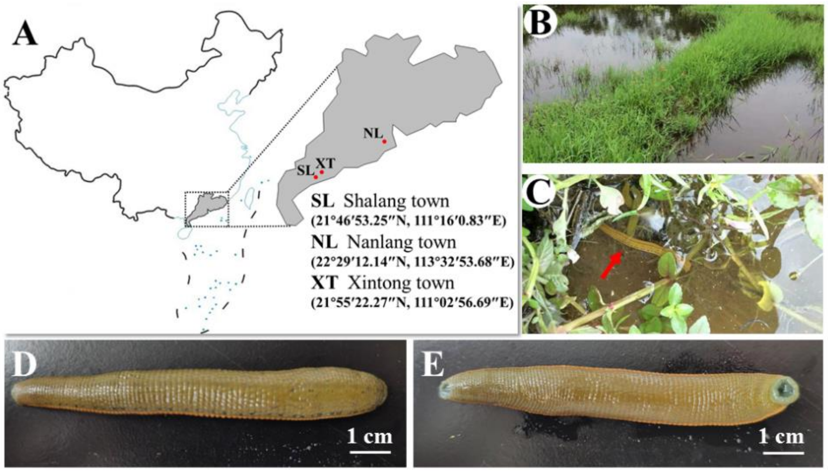


Fig. 1. Sampling sites and morphological identification of leeches (*Hirudinaria manillensis*). (A) Locations of sampling sites in Guangdong province, P. R. China. (B, C) Pictures of the types of environments where *H. manillensis* were collected (Shalang town), the red arrow shows a leech in the natural water body. (D, E) The morphology of *H. manillensis*. The anterior end is to the left, (D) the dorsal view and (E) the ventral view.

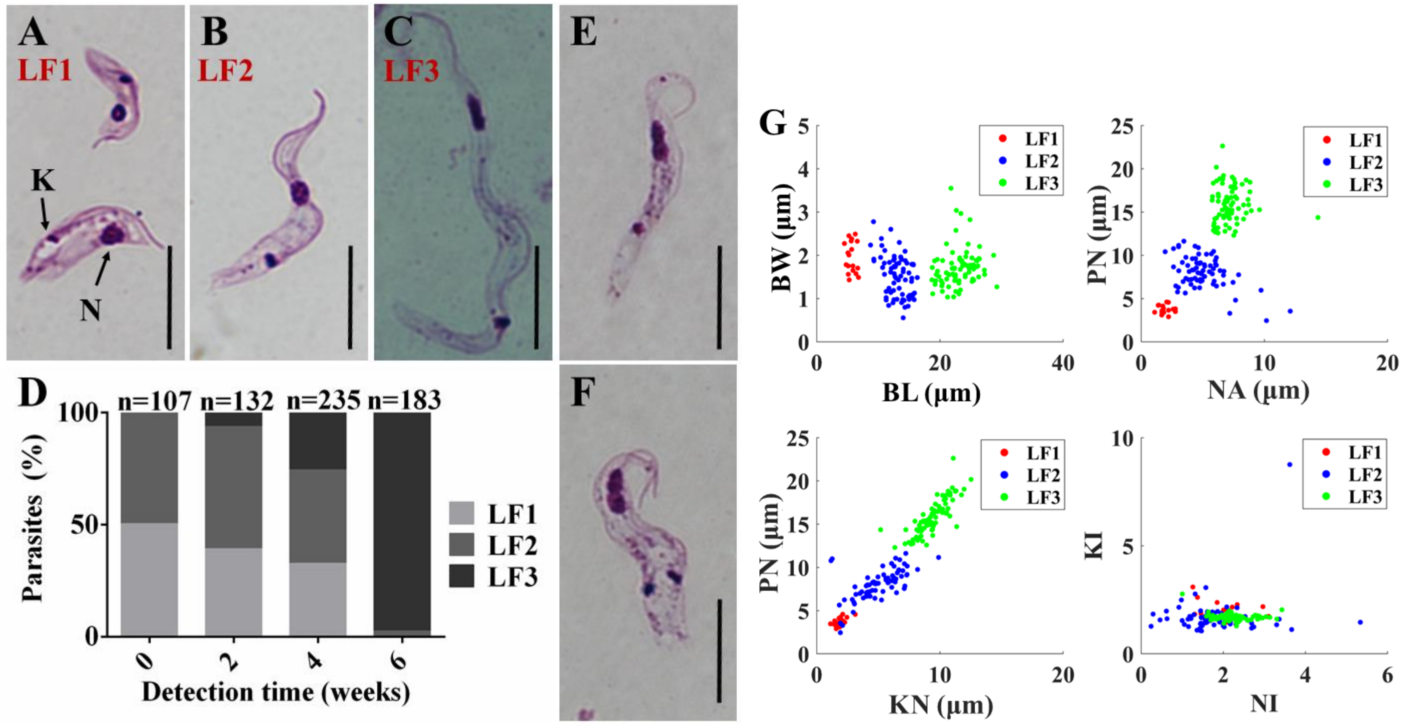


Fig. 2. Photomicrographs and morphological indices of Giemsa-stained trypanosomes from leech crop contents. (A) leech form 1 (LF1); (B) leech form 2 (LF2); (C) leech form 3 (LF3) and (E, F) dividing forms. N, nucleus; K, kinetoplast. (D) The percentages of each form of trypanosomes from the Shalang leeches are indicated at time points. (G) Morphological indices of each form. BW, maximum body width; BL, body length; PN, posterior end to mid-nucleus; NA, anterior end to mid-nucleus; KN, kinetoplast to mid-nucleus; NI, nuclear index; KI, kinetoplastic index. Bars correspond to 10 μm.

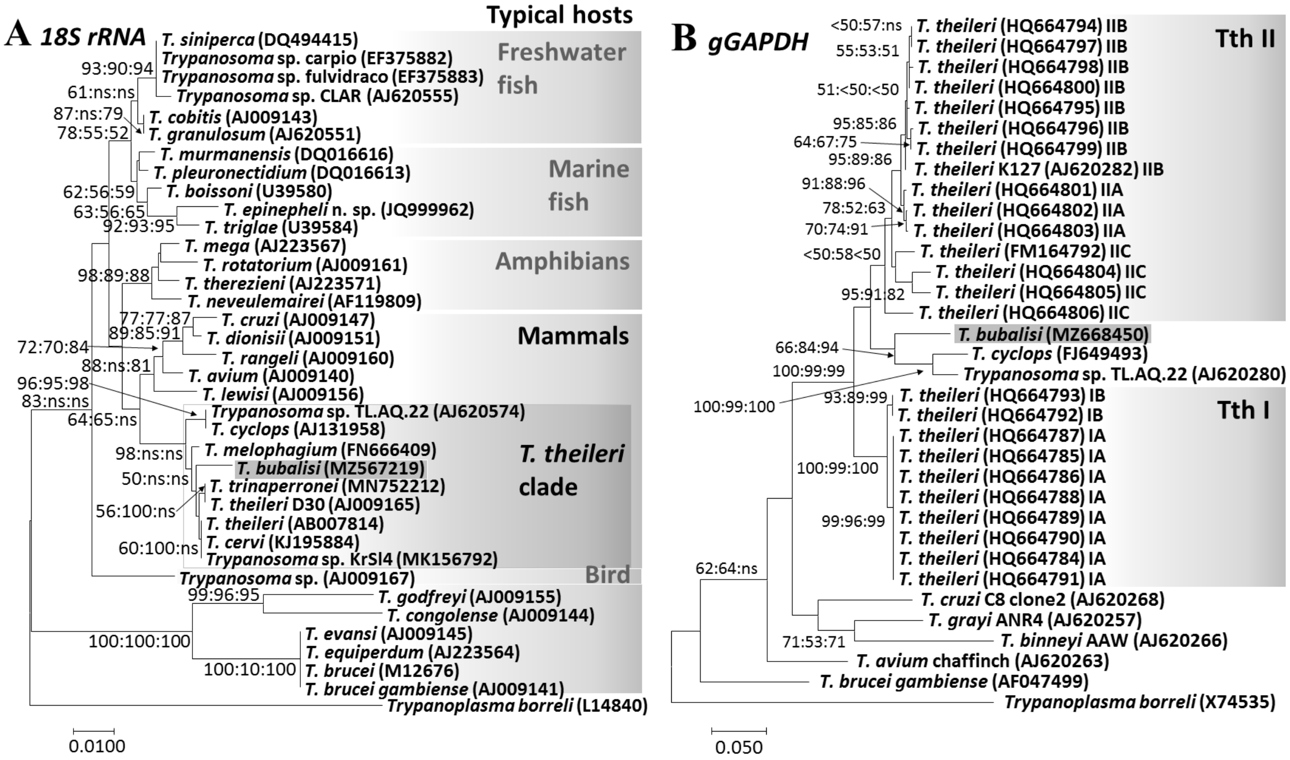


Fig. 3. Phylogenetic trees of trypanosomes based on 18S rRNA and gGAPDH sequences. (A) Phylogenetic trees of trypanosomes based on 18S rRNA sequences, including 36 trypanosome sequences of aquatic and terrestrial lineages. (B) Phylogenetic trees of trypanosomes based on gGAPDH sequences, including 33 trypanosome sequences of phylogenetic lineages of TthI, TthII and the *Trypanosoma cyclops* clade trypanosomes. *Trypanoplasma borreli* was used as outgroup and the *Trypanosoma bubalisi* from our work is shaded. Numbers at nodes are the support values for the major branches derived from 1000 replicates, respectively, for Neighbour Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses. ns, not supported. Accession numbers of sequences in GenBank are showed in parentheses.

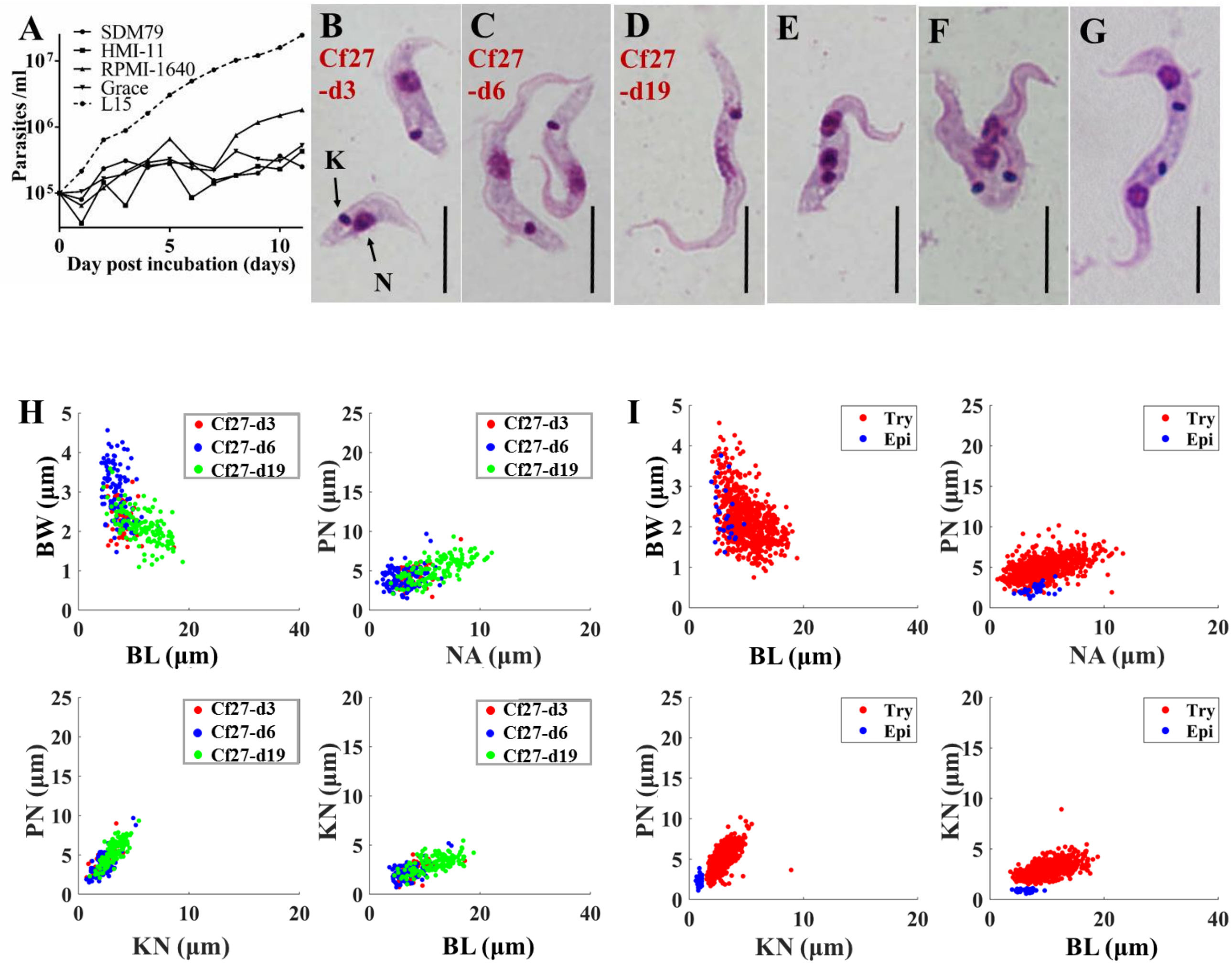


Fig. 4. The growth curves, photomicrographs and morphological indices of trypanosomes cultured at 27 C in vitro. (A) The growth curve of trypanosomes in SDM79, HMI-11, RPMI-1640, Grace's and L15 medium at 27 C for 11 days. (B-D) Photomicrographs of Giemsa-stained main forms of trypanosomes in L15 medium supplemented with 10% FBS and 1% penicillin–streptomycin at 27 C. (E-G) Suggested progression of cell division. N, nucleus; K, kinetoplast. Bars correspond to 10 lm. (H) Morphological indices of trypanosomes cultured at 27 C for 3 days (Cf27-d3), 6 days (Cf27-d6) and 19 days (Cf27-d19). (I) Morphological indices of different forms of trypanosomes. Try, trypomastigotes; Epi, epimastigotes. BW, maximum body width; BL, body length; PN, posterior end to mid-nucleus; NA, anterior end to mid-nucleus; KN, kinetoplast to midnucleus; NI, nuclear index; KI, kinetoplastic index.

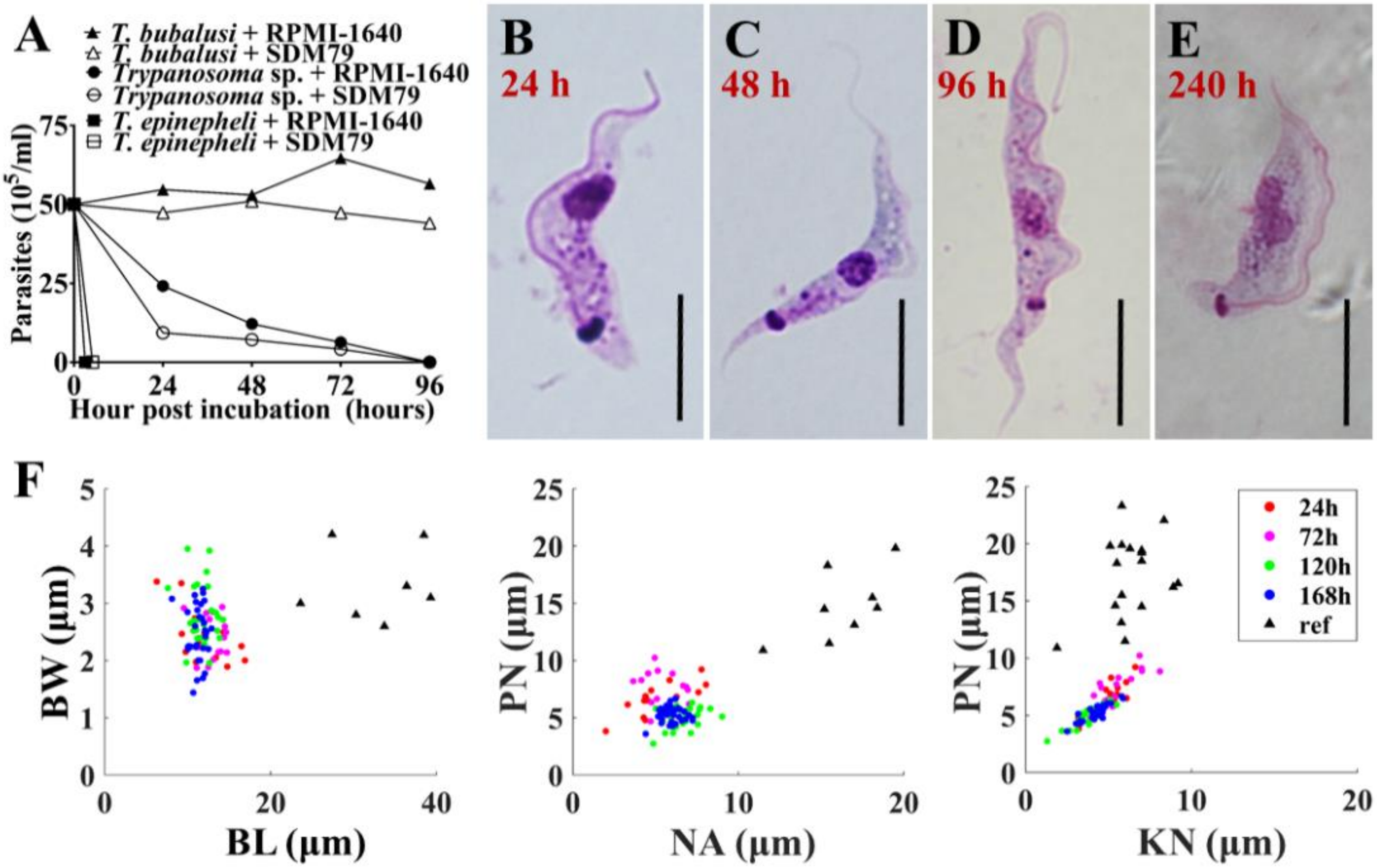
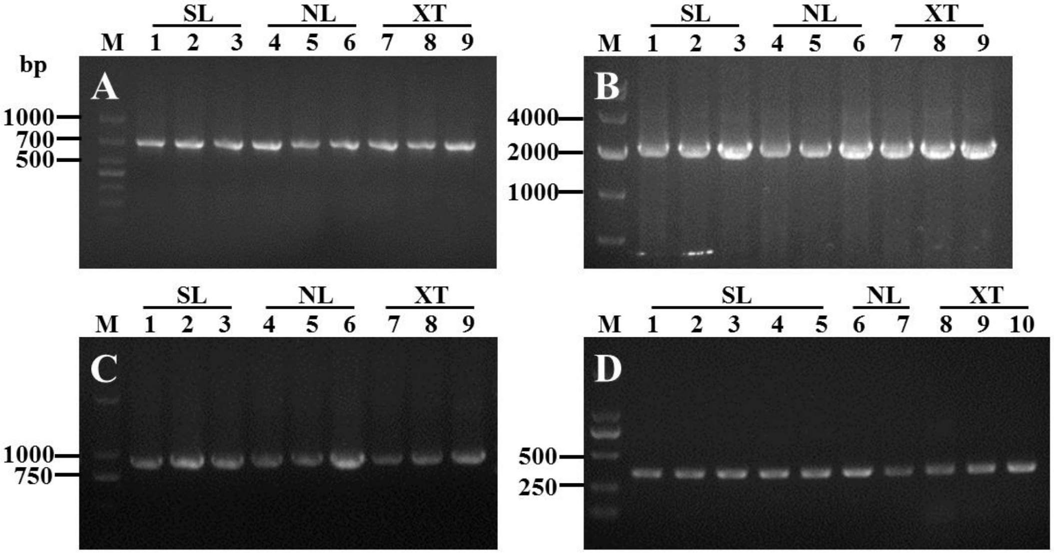
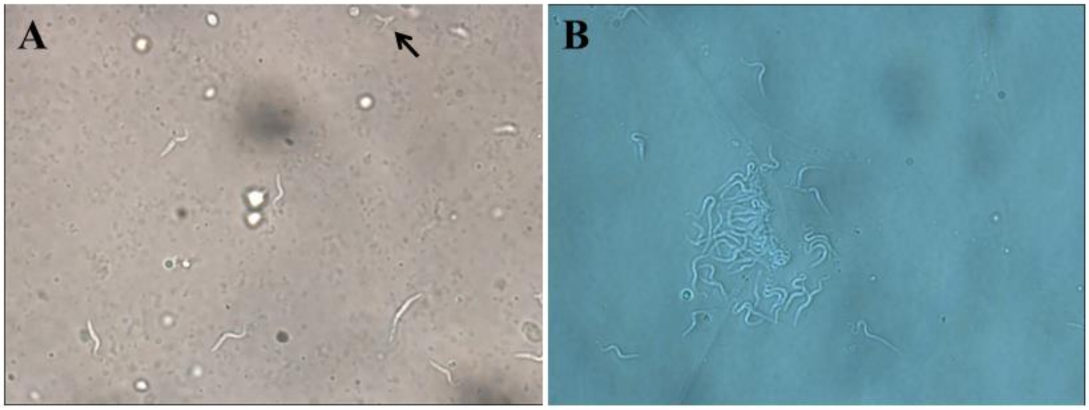


Fig. 5. The growth curves, photomicrographs and morphological indices of trypanosomes cultured at 37 C in vitro. (A) The growth curve of *Trypanosoma bubalisi* and *Trypanosoma* sp. from *Micropterus salmoides* in RPMI-1640 and SDM79 medium at 37 C for 96 h. Photomicrographs of Giemsa-stained trypanosomes at 37 C for 24 h (B), 48 h (C), 72 h (D) and the dividing form at 240 h (E). Bars correspond to 10 lm. (F) Morphological indices of *T. bubalisi* and other reference *Trypanosoma theileri* and *T. theileri*-like at 37 C (Woo et al., 1970; Kingston and Morton, 1975; Matthews et al., 1977; Kingston et al., 1982; Büscher and Friedhoff, 1984; Kingston et al., 1992; Saisawa et al., 1933; Nalbantoglu and Karaer, 2008). BW, maximum body width; BL, body length; PN, posterior end to mid-nucleus; NA, anterior end to mid-nucleus; KN, kinetoplast to midnucleus.



Supplementary Fig. 1. Electrophoresis of example PCR amplified fragments from leeches and trypanosomes from their crop contents. (A) The PCR amplified fragments of eukaryotic small subunit ribosomal RNA (*eSSU rRNA*) genes from posterior sucker of *H. manillensis* individuals; (B) PCR amplified fragments of Trypanosomatidae *SSU rRNA* gene (*tSSU rRNA*) and (C) *T. theileri* glycosomal glyceraldehyde phosphate dehydrogenase gene (*tgGAPDH*) of trypanosomes isolated from *H. manillensis*. (D) The PCR amplified fragments of mammalian mitochondrial cytochrome *b* gene (m*Cyt b*) from the crop contents of *H. manillensis*. SL, NL and XT represents the three sites, Shalang, Nanlang and Xintong, respectively, where the freshwater leeches were collected. M, DNA marker.



Supplementary Video 1. The wet slide of leech crop contents with PBS buffer viewed directly under a microscope. (A) Various forms of trypanosomes can be seen simultaneously in the crop contents of a 0 week 0 group leech, the black arrow shows a dividing cell. (B) Trypanosomes from a week 4 group leech.